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In Vivo Evaluations of Emulsion O/W for a New Topical Anti-Aging Formulation: Short-Term and Long-Term Efficacy

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Abstract

This study aims to evaluate the *in vivo* efficacy in terms of elasticity, hydration and anti-wrinkle enhancement of a new cosmetic cream formulation: Apollinea anti-ageing facial cream. The latter is O/W (oil/water) emulsion with high volume percentages of snail secretion filtrate and donkey milk serum in its composition. The investigation has been performed on (twelve) healthy subjects: 8 (eight) females, and 4 (four) males, all presenting evident skin wrinkles in typical facial areas (e.g. forehead and eye contour). The subjects had to apply about 2 grams of Apollinea anti-ageing facial cream every day. The efficacy of the new cosmetic formulation was assessed before and after the cosmetic treatment, precisely 2 hours, to assess the short-term efficacy, and 40 days after, this to evaluate the product long-term efficacy. Elasticity enhancements have been tested through measurements of the viscoelastic properties of the skin, whereas the hydration parameter through measurements of the skin and subcutaneous tissues dielectric constant. All the latter measurements have been performed with specific instrumentation, as discussed more in detail in the following. To evaluate the anti-wrinkles efficacy instead, we proceeded with digital camera imaging and image analysis processed through ImageJ software. The new Apollinea facial cream cosmetic formulation, based on donkey milk whey and snail secretion filtrate from Helix Aspersa Müller, showed significant enhancement effects on skin elasticity and hydration as well as an important skin-wrinkles-reduction.

Keywords

Skin, Wrinkles, Anti-Aging, Snail Secretion Filtrate, Donkey Serum

1. Introduction

Skin ageing is influenced by a number of intrinsic and extrinsic factors, promoting the accumulation, in the cutaneous structure, of physiochemical alterations [1] [2] leading to a progressive loss of structural integrity and physiological functions [3]. Such aforementioned alterations result in visible changes to the cutaneous tissue, of which few examples are wrinkling, dyschromia and elastosis [4]. Thus, a modern anti-aging cosmetic product must attain a variety of effects like moisturizing the skin, making it more elastic and reducing the visibility of fine and deep wrinkles. The aim of this study is to evaluate the short term (2 hours) and long term (40 days) efficacy of a new cosmetic formulation, Apollinea cosmetic cream (O/W emulsion), in skin elasticizing function, hydration and wrinkles reduction. The main ingredients in this new cosmetic formulation "Apollinea facial cream" are donkey milk serum, snail secretion filtrate and vegetable lipids, such as sweet almonds oil and Karite butter, all ingredients that strongly contribute to the anti-age efficacy of the final product. Among the latter donkey milk is the most suited for a dermo-cosmetic application given its high percentage of milk serum proteins and other milk compounds, well known for their high degree of uptake and water retention that promotes skin hydration and prevention of epidermis cells degeneration [5] [6] [7]. Prominent donkey milk serum-proteins are β-lactoglobulin (β-LG), α-lactalbumin (α-La), and lysozyme, with an average percentage respect to the total serum-proteins of 29.8%, 22.6% and 21.0%, respectively. The remaining of serum-proteins is the sum of minor compounds average percentages as lactoferrin, serum-albumin and immunoglobulin [8]. The latter has a high iron-chelating property that prevents free radical production and the degradation of the epidermal cells [5] [6] as well as anti-microbial and anti-viral properties [9]. Donkey milk serum contains 3.75 g/L of β-LG which is a protein that binds and carries various small hydrophobic molecules, and 1.8 g/L of α-La, which concentration is very close to the one found in human milk. The lysozyme is an anti-bacterial agent belonging to the hydrolase class and specifically is a glucosidase [9]. Its concentration in donkey milk serum spans from 0.76 - 3.75 g/L but this strongly depends on the analytic method applied for the measure [10] [11] [12]. As already reported from J. Cotte back in 1991, α-La and β-LG have been widely applied in cosmetics as hydrating agents that makes them effective in temporarily masking cutaneous wrinkles [13]. The capability in calming irritation symptoms [14] [15] and in restructuring skin-ageing processes [16] [17] of Serum-proteins as β-lactoglobulin and lysozyme and essential amino acids beautifying properties are also well known [18].

The second key ingredient of the cosmetic product under investigation is snail secretion filtrate, precisely from Helix Aspersa Muller, snail belonging to the gasteropods class and native of the Mediterranean area [19]. Snails are induced into mucus secretion through a harmless process. The clammy secretion covering the snail outer surface may serve in preventing the moisture evaporation, in

helping smooth movements and in protecting the body from mechanical injuries [20]. In 1961 Campion [21], carried out a histological and histochemical study of the Helix Aspersa mantle collar glands that secrete drool. He spotted at least eight different kinds of glands, four extruding different kinds of mucus, one rich in proteins, one in calcium carbonate granules, one is a pigmented secretion with a flavone, and one containing fat-globules. Campion observed that the mantel collar glands were producing mucus combined with proteins [21] [22]. In recent years, scientific studies of the secretions of the snail Helix Aspersa, have shown that the mucus contains a complex combination of natural substances, including allantoin and glycolic acid [23] [24] [25] [26]. According to USA Food and Drug Administration (FDA), allantoin is a safe and effective active compound for skin protection [27], is a well-known anti-irritating and hydrating [28] agent as well as a promoter of epithelial stimulation [29]. In particular allantolin promotes keratolytic activity [30] [31] [32] [33] as well as glycolic acid, an alpha-hydroxy-acid, effective as a peeling agent. It eliminates superficial dead skin cells and promotes their replacement with new corpuscles [32] [34].

Skin ageing is indeed an inevitable biological phenomenon affecting both dermis and epidermis through changes in the integumental system, which determines its most common manifestations [35]. The most reproducible and recognizable feature of skin ageing is the flattening of the dermo-epidermic junction [36] [37]. There happens a general atrophy of the extracellular matrix, reflecting in a decrease in the number of fibroblasts and in low levels of collagen [38] and elastin thus resulting in a compromised arrangement of the latter [37]. Such changes are partially the result of cumulative endogen damage due to the increasing formation of oxygen-reactive species (ROS) generated during the cellular oxidative metabolic process [39]. Moreover, ageing is speed up on the skin areas often exposed to sun light, also called photo-ageing [40]. To clarify photo-ageing dynamics, Brieva *et al.*, via in vivo studies, have shown that snail secretion has anti-oxidant efficacy through the superoxide dismutase (SOD), as well as Glutathione-S-transferase (GST) [41] [42] [43] [44]. Other studies carried out on snail drool have shed light on the possible mechanisms behind its regenerative properties [42] [43]. SOD is a key enzyme in the radical superoxide anion ($O2^-$) as well as hydrogen peroxide (H_2O_2) deactivation process, while GST is one of the enzymes responsible of the detoxification from xenobiotic substances and free radicals. Snail drool promotes fibroblasts proliferation, the rearrangement of the actin-cytoskeleton and favors the extra-cellular matrix (ECM) assembly [42] [43] thus reducing the progressive degeneration of the cutaneous mantel [41] [42] [43] [44] [45].

In the formulation of the O/W "Apollinea" emulsion an abundant portion of lipid compounds has been added, mainly consisting in butters and vegetable oils with noticeable protective and emollient effects.

The main lipid compound of the product on test is Karite butter. The latter contains fat acids such as: stearic, oleic, linoleic, arachidic, eicosenoic, docosanoic

and tetracosanoic acid [46] [47]. In particular is made 90% of triglycerides (saponifiable fraction) and 10% of non-triglycerides (unsaponifiable fraction) [47] [48].

Karité butter therapeutic properties belong to the fraction of compounds that cannot be saponified thanks to the presence of tocopherols, catechins, triterpenes, phenols, sterols. The latter possess strong anti-inflammatory and antioxidant properties. The triterpene alfa-amirin [47] [49], has anti-reddening and pruritus releaf efficacy [47] while Karite butter is also a very effective UV-screen [47] [49], as well as hydrating, regenerating and anti-wrinkles agent [49] [50].

The current study aims to evaluate the skin-care effects of the product under investigation taking carefully into account the specific combination of ingredients.

2. Materials and Methods

2.1. Snail Secretion Filtrate: How to Collect It

The snail secretion filtrate used as a dispersing medium in the formulation of the cosmetic product Apollinea O/W emulsion, is delivered by "Apollinea Lab. S.r.l., Masseria Cosentino, Lauria (PZ), Italy". The collection of snail drool is performed without any addition of salts or chemical compounds and at temperatures and humidity levels completely adequate for the snails. Secretion is obtained by a gentle shaking the snails with an appropriate equipment made of stainless steel, that simulates the stimulation normally performed manually. The mechanized procedure does not create any harm to the snails while instead enhances efficacy and productivity per kg of snails getting treated, which are then released back to the nurture. Increasing the humidity levels works like a switch for snail into drool secretion; this is achieved simply vaporizing water onto the snails while they lay inside a broached basket through which the drool is collected into an underlying container. After collection the snail secretion undergoes four stages of filtration: through 70 um and 1 um filters and then micro-filtration through 0.6 and 0.22 um filters respectively. After the filtration process snail secretion appears like a clear ambered fluid. The latter is completely miscible in water, displays a limited compatibility with Ethanol but it is insoluble in vegetable or mineral oils and its pH may vary between 6.5 and 7.5.

2.2. Donkey Milk and Its Serum Fraction

To obtain the donkey milk serum, used in the formulation of the cosmetic product Apollinea O/W emulsion, the donkey milk is centrifuged at first to eliminate the lipid fraction. The second step of the process consists in precipitating the casein fraction by reducing the pH to the exact value of 4.2, value at which the serum proteins, instead, are still stable and well dispersed.

2.3. Anti-Age Facial Cream Formulation

The dermo-cosmetic product under evaluation has been prepared in agreement

with the admitted substances listed for cosmetic and personal hygiene formulation pf the CEE legislation. The preservatives used in the aforementioned Apollinea—anti-aging facial cream formulation is listed in as CEE substances and within the expected concentrations limits as reported in the attachments related to the CEE legislations 1223/2009 and followings. The **INCI** (International Nomenclature of Cosmetic Ingredients) is: Donkey milk*, Snail secretion filtrate, Butyrospermum parkii butter, Glyceryl stearate, Caprylic/capric triglyceride, Cetearyl alcohol, Prunus amygdalus dulcis oil, Glycerin, Olea europea oil, Cera alba, Hydrogenated olive oil, Ethylhexylglycerin, Benzyl alcohol, Xanthan gum, Tocopheryl acetate, Hydrogenated castor oil, Sodium ascorbyl phosphate, Phenethyl alcohol, Potassium pamitoyl hydrolyzed wheat protein, Ethyl lauroyl arginate HCl, Parfum, Tetrasodium glutamate diacetate, Cinnamic acid, Ascorbyl palmitate, Nisin.

*Donkey milk serum

2.4. Experimental Study

The aim of the study was to evaluate the anti-wrinkle efficacy (reduction of facial skin roughness), hydration and elasticity of the skin of the face, of a dermo-cosmetic treatment, which involves the distribution until complete absorption on the anatomical districts of the face skin tested for efficacy, about 2 (two grams) of the O/W Apollinea-anti-aging facial facial cream over specific anatomic districts, subject to efficacy evaluation.

To this end, a study conducted in 12 healty subjects: 8 females and 4 males, with facial skin characterized by skin wrinkles. Thus, the study aims to investigate the "*in vivo*" efficacy of the Apollinea-anti-aging facial cream formulation on the short-term (2 hours) and long-term (40 days), after the treatment through non-invasive techniques able to quantify skin elasticity and hydration enhancement as compared to a reduction of the skin wrinkles. The current experimental investigation has been held in agreement with the Helsinki declaration (Ethical principles for medical research involving human subjects). The efficacy of the cosmetic product has been evaluated 2 hours and 40 days after the treatment through non-invasive techniques able to quantify skin elasticity and hydration enhancement as opposed to a reduction of the skin wrinkles.

Twelve healthy volunteers between 35 and 67 in age, have been selected with the following inclusion criteria:

- To be generally in good health;
- Without any pathological skin condition;
- With wrinkles and dermal laxity around the eyes;
- Subjects which do not show pigmentary lesions or other kind of lesions on the interested areas;

  which may interfere with the experimental results;
- Subjects which do not show history of hyper-sensibility against the most common compounds in cosmetic formulations;
- Subjects which are not pregnant nor breastfeeding;

- Subjects willing to use solely the cosmetic product being tested through all the test time in the skin areas devoted to the cosmetic evaluation;
- Subjects willing to sign the informed agreement.

And the following exclusion criteria:

- Subjects not belonging in the abovementioned criteria;
- Subjects under a specific pharmaceutical treatment which may interfere with the experimental study in question;
- Participation to a similar test study within the last 60 days;
- Subjects allergic to compounds in the proposed cosmetic formulation.

2.5. Topical Application

The product is applied by the experimenter massaging till complete absorption over the anatomic zones subject to evaluation. The levels of skin elasticity, hydration and rugosity were measured before the treatment (thus at time t = 0), t = 2 hours after and t = 40 days after in order to check on the efficacy of the aforementioned cosmetic formulation. On the first day, the experimenter provides to instruct the participants to the study about how to correctly apply the emulsion and in particular:

1) The facial skin must be thoroughly cleansed so to remove any other cosmetic eventually used before;
2) The emulsion is applied till complete absorption (t = 40 days) in wrinkles areas, through a smooth massage;
3) The experimenter the first day makes an assessment of skin elasticity, hydration and rugosity in order to evaluate the efficacy of the applied cosmetic product on the short-term (t = 2 hours).

2.6. Evaluation of the Skin Elasticity

The measurement of the skin elasticity level has been carried out through "soft-plus" instrumentation from Callegari S.p.A. Parma (Italy), provided with single measurement probes.

Measurement Principle: the evaluation of the cutaneous elasticity allows the monitoring of the skin viscous-elastic properties respect to immediate deformation and its recovery capability.

Through the measurement of the skin deformation under exerting negative pressure from probe, the index of skin elasticity can be determined.

2.7. Hydration Parameter Evaluation

The measurement of skin hydration has been carried out using a specific probe for single measurement, provided from the "soft plus" instrumentation form Callegari company S.p.A. (Italy).

Measurement principle: the measurement of the hydration level happens through a capacitor sensor effectively measuring the dielectric constant of the skin and under-laying tissues.

2.8. Anti-Wrinkles Efficacy Evaluation via Imaging Data Analysis

The anti-wrinkles efficacy evaluation has been carried out using a micro-camera, provided from the "soft plus" instrumentation form Callegari company s.p.A. (Italy), to obtain digital images of the skin areas of interest for such study, that can be used for further imaging-data analysis. The latter micro-camera is provided with a 100× magnification lenses and a ×12 ocular aperture, while the withe leds on top are needed to provide haven illumination on the selected skin areas. Pictures have been taken keeping the camera always at the same distance thank to a non-tunable spacer, part of the micro-camera equipment. Such software allows acquiring information about the height (H) and the width (W) of the skin-wrinkles. To confirm the validity of such equipment images have been taken also with another type of camera, a high definition digital camera, on the same anatomic skin-districts chosen for the abovementioned experiment and analyzed. The data processing and evaluation has been carried out with ImageJ software, a very popular open source software programmed in Java that allows visualization, modification, analysis, saving and printing of 8 - 16 and 32-bit images. The supported formats being TIFF, GIF, JPEG, BMP, DICOM, FITS and "raw". For a surely objective evaluation, ImageJ allows for pixels evaluation in grey tones and has a memory for selected areas in pictures which can thus be tracked in time as well. Pixels measurements of the area, height and integral density have been performed along each wrinkles profile chosen. The skin quality of every subject participating to this assessment has been tested before the cosmetic treatment, and after it, in the short run (t = 2 hours) and in the long run (t = 40 days).

2.9. Statistical Analysis

Skin elasticity, hydration and wrinkles-profile have been analyzed through an ANOVA 1-way test, given the three different sampling times: t = 0, thus before treatment, and t = 2 hours, t = 40 days, after treatment.

Test will outcome: n.s non-significant if $p > 0.05$; * significant if $p < 0.05$.

3. Results

3.1. Skin Elasticity Parameter

The skin elasticity parameter evaluates the skin viscous-elastic properties as a response to an immediate deformation of the skin surface. Figure 1 and Figure 2, show the difference in elasticity for direct instantaneous evaluation after applying a negative pressure to t = 0, t = 2 hours and t = 40 days (Table 1 and Table 2).

Measurements show an increase in skin elasticity at short term (t = 2 hr) of 30.80% and 49.60% at long-term (t = 40 days). The p-value corresponding to the F-statistic of one-way ANOVA is lower than 0.05, suggesting that the treatments are significantly different.

Table 1. Skin elasticity measurements: Pre-treatment t = 0, short-term treatment t = 2 hr and long-term treatment t = 40 days.

SUBJECT	t = 0	t = 2 hr	t = 40 days
AR1	12	39	44
AR2	29	33	39
AR3	22	33	45
AR4	42	42	37
AR5	45	48	50
AR6	38	43	44
AR7	29	39	43
AR8	17	24	36
AR9	12	39	42
AR10	24	28	44
AR11	29	34	42
AR12	23	49	50
Media	28.75	37.41667	43

Table 2. Skin elasticity—One-way ANOVA—3 independent treatment.

source	sum of squares SS	degrees of freedom ν	mean square MS	F statistic	p-value
Treatment	1625.0556	2	812.5278	12.6802	8.2133e-05
Error	2114.5833	33	64.0783		
Total	3379.6389	35			

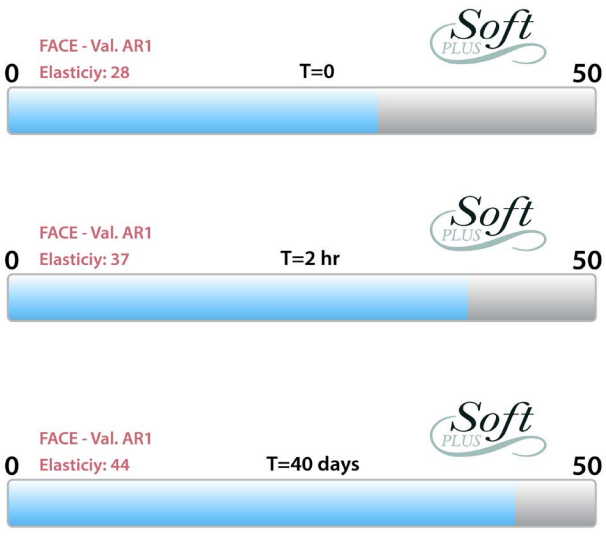


Figure 1. Typical representation of the elasticity parameter, generated by the probe, before and after treatment. The measure is expressed as arbitrary scale (0 to 50).

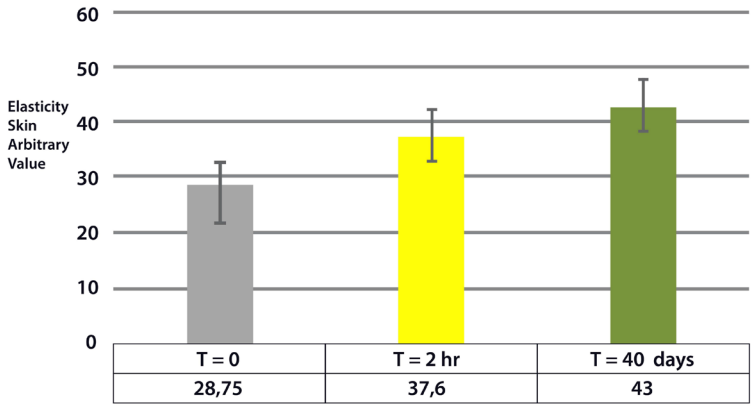


Figure 2. Histogram of skin elasticity (12 subjects): the parameter is significant t = 2 hours and t = 40 days.

3.2. Skin Hydration Parameter

The measurements about skin hydration have been carried out through a capacitor sensor able to monitor the dielectric constant of the skin and underlying skin layers. The latter measurements are directly proportional to the water content in the tissues under examination (**Figure 3** and **Figure 4**, **Table 3** and **Table 4**).

The measurement of the skin hydration parameter via capacitor sensor (skin and underlying tissues dielectric constant) shows an increase in facial skin hydration at short-term (t = 2 hr) of 30.67% and 68.70% at long-term (t = 40 day). The p-value corresponding to the F-statistic of one-way ANOVA is lower than 0.05, suggesting that the treatments are significantly different.

3.3. Anti-Wrinkles Efficacy Evaluation

For the objective evaluation of the images analyzed, Image J allows you to select the same skin portions, peculiarly, pixel measurements on the wrinkled profile in question: area, height, and integral density, which is equivalent to the average product of the mean for the areas (**Figures 5-7**, **Table 5** and **Table 6**).

Measurements show a decrease the wrinkle height at short-term (t = 2 hr) of 35.44% and 62.44% at long-term (40 day). The p-value corresponding to the F-statistic of one-way ANOVA is lower than 0.05, suggesting that the treatments are significantly different.

4. Discussion

Emulsion O/W care product were tested on 12 healthy subjects: 8 females and 4 males, with facial skin characterized by skin wrinkles. The efficacy of the cosmetic product has been evaluated 2 hours and 40 days after the treatment through non-invasive techniques whit significant results. In particular, the hydration and elasticity measures of the facial skin, increase respectively by 30.67% and 30.80% at short-term (2 hr) while at long-term (40 days) of 68.70% and 49.60%. In addition, the analysis of the rough profile showed a decrease in wrinkle height of 35.44% at short-term (2 hr) and 62.44% at long-term (40 days). The

Table 3. Skin Hydration measurements: Pre-treatment t = 0, short-term treatment t = 2 hr and long-term treatment t = 40 days.

SUBJECT	t = 0	t = 2 hr	t = 40 days
Val AR1	36	48	60
Val AR2	50	58	68
Val AR3	36	56	72
Val AR4	29	44	71
Val AR5	44	52	60
Val AR6	14	30	47
Val AR7	25	30	42
Val AR8	48	71	82
Val AR9	44	62	79
Val AR10	33	44	53
Val AR11	42	47	71
VAL AR 12	26	54	60
MEDIA	38,083	49,667	63,75

Table 4. Hydrating skin—One-way ANOVA—3 independent treatments.

Source	sum of squares SS	degrees of freedom vv	mean square MS	F statistic	p-value
Treatment	4760.1667	2	2380.0833	17.3697	7.02E-06
Error	4521.8333	33	137.0253		
Total	9282.0000	35			

Figure 3. Typical representation of the hydration parameter, generated by the probe, before and after treatment. The measure is expressed as arbitrary scale (0 to 100).

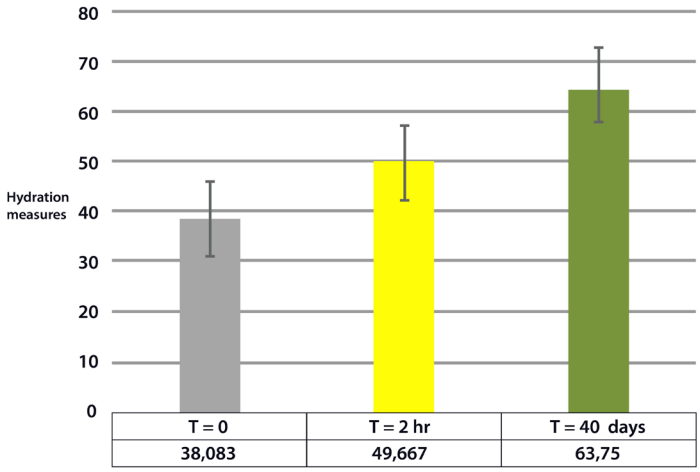


Figure 4. Histogram of hydration skin measures (12 subjects): the parameter is significant t = 2 hours and t = 40 days vs. t = 0, (p -value = 7.0243e-06).

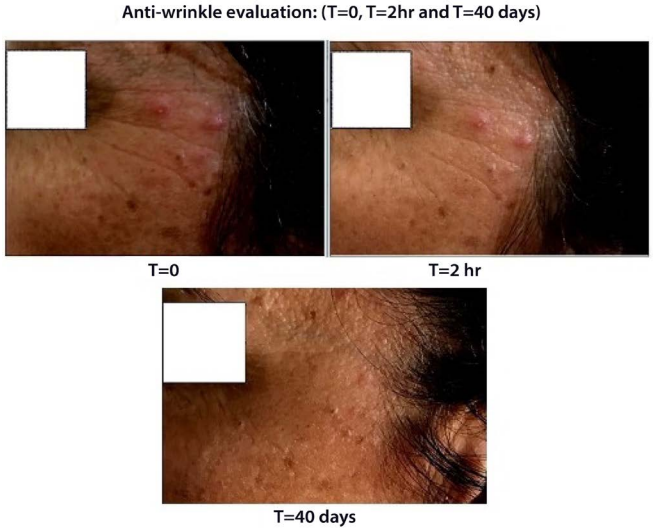
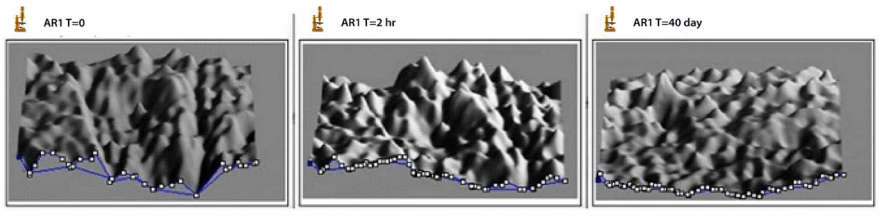


Figure 5. Periocular wrinkles: typical efficacy evaluation after 2 hr and 40 days of application.



Results

File	Edit	Font	Results			
	Label		Area	Mean	Height	IntDen
1	AR1 T=0.tif		1265	118.524	41	149933
2	AR1 T=2 hr.tif		525	114.122	31	59914
3	AR1 T=40 day.tif		385	101.699	22	39154

Figure 6. wrinkle profile t = 0, 2 hr, 40 days: measure (pixel) area, height, density integral: a typical trace.

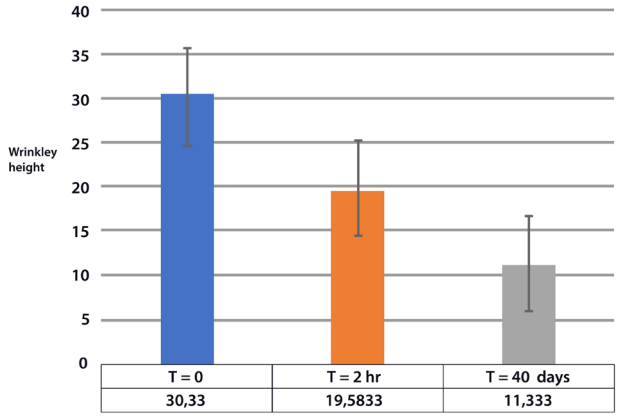


Figure 7. Histogram, parameter (Height). Average value of the wrinkle height (12 subjects). p-value = 6.7200E-07.

Table 5. Periocular wrinkles height measurements: Pre-treatment t = 0, short-term (T = 2 hr) and long-term (T = 40 day).

SUBJECT	T = 0	T = 2 hr	T = 40 days
AR1	41	31	22
AR2	34	28	12
AR3	29	18	11
AR4	54	38	14
AR5	28	17	12
AR6	31	21	11
AR7	29	16	10
AR8	22	13	9
AR9	28	17	8
AR10	30	19	14
AR11	27	16	10
AR12	22	12	8
MEDIA	30,333	19,583	11,333

Table 6. Decrease wrinkle height—One-way ANOVA—3 independent treatments (t = 0, t = 2hr, t = 40 day).

source	sum of squares SS	degrees of freedom νν	mean square MS	F statistic	p-value
treatment	2289.5000	2	1144.7500	22.5466	6.7200E-07
Error	1675.5000	33	50.7727		
Total	3965.0000	35			

result obtained in the short term indicates that the improvement is evident already two hours after the application of the emulsion is due to the presence of active serum donkey milk and snail secrete filtrate, also from the lipid fraction of

the emulsion, mainly consisting of sweet almond oil and shea butter. Shea butter has a high fraction of triglycerides and unsaponifiables. It is used in skin care for its emollient, protective and moisturizing properties [47] [48] [49] [50]. Almonds oil showed high efficacy in the treatment of dermatological pathologies such as psoriasis and eczema. Morover, thanks to its moisturizing properties, smoothes the skin and promotes cicatrization [51].

5. Conclusion

The tested product has proven to be effective and safe as each ingredient used in its formulation. Notably, the donkey milk serum promotes skin hydration and smoothening visibly contrasting skin wrinkles formation. The karité butter, thanks to its unsaponifiable components (tocopherols and catechin, triterpenes and sterols, Vanillin, Karitene and Allantolin) also displays hydrating, protecting, anti-inflammatory and anti-wrinkles properties. At last, the snail secretion promotes fibroblasts longevity and the assembly of the extracellular matrix thus minimizing the manifestations of skin ageing. Moreover, thanks to its unique and complex composition, the cosmetic product under investigation, favors the natural skin equilibrium as showed out from the testing over 12 volunteers that revealed a substantial increase in skin elasticity and hydration together with a sensible reduction of the skin-wrinkles.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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