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Formulation of an anti-aging cream containing *Alteromonas* ferment extract and evaluation of the effect on collagen concentration

Gulsah GEDIK [1] *

[1] Department of Pharmaceutical Technology, Faculty of Pharmacy, Trakya University, Edirne, Turkey

* Corresponding Author. E-mail: gulsahgedik@trakya.edu.tr (G.G.); Tel. +90-505-800 30 72.

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ABSTRACT: This study relates to the Alteromonas ferment extract, a by-product of a marine bacteria, *Alteromonas macleodii,* is used in anti-aging cream formulation. Alteromonas ferment extract is an innovative anti-pollution ingredient that chelates heavy metals and has non-toxic anionic polysaccharides, which are used as thickening or structuring agent in tissue engineering, wound management or drug delivery applications. Aging is a physiological process, it is undesirable especially on the skin. Aging leads to weakening of facial muscles and loss of subcutaneous fat and skin laxity, along with wrinkles, prominent lines and sagging skin. Changes in collagen play an important role in reducing skin elasticity. In this case, fibroblast decreases, matrix metalloproteinase increases (responsible for collagen breakdown), and glycosaminoglycans decrease. The objective of the study was to evaluate effects of the anti-aging cosmetic face cream including Alteromonas ferment extract on human dermal fibroblast cells. The outcomes of the characterization analysis have indicated the development of a successful cream formulation with optimum characteristics. No microbial growth was observed. Given the results obtained in the tested conditions, the anti- aging cream is not cytotoxic against fibroblasts 0.5% or less concentration. The dilutions of 0.1% and 0.2% were chosen for evaluation of collagen concentration, because they were the most appropriate concentrations in terms of solubility and lack of cytotoxicity. The treatment with anti-aging cream formulation at 0.1% and 0.2% increased collagen synthesis 57% and 67%, respectively in cell culture.

KEYWORDS: *Alteromonas* ferment extract; anionic polysaccharides; collagen synthesis; anti-aging cream; skin aging.

1. INTRODUCTION

Aging is a physiological process, it is undesirable especially on the skin. Skin has the potential to reflect an image of health and vitality, and the mismatch between a person's chronological age and their apparent age is understandably undesirable [1,2]. Aging leads to weakening of facial muscles and loss of subcutaneous fat and skin laxity, along with wrinkles, prominent lines and sagging skin [3]. Changes in collagen play an important role in reducing skin elasticity [4]. In this case, fibroblast decreases, matrix metalloproteinase increases (responsible for collagen breakdown), and glycosaminoglycans decrease, resulting in decreased skin turgor [4-6]. The main factors considered to influence skin aging are UV radiation and environmental pollutants [7,8]. Exposure to infrared rays has been shown to increase MMP1 levels [5]. Reactive oxygen species increase dramatically in response to UV radiation and damage cell DNA, RNA, proteins and lipids [9].

Non-surgical options such as fillers and facial threads are among the most popular and new cosmetic anti-aging options in recent years [10]. Although thought to be safe and effective [11,12], for many consumers, such techniques are still unacceptably invasive, and the cosmetic cream option is far more attractive and practical than a needle [13].

The marine is a rich source of polysaccharides which has got anti-microbial, anti-inflammatory, antioxidant and wound-healing abilities produced by marine bacteria [14,15]. The marine bacteria *Alteromonas macleodii* is composed of glucuronic acid, mannose, galactose, glucose and galacturonic acid which were present as(1 → 4)-linked residues, with branches or substitutions at positions 2 and/or 3 [16]. Alteromonas

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ferment extract is a by-product of the fermentation of the marine bacteria, Alteromonas macleodii. This bacteria is obtained from extremely hot and high pressure areas. The location allows organisms to evolve into special polysaccharides. Alteromonas ferment extract is an innovative anti-pollution ingredient that chelates heavy metals [17].

A study reported that Alteromonas ferment extract protected against UV-induced lipid peroxidation in keratinocytes. It has also been shown to potently chelate Cd and Pb have a barrier function on the skin [18]. Therefore, it is thought that Alteromonas extract can prevent pigmentation changes in the skin. Another study investigated the anti-aging effects of a cream formulation containing 0.2% carnosine, 0.06% sodium hyaluronate, 0.02% Alteromonas ferment extract, and 0.0005% a tripeptide after 56 days of use in 33 women aged 45 to 65 years. As a result, a redefined facial contour and improved skin were observed [19].

These polysaccarides have 1.4 million dalton molecular weight and are biocompatible and biodegradable [20,21]. High molecular weight gives them a particular affinity for the skin and potential for use in several applications including in the cosmetic, biomedicine, pharmaceutical, and therapeutics fields [22]. They might be a suitable thickening or structuring agent in tissue engineering, wound management, or drug delivery applications [16, 21] and soothesensitive skin against chemical (such as drying acne treatments or strong chemical exfoliants), mechanical (such as micro-cuts after shaving) and UVB aggressors and reduces irritation

The polysaccharide structure is quite crucial for our skin's natural ability to hydrate and retain moisture. It stimulates the production of natural hyaluronic acid and lipids in the skin. This structure allows for skin renewal, tissue repair, and protection.

This article presents the results of the type 1 collagen production effects of an anti-aging cosmetic face cream including Alteromonas ferment extract on human dermal fibroblast cells. Firstly, the sensorial parameters and the physicochemical parameters were investigated. Then, microbial contamination test and cytotoxicity studies were performed. Finally, in vitro type 1 collagen concentraction was evaluated with immunofluorescence labelling method.

2. RESULTS AND DISCUSSION

In this study it was aimed to show the effect of the anti-aging cosmetic face cream including Alteromonas ferment extract on type 1 collagen production on human dermal fibroblast cells. Cream formulations are safe and effective delivery systems that are frequently used for the delivery of active substances to the skin and tissues. The combination of polymers and oils and the concentration of these ingredients can pose a challenge in the development of stable and effective cream formulations with enhanced sensorial properties. The oily content of the formulations appears to have a high influence on their physical properties [23,24]. Therefore, the use of oil-containing substances has been limited while developing the formulation.

The physicochemical and sensorial characterization parameters of the formulations were reported in Table 1. The physical and sensory parameters were also evaluated by 3-month accelerated stability studies. The anti-aging cream formulation was white, smooth and had a characteristic odor. No phase separation/visual difference was observed. The formulation was found suitable for skin application considerin the pH, density, viscosity, grittiness and homogenity value. Although the pH is basic, it is at a tolerable level for the skin [25, 26].

Table 1. Physicochemical and sensorial characterization parameters of the formulation

Time (t)	**Initial**	**1st month**	**3rd month**
pH	6,09 ± 0,01	5,97 ± 0,02	5,82 ± 0,01
Viscosity (cP)	100.00 ± 0,41	120.00 ± 0,62	131.00 ± 0,17
Density (g/ml)	0,98 ± 0,02	1,01 ± 0,05	1,12 ± 0,20
Appearance	Cream	Cream	Cream
Odor	Characteristic	Characteristic	Characteristic
Color	White	White	White
Grittiness	None	None	None
Homogenity	Yes	Yes	Yes
Phase separation	None	None	None

The microbial contamination test results were given in Table 2. No microbial growth was observed. It was determined that the prepared formulations were microbiologically suitable for cell culture and dermatological experiments.

Table 2. Microbial test results are repored.

Test Microorganisms	Microbiological Parameters
Total *Bacteria*	<100 CFU*/mL
Yeast and *Mould*	None
Escherichia coli	None
Staphylococcus aureus	None
Pseudomonas aeruginosa	None
Candida albicans	None

* CFU: colony-forming unit

Given the results obtained in the tested conditions, the anti-aging cream is not cytotoxic against fibroblasts at 0.5% or less concentration. The dilutions of 0.1% and 0.2% were chosen for evaluation of collagen concentration because they were the most appropriate concentrations in terms of solubility and lack of cytotoxicity. (Figure 1).

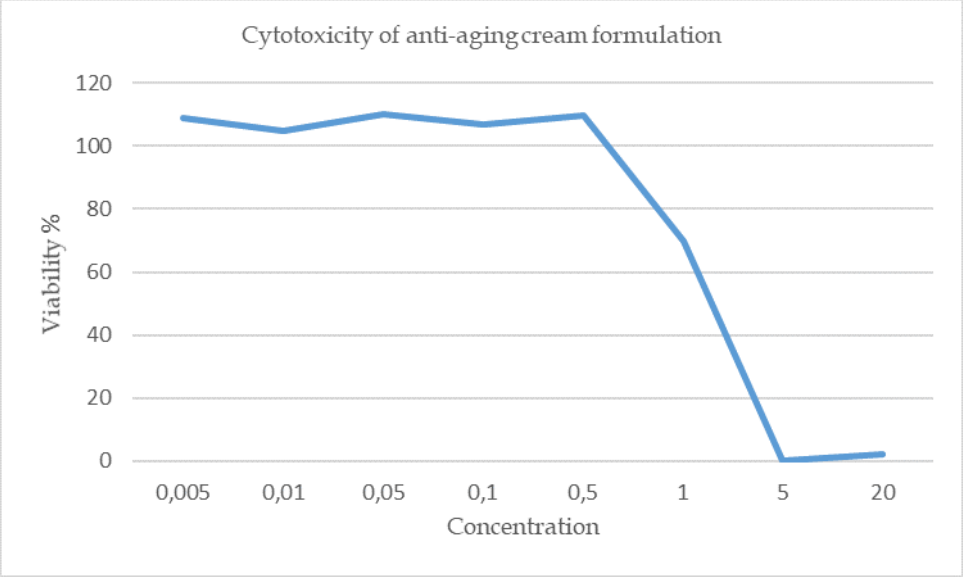


Figure 1. Cytotoxicity of anti-aging cream formulation is reported.

The video-microscobic observation is conducted to visualize collagen I (green) in 2D human dermal fibroblast culture (Figure 2 (A and C)). Without treatment, the collagen I concentration is low in cells (Figure 2 (A and B)). Fibroblasts treated with hyaluronic acid produced more collagen I by 869% in cells. (Figure 2 (C and D)). This result was expected and validated the test. Image analysis was carried out in order to quantify collagen I (Figure 2 (B and D)). The results are shown in the graph below (Figure 3).

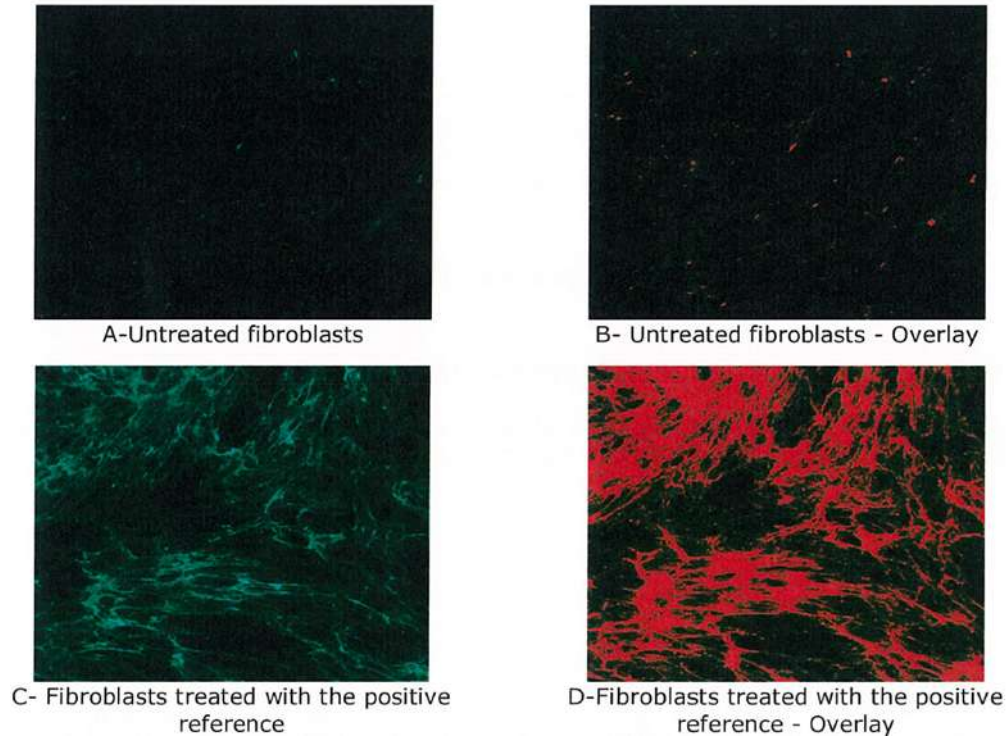


Figure 2. Immunofluorescence of untreated human dermal fibroblasts (A and B) or treated with positive control(C and D)

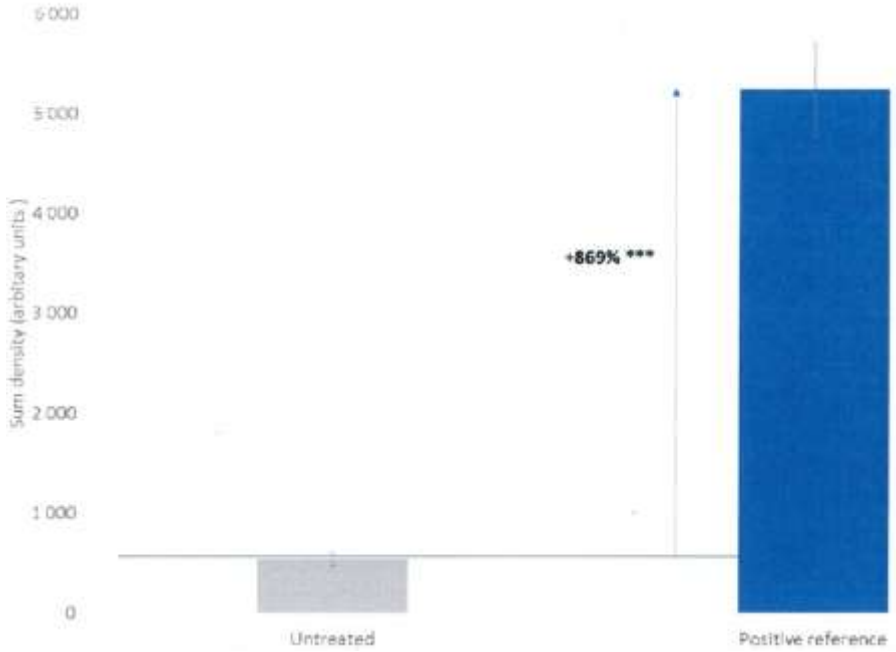
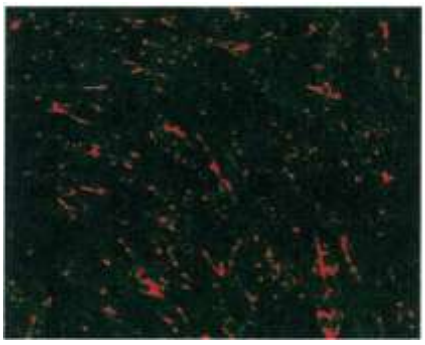


Figure 3. Effect of the positive control (hyaluronic acid) on type I collagen synthesis are reported.***p< 0.001 (student t test)

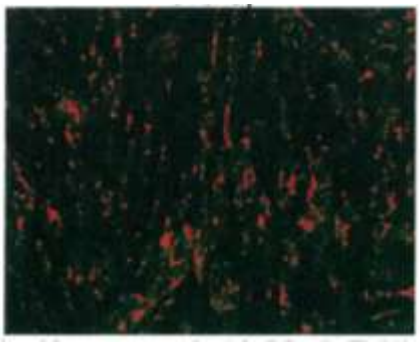
The treatment with the anti-aging cream significantly increases type I collagen synthesis . Image analysis was carried out in order to quantify collagen (collagen in red(Figure 4 (F and H)). The results are shown in the graph below (Figure 5).

E-Fibroblasts treated with cream at 0.1%



F-Fibroblasts treated with cream at 0.1%- Overlay

G-Fibroblasts treated with cream at 0.2%



H-Fibroblasts treated with cream at 0.2%- Overlay

Figure 4. Immunofluorescence of treated with the anti-aging cream formulation at 0.1% (E and F) and 0.2% (G and H) in human dermal fibroblasts.are reported.

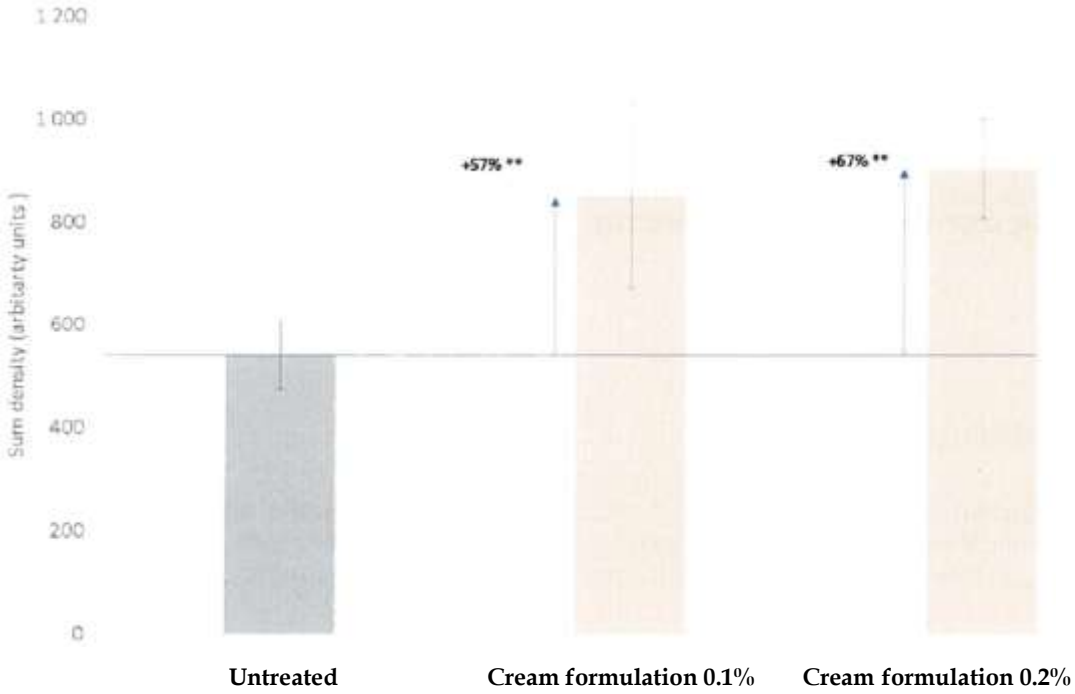


Figure 5. Effect of the the anti-aging cream formulation on type I collagen synthesis ***p<0.001 (student t test)

The treatment with anti-aging cream formulation at 0.1% and 0.2% increases collagen synthesis by respectively 57% and 67% in cell culture. According to the results obtained under the conditions of the test, the anti-aging cream formulation used at the non cytotoxic concentrations tends to increase collagen I production in human dermal fibroblast cells. In our previous study on eye cream containing hydrolyzed algin,

another anionic polysaccharide, we observed that it increased the collagen concentration [26]. Park et al. suggested that alginate oligosaccharides might have the potential to prevent skin aging by promoting collagen synthesis through the inhibition of collagen degrading enzyme [27].

Hyaluronic acid has been shown to increase proliferation and collagen synthesis in human dermal cells. High molecular weight hyaluronic acid over 1,250 kDa promotes the anti-inflammatory response in macrophages. The macrophages are responsible for secretion of soluble mediatorsand growth factors to trigger cellular cascades necessary to initiate proliferation and re-modelling [28]. High molecular weight hyaluronic acid was chosen as a positive control, considering that it would increase collagen production in fibroblasts in limited and stressful conditions more than other molecular weight hyaluronic acids [29].

The polysaccharides activates, stabilizes and localizes growth factors and cytokines [30]. In addition, polysaccharides obtained from marine microbiology have immunomodulatory, angiogenic, cell proliferative properties suitable for cosmetics and pharmaceuticals [31-33]. It is reported that species belonging to the genus Alteromonas have produced high molecular weight polysaccharides of industrial importance [34].

The presence of sulfates, phosphates and uronic acid in polysaccharides other than carbohydrates, makes this structure anionic. Therefore, they easily bind to cations in the marine environment and increase the availability of these micronutrients by bacteria for their nutritional requirements, survival and growth [35].

In a study, it was shown that the polysaccharid named EPS-A28 obtained from Alteromonas increased cell proliferative, migration and macrophage activation activities and it has been identified as a suitable substance for the wound care industry [34].

As a skin hydrator, polysaccharides help the skin reap the benefits of good hydration. Well-hydrated skin is well protected from damage caused by sun exposure and other environmental factors like pollution and dry weather.

In our study, it was shown that the cream formulation containing 2% (w/w) Alteromonas fermented extract significantly increased collagen production in fibroblasts. Electrostatic interactions of existing charges due to uronic acid residues, amine groups, sulfates, and phosphates present in the polysaccharides in the extract may facilitate binding to chemokines and other growth factors and trigger signaling cascades that initiate cellular events [36]. The sulfate groups are also responsible for the immunomodulatory activities of sulfated polysaccharides [37]. These findings point to the role of polycarides in the healing process, but their exact mechanisms of action need to be further investigated using different in vivo models.

3. CONCLUSION

As the world population ages, the demand for highly user-friendly, effective and sustainable anti-aging, anti-pollution and moustrizing products is increasing.

With our study, we have contributed to the limited literature describing the effects of Alteromonas ferment extract on the skin by revealing the effects of this subtance on collagen synthesis, and at the same time, we have developed a product suitable for consumer demand. Our results for this finished product provide cellular support to the evidence for Alteromonas ferment extract. This cream was shown to be safe and effective for its anti-aging property on the human dermal cells. The formulation offers an entirely non-invasive, safe and effective approach to counteract skin aging. Future controlled clinical trials are needed to evaluate the efficacy of the cream.

4. MATERIALS AND METHODS

4.1. Materials

All chemicals were of reagent and analytical grade. Cell lines, MTT(3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), phosphate buffer saline (PBS), formaldehyde, bovine serum albumin, hyaluronic acid (1250 kDa), Dulbecco's Modified Eagle Medium (DMEM), antibiotic /antimycotic solution, antibody solutions and microbial test kits were obtained from ARERKO Inc. Double-distilled water was used throughout the study. The CAS No and functions of all chemicals of the formulation were given in Table 3.

Table 3. The complete formulation of anti-aging cream.

Chemical Analysis Properties			
Components	**% w/w**	**CAS No**	**Function**
Aqua (Water)	q.s.	7732-18-5	solvent
Alteromonas ferment extract	2.000	267233-41-0	skin conditioning
Phenethyl alcohol	2.000	60-12-8	masking
Magnesium Aluminum Silicate	1.000	1327-43-1	viscosity controlling
Citric Acid	0.100	77-92-9	buffering
Phenoxyethanol	0.720	122-99-6	preservative
Ethylhexylglycerin	0.080	70445-33-9	skin conditioning
Sodium Polyacryloyldimethyl Taurate	1.500	-	viscosity controlling
Hydrogenated Polydecene		68037-01-4	emollient
Trideceth- 10		24938-91-8	cleansing
Xanthan Gum	0.250	11138-66-2	viscosity controlling
Tocopheryl Acetate	0.001	7695-91-2	antioxidant

4.2. Preparation of the formulation

The complete formulation was given in Table 1. The extract was added lastly and all the formulations were prepared by cold-homogenization. The formulation was kept at 25±1°C for 48 hours to see if there was a possible phase separation.

4.3. Characterization of Formulation

The formulation was evaluated in terms of sensorial and physicochemical parameters. The pH of the formulation was determined by a digital pH-meter (Mettler Toledo, S 220, Switzerland). The density was determined by a pycnometer (Mettler Toledo, 30330857, Switzerland), and the viscosity measurements were performed with a viscometer (Brookfield, DV-1, USA). The experiments were repeated three times at 25°C.

4.4. Stability Studies

Formulations were held in the stability cabinet (SWGC-1000, Daihan Scientific, South Korea). Physicochemical and sensorial parameters were also evaluated in accelerated stability conditions (40°C / 75% relative humidty) for 3 months.

4.5. Microbial Contamination Tests

The microbiological contamination of formulation was evaluated by validated tests methods TS EN ISO 22718, 22717, 21149, 16212, 18416 [38].

4.6. Cell Culture Studies and MTT Assay Test

Assessment of cytotoxicity of formulation was performed in fibroblast cell line at passage number 14 for 48 hours. MTT assay test was used. The logarithmic concentrations of samples were plotted against the percentage viability for curve fitting. The viability of untreated cultures is assumed to be 100%. The trials were performed in triplicate [39].

4.7. Evaluation of The Effect on The Collagen Concentraction

4.7.1. Method for Labelling of Collagen I

The fibroblasts (human dermal fibroblasts, from a donor aged of 30 years used at passage number 16 were seeded in a suitable culture medium for 5 days (1000 cells per well). At the end of these 5 days, the cells were treated or not (control) -with the studied formulations for 6 days, with renewal of the treatment at day 3.After incubation at 37 °C, 5% CO_2 atmosphere, the cells are fixed and treated for immunofluorescence labelling of the collagen type I, by carrying out the following steps: rinsing in PBS, fixation of the cells with formaldehyde 3.7%, incubation for 1h30min at +4°C, then washing with PBS, blocking of non-specific sites with a 1 % PBS/ BSA solution, incubation for 30 min at room temperature (RT), addition of the anti-collagen 1 primary antibody solution (AB758 Sigma-Aldrich), incubation for 1h at RT, then washing with PBS, addition of the secondary antibody solution (Donkey Anti-rabbit Alexa 488 (1/100)), incubation for 1h at RT, then washing with PBS. Before the observation, they were kept overnight at 4°C.

4.7.2. Observation of Collagen I

A microscopic observation was made with a microscope (NIKON TI Eclipse) equipped with a camera and two photographs were taken by experimental conditions. An image analysis (NIS-Elements Ar, Nikon) was then performed on the photographs in order to quantify type I collagen. Hyaluronic acid (0,1 mg/ml) with nominal molecular weights of 1250 kDa used for positive control [29].

4.8 Statistical Analysis

The raw data were transferred and processed using MS Excel Software. The different conditions were compared using the Student's t-test. A difference between the two groups was considered statistically significant if the p-value was less than 0.05, which was noted *p<0.05. If the p-value is lower than 0.01 and 0.001, it was noted **p<0.01 and ***p<0.001, respectively.

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