DESIGN OF NEW CREAM FORMULATIONS AND ASSESSING THEIR EFFECTIVENESS ON WOUND HEALING BY USING *IN-VIVO* ANIMAL MODEL

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To my precious family…

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ABSTRACT

Some of the beneficial ingredients that are traditionally used in wound healing are obtained from some plants, but their effectiveness has not been scientifically evaluated yet. In this study, cream formulations (levant storax and complex creams) with the same cream base (placebo cream) were developed. Addition to the ingredients used in the cream base, levant storax cream contained balsam of oriental sweet gum, while complex cream contained calendula oil, St. John's wort extract, escin, freeze dried powder of *Aloe vera* (L.) Burm.f. leaf juice and allantoin. Following the development of cream formulations of levant storax, complex and placebo, the characterization and stability tests were performed at predetermined time and conditions. The stability studies indicated that, physical properties of all formulations were unchanged during the test period of two months. In order to analyse the concentrations of herbal actives, hyperoside and escin, HPLC methods were studied and validated. The validated test methods were also suitable to test concentration of hyperoside and escin in complex cream. The wound healing potential of levant storax and complex creams were tested against a reference cream Madecassol®, negative control and placebo cream by employing an *in-vivo* excision wound model on rats. The studies indicated that levant storax cream treated rats had the best healing rates compared to all the other groups, whereas the group that was treated with complex cream showed a better healing rate than control and placebo groups. However no significant difference was found between the complex and the reference groups.

Key words: Wound cream, stability of cream, *in-vivo* excision wound model, wound healing, balsam of oriental sweet gum, hyperoside, escin, calendula oil.

ÖZET

Bitkilerden elde edilen birçok yararlı bileşik yara tedavisinde kullanılmaktadır; fakat geleneksel olarak kullanılan bu bileşiklerin yararlı etkileri bilimsel olarak açıklanmamıştır. Bu çalışmada, aynı krem bazı kullanılarak yeni krem formülasyonları (levant storax ve complex krem) geliştirilmiştir. Krem bazında kullanılan maddelere ilave olarak levant storax kremi sığla ağacından elde edilen balsam (sığla yağı), complex krem ise kalendula yağı, essin, sarı kantaron ekstraktı, *Aloe vera* yapraklarında bulunan sıvının dondurulup kurutulmasından elde edilen toz madde ve allantoin içermektedir. Levant storax, complex ve placebo kremlerinin geliştirmesinden sonra, karakterizasyon çalışmaları ve önceden belirlenmiş koşullarda ve sürelerde stabilite çalışmaları yapılmıştır. Stabilite çalışmaları sonucunda kullanılan bu formüllerin fiziksel özelliklerinin belirlenmiş bu koşullarda ve iki ay süre ile anlamlı bir değişikliğe uğramadığı gözlenmiştir. Ayrıca, hiperozit ve essin analizleri için HPLC test yöntemleri çalışılmış ve validasyonları yapılmıştır. Aynı test yöntemlerinin krem içerisindeki essin ve hiperozit miktarlarının tayini içinde de uygun olduğu gösterilmiştir. Levant storax ve complex kremlerinin yara iyileşmesi üzerindeki etkisi ise, referans ilaç Madecassol®, negatif kontrol ve krem bazı ile (placebo krem) karşılaştırmalı çalışmalarla, sıçanlarda *in-vivo* eksizyonel yara modeli kullanılarak incelenmiştir. *In-vivo* çalışmalar sonucunda levant storax kreminin diğer kremlere göre yara iyileşmesi süreci üzerinde en etkili formülasyon olduğu gözlenmiştir. Complex krem ise kontrol ve placebo grubuna göre daha etkili bulunurken, referans krem ile arasında anlamlı bir fark gözlenmemiştir.

Anahtar kelimeler: Yara kremi, kremin stabilitesi, *in-vivo* eksizyon yara modeli, sığla yağı, hiperozit, essin, kalendula yağı.

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ABBREVIATIONS

TEWL: Trans Epidermal Water Loss

MMPs: Matrix Metalloproteinases

ECM: Extra Cellular Matrix

MDT: Maggot Debridement Therapy

PVP-I: Povidone Iodine

DS: Dakin's Solution

SS: Silver Sulfadiazine

B: Bacitracin

G: Gentamicin

N: Neomycin

M: Mupirocin

O/W: Oil in Water

W/O: Water in Oil

W/O/W: Water-in-Oil-in-Water

O/W/O: Oil-in-Water-in-Oil

HLB: Hydrophilic-Lipophilic Balance

PIT: Phase Inversion Temperature

ICH: International Conference on Harmonisation

EMA: The European Medicines Agency

WHO: The World Health Organisation

RH: Relative Humidity

INCI: The International Nomenculture of Cosmetic Ingredients

SDA: Saborud Dextrose Agar

TSA: Tryptic Soya Agar

HPLC: High-Performance Liquid Chromatography

GC-MS: Gas Chromatography–Mass Spectrometry

1 INTRODUCTION AND THE AIM OF THE STUDY

Wound types are very diverse from acute to chronic wounds, also reason why the wound occurred differs from disease related to traumatic. Depending upon the type of the wound, wound management treatment method and the products used for this varies as well. As summarized by a market intelligence company, MedMarket Diligence LLC, prevalence of the wound types is rather different (Table 1). Surgical wounds have the highest prevalence and require some wound management treatment and closure materials such as sutures, staples, tapes are used, sometimes together with hemostasis (blood clotting) promoters, fabric bandages and surgical dressings. Lacerations are also common, generally minor in nature and require only cleansing and dressing for a shorter period. Lacerations can usually be treated by a doctor in outpatient medical center, or hospital accident and emergency department, but sometimes care is given only at home. Similarly some of the burn wounds are treated at home never entering the formal health care system. Approximately 3.4 million patients with burn wounds receive some level of medical care and treated with some professional wound care products or, alternately, with consumer-based products (1).

Chronic wounds generally take longer to heal, and care required is enormously variable, as is the healing time. Pressure ulcers, venous and diabetic ulcers require treatment every year. As part of the treatment process of chronic wounds, underlying problem has to be considered as well. Chronic wounds are growing in incidence due to the increasing numbers of elderly people in the population.

Wound healing is a complex biological process which starts from the moment of injury and it continues at varying time length depending on the severity of the wound. The healing cascade is divided into four phase; hemostasis phase, inflammatory phase, proliferative phase and maturational or remodeling phase.

Since ancient times, people have used plants and herbal preparations in order to accelerate the wound healing process. Often their use is merely based on tradition, without any scientific evidence of efficacy and little knowledge about active compounds' properties or their mode of actions. Some of the many potentially beneficial natural and traditionally used products include *Aloe vera* gel, calendula oil, horse chestnut and St. John's wort extracts, allantoin and Levant storax.

Aim of the present study was to formulate stable wound healing creams and to evaluate the wound healing potential of these formulations by using *in-vivo* excision wound model on rats.

The balsam (Levant storax), produced by injuring the oriental sweeet gum trees was thought to be effective in wound healing and widely used traditionally for this purpose. In the sout-eastern part of Turkey, people use Levant storax orally to treat their gastric ulcer or topically for many different kinds of wounds. Although some studies were conducted to see the healing properties of Levant storax for gastric ulcers, there was no study available to demonstrate its effects on wounds (2, 3, 4, 5, 6).

Topically used creams do contribute to wound healing process in many ways: as a base to carry the active ingredients, as a delivery system to facilitate the penetration of the actives through the skin, and also as a barrier against TEWL (Trans Epidermal Water Loss) which in turn facilitate wound healing.

Table 1 Worldwide Wound Prevalence by Etiology, 2009 (1).

Wound Type	**Worldwide Prevalence (Mlns)**	**Healing Time (Days)**	**CAGR 2007-2016**
Surgical wounds	110.3	14	3.6%
Traumatic wounds	1.6	28	1.7%
Lacerations	20.4	14	1.2%
Burn wounds (outpatient)	3.4	21	1.0%
Burn wounds (medically treated)	6.5	21	1.3%
Burn wounds (hospitalized)	0.2	50	1.1%
Pressure ulcers	8.5	–	6.9%
Venous ulcers	12.5	–	6.7%
Diabetic ulcers	13.5	–	9.3%
Amputations	0.2	–	1.2%
Carcinomas	0.6	14	3.0%
Melanoma	0.1	14	3.2%
Complicated skin cancer	0.1	28	3.1%

An important part of this study was designing stable cream formulations which contain selected botanical extracts with wound healing properties. Therefore characterization and testing the stability of the creams was also planned.

The expected contribution of this study was development of stable creams with healing properties. Healing properties of the creams were planned to be demonstrated by *in-vivo* animal testing.

2 GENERAL INFORMATION

2.1 EMULSIONS

2.1.1 Introduction

Historically, cosmetic emulsions represent the oldest examples of emulsions. Emollient and cosmetic creams have been known for thousands of years and invention of cold cream is ascribed to the Greek Physican Galen in the second century. Nowadays, emulsions find wide applications in many different fields ranging froom foods, cosmetics, pharmaceuticals, to polishes, and points (7).

Up to today, many different definitions have been made to describe an emulsion, many of which have common parts, while some of the important features are not mentioned by all. One of the very compressive and generally accepted emulsion defination was given by Becher: ‘An emulsion is a heterogeneous system, consistig of at least one immiscible liquid intimately dispersed in another in the form of droplets whose diameters, in general, exceed 0.1 μm. Such systems possess minimal stability which may be accentuated by such additives as surface-active agents, finally divided solids, etc.’ (7).

Generally, the two immiscible liquid phases are classified as oil (lipophylic) and aqueous (hydrophylic) phase. There are two kinds of simple emulsions such as, oil in water (O/W) emulsion in which oil droplets are dispersed in an an aqueous continuous phase and water in oil emulsion (W/O) in which water droplets are dispersed in oily continuous phase. These systems are thermodynamically unstable collodial systems and require the addition of at least a third component called an emulsifier, such as a surface-active agent, to impart stability to the system (7).

Also, multiple emulsions are defined; for example, water-in-oil-in-water (W/O/W) emulsions and oil-in-water-in-oil (O/W/O) emulsions. In order to stabilize multiple emulsions, two emulsifiers are invariably used, with a hydrophobic emulsifier designed to

stabilize the interface of the W/O internal emulsion and a hydrophilic emulsifiers to stabilize the external interface of the oil globules for W/O/W multiple emulsions, and the converse is true for O/W/O multiple emulsions. Multiple emulsions contain more interface that needs stabilization, therefore they are thermodynamically more unstable than the other simple emulsions (8, 9).

2.1.2 Advantages of Emulsions

The advantages and common uses of emulsions can simply be listed as follows:

- Compared to solid pharmaceutical forms, the active materials absorption could be increased.
- Active material penetration can be enhanced or prolonged, in other words controlled (10,11).
- Absorbtion of some macromolecules can be made possible like peptides.
- Stability of some actives could be increased when formulated in an emulsion form (12, 13, 14).
- Emulsions are used as adjuvants for vaccines or other sub-cutaneously used antigens and are protein like albumin (12).

2.1.3 Emulsion Applications

Emulsions are used mainly in many major chemical industries such as:

- Personal care and cosmetic emulsions: For examples of such cosmetic emulsion are lotions, creams, spray and sunscreens (7, 15).
- Pharmaceutical emulsions: As drug delivery systems, emulsions enhance or prolong drug absorption or deliver the drug to the specific sites. O/W emulsion anesthetics, lipid emulsions, topical creams are common examples. (7, 15).
- Food emulsions, such as mayonnaise, salad dressings, beverages and deserts (15).
- Agricultural sprays: The emulsions are used as insecticides for plants and animals (7).

- Paints, such as emulsions of alkyd resins and latex emulsions (15).
- Asphalt emulsions: These emulsions are employed in road construction, in roofs and floors, for paper and fabric impregnation, electrical and heat insulation, and as binders for other insulting materials, such as cork or asbestos (7).

2.1.4 Surface Activity

In the bulk of a liquid, the molecules are close to each other and short-range attractive forces (van der Waals) exist between molecules. Since these forces are equal on all directions, attractive forces are balanced. Also attractive forces are sufficiently great to keep all molecules together except for some molecules escaping into the air (vapour state). The molecules in the surface region are subject to unbalanced attraction, since they are not surrounded by other molecules from all sides, therefore the net effect of the forces will be directed inwards normal to the surface. The smaller the surface, the lower is the net force, meaning that lower the surface area lower is the energy required to keep the molecules together. This explains the existance of the surface tension.When two immisible liquids are placed together, an interface results. The net attractive forces affective in the interface is different than simple surfaces van der Waals' forces effective in both liquids are different, also the van der Waals' interaction between the two different liquids play a role. Due to the adhesive forces between two different molecules and the cohesive forces between bulk molecules, the interfacial tension lie between the individual surface tension. The force causing each liquid to resist breaking up into smaller globules is due to the interfacial tension, therefore an energy is needed to emulsify the two immiscible liquid (16, 17, 18).

2.1.5 Emulsifier

Emulsifiers are compound whose molecule exhibits both hydrophilic (water-loving) and lipophilic (oil-loving) characters (19). The hydrophilic group (polar group) contain heteroatoms such as O, S, N, P as part of some functional group such as carboxylate, sulfonate, sulfate, ammonium, phosphate, etc. Nonionic polar groups such as –OH group, which are not very polar, can easily hydrogen-bond to enhance water solubility of the entire molecule (17,19). The lipophilic groups are made of long hydrocarbon chains which contain

ten or more carbon atoms (17). Nonpolar (lipophilic) chain associates itself with oily materials, while the polar head group is attracted to water (19). Emulsifier located at the interface between the two phases (Figure 1) cause reduction in interfacial tension. By reducing the tension, emulsifier allows to disperse those two immiscible liquids (20).

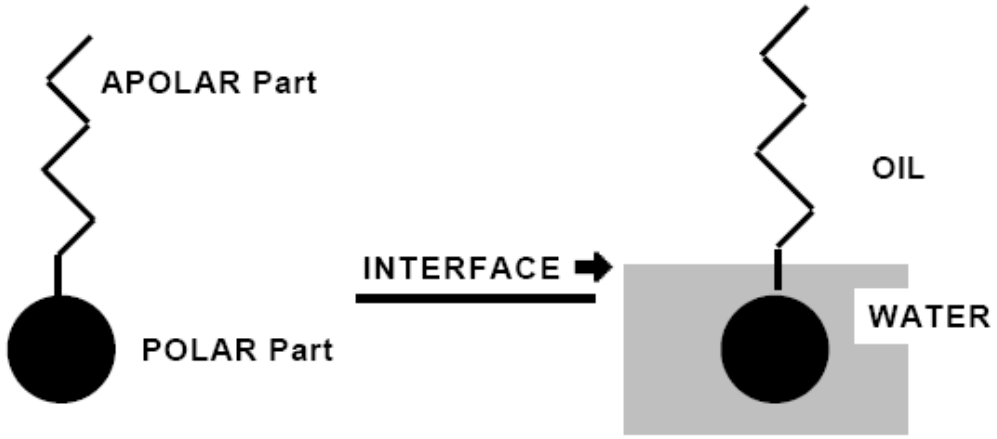

Figure 1 Positioning of emulsifier molecule at interface (17).

2.1.5.1 Classification of Emulsifiers

Emulsifiers are grouped by their different features. One of the grouping is based on where or how the emulsifier positioning itself at the interface. According to this criteria, emulsifiers are grouped into three (Figure 2):

1. Surface active agent: adsorbed at oil/water interface to form monomolecular film to reduce the interfacial tension, for example; potassium laurate, Tween 80.
2. Hydrophilic colloids: forming a multimolecular film around the dispersed droplet, for example; acacia gelatin.
3. Finely divided solid particles: they are adsorbed at the interface between two immiscible liquid phases to form particulate film, for example; kaolin (12).

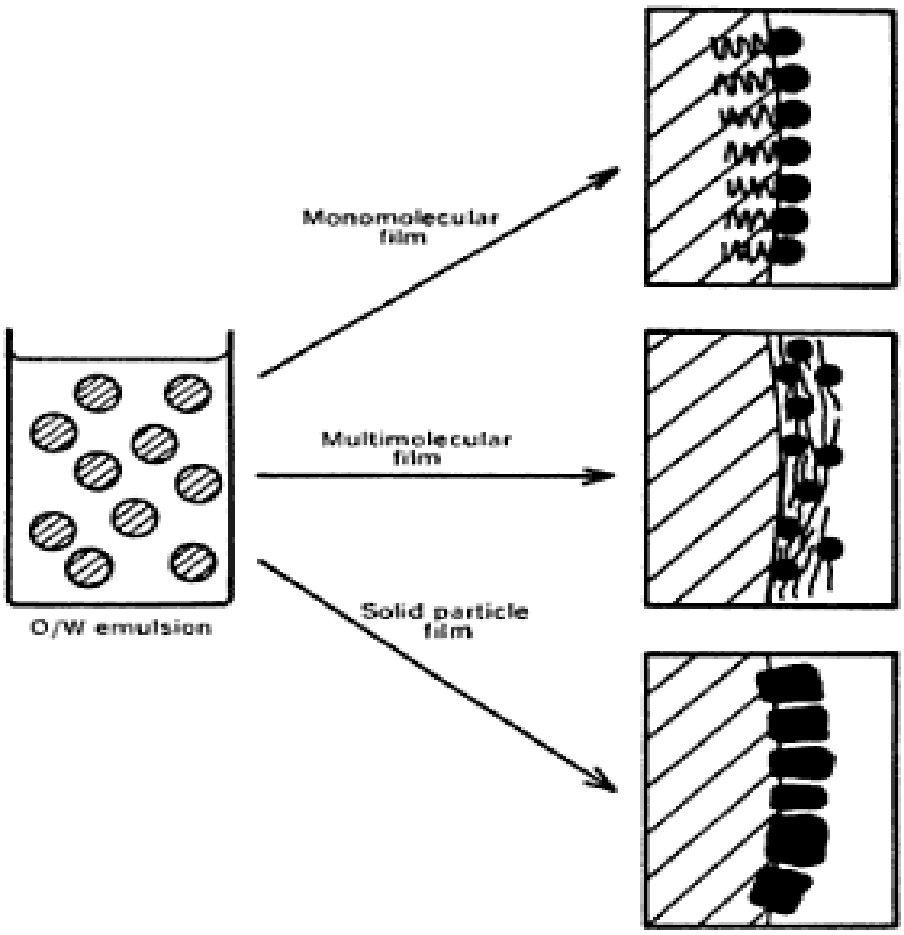

Figure 2 Types of films formed by emulsifying agents at the oil-water interface (12).

Another grouping is based on the ionic properties of the hyrophilic group in the molecule;

1. Anionics (negative charged emulsifiers, for example; Alkali salts),
2. Cationics (positive charged emulsifiers, for example; Quaternary ammonium compounds)
3. Nonionics (no charged emulsifiers, for example; Tween; polyethylene fatty acid ester) and
4. Amphoterics (cationic in acidic solutions and anionic in basic solutions, for example; lecithin) (19).

2.1.6 Choice of the emulsifier

Depending upon which type of droplet is stabilized, i.e. water droplet in the oil phase, or oil droplet in the water phase, W/O or O/W emulsions are produced. Indeed, an emulsifier is more efficient to stabilize W/O emulsions if it possesses a higher solubility in water or vice versa. The phase in which an emulsifier is more soluble constitutes the continuous phase, this generalization is known as Bancroft's rule (20). However, most useful system for the selection of emulsifier is Griffin's HLB (Hydrophilic-Lipophilic Balance) system in which non-ionic emulsifier have been classified on a scale from 0 to 20. In this system, each emulsifier have a specific HLB number. Emulsifiers with a low HLB number (<10) are lipophilic emulsifiers stabilizing W/O emulsions, whereas emulsifiers with high HLB number (>10) are hydrophilic emulsifiers in favour of O/W emulsions (21). Emulsifier whose HLB number is 10, is balanced hydrophilically-lipophilically. Numerous methods have been developed to calculate the HLB number of emulsifiers (22, 23, 24).

2.1.7 Method of emulsification

Mode of addition of the emulsifier affects droplet size and the type of final emulsion. There are four different ways of preparing the emulsion depending on the mode of addition of emulsifier (7).

Agent-in-water method: In this method, the emulsifier is dissolved directly in the water phase and then, during mixing properly, the oil is added. This procedure is used for formation of O/W emulsions. Adding additional oil into the emulsion, phase inversion takes place and this produces W/O emulsion.

Agent-in-oil method: In this method, the emulsifier is dissolved directly in the oil phase and then, the water phase is added. Normally, this procedure yields W/O emulsions directly. If O/W is desired, phase inversion should be induced by addition of further water.

Alternate addition method: In this method, both the oil and the water phases are added alternatively in small portions to the emulsifier. This method is particularly suitable for the preparation of cosmetic and food emulsions, like mayonnaise and other vegetable oil emulsions (7,18).

Nascent soap (in situ) method is suitable for emulsions which are stabilized by soaps. By using in-situ method both types of emulsions are produced, fatty acid is dissolved in the oil phase, and alkanline part in the water phase, when two phases are mixed together, a soap is formed at the interface to stabilize the emulsion (7,25).

2.1.8 Emulsification

Energy input is necessary in order to produce emulsion. The energy is required to divide droplets into small droplets and in order to protect them against coalescence. Most common mechanical devices used to prepare emulsions are rotor-stator disperser, colloid mill., single-stage homogenizer and sonolater (Figure 3). In addition to mechanical processes, some physicochemical methods are used to produce emulsions by phase inversion, phase inversion temperature condensation or electric emulsification (7, 26, 27).

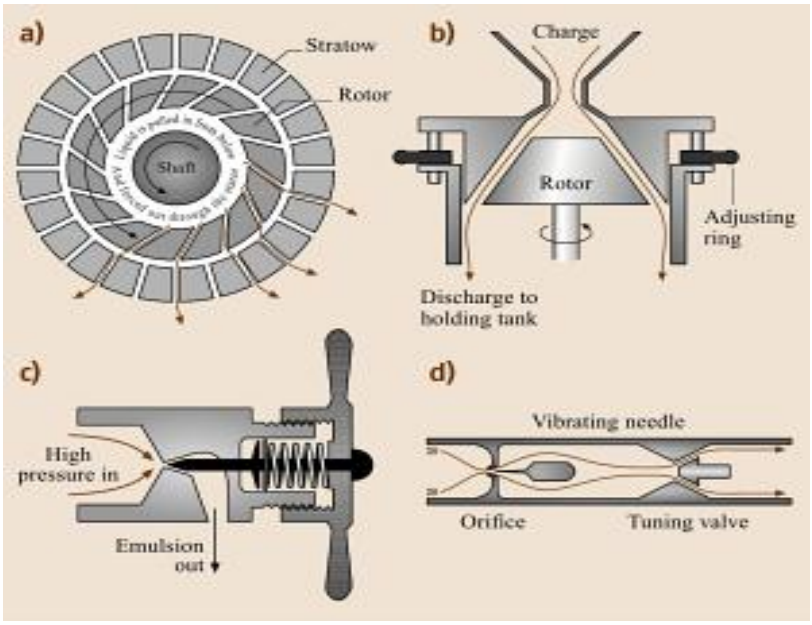

Figure 3 Mechanical devices for making emulsions: (a) Rotor-stator disperser, (b) colloid mill. (c) single-stage homogenizer, (d) sonolater (26).

2.1.9 Mechanism of emulsion instability

Emulsions are thermodinamically unstable and oil and water phase intent to go a lower energy state and phase separation occurs in time. Emulsions are seperated because of several break down processes (28).

'Break down' processes may be divided into six (Figure 5):

- Creaming
- Sedimentation
- Phase inversion
- Flocculation
- Coalescence
- Ostwald ripening

2.1.9.1 Creaming & Sedimentation

In process, because of differences in density of between continous phase and dispersed phase, sedimentation and creaming takes place. Emulsion separate into two emulsion, one of which is richer in dispersed phase, the other poorer than the original emulsion. The emulsion which is more concentrated emulsion is called the 'cream', and is located either at the top (creaming) or at the bottom (sedimentation) depending on the density of this phase. The instability condition can usually be reversed by simple mixing. Stoke's equation (Figure 4) is usually used to define the velocity of the creaming and sedimentation rate (29).

Stoke's equation

$$v = \frac{2r^2(\rho - \rho_0)g}{9\eta_0}$$

Figure 4 Stoke equation; v = rate of creaming or sedimentation, r = radius of droplets of dispersed phase, ρ, ρ_0 = density of dispersed and continuous phase respectively, g = gravitational rate constant, η = viscosity of continuous phase (29).

2.1.9.2 Phase inversion

The emulsions may change thier type from O/W to W/O, and vice versa, these conditions are called as phase inversion. Although reason of phase inversion is not well understood, some scientists believe that phase inversion is depended on the nature of the emulsifier, the emulsifier concentration, HLB value of emulsifier and concentration of dispersed phase (7).

2.1.9.3 Flocculation

In this case, because of the van der Waals forces, the droplets attend to produce clusters. Cluster's droplets number may vary in time. Flocculation of droplets can be reduced by creating energy barriers between droplets by using ionic or nonionic emulsifier or polymer. The instability condition can usually be reversed by simple mixing (30).

2.1.9.4 Ostwald ripening

In this case, large drops grow at the expense of those smaller because the chemical potential of the solute is higher in drops with greater interfacial area. Several methods may be applied to reduce ostwald ripening such as using the polymeric emulsifier (31, 32).

2.1.9.5 Coalescence

Coalescence is the irreversible fusion of two or more droplets to form a larger droplet. Coalescence will usually occur when a droplet approaches a fluid-fluid interface, where an interfacial film forms, drains to a certain thickness, and then ruptures. Film rupture is a statistical process whose probability depends on the frequency of surface fluctuations and on the elastic properties of the emulsifiers, used to form the interfacial film (31).

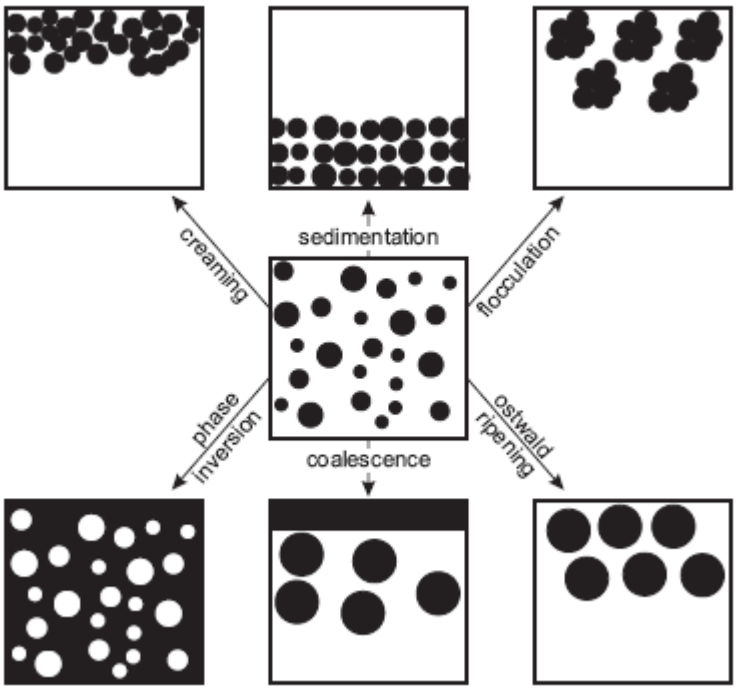

Figure 5 Schematic representation of the break-down processes in emulsions (30).

2.1.10 Auxiliary emulsifying agents (Co-emulsifiers)

In most of the cases, a fourth component becomes essential to maintain the stability of the emulsion droplets. These are called auxiliary emulsifying agents. These are those compounds which normally can not form an emulsion on their own but can function a supporting material to stabilize the emulsion. Eventhough small chain alkyl halides and amines could be used as auxiliary emulsifying agents, shortly channed n-alkanols are used widely. Short hydrophobic chain and terminal hydroxyl group of n-alkanols, makes it to interact with emulsifier

monolayers at the interface, affect the packing of the interface, and in turn influence the curvature of the interface and interfacial energy. They can promote oil solubility. In addition, formation of separate lipophilic network in the emulsions, n-alkanols increase the emulsions viscosity which will lead to a higher physico-chemical stability of the formulation (33, 34).

2.1.11 Preservation of Emulsions

Microbial contamination of emulsion may form because of contamination during manufacturing process, usage of impure raw materials, poor sanitation, contamination by consumer. In order to preserve the emulsions against microbial contamination and proliferation during storage in normal conditions and proper use, antimicrobial agents should be used. The preservative system must be effective and sufficient against a variety of pathogenic organisms in order to protect the product during its shelf-life and its use by consumer. The selected preservative must be less toxic and stable to heat and storage. Benzoic acid derivatives, benzalkonium chloride and cetyl trimethyl ammonium bromide are some of the examples of the antimicrobial agents used for emulsions (35, 36, 37).

2.1.12 Antioxidants

Emulsion oxidation is developed at the inteface between two phases by both the presence of pro-oxidant and the interactions between the different components within the emulsion. Many organic compounds come under autoxidation during exposure to air. Therefore emulsified lipids may particularly result in decomposition. The choice of the antioxidant molecules to add to the emulsion system is important because of the interfacial phenomena. According to the ‘Polar Paradox’ hydrophilic antioxidants are expected to concentrate at the oil-air interface and protect the lipids from oxidation. On the other hand, in oil in water emulsions, lipophilic oxidants locate preferentially in the oil-water interfaces and prevent lipid oxidation. Butylated hydroxyl toluene, gallic acid and ascorbic acid are some of the examples of the antioxidants (38, 39).

2.1.13 Characterization of Emulsions and Stability Testing

Emulsions is thermodinamically unstable, because of interfacial tension and gravity, water and oil phase form two layers one above another in time. In addition, stability of emulsion can be affected by environmental conditions such as; temperature, chemical, physical, microbiological stress. Physicochemical tests such as the determination of droplet size, viscosity, pH, conductivity, zeta potential of emulsions, and accelerated aging tests methods like centrifugation and freeze thawing have been traditionally used to assure the formulation of desired stability (7, 8, 15, 40).

2.1.13.1 Droplet-size distribution

In studies of stability, the change in the globule size distrubition with time is an important parameter. Because of the processes explained earlier, droplets of emulsions tend to form bigger droplets, therefore instability problems are emerged with time. There are four different methods in order to obtain information about the globule size distrubition; by microscopic observation (either direct or photographic), by sedimentation techniques, by light scattering measurements and by the instrumental counting (7, 40).

2.1.13.2 Viscosity

Viscosity of an emulsion is critical for many reasons during application. It gives a information about the flow properties of the emulsion; ease of application, spreadibility and skin feel. The viscosity of the continuous phase is critical due to its effects on the rate of flocculation and/or creaming. Droplet stability of moving towards each other can be greatly affected by the structure of the external phase; more viscous and network like structure increases the physical stability. Therefore the viscosity of an emulsion is important factor in determining its stability (7).

2.1.13.3 pH

Healthy human skin reported to have surface pH that ranges between 4-6 and a pH gradient exists within the skin. Therefore, the formulations intended for application to skin should have pH close to this range. The pH of emulsion has been reported to affect the extent of dissociation of ionisable ingredients and therefore the thermodynamic activity of the ingredients; partitioning and skin penetration. The pH changes during shelf life lead or guide the stability of emulsion, therefore the pH of an emulsion is an important characteristic also to determine its stability (41).

2.1.13.4 Conductivity

While aqueous systems are good conductors, most oils are poor conductors. Therefore conductivity measurement may serve to identify the external phase characteristics. In some cases, oil in water emulsions have poor conductivity because of nonionic emulsifiers used, addition of small amount of sodium chloride may help to solve the poor conductivity problem. It might also be noted that some W/O emulsions in which dispersed phase concentration is higher than sixty percent, may have moderately good conductivity (7). Conductivity also gives information about instability of the emulsions such as phase inversion.

2.1.13.5 Zeta potential

Electrostatic potential near the surface of droplets is called the *zeta potential.* The potential indicates if droplets are charged or non-charged and gives an information about stability of the emulsion. A high negative or positive zeta potential leads in the repulsion of the droplets among themselves and prevents droplets from folucculation. Droplets with zeta potential above 30 Mv (positive or negative) are normally considered stable, but density difference of phases and the pH value of the emulsion are important factors affecting the zeta potential and also the stability of emulsion (42).

2.1.13.6 Centrifugation

Centrifugation is one of the commonly used method to determine the stability of emulsion. The centrifugation is a relatively simple method and allows one to accumulate a large set of data for a relatively short period of time. For example, centrifugation at 3750 rpm (round per minute) in a 10 cm radius centrifuge for a period of five hours would represent effect of gravity for about one year (7).

2.1.13.7 Freeze and Thawing

Freeze thaw testing is a type of stability test in which you freeze your formula, then thaw it out, and test to see what result the process has on your emulsion. During freeze-thaw test, the test is repeated a few times, for example 3-5 cycles. To increase the environmental stress conditions even more, freeze-thawing followed by a storage at high temperature sort of cycling is also applied (43).

2.1.14 Determination of Emulsion Stability

The purpose of determination of emulsion stability is to ensure that a new emulsion meets the intended physical, chemical and microbiological quality standards when stored under appropriate conditions. Accelerated stability testing is performed at 25°C ±2°C with 60% ± 5% relative humidity, at 30°C ±2°C with 65% ± 5% relative humidity, at 40°C ±2°C with 75% ±5% relative humidity and +4-8°C (in refrigerator). For herbal preparations, which are not intended for storage at higher temperatures, some exceptions have been made and are set out in the 'Guideline on stability testing: stability testing of existing active substances and related finished products' (44, 45, 46).

In particular, temperature sensitive substances should be stored under an alternative, lower temperature condition which may then become the designated long term testing storage temperature. The long term and accelerated stability testing should be also continued for a sufficient period of time and frequency of testing should be sufficient to establish the stability characteristics of the emulsion. Examples of test conditions and intervals are summarized in

Table 2 (44, 45). Freeze/thaw stability test which involves cycling the product through 24 hours of freezing then 24 hours of thawing is also performed commonly. During stability studies, characteristic of the emulsion like droplet-size distribution, viscosity, pH, conductivity and zeta potential is monitored at pre-determined time intervals on the samples which are kept at normal and accelerated conditions (Table 2).

Table 2 Recommended test conditions and intervals (44).

	Storage condition	Frequency of testing
Long term stability	25°C ±2°C with 60% ± 5% relative huminity	0, 3, 6, 9, 12 th months
Accelerated stability	40°C ±2°C with 75% ± 5% relative huminity	0, 1, 2, 3 th months

2.2 WOUNDS

2.2.1 Wound Definations

The term **wound** has been defined as a disruption of normal anatomical structure and, more importantly, function. Therefore, healing is a complex and dynamic process that results in the restoration of anatomical continuity and function (47). Table 3 summarizes main types of wounds and definitions used in wound management.

Table 3 Types and definitions of wounds.

Term	Definition
Wound	Disruption of normal anatomical structure and, more importantly, function (47).
Acute wound	Wounds refer to those wounds, such as burns, other traumatic injuries, and surgically created wounds, that heal in a timely fashion (48).
Chronic wound	Wounds, which have failed to proceed through an orderly and timely reparative process to produce anatomic and functional integrity over a period of 3 months (49).
Abrasion	Loss of superficial skin (usually epithelium) that exposes nerve endings resulting in a painful injury like burns.
Incision	Wounds caused by a sharp object such as a bistoury or scalpel that have no tissue loss and minimal tissue damage.
Ulcer	Wounds which have loss of an epithelial surface together with a variable degree of underlying connective tissue (50).
Surgical Excision	Wounds caused by a doctor or surgeon using a scalpel (sharp knife) or other cutting instrument and the wound are left open.

Depending of the extent of tissue loss, wound closure and healing occur by ***primary, secondary and tertiary intention***.

- Healing of a clean, uninfected surgical insicion in which skin adges are approximated by surgical sutures has been termed healing by ***primary intention,*** for example; surgical incisions (51).
- Healing of open wounds have more extensive loss of cells and tissue, as occurs in infarction, inflammatory ulceration, abcess formation is sometimes termed healing by ***secondary intention***, for example: chronic ulcers (51).
- Healing related to wounds that are usually infected or dehisced surgical wounds is termed ***tertiary intention.*** In such wounds, healing is promoted by leaving the wound open in order to avoid the contamination or infection and to allow for growth of new tissue before approximating the skin edges for a primary closure. Wounds managed with delayed closure are classified as healing by ***tertiary intention,*** for example: abdominal insicions (50, 51).

2.2.2 Phases of Wound Healing

Wound healing is commonly divided into four phases (50):

- The hemostasis phase,
- The inflammatory phase,
- The proliferative phase,
- The maturational or remodeling phase.

2.2.2.1 The hemostasis phase

Hemostasis is the first phase of wound healing. This phase occurs right after initial injury. At the time of the injury, usually bleeding occurs. Bleeding activates hemostasis. At the site of vascular injury, vasoconstriction is started immediately by neurogenic mechanisms and secretion of endothelin. This vasoconstriction prevents the further loss of blood while the fibrin clot forms a temporariyl seal over the injury site and prevent the influx of microorganisms.

After an injury, platelets are the first cells appear within injury site. Injury site exposes extracellular matrix proteins, such as fibrillar collagen, fibronectin and other adhesive proteins which allow platelets to adhere and become activated. During adhesion platelets undergo aggregation and at the same time release many mediators, such as seratonin, adenosine diphosphate, and tromboxane, and also release adhesive proteins, such as fibrinogen, fibronectin, thrombospondin, and von Willebrand factor VIII. These mediators and locally generated thrombin induce further platelet aggregation and secretion, and form the platelet plug. During platelet aggregation, thrombin converts soluble circulating fibrinogen to insoluble fibrin which in turn traps to form the physical entity of the hemostasis plug; this is the process of primary hemostasis (48, 50, 51).

The blood coagulation pathways are divided into extrinsic and intrinsic pathways, converging where factor X is activated (51). Platelet aggregation also triggered a specific enzyme in blood known as Hagemen factor XII, to initiate the intrinsic cagulation pathway. In this cascade, by activation of some proenzyme, prothrombin is converted to thrombin. In the mean time, extrinsic caugulation pathway is activated by tissue factor, a cellular lipoprotein exposed at sites of tissue injury. There are interconnections between intrinsic and extrinsic pathway. For example, a tissue factor-factor VIIa complex also activated factor IX in the intrinsic pathway (48, 51). In addition, platelets also produce cytokines, such as platelet derived growth factors that call in cells to participate in later phases of healing. For example, they are responsible for some processes including the synthesis of collagen, influx of fibroblasts and regulation of cell migration (52).

2.2.2.2 The inflammatory phase

The second phase of wound healing is inflammatory phase that starts immediately after haemostasis, and last about 4-6 days. Vasodilation follows the initial vasoconstriction that increase vascular permeability in response to histamine. Vasodilation allows the leakage of vascular fluid from intravascular space to the extravascular compartment (53). By vasodilation, neutrophils, lymphocytes, and monocyte migrate into injury site (50). Neutrophils predominant for the first few days and then disappear if the wound does not become infected. Neutrophil also initiate wound repair by activating local fibroblasts and epithelial cells. Later in inflammation, monocytes differentiate into macrophages and become major phagocytic cell at the injury site (48, 50). Both neutrophils and macrophages have surface receptors that permit them to recognize, bind and engulf foreign materials such as bacteria and tissue debris. After engulfing, bacteria and debris are digested by the inflammatory cells.

Beside the having phagocytic propety of macrophages, the cells synthesize some cytokines including growth factors. These are involved in the migration, proliferation and organization for tissue repair (54). Macrophages also produce special enzymes called matrix metalloproteinases (MMPs) such as collagenase and elastase into the wounded area. Collagenase plays an important role in wound debriment and shaping of connective tissue (50).

2.2.2.3 The proliferative phase

This phase is characterized by three critical changes in the injured site namely re-epithelization, neovascularization and granulation (50). During this phase, fibroblasts stimulate the production of collagen, which gives the tissue its tensile strenght and its structure (52). Wounds in a moist environment demonstrate a faster and more direct course of epithelization.

2.2.2.3.1 Re-epithelization

Re-epithelization is the process of covering of the surface of the skin defect with keratinocytes. Locally released growth factors stimulate keratinocytes to proliferate and begin their migration across the wound bed within 12 to 24 hours after injury. Migration requires a fluid environment and involves a series of activities. Several elements have been implicated in keratinocyte migration, including extracellular matrix, integrin receptors, matrix metalloproteinases (MMPs) and growth factors (48).

During migration, keratinocyte elongate itself in the direction of growth. By using surface integrin receptors, edge of elongated keratinocye bind to a new spot in the wound bed. This binding permit the keratinocyte to pull itself forward across the wound surfaces. Until the migrating cells from opposing sites of the wound touch each other, this process is repeated (48, 50). As mentioned earlier, keeping the wound base moist is very important, when the wound base is covered with dry exudate and scab migration does not take place, beacuse of the keranocytes produces proteolytic enzymes that provide it to burrow downword to find moisture (50). This explains well how occlusive or/and semiocclusive dressing or cream base help re-epithelization. Another migration process is leap frogging occurs when a single cell moves only 2 or 3 cell lengths and then stops, allowing consecutive cells to climb over. Proliferation of keratinocye is increased to ensure an adequate supply of cells to migrate and cover the wound. Epidermal growth factor, keratinocyte growth factor and TGF-α stimulate keratinocyte migration, proliferation and re-epithelization (50).

2.2.2.3.2 Neovascularization

Restoring the vascular network is stimulated by some factors, such as growth factors, high lactate levels, acidic pH and decreased oxygen tension; tissue hypoxia and this process called angiogenesis or neovascularization. Due to distruption of native vasculature and increased oxygen consumption by the cells in the wound environment, there are severe degree of hypoxia in the injured tissue.

Endothelial cells release platelet and macrophage-derivatived growth factors, such as fibroblast growth factor and vascular endothelial growth factor which stimulate angiogenesis along the wound edges. The new capillary vessels bud from intact vessels in the dermis, buds join to from loops, therefore establish blood flow within the wound (50). Similar to the migration of keratinocytes, endothelial cells at the tip of capillaries migrate into the wound but do not undergo active proliferation. Newly formed vessels join a granulation tissue formation providing nutrition and oxygen to growing tissue formation (48).

2.2.2.3.3 Granulation

The final phase of proliferation is the development of granulation tissue. Replacement of fibrin/fibronectin with granulation tissue matrix stars to appear about 4 days after injury. Granulation tissue which is rich in hyaluronan, fibronectin, and other extracellular matrix (ECM) compunds replaces the fibrin clot scaffold. During granulation, proliferation of variety of cells and proteins are supported. Fibroblasts are the predominant cell type in granulation tissue. The cells release collagen and variety of other substances, such as fibronectin, glycoaminoglycans hyaluronan, proteoglycans and elastin. These substance promote adhesion, and migration promote tissue strenght. Granulation tissue includes 19 different types of collagen. For example, skin collagen is 80% type I and 20% type III. The new granulation tissue contains type I, III and V collagen fiber (50).

2.2.2.4 The maturational or remodeling phase

During this phase, the loose granulation tissue differentiates into stable extracellular matrix. Collagen fibers reorganize, remodel and mature, and the wound gaining its final tensile strenght. Macrophages and fibroblasts which activate ECM-bound growth factor and MMPs play a vital role in this phase.

At the end of the granulation phase, myofibroblasts (differentiated fibroblasts) are activated and the wound starts to contract. With wound closure, Type III collagen undergoes degradation and type I collagen synthesis peak. This process is controlled synthesis of new collagen and lysis of old collagen by the actions of MMPs. The MMPs is controlled by tissue

inhibitors of metalloproteinases. There must be balance between tissue inhibitor of metalloproteinases and MMPs in wound remodeling process (48). During new connective tissue formation, fibronectin and hyaluronan are replaced, collagen bundles grow in site and strenght neovascularization stops, and metabolic activity within the ECM diminishes. The density of cells such as macrophages, keratinocytes, fibroblasts and myofibroblasts are reduced by apoptosis. The balance between the synthesis of new collagen and the degradation of old is also important to wound repair and remodeling. At the end of the remodeling phase, the new connective tissue matures and changes from pink-red to white color (50).

2.2.3 Topical Wound Care

2.2.3.1 Antiseptics

Antiseptics have a broad spectrum of activity against bacteria, fungi, viruses and protozoa. Several antiseptic categories exist, including alcohols (ethanol), anilides (triclocarban), biguanides (chlorhexidine), bisphenols (triclosan), chlorine compounds, iodine and silver compounds, peroxygens, and quaternary ammonium compounds (55, 56). The most commonly used antiseptics in clinical practice today include hydrogen peroxide and povidone iodine (56).

Hydrogen peroxide is commonly utilised as a wound antiseptic in the form of three percent solution. At this strenght, hydrogen peroxide solution displays *in-vitro* broad-spectrum efficacy. However, potential cytotoxicity of hydrogen peroxide, particularly to fibroblasts, over top its bactericidal effect (55).

Iodine has bactericidal and bacteriostatic effects. Polyvinyl–pyrrilidone iodophores (also known as polyvinyl–pyrrolidine–iodine, povidone iodine or PVP-I) and cadexomer iodine are the most common forms of iodine available for wound managements (56). Povidone-iodine is the form of iodine and polyvinyl/pyrrolidone combination. It is available in several forms (solution, cream, ointment, scrub) and the dosage forms is used as a range of 1% to 10% on wound surfaces (57). Cadexomer iodine consists of spherical hydrophilic beads of cadexomer-starch, which includes 0.9% iodine (w/w), available as an ointment (56).

Chlorhexidine is used as an antiseptic with broad-spectrum antimicrobial activity. It is best used at a concentration of 0.02% and commonly used as a surgical irrigation solution (57). Although it has no apparent independent effect on wound healing, it may favor improved healing times by limiting wound infection (55).

Acetic acid is frequently used as an antiseptic in wounds. As a 0.25% to 0.5% solution, it is bactericidal against many gram-positive and gram-negative organisms especially *Pseudomonas aeruginosa* which is sensitive to low-pH acidic environments (56).

Dakin's solution (DS) is frequently used for cleansing and reducing bacterial concentrations in wounds. Advantage that DS has over povidone iodine and chlohexidine is releasing chlorine and oxygen to kill *Staphylococcus aureus*. DS is toxic to fibroblasts. It is recommended that it should be diluted to one-quarter strength in order to use as a debridement (0.125%) (55).

2.2.3.2 Topical Antibiotics

In wound care, antibiotics are effective when applied within three hours after wounding. The most frequently used topical antibiotic agents in clinical practice today include Silver sulfadiazine (SS), Bacitracin (B), Gentamicin (G), Neomycin (N), Mupirocin (M) (55, 58, 59).

Silver sulfadiazine (SS) is used as a topical agent, prepared to combine oligodynamic effect of silver with antibacterial effect of sulfadiazine. Sulfadiazine has no antibacterial effect in low concentration but, sulfadiazine and silver have synergistic effect on antibacterial activity (60). SS has a good antimicrobial spectrum against some bacteria and fungi (55). It is especially used for burns and mild infections (58).

Bacitracin is bactericidal for a wide range of gram-positive bacteria and gram-negative bacteria (58). B inhibites bacterial cell-wall synthesis. Topically applied B results reduction in

the absorption, therefore systemic toxicity is rare (61). B may be used to prevent and to treat local infections, burn and operative wounds.

Gentamicin is bactericidal for a broad range of gram-positive and gram-negative bacteria G inhibites bacterial protein synthesis (58, 62). Because of hypersensivity and development of bacterial resistance to the antibiotic, it is suggested that G is used only when there are no treatment options and G should not be used prophylactically (61).

Neomycin is an aminoglycoside antibiotic which is bactericidal against most gram-negative bacteria except anaerobes and *Pseudomonas aeruginosa*. It is active against some gram-positive bacteria with the exception of streptococci. It works by binding with ribosomal RNA, thereby inhibiting protein synthesis (63). N controls the proliferation of bacteria on the wound surface (61). The incidence of hypersensitivity and systemic toxicity N itself is high. Bacterial resistance can be expected (63).

Mupirocin is obtained by fermentation of Pseudomonas fluorescens known as pseudomonic acid. It prevents bacterial protein synthesis by inhibition of activity of bacterial isoleucyl tRNA synthetase (58, 64). Mupirocin is highly effective against aerobic Gram-positive cocci especially *S. aureus*, *S. epidermidis*, and hemolytic streptococci, and some Gram-negative cocci. The efficacy of the cream formulation of mupirocin in mouse surgical models with primary and secondary wounds infected with *S. aureus* and *S. pyogenes* was recently evaluated (63).

2.2.3.3 Wound dressing products

The balance of moisture in the wound environment improves wound healing and can potentially enhance the rate of reepithelialization (50, 65). The balance of moisture is important to facilitate the action of growth factors, cytokines and migration of cells including fibroblasts and keratinocytes (66). Excess moisture in the wound bed can result in to periwound maceration. On the other hand, inadequate moisture in the wound bad promotes desiccation of wound, necrosis, and eschar formation, and impede the healing process (67). Since the evolution of the benefits of moist wound healing, a wide variety of new wound

dressing materials have been developed (66). The important criteria for optimal wound dressings include the ability to: absorb excess exudate and toxic components; facilitate autolytic debridement; maintain a moist wound environment; allow gaseous exchange; provide thermal insulation; provide a bacterial barrier; be not harm the wound; allow removal without trauma at dressing change; be comformable and be sterilizable (68). Wound dressing products can be grouped as alginates, films, foams, hydrogels and hydrocolloids.

2.2.3.4 Wound debridement

Debridement is commonly described as the removal of necrotic tissue, exudate, cellular waste, harmful exudate, and other metabolic wastes that impede wound healing (69). Debridement is considered a necessary component of wound bed preparation, especially in nonhealing wounds. This method provide prevention of wound infection because necrotic tissue and its elements may serve as a culture medium for bacteria. When dead tissue is removed, the wound may start to granulation phase. Several types of debridement are available, including surgical (sharp), mechanical, enzymatical, biological and autolytic (70).

2.2.4 Wound Healing Effect of Some Plants and Their Active Ingredients

Since ancient times, people have utilized plants and their preparations in order to treat their wounds. Often their use is merely based on tradition, without any scientific evidence of efficacy and little knowledge about default active compounds or their mode of actions. As wound healing is a complex biological process several *in vitro* and *in vivo* assays are available. Some of the many potentially beneficial natural products are obtained from some plants such as, *Aloe vera*, *Hypericum perforatum*, *Momordica charantina* L.*, Matricaria recutita* L., *Calendula officinalis*, *Aesculus hippocastanum*, *Symphytum officinale*, *Centella asiatica*, *Liquidambar orientalis* Mill..

2.2.4.1 Aloe vera

Aloe vera is a tropical plant belongs to the Lilliaceae family. It has beneficial effects in variety of diseases. Traditionally, *Aloe vera* gel has been used in the treatment of a variety of

disorders such as arthritis, gout, acne, dermatitis, etc. and of wounds such as peptic ulcers and burns (71). The gel extract of aloe vera has been reported to have various curative effects, such as wound, burn, and frost-bite healing, in addition to having antiinflammatory, antifungal, hypoglycemic, and gastroprotective properties. Of those claims, aloe vera's antiinflammatory and wound healing has been the most extensively studied. It was found to have antiinflammatory activity on carrageenan-induced edema in rat paws. Moreover, it was found to promote wound tensile strength and antiinflammation (4, 72).

2.2.4.2 *Hypericum perforatum* (St. John's wort)

Hypericum perforatum have long been utilized for healing of wounds and burns in the folk medicine of some countries. The flowering tops include resin and essence, therefore it has vulneary and epithelising properties. Because of these properties, it is used against ulceration and burns (73). According to chromatographical data, oily extract of *Hypericum perforatum* includes hyperforin that is active ingredient responsible from wound healing effect. Wound healing activity of the oily extract of *Hypericum perforatum* was found by *in vivo* excision and incision wound models (74). *In-vitro* studies, it was shown that extract of *Hypericum perforatum* was found to result in fibroblast migration and stimulation of collagen synthesis (75).

2.2.4.3 *Momordica charantina* L.

Momordica charantina L. has long been used traditionally for healing of wounds in many developing countries like Brazil, China, Colombia, Cuba, Ghana (72). *In-vivo* studies, it was shown that fruit powder of *Momordica charantina* L. improves wound tensile strength and epithelization of wound (77).

2.2.4.4 *Matricaria recutita* L. (German chamomile)

German chamomile is one of the most commonly used herb for gastrointestinal ailments and skin injuries and problems. Extracts obtained by different methods contain proazulenes and azulenes, bisaboloids and flavonoids like apigenin. Because of the ingredients, the

extracts have antiinflammatory, antispasmodic, sedative, antimicrobial, antiulcerogenic and wound healing effects (78). *In-vivo* animal studies, showed that *Matricaria recutita* L. extract increased the rate of wound contraction and wound-breaking strength (79).

2.2.4.5 Calendula officinalis

Calendula officinalis (Marigold) preparations have been used for its anti-inflammatory effect which has been proven with several animal models. Due to anti-inflammatory property, such preparations are thought to have wound healing effect (4). *In-vitro* experiments, marigold was also found to contribute to the proliferation and migration of fibroblasts (80). The German Commission E monograph describes the anti-inflammatory and healing effects of marigold, which is used only for external applications (4).

2.2.4.6 Aesculus hippocastanum

Historically, the seed extract of *Aesculus hippocastanum* (horse chestnut) was used as a treatment for many diseases, including rheumatism, gastrointestinal disorders, fever, hemorrhoids, and leg cramps. Currently, horse chestnut seed extract is widely used in Europe for chronic venous insufficiency, hemorrhoids, post-operative edema, and topically for clearing the skin. Horse chestnut includes about 30 individual compounds of saponins. Among them the major ones include four escins; escin Ia, escin Ib, isoescin Ia and isoescin Ib. Escin acts as an anti-inflammatory and reduces edema (swelling with fluid) following trauma (81).

2.2.4.7 Symphytum officinale

The comfrey (*Symphytum officinale*) is a perennial plant belonging to the Boraginaceae family. Its root contains allantoin which, apart from being an antibacterial, is said to be an excellent healing agent, promoting granulation tissue and being able to promote the healing of fractures (82). The German Commission E monograph recognizes that comfrey root has healing properties and describes that anti-inflammatory effects have been observed only after local application (4).

2.2.4.8 Centella asiatica

Centella asiatica, a small herb, has been used widely in indian medicine system as a tonic in skin diseases and leprosy. In some *in-vitro* and *in-vivo* studies show that *Centella asiatica* active ingredient, *asiaticoside,* promotes fibroblast proliferation and collagen synthesis and, have antiulcer and wound healing activity (83, 84, 85).

2.2.4.9 *Liquidambar orientalis* Mill.

Liquidambar orientalis is one of the important species of Hamamelidaceae family, has a local distribution in coastal district of Turkey, especially growing in Köyceğiz, Fethiye, Marmaris and Ula. The name in Turkish for the particular species is 'Sığla (Günlük) ağacı*'*, a name also used in sole reference to sweeet gum tree itself (4, 5).

The balsam produced by injuring of the oriental sweeet gum is known in commerce as Levant Storax while the resin obtained from *Liquidambar sytraciflua* is known as American storax. Balsam production from the living trees requires a special procedure with many stages. The outer bark of the tree is readily scraped, is mechanically ruptured by special spoon in early summer, the ruptured bark is called as 'damar'. After one week of this process, the 'damar' is ruptured again in order to prevent its closing. This wounding process is called 'sur'. Two weeks later of these traumas, first collection process of thick liquid, named as 'sur arkası', is started. The thick liquid containing parts of bark and Levant Storax is called 'Sıyrıntı or Kapçık'.The mixture is collected from trees twice in a month from the begining of July till late November.

The collected mixture is put into a kettle which contains hot water and then it is boiled while stirring. After the boiling process, the mixture is subjected to pressure with mechanic or hydraulic press in order to separate oil and water from oddments, including cuts of bark with a little amount of oil which is called shallaki (buhur). Water and oil phases are separated from each other easily because oil phase settle to the bottom in two phase or water phase merge and rise to the top of two phase, therefore water phase is simply poured out.

Storax is a semi-solid, sticky material characterized by a balsamic and styrene-like odor and bitter taste; it is used in perfumes as fixative of flavours in perfume industry (5). Levant storax have long been used for centuries in the Turkish folk medicine for healing of skin diseases, disease of respiratory system and gastric ulcers (5, 6).

3 MATERIAL & METHODS

3.1 Studies On Emulsions

3.1.1 Chemicals

Table 4 Chemicals used in the studies on the emulsions and their suppliers.

INCI Names	Trade Name	Manufacturer/Donor
Shea butter	Cetiol ® SB 45	BASF East Asia Regional Headquarters Ltd., Hong Kong
Squalene	Fitoderm®	BASF East Asia Regional Headquarters Ltd., Hong Kong
Cetearyl olivate and sorbitan olivate	Olivem ® 1000 Crystal Skin	B & T Company 20043 Arcore (MI) - Via Rossi Ernesto,
Cetostearyl alcohol	LANETTE ® O	BASF East Asia Regional Headquarters Ltd., Hong Kong
Caprylic/capric triglyceride	MYRITOL®318	BASF East Asia Regional Headquarters Ltd., Hong Kong
Petroleum jelly	VASELINE®	Sigma-Aldrich, Germany
Glycerine	PRICERINE™ 9091	Croda Chemicals Europe Ltd., UK
Ethylenediaminetetraacetic acid	EDTA	Merck KGaA, Darmstadt, Germany
Escin 90 %	Horsechestnut Extract 90 %	Sami Labs Limited, India
Calendula Oil	Calendula Oil	Provital Group, Spain
***Aloe Barbadensis* leaf juice and maltodextrin**	Terry-Spray *Aloe vera* Powder 100X	Terry Laboratories, U.S.A.
Allantoin	Allantoin	Sigma-Aldrich, Germany (Fluka)
Glycerine, Water, *Hypericum perforatum* extract	Phytami® St. John's wort	Alban Muller Int'l, France
Methylparaben,Ethylparaben, Propylparaben, Butylparaben, Isobutylparaben, Phenoxyethanol	UNİPHEN P 23	Induchem, Switzerland
Acetonitrile	Acetonitrile CHROMASOLV®	Sigma Aldrich, U.S.A.
Ortho-phosphoric acid (85%)	Ortho-phosphoric acid (85%)	Merck, Germany
Escin	Escin standard (%96)	Sami Labs Limited, Germany.
Methanol	Methanol CHROMASOLV®	Sigma Aldrich, U.S.A.
Glacial acetic acid	Glacial acetic acid	Sigma Aldrich Co. Ltd., U.S.A
Ethanol	Ethanol CHROMASOLV®	Sigma Aldrich Co. Ltd., U.S.A
Hyperoside	Hyperoside standard (≥ 97.0 %)	Sigma Aldrich Co. Ltd., U.S.A

3.1.2 Equipments

Table 5 Equipments used in the studies on the emulsions and their suppliers

• Equipment name and model	• Company name
Termostated Water bath GFL AO42	Gesellschaft für Labortechnik mbH,Germany
Viscometer, Model-RVDII+	Brookfield ENG Labs Inc.
pH meter SevenMelti S47	Mettler Toledo
Conductivity meter SevenMelti S47	Mettler Toledo
Zetasizer nano-Zs	Malvern Instruments
Leica CTR 6000 microscope fitted with a DFC 350 FXR2 camera	Leica microsystems
Analytical balance	Ohaus Explorer
Silverson L4R homogenizer	Silverson Machines Ltd.
Binder incubator, KBF-P E5-2 720	Binder GmbH
Turbiscan™ technology	Formulaction
Agilent 1100 Series HPLC with diode-array detector	Agilent Technologies
Labofuge 200 Heraeus Sepatech centrifuge	Heraeus Centrifuges
Ultra sound bath, Sonorex Super RK 156 BH	Bandelin Sonorex
Ultra pure water system, Millipore Simplicity	Millipore
Zorbax Extend RP-18 column (4.6 mm × 250 mm)	Agilent Technologies
0.22 μm membrane filters	Sartorius
Agilent 5975C GC-MS	Agilent Technologies
DB-WAX GC column, 60 m × 0.25 mm id, 0.25 µm film thickness	Agilent Technologies

3.1.3 Methods

3.1.3.1 HPLC Analysis of Hyperoside in Complex Cream

3.1.3.1.1 Preparation of Standard Solutions and Calibration Studies

In order to prepare a stock solution, 2.5 mg standard hyperoside was accurately weighed into a 25-ml volumetric flask and dissolved in ethanol under 5 minutes (min) sonication. A series of concentrations, namely 2, 4, 6 and 8 % (mg/ml) were prepared by diluting the standard stock solution with ethanol. All solutions were filtered through 0.22 μm membrane filters. The standard solutions were analysed by Agilent 1100 series HPLC with diode array

detector. Linear regression equations and correlation coefficients were obtained from plots of concentration versus peak height ratio of the standard solutions. The equation of calibration curve and correlation coefficients are used to evaluate the linearity of the calibration curve.

3.1.3.1.2 Analysis Conditions

The chromatography column used was 5 μm Zorbax Extend RP-18 column (4.6 mm × 250 mm); mobile phase was 2 % v/v acetic acid and acetonitrile, gradient system was used as shown in Table 6. The flow rate was 1.0 ml/min, and the detective wavelength was 340 nm. Injection value for each time was 20 μl and separations were carried out isothermally at 30 ºC in a heated chamber.

Table 6 Gradient profile for hyperoside analysis.

Time (Minute)	Acetonitrile	Acetic acid %2
0	15	85
25	60	40
26	15	85
35	15	85

3.1.3.1.3 Identification and quantification of hyperoside in complex cream

12 g of the complex cream was weighed in a flask, dissolved in 10 ml ethanol under sonication (5 min) and centrifuged for 10 minutes at 5000 rpm. The ethanol phase was filtered through 0.45 μm filter and the filtrate was analyzed in HPLC as three replicates. By comparing the ratio of the peak height of the sample to that of the hyperoside standard and by using the calibration curve equation, we obtained the concentration of hyperoside in complex cream.

3.1.3.1.4 Method Validation

3.1.3.1.4.1 Linearity

The linearity of an analytical method is its ability to produce test results which are directly proportional the amount of analyte in the sample in the given concentration. The standard solutions of hyperoside were prepared from the stock solution to give a concentration in the range of 2-10 (mg/ml). The standard solutions were prepared in triplicates. Calibration curve of hyperoside standards was constructed by plotting peak height (y-axis) versus the concentrations (x-axis). Correlation coefficients (r) between peak height and the concentrations were calculated.

3.1.3.1.4.2 Specificity

The specificity of the method was determined by comparing the results of hyperoside analysis obtained for placebo cream (formulation containing only the excipients) with those results obtained for the standard hyperoside solutions.

3.1.3.1.4.3 Precision

Within the standard calibration curve range, the standard stock solutions (% 10) were quantified precisely and diluted with ethanol to obtain concentrations of 2, 4, 6, and 8 mg/ml. Each solution was injected into HPLC for analysis three times on the same day and the successive five days. The standard deviations (S.D.) and relative standard deviations (R.S.D.) were calculated.

3.1.3.1.4.4 Accuracy

Known concentrations of hyperoside standard were added into the placebo substrate prepared with the placebo cream and after filtrating each solution as described above. These

solutions were injected into HPLC for analysis. The recovery rates and accuracies were calculated. 12 g hyperoside free cream substrate obtained from the placebo cream was weighed in a 10 ml flask. Standard stock solutions were added to the flask to obtain solutions of 3, 4 and 5 mg/ml hyperoside solutions. These solutions were centrifuged for 10 minutes at 5000 rpm. Each supernate was filtered through 0.45 μm filter and the filtrates were analyzed by using HPLC. All samples were prepared in three replicates. The recovery rate was calculated from average peak height ratio of samples by using the calibration curve equation.

3.1.3.1.4.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined according to the ICH guidelines for Analytical Method Validation (86). A signal to noise ratio between 3 and 10 are generally considered acceptable for estimating the LOD and LOQ respectively.

3.1.3.2 HPLC Analysis of Escin in Complex Cream

3.1.3.2.1 Preparation of Standard Solutions and Calibration Studies

In order to prepare a stock solution, 25 mg standard escin was accurately weighed into a 25-ml volumetric flask and dissolved in methanol under 5 minutes sonication. A series of concentrations, namely 2, 4, 6 and 8 % (mg/ml) were prepared by diluting the standard stock solution with methanol. All solutions were filtered through 0.22 μm membrane filters. The standard solutions were analysed by Agilent 1100 series HPLC with diode array detector. Linear regression equations and correlation coefficients were obtained from plots of concentration versus peak height ratio of the standard solutions. The equation of calibration curve and correlation coefficients are used to evaluate the linearity of the calibration curve.

3.1.3.2.2 Analysis Condition

The chromatography column used was 5 μm Zorbax Extend RP-18 column (4.6 mm × 250 mm) which was eluted isocratically with a binary mixture of acetonitrile and 0.10 %

ortho-phosphoric acid solution (volume ratio 42:58) at a total flow rate of 1.0 ml/min. Elution was monitored at 203 nm on the diode array detector. The injection volume was 10µL, and separations were carried out isothermally at 30 ºC in a heated chamber.

3.1.3.2.3 Identification and quantification of escin in complex cream

0.2 g of the complex cream was weighed in a flask, dissolved in 10 ml methanol under 5 minutes sonication and centrifuged for 10 minutes at 5000 rpm. The methanol phase was filtered through 0.45 µm filter and the filtrate was and analyzed in HPLC in three replicates. By comparing the ratio of the peak height of the sample with the escin standard and calibration curve, the concentration of escin in complex cream was calculated.

3.1.3.2.4 Method Validation

3.1.3.2.4.1 Linearity

The linearity of an analytical method is its ability to produce test results which are directly proportional the amount of analyte in the sample in the given concentration. The standard solutions of escin were prepared from the stock solution to give a set of concentrations in the range of 0.2-1 mg/10 ml. The standard solutions were prepared in triplicates. Calibration curve of the escin standards was constructed by plotting peak height (y-axis) versus the concentrations (x-axis). Correlation coefficients (r) between the peak heights and the concentrations were calculated.

3.1.3.2.4.2 Specificity

The specificity of the method was determined by comparing the results obtained from the analysis of the placebo (formulation containing only the excipients) with those obtained from the analysis of a standard solution containing only escin.

3.1.3.2.4.3 Precision

Within the standard calibration range, the standard stock solution (1 mg/ml) was diluted precisely with methanol to give concentrations of 0.2, 0.4, 0.6, and 0.8 mg/1 ml. These solutions were injected into HPLC for analysis three times on the same day and during the successive five days. The standard deviation (S.D.) and relative standard deviation (R.S.D.) were calculated.

3.1.3.2.4.4 Accuracy

Known concentrations of escin were added into the solution prepared with placebo cream. These solutions were filtered and analysed by injecting them into HPLC. The recovery rates and accuracies were calculated.

0.2 g escin free cream substrate (prepared with placebo cream) was weighed in a flask. Standard stock solutions were added to the flask to give concentrations of 0.2, 0.4 and 0.5 mg/1ml escin. The solutions were centrifuged for 10 minutes at 5000 rpm. The supernates were filtered through 0.45 μm filter and the filtrates were analyzed in HPLC in two replicates. The recovery rates were calculated from the average peak height ratios of these samples and by using the calibration curve equation.

3.1.3.2.4.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined according to the ICH guidelines about The Analytical Method validation (86). LOD is defined as three times the signal-to-noise ratio and LOQ as 10 times of the same signal.

3.1.3.3 Analytical investigation on Levant storax essential oils by Gas Chromatography–Mass Spectrometry (GC–MS)

Liquidambar orientalis balsam was used to identify and quantify the essential oils contained in the balsam. The following steps were used for this study;

1- Gas chromatographic analysis was performed on the Agilent (7890A) - mass spectrometer (Agilent 5975C (GC-MS) equipped with a DB-WAX GC column (60 m × 0.25 mm id, 0.25 μm film thickness, USA).
2- The oven temperature was programmed to be isothermal at 40 °C for 4 min., then raised to 220°C at a rate of 4 °C /min and held at this temperature for 30 min.
3- Identification of the compounds within the balsam was accomplished by using the company's mass spectra library (Elso Kimya Sanayi ve Ticaret A.Ş., Turkey).

3.1.3.4 Formulation studies and preparation of the cream formulations

3.1.3.4.1 Formulation studies

Placebo cream was the first formulation developed for this study. During the pre-formulation studies, the following process steps were followed:

1- The candidate formulations were manufactured carefully by recording all of the precise details of the manufacturing steps.
2- Each of the candidate product was examined for its organoleptic characteristics, such as:
    - Appearance
    - Colour
    - Odour
    - Viscosity
    - Feel on the skin and spreadability

3- About 5 g sample was centrifuged at 3000 rpm for 30 minutes. Any formulation which showed a sign of separation (an oil film at the top or aqueous phase separation at the bottom of the tube) was considered as instability and no further testing was carried out with this formulation.

4- About 10 g of product was placed in the freezer for freeze-thawing cycle tests. When the candidate product was thawed, any signs of separation observed was regarded as instability and this formulation was discarded. Table 7 summarizes the placebo cream formulation trails and their compositions.

5- After selecting the "placebo formulation", complex cream and levant storax cream formulations were developed by using the same excipients. The same manufacturing and the pre-testing steps were followed as described above to develop the creams with active materials, complex cream and levant storax cream.

3.1.3.4.2 Preparation of the cream formulations

3.1.3.4.2.1 Aqueous phase

EDTA and glycerine were dissolved in water in the aqueous phase beaker (2000 ml). The mixture was heated up to 70°C in a thermostated water bath (GFL 1042, Gesellschaft fur Labortechnik mbH, Burgwedel, Germany) that was previously heated up to the required temperature. The temperature of the aqueous phase was maintained at 70°C until it was mixed with oil phase.

Table 7 Percentage composition of placebo cream formulations developed and assessed[a].

Ingredients(INCI names)	P001 (w/w)	P002 (w/w)	P003 (w/w)	P004 (w/w)	P005 (w/w)	P006 (w/w)	P007 (w/w)	P008 (w/w)	P009 (w/w)
Shea butter	1.00	2.00	2.80	2.80	2.80	2.80	2.80	2.80	2.80
Squalene	1.50	2.00	2.80	2.80	2.80	2.80	2.80	2.80	2.80
Cetearyl olivate and sorbitan olivate	6.00	6.00	8.40	8.40	8.40	8.40	8.40	6.00	6.00
Cetostearyl alcohol	-------	2.00	3.50	3.50	2.80	2.10	2.00	2.00	2.00
Caprylic/capric triglyceride	3.00	5.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00
Petroleum jelly	------	2.00	5.00	7.00	7.00	7.00	7.00	7.00	7.00
Glycerine	10.00	10.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00
Ethylenediaminetetraacetic acid	0.10	0.10	0.14	0.14	0.14	0.14	0.14	0.14	0.10
Methylparaben, Ethylparaben, Propylparaben, Butylparaben, Isobutylparaben, Phenoxyethanol	0.80	0.80	0.80	0.80	1.12	1.12	0.80	0.80	0.80
Water	77.60	70.10	55.56	53.56	53.94	54.64	54.74	57.46	57.50
Result based on pre-testing	F	F	F	F	F	F	F	F	A

[a]F: Failed, A: Accepted

3.1.3.4.2.2 Oil Phase

Shea butter, squalene, cetostearyl alcohol, cetearyl olivate and sorbitan olivate, petroleum jelly and caprylic/capric triglyceride were mixed together in a beaker as the oil phase (250 ml) and the mixture was heated up to 70°C in the thermostated water bath which was previously heated up to 70°C. The temperature of the oil phase was maintained at 70°C until it was added to aqueous phase.

3.1.3.4.2.3 Mixing of the phases to form the emulsion

Preheated aqueous phase at 70°C was added into the oil phase which was maintained at 70°C while mixing with high shear Silverson L4R homogenizer (Silverson Machines, Ltd., Waterside, Chesham, Buckinghamshire, England) to produce an emulsion. The emulsion was homogenized at 5th level for 2 minutes and at 3rd level for 12 minutes by using the same homogenizer. The temperature of the emulsion was allowed to cool down to 50-60°C while

homogenising. The cooling was facilitated by putting the beaker in a water bath at 50°C, first and then at 30°C.

3.1.3.4.2.4 Addition of temperature sensitive and active ingredients to complete the preparation of the emulsions

The emulsion pre-mix was cooled down until 30°C with continuous manual stirring while keeping the beaker in a water bath at 20°C. When the temperature was at 30 °C, all remaining ingredients were added into the emulsion pre-mix according to the formulation that is being processed. The mixture was homogenized at the 3rd level mixing speed for another 1.5 minutes using the same homogenizer in order to finish up the preparation of the emulsion. The creams were filled in the ointment jars, capped and labelled. Three batches were prepared for each cream formulation in order to validate the preparation method of the emulsions. All of the batches were put under the stability testing by using the same conditions and testing plan. The formulae of the creams were summarized in Table 8.

3.1.3.5 The Characterization of the Formulations

Each of the cream samples (placebo cream, levant storax cream and complex cream) were evaluated for characterization in triplicates. Organoleptic properties (appearance, colour, odour, feel/spreadability) are evaluated and pH, viscosity, conductivity, zeta-potential, average particle size and particle size distribution measurements were made for each sample cream. Also phase separation properties at centrifugation and freeze-thawing were investigated and microbiological quality was determined.

Table 8 The formulae of the final creams [a].

Ingredients(INCI names)	Trade Names	Placebo cream (w/w)	Complex cream (w/w)	Levant storax cream (w/w)
Shea butter	Cetiol ® SB 45	2.80	2.80	2.80
Squalene	Fitoderm®	2.80	2.80	2.80
Cetearyl olivate and sorbitan olivate	Olivem ® 1000 Crystal Skin	6.00	6.00	6.00
Cetostearyl alcohol	LANETTE ® O	2.00	2.00	2.00
Caprylic/capric triglyceride	MYRITOL®318	7.00	7.00	7.00
Petroleum jelly	VASELINE®	7.00	7.00	7.00
Glycerine	PRICERINE™ 9091	14.00	14.00	14.00
Ethylenediaminetetraacetic acid	EDTA	0.10	0.10	0.10
Escin 90 %	Horsechestnut Extract 90 %	--------	xxxxxx	--------
Calendula Oil	Calendula Oil	--------	xxxxxx	--------
***Aloe Barbadensis* leaf juice and maltodextrin**	Terry-Spray *Aloe vera* Powder 100X	--------	xxxxxx	--------
Allantoin	Allantoin	--------	xxxxxx	--------
***Glycerine, Water, Hypericum perforatum* extract**	Phytami® St. John's wort	--------	xxxxxx	--------
Balsam of oriental sweet gum	Levant storax	--------	--------	xxxxxx
Methylparaben, Ethylparaben, Propylparaben, Butylparaben, Isobutylparaben, Phenoxyethanol	UNIPHEN P23	0.80	0.80	0.80
Water	***qs***	100	100	100

[a] '--------' means; the ingredient was not used, 'xxxxxx' means; appropriate amount.

3.1.3.5.1 Determination of Organoleptic Properties

Each cream sample is evaluated carefully for its following features, at room temperature (25°C):

- Appearance
- Color
- Odour
- Feel of flow characteristics
- Spreadability on the skin (by applying on the skin).

3.1.3.5.2 Viscosity measurements

The viscosity of the cream samples was measured by using a Brookfield Viscometer Model-RVDII + (Brookfield ENG Labs Inc., Stoughton, MA, USA) with spindle number 29 at 22.5 °C. The viscosity of each sample was measured within twenty-four hours of manufacture and after pre-determined time points and stability storage conditions. In order to obtain accurate result, a minimum of two readings were taken for each batch of cream and average viscosity values were calculated (6 measurements for each cream formulation, at each time point).

3.1.3.5.3 pH measurements

The pH of the cream samples were measured by using pH meter (model of SevenMulti™ S47 Mettler Toledo), at 22.5 °C. The pH of each sample was measured within twenty-four hours of manufacture and after pre-determined time points and stability storage conditions. The pH measurements were taken twice to ensure accurate values for each sample (6 measurements for each cream formulation, at each time point).

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3.1.3.5.4 Conductivity measurements

Conductivities of the creams were measured at the predetermined time points by using conductivity meter (Mettler Toledo SevenMulti™ S47) at 22.5 °C. The conductivity measurements were taken for each sample within twenty-four hours of manufacture and after pre-determined time points and stability storage conditions. The conductivity measurements were made twice for each formulation, in order to ensure accurate measurements (6 measurements for each cream formulation, at each time point).

3.1.3.5.5 Zeta Potential measurements

For determination of the zeta potential of emulsion droplets, a Zetasizer (nano-ZS from Malvern Instruments) was used. Emulsions were placed in a folded capillary after being diluted in water phase described in Section 3.1.3.4.2.1. After dilution, zeta potential of emulsion samples was measured immediately. The zeta measurements were made for each sample within twenty-four hours of manufacture and after pre-determined time points under each stability storage conditions. In order to ensure accurate measurements, two successive measurements were taken at every measurement point (6 measurements for each cream formulation, at each time point).

3.1.3.5.6 Particle (globule) size and Particle size distribution determinations

Droplet size was investigated by two different methods.

3.1.3.5.6.1 Microscopic examination and microphotograph counts

Arithmetic average particle size of each cream sample (placebo, complex and levant storax creams) was determined to characterize the creams and also to monitor their stabilities.

Microscope slides were prepared by spreading a very thin layer of the cream on the specimen slide and pressing this layer well with a cover slip. The slides were observed with a Leica CTR 6000 microscope fitted with a DFC 350 FXR2 camera. The images of the slides were transmitted on to the monitor with the help of Leica software for image analysis. The software enabled recording of the images, therefore offering the advantage of not only having a permanent record of the images for analysing later, but also making the particle size measurement and counts on a print at a convenient time.

Normal transmitted light was used to visualize the images, the slides were viewed under 100-fold magnification and photomicrographs were taken for the counting and sizing at 400-fold or 200-fold magnification. The images did have a calibrated scale bar on them which enabled the calibration of a standard ruler for the globule size measurements. For each sample, about 1000 globules were counted and the arithmetic mean diameter was calculated. The arithmetic mean diameter is the sum of each particle diameter, divided by the number of particles (87).

3.1.3.5.6.2 Measurements of particle sizes with Turboscan™ technology

The **Turbiscan™ technology** is one of the light scattering methods used to analyse the stability of concentrated dispersions such as emulsions, suspensions and foams quickly and accurately. Turbiscan™ technology consists in measuring the Backscattering (BS) and Transmission intensities versus the sample height in order to detect particle size change (coalescence, flocculation) and phase separation (sedimentation, creaming) (88). A sample output representing the measurement, is shown in Figure 6, a pattern of intensity versus sample height.

Multiple Light Scattering (MLS) consists of sending photons of Near Infrared (NIR) light source at 880 nm into the sample. These photons, after being scattered many times by

the particles (or droplets) in the dispersion emerge from the sample and are detected by the 2 detectors of the Turbiscan™ reading head (Figure 6).

Backscattering is directly related to the photon transport mean free path. Thus Backscattering intensity depends on particle size and concentration.

From Backscattering measurement it is possible to:

- Monitor size change versus time
- Monitor local concentration change versus time (with the Turbiscan™ scanning reading head)
- Measure Particle mean diameter or concentration.

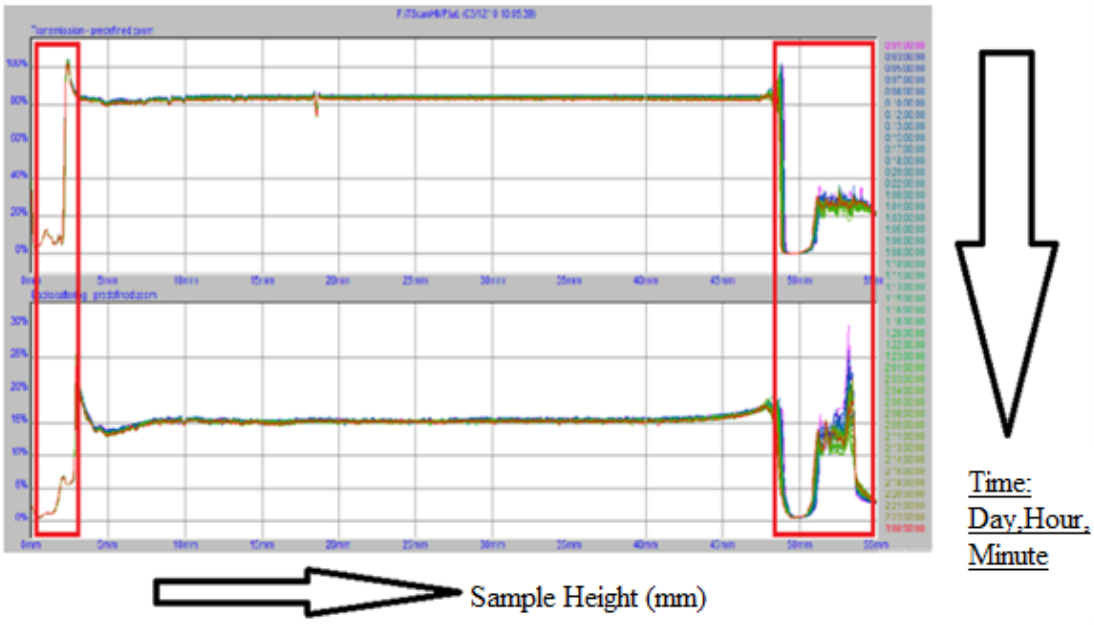

Figure 6 A pattern of intensity versus sample height was obtained.

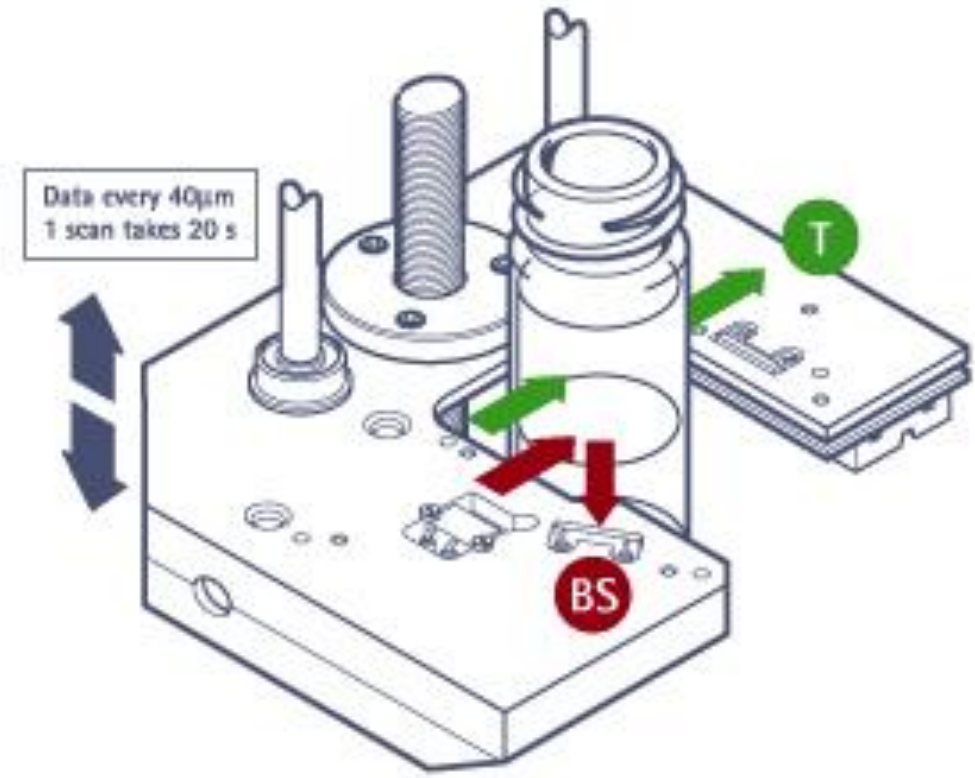

Figure 7 Turbiscan device (Formulaction, France).

Changes in the transmission (upper graph) and backscattering (lower graph) values as shown in Figure 7 can be analysed easily.

Separation and creaming result in changes of the left hand and right hand sides of the graphs. On the contrary, particle size change is observed along the length of the test tube. Therefore changes in Delta BS graphs need to be assessed carefully.

In this study, as an alternative method Turbiscan measurements were used in order to follow the particle size measurement changes. Delta change (Δ change) in the amount of Backscattered light in Multiple Light Scattering reflects the globule size changes in an emulsion. Only one batch of each cream was tested in Formulaction™ Lab device for 2 days by recording the amount of transmitted and backscattered light at every hour. The Δ change in the amount of the backscattered light was analyzed to monitor the size changes of the globules in the emulsions.

3.1.3.5.7 Centrifugation process

The resistance of the creams to centrifugation was tested immediately after manufacturing and at predetermined times during stability testing with the samples kept at different conditions. A Labofuge 200 Heraeus centrifuge (Germany) was used to centrifuge each cream sample at 3000 rpm for 30 minutes. The test was run in triplicate samples, at each test point.

3.1.3.5.8 Freeze-Thawing Studies

3.1.3.5.8.1 Standard Freeze-Thawing cycles

6 Freeze-Thawing cycles are performed as follows:

1- Samples of six sets of each product (3 creams and 3 batches of each cream) were prepared in capped centrifuge tubes.
2- All of the samples were placed in the freezer chamber of a fridge and let to freeze for 24 hours.
3- The frozen samples were taken out of the freezer and thawed at room temperature for 24 hours. All tubes were examined very carefully for color, odour, and appearance changes as well as for any sign of phase separation.
4- One sample from each batch of each product was centrifuged at 3000 rpm for 30 minutes. The samples were examined again very carefully for phase separation and any other visible changes.
5- Except for the centrifuged samples, all others were placed in the freezer again for the next cycle.
6- The steps of 2-5 were repeated exactly the same way for each freeze-thawing cycle until 6 cycles were completed.

3.1.3.5.8.2 Freeze-Thawing and Heating cycles

6 Freeze-Thawing and Heating cycles are performed as described below:

1- Samples of six sets of each product (3 creams and 3 batches of each cream) were prepared in capped centrifuge tubes.
2- All of the samples were placed in the freezer and let to freeze for 24 hours.
3- The frozen samples were taken out of the freezer and thawed at room temperature for 24 hours. All tubes were examined very carefully for color, odour, and appearance changes as well as for any sign of phase separation.
4- All of the samples were placed in an oven at 40ºC and kept there for 24 hours.
5- The samples were taken out of the oven and conditioned at room temperature. After conditioning, all samples were examined carefully for any changes.
6- One sample from each batch of each product was centrifuged at 3000 rpm for 30 minutes. The samples were examined very carefully for phase separation and any other visible change.
7- Except for the centrifuged samples, all others were placed in the freezer again for the next cycle.
8- The steps 2-7 were repeated as given above for each cycle until 6 cycles were completed.

3.1.3.5.9 Microbiological compliance tests

All products prepared were tested for microbiologic compliance by using the standard methods as described in Colipa Guidelines (89).

Briefly, the following method was used for microbiological compliance testing:

1- A neutralizing solution was used to dilute 10 ml cream in 40 ml dilution solution
2- 1 ml of the diluted solution as described in item 1 was put in 4 sterile plates (in pairs)

3- Two Petri plates were used for adding SDA (Saboroud Dextrose Agar) which is a nutrient media to support the growth of yeasts and moulds.
4- Two other Petri plates were used for TSA (Tryptic Soy Agar) which is a nutrient media that supports all bacteria.
5- About 8-10 ml of the nutrient media was added and carefully mixed with the product samples by swirling them carefully.
6- Plates were incubated at 37 ºC for at least 5 days.
7- Plates were examined for growth, if any, the number of colony forming units were counted.
8- If there was any growth in SDA and TSA plates, diagnostic tests were required to identify the microorganisms to ensure that there were no pathogenic organisms.

3.1.3.6 Stability Studies

In this study, the guidelines that were published by ICH (44), The European Medicines Agency (EMA) (90) and The World Health Organization (WHO) (91) were used to design the stability test protocol used for the assessment of the stability of the formulations developed.

3.1.3.6.1 Stability test protocol

The stability test protocol designed for this study included the following information:

1) Number of batches,

2) Container closure system,

3) Sampling frequency,

4) Sampling plan,

5) Test storage conditions,

6) Specifications, and

7) Test procedure.

3.1.3.6.2 Number of batches

Current international guidelines on stability evaluation of active substances and/or related finished products recommend the testing of a minimum of three batches (44, 90, 91). In this study, three successive batches were manufactured by using the same process steps as described in Section 3.1.3 and these batches were used for stability test. The size of each batch that was produced was 720 g.

3.1.3.6.3 Container-closure system

According the ICH guidelines, the stability studies should be conducted using the packaging that is the same or simulates the packaging proposed for storage or distribution of the product (44, 90, 91). However, ICH also suggests that the other containers which is suitable for the accelerated testing of the product can be used during the stability tests (40).

In this study, the formulations were packed into 90 g glass ointment jars with tightly fitting caps.

3.1.3.6.4 Testing frequency

Accelerated stability studies may be undertaken for a minimum of six months. During the six months test period, the samples should be tested at a minimum of three time points including the initial and final time points, (e.g., 0, 3, and 6 months) (44, 90, 91).

However, in order to determine whether the formulations are a potentially stable, our stability studies were conducted for a period of only two months and the sampling time points were at 0,1, 4 and 8 weeks.

3.1.3.6.5 Sampling plan

To satisfy the pre-determined test requirements, namely, the stability conditions and the testing time points, three samples were packed in 90 g glass ointment jars for each test condition (3 creams and 3 batches of each cream).

3.1.3.6.6 Accelerated Test storage conditions

According to the climatic conditions, all countries around the world have been divided into four climatic zones. Turkey falls into Zones II (1, 2). For countries which are located in Zone I-II, accelerated stability testing should be conducted at 40 °C ±2°C /75% RH ±5%. However, the formulations that contain some herbal extracts such as essential oils, lower temperature set-up is recommended, because essential oils may be degraded at 40 °C ±2°C /75% RH ±5% (46). In such cases, the stability test should be conducted at an intermediate condition (such as 30°C ±2°C /60 % RH ±5%). Due to the lack of a controlled stability chamber set at 30°C ±2°C and 60 % RH ±5% in our laboratory, stability studies were

conducted at 25 ±2°C and 60 ±5% RH, 4°-8°C (in refrigerator) and at room temperature / at dark.

3.1.3.6.7 Specifications

In this study, during the stability tests, the formulations were evaluated in terms of the specifications set for each of them at the zero time point. The features (specifications) tested for all samples under the accelerated storage conditions at the pre-determined time points were explained in Section 3.1.4.

3.2 Excisional wound model and wound healing rate monitoring

3.2.1 Animals

- Male, Sprague–Dawley rats weighing 263–299 g were used.
- The animals were maintained on standard pellet diet and water ad libitum throughout the experiments.
- This study was approved by the Experimental Animals Ethics Committee at Yeditepe University Medical Faculty (Ethical comitte desicion no: 129).
- The animals were devided in five groups (six animals placed in each group);

    - Control group
    - Placebo group
    - Reference group (Madecassol®, 10 mg/1g *Centella asiatica* extract)
    - Complex group
    - Levant storax group see Table 9.

3.2.2 Material of excisional wound model

- Anaesthesia solutions: Ketalar® (Ketamine hydrochloride 10%) and Rompun® (xylazine-hydrochloride)
- Ethanol (% 96 v/v solution in water).
- Single use, sterile, nontoxic, non-pyrogenic syringes (1 ml).
- Single use, sterile, nontoxic, non-pyrogenic needles.
- Examination gloves, single-use razor (Permatik®), paper towels.
- 5 mm punch
- Formaldehyde (% 10)
- 5 mm biopsy punch, forceps, scissors.

3.2.3 Method of excisional wound model

For each animal;

- Single-use syringe with Ketalar® (100 mg/kg) and Rompun® (10 mg/kg) for anaesthesia was prepared .
- Anaesthesia solution was injected intraperitoneally.
- The rat was put back in a cage, so that it can rest. The effect of the anaesthesia is seen in 5-10 minutes.
- The back of the anesthetized rat was shaved by using the single-use razor.
- The anesthetized and shaved rat was placed on a paper towel.
- The shaved back of the animal was wiped with sufficient amount of 96 % EtOH.
- The skin of the back of the rat was lifted by using forceps from two points and lifting it up to enable the easy punching.
- The skin of the rat was punched by using 5 mm punch.
- Punching was repeated to create a total of six wounds on the back of the each animal see Figure 8.

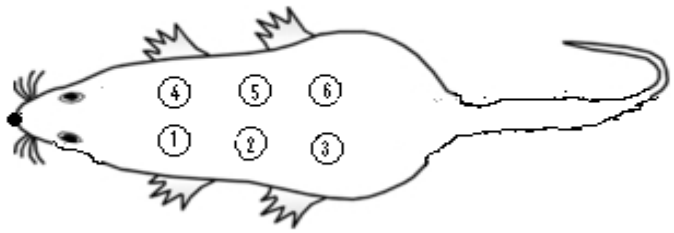

Figure 8 Template for wound numbering on the back of the animals. 1; First wound, 2; Second wound, 3; Third wound, 4; Fourth wound, 5; Fifth wound, 6; Sixth wound.

3.2.4 Wound care plan

1. After completion of excisional wounding, wounds of control group were untreated.
2. Levant storax cream, reference drug (Madecassol®) and placebo creams were applied topically once a day till the wounds of one of the group were completely healed Table 9 summarizes the groups and the wound care plan for the groups.

Table 9 Animal groups and summary of their wound care plans.

Animal Group Name	Number of Animal	Feeding method	Wound care plan
Control group	6	Standard pellet diet and water ad libitum	Untreated
Placebo group	6	Standard pellet diet and water ad libitum	Placebo cream was applied topically once a day
Reference group (Madecassol®)	6	Standard pellet diet and water ad libitum	The reference (Madecassol®) cream was applied topically once a day
Complex group	6	Standard pellet diet and water ad libitum	Complex cream was applied topically once a day
Levant storax group	6	Standard pellet diet and water ad libitum	Levant storax cream was applied topically once a day

3.2.5 Termination of excisional wound model

- At the end of the treatment schedule, on the 9th day, all groups were sacrificed by injection of high dose anaesthesia. Tissue samples were isolated from the healed skin of each animal by biopsy punches.
- Healed skin specimens were belonging to the wounds numbered 1, 2 and 3 were isolated and fixed in 10% buffered formaldehyde for the histopathological examination and evaluation of full thickness skin.

- Fourth, fifth and sixth healed wounds were isolated and snap-frozen on dry ice and stored at $-80°C$ for other investigations.
- Also, three intact tissue samples were isolated from nearby tissue of wounds numbered 1, 2, 3 by biopsy punch for evaluation of full skin thickness (Figure 9).

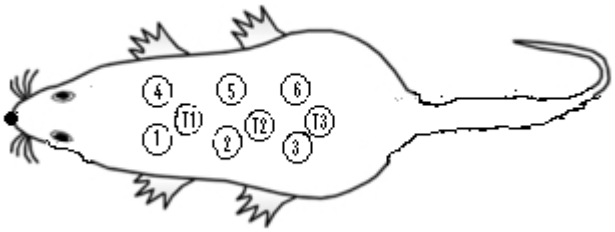

Figure 9 Template of biopsy places for evaluation of full skin thickness. T1; Biopsy of intact tissue which was compared with first wound, T2; Biopsy of intact tissue which was compared with second wound T3; Biopsy of intact tissue which was compared with the third wound.

3.2.6 Determination of wound contraction rate

- The progressive changes in wound area were measured by the means of the photographs taken from the wounds by a camera (Nicon®) every other day. A standard reference ruler was placed near the wound when the pictures were taken in order to facilitate calibration.
- All wounds were photographed also by DLite Analog Microscope on the first and last day (tenth day) of the study, in the presence of the standard reference ruler.
- The photographs were used to compute the wound contraction rates. Image J software was used for the calculations. The wound contraction rates were calculated as the percentage of reduction in the wound area. The wound contraction rates were compared statistically between the groups for significance.

3.2.7 Histopathological Evaluations

- The healed skin specimens from the wounds numbered 1, 2, 3 were isolated and fixed in 10% buffered formaldehyde for the histopathological examination.

- After routine fixation procedures, tissues were embedded in paraffin wax. Serial sections of paraffin embedded tissues of 5μm thickness were cut. Haematoxylin and eosin stained preparations were examined under Leica CTR 6000 microscope fitted with a DFC 350 FXR2 camera for the assessment of vascularization, active and chronic inflammation, fibroblastic activity, fibrosis and hair follicle development processes.

3.2.8 Evaluation of full skin thickness

- Intact tissues (Figure 9) were stained with hematoxylin and eosin (HE).
- Healed skin of first, second, third numbered wounds and sections of intact tissues were photographed in the presence of a standard reference ruler by DLite Analog Microscope which were plugged into computer.
- Skin thickness of the wounds from healed and intact nearby tissues were measured by using Image J software and the results were compared statistically for significance.

3.2.9 Analysis of data

One-way analysis of variance (ANOVA) and repeated measure ANOVA were used in order to statistically analyse the wound contraction rates and full skin thickness development rates. Data obtained from the histopathological evaluations were statistically analyzed by using Kruscall Wallis with Mann-Whitney U tests. $P<0.05$ were considered significant. SPSS program was used for statistical data analysis.

4 RESULTS

4.1 Studies On Emulsions

4.1.1 Analysis of Hyperoside in Complex Cream

4.1.1.1 Calibration curve

Within the range of concentration of 2-10 mg/100 ml, all points on the calibration curve were in good linear correlation with a correlation coefficient of 0.999 (Figure 10). The equation of the calibration curve was shown on the graph.

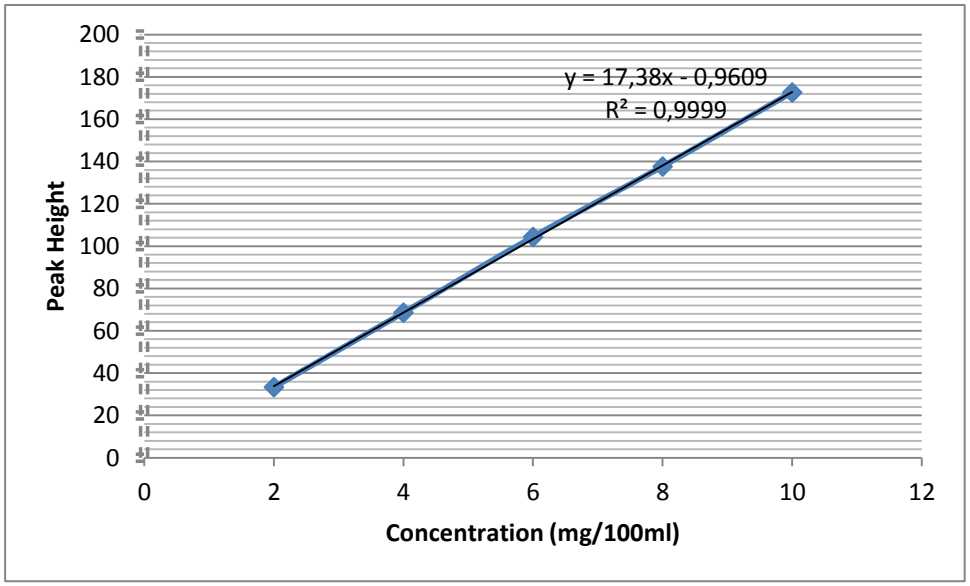

Figure 10 Calibration curve of hyperoside standard.

4.1.1.2 Identification and quantification of hyperoside in complex cream

The test was performed according to section 3.1.3.1 and retention time of hyperoside standard was found as ~ 6.90 minute (Figure 11). The chromatogram of the complex cream was shown in Figure 12 which also agreed with the standard solution of hyperoside. When calculated amount of Hyperoside is calculated in 12 g of freshly prepared complex cream, as

it was shown in Table 10, the result was as expected. This result clearly showed that, the method described in section 3.1.3.1 is a method which can be used to measure hyperoside content of our complex cream, ± SD level was reasonably low.

Table 10 The amount of hyperoside in complex cream[a].

Analysis Day		Sample No			Average ± SD
		1	2	3	
T0	Hyperoside amount in 12 g of complex cream (mg)	0,278	0,280	0,264	0,274 ± 0,006

[a]n:3, Repeat injection three times.

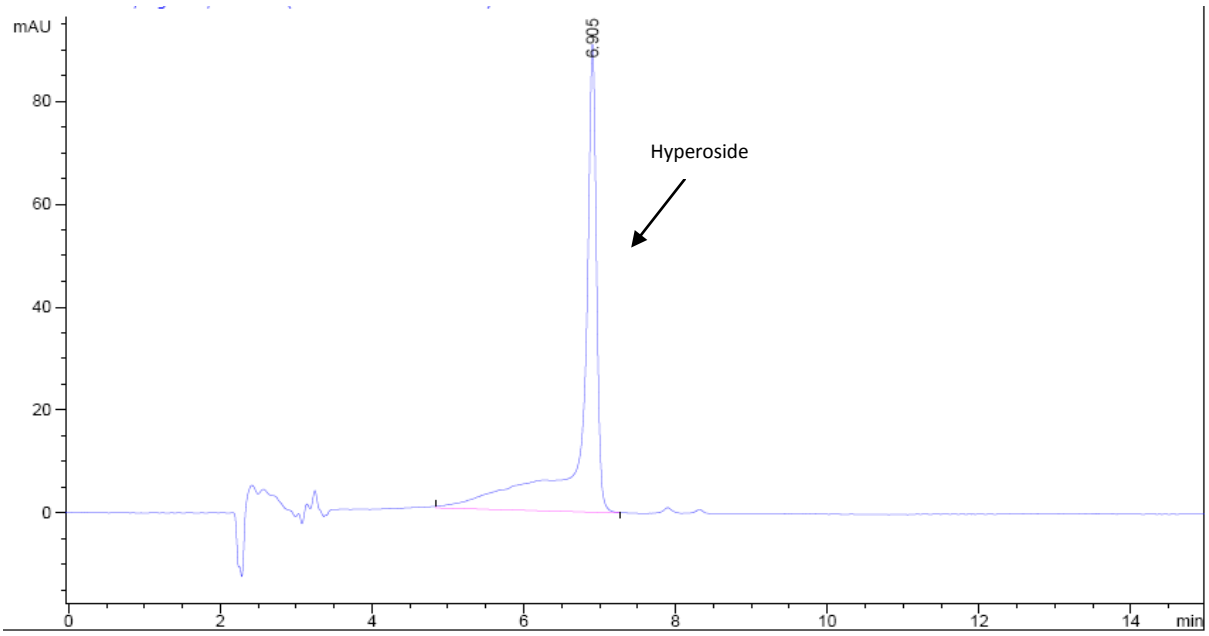

Figure 11 HPLC chromatogram of hyperoside standard.
Conditions: column; 5 µm Zobax Extend RP-18 column (4.6 mm × 250 mm); mobile phase, 2% glacial acetic acid and acetonitrile, flow-rate, 1 ml/min.

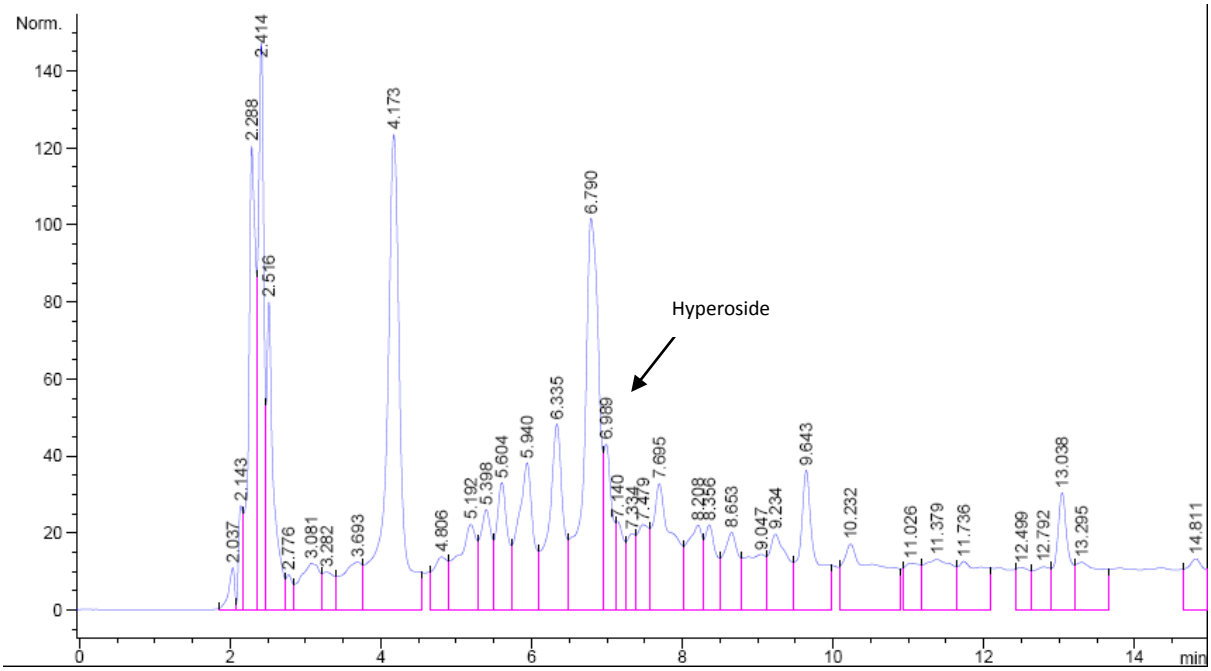

Figure 12 HPLC chromatograms of complex cream.
Conditions: column 5 µm Zobax Extend RP-18 column (4.6 mm × 250 mm); mobile phase, 2% glacial acetic acid and acetonitrile; flow-rate, 1 ml/min.

4.1.1.3 Results of Method Validation

4.1.1.3.1 Linearity

As given in section 3.1.3.1.4.1, when peak height ratios vs. concentrations of all standard solutions are plotted, the calibration curve showed a good linearity within the concentration ranges stated above (Figure 10). The correlation coefficient computed was high, indicating that the equation of the curve would be used to predict concentrations of solutions, if they are prepared as explained in section 3.1.3.1.3.

4.1.1.3.2 Specificity

The chromatogram of hyperoside free cream substrate is shown Figure 13. As it can be seen, there was no peak observed at the same retention time with standard hyperoside solution, showing the specifity of the method.

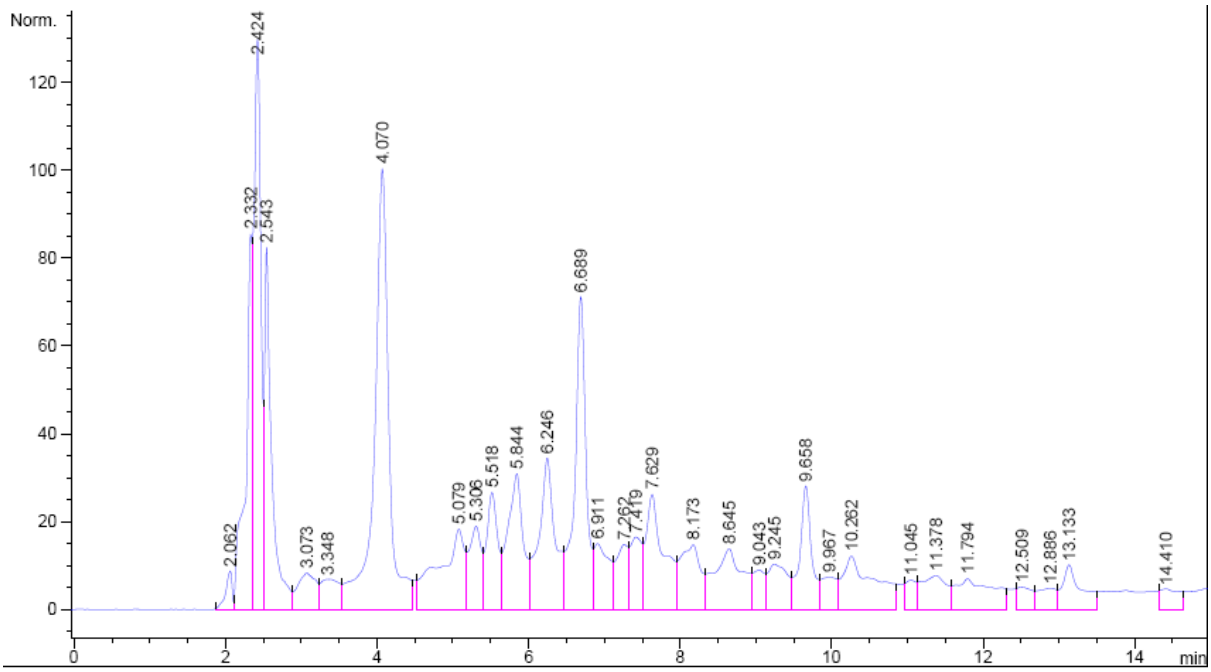

Figure 13 HPLC Chromatograms of hyperoside free cream substrate.
Conditions: column, 5 µm Zobax Extend RP-18 column (4.6 mm × 250 mm); mobile phase, 2% acetic acid and acetonitrile; flow-rate, 1 ml/min.

4.1.1.3.3 Precision & Accuracy

The results of the interday and intraday readings of hyperoside were listed in Table 11. The relative standard deviation of the intraday and interday runs were between 1.549~2.350 % and 0.253 ~1.721 % respectively. Recoveries of hyperoside in hyperoside free cream substrate are shown in Table 12. The recovery rates of hyperoside in the complex cream were 91.849-103.225.

Table 11 The relative standard deviations of intraday and interday run of hyperoside.

Hyperoside Concentration % (mg/ml)	Mean ± S.D. (R.S.D. %)	
	Intraday[a]	Interday[b]
2	1,964 ± 0,038 (1,967)	1,950 ± 0,033 (1,721)
4	3,968 ± 0,093 (2,350)	4,132 ± 0,067 (1,627)
6	6,002 ± 0,094 (1,578)	6,182 ± 0,020 (0,337)
8	7,971 ± 0,124 (1,559)	8,221 ± 0,020 (0,253)
10	9,884 ± 0,153 (1,549)	9,968 ± 0,118 (1,184)

[a] n=3, Repeat injection three times on the same day.
[b] n=15, Repeat injection three times each day and a successive five-day.

Table 12 Recoveries of hyperoside in hyperoside free cream substrate[a].

Theoretical conc. (% mg/ml)	Estimated conc. (% mg/ml)	Recovery (%)
3,000	2,755	91,849
4,000	4,027	100,678
5,000	5,161	103,225

[a]n= 2, Repeat injection two times on the same day

4.1.1.3.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

As explained in section 3.1.3.1.4.5, the limit of detection and quantification were determined and found to be as 0,058 mg/100 ml and 0,178 mg/100 ml respectively.

4.1.2 Results of HPLC Analysis of Escin in Complex Cream

4.1.2.1 Calibration curve

As indicated in section 3.1.3.2.1, the calibration curve for escin standard was prepared. Within the range of concentrations of 0,200-1,000 mg/ml, the calibration curve was linear with a correlation coefficient of 0,999 (Figure 14).

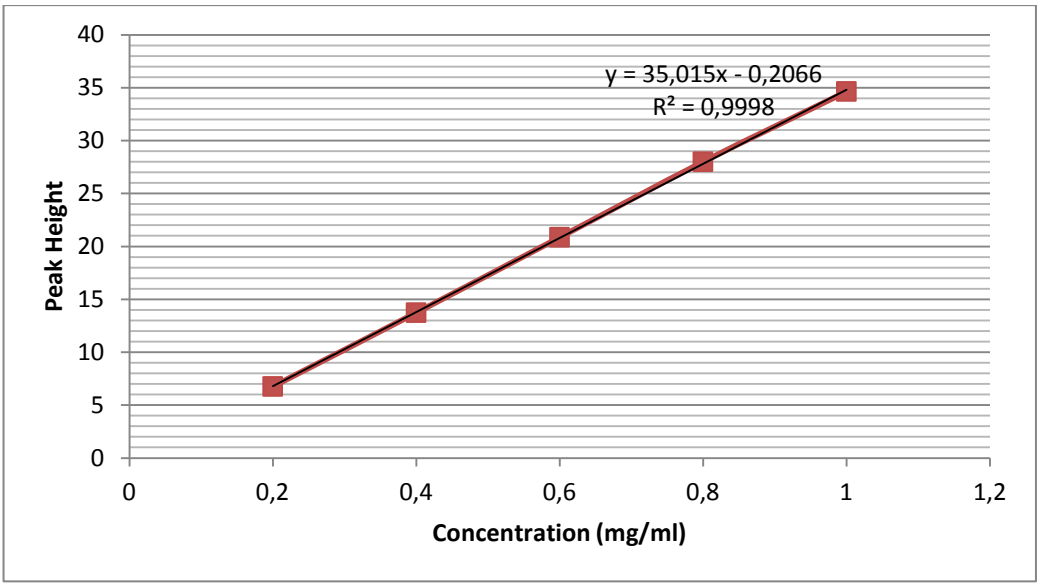

Figure 14 Calibration curve of escin standard.

4.1.2.2 Identification and quantification of escin in complex cream

The chromatograms of escin standard and complex cream are shown in Figure 15 and 16 respectively. The amount of escin in freshly prepared complex cream was analysed as shown in Table 13. These results clearly indicated that the method described in section 3.1.3.2 is a valid method to use in determining the quantity of escin in complex cream formulation.

Table 13 The amount of escin in complex cream[a].

Analysis Day		Sample No			Average ± SD
		1	2	3	
T0	Escin amount in 0,200 g of complex cream (mg)	0,319	0,296	0,324	0,313 ± 0,015

[a]n:3, Repeat injection three times.

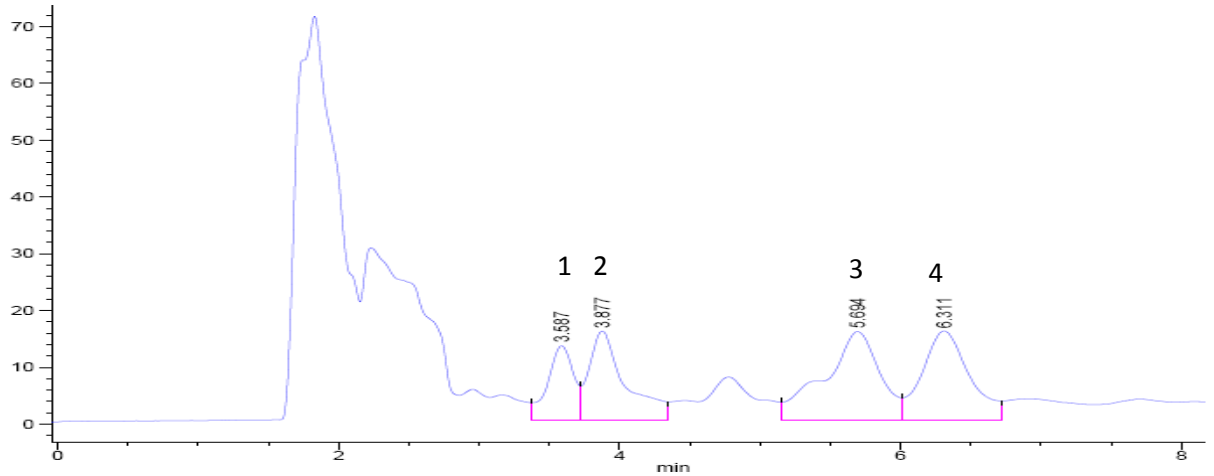

Figure 15 HPLC chromatogram of standard solution of four escin saponins.
Conditions:column, 5 μm Zobax Extend RP-18 column (4.6 mm × 250 mm); mobile phase, acetonitrile and 0.10% phosphoric acid solution (volume ratio 42:58); flow-rate, 1 ml/min.

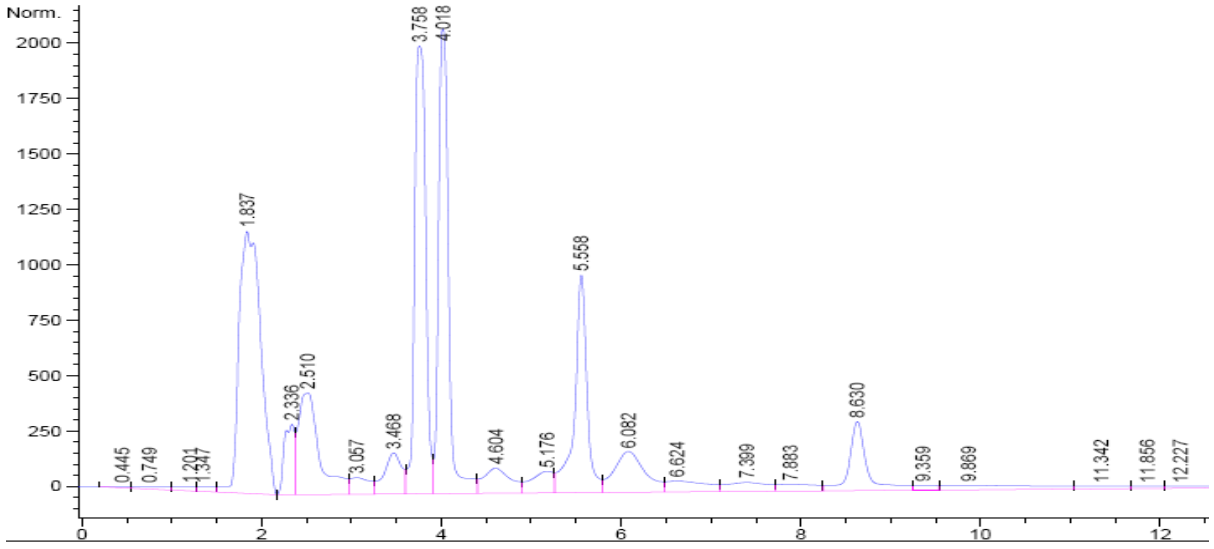

Figure 16 HPLC chromatogram of complex cream.
Conditions:column, 5 μm Zobax Extend RP-18 column (4.6 mm × 250 mm); mobile phase, acetonitrile and 0.10 % phosphoric acid solution (volume ratio 42:58); flow-rate, 1 ml/min.

4.1.2.3 Method Validation Results

4.1.2.3.1 Linearity

The plot of peak height ratios vs. concentrations of all standard solutions were found to be linear within the concentration ranges as stated in section 3.1.3.2.1 (Figure 14). The correlation coefficient was 0,999.

4.1.2.3.2 Specificity

The chromatograms of escin free cream substrate and complex cream was shown Figure 17. The escin free cream substrate contained three peaks whose retention times were overlapped with three peaks of escin isomers. There was one peak identified which was different from the escin free cream substrate chromatogram, therefore this peak was used in order to analyse the escin amount in the complex cream. This peak had a retention time of about ~ 6,20 minute (Figure 17).

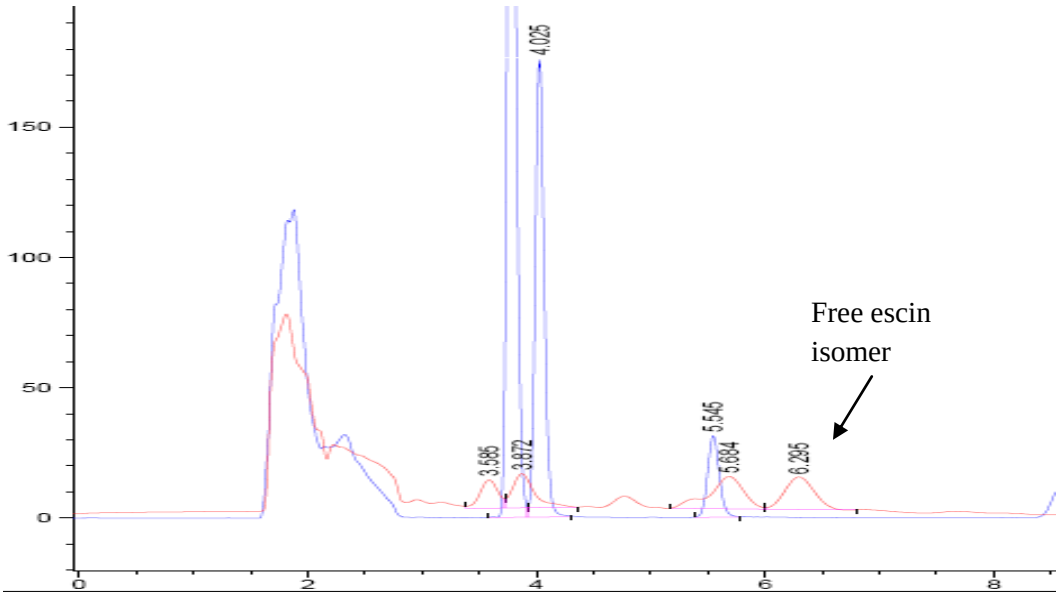

Figure 17 HPLC chromatograms of complex cream and the escin free cream substrate (Placebo).
Conditions:column, 5 µm Zobax Extend RP-18 column (4.6 mm × 250 mm); mobile phase, acetonitrile and 0.10% phosphoric acid solution (volume ratio 42:58); flow-rate, 1 ml/min.

4.1.2.3.3 Precision & Accuracy

The results of the interday and intraday tests of escin were listed in Table 14. The relative standard deviation of the intraday and interday test results were between 0,888~6,694 % and 1,597~7,092 %. Recoveries of escin incorporated in escin free cream substrate as described in section 3.1.3.2.4.4 were shown in Table 15. The recovery rates of escin in the complex cream were 96,735 -107,067.

Table 14 The relative standard deviations of intraday and interday run of escin.

Escin Concentration (mg/mL)	Mean ± S.D.(R.S.D.%)	
	Intraday[a]	Interday[b]
0,2	0,193 ± 0,013 (6,694)	0,196 ± 0,013 (7,092)
0,4	0,417 ± 0,007 (1,848)	0,410 ± 0,011 (2,709)
0,6	0,615 ± 0,009 (1,569)	0,622 ± 0,017 (2,807)
0,8	0,816 ± 0,026 (3,188)	0.810 ± 0,017 (2,09)
1	0,992 ± 0,008 (0,888)	1,014 ± 0,016 (1,597)

[a] n=3, Repeat injection three times on the same day.
[b] n=15, Repeat injection three times each day and a successive five-day.

Table 15 Recoveries of escin in escin free cream substrate[a].

Theoretical conc. (mg/mL)	Estimated conc. (mg/mL)	Recovery (%)
0,20	0.214	107,067
0,40	0,386	96,735
0,50	0,485	97,127

[a]n= 3

4.1.2.3.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection and quantification were 0,02 mg /mL and 0.08 mg / mL.

4.1.3 Results of GC analysis of the Levant storax

Results of GC analysis of the Levant storax were shown in Figure 18. According to these results, Levant storax (balsam of *Liquidambar orientalis* Mill.) contains the following ingredients: a-pinene, b-pinene, styrene, d-isomenthol, benzaldehyde, menthol crystal, germacrene d, hexanoic acid, benzyl alcohol, phenol, phenyl propyl alcohol and cinnamyl alcohol.

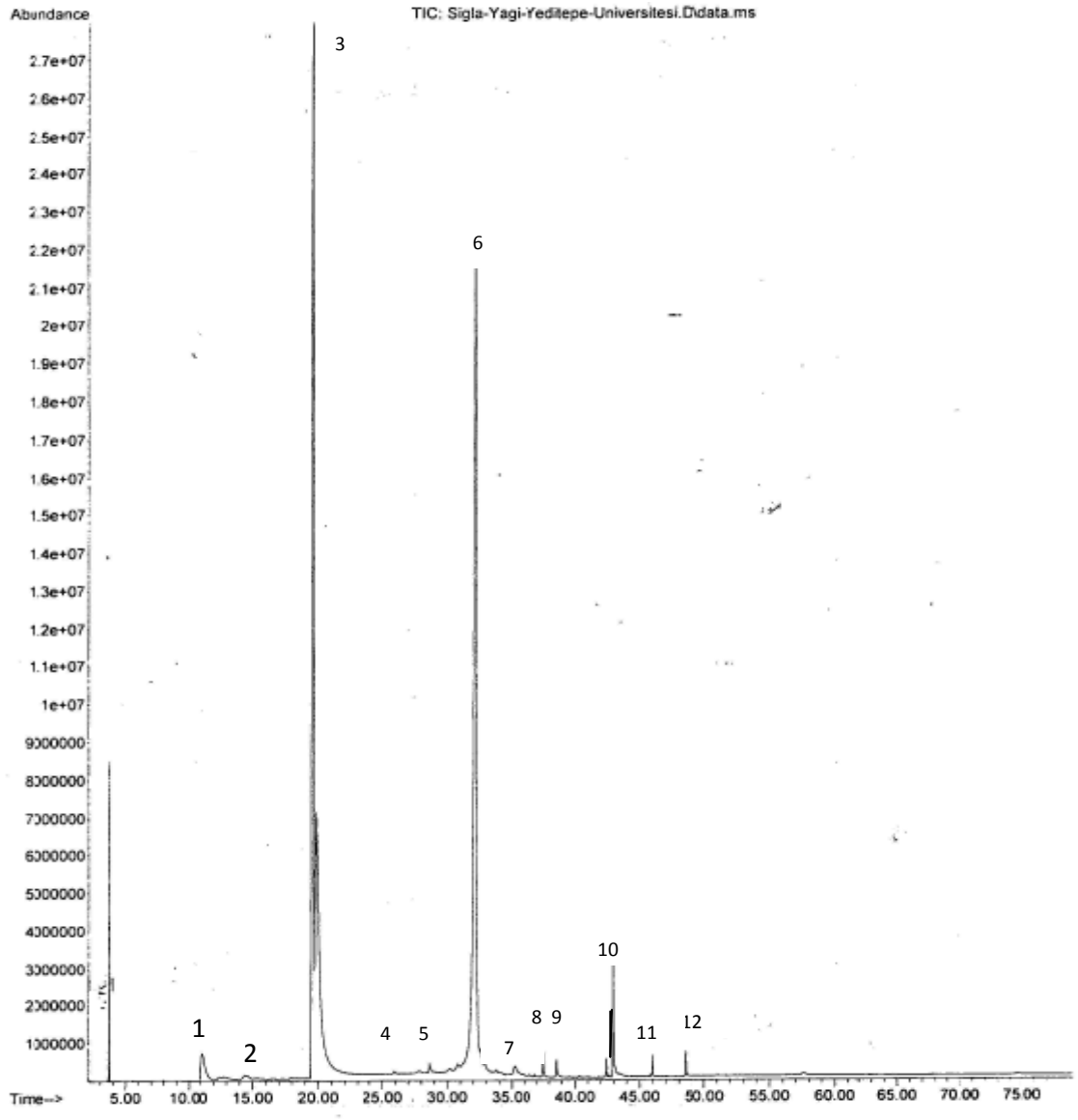

Figure 18 Gas chromatogram of Levant storax: (1) a-pinene; (2) b-pinene; (3) styrene; (4) d-isomenthol; (5) benzaldehyde ; (6) menthol crystal; (7) germacrene d; (8) hexanoic acid; (9) benzyl alcohol; (10) phenyl propyl alcohol; (11) phenol; (12) cinnamyl alcohol.

4.1.4 Results of Formulation studies

4.1.4.1 Placebo cream

Nine formulations were tried in small amounts (140 g) as shown in Table 7. At the end of the formulation studies, placebo cream code P009 was selected as the best placebo formulation which met the requirements. Placebo cream P009 passed the stability criteria (pre-tests) and also the viscosity, spreadability, feel on the skin expectations.

4.1.4.2 Complex cream formulation

Complex cream was formulated by using the same cream base formulation as placebo cream. Herbal extracts as listed in Table 8 were incorporated in the base placebo cream. Although some difficulties in formulating complex cream was expected due to the high number and amount of the extracts, no further adjustments were required to finalize the complex cream formulation.

4.1.4.3 Levant storax cream formulation

Similarly, levant storax cream formulation did not present any problems although the subject balsam had to be included in the base formulation. The base cream formulation (placebo cream) took up the balsam without any problem, therefore levant storax cream was proved to be a reasonably easy formulation (Table 8).

4.1.5 Characterization of The Cream Formulations

Organoleptic characteristics of these three creams were presented in Table 16. All formulations were centrifuged as soon as they were prepared as described in Section 3.1.3.5.7. No phase separation was observed in any of the formulations. Viscosity, pH, conductivity and zeta potential measurements were made as described in section 3.1.3.5. Results of these

measurements were shown in Table 17. The results of the first measurements which represent the values, are represented as mean values ± S.D.

Table 16 The organoleptic characteristics of the creams

Product parameters	Placebo	Complex	Levant storax
Appearance	Opaque	Opaque	Opaque
Colour	White	Light Mustard colour	Greyish white
Odour	Odorless	Characteristic odour	Characteristic odour

Table 17 pH, viscosity and conductivity measurements of the cream formulations.

Name of Cream Formulation	pH values	Conductivity values (µs/cm)	Viscosity values (kcps)	Zeta Potential (mV)
Placebo cream	4.85 ± 0.155	52.10 ± 3.535	33.94 ± 0.268	41.65 ± 1.626
Complex cream	4.18 ± 0.049	111.00 ± 1.414	39.23 ± 2.269	36.10 ± 1.131
Levant storax cream	4.47 ± 0.424	70.88 ± 4.065	71.45 ± 0.926	37.70 ± 0.600

4.1.6 Stability Studies

4.1.6.1 Qualitative analysis

There were no noticeable changes in the organoleptic properties of the formulations in terms of appearance, colour and odour (Table 18) over the entire stability test period.

Table 18 Summary of the qualitative parameters of all cream formulations during storage at all test conditions. '√' means no change was observed in appearance, colour, odour, any signs of phase separation [a].

		T0				1th week				4th week				8th week			
		A.,	C.,	O.,	PS	A.,	C.,	O.,	PS.	A.,	C.,	O.,	PS.	A.,	C.,	O.,	PS
+4-8 ºC	PLACEBO	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	COMPLEX	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	LEVANT STORAX	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
25ºC ±2 ºC with 65%RH	PLACEBO	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	COMPLEX	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	LEVANT STORAX	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Room Temperature	PLACEBO	√	√	√	√	~	~	~	~	~	~	~	~	√	√	√	√
	COMPLEX	√	√	√	√	~	~	~	~	~	~	~	~	√	√	√	√
	LEVANT STORAX	√	√	√	√	~	~	~	~	~	~	~	~	√	√	√	√

[a]A; appearance, C; colour, O; odour, PS; phase separation, √; no change, ~; not tested.

4.1.6.2 Quantitative analysis

The stability test data obtained from the three batches of the placebo, complex and levant storax creams were summarized in Table 19, 20 and 21. Measurement results of pH, viscosity, conductivity, zeta potential were taken as described in sections of 3.1.3.5.2, 3.1.3.5.3, 3.1.3.5.4 and 3.1.3.5.5. Particle size distribution and arithmetic average particle size calculations were made as described in section 3.1.3.5.6. All of the results were presented as mean values ± S.D. of two measurements. As described earlier, each of the cream formulations were prepared in 3 batches.

4.1.6.2.1 pH

The pH data shown in Table 19, 20 and 21 indicated that pH of the cream formulations remained within the specification limits over the entire test period. However, it appeared that

the pH of the placebo cream batches fluctuated slightly after exposure to the stability conditions within the first week, but remained almost stable during the rest of the time. pH of the complex cream and levant storax batches decreased slightly at +25°C ± 2 °C 65 % RH during the stability studies.

Table 19 Stability data summary for placebo cream. P and B denote "Placebo cream" and "Batch" respectively.

Sampling time (week)	+4-8 °C			+25°C ± 2 °C 65 % RH			Room Temperature		
	PB1	PB2	PB3	PB1	PB2	PB3	PB1	PB2	PB3
	pH values								
0	4.96 ± 0.001	4.74 ± 0.028	4.76 ± 0.019	4.96 ± 0.001	4.73 ± 0.028	4.76 ± 0.019	4.96 ± 0.001	4.74 ± 0.028	4.76 ± 0.019
1	4.84 ± 0.000	4.85 ± 0.026	4.83 ± 0.001	4.83 ± 0.000	4.86 ± 0.007	4.85 ± 0.001			
4	4.84 ± 0.000	4.84 ± 0.003	4.84 ± 0.000	4.84 ± 0.002	4.84 ± 0.002	4.82 ± 0.004			
8	4.84 ± 0.014	4.84 ± 0.000	4.86 ± 1.414	4.80 ± 0.002	4.86 ± 0.001	4.84 ± 1.414	4.95 ± 0.009	4.89 ± 0.004	4.87 ± 3.535
	Conductivity values (µs/cm)								
0	36.70 ± 0.282	40.80 ± 0.494	61.30 ± 0.565	36.70 ± 0.282	40.80 ± 0.494	61.30 ± 0.565	40.80 ± 0.494	89.30 ± 0.777	61.30 ± 0.565
1	62.90 ± 1.131	54.80 ± 1.414	52.50 ± 0.707	47.85 ± 0.494	54.30 ± 0.353	55.15 ± 0.636			
4	53.90 ± 0.707	52.00 ± 0.707	52.4 ± 0.424	51.30 ± 0.000	45.40 ± 0.282	44.25 ± 1.767			
8	53.20 ± 0.707	59.95 ± 0.353	61.00 ± 0.707	57.55 ± 0.494	53.40 ± 0.848	47.00 ± 0.636	51.10 ± 0.282	85.40 ± 0.848	57.00 ± 0.070
	Viscosity values (kcps)								
0	33.75 ± 0.945	34.13 ±1.366	26.41 ± 0.473	33.75 ± 0.945	34.13 ±1.366	26.41 ± 0.473	34.13 ±1.366	40.83 ± 1.178	26.41 ± 0.473
1	32.80 ± 0.424	32.40 ± 0.565	27.60 ± 0.707	35.10 ± 1.414	34.68 ± 0.813	28.94 ± 0.197			
4	32.50 ± 1.414	33.55 ± 0.424	28.21 ± 0.058	34.50 ± 1.414	35.05 ± 0.070	29.03 ± 0.035			
8	31.65 ± 0.212	33.20 ± 0566	30.41 ± 0.118	32.5 ± 0.707	34.23 ± 0.094	28.63 ± 0.176	31.10 ± 0.141	41.92 ± 0.589	29.00 ± 0.070
	Zeta Potential (mV)								
0	41.65 ± 1.626	37.55 ± 0.353	40.90 ± 2.121	41.65 ± 1.626	37.55 ± 0.353	40.90 ± 2.121	41.65 ± 1.626	37.55 ± 0.353	40.90 ± 2.121
4	45.65 ± 0.212	41.55 ± 0.070	39.10 ± 2.969	32.90 ± 3.535	45.10 ± 1.414	43.70± 0.565			
8	45.15 ± 0.777	48.30 ± 1.979	46.10 ± 0.565	50.80 ± 1.131	56.25 ± 1.202	38.75 ± 1.484	50.00 ± 1.131	42.20 ± 1.131	47.85 ± 1.767

Table 20 Stability data summary for complex cream. C and B denote “Complex cream” and “Batch” respectively.

Sampling time (week)	+4-8 °C			+25°C ± 2 °C 65 % RH			Room Temperature		
	CB1	CB2	CB3	CB1	CB2	CB3	CB1	CB2	CB3
	pH values								
0	4.22 ± 0.022	4.15 ± 0.000	4.17 ± 0.000	4.22 ± 0.022	4.15 ± 0.000	4.17 ± 0.000	4.22 ± 0.022	4.15 ± 0.000	4.17 ± 0.000
1	4.17 ± 0.009	4.11 ± 0.002	4.20 ± 0.003	4.19 ± 0.005	4.20 ± 0.003	4.17 ± 0.000			
4	4.17 ± 0.000	4.17 ± 0.000	4.17 ± 0.000	4.17 ± 0.002	4.17 ± 0.001	4.17 ± 0.003			
8	4.16 ± 0.000	4.16 ± 0.000	4.17 ± 0.000	4.18 ± 0.005	4.15 ± 0.001	4.15 ± 0.001	4.14 ± 0.000	4.14 ± 0.000	4.14 ± 0.001
	Conductivity values (µs/cm)								
0	89.30 ± 0.777	126.60 ± 1.697	121.10 ± 0.424	89.30 ± 0.777	126.60 ± 1.697	121.10 ± 0.424	89.30 ± 0.777	126.60 ± 1.697	121.10 ± 0.424
1	84.85 ± 0.070	92.35 ± 0.777	95.90 ± 0.424	81.00 ± 0.424	94.10 ± 0.565	93.75 ± 3.606			
4	85.75 ± 0.707	91.35 ± 0.494	94.85 ± 2.474	81.65 ± 1.202	92.15 ± 0.494	85.85 ± 1.343			
8	95.40 ± 3.959	97.50 ± 0.141	105.60 ± 3.536	64.4 ± 1.131	92.55 ±1.060	94.50 ± 0.707	85.40 ± 0.848	91.65 ± 0.919	97.90 ± 0.778
	Viscosity values(kcps)								
0	40.83 ± 1.178	37.62 ± 1.555	36.75 ± 1.060	40.83 ± 1.178	37.62 ± 1.555	36.75 ± 1.060	40.83 ± 1.178	30.90 ± 1.555	28.50 ± 1.650
1	41.25 ± 0.353	34.75 ± 0.353	38.00 ± 0.000	39.80 ± 0.989	36.75 ± 1.060	35.65 ± 0.494			
4	41.50 ± 0.707	34.25 ± 1.060	39.83 ± 0.235	42.25 ± 0.353	41.50 ± 0.707	39.94 ± 0.082			
8	41.25 ± 0.354	37.33 ± 0.471	40.23 ± 0.613	41.25 ± 0.353	39.08 ± 0.118	38.40 ± 0.141	41.92 ± 0.589	41.00 ± 0.707	40.00 ± 0.354
	Zeta Potential (mV)								
0	36.10 ±1.131	37.65 ± 0.494	34.20 ± 0.282	36.10 ± 1.131	37.65 ± 0.494	34.20 ± 0.282	36.10 ±1.131	37.65 ± 0.494	34.20 ± 0.282
4	30.95 ± 0.353	35.65 ± 0.122	35.80 ± 0.565	33.80 ± 2.828	37.90 ± 0.000	34.80 ± 0.000			
8	44.95 ± 0.636	41.95 ± 0.636	42.30 ± 1.697	41.25 ± 1.774	45.05 ± 0.070	46.15 ± 3.040	44.35 ± 2.757	41.05 ± 1.484	44.20 ± 1.979

Table 21 Stability data summary for levant storax cream. LS and B denote “levant storax” and “Batch” respectively.

Sampling time (week)	+4-8 °C			+25°C ± 2 °C 65 % RH			Room Temperature		
	LSB1	LSB2	LSB3	LSB1	LSB2	LSB3	LSB1	LSB2	LSB3
	pH values								
0	4.44 ± 0.007	4.44 ± 0.016	4.50 ± 0.017	4.44 ± 0.007	4.44 ± 0.016	4.50 ± 0.017	4.44 ± 0.007	4.44 ± 0.016	4.50 ± 0.017
1	4.40 ± 0.043	4.38 ±0.005	4.49 ± 0.003	4.40 ± 0.004	4.38 ± 0.012	4.50 ± 0.005			
4	4.40 ± 0.002	4.37 ± 0.001	4.40 ± 0.002	4.39 ± 0.003	4.36 ± 0.007	4.42 ± 0.002			
8	4.38 ± 0.000	4.36 ± 0.002	4,44 ± 0.001	4.33 ± 0.000	4.32 ± 0.000	4.39 ± 0.002	4.38 ± 0.000	4.32 ± 0.002	4.38 ± 0.001
	Conductivity values (µs/cm)								
0	73.75 ± 0.636	71.50 ± 2.969	58.25 ± 0.707	73.75 ± 0.636	71.50 ± 2.969	58.25 ± 0.707	73.75 ± 0.636	71.50 ± 2.969	58.25 ± 0.707
1	72.85 ± 0.777	59.80 ± 0.565	51.10 ± 0.424	72.45 ± 0.919	61.45 ± 0.636	48.50 ± 0.424			
4	75.35 ± 0.777	60.70 ± 0.565	53.70 ± 0.989	71.60 ± 0.989	61.45 ± 0.777	51.15 ± 0.494			
8	82.05 ± 0.919	69.35 ± 1.060	61.55 ± 0.212	68.90 ± 0.848	68.9 ± 5.798	55.75 ± 0.495	68.25 ± 0.919	62.95 ± 1.343	50.85 ± 0.919
	Viscosity values(kcps)								
0	70.59 ± 2.001	71.90 ± 1.548	50.40 ± 2.262	70.585 ± 2.001	71.91 ± 1.548	50.40 ± 2.262	70.59 ± 2.001	71.91 ± 1.548	50.40 ± 2.262
1	73.25 ± 0.212	72.40 ± 0.424	48.55 ± 0.636	70.95 ± 0.212	73.26 ± 0.770	50.75 ± 0.353			
4	64.95 ± 0.636	66.25 ± 0.353	42.50 ± 0.707	68.40 ± 1.014	72.05 ± 0.353	47.75 ± 0.353			
8	72.12 ± 0.304	71.83 ± 0.471	48.75 ± 1.061	71.25 ± 0.306	73.17± 0.235	48.75 ± 0.471	67.10 ± 0.141	72.75 ± 0.353	52.50 ± 0.707
	Zeta Potential (mV)								
0	37.70 ± 0.600	34.65 ± 1.484	34.6 ± 1.414	37.70 ± 0.600	34.65 ± 1.484	34.60 ± 1.414	37.70 ± 0.600	34.65 ± 1.484	34.60 ± 1.414
4	45.15 ± 1.484	40.80 ± 1.131	46.70 ± 1.272	42.50 ± 0.989	37.65 ± 1.767	45.85 ± 0.353			
8	46.25 ± 1.060	48.60 ± 0.282	46.40 ± 2.545	37.40 ± 1.697	46.80 ± 1.272	49.90 ± 0.000	45.15 ± 1.484	48.35 ± 2.050	45.40 ± 0.282

4.1.6.2.2 Conductivity

Electrical conductivity values of the cream formulations that were kept at different storage conditions were presented in Table 19, 20 and 21. Based on these results, we can conclude that the creams were still oil in water (O/W) emulsions and there were no major structural changes which could have affected the conductivity during the stability tests.

4.1.6.2.3 Viscosity

The viscosity measurements are summarized in Table 19, 20 and 21 showing that the viscosity of the creams remained unchanged within the established specification ranges over the stability test period. Based on these results, the cream formulations are considered stable for the 2 months test period under investigation.

4.1.6.2.4 Zeta Potential

Zeta potential results are summarized in Table 19, 20 and 21.

4.1.6.2.5 Particle (globule) size and Particle size distribution determinations

4.1.6.2.5.1 Microscopic examination and microphotograph counts

The changes in avarage particle size and the actual size distribution of the creams during storage are presented in Table 22, 23, 24.

Table 22 Calculated aritmetic mean diameters of particle size for placebo cream batches at different conditions and time intervals. P; Placebo, B; Batch.

Sampling time (week)	+4-8 °C			+25°C ± 2 °C 65 % RH			Room Temperature		
	PB1	PB2	PB3	PB1	PB2	PB3	PB1	PB2	PB3
	Arithmetic mean diameter (µm)								
0	0,04	0,02	0,03	0,04	0,02	0,03	0.04	0,03	0,03
4	0,10	0,10	0,11	0,13	0,11	0,15			
8	0,21	0,10	0,14	0,16	0,20	0,21	0.16	0,17	0,13

Table 23 Calculated aritmetic mean diameters of particle size for complex cream batches at different conditions and time intervals. C; Complex, B; Batch.

Sampling time (week)	+4-8 °C			+25°C ± 2 °C 65 % RH			Room Temperature		
	CB1	CB2	CB3	CB1	CB2	CB3	CB1	CB2	CB3
	Arithmetic mean diameter (µm)								
0	0,19	0,06	0,22	0,19	0,06	0,22	0,19	0,06	0,22
4	0,18	0,17	0,18	0,17	0,15	0,17			
8	0,17	0,14	0,24	0,16	0,11	0,18	0,18	0,16	0,18

Table 24 Calculated aritmetic mean diameters of particle size for levant storax cream batches at different conditions and time intervals. LS; Levant storax, B; Batch.

Sampling time (week)	+4-8 °C			+25°C ± 2 °C 65 % RH			Room Temperature		
	LSB1	LSB2	LSB3	LSB1	LSB2	LSB3	LSB1	LSB2	LSB3
	Arithmetic mean diameter (µm)								
0	0,12	0,17	0,24	0,12	0,17	0,24	0,19	0,12	0,17
4	0,15	0,16	0,19	0,21	0,21	0,30			
8	0,25	0,24	0,24	0,20	0,17	0,22	0,18	0,20	0,17

4.1.6.2.5.2 Measurements of particle sizes with Turboscan™ technology

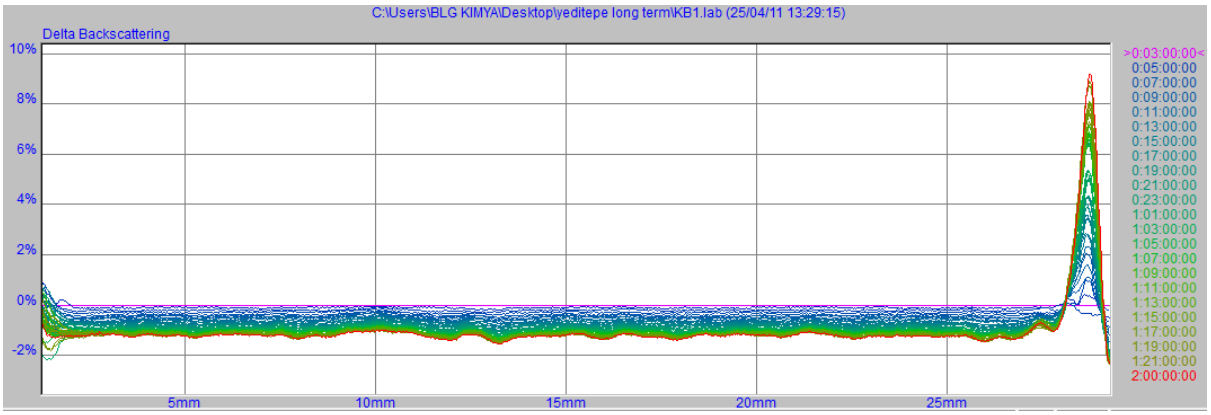

Figure 19 Delta Backscattering results of complex cream batch 1.

Figure 19 shows that there is some creaming and a particle size growth.

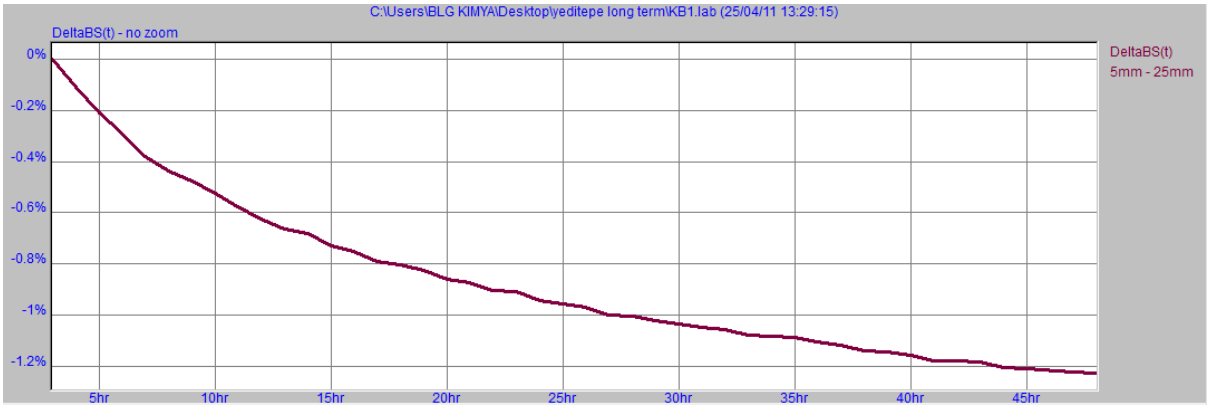

Figure 20 Delta BS (t) graph of complex cream batch 1.

In order to understand particle size growth, Delta BS (t) graph needs to be analyzed. According to the graph, there is about 1,2 % change in the 2 days experiment, and growth rate becomes slower as seen from the slope of the graph (Figure 20).

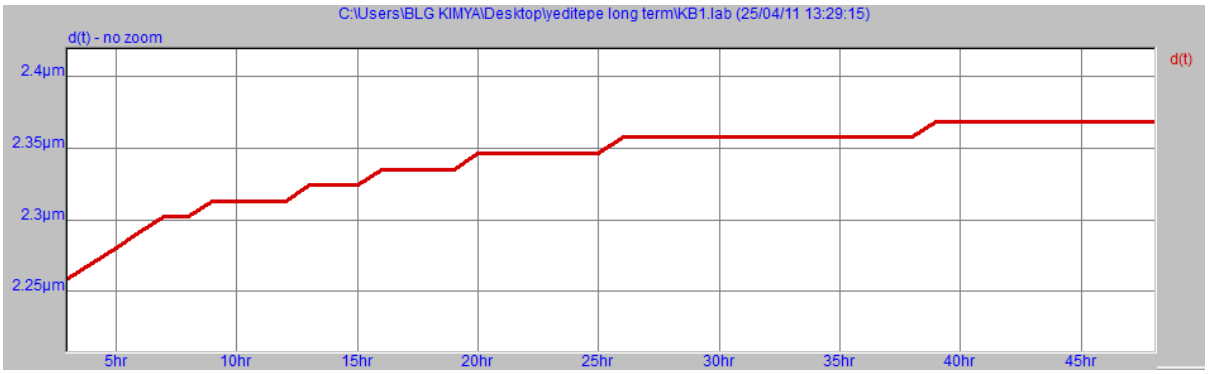

Figure 21 Change in particle diameter of complex cream batch 1.

Figure 21 shows the changes on avarage diameter. Particle diameter increased from 2,26 µm to 2, 37 µm during measurement period. Furthermore the graph shows a reduction on increment rate.

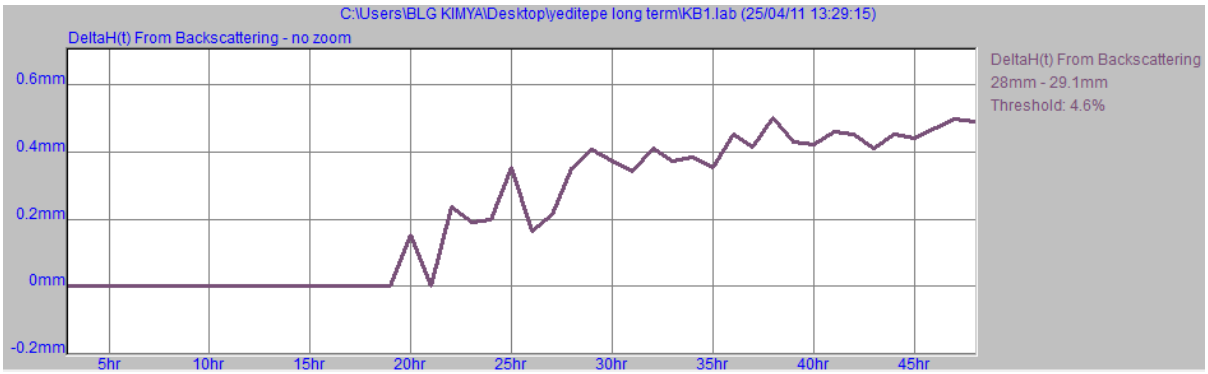

Figure 22 Graph of DeltaH(t) of complex cream batch 1.

In order to investigate the creaming against time we use graph of DeltaH(t) (Figure 22). After two days creaming increased to 0.5 mm width. At the end of the measurement period, creaming decreased and creaming width is fixed which means that the sample is stabilised.

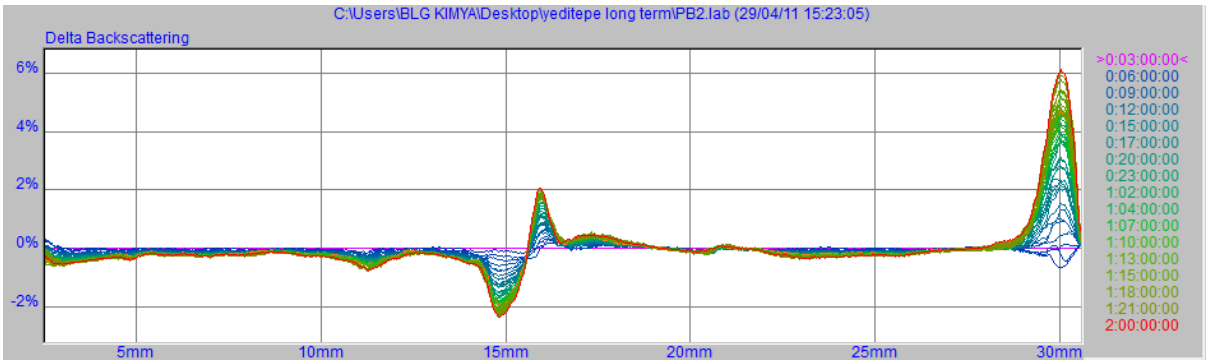

Figure 23 Delta Backscattering results of placebo cream batch 1.

In Figure 23, Delta Backscattering graph shows, there is slight creaming.

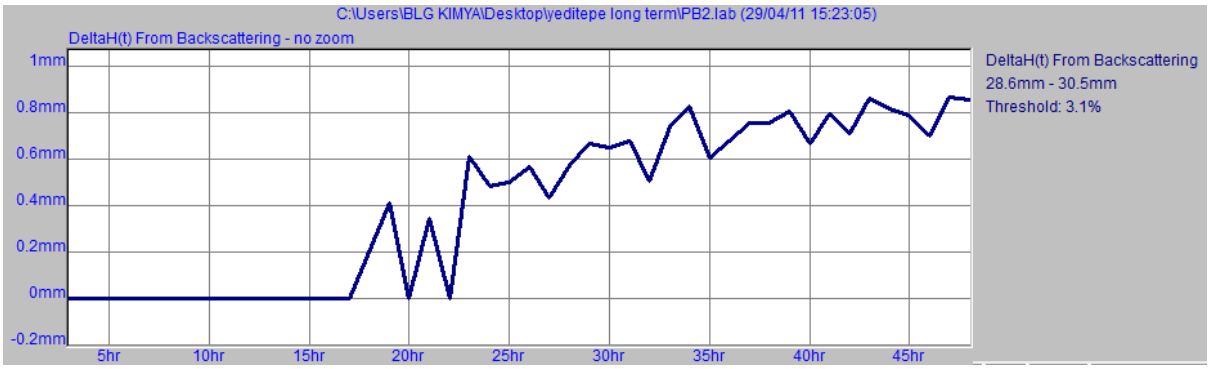

Figure 24 Delta BS (t) graph of placebo cream batch 1.

At the end of the second day, creaming was about 0,8 mm on the top of the tube, however creamin rate was higher in the initial hours, and slower and then linear in the later hours (Figure 24).

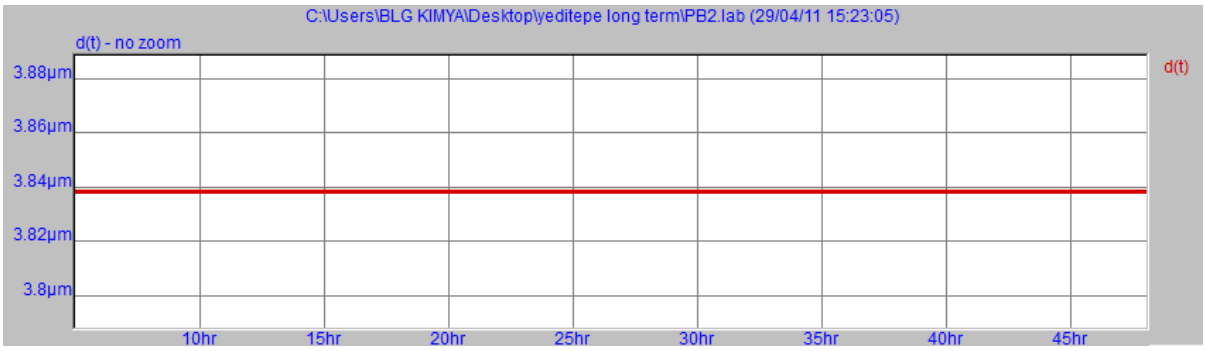

Figure 25 Change in particle diameter of placebo cream batch 1.

Avarage particle diameter remains unchanged (Figure 25).

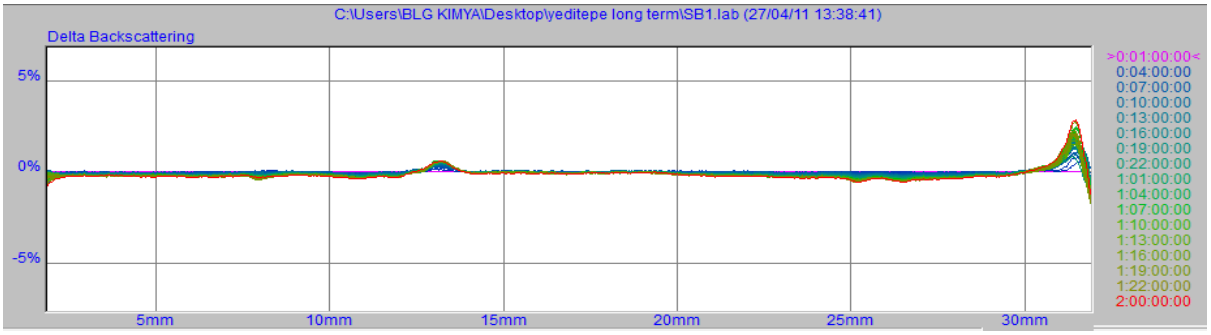

Figure 26 Delta Backscattering results of levant storax cream batch 1.

At the end of 2 days, creaming reaches about the thickness of 0,3 mm, creaming rate slows down and stabilized towards the end of the experiment (Figure 26).

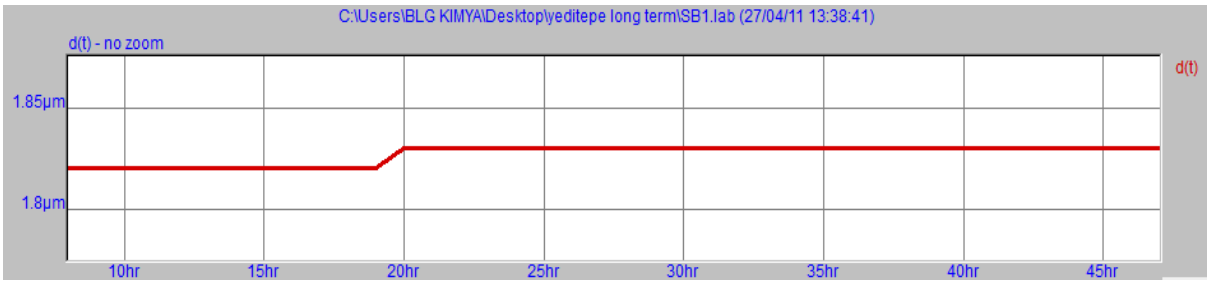

Figure 27 Change in particle diameter of levant storax cream batch 1.

Avarage particle diameter remains unchanged (Figure 27).

4.1.6.2.6 Freeze Thawing Studies

During the freeze thawing studies, no phase separation was observed during each of the cycles (Table 25). This is true for standard as well as stressed freeze-thawing conditions.

Table 25 The results of freeze thawing studies . '√' means no phase separation.

Thawing Temperature	Group	1th cycle	2th cycle	3th cycle	4th cycle	5th cycle	6th cycle
40 °c	PLACEBO	√	√	√	√	√	√
	COMPLEX	√	√	√	√	√	√
	LEVANT STORAX	√	√	√	√	√	√
Room Temperature	PLACEBO	√	√	√	√	√	√
	COMPLEX	√	√	√	√	√	√
	LEVANT STORAX	√	√	√	√	√	√

4.1.6.2.7 Microbiological compliance tests

No contamination could be detected for all formulations in the SDA and TSA plates during incubator periods both at the beginning and the end of the stability studies, for all conditions (Figure 28, 29).

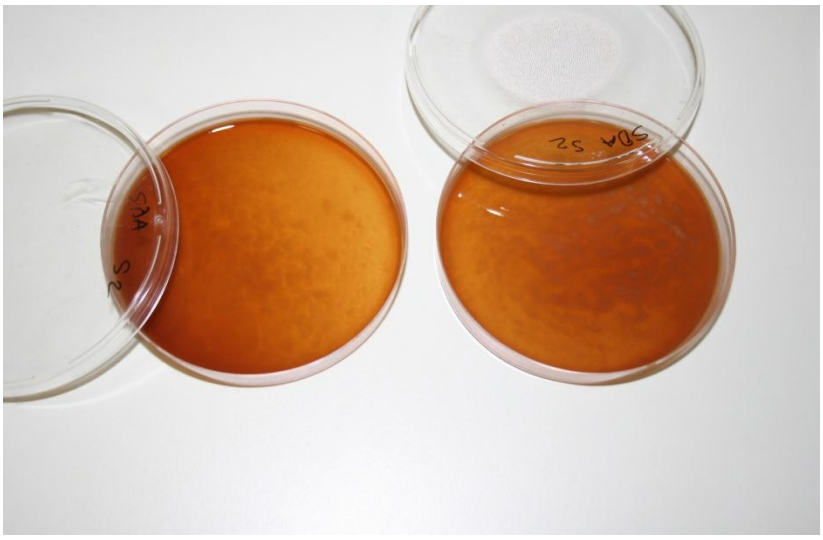

Figure 28 Petri dishes for Sabouraud Dextrose Agar (SDA) after 5 days of storage at 37ºC.

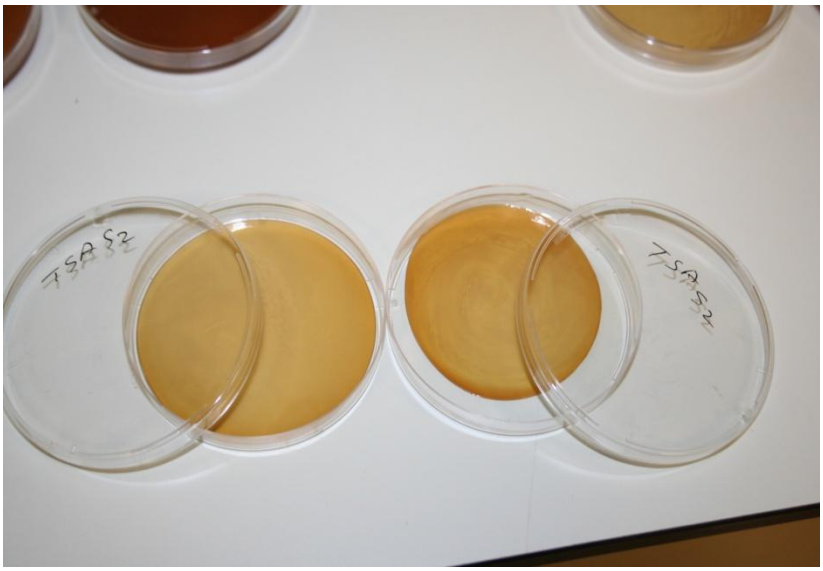

Figure 29 Petri dishes for Tryptic Soy Agar (TSA) after 5 days of storage at 37ºC.

4.2 Results for the wound healing activity

4.2.1 Wound contraction rates

The data obtained from Image J computations are represented in Table 26, 27 and these results are represented on Figure 30 and 31. The percentage wound healing results are statistically analyzed by using repeated measure ANOVA for variation between different time intervals and groups (Appendix 2 to Appendix 6). Also, one way ANOVA was used in order to analyze the measured values obtained by DLite Analog Microscope for variation between different groups according to percentage change of wound area on day 9 (Appendix 7 to 11). $P<0.05$ was considered significant. When repeated measure ANOVA was applied to check differences between time intervals and groups, it gave insignificant changes. And, when one way ANOVA with Tukey post-hoc tests were used to analyze differences between groups, it gave significant changes.

The levant storax group showed the best significant activity among all other groups. The complex and the reference groups demonstrated significant activity when the groups were compared with the control group.

Table 26 Progressive changes of wound area of all groups monitored by camera (Nicon®).

DAY	CONTROL	PLACEBO	REFERENCE	COMPLEX	LEVANT STORAX
	WOUND AREA(mm^2)				
0	14,083 ± 2,025	13,952 ± 1,396	14,447 ± 2,004	14,540 ± 1,699	14,482 ± 1,597
2	12,305 ± 1,653	11,771 ± 1,968	12,257 ± 2,157	12,439 ± 2,653	11,807 ± 2,104
4	10,836 ± 1,861	10,011 ± 1,946	10,497 ± 2,042	10,651 ± 2,455	10,018 ± 2,269
6	7,382± 2,499	7,164 ± 1,864	6,270 ± 3,129	6,632 ± 2,190	5,982 ± 2,795
9	2,939 ± 1,515	2,556 ± 2,844	2,000 ± 1,615	1,812 ± 1,671	1,504 ± 1,511

Values are expressed as mean ± SD, N: ~ 30

Table 27 Progressive changes of wound area of all groups monitored by DLite Analog Microscope on day 0 and 9.

DAY	CONTROL	PLACEBO	REFERENCE	COMPLEX	LEVANT STORAX
	WOUND AREA(mm^2)				
0	13,045 ± 2,290	14,736 ± 1,877	15,615 ± 2,442	14,716 ± 2,711	15,998 ± 2,349
9	3,134 ± 1,610	2,853 ± 1,993	2,515 ± 1,814	2,398 ± 1,370	1,224 ± 1,100

Values are expressed as mean ± SD, N: ~ 30

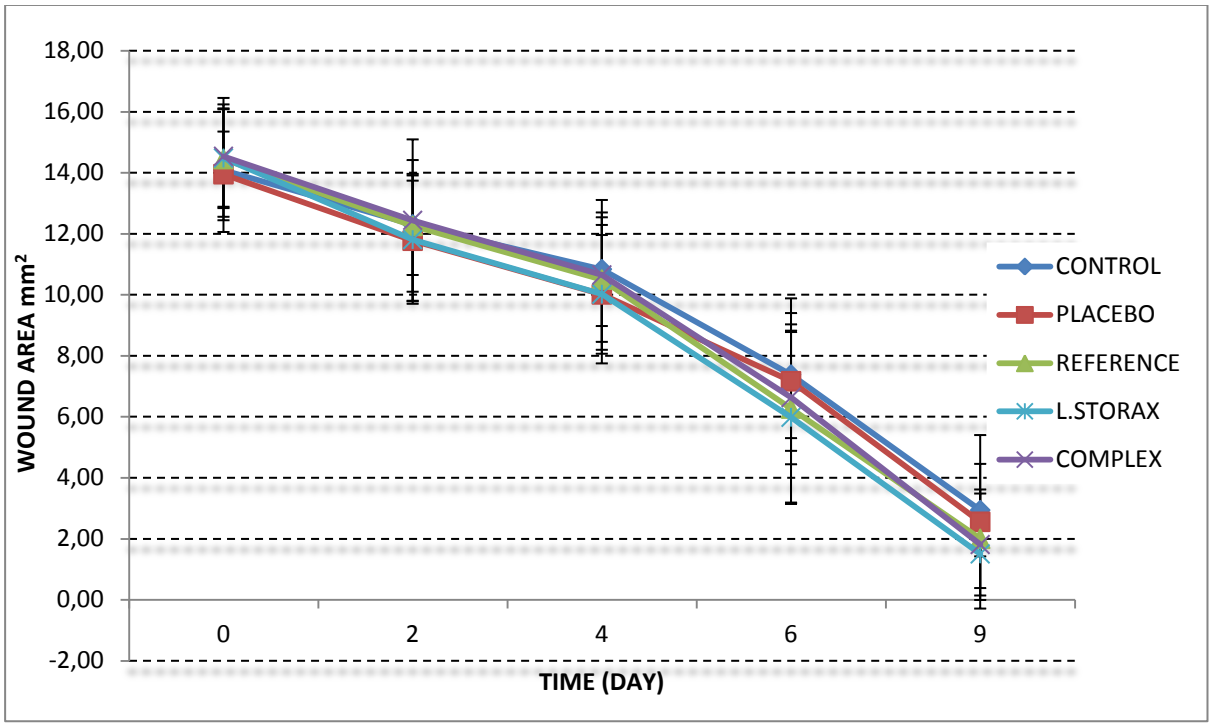

Figure 30 Wound contraction rates of all groups according calculated based on photograph of the camera.

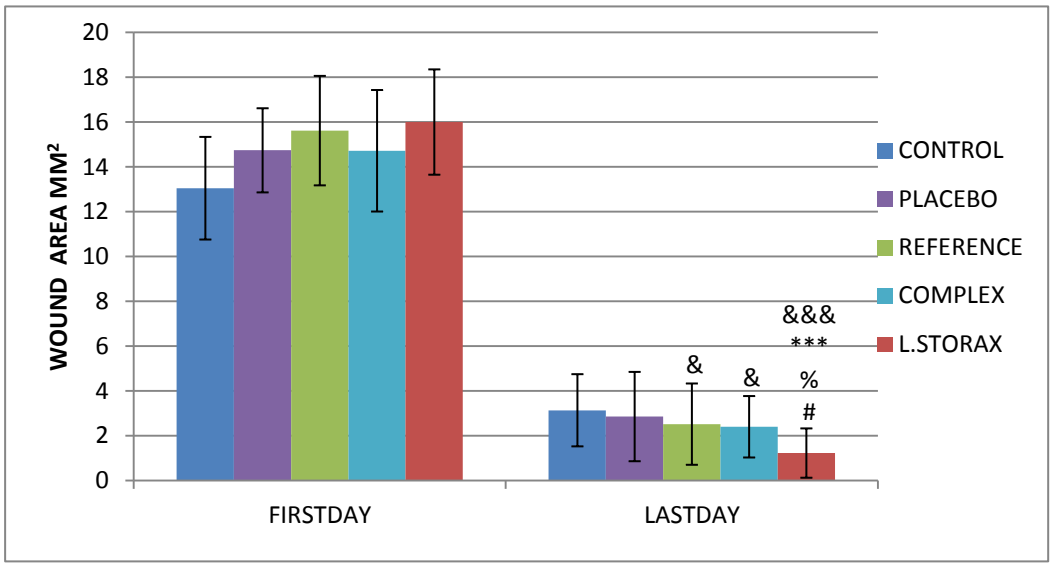

Figure 31 Wound contraction of all groups according to photographs of DLite Analog Microscope.'&'; compared with the control group, '*'; compared with the placebo group, '%'; compared with the reference group, '#'; compared with the complex group.
&,#, % p< 0,05
***, &&& p< 0,001

4.2.2 Full Thickness Skin Development

Treated skin thicknesses of all groups according to percentage size differences between intact and treated skin tissue are shown Table 28. The percentage data obtained from measurement of treated and intact skin thicknesses were statistically analyzed using one way ANOVA for variation between different groups, $P<0.05$ was considered significant. When one way ANOVA with Tukey post-hoc tests were used to analyze differences between the groups, results showed differences. Levant storax cream group showed significant activity when the group was compared with the control, placebo and reference groups. Complex cream group also demonstrated significant results against the placebo group (Figure 32, Appendix 12).

Table 28 The 9th day skin thickness results according to percentage differences between intact and treated skin tissue.

CONTROL	PLACEBO	REFERENCE	COMPLEX	LEVANT STORAX
Thickness of wounds (%)				
75,258 ± 9,643	63,411 ± 11,033	76,179 ± 10,984	80,553 ± 5,644	93,520 ± 15,522

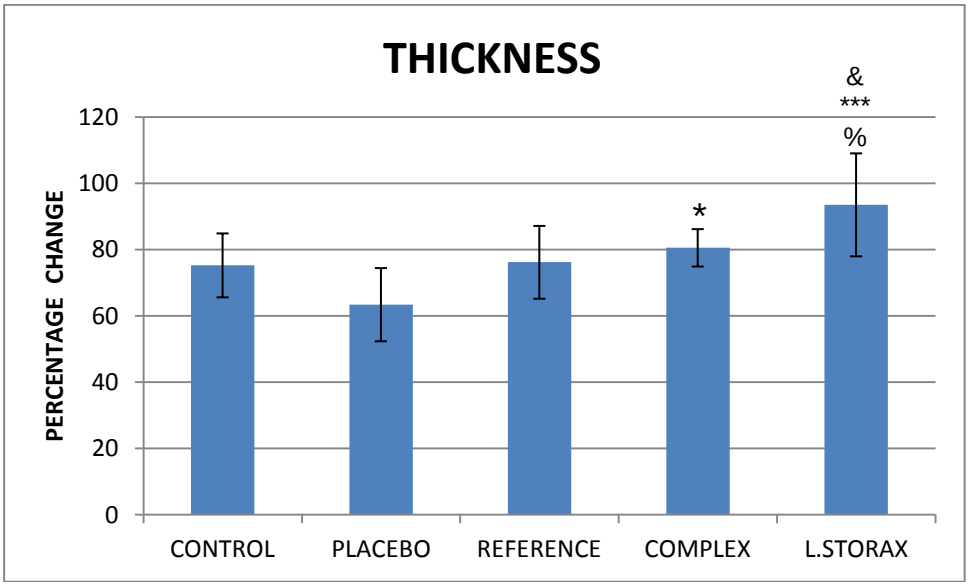

Figure 32 Percentage changes of full thickness of all groups. '&'; compared with the control group, '*'; compared with the placebo group, '%'; compared with the reference group.
&,*, % p< 0,05
***p< 0,001

4.2.3 Histopathological Evaluations

In order to evaluate histopathological parameters such as active and chronic inflammation, neovascularization, fibrosis and fibroblastic activity levels, findings were scored 0 to 3 scale meaning nil (0), mild (1), moderate (2) and severe (3). The mean scores for all groups were calculated and shown in Table 29. Histopathological views of wound healing in the different groups are shown in Figure 33, 34, 35, 36, 37. All data obtained from histopathologic observations were analyzed by using:

- Kruscall Wallis test for all histopathologic parameters (Appendix 13).
- Mann-Whitney U test for variations between two groups.

In both analysis, the differences were significant in terms of active and chronic inflammation, vascularization level, fibrosis and fibroblastic activity. Levant storax cream was the best in all favourable scores as healing indications. As shown in Table 29, healing phase was complete for levant storax cream. These results indicate that histopathologically both experimental creams performed better than the reference cream, placebo cream and of course the control group.

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Table 29 Histopathological evaluation of the wound sections treated with test creams on the wound healing processes and healing phases[a].

GROUP NAME	NEUTROPHIL	MONONUCLEAR-CELLS	FIBROBLASTIC ACTIVITY	NEOVASCULARIZATION	FIBROSIS	HAIR FOLLICLE FORMATION	HEALING PHASE
CONTROL	1.000 ± 0.471	1.277 ± 0.375	1.833 ± 0.537	1.777 ± 0.611	0.555 ± 0.455	0.472 ± 0.267	2.444 ± 0.467
PLACEBO	0.600 ± 0.383	1.333 ± 0.311	1.766 ± 0.560	1.600 ± 0.383	0.133 ± 0.182	0.533 ± 0.182	2.066 ± 0.547
REFERENCE	0.388 ± 0.490	0.777 ± 0.490	1.194 ± 0.871	1.111 ± 0.854	0.666 ± 0.298	0.333 ± 0.298	2.666 ± 0.421
COMPLEX	0.222 ± 0.403	0.527 ± 0.371	0.777 ± 0.201	0.527 ± 0.400	0.416 ± 0.361	0.555 ± 0.172	3.000 ± 0.000
LEVANT STORAX	0.111 ± 0.272	0.361 ± 0.125	0.138 ± 0.194	0.222 ± 0.13	0.277 ± 0.136	0.944 ± 0.136	3.000 ± 0.000

[a]Hematoxylin and eosin (HE) stained sections were scored as nil (0), mild (1), moderate (2) and severe (3) for epidermal and/or dermal re-modelining.

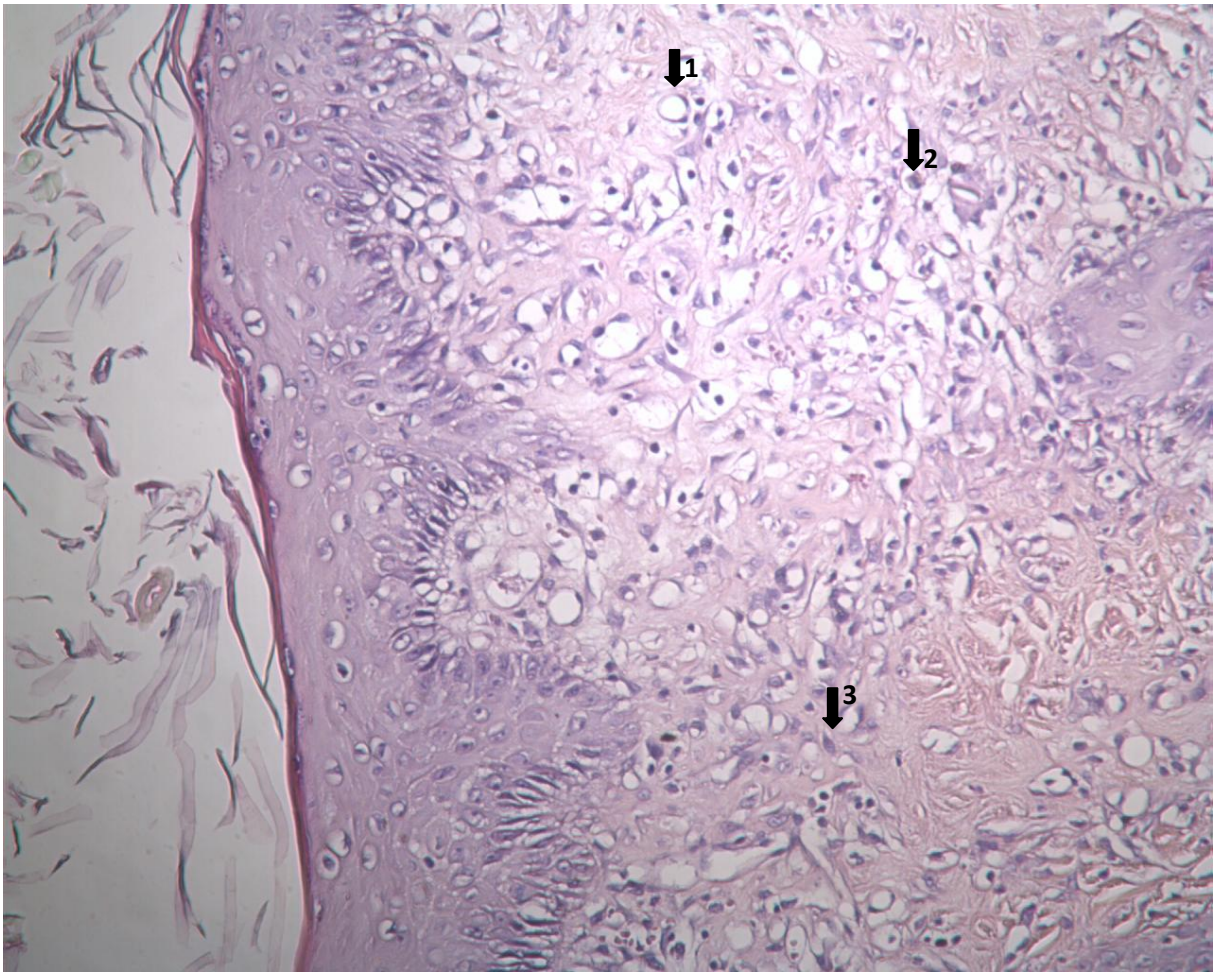


Figure 33 Histopathological view of wound healing in the control group. The skin sections show the hematoxylin and eosin stained epidermis and dermis. Arrows pointing events during wound healing; 1, neovascularization; 2, mononuclear cell; 3, fibroblast.

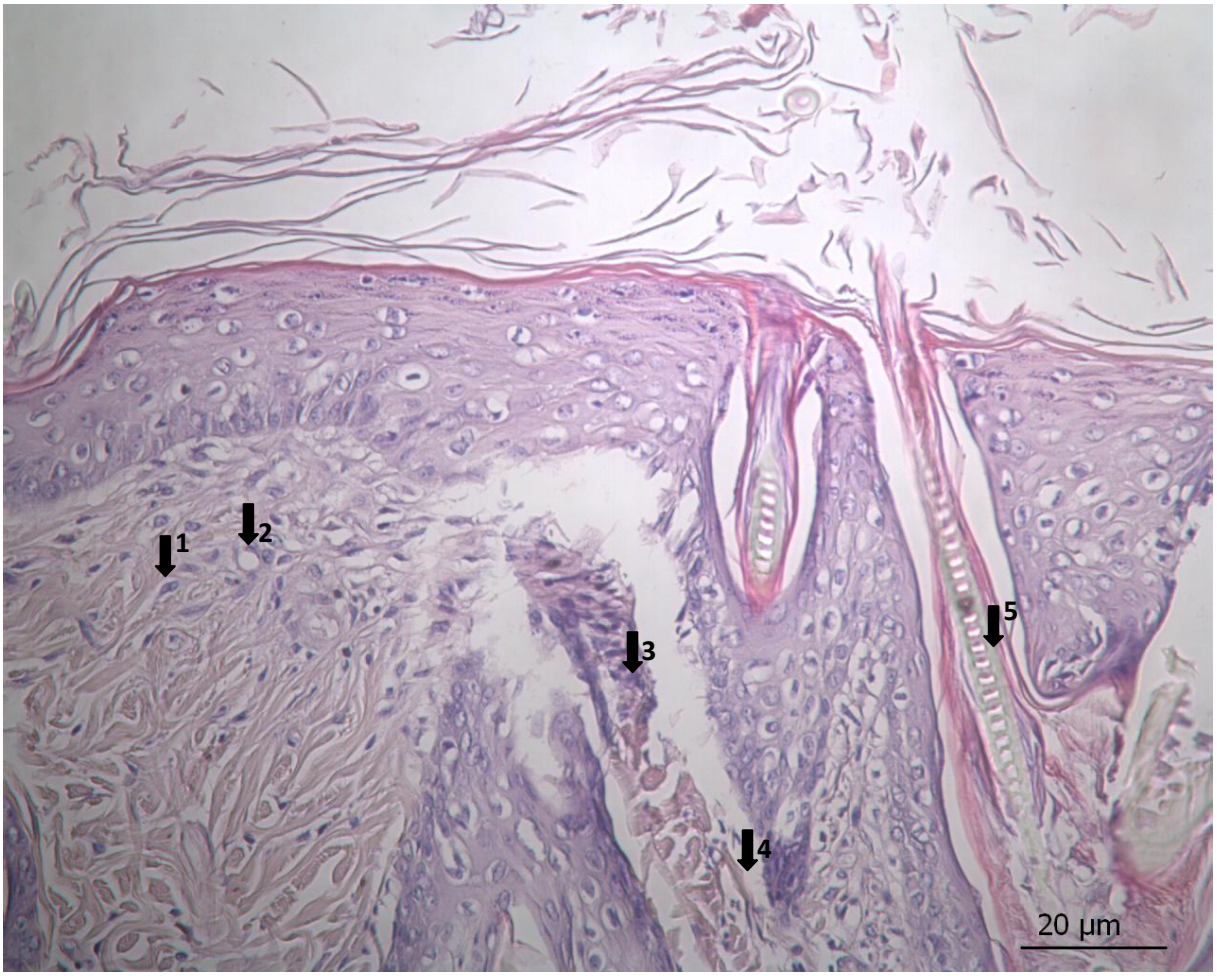


Figure 34 Histopathological view of wound healing in the placebo group. The skin section shows the hematoxylin and eosin stained epidermis and dermis. Arrows pointing events during wound healing; 1, fibroblast; 2, neovascularization; 3, neutrophil; 4, congestion; 5, hair follicle.

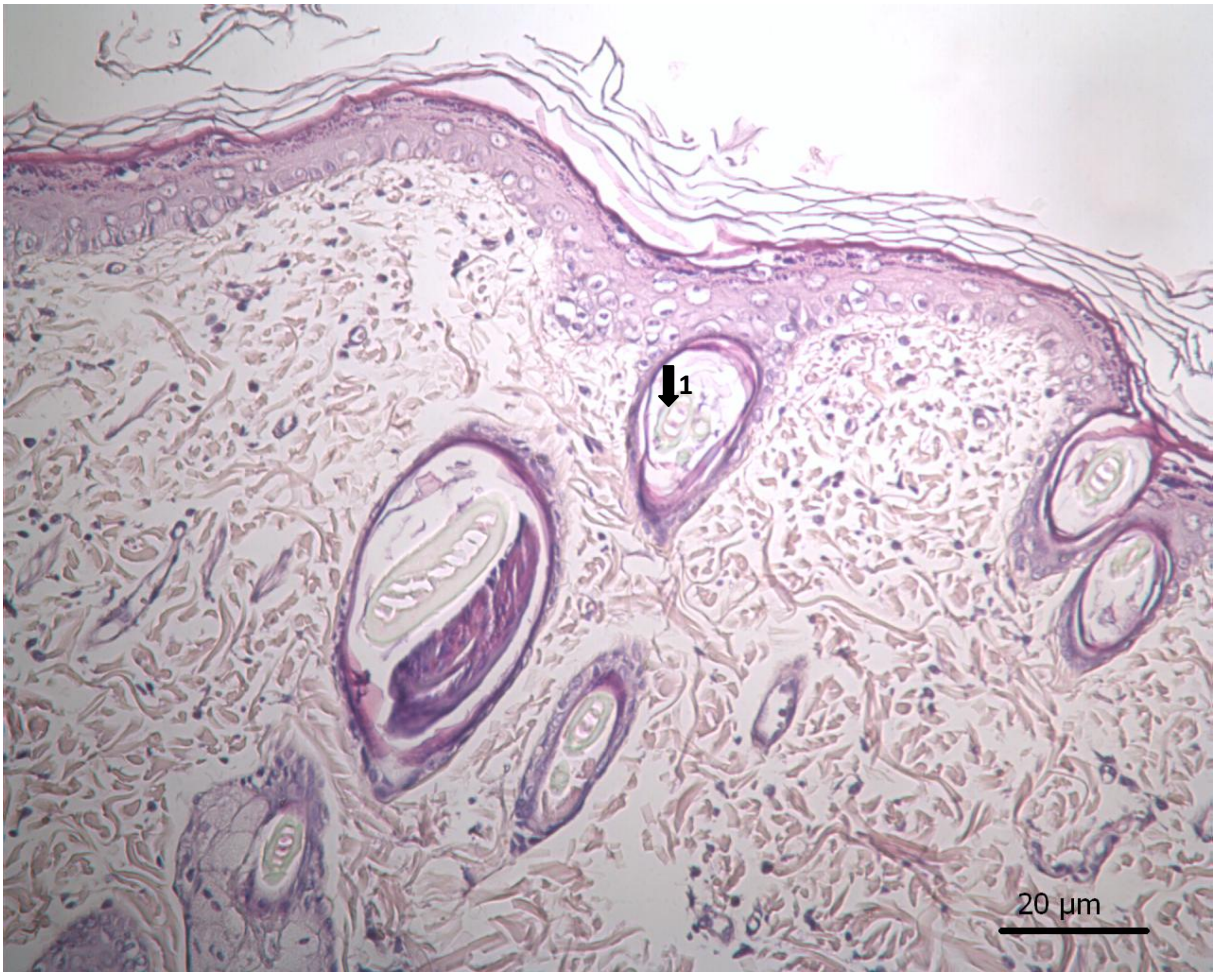


Figure 35 Histopathological view of wound healing in the reference group. The skin section shows the hematoxylin and eosin stained epidermis and dermis. Arrows pointing events during wound healing. Arrows pointing events during wound ealing; 1, hair follicle.

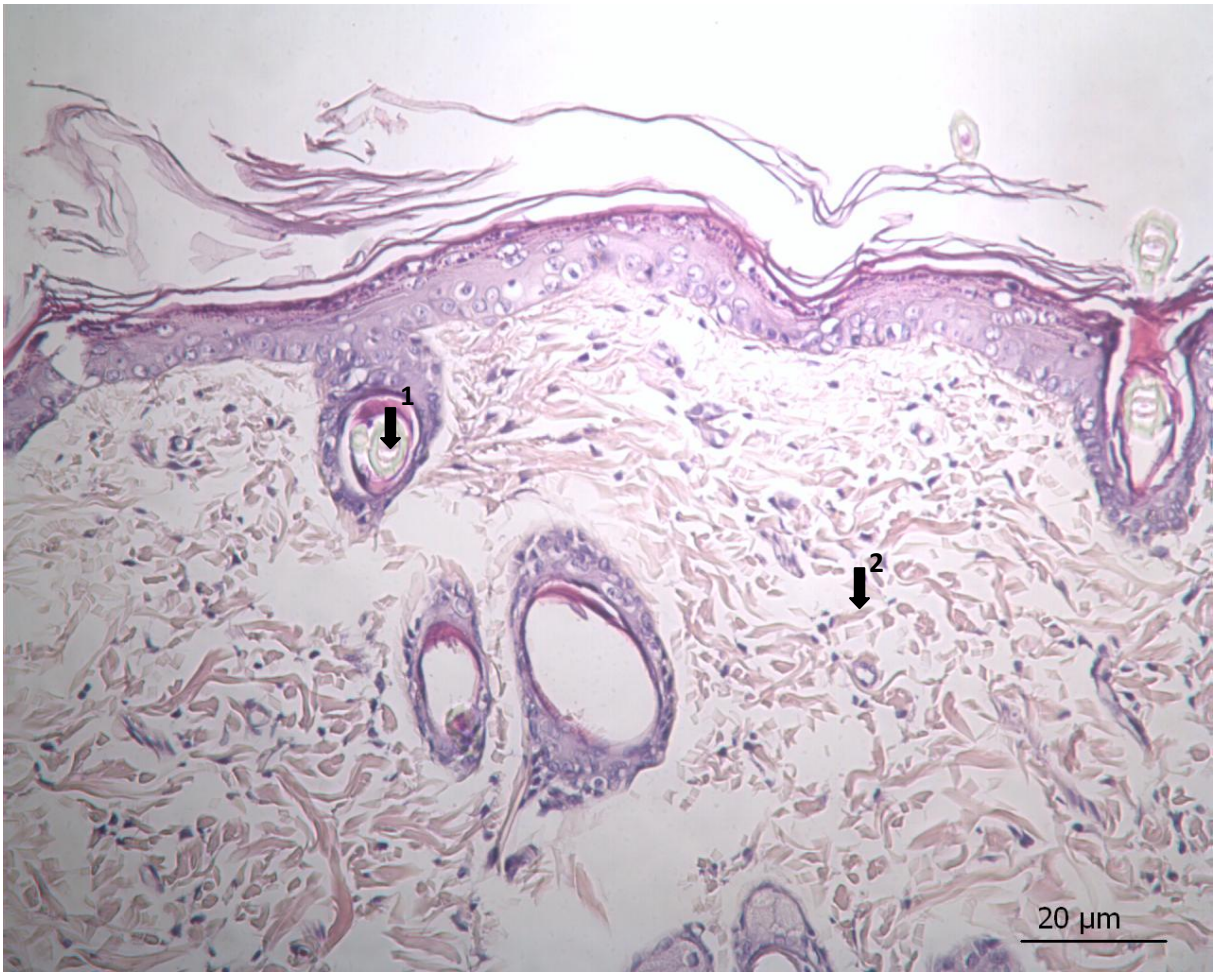


Figure 36 Histopathological view of wound healing in the complex group. The skin section shows the hematoxylin and eosin stained epidermis and dermis. Arrows pointing events during wound healing; 1, hair follicle; 2, collagen fibre.

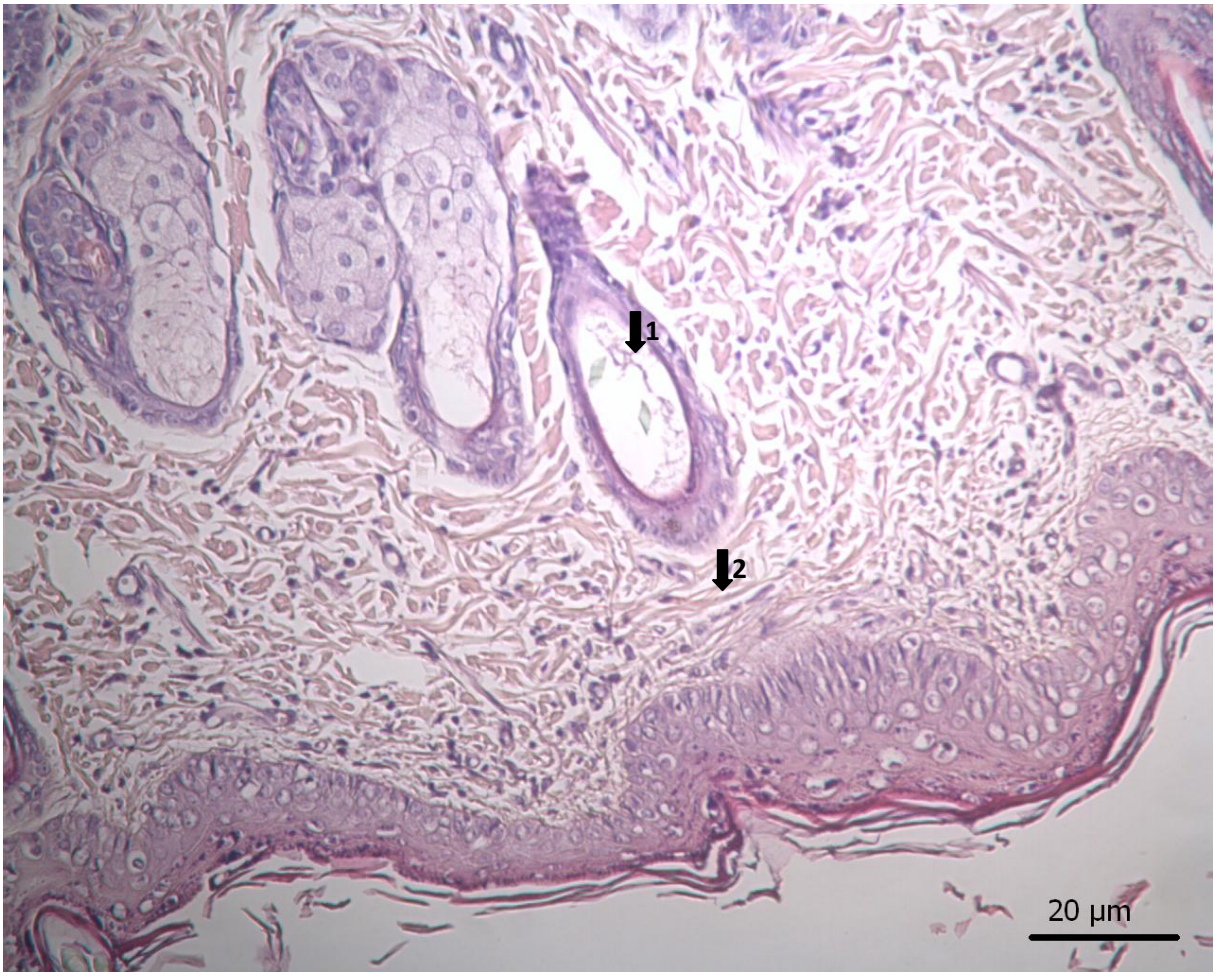


Figure 37 Histopathological view of wound healing in the levant storax group. The skin section shows the hematoxylin and eosin stained epidermis and dermis. Arrows pointing events during wound healing; 1, hair follicle; 2, collagen fibre.

4.2.3.1 Active Inflammation

By using Kruscall Wallis test at the 5% level of significance, it was found that the change in active inflammation of different groups was significant. However, when Mann-Whitney U test was conducted in order to find differences between two groups, it gave insignificant results demonstrated in Figure 38.

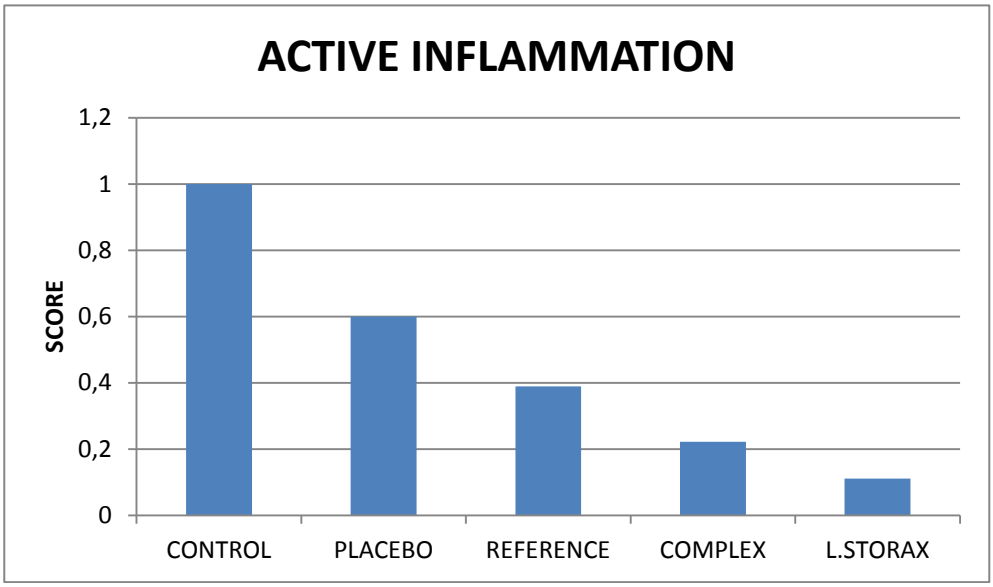


Figure 38 Difference in active inflammation between all groups.

4.2.3.2 Chronic Inflammation

When Kruscall Wallist test was conducted to check the differences in chronic inflammation of different groups, it gave significant results. According to results of Mann-Whitney U test, the levant storax and complex groups had significant differences against the control and placebo groups as shown in Figure 39.

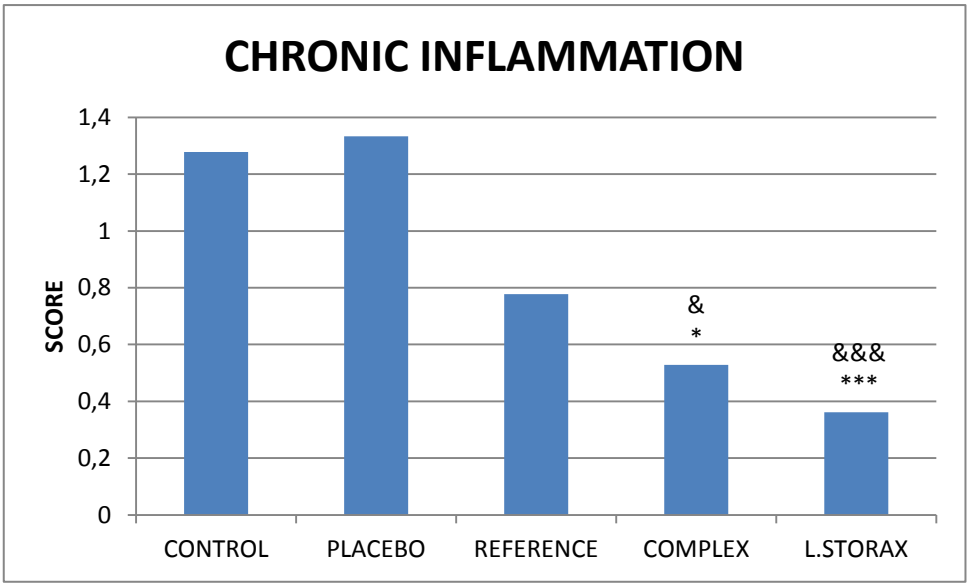


Figure 39 Difference in chronic inflammation between all groups; '&' compared with the control group, '*' compared with the placebo group.
&,* p< 0,05
***, &&& p< 0,001

4.2.3.3 Fibroblastic Activity

When Kruscall Wallist test was performed to identify the differences among the groups about fibroblastic activity, results showed significant difference. When Mann-Whitney U test was conducted, it showed that the results of the levant storax was significant different from all other groups as shown in Figure 40.

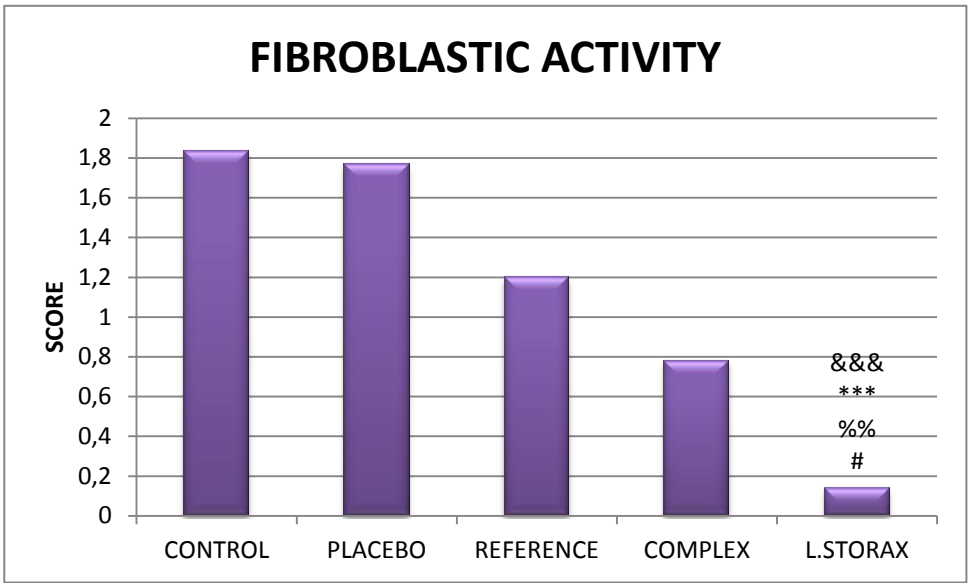


Figure 40 Difference in fibroblastic activity for all groups; '&'; compared with the control group, '*'; compared with placebo the group, '%'; compared with the reference group, '#'; compared with the complex group.
# p< 0,05
%% p< 0,01
***, &&& p< 0,001

4.2.3.4 Neovascularization

When Kruscall Wallist test was conducted to check the difference in vascularization of different groups, it resulted in significant results. According to the results of Mann-Whitney U test, the levant storax and complex groups had significant differences in vascularization compared to the control and placebo groups as shown in Figure 41.

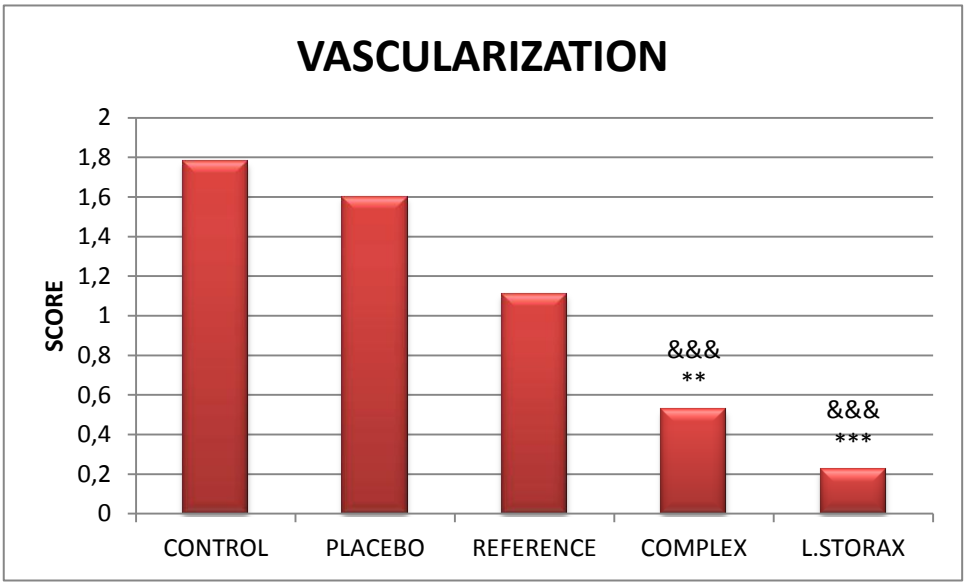


Figure 41 Difference in vascularization for all groups; '&'; compared with the control group, '*'; compared with the placebo group,
** p< 0,01
***, &&& p< 0,001

4.2.3.5 Fibrosis

When Mann-Whitney U test was conducted to check the differences about fibrosis distribution between the scores of two groups, it showed that the levant storax and placebo groups were significantly different from the reference group as shown in Figure 42.

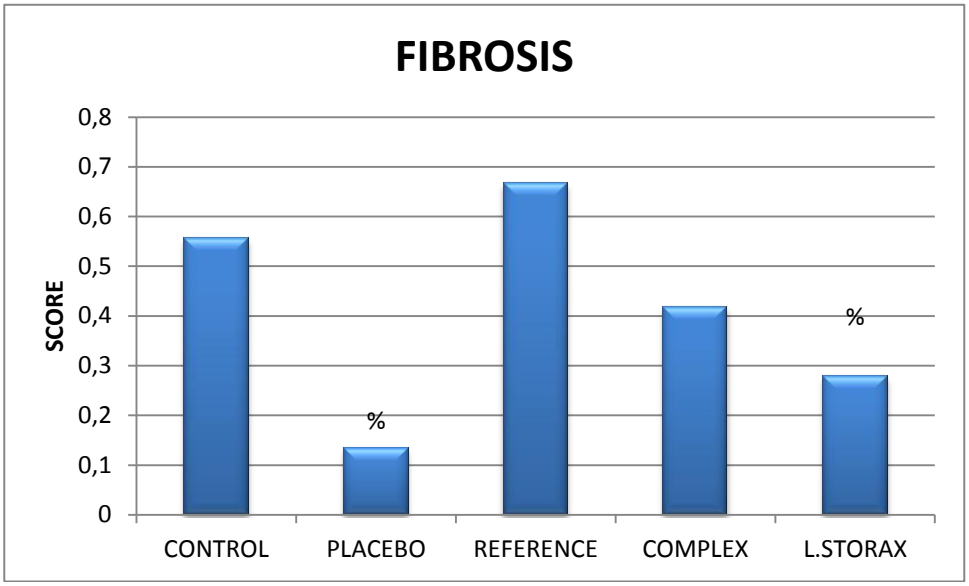


Figure 42 Difference in fibrosis for all groups; '&'; compared with the reference group. % $p < 0,05$

4.2.3.6 Hair follicle formation

When Kruscall Wallist test was conducted to check the difference in hair follicle formation in different groups, it produced significantly different results. According to the results of Mann-Whitney U test, the levant storax group had significant differences against the control and reference groups as shown in Figure 43. The data revealed that levant storax cream has more regenerative effect on hair follicle formation than the other groups**.**

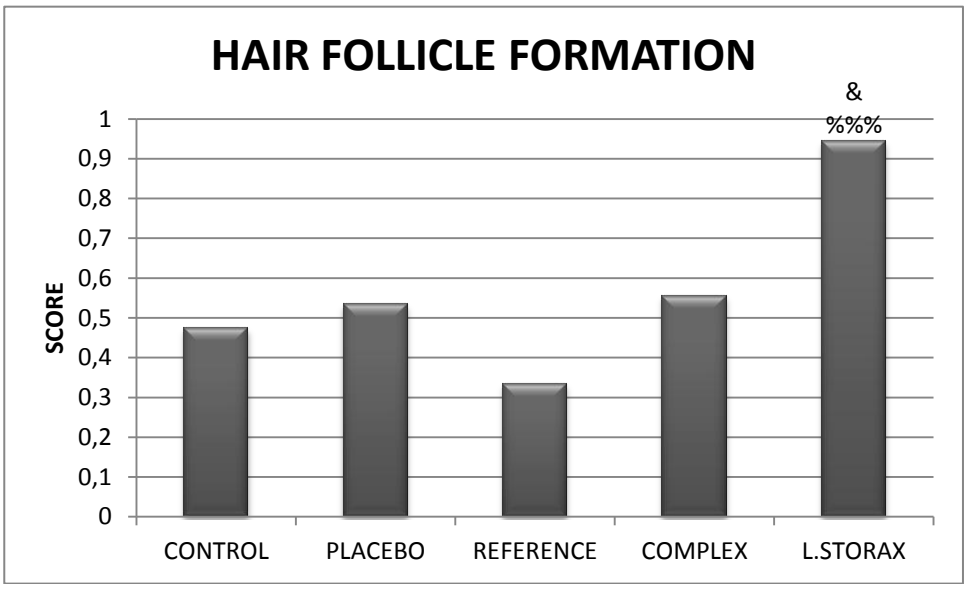


Figure 43 Difference in hair follicle formation; '&'; compared with the control group, '%'; compared with the reference group.
& p< 0,05
%%% p< 0,001

4.2.3.7 Healing Phase

When Kruscall Wallist test was conducted to check the variation in healing phase of different groups, it showed significant differences. According to the results of Mann-Whitney U test, the levant storax and complex groups were significantly different than the control and placebo groups, and the reference group was different against placebo group shown Figure 44.

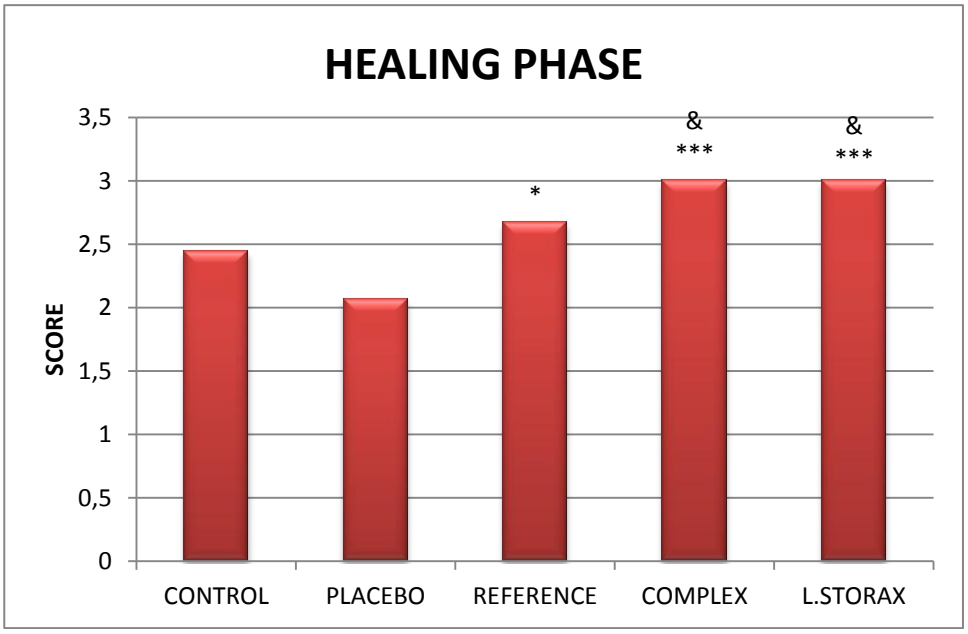


Figure 44 Change in healing phase; '&'; compared with the control group, '%'; compared with the placebo group.
&,* p< 0,05
*** p< 0,001

5 DISCUSSION AND CONCLUSION

5.1 Studies on Emulsions

The aim of this work was first to develop two stable emulsions which would be used for wound healing and second to assess the effects of these creams on wound healing by using *in-vivo* animal models. During the emulsion development studies, a good placebo cream formulation was produced as a base cream. In order to find a base formulation that meets the initial requirements, nine formulations were prepared by changing the excipients and/or adjusting the amounts of the excipients. Since inadequate moisture in the wound bed would impede the healing process (67), the wound care product was expected to cover the wound and remain in place for at least some time, like a wound dressing product. Therefore, viscosity of the product was regarded as an important parameter as much as the results of the initial stability tests. At the end of the formulation and preliminary studies, placebo cream code P009 was selected as the best placebo formulation (Table 7). After selecting the formulation of 'the placebo cream', complex cream and levant storax cream formulations were developed by using the same cream base formulation. The centrifugation and freeze–thawing cycles of placebo cream indicated that this base cream would be stable enough to take up the herbal extracts that were planned as the active materials for complex cream and levant storax cream. It is well known and shown by many researchers that herbal extracts may act like electrolytes and can cause emulsion instability and phase separation in a short time (92). In this study, none of the formulations had a major problem in terms of stability, at least during the limited time period the tests were conducted.

Calendula oil (73, 80, 93, 94, 95), St. John's wort extract (74, 75, 94, 95), escin (94, 95, 96), freeze dried *Aloe vera* powder (72, 94, 95, 97) and allantoin (94, 95) were selected as functional actives to formulate complex cream (Table 8) after studying many of their features, including physical and chemical characteristics, and pharmacological effects. The appropriate concentrations of each ingredient was decided based on

suppliers' recommendations and literature reviews. Similarly, Levant storax (the balsam of *Liquidambar orientalis*) concentration range in levant storax cream was decided according to literature (2, 4).

In characterization studies, all three formulations were evaluated organoleptically for their appearance, colour, odour, feel/spreadability as well as pH, viscosity, conductivity, zeta-potential measurements. The particle size and particle size distribution analysis were conducted in order to understand poly-dispersity of the emulsions and their tendency towards phase separation under stress conditions like centrifugation and freeze-thawing cycles.

The balsam of *Liquidambar orientalis* is very viscous liquid therefore, viscosity of the levant storax cream was the highest compared to the placebo and complex creams (Table 17). The Complex and levant storax creams had higher conductivity values than the placebo cream, because the cream formulations contained active ingredients which acted as electrolytes. Because of the different active ingredients used in the formulations, conductivity values were specific to the formulations. Conductivity measurements also indicated that all of the the cream formulations were oil in water emulsion (7), and they remained as oil in water emulsions during the stability test period (Table 17).

Healthy human skin has a surface pH that ranges between 4-6 and a pH gradient exists within the skin layers. The skin's natural acidic environment is corrupted when wounded. Most human pathogenic bacteria and yeast survive better in alkali millieu, whereas the pH values below 6, their growth is obstructed. Therefore, in order to reduce the bacterial load on the skin surface, acidic millue of the skin should be established and maintained around and on the wounded areas. Although it is not widely investigated yet, there have been some studies indicating that the pH value can influence wound healing. pH seems to be important not only because of its effects on controlling the growth of bacteria, but also to influence the healing process together with many other endogenous and exogenous factors (98). The formulations studied only intended to maintain the

physiological pH of the skin, thus the pH values of all cream formulations designed for this study were acidic (Table 17), although there were slight differences between them due to the composition differences.

During the stability studies, the formulations did not show any signs of physical instability such as phase separation, odour and colour change (Table 18). Based on the pH, viscosity and conductivity evaluation results, it was concluded that the creams were stable for 2 months test period (Table 19, 20 and 21). The emulsifier molecules surrounding the oil globules seemed to created a stable oil/water interfacial film. The type of the emulsifiers as well as the other ingredients available in the formula contributed to the surface of the globules and in turn to the development of zeta potential. Non-ionic emulsifiers used as the main emulsifiers created a negatively-charged surface as it has been experienced by many researchers (99), which is affected by the presence of the electrolytes. Studies have shown that rather than the charge, the magnitude of the zeta potential is important in terms of the stability of the globules against flocculation, aggregation and coalescence. Zeta potential measurements were used widely for quantifying of the magnitude of the electrical charge at the double layer and there have been number of studies conducted in order to illustrate the relationship between the magnitude of zeta potential and the stability of the emulsions. Based on the data and general observations, zeta potential ranges from ± 30 to ± 40 mV is considered good for moderate stability and from ± 40 to ± 60 mV for good stability. In other words, droplets with zeta potential above 30 Mv (positive or negative) are normally considered stable because of the electrostatic repulsion between the oil droplets (42, 100, 101). In this study, non-ionic emulsifiers (cetearyl olivate and sorbitan olivate) were used as the surfactant mixture, thus zeta potential of all droplets (in all three types of the emulsions) were negatively charged and the zeta potential values of all cream formulations were above -30 mV. Although there was a tendency of slight increase by time, the value was always above 30 mV contributing to the stability of all products. This was in line with all other measurements that the emulsions were stable under the testing conditions. When the zeta potential increases were compared between the placebo cream, complex cream and levant storax cream, there was no major differences observed. Therefore, it was appropriate to conclude that the zeta potential increases

were not related to the actives added to the creams and all of the formulations were stable during the testing period. The increasing tendency of zeta potential values between T0, 4th and 8th weeks all lied within the zeta potential ranges (Table 19, 20, 21). that are regarded as favourable in terms of stability (42, 100, 101).

In the recent years, sedimentation and light scattering measurements are used more widely for many reasons. Using such equipment is very easy, and results are obtained in a very short-time. One of the light scattering technology, Turbiscan™ technology was used to evaluate the stability of emulsions quickly and accurately in order to detect particle size change (coalescence) and phase separation (sedimentation, creaming) (102). In this study, the light scattering technology was also used as an alternative method. According to the results of Turbiscan Technology analyses, the particle size of the all formulations remained unchanged throughout the experiments (Figure 19 to 27). During the stability studies, changes in particle size was also investigated by microscopic analyses. In microscopic analysis, there were slight changes in the particle sizes for all cream formulations (Table 22, 23 and 24). However, under each storage condition, no major size changes were observed and average particle size of all emulsions remained as sub-micron size (Appendix 1**).**

Emulsions are thermodynamically unstable, because they do not possess a minimum interfacial energy. In order to achieve the most stabile condition, two phases tend to separate. Accelerated stability tests conditions were used to understand the susceptibility of the emulsions towards instability. For this purpose, methods like centrifugation and freeze thawing have been traditionally used widely to quickly assess the properties of the emulsions and, to a certain degree, assure the formulation of desired stability of the emulsions designed (7, 8, 15). Therefore exertion of the emulsion under centrifugational forces is an effective method to evaluate the tendency of phases to separate. Sedimentation rate during centrifugation depends on average globule size of emulsion as well as the polydispersity of the globules. The emulsions containing larger globule size are less stabile, therefore centrifugation at different rpm levels provides some immediate indication about stability of emulsions (15).

During freeze-thaw cycles, emulsions are exposed to high stress conditions. During freezing breaking, therefore phase seperation of emulsions may be observed. Among the many reasons for phase seperation 'withdrawal of free and/or bound water from the film between adjacent droplets, by crystallization as ice, or by concentration of any solutes present; establishment of true contact between adjacent films of emulsifier(s), with loss of the orienting influence of water; diffusion of the emulsifier in the film away from these thick regions; and decrease in film area, resulting in coalescence of droplets as soon as thawing of the ice permits them to change shape' can be mentioned (43).

In our study, centrifugation and freze thawing studies were conducted as described in Section 3.1.3.5.7 and 3.1.3.5.8. There were no phase separation or organeleptic changes observed indicating that the cream formulations were stable under such accelerated stress conditions (Table 25).

Microbiological compliance tests at the beginning and at the end of two months demonstrated the ability of the preservatives to prohibit any microbial growth in the creams during normal storage. In this study, all formulations were preserved with Uniphen P 23 (Methylparaben, Ethylparaben, Propylparaben, Butylparaben, Isobutylparaben, Phenoxyethanol), since it had a strong bacteriostatic efficacy against numerous microorganisms according to the supplier's efficacy tests. In the compliance test, there was no growth on SDA and TSA plates meaning that there was no pathogenic or non-pathogenic microorganisms in any of the creams produced.

In addition to the emulsion development and physical stability studies, analytical method for quantitative analysis of hyperoside and escin was developed and validated. These active ingredients were used in complex cream, and more advanced stability tests should include the chemical analysis of actives and investigation of their degredation within the cream formulations.

Two validated HPLC methods were developed as part of this study. HPLC validation studies for hyperoside revealed that the recoveries were within the range of ±

10 % and the R.S.D. values of the interday and intraday run of hyperoside were better than 2,5 %. (Table 11 and 12). The limit of detection and quantification levels were 0,33 mg/100 ml and 1 mg/100 mL. These results demonstrate that the method is good and can be used to test hyperoside in the complex cream (103). Similarly, validation studies for an HPLC method for escin was completed. For this method for quantitative analysis of escin in complex cream had the recoveries within the range of ± 10 % and the R.S.D. values of the interday and intraday run better than 7.10 % (Table 14 and 15) The limit of detection and quantification were 0,02 mg /1ml and 0.08 mg/1 ml. These results also demonstrated that this method for escin was a reliable method (103). These studies established a feasible HPLC reverse phase analysis methods for escin and hyperoside in order to analyse and monitor these two active materials. If further quantitative studies are conducted, the validated HPLC methods could be used.

Levant storax balsam of *Liquidambar orientalis,* which was used in levant storax cream formulation. GC-MS analysis was performed to show the composition of the balsam (Figure 18). No further quantitative test methods were developed to test the amount of balsam or some of its constituents. In some studies, main components of oriental sweet gum were reported as styrene, a-pinene, and b-pinene (6). However, main components of our analysis were styrene, menthol crystal, phenyl propyl alcohol and cinnamyl alcohol as shown in section 4.1.3. Based on earlier data reported by other researchers, and our own findings, the constituents like cinnamyl alcohol, menthol crystal, benzyaldehyde, a-pinene, b-pinene and germacrene d, posses antibacterial activity (104, 105, 106, 107) which helped faster and better healing of the wounds. This could be one of the reason why levant storax cream showed the best wound healing efficacy in our studies.

5.2 *In-vivo* Animal Studies

Excisional wound model was employed on rats for the wound healing activity assessment of the creams. Wound contraction rates in the healing progression are shown in Table 26 and 27. Inadequate moisture in the wound bed promotes desiccation of

wound, necrosis,and eschar formation, and impede the healing process (67). Due to the balsam of *Liquidambar orientalis,* which is used in levant storax cream is more viscous and sticky cream than the other creams, it covered the wounds better and stayed in place longer, like a dressing material. Since this may have prevented water loss from wound area and wound bed, rate of epithelization and contraction of the wounds treated with this cream were better. Also, levant storax cream contained some essential oils with antimicrobial activities (Figure 18), therefore contributed the healing process at least by keeping the unwanted microorganisms and their possible negative effects on wound healing away. Because of better viscosity and antimicrobial activity of levant storax cream, its effects on contraction of the wounds was the best among all groups (Figure 31). Although traditional wound healing effect of balsam of *Liquidambar orientalis* was well recognized, other reasons, apart from the antimicrobial activity and better moisturizing effect of levant storax cream, that contributed on wound healing needs to be further investigated.

The complex cream contains some very well recognized functional actives such as escin, *Aloe vera,* allantoin and calendula oil. All these actives bring fibroblast stimulating, anti-inflammatory and anti-oedema properties to the complex cream which in turn resulted in statistically better contraction rates of complex cream compared to control group.

Results of the development of full skin thickness were shown in Table 28. In this study, it was clearly shown that the best effect on regenerated skin thickness was produced by the levant storax group. These results were also due to the same reasons discussed above. Complex cream also had significantly better effect on the renewal of skin thickness compared to placebo cream. This was probably be due to the fibroblast stimulation ability of the complex cream (Appendix 12).

Wound healing is divided into four main phases. These are homeostasis, inflammation, proliferation and remodelling (recovery) phases. In the histopathalogical studies, some indicators of these phases were used in order to determine the natural

healing phase of the groups. Neutrophils and mononuclear cells act as an indicator for the inflammation phase (48). The proliferation phase was investigated by fibroblastic activity and neovascularisation (48, 50, 51). The recovery phase was determined with fibrosis and hair follicle formation, decreasing in neovascularisation and disappearance of neutrophils and mononuclear cells (48, 50).

Histopathological results were shown in Table 29. Shortly after injury was generated, neutrophils migrated into the injury site and also inflammation phase was initiated. Neutrophils were predominant for the first few days and then disappear if the wound was not became infected (48, 50). Therefore, neutrophil was the best indicator to monitor active inflammation. In this study, for histopathological observations, neutrophil was used as an indicator for monitoring the active inflammation. Based on the results, there was almost no active inflammation was seen in any of the groups. All wounds progressed to the other steps of the healing process (Figure 38).

The main cells involved in chronic inflammation are macrophages and lymphocytes. Because both of these cells have a single nucleus, they are known as mononuclear cells (108). In such wound healing studies, mononuclear cells were used for detection of chronic inflammation. Figure 39 clearly showed that the levant storax cream and complex cream groups had almost no chronic inflammation whereas the control and placebo groups fell into chronic inflammation phase. These results were probably due to the effects of the active ingredients contained in levant storax and complex creams.

Fibroblasts are one of the important cells for healing process, they produce collagen and variety of other substances, such as fibronectin, glycoaminoglycans hyaluronan, proteoglycans and elastin. These substances promote cell adhesion and migration, and promote tissue strength (3). Based on the data given in Figure 40, levant storax cream had the highest fibroblastic activity compared to all of the other groups. This may be the result of the favourable wound healing activity of balsam of *Liquidambar orientalis.*

The process of restoring the vascular network is called neovascularisation or angiogenesis. Because of disturbed native vasculature and increased oxygen consumption, there is a severe degree of hypoxia in the wound area (50). Therefore neovascularisation is an important process for new tissue formation. However, during new connective tissue formation at recovery phase, strength of neovascularisation ceases. Figure 41 showed that restoring the vascular network in the wounds of levant storax and complex groups were better than the placebo and control groups. These results were also supporting that active ingredients of levant storax and complex creams are promoting the wound healing process in many different ways and mechanism.

The fibroblast is the connective tissue cell responsible for collagen deposition which is needed to repair wounds. In excessive healing, there is too much deposition of collagen that results in fibrosis. Keloids and hypertrophic scars in the skin are examples of fibrosis. Fibrosis can be defined as the replacement of the normal structural elements of the tissue by distorted, non-functional and excessive accumulation of scar tissue. Fibroblasts that are isolated from keloids produce about 2 to 3 times more collagen compared to fibroblasts isolated from normal skin in the same patients (109). As shown Figure 42, the placebo and levant storax groups had less fibrotic tissue produced than the other groups, resulting in less apparent scars.

Hair follicle formation occurs several days after re-epithelisation (110). Stem cells migrate from the hair follicle bulge to regenerate epithelium and then participate in skin re-epithelisation (111). Therefore, hair follicle formation is an important parameter in the investigation of wound healing process. As shown Figure 43, levant storax cream has more regenerative effect on hair follicle formation than all of the other groups**.**

The wounds which were treated with levant storax and complex cream were in the recovery phase while the control and the placebo groups were still in proliferation phase. The reference group’s wounds were almost in the recovery phase. These results all together indicate strongly that the wound healing activity of levant storax and complex cream were better than the other groups (Figure 44).

5.3 Conclusion

In this study, two emulsion formulations were developed for wound care. The characterization and stability studies showed that all formulations developed during this study were stable for the duration of the tests performed and under the conditions where the samples were stored.

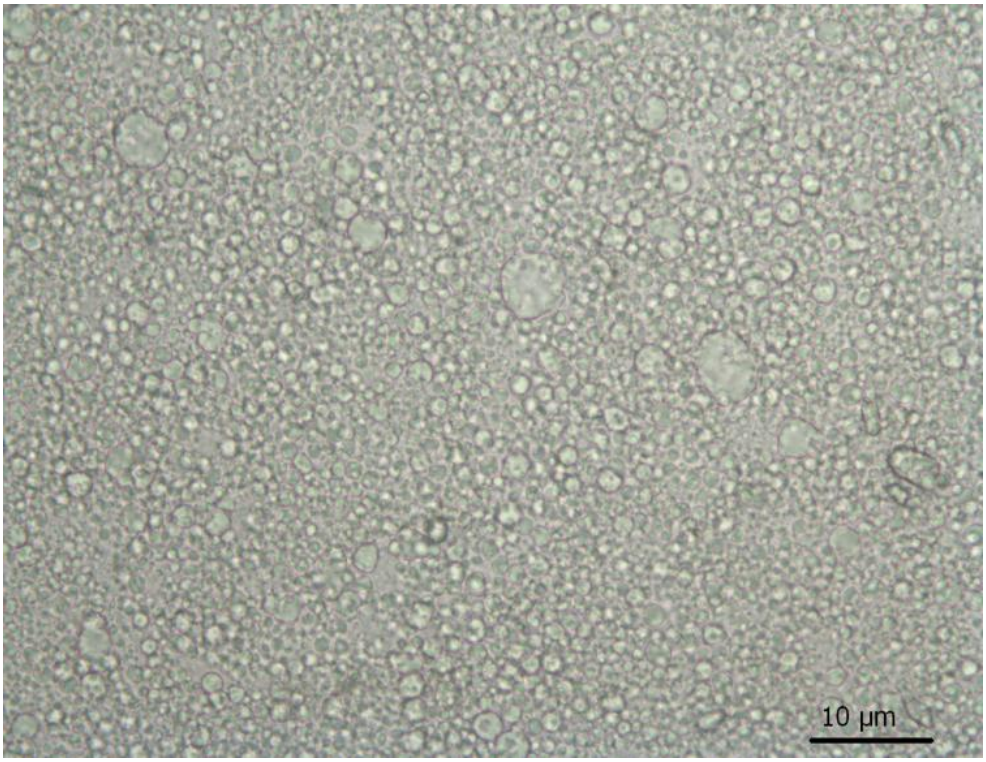
During stability studies for chemical analysis of complex cream, the amounts of both escin and hyperoside in complex cream were analysed with validated HPLC methods. These validated HPLC methods can provide a good, simple, and fast way for quantification and identification of escin and hyperoside in complex cream.

In-vivo wound care studies indicated that levant storax cream treated rats showed the best healing rates compared to the reference cream (Madecassol®) and all other treatment groups, whereas complex cream showed a better healing rate than the control and placebo groups. No significant difference was found between complex and reference cream groups.

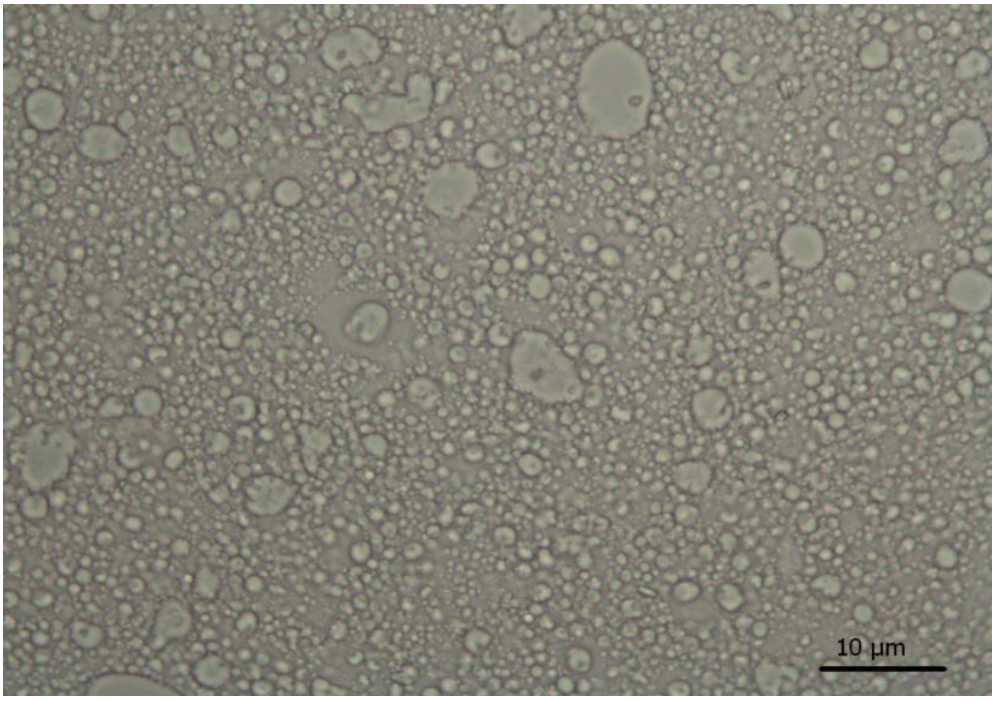
Additional supportive and detailed studies should be conducted in order to reveal other benefits of levant storax and complex creams which clearly showed a good wound healing effects.

6 APPENDICES

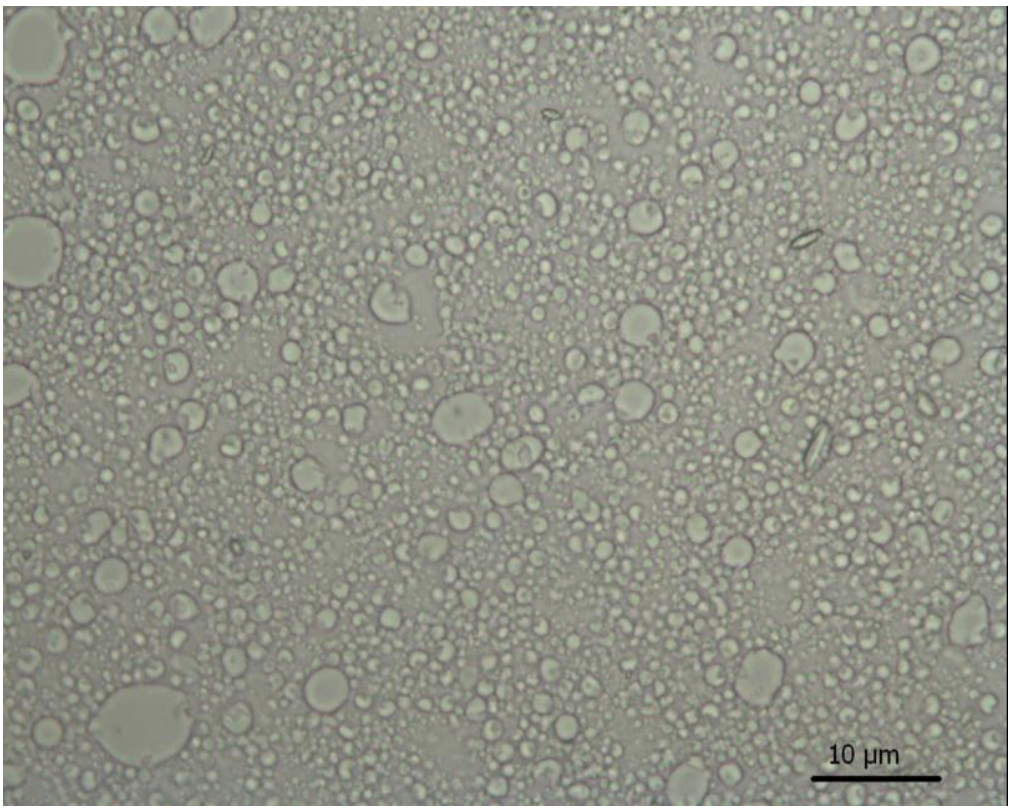
Appendix 1. Representative photograps of all cream formulations particle size were taken with a Leica CTR 6000 microscope fitted with a DFC 350 FXR2 camera, C; Complex, P:Placebo, L; Levant storax, B:Batch.



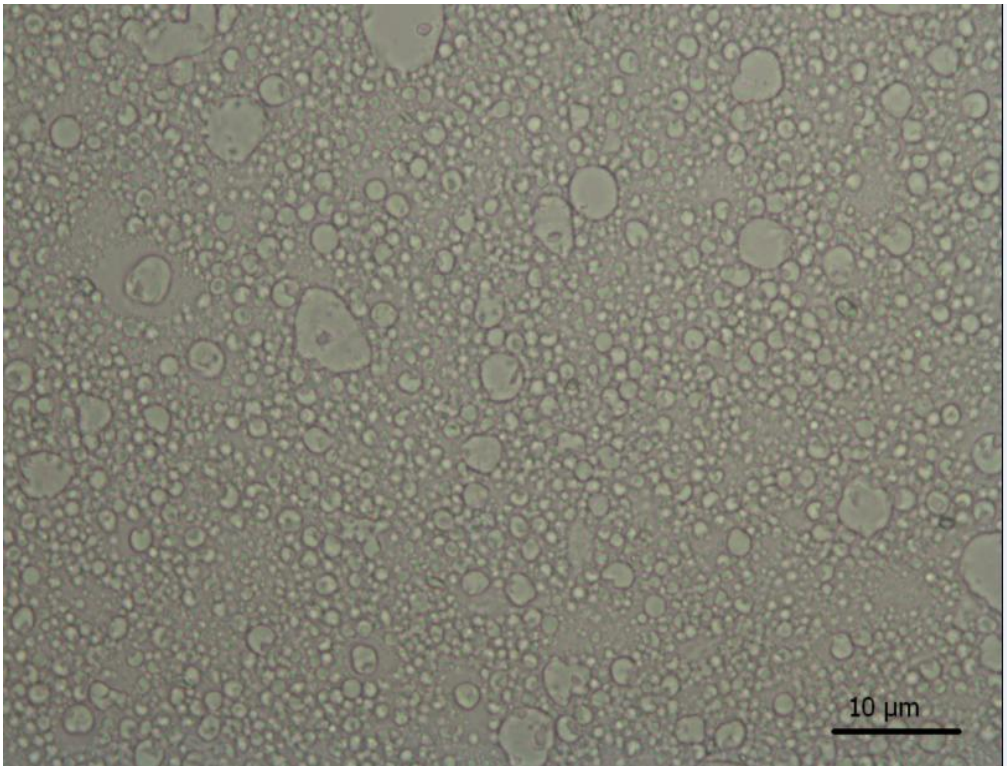
1-CB1, First Day



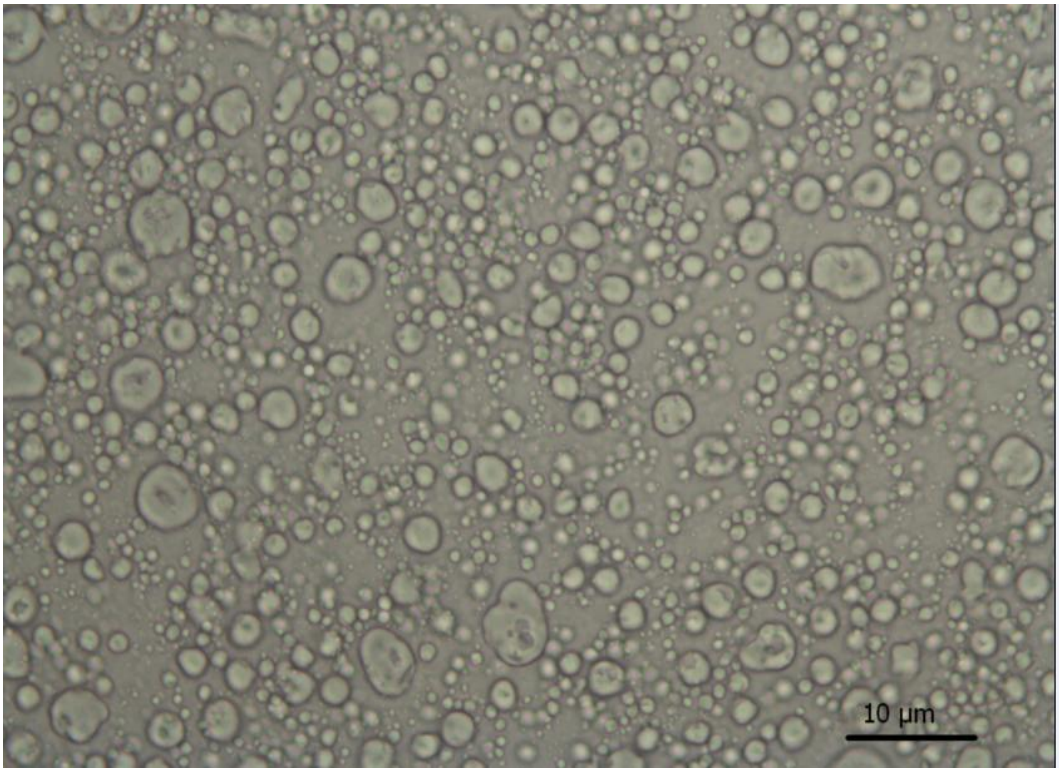
2- CB1, +4-8 °C, Last Day



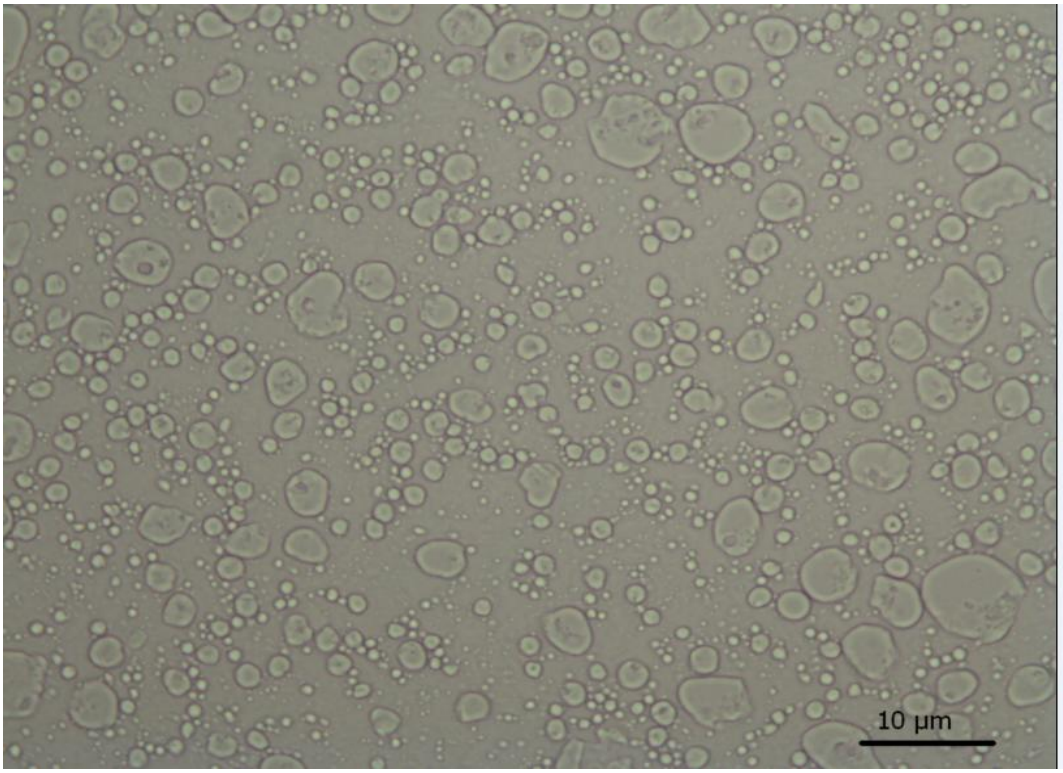
3-CB1, +25°C ± 2 °C 65 % RH, Last Day



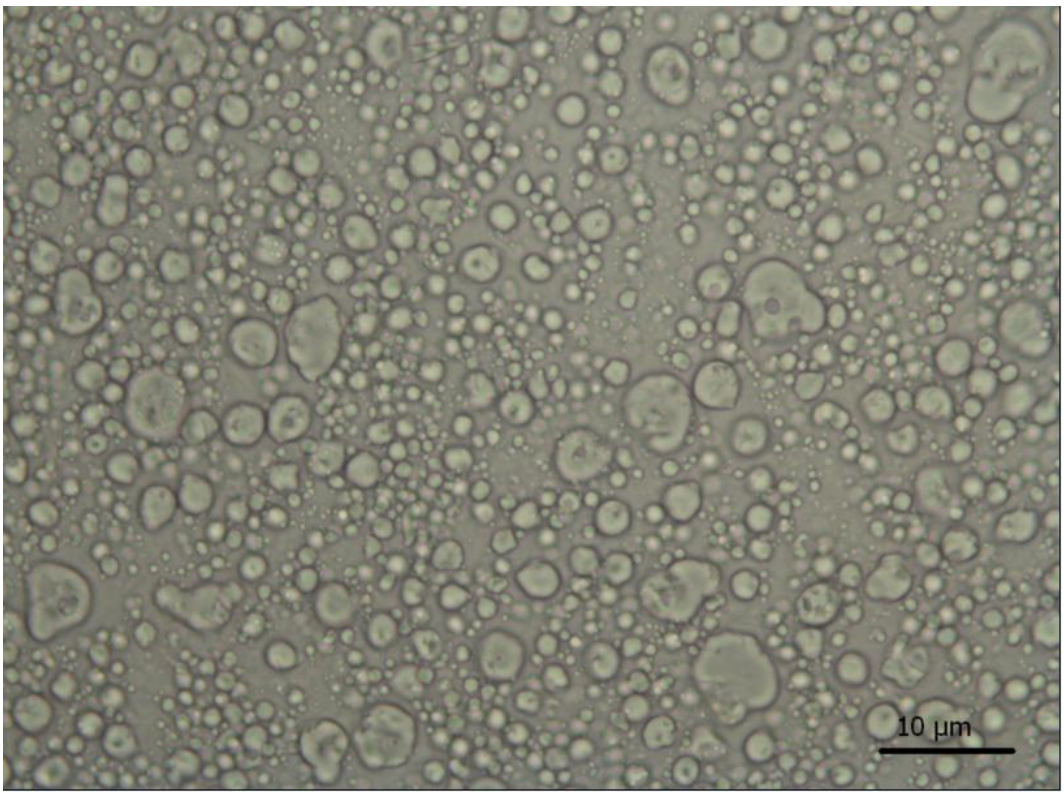
4-CB1, Room Temperature, Last Day



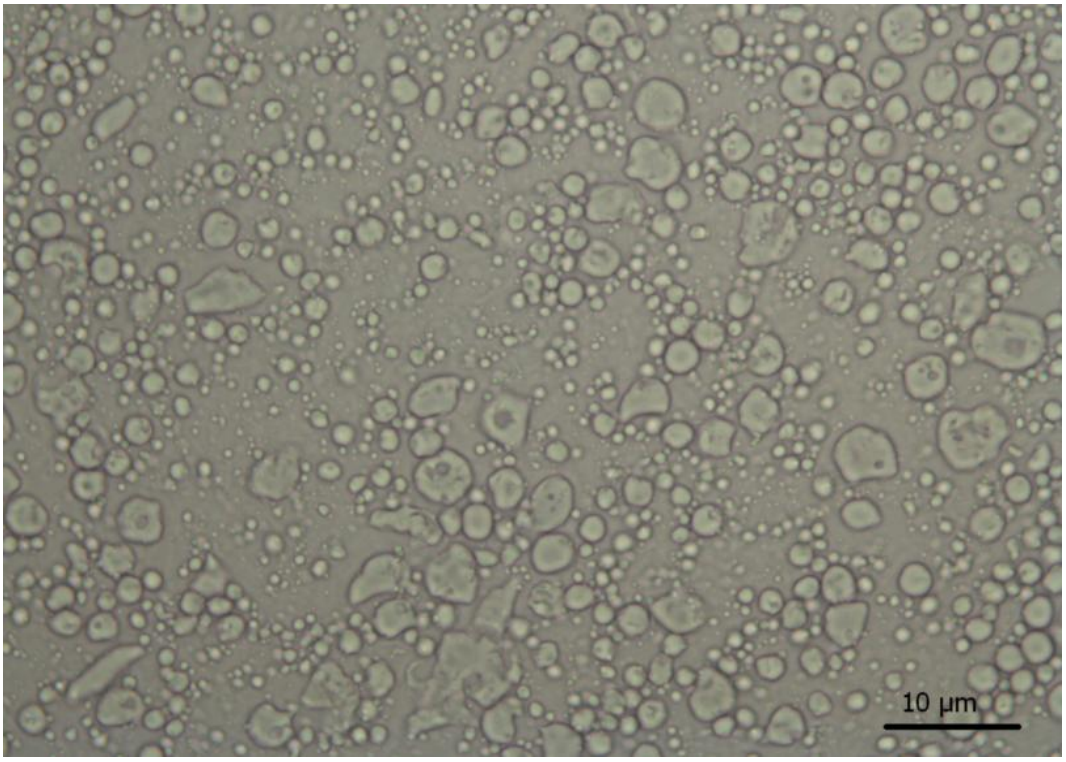
5-PB1, First Day



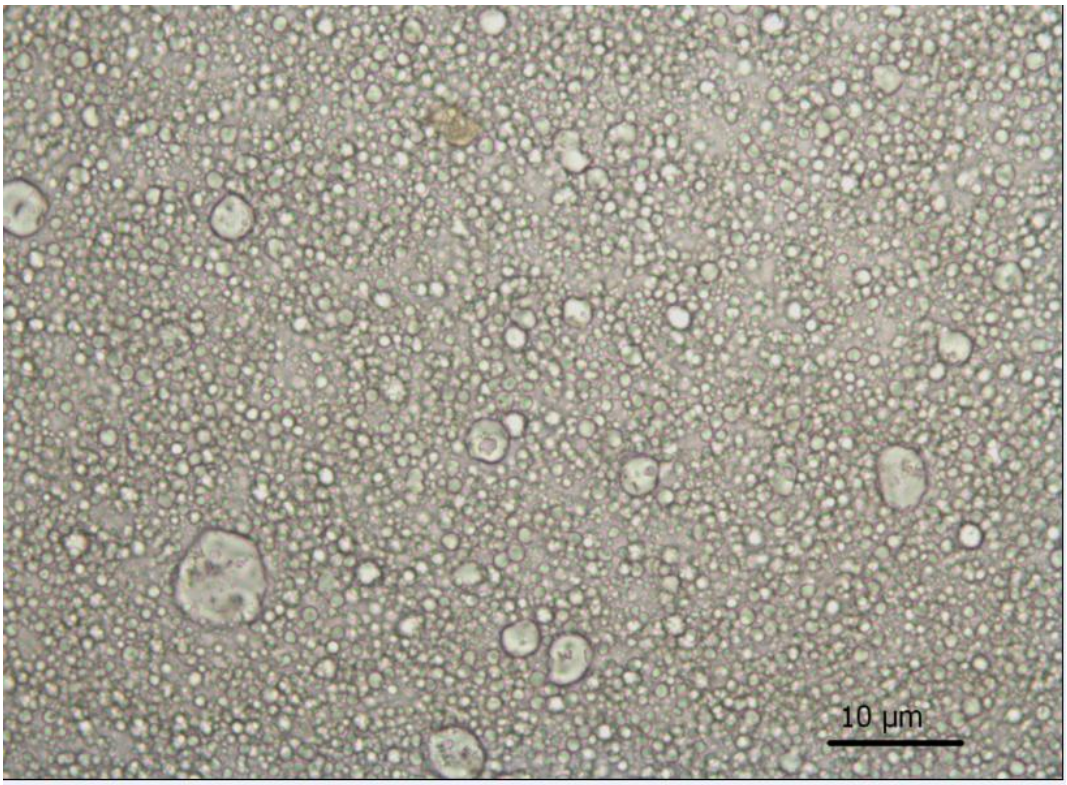
6-PB1, +4-8 °C, Last Day



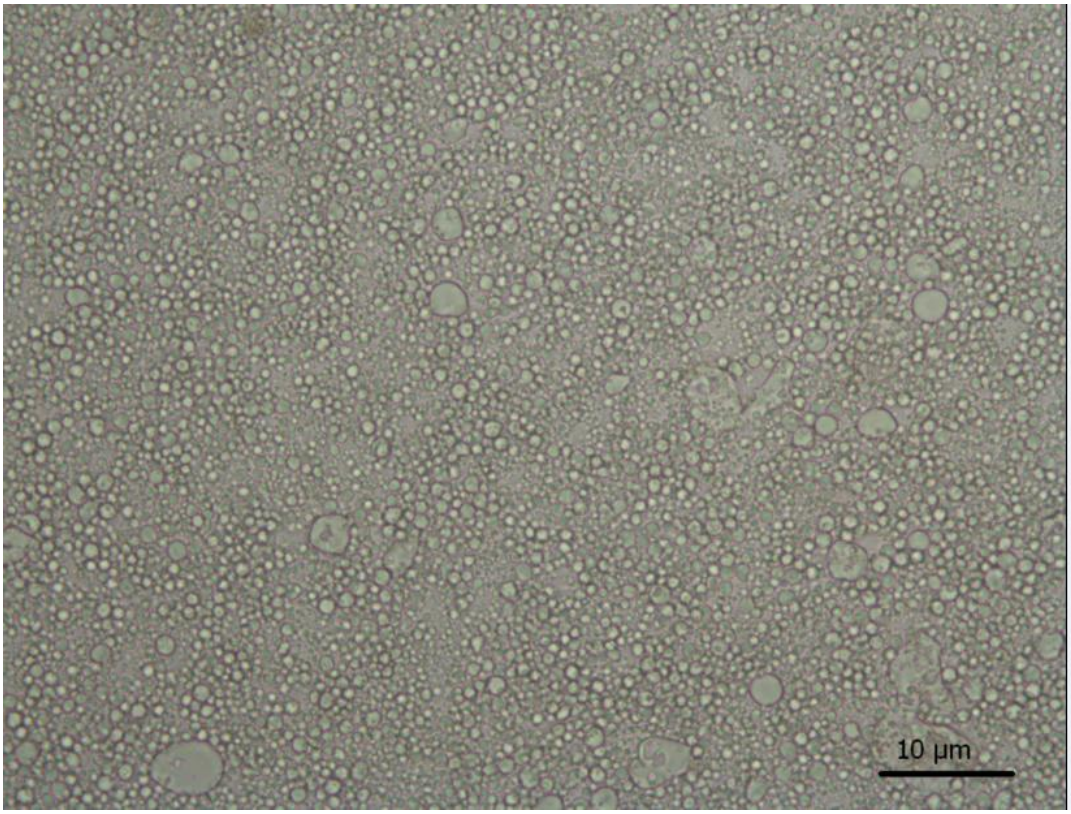
7-PB1, +25°C ± 2 °C 65 % RH, Last day



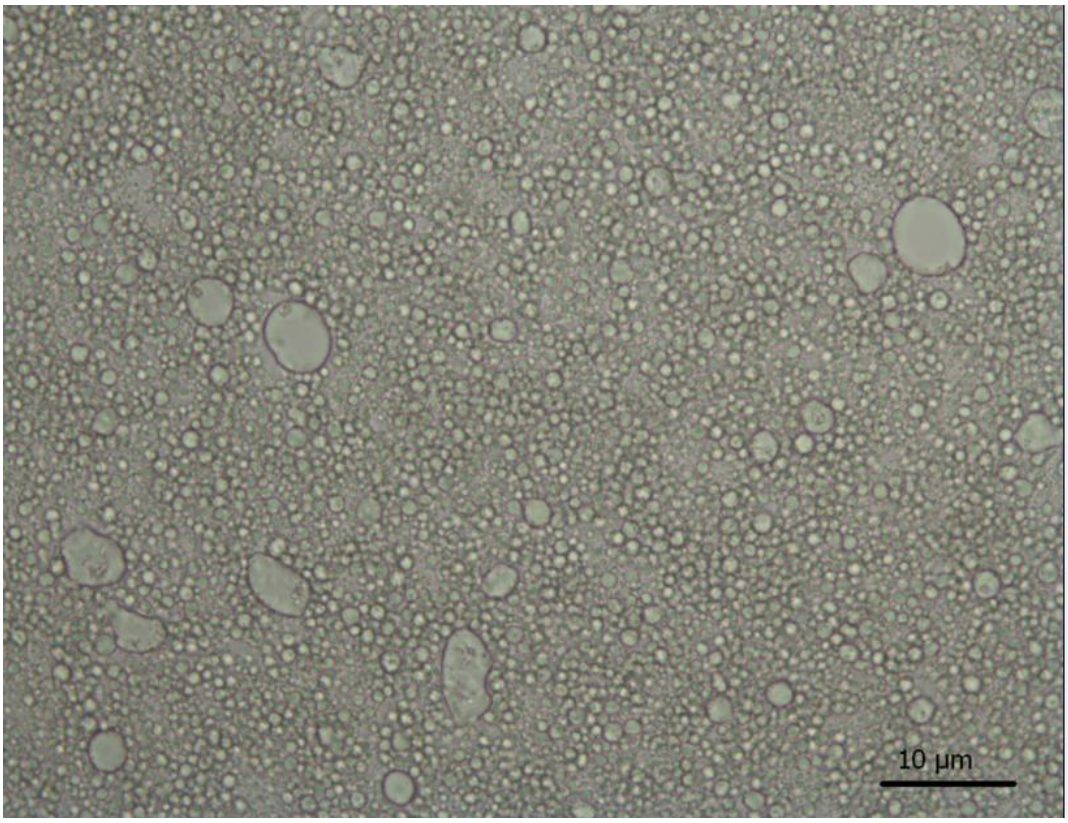
8-PB1, Room temperature, Last Day



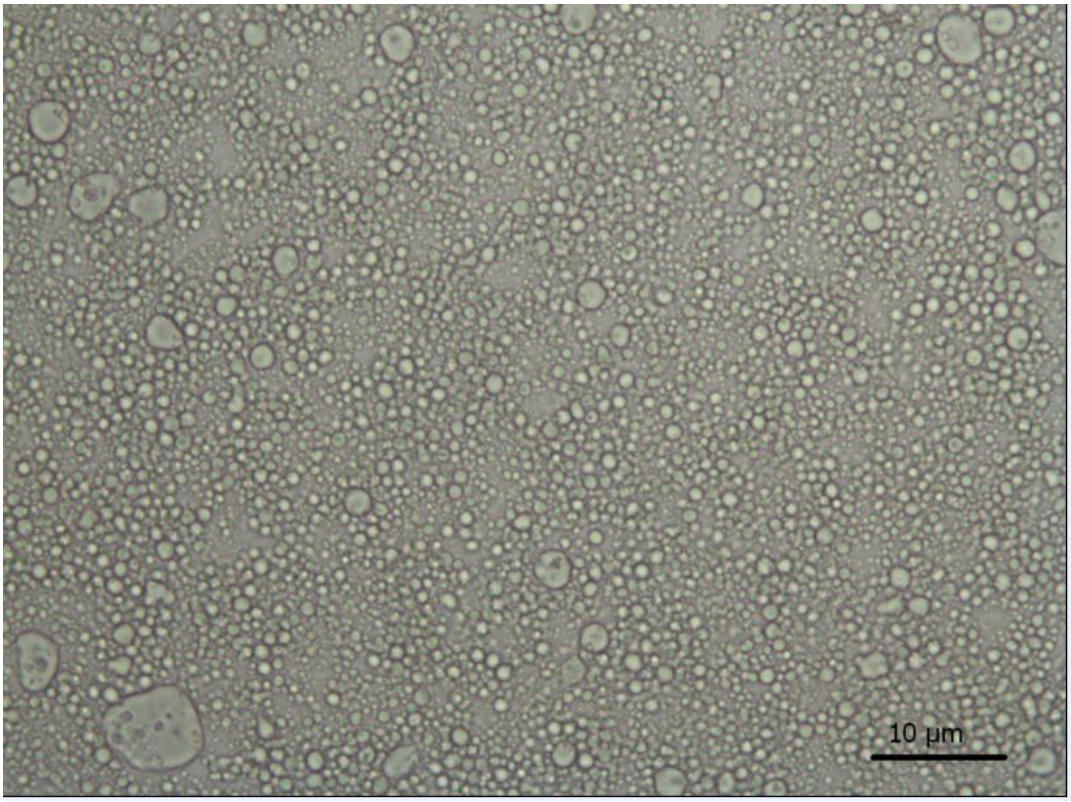
9- LSB1 First Day



10- LSB1+4-8 °C, Last day

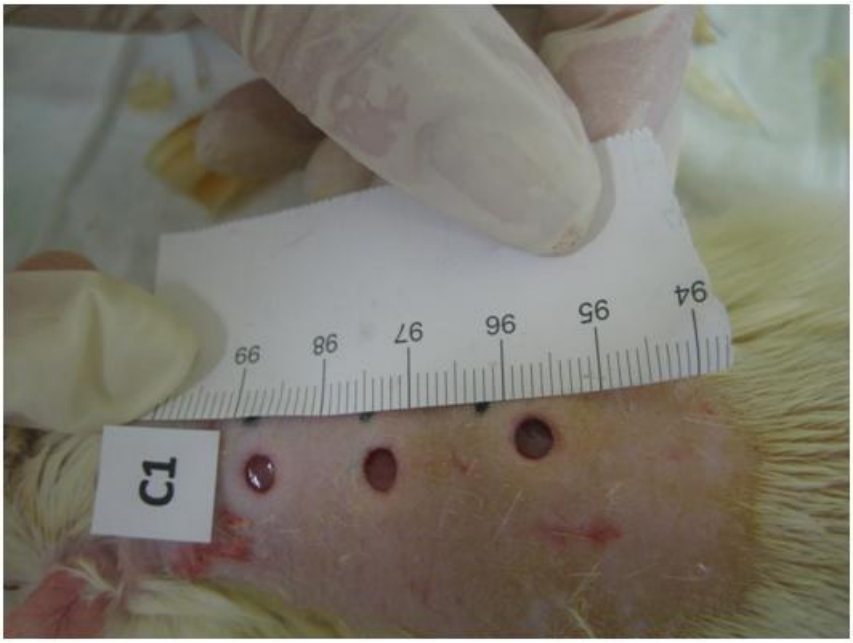


11- LSB1+25°C ± 2 °C 65 % RH, Last day

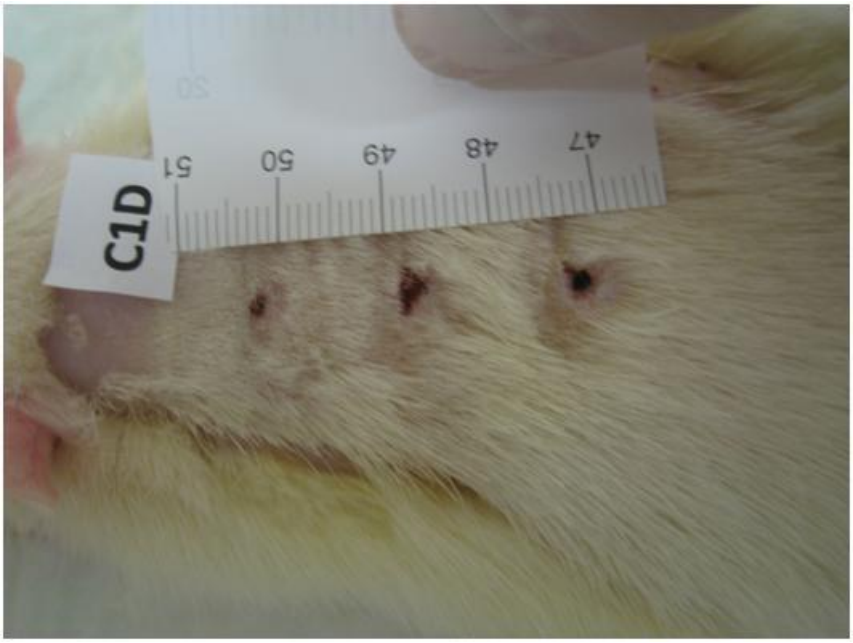


12- LSB1, Room Temperature, Last Day

Appendix 2 The progressive changes in one of the control group's animal wound area were measured by a standard reference ruler and monitored by a camera (Nicon®).

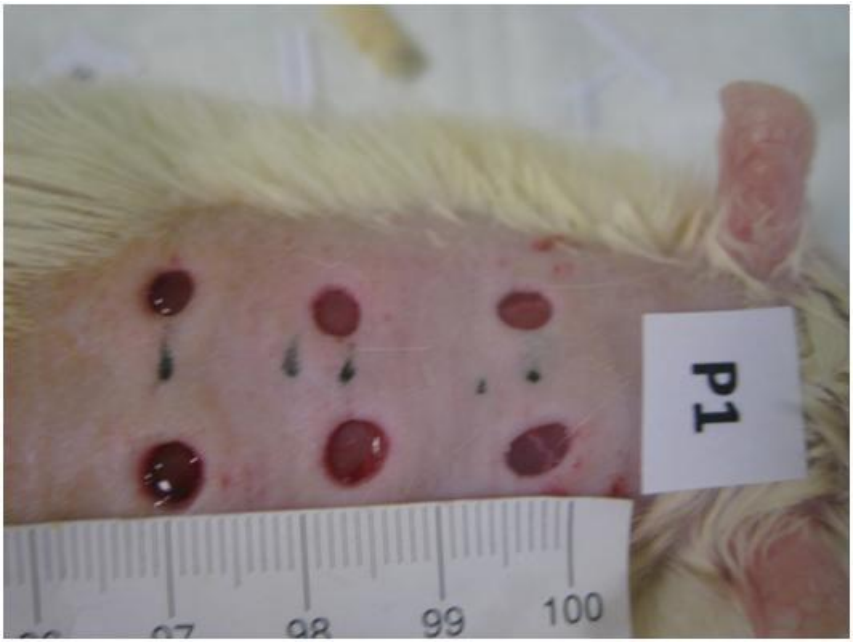


First Day

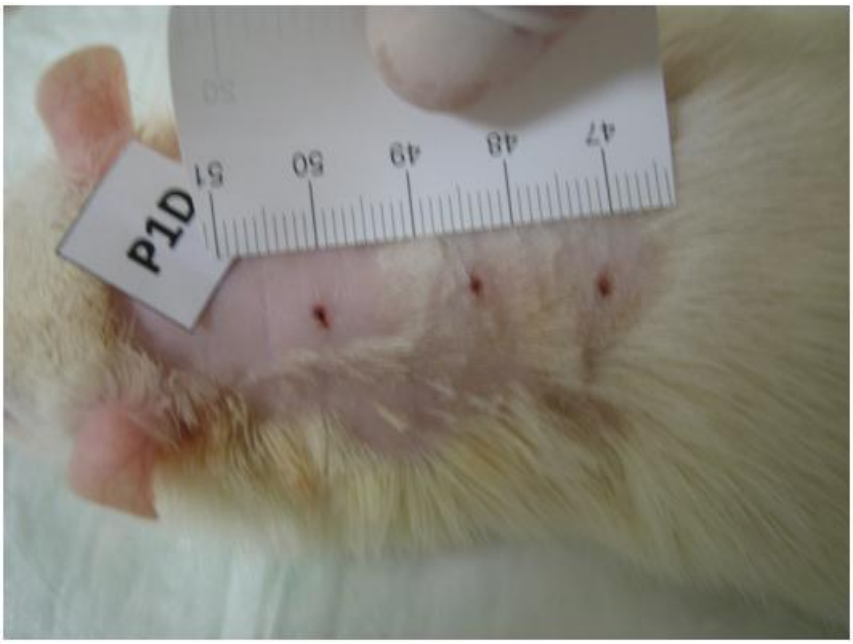


Last Day

Appendix 3 The progressive changes in one of the placebo group's animal wound area were measured by a standard reference ruler and monitored by a camera (Nicon®).

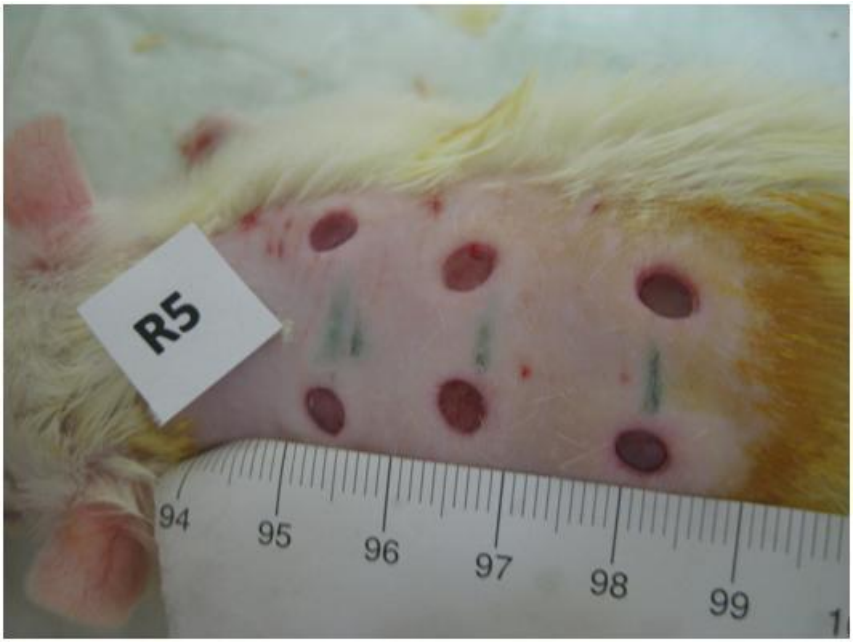


First Day

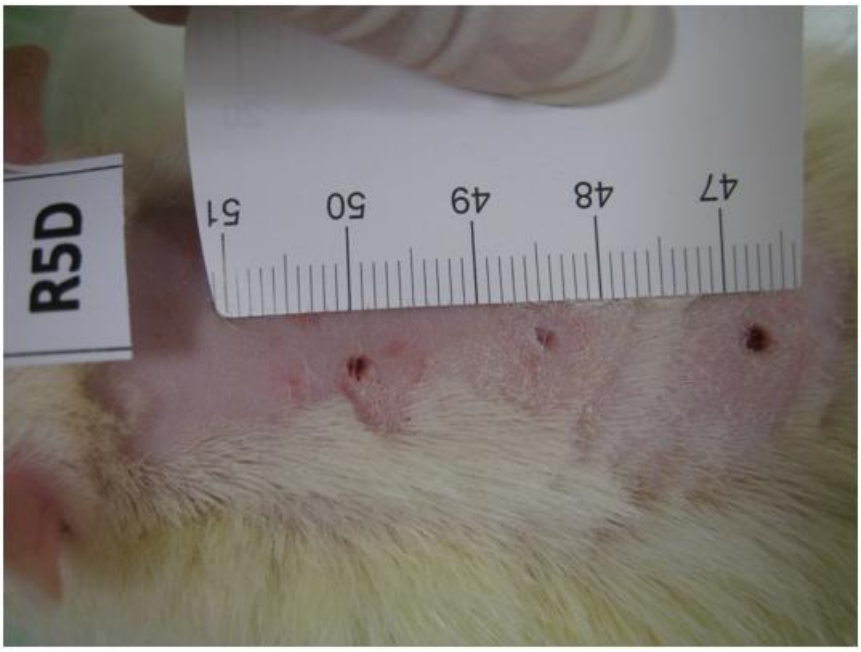


Last Day

Appendix 4 The progressive changes in one of the reference group's animal wound area were measured by a standard reference ruler and monitored by a camera (Nicon®).

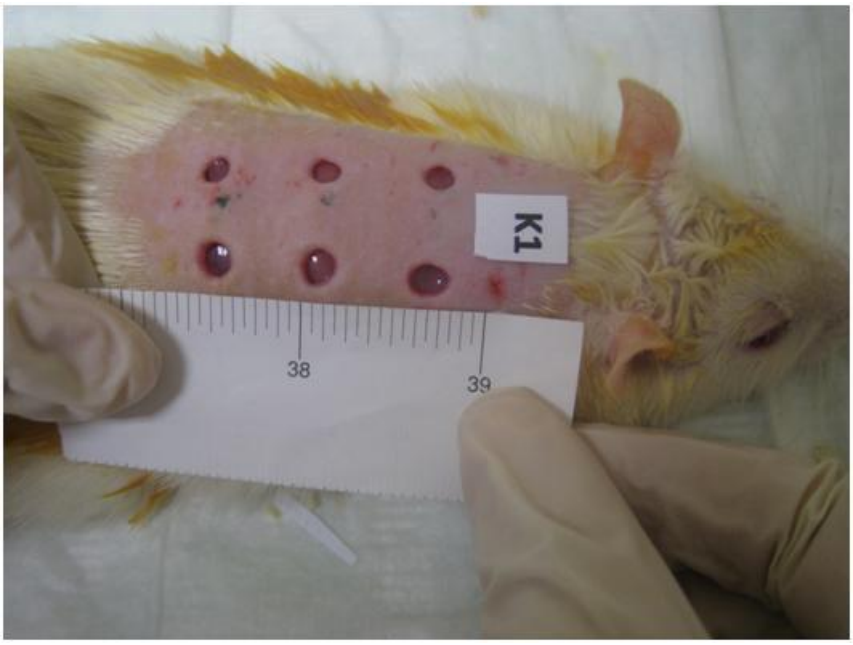


First Day

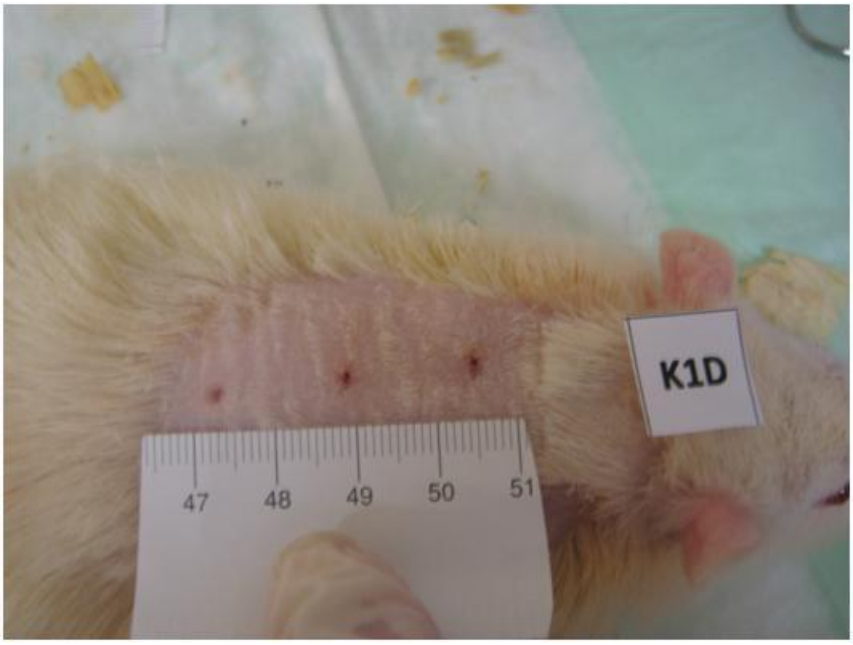


Last Day

Appendix 5 The progressive changes in one of the complex group's animal wound area were measured by a standard reference ruler and monitored by a camera (Nicon®).

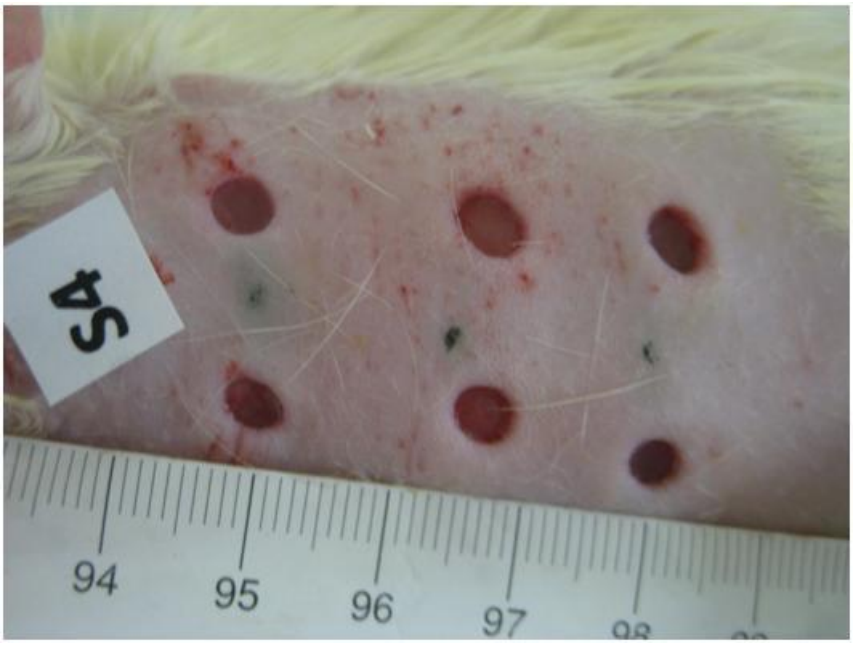


First Day

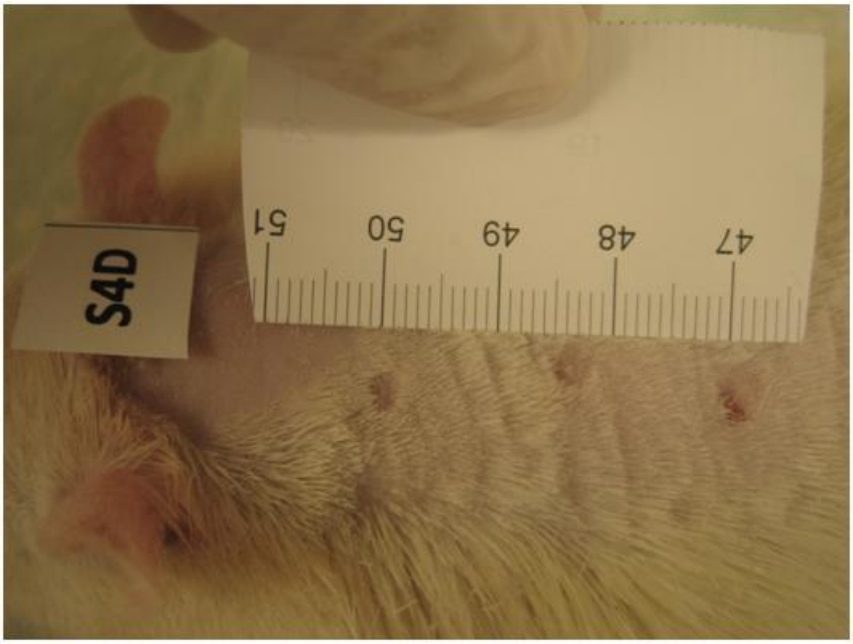


Last Day

Appendix 6 The progressive changes in one of the levant storax group's animal wound area were measured by a standard reference ruler and monitored by a camera (Nicon®).

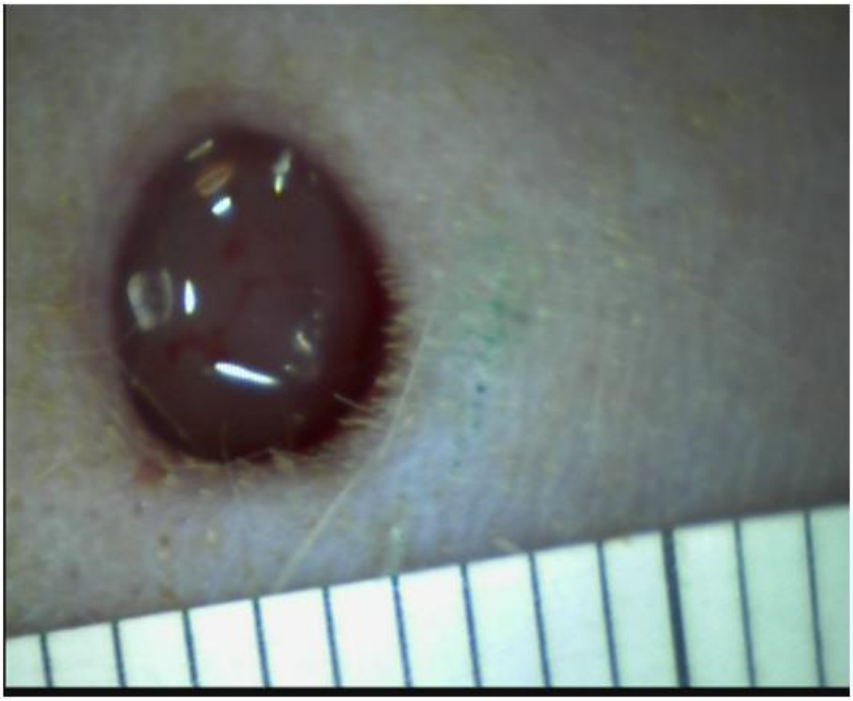


First Day

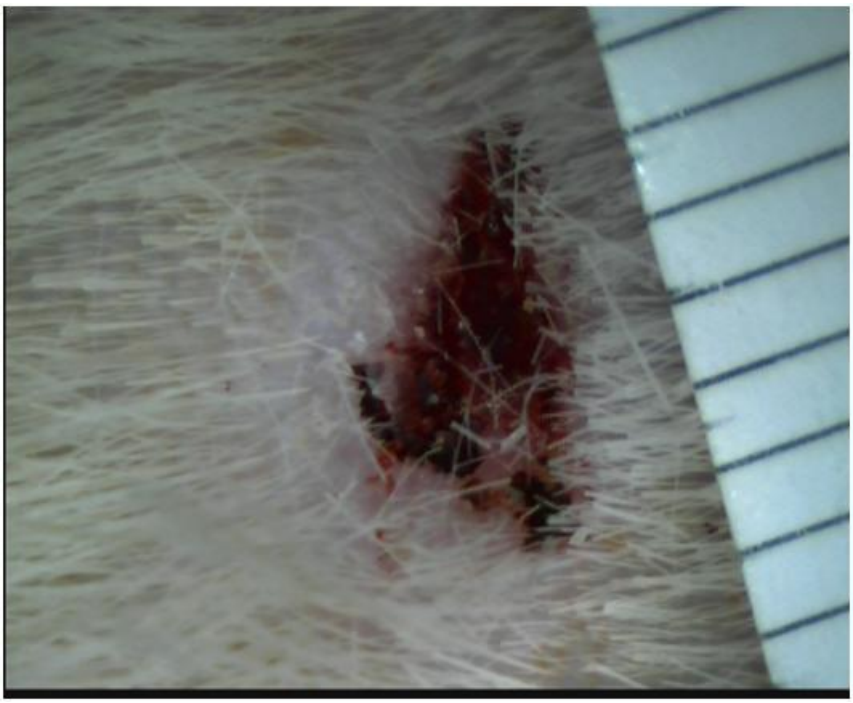


Last Day

Appendix 7 Representative wounds of the control group wound was photographed in the presence of the standard reference ruler by DLite Analog Microscope on the first and last day (nineth day) of the study.

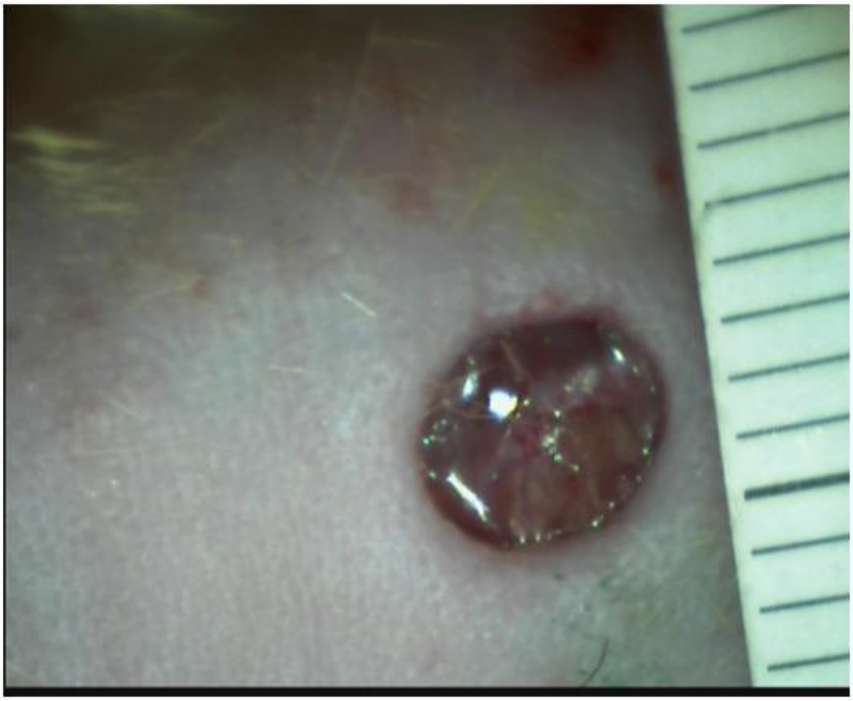


First Day

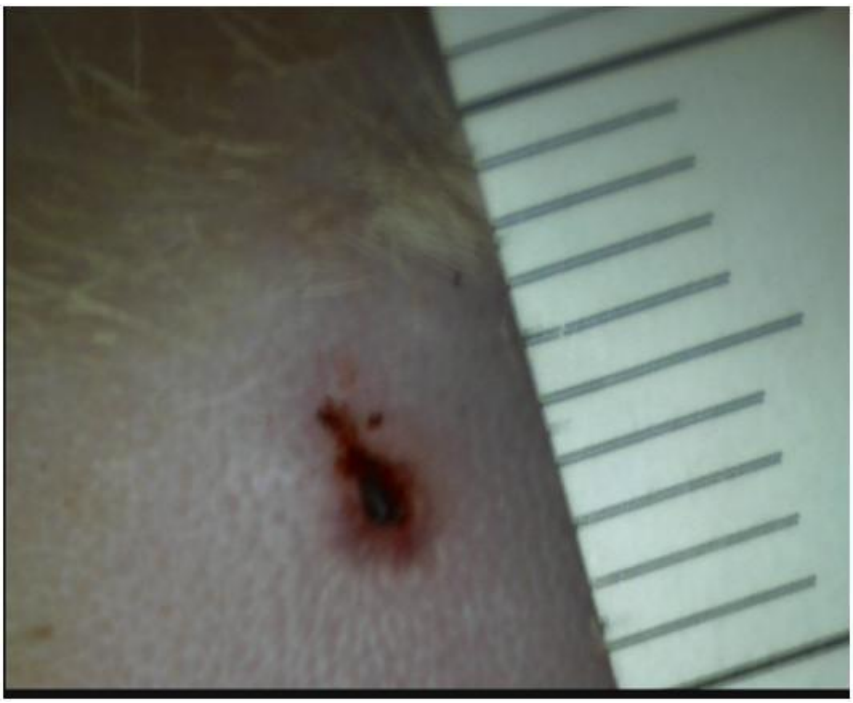


Last Day

Appendix 8 Representative wounds of the placebo group was photographed in the presence of the standard reference ruler by DLite Analog Microscope on the first and last day (nineth day) of the study.

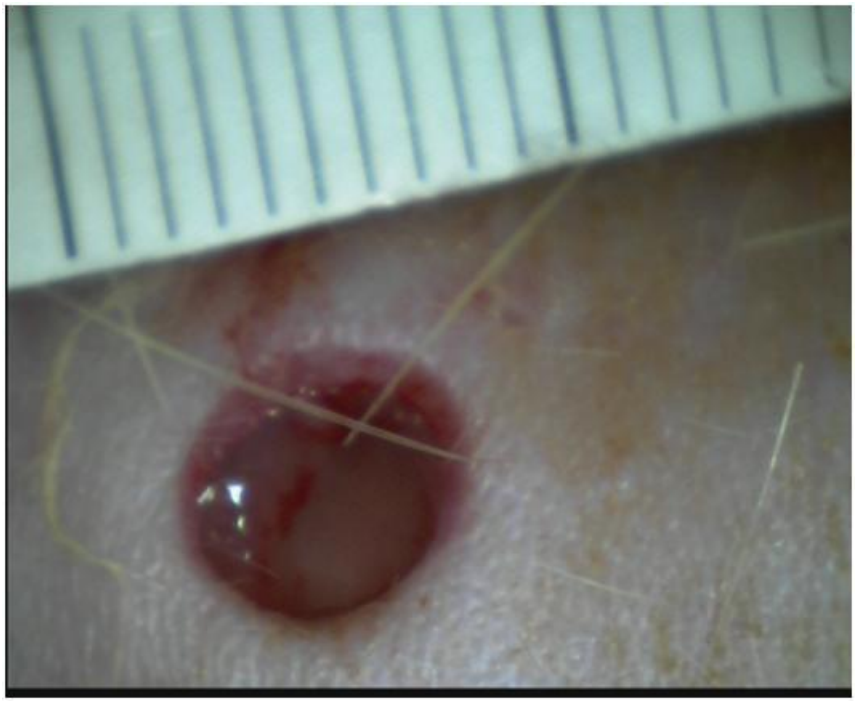


First Day

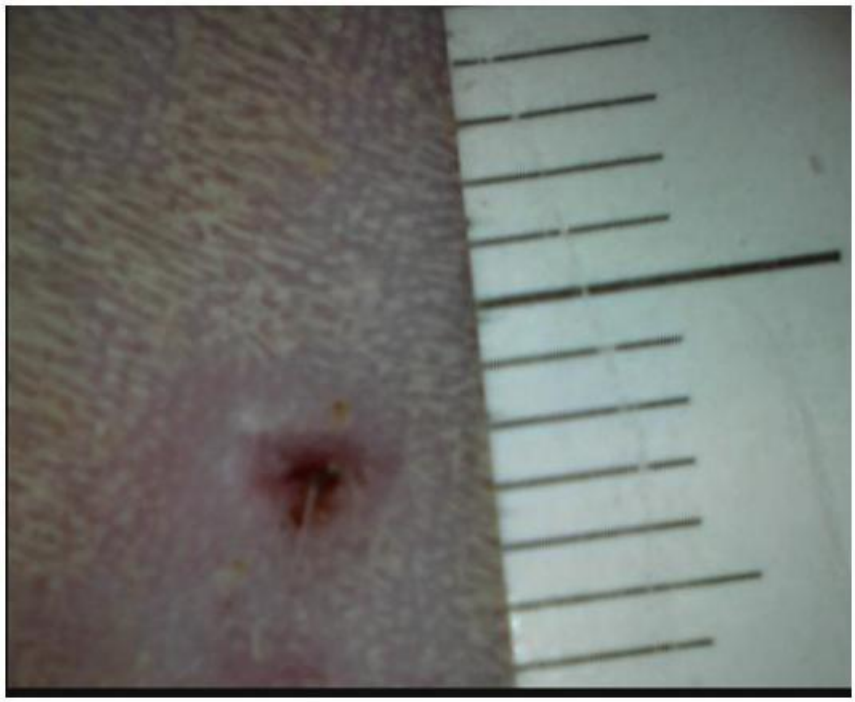


Last Day

Appendix 9 Representative wounds of the reference group was photographed in the presence of the standard reference ruler by DLite Analog Microscope on the first and last day (nineth day) of the study.

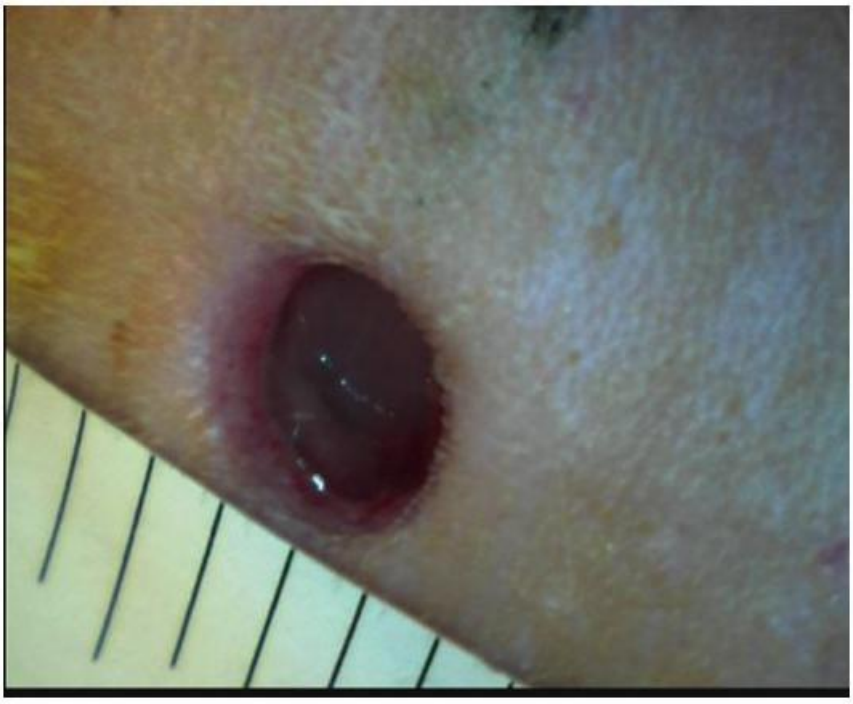


First Day

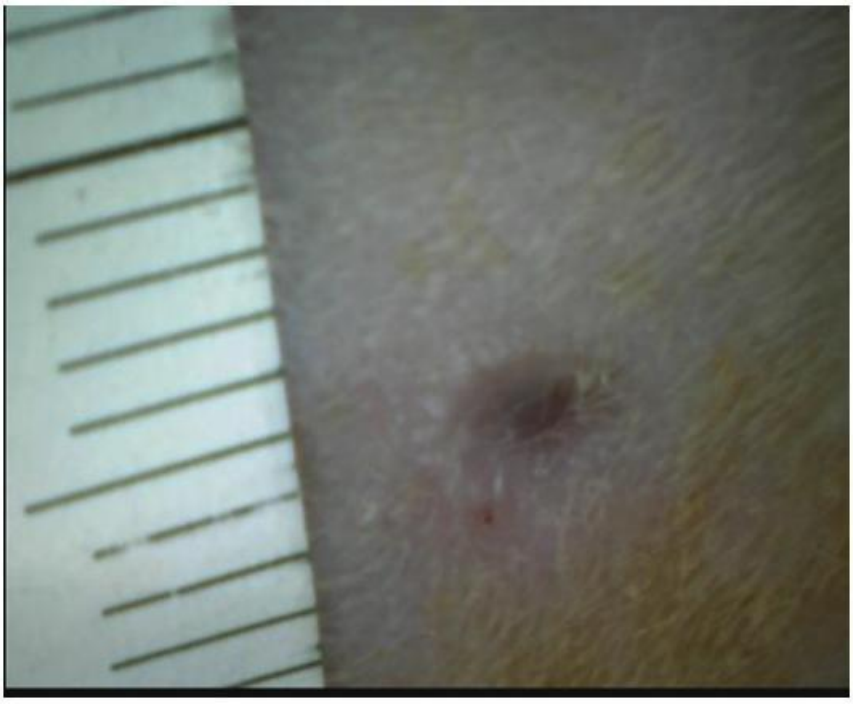


Last Day

Appendix 10 Representative wounds of the complex group was photographed in the presence of the standard reference ruler by DLite Analog Microscope on the first and last day (nineth day) of the study.

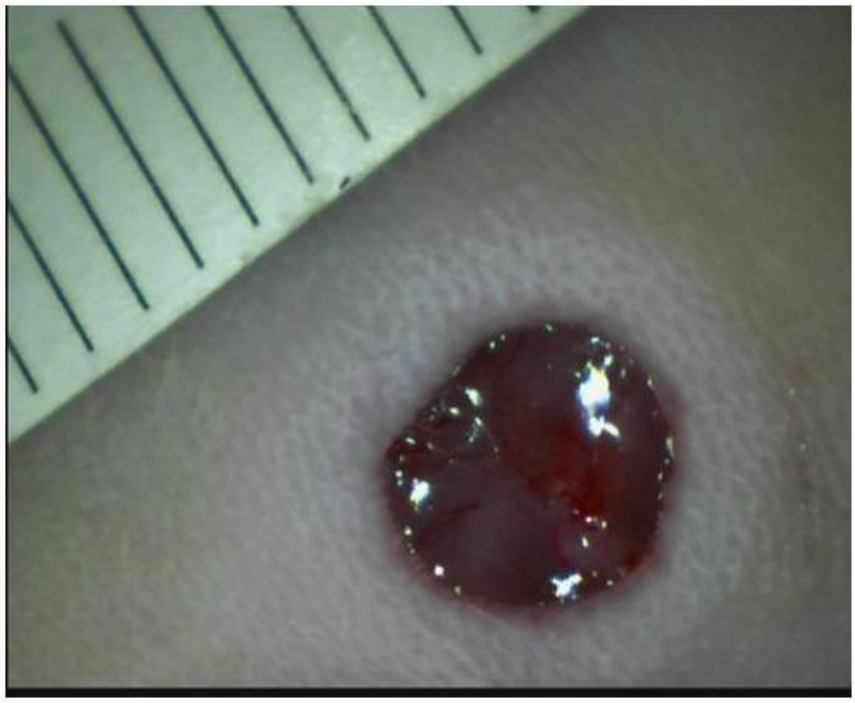


First Day

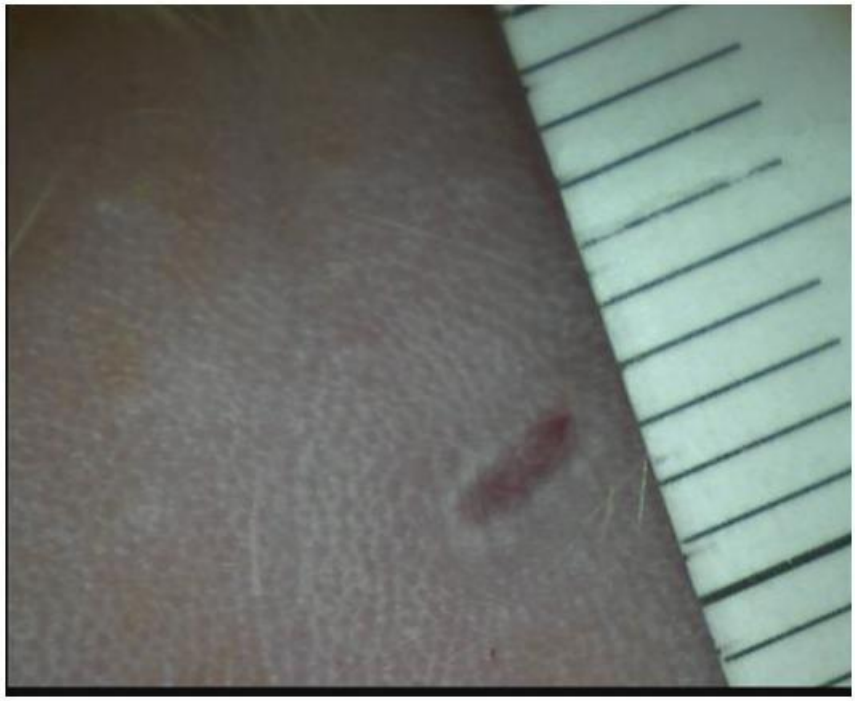


Last Day

Appendix 11 Representative wounds of the levant storax group wound was photographed in the presence of the standard reference ruler by DLite Analog Microscope on the first and last day (nineth day) of the study.



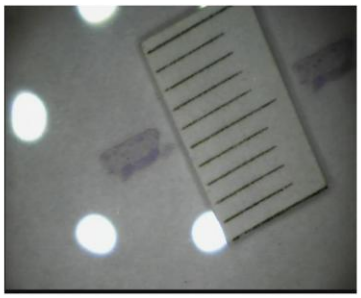
First Day



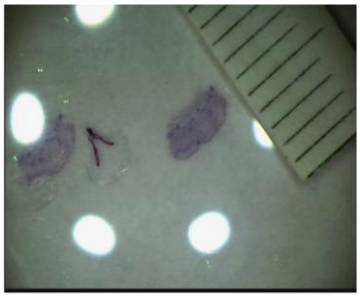
Last Day

Appendix 12 Representative intact tissue photographs were obtained by DLite Analog Microscope.

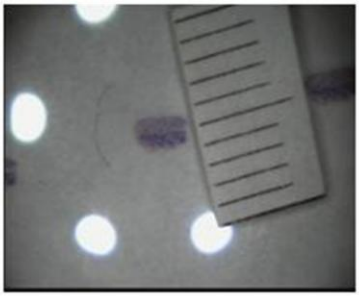
Control



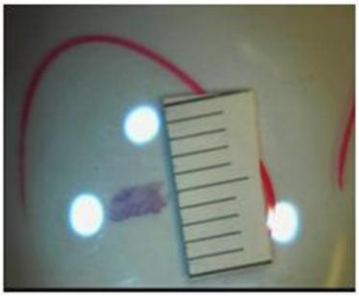
Placebo



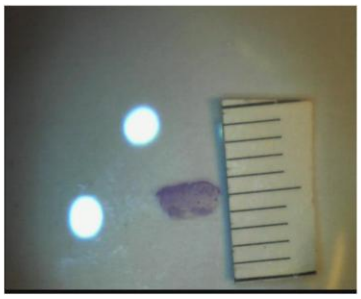
Reference



Complex



Levant Storax



Appendix 13 Results of Kruscall Wallis test for histopathological parameters.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of ACTIVEINFLAMMATION is the same across categories of GROUP.	Independent-Samples Kruskal-Wallis Test	.019	Reject the null hypothesis.
2	The distribution of CHRONICINFLAMMATION is the same across categories of GROUP.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
3	The distribution of FIBROBLASTICACTIVITY is the same across categories of GROUP.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
4	The distribution of NEOVASCULARIZATION is the same across categories of GROUP.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
5	The distribution of FIBROSIS is the same across categories of GROUP.	Independent-Samples Kruskal-Wallis Test	.009	Reject the null hypothesis.
6	The distribution of HAIRFOLLICLEFORMATION is the same across categories of GROUP.	Independent-Samples Kruskal-Wallis Test	.004	Reject the null hypothesis.
7	The distribution of HEALINGPHASE is the same across categories of GROUP.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

7 REFERENCES

1. Worldwide Wound Management, 2009: Established and Emerging Products, Technologies and Markets in the U.S., Europe, Japan and Rest of World.

2. Sezik E, Yeşilada E, Gürbüz İ. *Styrax liquidus*'un Antiülserjenik aktivitesi üzerinde çalışmalar. Gazi Üniversitesi Araştırma Fonu EF 02/2001-06, 2001.

3. Ozturk M, Parks CR, Coskun F, Gork G, Secmen O. Vanishing tertiary genetic heritage in the East Mediterranean: *Liquidambar orientalis*. Environews, 10: 6–8, 2004.

4. Bruneton J. Pharmacognosy Phytochemistry Medicinal Plants (2nd ed.) Lavoisier, Paris, pp 520, 1999.

5. Hafizoglu H, Reunanen M, Istek A. Chemical constituents of from *Liquidambar orientalis*. Holzforschung, 50: 116–117, 1996.

6. Lee YS, Kim J, Lee SG, Oh E, Shin SC, Park IK. Effects of plant essential oils and components from Oriental sweetgum (Liquidambar orientalis) on growth and morphogenesis of three phytopathogenic fungi. Pesticide Biochemistry and Physiology, 93: 138–143, 2009.

7. Becker P. *Emulsions*: *Theory and Practice.* (2nd ed.) Reinhold Publ. Co., New York, pp 1-4, 1965.

8. Laba D. *Rheological Properties of Cosmetics and Toiletries*. Marcel Dekker Inc., New York, pp 318-319, 1993.

9. Graaf S, Schroen CGPH, Boom RM. Preparation of double emulsions by membrane emulsification—a review. Journal of Membrane Science, 251: 7–15, 2005.

10. Ozer O, Mutlu B, Tömek S. Development and *in vitro* evaluation of topically applied cinnamic acid formulation. An international Journal of Pharmaceutical Sciences, 62: 138-144, 2007.

11. Ozer O, Ozyazici M, Tedajo M, Taner MS, Köseoglu K. W/O/W Multiple Emulsions Containing Nitroimidazole Derivates for Vaginal Delivery. Drug Delivery, 14: 139-145, 2007.

12. Gerbino PP. Remington: The Science and Practice of Pharmacy. (21st ed.) Lippincott Williams and Wilkins, Philadelphia pp 325-329, 2005.

13. Jorgensen L. Physical Stability of Proteins in Emulsions. SciTopics, Retrieved June 26, 2011.

14. Stevens J, Mims P, Coles N. Lipid Emulsions as Drug Delivery Systems. Business Briefing, Pharmatech: 1-4, 2003.

15. Tadros TF. Emulsion science and technology: A general introduction. Wiley-VCH Verlag GmbH & KgaA, Weinheim, pp 3-4, 2009.

16. Salager JL. Interfacial Phenomena in Dispersed Systems. FIRP, Merida-Venezuela, pp 2-24, 1994.

17. Prochaska FO. A study of Interfacial Tension In Ternary Systems. Faculty of Texas Technological Collage, Master Thesis, Texas, 1966.

18. Birdi KS. Surface and Colloid Chemistry, Handbook of Surface and Colloid Chemistry. Taylor & Francis Group, pp 1-40, 2009.

19. Schueller R, Romanowski P. Beginning Cosmetic Chemistry. Allured Publ. Co.: 39-41, 1999.

20. Ruckenstein E. Microemulsions, macroemulsions, and the Bancroft rule. Langmuir, 12: 6351-6353, 1996.

21. Balsam MS, Sagarin E. *Cosmetics*: *Science and Technology*. (2nd *ed*) New Wiley-Interscience, New York, pp 583-598, 1974.

22. Sajjadi S, Zerfa M, Brooks BW. Phase inversion in p-xylene/water emulsions with the non-ionic surfactant pair sorbitan monolaurate/polyoxyethylene sorbitan monolaurate (Span 20/Tween 20). Colloids and Surfaces A: Physicochem. Eng. Aspects 218: 241-254, 2003.

23. Boyd J, Parkinson C, Sherman P. Factors Affecting Emulsion Stability, and HLB Concept. Journal of Colloid and İnterface Science, 2: 1972.

24. Opawale FO, Burgess DJ. Influence of Interfacial Properties of Lipophilic Surfactans on Water-in-Oil Emulsion Stability. Journal of Colloid and Interface Science, 197: 142-150, 1998.

25. Lin TJ. Effect of Initial Surfactant Locations on the Viscosity of Emulsions. J. Soc. Cosmetic Chemists, 19: 683-697, 1968.

26. Tropea C, Yarin AL, Foss JF. Springer handbook of experimental fluid mechanics. Springer-Verlag, Berlin: 717-718, 2007.

27. James-Smith MA, Alford K, Shah DO. A novel method to quantify the amount of surfactant at the oil/water interface and to determine total interfacial area of emulsions. Journal of Colloid and Interface Science, 310: 590-598, 2007.

28. Roland I, Piel G, Delattre L, Evrard B. Systemic characterization of oil-in-water emulsions for formulation design. International Journal of Pharmaceutics, 263: 85-94, 2003.

29. Chanamai R, McClements DJ. Dependence of creaming and rheology of monodisperse oil-in-water emulsions on droplet size and concentration. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 172: 79–86, 2000.

30. Tadros T. Application of rheology for assessment and prediction of the long-term physical stability of emulsions. Advances in Colloid and Interface Science, 108: 227–258, 2004.

31. Dreher TM, Glass J, O'Connor AJ, Stevens GW. Effect of Rheology on Coalescence Rates and Emulsion Stability. AIChE Journal, 45: 1182-1190, 1999.

32. Jiao J. and Burgess DJ. Ostwald ripening of water-in-hydrocarbon emulsions. Journal of Colloid and Interface Science, 264: 509–516, 2003.

33. Paul S, Panda AK. Physico-Chemical Studies on Microemulsion: Effect of Cosurfactant Chain Length on the Phase Behavior, Formation Dynamics, Structural Parameters and Viscosity of Water/ (Polysorbate-20 + n-Alkanol)/n-Heptane Water-in-Oil Microemulsion. Journal of Surfactants and Detergents, 2011.

34. Eccleston GM. Functions of mixed emulsifiers and emulsifiying waxes in dermatological lotions and creams. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 123-124: 169-182, 1997.

35. Han J, Washington C. Partition of antimicrobial additives in an intravenous emulsion and their effect on emulsion physical stability. International Journal of Pharmaceutics, 288: 263–271, 2005.

36. Jumaa M, Furkert FH, Muller BW. A new lipid emulsion formulation with high antimicrobial efficacy using chitosan. European Journal of Pharmaceutics and Biopharmaceutics, 53: 115–123, 2002.

37. Sznitowska M, Janicki S, Dabrowska EA, Gajewska M. Physicochemical screening of antimicrobial agents as potential preservatives for submicron emulsions. European Journal of Pharmaceutical Sciences, 15: 489–495, 2002.

38. Ramful D, Aumjaud B, Neergheen VS, Soobrattee MA, Googoolye K, Aruoma OI, Bahorun T. Polyphenolic content and antioxidant activity of Eugenia pollicina leaf extract in vitro and in model emulsion systems. Food Research International, 44: 1190–1196, 2011.

39. Mattia CDD, Sacchetti G, Mastrocola D, Pittia P. Effect of phenolic antioxidants on the dispersion state and chemical stability of olive oil O/W emulsions. Food Research International, 42: 1163–1170, 2009.

40. Roland I, Piel G, Delattre L, Evrard B. Systemic characterization of oil-in-water emulsions for formulation design. International Journal of Pharmaceutics, 263: 85-94, 2003.

41. Waqas MK, Akhtar N, Ahmad M, Murtaza G, Khan HMS, Iqbal M, RASUL A, Bhatti NS. Formulation and characterization of a cream containing extract of fenugreek seeds. Acta Poloniae Pharmaceutica ñ Drug Research, 67: 173-178, 2010.

42. Zeta Potential An Introduction in 30 Minutes, Malvern Instruments.

43. Truong L, Phillips C. Freezing of Oil-Water and Oil-Saline Emulsions. Environmental Science Technology, 10: 482-485, 1976.

44. ICH Q1A(R2) Guideline: stability testing of new drug substances and products: CPMP/ICH/2736/99. International Conference on Harmonisation, London, pp 1-20, 2003.

45. Guidelines on Stability Testing of Cosmetic Products. COLIPA, pp 1-8, 2004.

46. Questions & answers on quality of herbal medicinal products/traditional herbal medicinal products. EMEA Committee on Herbal Medicinal Products, 41500: 13, 2010.

47. Lazarus GS, Cooper DM, Knighton DR, Margolis DJ, Pecoraro RE, Rodeheaver G, Robson MC. Definitions and guidelines for assessment of wounds and evaluation of healing. Arch Dermatol, 130: 489-493,1994.

48. Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. Clinics in Dermatology, 25: 9–18, 2007.

49. Werdin F, Tennenhaus M, Schaller HA, Rennekampff HO. Evidence-based Management Strategies for Treatment of Chronic Wounds. Eplasty, 9: 169-179, 2009.

50. Strodtbeck F. Physiology of Wound Healing. Newborn and Infant Nursing Reviews, 1: 43–52, 2001.

51. Cotran RS, Kumar V, Collins T. Robbins Pathologic Basis of Disease (2nd ed.). W.B. Saunders Company, USA, pp 107-109, 1999.

52. Hess CT, Kirsner RS. Orchestrating wound healing: assessing and preparing the wound bed. Advances In Skin & Wound Care, 16: 246-257, 2003.

53. Phillips SJ. Physiology of Wound Healing and Surgical Wound Care. ASAIO Journal, 46: 2-5, 2000.

54. Monaco JL, Lawrence WT. Acute wound healing An overview. Clinics Plastic Surgery, 30: 1-12, 2003.

55. Farstvedt E, Stashak TS, Othica A. Update on Topical Wound Medications. Clinical Techniquies in Equine Practice, 3: 164-172, 2004.

56. Hamm RL. Antibacterial Dressings. Advance in wound care, 1: 148-154, 2010.

57. Kramer SA. Effect of povidone-iodine on wound healing: A review. Journal of Vascular Nursing, 17: 17-23, 1999.

58. Spann CT, Tutrone WD, Weingberg JM, Scheinfeld N, Ross B. Topical Antibacterial Agents for Wound Care: A Primer. Dermatol Surg, 6: 620-623, 2003.

59. Lucas VS, King AW. Wound Care for the Plastic Surgery Nurse. Plastic Surgical Nursing, 30: 158-169, 2010.

60. Fox CL, Modak SM. Mechanism of Silver Sulfadiazine Action on Burn Wound Infections. Antimicrobial Agents and Chemotherapy, 5: 582-588, 1974.

61. Ward RS, Sat JR. Topical Agents in Burn and Wound Care. Physical Therapy, 75: 526-538, 1995.

62. Moukaddemm M, Tangy F, Capmau ML, Le GF. Effects of Cations, Polyamines and Other Aminoglycosides on Gentamicin C. Binding to Ribosomes from Sensitive and Resistant Escherichia Coli Strain. The Journal Of Antibiotics, 39: 136-140, 1986.

63. Spann CT, Taylor SC, Weinberg JM. Topical Antimicrobial Agents in Dermatology. Disease-a-Month , 50: 407-421, 2004.

64. Rode H, Hanslo D, De Wet PM, Millar AJW, Cywes S. Efficacy of Mupirocin in Methicillin-Resistant Staphylococcus aureus Burn Wound Infection. Antimicrobial Agents and Chemotherapy, 33: 1358-1361, 1989.

65. Harding KG,Jones V, Price P. Topical treatment: which dressing to choose. Diabetes/Metabolism Research and Reviews 1 (Suppl): 47-50, 2000.

66. Sharman D. Moist wound healing: a review of evidence, application and outcome. The Diabetic Foot, 6: 112-120, 2003.

67. Okan D,Woo K, Ayello EA, Sibbald G. The Role of Moisture Balance in Wound Healing. Advances in Skin & Wound Care, 20: 39-53, 2007.

68. Stashak TS, Farstvedt E, Othic A. Update on Wound Dressings: Indications and Best Use. Clinical Techniques in Equine Practice, 3: 148-163, 2004.

69. Singhal A, Reis ED, Kerstein MD. Options for nonsurgical debridement of necrotic wounds. Adv Skin Wound Care, 14: 96–101, 2001.

70. Steed DL. Debridement. The American Journal of Surgery 1(Suppl): 71-74, 2004.

71. Pandarinathan C, Sajithlal GB, Chandrakasan G. Influence of *Aloe vera* on collagen characteristics in healing dermal wounds in rats. Molecular and Cellular Biochemistry, 181: 71–76, 1998.

72. Choı S, Chung MH. A review on the relationship between *Aloe vera* components and their biologic effects. Seminars in Integrative Medicine, 1: 53-62, 2003.

73. Lavagna SM, Secci D, Chimenti P, Bonsignore L, Ottaviani A, Bizzarri B. Efficacy of *Hypericum* and *Calendula* oils in the epithelial reconstruction of surgical wounds in childbirth with caesarean section. Farmaco, 56: 451–453, 2001.

74. Suntar IP, Akkol EK, Yılmazer D, Baykal T, Kırmızıbekmez H, Alper M, Yesilada E. Investigations on the in vivo wound healing potential of *Hypericum perforatum* L.. Journal of Ethnopharmacology, 127: 468–477, 2010.

75. Öztürk N, Korkmaz S, Öztürk Y. Wound-healing activity of St. John's Wort (*Hypericum perforatum* L.) on chicken embryonic fibroblasts. Journal of Ethnopharmacology, 111: 33-39, 2007.

76. Grover JK, Yadav SP. Pharmacological actions and potential uses of *Momordica charantia*: a review. Journal of Ethnopharmacology, 93: 123–132, 2004.

77. Prasad V, Jain V, Girish D, Dorle AK. Wound-healing property of *Momordica charantia* L. fruit powder. Journal of herbal pharmacotherapy, 6: 105-15, 2006.

78. Oehler C et al. Chamavioline Antiedematous, but not a constituent of Matricaria recutita. Phytochemistry Letters, 2: 171–175, 2009.

79. Shivananda B, Sivachandra RS, Chalapathi RAV. Wound healing activity of Matricaria recutita L. extract. Journal of Wound Care, 16: 298–302, 2007.

80. M. Fronza et al. Determination of the wound healing effect of Calendula extracts using the scratch assay with 3T3 fibroblasts. Journal of Ethnopharmacology, 126: 463–467, 2009.

81. *Aesculus Hippocastanum* (Horse chestnut), Monograph, Alternative medicine review. A journal of clinical therapeutic, 14: 278-83, 2009.

82. Forrest RD. Early history of wound treatment. Journal of the Royal Society of Medicine, 75: 198-205, 1982

83. Somashekar Shetty B, Udupa SL, Udupa AL, Somayaji SN. Effect of *Centella asiatica* L. (Umbelliferae) on Normal and Dexamethasone-Suppressed Wound Healing in Wistar Albino Rats. Lower extremity wounds, 5: 137-143, 2006.

84. Shukla A et al. *In vitro* and *in vivo* wound healing activity of asiaticoside isolated from *Centella asiatica*. Journal of Ethnopharmacology, 65: 1–11, 1999.

85. Rougier A. Clinical efficacy on epidermal wound healing of topically applied madecassoside associated with copper/zinc/manganese salts. Journal of the American Academy of Dermatology, 56: 28, 2007.

86. ICH Guideline: Q2(R1): CPMP/ICH/381/95. International Conference on Harmonisation, November 1994.

87. Ursica L, Tita D, Palici I, Tita B, Vlaia V. Particle size analysis of some water/oil/water multiple emulsions. Journal of Pharmaceutical and Biomedical Analysis, 37: 931–936, 2005.

88. Bendjaballah M, Canselier JP, Oumeddour R. Optimization of Oil-in-Water Emulsion Stability: Experimental Design, Multiple Light Scattering,and Acoustic Attenuation Spectroscopy. Journal of Dispersion Science and Technology, 31: 1260–1272, 2010.

89. Colipa Guidelines on Microbial Quality Management, (MQM), 1997, (www.colipa.com).

90. The European Agency for the Evaluation of Medicinal Products Evaluation of Medicines for Human Use: CPMP/QWP/122/02, London, pp 4-18, 2003.

91. Regional Guideline for The WHO Eastern Mediterranean Region, Stability Testing of Active Substances and Pharmaceutical Products: QAS/06.179, World Health Organization, Geneva, pp 1-33, 2006.

92. Marti-Mestres G, Nielloud F. Emulsion in health care applications-An overview. J Dispers Sci Techno, 23: 419–39, 2002.

93. Bernatoniene J, Masteikova J, Davalgiene J, Peciura R, Gauryliene R, Bernatoniene R, Majiene D, Lazauskas R, Civinskiene, G, Velziene S, Muselik J, Chalupova Z. Topical application of *Calendula officinalis* (L.): Formulation and evaluation of hydrophilic cream with antioxidant activity. Journal of Medicinal Plants Research, 5: 868-877, 2011.

94. B Burlando, L Verotta, L Comara. Herbal Principles in Cosmetics: Properties and Mechanisms of Action. Taylor & Francis Group, 2010.

95. Burford G, Bodeker G, Ryan TJ. Skin and wound care: traditional, complementary and alternative medicine in public health dermatology. Traditional, Complementary & Alternative Medicine: Policy and Public Health Perspectives, Imperial College Press, London.

96. Wilkinson JA., Brown MG. Horse Chestnut – Aesculus Hippocastanum: Potential Applications in Cosmetic Skin-care Products. International Journal of Cosmetic Science, 21: 437–447, 1999.

97. Maenthaisong R, Chaiyakunapruk N, Niruntraporn S, Kongkaew C. The efficacy of aloe vera used for burn wound healing: A systematic review. Burns 33: 713-718, 2007.

98. Schneider L, Korber A, Grabbe S, Dissemond,J. Influence of pH on wound-healing: a new perspective for wound-therapy. Archives of Dermatological Research, 298: 413-420, 2007.

99. Cambiella A, Benito JM, Pazos C, Coca F. Interfacial properties of oil-in-water emulsions designed to be used as metalworking fluids. Colloids and Surface, 30: 112-119, 2007.

100. Stachurski J, MichaLek M. The Effect of the Potential on the Stability of a Non-Polar Oil-in-Water Emulsion. Journal of Colloid and Interface Science, 184: 433-436, 1996.

101. Wiacek A, Chibowski E. Zeta potential, effective diameter and multimodal size distribution in oil/water emulsion. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 159: 253-261 ,1999.

102. Bendjaballah M, Canselier JP, Oumeddour R. Optimization of Oil-in-Water Emulsion Stability: Experimental Design, Multiple Light Scattering,and Acoustic Attenuation Spectroscopy. Journal of Dispersion Science and Technology, 31: 1260–1272, 2010.

103. http://uwadmnweb.uwyo.edu/vetsci/Courses/PATB_4130/Fall09/(15)%20NOTES%20CHRONIC_INFLAMMATION.pdf

104. Chang ST, Chen PF, Chang SC. Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum*. Journal of Ethnopharmacology, 77: 123–127, 2001.

105. Amor ILB, Neffati A, Sgaier MB. Antimicrobial Activity of Essential Oils Isolated from Phlomis crinita Cav. ssp. mauritanica Munby. J Am Oil Chem Soc, 85: 845–849, 2008.

106. Palazzo MC, Wright HL. Chemical Compositions and Biological Activities of Leaf Essential Oils of Six Species of Annonaceae from Monteverde. Costa Rica. *Rec. Nat. Prod*., 3: 153-160, 2009.

107. Kubo I, Muroi H, and Kubo A. Antibacterial Activity of Long-Chain Alcohols against *Streptococcus mutans*. J. Agrlc. Food Chem, 41*:* 2447-2450, 1993.

108. http://uwadmnweb.uwyo.edu/vetsci/Courses/PATB_4130/Fall09/(15)%20NOTES%20CHRONIC_INFLAMMATION.pdf

109. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. Frontiers in Bioscience, 9: 283-289, 2004.

110. Fan C, Luedtke MA, Prouty SM, Burrows M, Kollias N, Cotsarelis G. Characterization and quantification of wound-induced hair follicle neogenesis using *in vivo* confocal scanning laser microscopy. Skin Research and Technology, 2011.

111. Zhu H, Wei X, Bian K, Murad F. Effects of nitric oxide on skin burn wound healing. Journal of Burn Care Research, 29: 804-814, 2008.

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Yrd. Doç. Dr. Yasemin Yağan Uzuner, YÜ Eczacılık Fakültesi

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