Application of l-Ascorbic Acid and its Derivatives (Sodium Ascorbyl Phosphate and Magnesium Ascorbyl Phosphate) in Topical Cosmetic Formulations: Stability Studies

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Summary: The present study aimed to formulate and subsequently evaluate a topical skin-care cream (o/w emulsion) from l-ascorbic acid and its derivatives (sodium ascorbyl phosphate and magnesium ascorbyl phosphate) at 2% versus its vehicle (Control). Formulations were developed by entrapping it in the oily phase of o/w emulsion and were stored at 8°C, 25°C and 40°C (in incubator) for a period of four weeks to investigate their stability. In the physical analysis, the evaluation parameters consisted of color, smell, phase separation, centrifugation, and liquefaction. Chemical stability of both derivatives was established by HPLC analysis. In the chemical analysis, the formulation with sodium ascorbyl phosphate was more stable than those with magnesium ascorbyl phosphate and l-ascorbic acid. The microbiological stability of the formulations was also evaluated. The findings indicated that the formulations with l-ascorbic acid and its derivatives were efficient against the proliferation of various spoilage microorganisms, including aerobic plate counts as well as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and yeast and mold counts. The results presented in this work showed good stability throughout the experimental period. Newly formulated emulsion proved to exhibit a number of promising properties and attributes that might open new opportunities for the construction of more efficient, safe, and cost-effective skin-care, cosmetic, and pharmaceutical products.

Keywords: Ascorbic acid; Sodium ascorbyl phosphate; Magnesium ascorbyl phosphate; Stability.

Introduction

An emulsion is a complex mixture of two immiscible phases, with one phase dispersed in another. The macroscopic separation of the phases is prevented by the addition of a suitable surfactant [1]. In an emulsion, the therapeutic properties and spreading ability of the constituents are increased [2]. In fact, the development of liquid-liquid emulsion is a common practice in the food and pharmaceutical industries. A system that consists of oil droplets dispersed in an aqueous phase is called oil-in-water or O/W emulsion; a system that consists of water droplets dispersed in an oil phase is called water-in-oil or W/O emulsion [3]. While O/W emulsions are commonly used as water-washable drug bases and for general cosmetic purposes, W/O emulsions are widely used as emollients and for dry skin treatment [4]. Additional value can be conferred to these formulations by including active ingredients with specific cosmetic effects. Particularly advantageous cosmetic emulsion preparations are obtained when antioxidants are used as active ingredients [5]. Due to their beneficial and therapeutic properties, l-Ascorbic acid (Vitamin C) and its derivatives have often been incorporated in the form of emulsions in recent pharmaceutical and cosmetics formulations and preparations.

L-Ascorbic acid is an important antioxidant that protects the skin by scavenging and destroying free radicals and reactive oxygen-derived species [6]. It could improve the morphogenesis of dermal epidermal junction, and is also known for its skin lightening properties [7, 8]. As an UV photoprotection agent, it also has a synergistic effect when used in conjunction with vitamin E, a lipophilic vitamin [9]. L-Ascorbic acid is also used topically because of its ability to reduce wrinkles by promoting collagen synthesis [10] and its skin-depigmenting activity [11]. Because of these favorable effects, L-Ascorbic acid has long been used in pharmaceutical and cosmetic preparations [12]. The use of ascorbic acid in cosmetic and pharmaceutical products is however limited due to its low stability. Under aerobic conditions it is reversibly oxidized to l-dehydro ascorbic acid, which can be irreversibly degraded to oxalic acid [13]. To solve this problem of stability, derivatives of vitamin C, some esters such as sodium ascorbyl phosphate and magnesium

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ascorbyl phosphate, have been synthesized having an action similar to ascorbic acid but with improved chemical stability.

Sodium ascorbyl phosphate is one of the most effective free radical quenchers, and has the greatest potential for slowing down the detrimental effects resulting from photodamage [14]. Sodium ascorbyl phosphate is a stable Vitamin C derivative, which is cleaved by enzymes in the skin to release ascorbic acid. It protects cells against free radicals, promotes collagen formation and acts on the melanine formation process [15].

Magnesium ascorbyl phosphate (magnesium-l-ascorbyl-2-phosphate) is a more-stable water-soluble derivative of l-ascorbic acid that is used as an excellent additive in the modern functional whitening cosmetics [16]. It appears to have the same potential as Vitamin C to promote skin collagen synthesis, prevent free radical damage and inhibit the melanic [17].

Given the low stability of ascorbic acid dissolved in aqueous vehicles, these new forms may be considered of great potential value.

Accordingly, the present study was undertaken to investigate the physical, chemical and microbiological stability of O/W emulsions containing ascorbic acid and its esters: sodium ascorbyl phosphate and magnesium ascorbyl phosphate in order to make a comparative assessment of these active principles.

Results and Discussion

Physical Stability of Formulated Smulsions

Several mechanisms may deteriorate emulsions, including the swell of internal drops due to osmotic pressure leading to passage of water from external phase to internal phase, the rupture of the oil layer or coalescence of the oil globules, or the coalescence of the internal water droplets [18].

In this study, formulations were placed in different storage conditions i.e. 8+2°C, 25+2°C and 40+2°C for a period of four weeks in stability chambers. The samples were observed for change in color, liquefaction, and phase separation, as presented in Table-1 and 2.

Color

The findings revealed that the freshly prepared emulsions were white and pale yellow in color for F_1, F_2, F_3 and F_4. Little changes in color were observed for emulsions F_1, F_2 and F_3, well the end of storage is marked by the following colors: yellowish white and soft yellowish white (Table-1 and 2). For example, for F_1, the change in color appeared from the 21st day and persisted up to the 28th day of the analyses period. The change in color at the end of the observation period was presumably due to the oily phase separation which was promoted at higher temperature. Interestingly, no change in color was observed for F_4 at the different storage conditions, i.e., 8 $\pm$2°C, 25 $\pm$2°C and 40 $\pm$2°C, up to 28 days of observation.

Liquefaction

The viscosity of emulsion is often reported to play a vital role in its flow properties [19]. Starting from the emulsion preparation, the time and temperature processes begin to affect its separation, leading to a decrease in viscosity which, in turn, results in liquefaction increase [2]. In fact, liquefaction is a sign of instability, and might attribute to the passage of water from the internal phase to external phase as described in several reports in the literature [20]. Slight liquefaction was observed in the samples kept at 40 °C on 21st day for F_1 (control) and F_2 (l-ascorbic acid). No liquefaction was observed for the emulsions in any of the storage conditions under investigation, i.e., 8 +2°C, 25 +2°C and 40 +2°C throughout the 28 days of observation for F_3 and F_4. The absence of liquefaction provided strong evidence for the stability of the emulsions under investigation.

Centrifugation Test

The centrifugation test is based on the principle of using centrifugal force to separate two or more substances of varied densities, such as two different liquids or a liquid and a solid, and is a useful tool for assessing and predicting the shelf life of emulsions [21]. No phase separation was observed after centrifugation in any of the samples kept at different storage conditions up to 21 days. A slight phase separation was, however, recorded on centrifugation from the 21st day and up to the 28th day of observation in the samples F_1 and F_2 kept at 40°C. No other phase separations were observed till the end of the experimental period. This was presumably due to the proper homogenization speed during emulsion formulation which might have prevented the breakage of the formulations during testing [22].

Table-1: Physical characteristics of F_1 and F_2, formulations kept at 8 ±2°C, 25 ±2°C and 40 ±2°C.

		Fresh		24h		3 day		7 day		14 day		21 day		28 day	
		F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2
Liquefaction	8°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40°C	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Color	8°C	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	25°C	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	40°C	W	W	W	W	W	W	W	W	YW	W	YW	YW	YW	YW
Phase separation	8°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40°C	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Centrifugation	8°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40°C	-	-	-	-	-	-	-	-	-	-	+	+	+	+

- =No Change; + =Slight Change; PY= Pale yellow; SYW= Soft yellowish white; YW= Yellowish White; Y= Yellow; W= White.

Table-2: Physical characteristics of F_3 and F_4, formulations kept at 8 ±2°C, 25 ±2°C and 40 ±2°C.

		Fresh		24h		3 day		7 day		14 day		21 day		28 day	
		F_3	F_4	F_3	F_4	F_3	F_4	F_3	F_4	F_3	F_4	F_3	F_4	F_3	F_4
Liquefaction	8°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Color	8°C	PY	YW	PY	YW	PY	YW	PY	YW	PY	YW	PY	YW	PY	YW
	25°C	PY	YW	PY	YW	PY	YW	PY	YW	PY	YW	PY	YW	PY	YW
	40°C	PY	YW	PY	YW	PY	YW	PY	YW	PY	YW	SYW	YW	SYW	YW
Phase separation	8°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Centrifugation	8°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- =No Change; + =Slight Change; PY= Pale yellow; SYW= Soft yellowish white; YW= Yellowish White; Y= Yellow; W= White.

Evaluation of Skin Measurements

Only the formulation containing ascorbic acid (F_2) led to an enhancement in TEWL values (Fig. 1) contributing alteration of skin barrier function. During the process of terminal differentiation, loss of water and several biochemicals changes and occur on the skin surface [23] and because ascorbic acid led to an increase in TEWL, we have an indication that it enhanced the epidermal cell renewal process on human skin. For F3 and F4, the values of TEWL remain constant during storage.

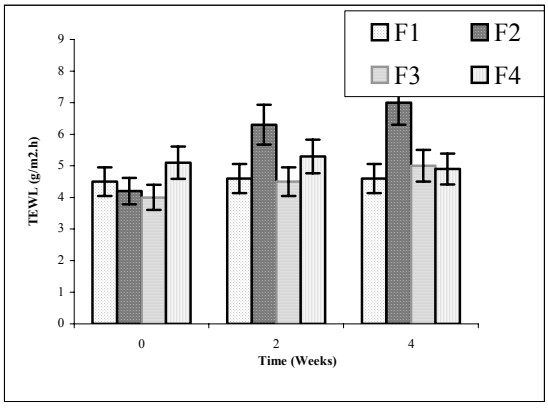

Fig. 1: Transepidermal water loss (TEWL) of the formulations studied (F_1, F_2, F_3 and F_4) at 0, 2 and 4 week period of application.

Chemical Stability

Chromatographic Analysis of Sodium Ascorbyl Phosphate (F_3) and Magnesium Ascorbyl Phosphate (F_4)

Several chromatographic analytical methods for quantifying the amount of sodium ascorbyl phosphate and magnesium ascorbyl phosphate are described in the literature [15, 24]. We used therefore different chromatographic conditions as described in Section 2.3.1, which proved to be suitable for systems under study.

The chemical stability was tested by HPLC and the results suggested that l-ascorbic acid and magnesium ascorbyl phosphate degradation follows first-order kinetics, corroborating the results of Gallarate *et al* [25].

The instability of magnesium ascorbyl phosphate is a result of its oxidative degradation. Generally, the kinetics of oxidative reactions are second order, but can usually be simplified to pseudo first order if oxygen is in excess. They also occur in larger extent in more dilute systems, indicating that the initial concentration of the active ingredient is an important factor concerning the extent of its degradation [26]. The influence of the initial concentration of magnesium ascorbyl phosphate on its stability for o/w emulsion was therefore studied at

the beginning of our work. It is known that the concentration range of magnesium ascorbyl phosphate is in general ranged from 0.05 to 0.1%, when used as an antioxidant to stabilize formulation; if used as an active ingredient the concentrations are higher, usually about 1–2%. In our study, magnesium ascorbyl phosphate was incorporated in o/w emulsion in a concentration of 2.00%. Fractions of nondegraded magnesium ascorbyl phosphate determined at different time intervals of storage in the dark are listed in Table-3. After 28 days less than 40% of active ingredient remained in F_4. It was further confirmed that the initial concentration significantly influences the degradation of the compound, 2.00% of magnesium ascorbyl phosphate as a rule reducing the extent of its degradation.

The stability of sodium ascorbyl phosphate in o/w emulsion at an initial concentration 2.00% is determined. The fractions of nondegraded sodium ascorbyl phosphate were determined by HPLC. As expected, sodium ascorbyl phosphate was stable.

Compared to magnesium ascorbyl phosphate, sodium ascorbyl phosphate was significantly more stable. After 2 months, more than 95% of nondegraded compound remained in both microemulsions. These results support the use of sodium ascorbyl phosphate as an active ingredient in cosmetic and pharmaceutical preparations.

Microbilogical Stability of Formulated Emulsions

Aerobic Plate Count

The increase in storage time resulted in significant proliferations in Aerobic plate counts regardless of the type of treatment being applied (Table-4). The log mean count recorded for the Aerobic plate count of samples on day 0 was about 2 log10 CFU/ g. On day 28 of storage, the log mean count of Aerobic plate count reached 3.13, 2.93, 2.95 and 2.33 for F_1, F_2, F_3 and F_4, respectively, which did not approximate the maximum limit of 6,9 log10 CFU/ g for Aerobic plate count recommended by AFNOR, ISO NF- 21149 (2006) [27] in processed cosmetics.

Pseudomonas aeruginosa and Staphylococcus aureus Counts

The results from the *Pseudomonas* and *Staphylococcus aureus* detection tests were negative, thus confirming that all the treated samples met the conventional standards specified with regards to fitness for human consumption [28, 29] (Table-4).

Yeast and Mold Counts

Yeast and mold have often been tested in cosmetic products to assess microbiological safety, sanitation conditions, and product quality during processing and storage [30]. Despite the presence of yeast and mold in the samples, the levels of these microorganisms were noted to remain under the standard limit. In fact, the initial yeast and mold counts recorded for all treatments were below the detection limit [30].

Moreover, the yeast and mold counts values recorded for the formulated sample F_4 were noted to show delayed growth when compared to F_1, F_2 and F_3 (Table-4).

In conclusion, L-ascorbic acid and its derivatives: sodium ascorbyl phosphate and magnesium ascorbyl phosphate seems to be very interesting since it preserved physicochemical properties of the product and was efficient against the proliferation of various spoilage microorganisms. In fact, the stability of L-ascorbic acid and its derivatives has been determined in o/w emulsions. As expected, the sodium ascorbyl phosphate is more stable than the magnesium ascorbyl phosphate at the initial concentration used (2.00%). Nevertheless magnesium ascorbyl phosphate is still convenient as an antioxidant to stabilize formulations. On the contrary sodium ascorbyl phosphate can be used as an active ingredient in cosmetic and pharmaceutical preparations on the basis of its stability. The newly formulated cream was proved to exhibit a number of promising properties and attributes that might open new opportunities for the construction of more efficient, safe, and cost-effective skin-care, cosmetic, and pharmaceutical products.

Table-3: Percentages of nondegraded sodium ascorbyl phosphate (F_3) and magnesium ascorbyl phosphate (F_4) (n=3) in o/w emulsions at 2.00%.

Formulation	Day						
	0	1	2	3	7	14	28
F_3	100	91.11±6.96	88±5.44	73.41±7.82	49.73±8.78	36.74±1.39	28.46±1.26
F_4	100	99.91±4.26	99.01±8.86	98.77±9.23	98.11±6.66	98.04±5.46	97.81±8.06

Table-4: Microbial load of aerobic plate count, *Pseudomonas* spp, *Staphylococcus aureus* and Yeast and molds count of F1, F2, F3 and F4 during 28 days of storage at 25 ± 2°C.

	Days of storage at 25 ± 2°C				
	0	**7**	**14**	**21**	**28**
			Aerobic plate count		
F1	**2.0±0.30** [a]	**2.43±0.34** [c]	**2.55±0.26** [b]	**2.89±0.19** [b]	**3.13±0.30** [c]
F2	**2.02±0.31** [a]	**2.15±0.37** [a]	**2.26±0.18** [a]	**2.88±0.22** [a]	**2.93±0.29** [b]
F3	**2.04±0.25** [a]	**2.18±0.29** [b]	**2.28±0.17**[b]	**2.45±0.18** [b]	**2.59±0.18** [c]
F4	**2.01±0.27** [a]	**2.11±0.19** [a]	**2.24±0.15** [a]	**2.36±0.15** [a]	**2.33±0.11** [a]
			***Pseudomonas* spp**		
F1	**<1**	**<1**	**<1**	**<1**	**<1**
F2	**<1**	**<1**	**<1**	**<1**	**<1**
F3	**<1**	**<1**	**<1**	**<1**	**<1**
F4	**<1**	**<1**	**<1**	**<1**	**<1**
			Staphylococcus aureus		
F1	**<1**	**<1**	**<1**	**<1**	**<1**
F2	**<1**	**<1**	**<1**	**<1**	**<1**
F3	**<1**	**<1**	**<1**	**<1**	**<1**
F4	**<1**	**<1**	**<1**	**<1**	**<1**
			Yeast and molds		
F1	**1.12±0.28** [a]	**1.32±0.22** [a]	**1.56±0.15** [a]	**1.69±0.39** [a,b]	**1.8±0.22** [a]
F2	**1.14±0.16** [a]	**1.25±0.22** [b]	**1.48±0.14** [c]	**1.58±0.27** [a]	**1.68±0.27**[c]
F3	**1.11±0.11** [a]	**1.2±0.15** [a]	**1. 41±0.16** [a]	**1.52±0.17** [a]	**1.58±0.23** [b]
F4	**1.13±0.16** [a]	**1.17±0.22** [a]	**1.29±0.14** [b]	**1.32±0.27** [b]	**1.39±0.27** [a]

±: Standard deviation of three replicates
CFU: Colony -forming units
a–c: Averages for different microbial analyses with different letters in the same column are different (P<0.05).

Experimental

Formulations

Table-5 show the components and concentrations of the formulations (F_1, F_2, F_3 and F_4) used in the study. F_1 (control) is composed of: glycerol monostearate, ceteareth-25, isopropyl palmitate, cetearyl alcohol, ethyl paraben, polyethylene glycol, cetyl lactate, laureth 7 and disodium EDTA. With this basic formulation (control), 2% of l- ascorbic acid (F_2), 2% of sodium ascorbyl phosphate (F_3) and magnesium ascorbyl phosphate (F_4) were added at pH 5.5, the active principle optimal pH. The O/W formulations were prepared and mixed in a Heidolph RZR 2021 shaker at 600 rpm.

Table-5: Components and concentrations of the formulations.

Ingredients	**Formulations**			
	F_1	**F_2**	**F_3**	**F_4**
Glycerol monostearate	**3%**	**3%**	**3%**	**3%**
Ceteareth-25	**1%**	**1%**	**1%**	**1%**
Isopropyl palmitate	**1%**	**1%**	**1%**	**1%**
Cetearyl alcohol	**3%**	**3%**	**3%**	**3%**
Ethyl paraben	**0.5%**	**0.5%**	**0.5%**	**0.5%**
Polyethylene glycol	**6%**	**6%**	**6%**	**6%**
Cetyl lactate	**2%**	**2%**	**2%**	**2%**
Laureth 7	**2%**	**2%**	**2%**	**2%**
Disodium EDTA	**0.5%**	**0.5%**	**0.5%**	**0.5%**
Sodium dithionite	**0.5%**	**0.5%**	**0.5%**	**0.5%**
L- ascorbic acid	**-**	**2%**	**-**	**-**
Sodium ascorbyl phosphate	**-**	**-**	**2%**	**-**
Magnesium ascorbyl phosphate	**-**	**-**	**-**	**2%**
Water	**81%**	**79%**	**79%**	**79%**

Physical Stability

The obtained emulsion was submitted to a set of organoleptic (color, thickness, look, feel) and physical (creaming and phase separation) analyses.

Stability Tests

Stability tests were performed at different conditions for emulsions to investigate the effect of these conditions on the storage of emulsions. These tests were performed on samples kept at 8°C ±2°C (in refrigerator), 25°C ±2°C (in incubator) and 40°C ±2°C (in incubator). The physical, i.e. color, and the organoleptic, i.e. liquefaction and phase separation, characteristics of emulsions were observed at various intervals for 28 days.

Centrifugation Tests

Centrifugal tests were performed for emulsions immediately after preparation. Those tests were repeated for emulsions after 24 hours, 7 days, 14 days, 21 days, and 28 days of preparation. They were performed at 5000 rpm and 25°C for 10 minutes by placing 10g of the sample in centrifugal tubes.

Skin Measurement (Measurement of Transepidermal Water Loss)

The transepidermal water loss (TEWL) is determined using skin biophysical technique, Tewameter® CM 210 from Courage1Khazaka (Cologne, Germany). TEWL, which is related to skin barrier function, was expressed as g/m^2**.** h [31].

Chemical Stability

All samples were stored in well-closed 25 ml glass flasks. During storage samples were kept at room temperature (22±1 °C) in the dark, except for those used for studying the influence of light. The

amount of nondegraded active ingredient in samples was determined quantitatively at the beginning of storage and subsequently on the 1st, 2nd, 3rd, 7th, 14th and 28th day

Chromatographic Conditions

The HPLC apparatus consisted of JASCO HPLC, a sample injector with a 20 µl sample loop and a wavelength detector. For sodium ascorbyl phosphate the stationary phase was a 250×4 mm i.d column packed with 100 µm Nucleosil NH2, the mobile phase acetonitrile-0.3 M phosphate buffer pH 4 (40:60). The flow rate was 0.8 ml min^{-1} and UV detection at 258 nm.

For Magnesium ascorbyl phosphate the stationary phase was a 120×4 mm i.d column packed with 5 µm Eurospher C 18, the mobile phase methanol-acetonitrile-0.02 M phosphate buffer pH 2.5 (75:10:15). The flow rate was 1.5 ml min^{-1} and UV detection at 254 nm.

All analyses were performed at ambient temperature.

Sample Preparation for Stability

The stability of sodium ascorbyl phosphate and Magnesium ascorbyl phosphate were determined by HPLC in samples kept at room temperature (22±1 °C) in the dark for 4 weeks. For Magnesium ascorbyl phosphate 100 µl of microemulsion were diluted 1:100 (v/v) with methanol; for sodium ascorbyl phosphate 100 µl of microemulsion were diluted 1:100 (v/v) with tetrahydrofuran-0.3 M phosphate buffer pH 4 and then further diluted with 0.3 M phosphate buffer pH 4 to a final dilution 1:1000 (v/v).

Microbiological Stability

In order to assess the degree of contamination, 1 g of material was dispersed in a 4ml sterile Ringer solution containing 0.25% tween 80. Appropriate dilutions were made in the same dispersing vehicle, and 0.1 ml was plated out on the appropriate solid medium using the surface viable method. Emergent colonies were counted after the necessary incubation. All operations were carried out in duplicates [30].

Aerobic Plate Count

Aerobic plate counts (APC) were determined by inoculating 0.1 mL of the homogenate sample onto triplicate sterile plates of pre-poured and dried Standard Methods Agar using the surface spread technique. The plates were then incubated for 48 h at 35°C [27]. The Standard Methods Agar is a standardized medium for the enumeration of microorganisms from materials of sanitary importance. Duplicates of each dilution (1 mL) of neutralized and non-neutralized samples were pour-plated using Standard Methods Agar (Oxoid, Basingstoke, Hampshire, England) and incubated at 30 ± 1°C for 48 ± 3 h. Plates containing 25–250 colonies were selected and counted, and the average number of CFU/mL was calculated.

Pseudomonas aeruginosa Count

Pseudomonas aeruginosa were enumerated on Pseudomonas Agar Base (CM 559, Oxoid) supplemented with cetrimide, fucidin, and cephaloridine (CFC), providing a selective isolation medium for *Pseudomonas aeruginosa*. Colonies were counted after 2 days of incubation at 25°C [28].

Staphylococcus aureus

Surviving population of *Staphylococcus aureus* was determined by standard plating methods [28]. At each sampling time, colonies of *Staphylococcus* were selected, Gram-stained, and observed for catalase and oxidase reactions to confirm the presence of *Staphylococcus aureus*. Microbiological data were transformed into logarithms of the number of colony-forming units (CFU/g).

Yeast and Mold Counts

The method involved enumeration of colonies on Sabouraud dextrose chloramphenicol agar medium. Enumeration was carried out as a pour plate, surface spread, or membrane filtration method [30].

Microbiolgical tests were repeated for fresh and formulations at 25°C after 3, 5, 7, 10, 15, 20, 25, and 30 days of preparation

Statistical Analysis

All measurements were carried out in triplicates, and all microbial counts were converted into base-10 logarithms of colony forming units per ml of formulations (log10 CFU/ g). Data were subjected to analysis of variance (ANOVA) using the General Linear Models procedure of the Statistical Analysis System software of SAS Institute [32].

Differences among the mean values of the various treatments and storage periods were determined by the least significant difference (LSD) test, and the significance was defined at $P < 0.05$. The differences which are equal to or more than the identified LSD values are considered statistically significant.

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