*IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)*
*e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 10, Issue 6 Ver. I (Nov - Dec. 2015), PP 51-60*
*www.iosrjournals.org*

# Anti-aging potential of a cream containing herbal oils and honey: Formulation and in vivo evaluation of effectiveness using non-invasive biophysical techniques

Ebru Altuntaş and Gülgün Yener
*(Department of Pharmaceutical Technology, Faculty of Pharmacy, Istanbul University, Turkey)*

***Abstract:*** *A topical oil in water type (o/w) emulsion containing pomegranate seed oil, grape seed oil, sesame oil and flower honey was developed in this study. In order to determine stability, the selected formulations were kept at room temperature (25±2°C) and accelerated conditions (40±2°C ve %75 relative humidity). Physicochemical and microbiological stability of the formulations were evaluated. In order to determine anti-aging efficacy and safety of the selected formulation, various skin parameters such as moisture level, pH value, sebum content, roughness, mean size of skin pores and elasticity of the application area of 12 healthy female volunteers, 25-65 years old were assessed by non-invasive biophysical techniques. In addition to this, the panelists answered a preformed questionnaire to assess the performance of the product. A cosmetically acceptable and stable cream was developed regarding to the results of the stability studies. It was determined that the cream has not created any allergic reaction on volunteers based on the 48-hour patch test results. Results of the non-invasive biophysical tests have shown that the product increased skin moisture, elasticity and smoothness significantly (p<0,05), it protected skin pH and sebum content (p>0,05). In addition, the product decreased skin roughness and mean size of skin pores significantly (p<0,05).*

***Keywords:*** *Cosmetic cream, Herbal oils, Honey, Skin aging, Skin moisturization.*

## I. Introduction

Skin aging is a complex biological process which can be classified in two categories as intrinsic and extrinsic skin aging. Intrinsic skin aging (also known as the natural aging ) is a chronologically emerging and inevitable process accompanied by genetic changes.[1] It is characterized by fine wrinkles, roughness, dryness, sagging and epidermal thinning with seborrheic keratoses (benign neoplasms).[2] Extrinsic aging (often referred to as photoaging) is seen due to exposure to environmental factors which cause oxidative damage in skin. Hyperpigmentation is characterized by dry and rough skin as well as deep wrinkles. Environmental factors such as cigarette smoke, air pollution and ozone may contribute to premature skin aging. However, the main cause of extrinsic aging is exposure to ultraviolet radiation (UV).[3,4]

Exposure of skin to UV radiation induces photo-oxidative reaction which weakens the antioxidant defense system and increases reactive oxygen species (ROS) at cellular level. This occurrence suppresses the defense capacity of the skin thereby reducing the ability thereof for protecting itself from the harmful effects of UV. Consequently, damage occurs in the cutaneous tissues. As such, additional efforts are required to protect the skin from the destructive effects of UV radiation. One of the approaches employed with an eye to prevent skin damage, is supporting the endogenous photoprotection mechanism by topical or oral application of herbal antioxidants with photoprotective effect. In addition, herbal antioxidants stimulate collagen and elastin formation, and reduce the degradation of structural components thereby playing a role also in the repair of cutaneous photodamage.[5]

Studies conducted have revealed the fact that, components found in plants can reduce the damages induced by UV light. Herbal components fight against the harmful effects of UV radiation through following mechanisms:

1. By reducing sunburn and inflammation induced through UV,
2. By eliminating reactive oxygen species (ROS) and free radicals which are harmful for skin,
3. By adjusting the changed signal paths due to UV radiation.[5]

Grape (*Vitis vinifera L.*) seeds contain various polyphenols in the ratio of 5-8%. Many varieties contain polyphenolic proanthocyanidins. Antioxidant effect of procyanidins within the content thereof is 20 times more than vitamin E and 50 times more than vitamin C. Grape seeds inhibit lipid peroxidation thereby accelerating wound healing. Grape seeds also protect collagen and elastin without degradation. Grape seed extracts are included in anti-aging and skin lightening cosmetics inasmuch as they do tyrosinase inhibition.[6]

Pomegranate (*Punica granatum*) is an ancient fruit with extremely rich ethnomedical applications. Pomegranate seeds are rich in type 2 polyphenolic compounds. These are anthocyanidins (delphinidin, cyanidin and pelargonidin) and hydrolysable tannins. Pomegranate seeds have powerful antioxidant and anti-

inflammatory effects.[7,8] Pomegranate inhibits UVB-induced NF-κB activation and protein kinase pathways activated by mitogen and protects the skin against the side effects of UVB radiation. It also provides protection against the harmful effects of UV light.[8] It has been proven that pomegranate seed oil stimulates keratinocytes proliferation in monolayer culture. A slight thickening has been observed in the skin culture and the epidermis in parallel with this.[7]

It is known in ancient folklore that application of sesame (*Sesamum indicum L.*) oil by massaging the skin, prevents aging and eliminates wrinkles. This is partly due to a variety of antioxidants sesame oil.[9] Sesame oil has high antioxidant effect due to its content of components with lignin structure such as sesamin, sesamolin, sesaminol and sesamol as well as γ-tocopherol. The studies currently conducted have revealed that, sesame oil is a natural sun protector and can block 30% of UV rays.[10] In one study, it has been determined that sesamol which has antioxidant effect, has beneficial effects in the prevention of photodamage in skin of mice.[9, 11]

Honey is a viscous and supersaturated solution of sugars derived from the nectar collected and modified by Apis mellifera (honey bee).[12] Bee products such as honey, royal jelly and pollen are included the functional foods due to their naturally high antioxidant potential. Honey, in addition to sugar, includes amino acids and proteins, carotenoids, flavonoids and phenolic compounds, many components with antioxidant effect such as ascorbic acid and organic acids.[13]

An emulsion is a two phase colloidal systems when there is the mixture of two liquids that are normally immiscible with each other.[14] An emulsion is capable of penetrating the skin at high rates. Both water-in-oil (W/O) emulsions and oil-in-water (O/W) emulsions are widely used in cosmetics inasmuch as they have moisturizing and dry skin regenerating properties. O/W type emulsions are the most appropriate formulations for usage in general cosmetic purposes and as washable drug bases.[15]

The objective of this study is to develop a stable O/W type cream formulation including a variety of vegetable oils and flower honey which can be considered as a cosmetic and evaluate the anti-aging efficacy and safety thereof clinically with a non-invasive variety of biophysical techniques and subjective assessment.

## II. Materials And Methods

### 2.1. Materials

Flower honey were obtained from Balparmak (Turkey), pomegranate seed oil, grape seed oil and sesame oil were kindly donated from Zade Naturel (Turkey). Tween 20 Merck (Germany); Tocopherol acetate, ethyl alcohol, methyl paraben, propyl paraben, triethanolamine, butyl hydroxy anisole and butylated hydroxy toluene were purchased from Sigma-Aldrich (ABD). Carbopol 940, Lubrizol (France); Hidroksipropil metilselüloz (Methocel K15 M), Colorcon (England).

### 2.2. Methods

#### 2.2.1. Preparation of Formulations

HLB, hydrophilic-lipophilic balance, is the ratio of oil-soluble portion to the water-soluble portion of the molecule and firstly developed by Griffin. Griffin has directed his activities to select optimal non-ionic emulsifiers ensuring the stability of the emulsion. HLB value of the emulsifier combinations are selected in a way that it is almost equivalent to the substances to be emulsified. If more than one substance will be emulsified at the same time, average weighted HLB value will be calculated based on the HLB value they according to% compositions used in the mixtures of these substances and that the emulsifier is determined according to this HLB value.[16] Various formulations were prepared according to this information to include 4% and 6% non-ionic emulsifier in total. Water-soluble Tween 20 (HLB:16.7) and oil-soluble glyceryl monostearate (HLB: 3.8) combination was used as emulsifier. Emulsifiers were selected in three different ratios as a result of the calculations. (Table 1).

**Table 1: The Percentages of the Emulsifier Mixtures Used in the Formulations and HLB Values.**

| **Emulsifier mixtures % (w/w) (Tween 20: Glyceryl monostearate)** | 65:35 | 45:55 | 55:45 |
|---|---|---|---|
| **HLB values** | 12.2 | 9.6 | 10.9 |

Thickening polymers (Carbopol 940 and hydroxypropyl methylcellulose) were swelled with water for one day in 1/3 part of the distilled water. Gels were kept in ultrasonic water bath (Wiseclean, Germany) for 30 minutes.

The temperature of the oil and water phase was made 80°C by using the water bath (WiseBath, Germany). Firstly, the gel phase at the same temperature was combined with the water phase and then the oil phase was slowly added to the water phase. Trials were made with different stirring speeds (8000 rpm with ultraturrax, 500 rpm and 1000 rpm with a mechanical stirrer) and with different mixer types (Ultraturrax, X620 CAT M-ZippererGmbH, Germany, and mechanical stirrer, Wise Stir® HS-100D DAHIAN Scientific, Korea)

for 5 minutes to determine the most suitable preparation process for emulsion formation. When the formulations reached the room temperature, pH adjustment was made with 10% (w/w) of triethanolamine solution or a 10% (w/w) citric acid solution. Formulations and codes are shown below (Table 2).

**Table 2: The Contents of Formulations (% w/w).**

| | Ingredients (% w/w) | FORMULATIONS | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 | F11 | F12 | FS1 | FS2 | FS3 |
| Oil phase | Grape seed oil (GSO) | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | Pomegranate seed oil (PSO) | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | Sesame oil ( SO) | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | Glyceryl monostearate (GMS) | 2.2 | 1.8 | 1.4 | 3.9 | 3.3 | 2.7 | 3.9 | 3.3 | 2.7 | 3.9 | 3.3 | 2.7 | 3.9 | 3.3 | 2.7 |
| | Cetyl alcohol (SA) | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | Propyl paraben | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 |
| | Butyl hydroxyanisole (BHA) | - | - | - | - | - | - | - | - | - | - | - | - | 0.05 | 0.05 | 0.05 |
| | Butyl hydroxy toluene (BHT) | - | - | - | - | - | - | - | - | - | - | - | - | 0.05 | 0.05 | 0.05 |
| | Tocopherol acetate (TA) | - | - | - | - | - | - | - | - | - | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Water phase | Honey | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | Tween 20 (T20) | 1.8 | 2.2 | 2.6 | 2.1 | 2.7 | 3.3 | 2.1 | 2.7 | 3.3 | 2.1 | 2.7 | 3.3 | 2.1 | 2.7 | 3.3 |
| | Methyl paraben | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 |
| | Glycerin | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| | Hydroxypropyl methyl cellulose (HPMC) | - | - | - | - | - | - | - | - | - | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |
| | Carbopol 940 (CP 940) | - | - | - | - | - | - | 0.4 | 0.4 | 0.4 | - | - | - | - | - | - |
| | Triethanolamine | q.s. | q.s. | q.s. | q.s. | q.s | q.s. | q.s. | q.s. | q.s. | q.s. | q.s. | q.s. | q.s. | q.s. | q.s. |
| | Distilled water (d.w.) | 69.8 | 69.8 | 69.8 | 67.8 | 67.8 | 67.8 | 67.4 | 67.4 | 67.4 | 66.9 | 66.9 | 66.9 | 66.4 | 66.4 | 66.4 |

F: Formulation
FS: Selected formulation
q.s.: quantum satis

### 2.2.2. Emulsion Characterization and Stability Tests

#### 2.2.2.1. Determination of the Emulsion Type

The emulsion type was determined through utilization of dilution test based on the solubility of the emulsion's external phase in water or oil.[17] Briefly, a few drops of the prepared emulsion was added to the test tubes containing a small amount of water. If the outer phase of the emulsion was distributed homogeneously in water it was determined as O/W type and if it became separate as a layer it was determined as W/O type.

#### 2.2.2.2. Thermodynamic Stability Tests

The preliminary stability of the emulsions was evaluated by centrifugation and thermal stress tests within 24 hours. Stability was determined by macroscopic observation of the emulsions. The centrifuge test carried out for the purpose of accelerate possible stability issues, centrifugation at room temperature (25 ± 1°C) and at 3500 rpm speed was applied to 10 g emulsion for 30 minutes with a laboratory type centrifuge device (ThermoScientific, USA).[18] 10 g emulsion was exposed to different temperatures in thermostatic water bath test from 40°C to 80°C, with 5°C temperature increases in the thermal stress test. The formulations were kept for 30 minutes at each temperature point. Formulations in which any phase separation or creaming was not observed as a result of the two tests were continued to work with. At the later phase, characterizations of formulations which were found to be successful in the thermodynamic stability tests were performed. [19]

#### 2.2.2.3. Characterization

Emulsions selected as a result of the two preliminary stability tests were stored for 3 months at room temperature (25 ± 2°C) and climate chamber (Nüve TC120, Turkey) and at 40 ± 2°C and 75% relative humidity (RH). Organoleptic and physical controls and pH and viscosity measurements were made in the determined time intervals (1st day, 1st, 2nd and 3rd months) for physicochemical analyzes.

**Organoleptic and physical controls**

Organoleptic and physical controls (color, odor, appearance, liquefaction, creaming, phase separation) of the formulations were carried out under the determined conditions and time intervals.

**pH Measurements**

pH measurements were made at room temperature (25 ± 2°C) and climate chamber (at 40 ± 2°C and 75% relative humidity) under the determined conditions and time intervals. (n:3). Samples were diluted 1:10 with distilled water prior to the test.

**Viscosity Measurements**

Viscosity measurements of the formulations were determined using rotational-type viscometer (Brookfield DVII, Germany TA spindle, 25±1°C). Measurements were taken in 3 replications in 100 rpm (n:3). Viscosity values were recorded in centipoise (cP).

**Microbiological Stability Test**

Microbiological limit test of formulations (FS1, FS2, FS3) selected after studies of stability and characterization was carried out according to the procedures reported in the USP XXIX during 1 month with the aim of evaluating the microbiological stability thereof.

**2.2.3. Clinical Studies**

**2.2.3.1. Study design**

The single-blind in vivo studies, compliance with the Helsinki Declaration, has been approved by Ethics Committee of Medicine Clinical Research Department of Yeditepe University Faculty (Document number: 060-26.10.2010). The study was initiated on 12 healthy female volunteers between the ages of 25-65, after they signed a written informed consent form. Exclusion criteria for volunteers are as follows:

1) Pregnancy and lactation cases for women,
2) Use of any systemic or topical medication for skin diseases within the same period,
3) Knowing that the person had hypersensitivity previously to formulation content,
4) Persons with significant systemic story,
5) Persons who have started hormonal therapy before 12 weeks or less prior to the study,
6) Persons who cannot adapt to the study,
7) Persons with extreme sensitivity in the selected region.

**2.2.3.2. Dermatological Testing (Patch Test)**

0.2 grams of test product was placed in the patch test material (IQ chamber) before starting to use the product in order to determine if any adverse effect occurred on the skin of volunteers, by affixing in a way to contact the $1cm^2$ forearm region. The volunteer was informed to avoid from contact with water and direct sunlight during the 48-hour observation period. The patch test material was removed at the end of the 48-hour period and it was checked if a reaction such as erythema and edema occurred in the skin of the volunteers.

**2.2.3.3. Preparation of Test Environment**

If the tests are carried out in an environment receiving direct light, the skin warms up, sweating increases and the secretion of sebum decreases as a result. And as a result of this hydrolipidic film layer of the skin can vary greatly. Therefore it is avoided to make the measurement under exposure to direct light. Other environmental factors that can affect the measurement are humidity and temperature of the environment.

Therefore, the instrumental measurements were taken after the volunteers rested in the air-conditioned room at 20°C±2 temperature and at 40-60% relative humidity for 30 minutes.

**2.2.3.4. Biophysical Methods**

During the eight-week test period, the volunteers were not allowed to use any skin care product on the right forearm where the product would be used. The formulation coded FS1 which could be accepted as a cosmetic and stability of which was the most suitable was used by the volunteers twice a day for 8 weeks.

Measurements were taken three times as before starting to use the product (T0), in the 4th week (T4) and in the 8th week (T8) by non-invasive biophysical methods from the application regions of the volunteers and various parameters were evaluated. The moisture content, pH value, sebum content, elasticity, roughness and average pore size of the skin were determined by Corneometer CM 825® (Courage&Khazaka electronic GmbH, Cologne, Germany) device working according to capacitance method, SKIN-pH-METER 900® (Courage&Khazaka electronic GmbH, Cologne, Germany), Sebumeter® SM 815 (Courage&Khazaka electronic GmbH, Cologne, Germany) and Aramo TS skin diagnosis system (Aramhuvis Co., Ltd., Korea) respectively by taking 5 different measurements from the test area for each parameter.

**2.2.3.5. Subjective Evaluation**

A panel test was conducted to support the results of biophysical measurements after the 8-week period of product application of volunteers. Volunteers were asked seven questions with regard to the effect of the formulation on the skin. They were asked to reply these questions by giving scores between 0 and 5.

**2.2.3.6. Statistical Analysis**

The results obtained from clinical trials were evaluated through utilization of GraphPadPrism 5 program (GraphPad Software Inc., CA, USA). One-way analysis of variance (ANOVA) test was applied to determine eventual variation between different time intervals. Statistically, a significant difference was considered at a p value of less than 5% ($p < 0.05$)

## III. Results

Because it was determined in the preliminary tests of the formulation that 5 minutes of stirring with ultraturrax at 8000 rpm gave the best results macroscopically, it was continued to work with this method later.

Several experiments were made in preparation of the formulations, by using the combination of T20 and GSM in ratio of 4% (w/w) and 6% (w/w) in total. Consequently, it has been decided that consistency of formulations containing the 6% of the emulsifier mixture were higher than those containing 4% and they were more suitable for the desired creamy consistency.

All formulations prepared according to dilution test used for the determination of the emulsion type were determined as O/W type. As a result of centrifugal and thermal stress tests conducted, coalescence and phase separation was observed in formulations containing 4%, emulsifier mixture and less coalescence occurred in formulations containing 6% emulsifier. Therefore, two different polymers [CP940 (0.4% w/w) and HPMC (0.6% w/w)] were added to formulations containing a mixture of 6% emulsifier with the aim of both increasing emulsion viscosity and stability by ensuring the migration polymer to the oil-water interface surface.[20,21]

Emulsions found suitable in preliminary stability tests (F7-F12, FS1-FS3) were stored at room temperature (25±2°C) and in climate chamber at 40±2°C and at 75% relative humidity for 3 months and their physicochemical stability (organoleptic and physical controls, pH and viscosity measurements) was evaluated at specified time intervals. Findings as to their physicochemical stability parameters are presented below (Table 3-6).

**Table 3: Physical Controls of the Formulations.**

| Formulation Code | Physical stability | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 25°C±2 | | | | 40°C±2 (%75 R.H) | | | |
| | 24 hours | 1th month | 2th month | 3th month | 24 hours | 1th month | 2th month | 3th month |
| F7 | S | S | S | + | S | S | ++ | +++ |
| F8 | S | S | S | + | S | + | +++ | +++ |
| F9 | S | S | + | ++ | S | + | ++ | ++ |
| F10 | S | S | S | S | S | + | + | + |
| F11 | S | S | S | + | S | + | + | ++ |
| F12 | S | S | S | + | S | S | + | ++ |
| FS1 | S | S | S | S | S | S | S | S |
| FS2 | S | S | S | S | S | S | S | S |
| FS3 | S | S | S | S | S | S | S | S |

S: Stable form; +: Decrease in viscosity; ++: Coalescence; +++: Partial phase separation; +++++: Complete phase separation

**Table 4: Organoleptic controls of the formulations.**

| Formulation Code | Color | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 25°C±2 | | | | 40°C±2 (%75 R.H) | | | |
| | 24 hours | 1th month | 2th month | 3th month | 24 hours | 1th month | 2th month | 3th month |
| | | | | | | | | |
| F7 | W | W | W | YW | W | W | YW | Y |
| F8 | W | W | W | YW | W | W | YW | Y |
| F9 | W | W | W | YW | W | Y | Y | Y |
| F10 | W | W | W | W | W | W | W | W |
| F11 | W | W | W | W | W | W | W | W |
| F12 | W | W | W | W | W | W | W | YW |
| FS1 | W | W | W | W | W | W | W | W |
| FS2 | W | W | W | W | W | W | W | W |
| FS3 | W | W | W | W | W | W | W | W |

W:White; YW: Yellowish white; Y:Yellow

**Table 5: pH of the Prepared Formulations at Different Time Intervals.**

| Formulation Code | pH | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 25°C±2 | | | | 40°C±2 (%75 R.H) | | | |
| | 24 hours | 1th month | 2th month | 3th month | 24 hours | 1th month | 2th month | 3th month |
| F7 | 6.30 | 6.08 | - | - | 6.30 | 5.74 | - | - |
| F8 | 6.10 | 5.89 | - | - | 6.14 | 5.70 | - | - |
| F9 | 5.94 | 5.76 | - | - | 5.92 | 5.64 | - | - |
| F10 | 6.13 | 6.10 | 5.80 | - | 6.18 | 5.95 | 5.64 | - |
| F11 | 6.04 | 5.91 | 5.52 | - | 5.93 | 5.78 | 5.40 | - |
| F12 | 5.82 | 5.79 | 5.34 | - | 5.80 | 5.61 | 5.18 | - |
| FS1 | 6.57 | 6.51 | 6.31 | 6.29 | 6.57 | 6.38 | 6.36 | 6.36 |
| FS2 | 6.71 | 6.69 | 6.64 | 6.47 | 6.57 | 6.31 | 6.28 | 6.23 |
| FS3 | 6.73 | 6.61 | 6.46 | 6.43 | 6.73 | 6.35 | 6.24 | 5.86 |

-: Measurements were not taken due to the stability was not appropriate.

**Tablo 6: Viscosity of the Prepared Formulations at Different Time Intervals.**

| Formulation Code | Viscosity (cP) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 25°C±2 | | | | 40°C±2 (%75 R.H) | | | |
| | 24 hours | 1th month | 2th month | 3th month | 24 hours | 1th month | 2th month | 3th month |
| F7 | 23200 | 23876 | - | - | 23340 | 21760 | - | - |
| F8 | 21536 | 20452 | - | - | 22894 | 21458 | - | - |
| F9 | 20985 | 19920 | - | - | 20980 | 19861 | - | - |
| F10 | 22344 | 21345 | 21880 | - | 21765 | 19649 | 18840 | - |
| F11 | 22080 | 20860 | 19635 | - | 22100 | 18977 | 18035 | - |
| F12 | 19759 | 19652 | 18998 | - | 19978 | 19632 | 17143 | - |
| FS1 | 25600 | 24896 | 24754 | 24579 | 25600 | 24759 | 24748 | 23957 |
| FS2 | 20230 | 19600 | 19975 | 19925 | 20230 | 19840 | 19326 | 18900 |
| FS3 | 20430 | 19753 | 19355 | 18332 | 20430 | 19478 | 18653 | 18765 |

-: Measurements were not taken due to the stability was not appropriate.

Findings as to microbiological stability tests carried out on FS1, FS2 and FS3 formulations which were found to be cosmetically acceptable and the most durable as a result of the physicochemical stability tests are provided below (Table 7).

**Table 7: Microbiological Test Results of FS1, FS2 ve FS3 Formulations.**

| Formulations | Test Results |
|---|---|
| FS1 | <10 cfu/g of total aerobic bacteria and fungi were detected in the example. Bacteria from the Enterobacteriaceae family, Staphyloccocus aureus and Pseudomonas aeruginosa were not detected in the example. |
| FS2 | <10 cfu/g of total aerobic bacteria and fungi were detected in the example. Bacteria from the Enterobacteriaceae family, Staphyloccocus aureus and Pseudomonas aeruginosa were not detected in the example. |
| FS3 | $9{,}5\times10^2$ cfu/g of total aerobic bacteria and <10 cfu/g fungi were detected in the example. Staphyloccocus aureus was detected in the example. Bacteria from the Enterobacteriaceae family and Pseudomonas aeruginosa were not detected in the example. |

FS1 coded cream was selected as the most suitable formulation for clinical trial following the organoleptic controls and physicochemical and microbiological stability tests. Inasmuch as no redness, itching or irritation occurred on skin of volunteers as a result of the dermatological tests conducted before starting to use the product, all subjects were included in the 8-week clinical trial.

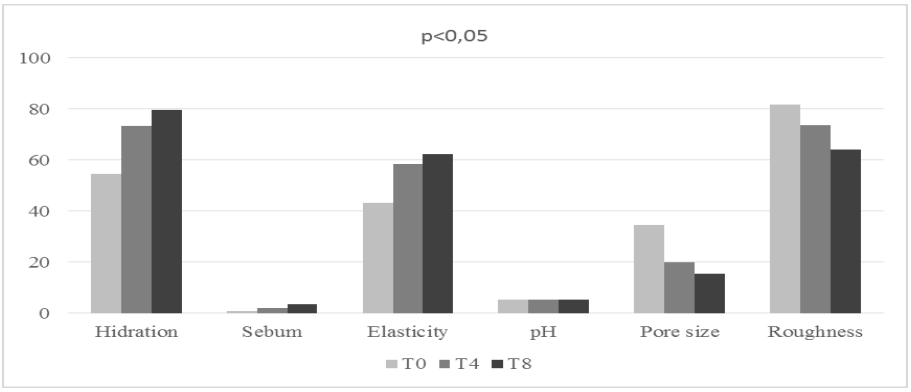
Findings as to measurements of sebum, elasticity, pH, the pore size and surface roughness measurement received from the application parts of the volunteers three times as before starting to use the product (T0), in the 4th week (T4) and in the 8th week (T8) by non-invasive biophysical methods are shown below (Table 8 and Fig.1).

**Table 8: Comparison of Biophysical Parameters of Skin (n:12).**

| Biophysical Parameters | T0 | T4 | | T8 | | Statistical significance (p<0.05) |
|---|---|---|---|---|---|---|
| | Mean | Mean | % Variation | Mean | % Variation | |
| Hidration (Corneometer unit) | 54.40±9.89 | 73.42±7.64 | 34.96 | 79.65±1.53 | 46.42 | Yes |
| Sebum (mg/cm$^2$) | 1.74±0.74 | 2.07±1.03 | 18.97 | 3.42±1.10 | 96.55 | Yes |
| Elasticity[a] | 43.13±11.44 | 58.32±9.26 | 35.22 | 62.25±1.74 | 44.33 | Yes |
| pH | 5.32±0.67 | 5.27±0.24 | -0.94 | 5.30±0.15 | 0.38 | No |
| Pore size[a] | 34.66±11.83 | 19.83±7.60 | -42.79 | 15.33±1.14 | -55.77 | Yes |
| Roughness[a] | 81.58±5.56 | 73.50±9.97 | -9.90 | 64.17±1.68 | -21.34 | Yes |

a: dimensionless

**Figure 1: Biophysical test values averaged over all 12 volunteers before and after application of FS1 formulation.**




Findings as to the survey questions asked to the volunteers and the answers given thereby after the biophysical measurements are given below (Table 9).

**Table 9: Questionnaire scores given by the panelists.**

| Questions | Score | SD |
|---|---|---|
| 1. Did the herbal cream that you used decrease your skin moisture loss? | 4.25 | 0.55 |
| 2. Did the herbal cream that you used provide an increase your skin brightness and vibrancy? | 3.60 | 0.75 |
| 3. Did the herbal cream that you used provide an increase your skin elasticity? | 3.55 | 0.51 |
| 4. Was the herbal cream that you used absorbed easily by your skin? | 4.15 | 0.81 |
| 5. Did the herbal cream that you used provide a reduction in the appearance of wrinkles? | 3.40 | 0.59 |
| 6. Did the herbal cream that you used give softness to your skin? | 4.10 | 0.64 |
| 7. Did the herbal cream that you used cause an increase your skin oiliness? | 0.35 | 0.48 |

Score: 0 not at all; 1 slightly; 2 few; 3 medium; 4 much; 5 too much

## IV. Discussion

Cosmetic products used against skin aging have to be products, reliability and efficiency of which are proven in various ways since they make some structural and functional changes on the skin. Much as the physical properties and stability of the product are important for the consumer, it is also very important for the product to show its claimed cosmetic activity. Any product having unpleasant appearance cosmetically and poor absorption and which is unstable or has irritant or allergic effects on the skin are not preferred by consumers even if it has efficiency.

Determination of an acceptable shelf life is very important in the development of emulsion formulations.[22] Pharmaceutical and cosmetic manufacturers desire their products to have a shelf life of at least 2 or 3 years.[23] A cosmetic cream must be able to remain stable during the shelf life without occurrence of phase separation and changes in color, odor and appearance. A stability test carried out under normal storage conditions is an effective way for determining the system's durability. However, accelerated stability tests in which the product is exposed to various stress conditions are carried out in order to shorten the cited period, to obtain information faster and more reliable related to stability. Measurement of the physicochemical properties of the product under accelerated conditions may reflect the long-term storage performance of the product.[24,25]

Creaming and flocculation, which are some of the stability problems observed in emulsions, are processes which occur slowly. Therefore, centrifugation and accelerated thermal stability tests were conducted in order to speed up the formulation studies at the first phase. It has been observed that usage of 4% and 6% emulsifier mixture (T20 + GMS), in preparation of emulsions containing a mixture of 15% vegetable oil is not sufficient in terms of stability as a result of these tests. It has been reported that addition of polymer to the water phase of the emulsion increases the viscosity and stability of the emulsion, because it increases the density of the water phase.[26] Accordingly, formulations containing 6% emulsifier mixture were prepared again by adding polymer (0.4% CP940 and 0.6% HPMC) to increase the stability and consistency. Because the resulting emulsions (F7-F12, FS1-FS3) were found to be appropriate in centrifugation and accelerated thermal stability tests, it has been observed that addition of polymers to formulations increase the stability as expected. F7-F12, FS1-FS3 coded formulations which were found to be successful were subjected to physicochemical stability test for 3 months located, at room temperature (25±2°C) and in a climate chamber (40±2°C and %75 R.H) at a later stage.

It was found in organoleptic controls that at the end of the 3rd month, the initial white color of the F7-F9 coded formulation containing CP940 darkened in both storage conditions. Mild yellowing was observed only in the sample of F12 coded formulation, among those containing HPMC, which was kept in the climate chamber in the 3rd month while all other formulations kept remaining in white color, homogeneous and in a stable condition. When the physical stability was evaluated, it was observed at the end of the 3rd month that FS1, FS2 and FS3 coded formulations kept their stable form at both storage conditions and it was detected that physical deterioration (phase separation and coalescence) began in the 2nd month in F7-F9 coded formulations containing CP940 months and in F10-12 coded formulations containing HPMC in the 3rd month which were kept in the air conditioning cabinet. Thus, it was decided that formulations containing CP940 were more susceptible to heat and humidity compared to the formulations containing HPMC.

Monitoring of pH is important in detecting the stability of the emulsions. pH changes which have occurred in the product over time reveal that chemical reactions take place while, it gives opinions on the product quality. Accelerated product performance test and kinetics of the pH profile are significant in terms of chemical stability.[27] pH measurements of FS1, FS2 and FS3 formulations could be followed for 3 months in this study. As a result, all of the formulations kept in both conditions showed a slight decrease in pH. This case can be based on the fact that, fatty acid esters are hydrolyzed to free fatty acids in the emulsions produced with vegetable oils.[19] However, it has been detected that all of the formulations are appropriate for the human skin pH range (4.5 to 6.5) 3 months in the desired way.[28]

Viscosity is a significant parameter commonly used in assessing the effect of stress conditions on emulsion stability. Moreover, viscosity is also related to quality characteristics such as filling the product into the packaging material, ease of use, physical appearance, consistency and skin-spreadability.[29,30] In this context, viscosity measurements of FS1, FS2 and FS3 coded formulations was monitored for 3 months. Viscosity values of the three formulations were within acceptable limits and no change was observed in homogeneity under all stability conditions.

Cosmetics products are not required to be sterile. However, they need to be protected sufficiently against microbial contaminations and degradation that may occur as a result of consumer use and production processes. The contamination due to production can be prevented reasonably while it is not possible to control contamination originating from consumer use. For this reason, use of antimicrobial preservatives is required in order to protect the product against bacteria, yeast and mold growth during its shelf life sufficiently.[31,32] In this study, methyl paraben (0.3% w/w) and propyl paraben (0.3% w/w) combination, which is the most commonly used preservative, has been selected for the prevention of microbial contaminations. Furthermore, TA (0.3% w/w), BHT (0.05% w/w) and BHA (0.05% w/w) combination was used at appropriate concentrations against oxidative degeneration that frequently occurs in the emulsions containing plant oils. As a result of the microbiological limit test of the selected formulations (FS1, FS2, FS3), all formulations were found to be eligible in terms of microbiological quality. In addition, pathogens such as bacteria from the Enterobacteriaceae family Staphyloccocusaureus and Pseudomonasaeruginos which are not allowed to be present in cosmetic products were not found in none of the formulations.

Non-invasive biophysical measurement techniques are methods which consumers and cosmetics researchers prefer more which are ethically more acceptable and which have major advantages such as reproducibility. They ensure detection of invisible changes on the skin or hair without causing pain and suffering. They are also appropriate for statistical evaluation.[33]

Hydration or water content of the outermost layer of skin (stratum corneum), is associated with intrinsic aging and menopause. It is considered that the reduction in the amount of glycosaminoglycans having a hydrophilic structure make a direct reduction in the water content of the skin. Moisturisers help to normalize the barrier function of the skin and create a smooth, flexible and healthier looking skin.[34] While the hydration value of the skin was 54.40 before the application of the product (T0), it was found to be 73.42 and 79.65 after 4 and

8 weeks of application respectively in the measurements taken from the right forearms of the volunteers by Corneometer CM 825® working with capacitance principle. The results revealed that application of the product for 4 and 8 weeks significantly increased the moisture content of the skin ($p < 0.05$). This case can be attributed to use of a combination containing PSO, GSO, SO with occlusive effect which reduce the evaporation of skin moisture and honey with humectant effect (moisture absorbing) in the composition of the formulation.

Sebum has amphiphilic properties due to its free fatty acids and waxes content. This causes a little hydration of skin. It is protective against intensive dehydration in the skin. Furthermore, it has a nutrient function for the useful bacterial species in the organism, while it ensures protection of fungistatic activity and the functional quality of hair.[35]

Sebum secretion rate reaches its highest level in the teenage years and decreases gradually thereafter. Sixty-five and older people, have a very low rate of sebum secretion. This reduction in sebum production accompanied by a decrease in antimicrobial fatty acids lead to the formation of skin infections in the elderly.[35]

According to measurements taken from volunteers by Sebumeter® SM 815, while the skin sebum content ($mg/cm^2$) was found 1.74 before application of the product, it was found to be 2.07 and 3.42 after 4 and 8 weeks of application respectively. The results revealed that application of the product for 8 weeks significantly increased the sebum content of the skin ($p < 0.05$). However, the normal sebum value in $mg/cm^2$ should be $> 6$ in the forearm where the product was applied. When taking into consideration this case, it is considered that the product may return sebum secretion to normal which decreas over time and is ideal for use in anti-aging.

The skin is a complex structure with elastic and viscous properties. These viscoelastic properties of the skin are dependant to collagen and elastin fibers in the dermis.[36] Cutometer probe of the Aramo TS skin analyzer device was used to measure skin elasticity which had a measurement principle based on measuring the skin deformation as a result of a vertical vacuum that was applied to the skin surface. While the elasticity value of the skin was 43.13 before the application of the product (T0), the elasticity value of the skin was found to be 58.31 and 62.25 after 4 and 8 weeks of application respectively in the measurements taken from the volunteers. The results revealed that application of the product for 4 and 8 weeks significantly increased the elasticity of the skin ($p < 0.05$).

The skin is covered with an acid mantle consisting of secretion of sweat and sebaceous glands. Acidic pH and natural flora of the skin plays an important role in maintaining healthy skin. Some external factors, such as soap, detergents and cosmetics can alter the normal pH of the skin. Changes in skin pH may lead to irritation, or inhibition of keratinization process.[37] According to measurements taken from volunteers by SKIN-pH-METER 900®, while the skin's pH value was 5.32 before the application of the product (T0), it was found to be 5.27 and 5.30 after 4 and 8 weeks of application respectively. The results revealed that application of the product for 8 weeks did not have skin irritation effects on the skin ($p < 0.05$).

Clinical signs of dry skin are roughness, redness, flaking and superficial skin loss. [38] Moisturizing and emollient products are gaining increasing importance in dry skin treatment, maintenance of daily care of normal skin as well as ancillary therapy of many skin diseases. Moisturizers smooth and hydrate the skin. Effectiveness of the moisturizers can be increased according to the targeted skin condition by selecting the proper moisturizing agents.[39] To this end, x60 triple lens of the Aramo TS skin analyzer was used to determine the average pore size and roughness of the application area. While the skin's average pore size and roughness value was 34.66 and 81.58 respectively before the application of the product (T0), it was found to be 19.83 and 73.50 after 4 weeks respectively and 15.33 and 64.17 respectively after 8 weeks of application. The obtained measurement results revealed that application of the product for 4 and 8 weeks has significantly decreased the elasticity of the skin ($p < 0.05$).

## Acknowledgements

This study is supported by Istanbul University Scientific Research Projects (Project number: 6393).

## References

[1] H. Assaf, M.A. Adly, M.R. Hussein, Aging and intrinsic aging: pathogenesis and manifestations, in M.A. Farage, K.W. Miller, H.I. Maibach, (Ed.), Textbook of aging skin, (Berlin: Springer, 2010) 129-138.

[2] F.C. Lau, M. Bagchi, S. Zafra-Stone, D. Bagchi, The benefits of antioxidant-rich fruits on skin health, in A. Tabor, R. Blair, (Ed.), Nutritional cosmetics, (Oxford: Elsevier, 2009) 217-232.

[3] N. Wilson, Market evolution of topical anti-aging treatments, in N. Dayan, (Ed.), Skin aging handbook: an integrated approach to biochemistry and product development, (NY: William Andrew 2008) 16-31.

[4] G. Yener, J. Hadgraft, W.J. Pugh, Penetration of two sunscreens in various formulations through a synthetic membrane as compared to human skin in vitro, Acta Pharmaceutica Turcica, 40(1), 1998, 27-32.

[5] M.A. Zaid, F. Afaq, D.N. Syed, H. Mukhtar, Botanical antioxidants for protection against damage from sunlight, in A. Tabor, R. Blair, (Ed.), Nutritional cosmetics, (Oxford: Elsevier 2009) 162-177.

[6] J. Shi, J. Yu, J.E. Pohorly, Y. Kakuda, Polyphenolics in grape seeds-biochemistry and functionality, Journal of Medicinal Food, 6(4), 2003, 291-299.

[7] M.N. Aslama, E.P. Lansky, J. Varani, Pomegranate as a cosmeceutical source: Pomegranate fractions promote proliferation and procollagen synthesis and inhibit matrix metalloproteinase-1 production in human skin cells, Journal of Ethnopharmacology, 103(3), 2006, 311-318.

[8] S. González, M. Fernández-Lorente, G.C. Yolanda, The latest on skin photoprotection, Clinics in Dermatology, 26(6), 2008, 614–26.

[9] S. Sharma, I.P. Kaur, Development and evaluation of sesamol as an antiaging agent, International Journal of Dermatology, 45(3), 2006, 200-208.

[10] R.R. Korać, K.M. Khambholja, Potential of herbs in skin protection from ultraviolet radiation, Pharmacogn Rev, 5(10), 2011, 164–173.

[11] X.H. Gao, L. Zhang, H. Wei , H.D. Chen, Efficacy and safety of innovative cosmeceuticals, Clinics in Dermatology, 26(4), 2008, 367-374.

[12] A.B. Jull, A. Rodgers, N. Walker, Honey as a topical treatment for wounds, Cochrane Database Syst Rev, 1(4), 2008, 1-48.

[13] M.V. Martos, Y.R. Navajas, J.F. L´opez, J.A. P´erez-´Alvarez, Functional properties of honey, propolis, and royal jelly, J Food Sci, 73(9), 2008, 117-124.

[14] A. Sharif, N. Akhtar, M.S. Khan, B. Menaa, B.A. Khan, F. Menaa, Development and optimization of dimethicone-based cream containing muscat Hamburg grape extract: In-vitro evaluation, J Pharma Care Health Sys, 1(1), 2014, doi:10.4172/jpchs.1000107.

[15] M.I. Mohamed, Optimization of chlorphenesin emulgel formulation, The AAPS Journal, 6(3), 2004, 1-7.

[16] M.J. Rosen, J.T. Kunjappu, Surfactants and Interfacial Phenomena (Hoboken, NJ:John Wiley & Sons, 2012).

[17] L.L. Schramm, Emulsions, Foams, and Suspensions: Fundamentals and Applications (Weinheim: Wiley-Vch Verlag, 2005).

[18] F. Shakeel, W. Ramadan, M.A. Ahmed, Investigation of true nanoemulsions for transdermal potential of indomethacin, Journal of Drug Targeting, 17(6), 2009, 435–441.

[19] D.S. Bernardi, T.A. Pereira, N.R. Maciel, J. Bortoloto, G.S. Viera, G.C. Oliveira, et al. , Nanoemulsions containing rice bran oil: in vitro and in vivo assessments, Journal of Nanobiotechnology Formation and stability of oil-in-water, 9(1), 2011, doi: 10.1186/1477-3155-9-44.

[20] J. Knowlton, S. Pearce, Handbook of Cosmetic Science and Technology (Oxford: Elsevier Advanced Technology, 1993).

[21] G. Yener, Cosmetic Carrier Systems, in Y. Yazan (Ed.), Kozmetik Bilimi, (İstanbul: Nobel Tıp Kitabevleri, 2004) 233-252.

[22] N. Çelebi, Emulsions, in F. Acartürk, İ. Ağabeyoğlu, N. Çelebi, T. Değim, Z. Değim, T. Doğanay, S. Takka, F. Tırnaksız (Ed.), Modern Farmasötik Teknoloji, (Ankara: Fersa Matbaacılık, 2007) 277-299.

[23] H.A. Lieberman, M.M. Rieger, G.S. Banker, Pharmacetical dosage forms: Disperse systems (New York: Marcel Dekker, 1998).

[24] Y.S. Cheng, K.W. Lam, K.M. Ng, R.K. Ko, C. Wibowo, An integrative approach to product development-A skin-care cream, Computers & Chemical Engineering, 33(5), 2009, 1097-1113.

[25] V. Nazarenko, A. Nych, B. Lev, Crystal structure in nematic emulsion, Phys Rev Lett, 87(7), 2001, doi: 10.1103/PhysRevLett.87.075504.

[26] Y. Xuan, D. Ma, M. Zhou, M. Gao, Significance of polymer on emulsion stability in surfactant-polymer flooding, J. Appl. Polym. Sci., 132(26), 2015, doi: 10.1002/APP.42171

[27] T.S. Issa, B. Philippe, H. Raymond, H. Michel, D. Jacques, Improved kinetic parameter estimation in pH-profile data, Int. J. Pharm, 198(1), 2000, 39-49.

[28] A. Rasul, N. Akhtar, B.A. Khan, T. Mahmood, S. Uz Zaman, H.M. Khan, Formulation development of a cream containing fennel extract: in vivo evaluation for anti-aging effects, Pharmazie, 67(1), 2012, 54-8.

[29] A.R. Rahatea, J.M. Nagarka, Emulsification of vegetable oils using a blend of nonionic surfactants for cosmetic applications, Journal of Dispersion Science and Technology, 28(7), 2007, 1077-80.

[30] S. Buchmann, Main cosmetic vehicles, in A.O. Barel, M. Paye, H.I. Maibach (Ed.), Handbook of Cosmetic Science and Technology, (New York: Marcel Dekker, 2001) 145-169.

[31] S.V.W. Sutton, M.A. Magee, D.K. Brannan, Preservative efficacy, microbial contenti and disinfectant testing, in D.K. Brannan (Ed.), Cosmetic Microbiology: A Practical Handbook, (Florida: CRC press LLC, 1997) 96-117.

[32] DK. Brannan, An preservation of personal care products, in F.F. Morpeth (Ed.), Preservation of Surfactant Formulations, (Dordrecht: Springer Science Business Media, 1995) 147-184.

[33] T. Miller, Clinical testing to uphold an anti-aging claim, in N. Dayan (Ed.), Skin aging handbook: an integrated approach to biochemistry and product development, (NY: William Andrew, 2008) 364-385.

[34] V. Couturaud, Biophysical characteristics of the skin in relation to race, sex, age, and site, in A.O. Barel, M. Paye, H.I. Maibach (Ed.), Handbook of Cosmetic Science and Technology (New York: Informa Healthcare USA, 2009) 5-24.

[35] P.W. Wertz, Changes in epidermal lipids and sebum secretion with aging, in N. Dayan (Ed.), Skin aging handbook: an integrated approach to biochemistry and product development (NY: William Andrew, 2008) 92-101.

[36] C. Galzote, M. Suero, R. Govindarajan, Noninvasive evaluation of skin in the cosmetic industry, in K.A. Walters, M.S. Roberts (Ed.), Dermatologic, Cosmeceutic, and Cosmetic Development Therapeutic and Novel Approaches (New York: Informa Healthcare, 2008) 467-486.

[37] S.A. Ansari, Skin pH and skin flor, in A.O. Barel, M. Paye, H.I. Maibach (Ed.), Handbook of Cosmetic Science and Technology (New York: Informa Healthcare USA, 2009) 221-232.

[38] D. Rudikoff, The effect of dryness on the skin, Clinics in Dermatology, 16(1), 1998, 99-107.

[39] N. Kaul, M. Sinaisky, E. Kohoot, Skin moisturizer's: Therapeutic potential and preventative maintenance of dry skin, American Academy of Dermatology, 58(2), 2008, AB51.