Nano- and Micro-emulsions with Natural Oil Blend

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Abstract:

Targeted active delivery via micro- and nano-emulsion systems in cosmetic products allow for active ingredients to penetrate dermal layers and benefit the skin. From a physical chemistry perspective, nano-emulsions are kinetically stable systems containing droplets in the range of 10-1000 nm, whereas microemulsions are thermodynamically stable systems with various morphologies like oil in water, bi-continuous and water in oil, with dispersed phase domain sizes range from 5-100 nm. While emulsions are commonly employed in cosmetics, microemulsions are comparatively scarce, primarily due to the need for high concentrations of emulsifiers, lack of wide temperature stability and sensitivity to active ingredient incorporation.

Oil plays a significant role in cosmetics, providing hydration through occlusion, active delivery via skin lipids interaction and improving the skin barrier. Plant derived vegetable oils are commonly used, but due to their high viscosity (> 40 mPa.s), render products heavy/greasy, with low spreadability, skin absorption and phyto-active solubility. At Almora Botanica, we developed oil blends with desired physico-chemical characteristics, e.g., with low viscosity (2-5 mPa.s), high solubility of phyto-actives (>5% by wt.), sub-zero freezing points and high spreadability on skin. With appropriate emulsifiers, micro- and nano-emulsions made with our oil blends displayed wide temperature stability, and were developed into multiple skin care products.

Described in this paper are three products, i.e., Serum for Dark Spots & Pigmentation, Serum for Fine lines and Restorative Cuticle Oil that were tested for efficacy using a combination of clinical trials and *in vitro* studies. The results of these studies reinforced the fact that nano-and micro-emulsion systems are powerful vehicles for active delivery, and create efficacious skincare products.

Key Words: *Wrinkle-reduction; Fine-line reduction; Melanin reduction; Cuticle care; Skin elasticity; Hydration.*

1. Introduction:

Skin care, e.g., anti-ageing treatment or therapy is very well documented in Ayurveda [1]. Some of the related classical Ayurvedic concepts are, *Vayasthapana* (age defying), *Varnya* (brighten skin glow), *Sandhaniya* (cell regeneration), *Vranaropana* (healing), *Tvachya* (nurturing), *Shotahara* (anti-inflammatory), *Tvachagnivardhini* (strengthening skin metabolism) and *Tvagrasayan*a (retarding ageing). Many *rasayana* plants, such as *Emblica officinalis* (Amla) and *Centella Asiatica* (Gotukola) are very well known today in the Beauty and Wellness industry. Despite such advances in therapeutic plant extracts, beauty product development using these extracts still face challenges in terms of product efficacy (bioavailability of active molecules), product sensory as well as product stability [2, 3]. With informed consumers today looking for products that are natural, sustainable and efficacious, there is also an increasing demand for all product and benefit information to be transparently presented. To cater to these requirements, many brands and their products are

now certified by the Cosmetic and Organic Standard (COSMOS), a global presence in the Beauty industry [4].

Almora Botanica is a COSMOS-certified, natural range of specialty skin care products that combines the wisdom of Ayurveda with advanced research and clinical evidence. In the present paper, we will present our product development process, which combines knowledge of Ayurvedic ingredients with product microstructures. Overall, our products seek to provide consumer benefits, usually attributable to a cosmeceutical product [5].

Another facet of our work covered in this paper is the importance of oil(s) in cosmetic formulations. Oil is an essential ingredient in beauty products with diverse functions: improved skin feel; active delivery into dermal layers; improved hydration etc.. [6]. Besides, oil also acts as a delivery medium for oil-soluble phyto-actives. In Ayurveda, oil (*tailam*) plays a significant role in topical applications [7].

Most often, in beauty or cosmetic products, oil is present as a dispersed phase, usually in the form of an emulsion. Emulsion stability, therefore, is extremely important for product/formulation development. Microemulsions, Nano-emulsions are such dispersions, which provide stability and are linked to underlying physico-chemical parameters, such as oil-water interfacial tension, γ_{ow}; Oil molecular weight (Equivalent Alkane Carbon Number, EACN); Hydrophilic-Lipophilic Balance (HLB) of emulsifiers etc. Another important aspect of such a dispersed system is to deliver phyto-actives into dermal layers [8, 9, 10, 11].

In the current paper, we discuss about: (i) the development of natural oil blends, which are extremely light (bulk viscosity in the range of 2-5 mPa.s), have high spreadability and are capable of solubilizing phyto-actives even at lower temperatures; (ii) Micro- and nanoemulsion formation with the oil blends and (iii) Biological efficacy of such dispersed systems.

2. Materials & Methods:

Oil Blend

Seven oils were considered for various oil blend preparations. These oils are, $C_{15\text{-}19}$ alkane (Emogreen, Seppic), Undecane & Tridecane (Cetiol Ultimate, BASF), Cetyl Ricinoleate (Tegosoft CR, Evonik), Caprylic/Capric triglycerides (Myritol, BASF), Coco caprylate (Cetiol C5, BASF), Dicaprylyl carbonate (Cetiol CC, BASF) and Isoamyl cocoate (Tegosoft AC, Evonik). These oils were used as received from respective suppliers.

Emulsifiers

Two emulsifiers were considered for our study: Polyglyceryl-10 Laurate (Syneth L15 K RSPO MB, Lonza) and Polyglyceryl -10 Oleate (Syneth O13 K RSPO MB, Lonza), and were used as received.

Phyto-actives

A list of phyto-actives used in our studies is given here: *Marrubium Vulgare* Extract (Citystem, CRODA/Sederma), *Centella Asiatica* Leaf Extract (Taladvance, Seppic), *Mangifera Indica* Fruit Extract (Mangoeco, Provital), *Lavandula* (Lavender) *Angustifolia* Oil (Statfold), *Morus Alba* Root Extract (Cosme-Phytami, Alban Muller), *Glycyrrhiza Glabra* (Licorice) Root Extract (Licorice Eco, Provital), Tocopherol & Tocotrienols (Davos Life E3 DVL95, Davos Life Science), *Cystoseira Tamariscifolia* Extract (Rainbow Algae, Cywhite, CODIF), *Apium Graveolens* (Celery) Seed Extract, (Neonyca MBAL, Croda) and *Melia Azadirachta* (Neem) Leaf Extract, Neem Leaf Liquid G, Ichimaru Pharcos).

Excipients

The list of ingredients, traditionally considered as excipients, used in our study, is listed here: Glycerin (vegetable glycerin, Univar), Citric Acid, Linalool, Elaeis Guineensis Oil, Glycine Soja Oil and Helianthus Annuus Seed Oil. Sodium Benzoate and Potassium Sorbate (Euxyl K 712, Schulke & Mayr) were used as preservatives.

Cell Models

EpiSkin Reconstructed Human Pigmented Epidermis (EpiSkin, France)

EpiSkin's Reconstructed Human Pigmented Epidermis (RHPE) is a cell model that represents three different phototypes of human skin, by cultivating normal human keratinocytes in the presence of phototype II, IV or VI melanocytes. The melanocytes are localised in the basal layer, wherein basal keratinocytes are also scattered. The RHPE model has various applications, including Pigmentation and Depigmentation, the main focuses of this study [12].

T-skin/Reconstructed Human Full Thickness Model (EpiSkin, France)

EpiSkin's T-skin, also known as Reconstructed Human Full Thickness Model, consists of human fibroblasts and keratinocytes cultured on an inert polycarbonate filter. T-skin represents a well differentiated epidermis of human skin, upon which markers of dermal cell differentiation, dermal-epidermal junction formation and maintenance, as well as proliferation can be analysed [13].

Experimental Methods:

Viscosity

Viscosities of all oil blends as well as products were measured by using a stress-controlled Rheometer (Anton Paar MCR 92) by doing a shear rate scan from 0.1 to 100 s^{-1}, with 50

data points and the time interval between data points was kept at 6 s. The total time elapsed for the measurement was 300 s. The viscosity values, discussed in the report, correspond to the shear rate of ~ 21 s^{-1}.

Spreadability

The spreading of individual oils as well as blends were measured by using ashless Whatman filter papers (medium-fast retention). A 20 µL sample of the composition was taken and added to the centre of the filter paper. After 10 minutes, the area within which the oil spread was measured. Typically, the composition spreads as a circle and its diameter is measured to obtain the area of the circle [14].

Solubilization of phyto-actives in oil

The active(s) were solubilised in our oil blend at an elevated temperature (45°C) by a hot plate cum magnetic stirrer to ensure a complete solubilisation and equilibrated overnight at 45°C using a stability chamber oven. The composition was then cooled down at a given rate (3.5℃/min) and the active precipitation was detected by measuring the storage and loss moduli at 1 Hz frequency using an Anton Paar MCR 92 rheometer. The following settings were used in the RheoCompass software: 0.1 % strain, No. of datapoints:100, Time interval between data points: 6 secs, Total time: 600 secs, Starting Temperature: 30 ℃. The precipitation temperature of the active was determined by noting the temperature at which storage modulus becomes larger than the loss modulus. This precipitation temperature is a direct measure of the temperature at which phase separation of the active(s) was observed. Higher temperature of phase separation of the active from oil was indicative of lower composition stability, while lower temperature phase separation indicated higher stability.

Microemulsion Phase Behaviour

Phase behaviour of micro-emulsions was studied by preparing cosmetic compositions and equilibrated at a range of temperatures. Cosmetic compositions (20 g) were dispensed into transparent, graduated Tarson tubes (Polyethylene made) and placed in a stability chamber (T= 30℃ and 45℃) for 24 hours. The stability chambers were maintained at temperatures with a SD of ± 1 ºC. Relative humidity of the stability chambers was maintained at 75 ± 1%. After 24 h, the samples were removed from the stability chamber to check for their visual appearance. Cloudy appearance is denoted by an opaque milky sample. In such a case, the graduations on the centrifuge tube cannot be seen when held against a light source (incandescent light, 40 W) and viewed from the opposite side of the tube. Slightly Cloudy appearance is denoted by a translucent brownish yellow sample. In such a case, the graduations on the centrifuge tube appear hazy when held against light and viewed from the opposite side of the tube. Clear appearance is denoted by a transparent sample. In such a case, the graduations on the centrifuge tube appear distinct when held against the light and viewed from the opposite side of the tube. The same process was followed to observe samples cooled overnight in a refrigerator at 6 ºC. For repeat measurements, the samples were shaken by hand before equilibrating at respective temperatures. The observations of

stability of the cosmetic composition are recorded using the following keywords: ‘Clear’, ‘Cloudy’ and ‘Phase Separated’.

Melanin Reduction Studies

EpiSkin’s RHPE (human live skin 3-D equivalent) was used to evaluate Almora Botanica’s Serum for Dark Spots & Pigmentation for melanin reduction. Non-GLP EpiSkin 12-well plates with human skin equivalent tissue inserts were used for experiments. All formulations, including controls, were applied on top of the 3D tissue inserts. Microbe-free test formulations were provided by Almora Botanica, which were tested against positive (Kojic acid) and negative (untreated and placebo) controls. Kojic acid was used as a positive control due to its well studied melanin reduction activity [15]. Untreated inserts had nothing applied to them, whereas placebo control inserts were treated with the serum’s base formulation, without any active ingredients.

Test formulations were applied once every two days (48 hrs) and following the third application, melanin was extracted from each tissue insert. Melanin content was observed using optical density at 490 nm, using BioTek’s Multiwell Plate Reader. Cellular cytotoxicity was also monitored using Invitrogen Thermofisher’s CyQUANT LDH Cytotoxicity Assay kit throughout the experiment, using 50 µL of spent media from each insert well. The study was carried out at Reagene Biosciences Pvt. Ltd., BioNEST, School of Life Sciences, University of Hyderabad, Telangana, India.

Gene Expression Studies for the Serum for Fine lines

EpiSkin’s T-skin (Full Thickness Model) was used to evaluate dermal gene activity after application of the Serum for Fine Lines. Non-GLP T-skin 6-well plates with inserts were used in conjunction with nylon meshes for testing formulations. Test formulations, including controls, were applied onto the nylon meshes, and placed on top of the inserts. Microbe-free test formulations were provided by Almora Botanica, which were tested against positive (Retinol) and negative (untreated and placebo) controls. Retinol was used due to its wide recognition, through literature review, for Anti-aging benefits in the skin [16]. Untreated inserts had nothing applied to them, whereas placebo control inserts were treated with Serum for Fine Lines, without any active ingredients.

Test formulations were applied once every two days (48 hrs), and 50 uL of media was tested for cytotoxicity using Invitrogen ThermoFisher’s CyQUANT LDH Cytotoxicity Assay kit. Pictures were taken every two days as well. Following the third application, nylon meshes were removed, and the inserts were gently washed with 1X cell culture PBS (Phosphate Buffered Saline), to remove any test formulation and debris stuck to the tissue insert surface. PBS was also washed off of the inserts as much as possible, before further experiments were done. Cleaned tissues were cut along the edges and used for RNA extraction.

Cells taken from the tissue inserts were lysed and RNA extraction was carried out using a QIAGEN kit. Following RNA extraction, an Agilent 2100 BioAnalyzer Lab-on-a-chip was

used to determine quantities of purified RNA. Following RNA quantification, samples were prepared for Microarray and qRTPCR studies. Microarray chips covered expression of over 1000s of genes, which were narrowed down to 50 potential genes of interest given their significance to skin health. Microarray assay used Agilent's Human genome wide coverage 8X60k microarray chips, as well as single colour hybridisation. Out of those 50 genes of interest in the microarray assay, eight genes were selected for qRTPCR analysis, Elastin 1 (ELN1), Matrix metalloproteinase (MMP1), Survivin (BRIC5), Epidermal growth factor receptor (EGFR), Peroredocin 4 (PRDX4), GLB1 and housekeeping genes Actin and GAPDH. Primers were designed by Tranalab and purchased via an external supplier.

Dermal colorimetric analysis, skin texture and wrinkle formation

For the Serum for Fine Lines, dermal colorimetric analysis, skin texture and wrinkle formation were studied by Antera 3D. Utilizing multi-directional illumination and digital skin surface reconstruction, the camera is able to light the skin surface from different angles and use multiple images to reconstruct a 3D skin view. Spectral and Spatial analysis allow for comprehensive skin topography and colorimetry assessment. Multi-spectral analysis further enables melanin mapping, along with precise colorimetric analysis for pigmentation, brown spots and skin phototype measurements [17]. The Antera 3D was used on volunteers' face, neck and hand areas.

Dermal elasticity and firmness

The Cutometer MPA 580 was used to analyse dermal elasticity and firmness after application of the Serum for Fine Lines. This instrument measures elasticity of skin through negative pressure suction. Deforming the skin mechanically, the skin's upper layers are drawn into the probe for a defined time, and then released. Skin penetration depth is measured by a non-contact optical system, consisting of a light source and receptor, with penetration depth varying with light intensity. Firmness (resistance of skin to negative pressure) and elasticity (ability of skin to return to original position) of skin are displayed in terms of penetration depth (mm/time) curves. The curves can be used to calculate R, F and Q parameters of the skin, which explain quantitatively the levels of elasticity, tiring effects, firmness and recovery, etc. details about the skin area of interest [18].

Dermal imaging

Imaging of volunteers' fine lines was carried out using the VISIA CR system. A comprehensive facial imaging tool, the VISIA CR system allows for seven different illumination modes for analysing various skin characteristics, including pigmentation, lesions, acne, fine lines and wrinkles, brightness, evenness and skin tone. The tool produces high-resolution images with rapid capture times, along with multiple preset lighting modes for capturing images of volunteers' in multiple lighting modes, such as Blue, UVA, etc. [19].

Onychoscopy

For analysing nail health improvement post-application of the Restorative Cuticle oil, DermaLab Combo's Video scope was used. The video scope was able to observe and record images of the nail and skin around the nail regions. A non-invasive method of observing nail health and changes to the nail plate and bed, Video Dermoscopy (VD), is a comprehensive tool to conduct onychoscopy, or dermoscopy of nails. Onychoscopy observes nail health parameters such as cornification, nail cohesion, cuticles, lateral nail folds and any signs of breakage and splitting. The instrument combines Non Polarising and Polarising light, enabling analysis of surface anatomy, as well as pigmentation and vascularity of the nails and surrounding areas [20].

Clinical Evaluation of Serum for Fine lines

The Serum for Fine Lines clinical trial was conducted at MS Clinicals (Bangalore, Karnataka, India) for a period of 12 weeks (85 days) from January - April 2021. Subjects were 38 healthy females aged 35-60 (inclusive), with Fitzpatrick skin type III-V. All volunteers were asked to refrain from the usage of anti-aging and moisturising products 2-4 weeks prior to starting of the clinical study. The Serum for FIne Lines was used twice daily (morning/night) by all volunteers, with instrumental and dermatological assessments carried out on Days 1, 15, 29 and 85 (4 visits to study centre).

Clinical Evaluation of Restorative Cuticle Oil

The Restorative Cuticle Oil clinical trial was conducted at Radiant Research Services Pvt Ltd (Bangalore, Karnataka, India) for a period of 45 days, during January - February 2021. Study subjects were 30 healthy Indian males and females aged 30-60 (inclusive), with Fitzpatrick skin type III-V. All volunteers were instructed to avoid using their own lotions, nail polish, nail lacquers, nail polish remover and nail oils/cream for the total 45 days duration of the study. Volunteers were asked to apply the Restorative Cuticle Oil once daily in the afternoon, on clean hands, nails and cuticle areas.

Subject feedback was assessed by subjective questionnaires and examination of areas of interest (nail and skin around nails) by Onychoscopy and video dermoscopy. Onychoscopy and video dermoscopy were conducted on Days 0, 21 and 45. Descriptive statistics was used to demonstrate subject feedback on reduction of dryness, splitting and fragility, as well as improvement in cuticle appearance, nail strength, smoothness, thickness and nourishment.

3. Results and Discussion:

Individual oils were chosen based on their literature viscosity value, Hansen solubility parameter values and their freezing point. Spreading parameters were not available for all of them. Among the chosen oils, $C_{15\text{-}19}$ alkane as well as decane & undecane ($C_{9\text{-}13}$ alkane) have similar solubility parameters, i.e., only the dispersion component $ó_D$, being 16.1. The electrical permittivity value is like that reported for hydrocarbons ($\varepsilon_r = 2$). The most polar

oil in the chosen group was caprylic capric triglyceride (CCTG), with $\varepsilon_r \sim 5$. Its solubility parameter values are, $ó_D = 18.2$, $ó_P = 5.4$ and $ó_H = 14.7$. The highest and lowest viscosity in the chosen group of oils was that of Cetyl Ricinoleate (47 mPa.S at 30 °C) and $C_{9\text{-}13}$ alkane (1 mPa.S at 25 °C) respectively.

Oil blends were prepared to create solubility parameter values with all the three contributions, i.e., dispersion, polar and H-bond. As an example, Oil blend A (Ref. Table 1) has $ó_D = 16.5$, $ó_P = 1.4$ and $ó_H = 1.8$. Table 1 provides the composition of three oil blends with their viscosity, average spreading coefficient and solidification temperature.

Table 1: Oil Blend compositions A, B and C

Oil	Oil Blend A	Oil Blend B	Oil Blend C
C 15-19 Alkane	30	35	70
C 9-13 Alkane	20	20	20
Cetyl Ricinoleate	5	0	10
Capric Caprylic Triglyceride	20	20	0
Coco Caprylate	10	10	0
Di-capyrolyl Carbonate	10	10	0
Isoamyl Cocoate	5	5	0

Table 2: Physical parameters of Oil Blends A, B and C. Phyto-active used for solidification point measurement was Bidens Pilosa extract (Revinage, Chemyunion) [21]. Bidens Pilosa Extract has a melting point of ~ 68 °C. The Spreading coefficient measurement was carried out at ambient conditions (~ 25 °C), whereas viscosity measurements were carried out at 30 °C

Oil Blend	Viscosity at 21 s^{-1}	Spreading Coefficient (mm^2/10 min)	Solidification of phyto-active (10% by wt.) ℃
A	5	531.2	4
B	5.1	573.8	-2
C	2.5	675.6	2.5

As may be seen from Tables 1 & 2, all oil blends demonstrated low viscosity values and spreading coefficient values better than respective values exhibited by individual oils [12]. For individual oils, the highest and lowest spreading coefficient (692 mm^2/10 min and 202 mm^2/10 min) was for Cococaprylate and Cetyl Ricinoleate respectively. The solidification temperature of Oil Blend A was observed to be 4 °C . Upon solubilisation of a phyto-active, Bidens Pilosa extract (Revinage, Chemyunion) with a melting point of ~ 68 °C , into our oil blend, no change to the solidification temperature was observed, indicating efficient solubilisation. As seen in Table 2, the solubility of the phyto-active in all oil blends was good without any precipitation at ambient conditions.

For all oil blends, microemulsion phase behaviour was explored along with chosen emulsifiers, Polyglyceryl-10-laurate and Polyglyceryl-10-oleate. Detailed description regarding emulsifier choice and microemulsion details can be accessed in a patent filed by Almora Botanica [22, 23]. Microemulsion phase behaviour was studied with all the oil blends. Figure 1 presents stability data of microemulsion compositions at three different temperatures: 6°C, 30°C and 45°C, where emulsifier concentration was varied. It may be seen that microemulsion formation occurs over a range of C_{12} G_{10} weight fractions, from 0.2 to 0.6. The microemulsion phase was monitored when oil:water = 1:1 by wt. composition, it may be said that the emulsifier compositions in Figure 1 are the most efficient for microemulsion formation. Usually, microemulsions with such wide temperature stability are uncommon and many approaches in the past have been made to achieve the same. Some of the approaches tried in the past made use of emulsifier mixtures, oil mixtures, and their combinations[10]. Commercial systems exhibiting microemulsion phases over a wide temperature range are uncommon.

Microemulsion system and Nail Benefits of Restorative Cuticle Oil:

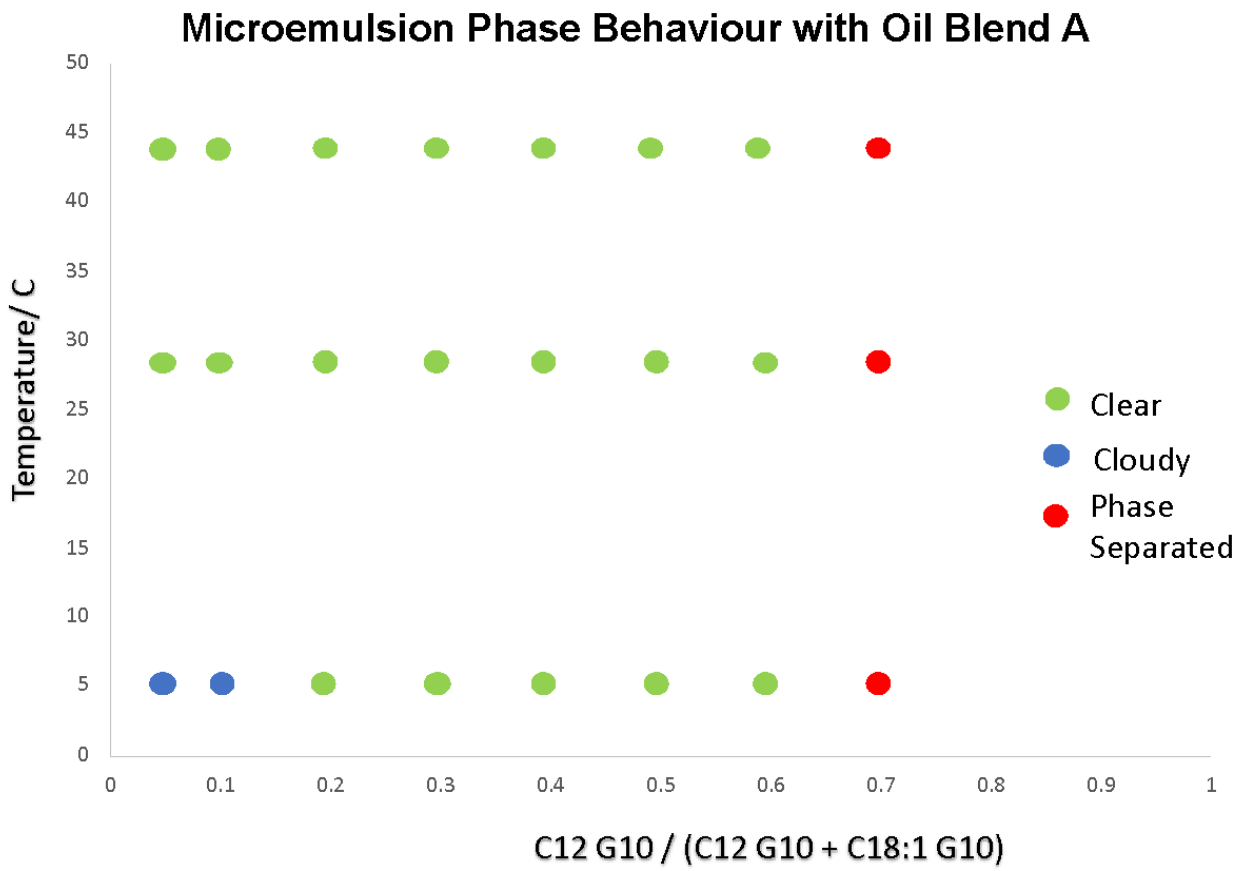

Figure 1. Microemulsion phase behaviour of Oil Blend A+ Emulsifiers + Water. Emulsifiers used were polyglyceryl-10-laurate (C_{12} G_{10}) and Polyglyceryl-10-Oleate ($C_{18:1}G_{10}$). The emulsifier concentration in the microemulsion was 28% by wt. The water: oil blend wt. ratio was 1:1.

We also studied microemulsion phase behaviour with oil blends B and C at 6°C, 30°C and 45°C. Oil blend B showed microemulsion phase behaviour with C_{12} G_{10} wt. fr. of 0.4, whereas with oil blend C, microemulsion phases could be observed with C_{12} G_{10} wt. fr. of 0.4 and 0.5. The observed differences in the microemulsion phase behaviour were attributed mostly due to the physico-chemical nature of the oil blend. In the present case, Oil blend A, containing seven oils, was the best oil blend, capable of demonstrating microemulsion phase behaviour over a wide composition range of the emulsifiers.

The above microemulsion system was the basis for the development of our Restorative Cuticle Oil. The objective of the cuticle oil was to have a look and feel of oil, but contain a significant aqueous phase for water-soluble Neem leaf extract. The presence of aqueous and oil phases in a single product ensures the penetration of actives into the nail plate and cuticles [24]. Structurally a bi-continuous phase, the product's high viscosity (500-800 mPa.s) allows for easier application.

Figure 2 shows a key results of the clinical study conducted with the Restorative Cuticle Oil, containing *Apium Graveolens* (Celery) Seed Extract, (Neonyca MBAL, Croda), *Melia*

Azadirachta (Neem) Leaf Extract, Neem Leaf Liquid G, Ichimaru Pharcos), along with oil blend A and 28% by wt emulsifiers (C_{12} G_{10} wt. fr. of 0.5) .

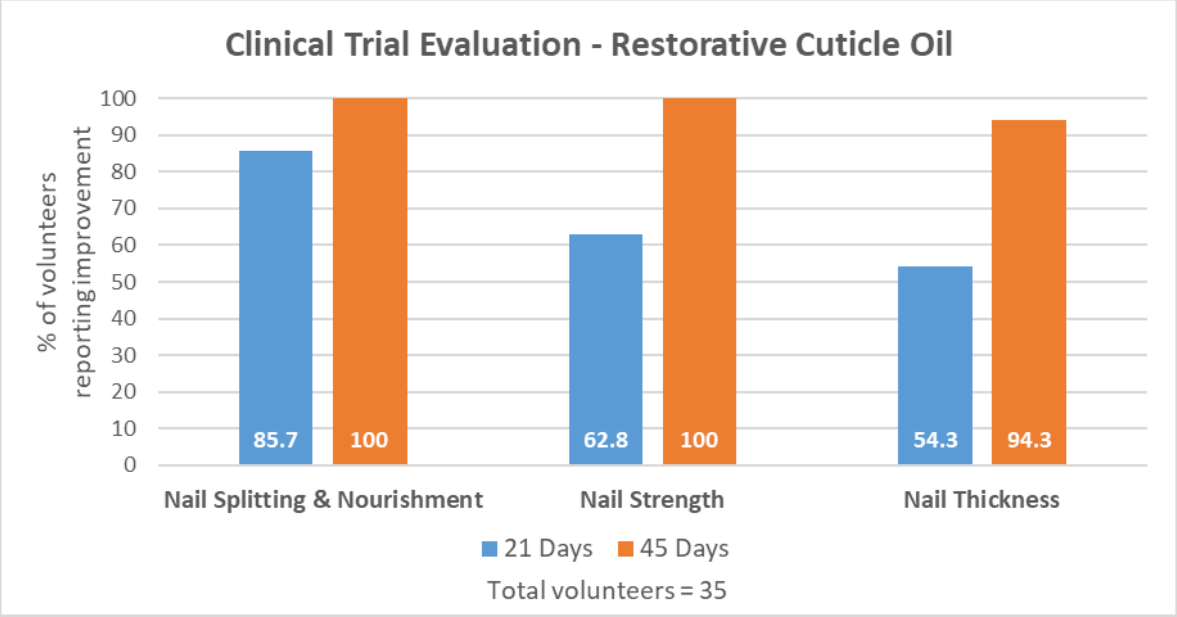

Figure 2. Improvement in various nail health attributes (Nail splitting, Nail strength and Nail thickness), as indicated by consumers after 21 and 45 days.

As seen in Figure 2, after 45 days of daily product application (once a day), 100% of volunteers perceived a reduction in nail splitting and an improvement in nail nourishment and strength. Similar perception by volunteers concerning nail thickness, with 94.3% of volunteers citing improvement. These results indicate the clear benefits perceived by consumers even after 3 weeks of application, continuing to 6 weeks.

Melanin reduction by Serum for Dark Spots & Pigmentation:

In addition to microemulsion formation, it was also found that for oil blend A and C_{12} G_{10} wt. fr. of 0.5 ($C_{12}G_{10}$: $C_{18:1}G_{10}$ = 1:1), nano emulsions form at low emulsifier concentrations (as low as 1% by wt.). These nano-emulsions exhibit oil droplets with diameters in the range of 30-70 nm, as determined by dynamic light scattering (DLS) measurement. Serum of Dark Spot & Pigmentation has the following actives: *Morus Alba* Root Extract (Cosme-Phytami, Alban Muller), *Glycyrrhiza Glabra* (Licorice, *Yashtimadhu in Ayurveda*) Root Extract (Licorice Eco, Provital), Rainbow Algae (*Cystoseira Tamariscifolia)* extract (Cywhite, CODIF) and Mango (*Mangifera Indica*) fruit extract.

Figure 3 shows the melanin content in Episkin skin models after nine days of application.

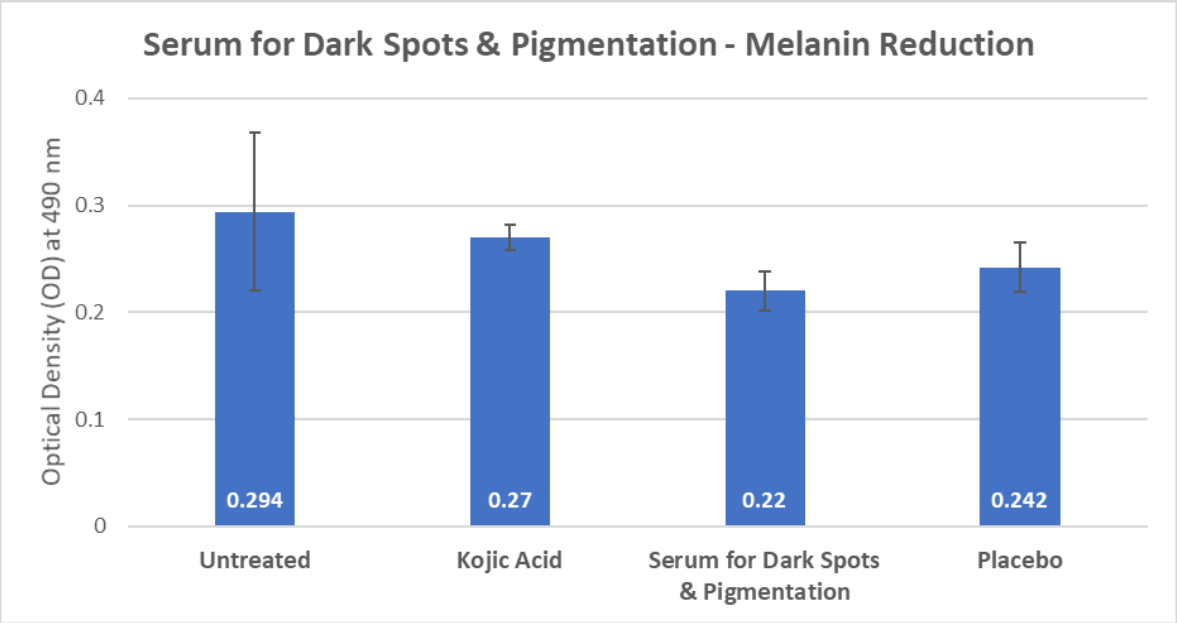

Figure 3. Serum samples' optical density readings at 490 nm, directly correlate with the melanin content. Kojic acid was used as a positive control. Untreated and Placebo treated samples were used as negative controls.

As displayed in Figure 3, as compared to untreated tissue inserts, the serum applied wells showed a 25% reduction in melanin content after 9 days. It may also be noticed that Kojic acid alone causes a 9% reduction in melanin, whereas the placebo, base serum formulation without active ingredients, causes a 17% reduction in melanin. One of the possible reasons for the placebo to be performing is the presence of tocotrienols and tocopherols at 0.1% in the placebo, which are potent antioxidants that provide stability to phyto-actives present. With the antioxidants being able to penetrate deep into dermal layers by being in a nanoemulsion format, it is likely that the vitamin E sources in the base formulation aid in Melanin reduction significantly. This significant benefit observed across the Serum and placebo formats could be achieved following a strategy of creating a nanoemulsion with actives partitioning into the oil phase as well into the continuous aqueous phase. The water-soluble actives interact with melanin in the keratinocytes, whereas the oil-soluble actives affect the melanosomes in the melanocyte. Possible mechanisms based on the data provided by the raw material supplier (CODIF), melanin reduction could occur in several ways: tyrosinase inhibition; slowing melanin transport as well as degradation of melanin in the keratinocyte [25].

Gene Expression studies with Serum for Fine Lines:

Several genes with functions relating to anti-ageing, inflammation, DNA damage, collagen breakdown, etc., were analysed for changes in expression post application of the Serum for Fine Lines in T-skin models. Negative controls were untreated tissue inserts, whereas Retinol was used as a positive control.

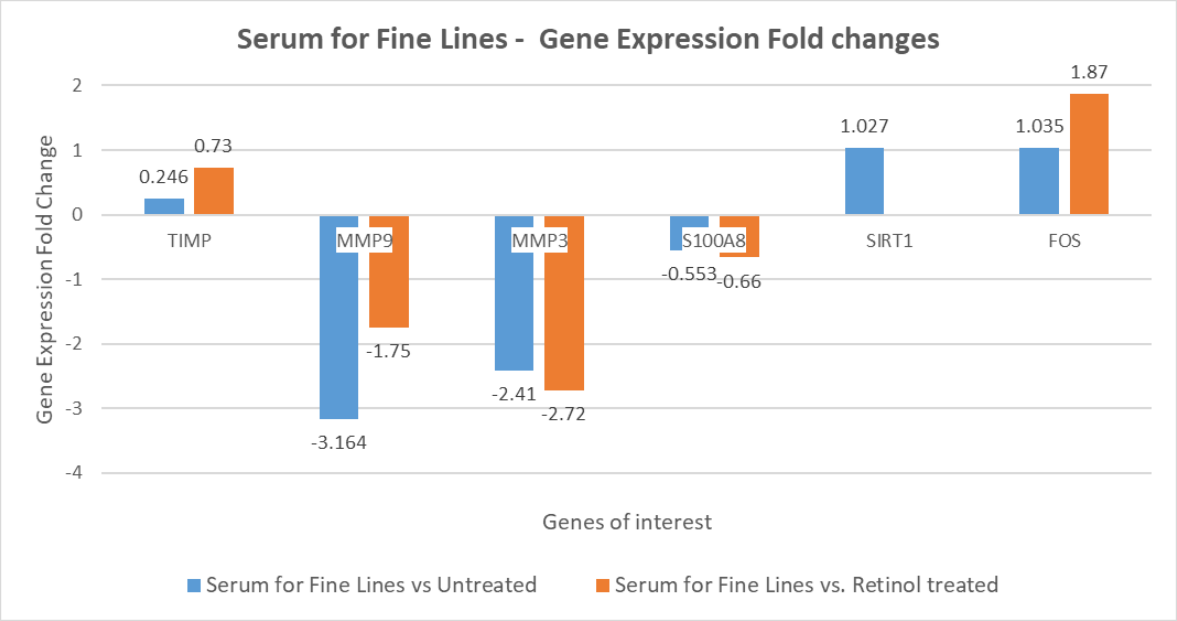

Figure 4. Gene regulation changes observed in T-skin models after treatment of Serum for fine lines. Retinol was used as a positive control.

Results shown in Figure 4 correspond to common gene expression results observed in a Microarray experiment comparing pools of data across Untreated and Retinol compared Serum samples. Fold changes indicate transcription level changes in gene expression activity, which correlates to the amount of downstream protein produced. We considered ±0.25 fold changes to be significant.

Tissue inhibitor of metalloproteinase-1 (TIMP-1) is one representative of the natural metallomatrix proteinase (MMP) inhibitor family. Its expression is decreased with fibroblast senescence, both ex vivo and in vivo, thus contributing to increased catabolic activity within the dermis. On the other hand, its upregulation arrests fibroblast senescence, thus strengthening the extracellular matrix (ECM). Besides, it exhibits keratinocyte and fibroblast growth factor-like activity and has been described as a cell survival factor [TIMP1.1] [26, 27]. As can be seen from Figure 4, after treatment with Serum for Fine Lines, TIMP1 is seen to be upregulated, when compared with untreated samples, as well as the one treated with Retinol.

MMPs are secreted as inactive pro-proteins which are activated when cleaved by extracellular proteinases. MMPs primarily function as collagenases, and also have a significant role in skin diseases [28, 29, 30, 31]. In Figure 4, Microarray results indicate that MMP9 and MMP3 activity were significantly downregulated with application of our Serum product. MMP9 degrades various substrates including gelatin, collagen types IV, V and elastin. Thus its upregulation indicates breakdown of the ECM in a normal physiological process like ageing. Down-regulation thus indicates inhibition of the enzyme and delayed breakdown of the ECM, thus slowing down the ageing process considerably. MMP3 degrades fibronectin, laminin, collagens III, IV, IX, X and cartilage proteoglycans

[11, 32]. Our results show that MMP3 activity was decreased by 2.41 fold as compared to Untreated samples, and by 2.72 fold as compared to Retinol.

During inflammation in the skin, S100A8 is released actively and exerts a critical role in modulating the inflammatory response by stimulating leukocyte recruitment and inducing cytokine secretion. It serves as an indicator of therapeutic responses to inflammation-associated disorders or events. Down-regulation of S100A8 indicates the benefits related to anti-inflammation and therapeutic skin benefits [33, 34]. Figure 4 shows down-regulation by 0.5 fold indicating the strong therapeutic effect of Serum for Fine Lines on human skin.

Silent Information Regulator Gene (SIRT1) expression is linked to cell longevity. It belongs to a family of enzymes implicated in gene-silencing, apoptosis, fatty acid metabolism, and regulation of cellular life spans of organisms. It is also associated with genes that coordinate and optimize the function of cells as cells smuggle to survive in a stressful environment, as it is the case for skin cells. [34, 35]. Figure 4 shows a 1.027 fold upregulation after the product application, when compared to untreated samples.

Lastly, the FOS gene is critical for cellular proliferation and differentiation, and is key in encoding downstream proteins that regulate cellular apoptosis [36]. Figure 4 displays upregulation of the FOS gene, a +1.035 fold change compared to untreated samples, and +1.87 compared to Retinol treated samples.

Clinical studies with Serum for Fine Lines:

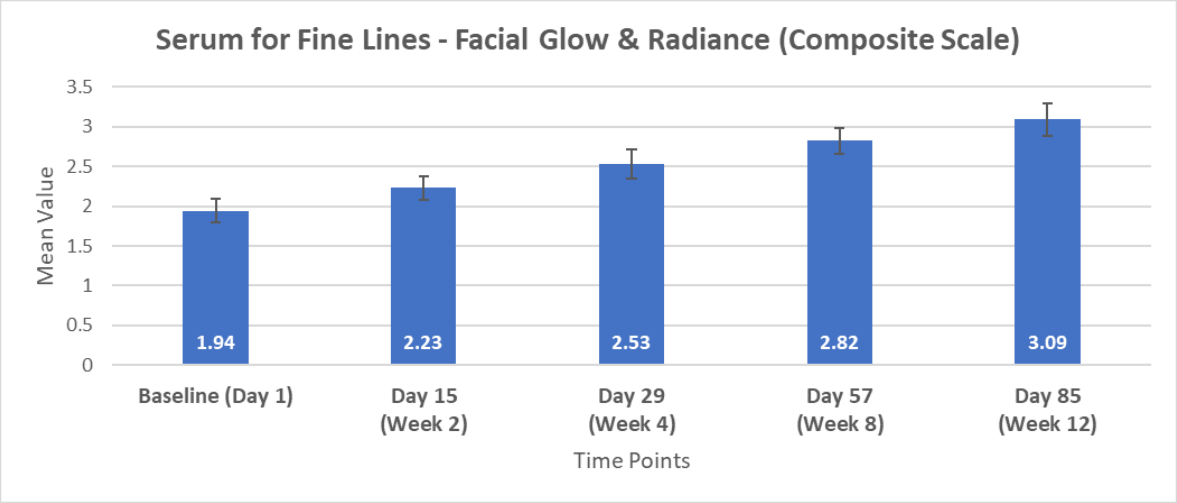

Figure 5. Improvement of Facial Glow and Radiance with usage of Almora's Serum for Fine Lines (12 week study)

A composite parameter, Facial Glow and Radiance defined as:

$$(0.1 * hydration) + (0.2 * texture) + (0.3 * clarity) + (0.3 * evenness) + (0.1 * colour), where$$

the individual parameters are mapped on a scale of 1 to 5. Hydration is defined with 1 as 'dry' and 5, as 'moisturized'. Skin texture is defined by skin smoothness in a sensorial evaluation with 1, as 'rough' and 5, as 'smooth'. Clarity is defined in terms of reflectance/luminance by visual assessment with 1, as 'unclear' and 5, as 'clear'. Evenness of skin tone is defined in terms of color variation across the test area, with 1, as 'uneven tone' and 5, as 'eventone'. Skin color is defined in terms of background color with 1, as 'very dark' and 5, as 'Fair'.

There was a 59.3% increase observed in the mean values of facial glow and radiance on face at all time points when compared to the baseline, implying that the test serum was efficacious in providing a significant improvement in skin glow and radiance after 3 months of regular use.

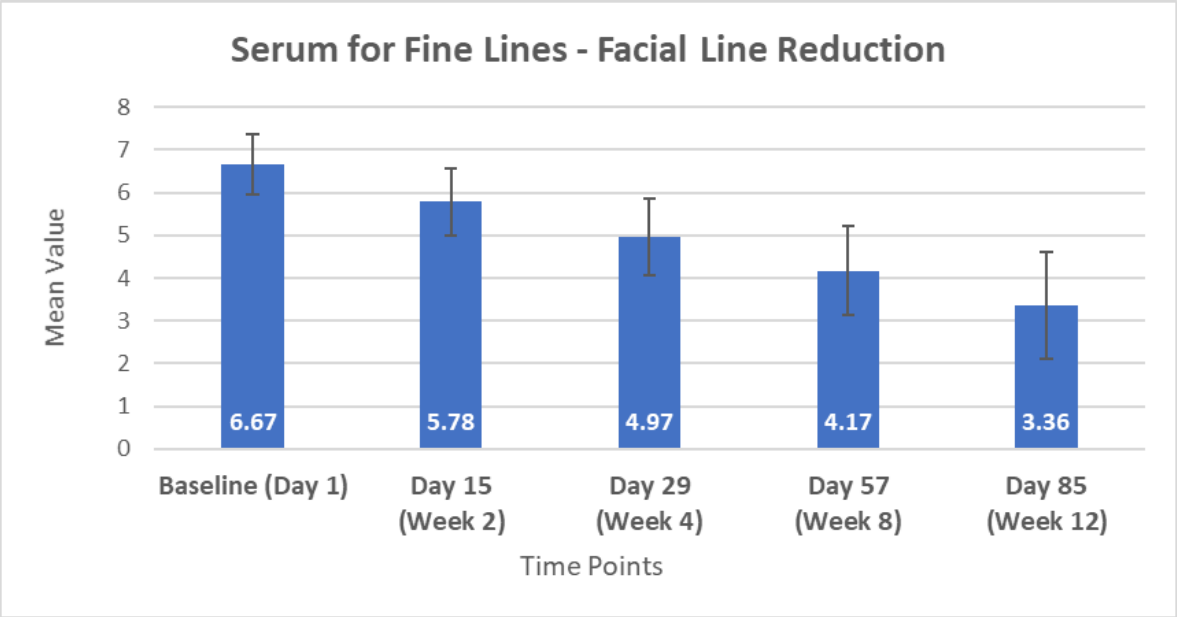

Figure 6: Reduction of facial fine lines after application of Serum for Fine lines (12 week study)

Danielle's 10 point scale of 0-9 was followed to assess fine lines on the face, the details of which are described in the Materials and Methods section. It may be noticed that a significant reduction in fine lines was observed after 2 weeks of application. At the end of 12 weeks, fine line density was reduced by 33% from baseline. After two weeks of regular application, a reduction of 10% was observed.

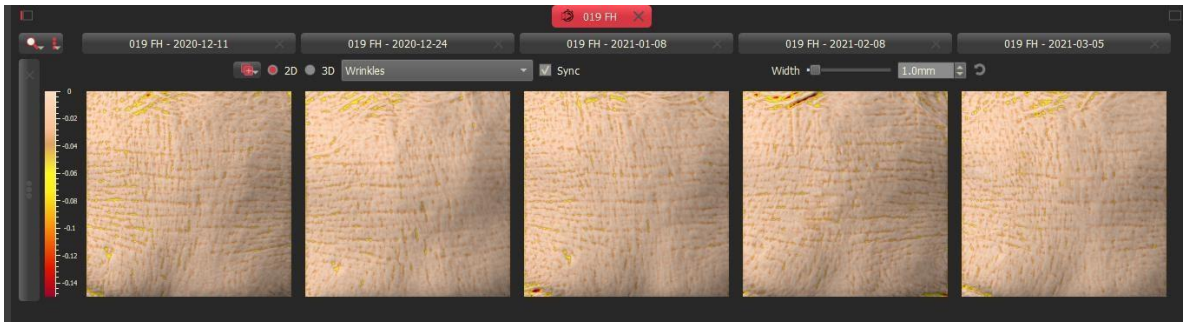

Figure 7. Forehead images taken by Antera 3D, displaying fine line images indicating fine reduction at different time points: 0, 2, 4, 8 and 12 weeks for one volunteer.

The forehead images depicting fine lines were recorded for a given consumer at different time points.

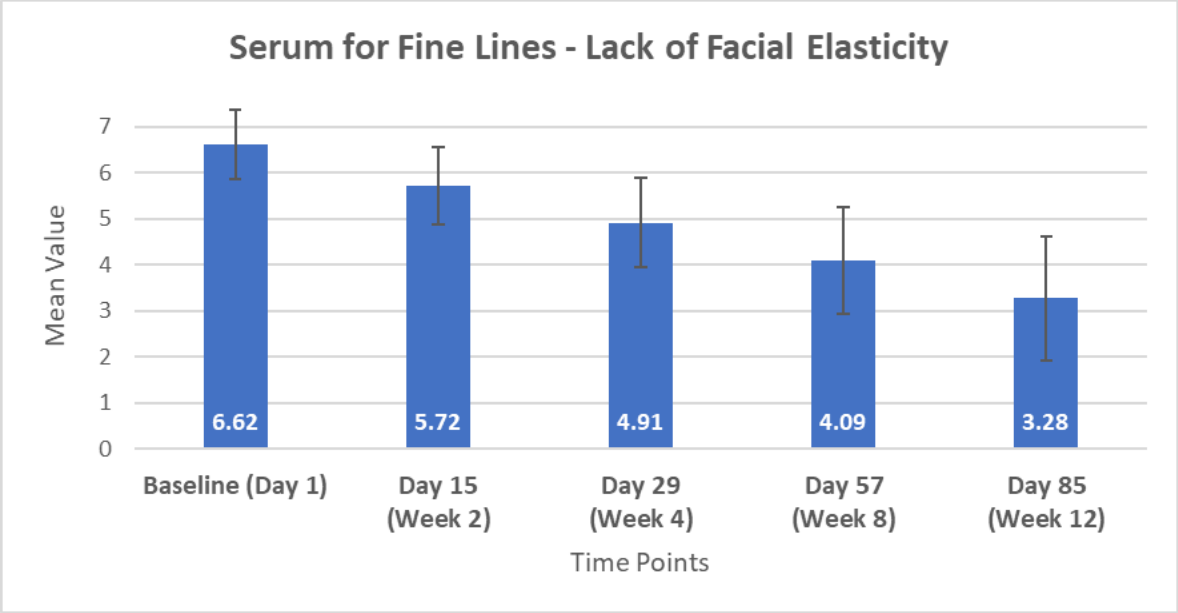

Figure 8: Improvement in the Skin Elasticity after application of Serum for Fine Lines (12 week study).

In the Dermatological evaluation, a scale of 0-9 was used to assess skin elasticity where 0 was considered to be elastic (no sagging); 1-3 was mild sagging (lack of elasticity); 4-6 was considered 'moderate sagging' (lack of elasticity) and 7-9 was 'severe sagging' (lack of elasticity). In this scale, it was seen that consumers with close to 'severe sagging' skin improved to 'mild sagging' at the end of 12 weeks of regular application.

4. Conclusion

An oil blend consisting of seven oils was developed with the following characteristics: very light (viscosity = 5 mPa.s); good spreadability and easily absorbed on skin; low freezing point (4 °C); capable of solubilising solid phyto-actives.

Micro-emulsions as well as nano-emulsions with the oil blend were developed using a mixture of emulsifiers, C_{12} G_{10} and $C_{18:1}$ G_{10}. These microemulsions as well as nano-emulsions formed the basis for several commercial products of Almora Botanica, i.e. Restorative Cuticle Oil, Serum for Fine Lines and Serum for Dark Spots & Pigmentation.

In vitro experiments using Episkin's RHPE as well as T-skin models, along with clinical studies, indicated a strong biological basis for product efficacy and sensorial benefit. By packaging specialized active ingredients into micro-emulsion and nano-emulsion product formats, targeted delivery and dermal nourishment was observed.

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Conflicts of Interest:

None.

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