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# Determination of the stability of cosmetic formulations with incorporation of natural products

**Amira Bouranen**

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Supervised by

**Dr. Maria joão Sousa**

**Dr. Vitor Manuel R. Martins**

**Dr. Habib Mosbah**

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# ABBREVIATIONS LIST

| | |
|---|---|
| **BP** | Bee pollen |
| **$CaCl_2$** | Calcium chloride |
| ***C. albicans*** | *Candida albicans* |
| **CAM** | Chorioallantoic membrane |
| **C30** | Chloraphenicol, Antibiotic disc susceptible to bacteria |
| **DMSO** | Dimethylsulfoxide |
| ***E. coli*** | *Escherichia coli* |
| **EO** | Essential oil |
| **EDCs** | Endocrine disrupting chemicals |
| **EDTA** | Ethylenediaminetetraaceticacid |
| **$\gamma$-rays** | Gamma rays |
| **F100** | Flucanosole , antibiotic disc susceptible to yeast |
| **G** | Gel |
| **GC** | Gas chromatography |
| **GC-MS** | Gas chromatography-mass spectrometry |
| **HET-CAM** | Hen's egg-test on the chorioallantoic membrane |
| **$H_2O_2$** | Hydrogen peroxide |
| **KCl** | Potassium chloride |
| **$LC_{50}$** | Lethal concentration, the concentration that kills 50% of Artemia |
| **M%** | Pourcentage of mortality |
| **$MgCl_2$** | Magnesium chloride |
| **$MgSO_4$** | Magnesium sulfate |
| **NaCl** | Sodium chloride |

| | |
|---|---|
| **$NaHCO_3$** | Sodium bicarbonate |
| **NaOH** | Sodium hydroxide |
| **$O_2-$** | Superoxide |
| **pH** | Potential of hydrogen |
| ***P. aeruginosa*** | *Pseudomonas aeruginosa* |
| **RAC** | Committee for Risk Assessment |
| **RI** | Retention index, relative to C9–C17 *n* alkanes on the DB-1 column. |
| **RPM** | Rotation per minute |
| **ROS** | Reactive Oxygen Species |
| ***S. aureus*** | *Staphylococcus aureus* |
| **SCCNFP** | Scientific committee on cosmetic products and non-food products intended for consumers |
| **Subsp** | Subspecies |
| **T** | Thymus |
| **T** | Trace (<0.05) |
| **UV** | Ultraviolet |
| **WHO** | World Health Organization |

## ABSTRACT

Nowadays, natural products are gaining increased popularity as cosmetic ingredients due to their active properties and the different roles they can play in a single cosmetic formula, acting as moisturizers, fragrances, surfactants and preservatives.

The aim of this study is to formulate and subsequently evaluate the stability of an anti-aging gel based on natural products namely, bee pollen and essential oils from two species of thymus, Portuguese species *Thymus zygis* subsp *zygis* and Tunisian species *Thymus capitatus* applied as natural preservatives.

The stability of the cosmetic formulations was evaluated through a series of physicochemical assays, such as pH, density and viscosity determinations, thermic stress tests, evaluation of the organoleptic characteristics (odor, color and general aspect), UV spectrophotometric analysis, centrifuge test, test of vibration, and also through light test. The formulation also evaluated for its resistance and activity against a variety of microorganisms such as: *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans*. The microbiological stability of the formulations was evaluated through agar diffusion assays using cultures of Escherichia *coli, Staphylococcus aureus, Candida albicans,* and *Pseudomonas aeruginosa.* The safety of the formulations were evaluated through the determination of the acute toxicity of the compounds incorporated in the base formulation using brine shrimp (*Artemia salina*) mobility assay and also through the evaluation of their ocular irritancy using Hen's egg-test on the chorioallantoic membrane test (HET-CAM test).

The cosmetic formulations prepared presented a pH value that seems adequate for topic application, and presented a non-Newtonian flow behaviour (shear thinning), which is frequently observed in cosmetic products, besides evidencing no phase separation after centrifuge except the formulation containing methylparaben and bee pollen. No phase separation in all the formulations tested during the vibration test. Organoleptic stability of the formulations was achieved during 2 weeks. The results presented in this study showed good stability throughout the experimental period. All the formulations tested showed a good physical and chemical stability. In addition, the findings suggested that the gel formulations with higher concentration of thyme oils and/or methylparaben have apromising antibacterial

effect against the proliferation of various microorganisms namely *S. aureus*, P. *aeruginosa*, *E. coli* and *C. albicans* evidencing bacteriostatic effect. *S. aureus* was the most sensitive bacteria with an inhibition zone ranging from 10 to 25 mm. Moreover, it was observed that the formulations in which bee pollen and thyme oils were incorporated did not caused any irritant reaction on the chorioallantoic membrane of chicken embryo. Also, the previous cited products did not show a toxic effect through *Artemia* assay with $LC_{50}$ higher than 1mg/mL (1.88 g/mL, 1.94 g/mL, and 17.78 g/mL for *Thymus zygis zygis* essential oil, *Thymus capitatus* essential oil, and for bee pollen, respectively).

Therefore, the tests performed suggested that the various cosmetic formulations prepared through the incorporation of bee pollen, *Thymus zygis zygis* and *Thymus capitatus* essential oils present characteristics suitable for topic use. Also, the incorporation of the natural products did not seem to influence negatively the stability of the cosmetic formulations.

During the present work, it was consistently observed a fungal contamination of the cosmetic formulations based on bee pollen. Different methodologies, such as aseptic practices, freeze-drying and heat treatment were used to overcome it. However only by increasing the preservatives concentration, a decrease in fungi growth was registered.

# RESUMO

Atualmente os produtos naturais estão a crescer em popularidade como ingredientes de cosméticos, devido ás suas propriedades ativas, e também, porque podem desempenhar diferentes papéis nas formulações cosméticas, podendo atuar como hidratantes, fragâncias, surfactantes e conservantes.

O principal objetivo deste trabalho foi desenvolver uma formulação de um gel anti-idadee subsequentemente avaliá-la em termos de estabilidade. O gel foi desenvolvido utilizando como princípios ativosprodutos naturais: pólen de abelha e óleos essenciais de duas plantas do género *Thymus*, uma espécie Portuguesa,característica da zona de Trás os Montes, *Thymus zygis zygis*, e uma espécie da flora Tunisina, o *Thymus capitatus*, que foram usados como conservantes naturais.

A estabilidade desta formulação cosmética foi avaliada através de uma série de ensaios físico-químicos, como determinações do pH, da densidade e da viscosidade, testes de stresse de temperatura (temperaturas elevadas por períodos de tempo específicos), avaliações das características organolépticas (odor, cor e aspecto geral), análises de espectrofotometria, teste de centrifugação, teste de vibração, Adicionalmente, a estabilidade microbiológica da formulação foi avaliadaatravés de ensaios de difusão em agar, recorrendo à utilização de culturas de *Escherichia coli, Staphylococcus aureus, Candida albicans*, e *Pseudomonas aeruginosa*. A formulação foi também avaliada quanto àresistência e actividade contra alguns microorganismos da pele como: *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, *Candida albicans.* A toxicidade aguda dos compostos incorporados na formulação foi determinada através da avaliação da mobilidade da *Artemia salina* e a irritabilidade ocular foi avaliada usando o teste de HET- CAM.

O pH da formulação desenvolvida apresenta valores adequadosà utilização tópica, e apresenta um comportamento de fluxo não-Newtoniano (reofluidificante), que é frequentemente observado em produtos cosméticos, assim como não apresentou separação de fases nos testes de centrifugação e de vibração, exceto a formulação contendo metilparabeno e pólen de abelha. Sem separação de fases em todas as formulações testadas durante os testes de vibração. A estabilidade organoléptica das formulações manteve-se durante as 2 semanas testadas. Os resultados apresentados neste estudo apresentaram boa estabilidade ao longo do

período experimental. Todas as formulações testadas apresentaram boa estabilidade física e química.

Em acréscimo, os resultados sugeriram que as formulações de gel com maior concentração de óleo de tomilho e/ou metilparabeno possuem atividade antibacterianas contra a proliferação de vários microrganismos, como *S. aureus, P. aeruginosa, E. coli* e *C. albicans* que evidenciam efeito bacteriostático. *S. aureus* foi a bactéria mais sensível com uma zona de inibição variando de 10 a 25 mm. Observou-se que as formulações nas quais o pólen de abelha e os óleos de tomilho foram incorporados não provocaram qualquer reação irritante na membrana corioalantóica do embrião de frango. Verificou-se ainda que, os produtos citados anteriormente não mostraram efeito tóxico (toxicidade aguda) através do teste de *Artemia* com CL50 superior a 1 mg/mL (1,88 g/ mL; 1,94 g/mL e 17,78 g/mL para óleo essencial de *Thymus zygis zygis*, óleo essencial *Thymus capitatus*, e para o pólen de abelha, respectivamente).

Assim sendo, os resultados obtidos sugerem que, as formulações cosméticas preparadas através da incorporação do pólen de abelha e óleos essenciais, apresentam características adequadas para uso tópico. Além disso, a incorporação dos produtos naturais não pareceu influenciar, negativamente, a estabilidade das formulações cosméticas.

Durante o presente trabalho, observou-se consistentemente uma contaminação por fungos das formulações cosméticas à base de pólen de abelha. Foram utilizadas diferentes metodologias, como práticas de assepsia, liofilização e tratamento térmico (pasteurização) para ultrapassa-la. No entanto, apenas aumentando a concentração de conservantes, foi registaada uma diminuição no crescimento de fungos.

# CHAPTER 1. INTRODUCTION

## 1. Valorization of natural products

*"When all the trees have been cut down, when all the animals have been hunted, when all the waters are polluted, when all the air is unsafe to breathe, only then will you discover you cannot eat money".*

*Indian quote* (http://www.coolnsmart.com/nature_quotes/).

Nature is not just a beautiful landscape to relax but rather it is a source of life for humans, furthermore, "the heart of the environment". Though silent, provides an everlasting, priceless service to the world. It gives everything that humans need to survive and to thrive for free of charge everyday and everywhere, such as the air we breathe, the food we eat and the water we drink, wildlife and tranquil surroundings, it provides man with wood, stone or earth to build his shelters and wide range of useful products as food, drugs, beauty products. (https://news.mongabay.com/2011/04/what-does-nature-give-us-a-special-earth-day-article/).

Plants do not only fulfill our necessities such as food and medicine, but, more important than this, plants act like "lungs" by controlling the oxygen and carbon dioxide balance in the nature contributing to fresh air. Moreover, plants play an essential role in the maintaining of biodiversity and sustainability to ensure stable environment through their reproduction.

Bees participate in this process by pollinating flowers. The flowers, which give beauty to the forest, also provide food to bees and this fascinating little insect offers us some valuable natural products foods with health-promoting benefits (Ediriweera & Premarathna, 2012).

## 2. Basis of cosmetics

The concept of beauty and cosmetics is as old as mankind and civilization, since quite long time people took great care in their appearance and cleanliness. Thus cosmetic products have a history covering many thousands of years with the use of many ingredients from plants, animals, and mineral sources (Jain & Chaudhri, 2009; Okereke*et al*., 2015). The word cosmetics have been derived from the Greek word "COSMETIKOS" meaning the skill to

decorate, so cosmetics can be considered as the art of decorating yourself to look beautiful (Sumit*et al*., 2012).

Cosmetics are used not only for developing an attractive external appearance, but also to achieve longevity. Hence, Directive 76/768/ EEC defines cosmetic products "*any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips, and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odors"* (European Commission Enterprise Directorate-General Pharmaceuticals and cosmetics, 1999).

Cosmetics have become part of our daily routine and their use has increased significantly in recent years. Hence, the cosmetic market greatly extended offering a wide range of products for millions of consumers worldwide.

### *2.1.Classification of cosmetic products*

Cosmetics can be classified according to their use and area of application into skin care cosmetics, makeup cosmetics, body cosmetics, hair care cosmetics, oral cosmetics and fragrances as described in **Table 1**.

Cosmetics comprise an extremely diversified set of products, including creams, powders, perfumes, lotions, washing products, and the wide sector of decorative cosmetics or makeup (**Table 1**).

**Table 1:** Broad categorization of cosmetics (Source: Ota & Yokoyama, 2010)

| **Classification** | | **Usage** | **Main products** |
|---|---|---|---|
| For skin | Skin care cosmetics | Cleansers | Face cleansing creams and foams |
| | | Conditioners | Lotions, packs, massage creams |
| | | Protectors | Milky lotions, moisture creams |
| | Makeup cosmetics | Base makeup | Foundations, face powders |
| | | Point makeup | Lipstick, blushers, eye shadow, eye liners |
| | | Nail care | Nail enamels, nail polish removers |
| | Body cosmetics | Bath | Soaps, liquid cleansers, bath preparations |
| | | Sun cares and suntans | Sunscreen creams, sun oils |
| | | Antiperspirants an deodorants | Deodorant sprays |
| | | Bleaching, depilatory | Bleaching creams, depilatory creams |
| | | Insect repellents | Insect repellent lotions and sprays |
| For hair and scalp | Hair care cosmetics | Cleansing | Shampoos |
| | | Treatments | Rinses, hair treatments |
| | Scalp care cosmetic | Hair styling | Hair mousses, hair liquids, pomades |

| | | | |
|---|---|---|---|
| | | Permanent waves | Permanent wave lotions |
| | | Hair colors and bleaches | Hair colors, hair bleaches, color rinses |
| | | Hair growth promoters | Promoters Hair growth, hair tonics |
| | | Treatments | Scalp treatments |
| Oral | Oral care cosmetic | Toothpastes | Toothpastes |
| | | Mouthwashes | Mouthwashes |
| | Fragrances | Fragrance | Perfumes |

### *2.2.Cosmetic raw materials*

The ingredients used in cosmetic products depend greatly on the type of formulation (cream, gel, emulsion, lotion, etc). However, the principal raw materials used to manufacture cosmetics are water, oily materials, such as oils, fats, and wax, surfactants, humectants, thickening agents, antioxidants, preservatives, coloring agents, such as dyes and pigments, along with vitamins, pharmaceutical agents such as plant extracts and fragrances (Mitsui, 1997)

#### 2.2.1. Oily materials

Oils (fatty acids) are widely used as a component of cosmetics due to their ability to dissolve fats as well as to control the evaporation of moisture from the skin. They are widely used in preparation of creams, lotions, brilliantine, hair oil, and lipsticks (Mitsui, 1997).

#### 2.2.2. Humectant

The skin is daily exposed to external aggressions, such as cold, heat, wind, and pollution, causing loss of moisture, which leads to dehydrated skin and subsequently to precocious skin aging. Thus, humectants play an important role in cosmetics by preventing loss of moisture, improving skin hydration, and thereby retaining the skin's natural moisture (Pandey & Wasule, 2017). These substances are used in wide range of products including shampoo, conditioner, lotions, creams, lip treatments, cleansers, after-sun lotion, and some soaps or body lotion. A wide variety of humectants are used in cosmetics including polyhydric alcohols like glycerin, propylene glycol, and sorbitol (Mitsui, 1997).

#### 2.2.3. Thickening agents

Thickening agents are used to adjust the viscosity of products, to make them easy to use, as well as to maintain the product stability. For instance, they are used to ensure the stability of

milky lotions and liquid formulations, by preventing the separation of emulsified particles and powders (Mitsui, 1997)

### 2.2.4. Preservatives

Preservatives are considered one of the most important ingredients that should be present in all type of cosmetic formulations. These chemical compounds are used to preserve the formulation preventing bacterial growth then prevent product spoilage and subsequently consumers' infections caused by harmful microorganisms (Kerdudo *et al*., 2016a). The esters of *p*-hydroxybenzoic acid, also known as parabens, are the group of antimicrobial preservative most widely used in cosmetic products (Garner *et al*., 2014). Whereas previous studies reported that parabens could pose serious risks in particular to human health. Therefore, there is a growing demand for cosmetics that are preservatives free. Alternative way to substitute paraben and solve the problem of microbial purity of cosmetics is the use of natural compounds either plant extract or essential oils (Herman *et al*., 2013; Kerdudo *et al*., 2016) which support our present work.

### 2.2.5. Surfactants

Surfactants are amphiphilic compounds containing hydrophobic and hydrophilic moieties which reduce the surface tension between two liquids of different polarity (or between a liquid and a solid), and may act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants (Lourith & Kanlayavattanakul, 2009).

There is a wide range of surfactants either synthetic divided into anionic, cationic, amphoteric, and non-ionic surfactants or derived from natural products classified into lipopeptides, phospholipids (lecithin), fatty acids and polymeric compounds (Lourith & Kanlayavattanakul, 2009; Mitsui, 1997).

### 2.2.6. Colorant agent

Color plays an important role in consumer preferences towards cosmetic products. Coloring agents are added in order to color the product itself or to color the skin, nails, hair, and eyelashes for decorative purposes (Azwanida *et al*., 2015).

The ingredients used in cosmetics can be synthetic or naturally occurring chemicals. However, many synthetic ingredients once believed safe are no longer permitted in cosmetics.

#### 2.2.7. Antioxidants

Skin is exposed daily to external factors (temperature, UV radiations, air pollution) and internal factors (stress) that induces a photooxidative reaction that weakens the antioxidant defense system and increases the reactive oxygen species (ROS) at the cellular level reducing the skin ability to protect. As result, damage occurs in the skin tissue therefore its premature aging (Altuntaş & Yener, 2015). For that, antioxidants molecules are added to cosmetic formulation to prevent and repair skin damage caused by oxidative stress by scavenging ROS species also antioxidants are added to cosmetic product to protect ingredients from oxidation. Enzymes such as catalase and vitamins including vitamin C and E can be used as antioxidants (Pham-Huy, He, & Pham-Huy, 2008). Nowadays, there is an increase on using natural components due to their high antioxidant potential.

### *2.3.Safety*

The cosmetic products should fulfill 3 crucial requisites: quality, effectiveness and safety for consumer satisfaction. A cosmetic product intended for the consumer must be safe for human health and should comply with the relevant cosmetic legislation "Directive 76/768/EEC (Art. 2) which requires that *"cosmetic product put on the market within the Community must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use."*

Natural products are incorporated in cosmetic formulations due to their richness in bioactive compounds and their use is increased in recent years. Hence, experimental screening of their toxicity is crucial to guarantee the safety of the consumers, since some plant extracts, or their Essential oils, may be toxic, or cause allergies, and must be assayed regarding their toxicity. Several tests are used to evaluate safety of cosmetic formulations, including, skin sensitization, repeated dose toxicity, mutagenicity /genotoxicity/ carcinogenicity, reproductive toxicity, toxicokinetics, photo-induced toxicity, acute toxicity, skin/eye irritation which are frequently used for evaluation of cosmetics toxicity (Pauwels & Rogiers, 2010).

In this present work, the safety of our formulations was evaluated through the following tests:

#### 2.3.1. Acute toxicity

The acute toxicity of raw materials is evaluated by standard technique using an invertebrate animal namely brine shrimp *Artemia salina* which is a marine microcrustacean that live in saline environments. Their eggs (actually cysts), which can be inexpensively purchased from pet stores, hatch quickly and the larvae, termed a *nauplius* (plural, *nauplii*) are sensitive to small doses of biologically active chemicals, frequently used in bioassays to evaluate cytotoxicity of chemicals and natural compounds (Arcanjo *et al*., 2012).

Brine shrimp lethality assays are extensively used in research and applied toxicology that screen a large number of extracts for drug discovery in medicinal plants (Rajabi *et al*., 2015).

Brine shrimp assay has number of advantages such as, experimental simplicity, sensitivity, reproducibility, ease of handing, lack of continuous culturing, short exposure time and lower costs (Mclaughlin *et al*., 1998; Arcanjo et al., 2012).

### 2.3.2. Ocular irritancy

The evaluation of irritation properties of chemicals and consumer products that might come into contact with human eyes is required in order to guarantee the product safety (Steiling *et al*., 1999). For that, hen's egg-test on the chorioallantoic membrane is used to evaluate the potential ocular irritancy of a substance, measured by its ability to induce toxicity in the chorioallantoic membrane of a chicken egg. It is one of the most robust and successful assays for the evaluation of the local compatibility of raw materials as well as the final products (Steiling *et al*., 1999).

## 3. Incorporation of natural products in cosmetics

Since immemorial time various civilizations have made extensive use of plants and bee products such as honey, pollen as traditional medicine, spices, home-remedies, health foods to preserve general good health and hygiene, also as cosmetic products to maintain and enhance human beauty and youthful look (Kaur *et al*., 2013; Mizrahi, 1997; Shivanand, Nilam, & Viral, 2010). Women have long used herbs such as Sandalwood and Turmeric for skin care, Henna to color the hair, honey, rose water, and natural oils to perfume their bodies.

Recently, the use of herbs and hive products has increased through many scientific studies that have been performed, in order to obtain novel bioingredients with high safety, nutritive and medical value, which interest pharmaceutical and biotechnology companies. This has led

to an increased effort from these companies in obtaining bioactive compounds from natural products through extraction and subsequent purification (Devappa, Rakshit, & Dekker, 2015).

### *3.1.Natural products versus synthetic products*

Nowadays, the demand of natural products from diverse sources is increasing rapidly due to their safety: lack of side effect, free from all the harmful synthetic compounds which may cause health hazard (Joshi LS & HA, 2015; Kumar*et al*., 2016) as well as they synthesize an amazingly vast set of bioactive compounds that have numerous specific pharmacological and technological values, such as natural antioxidants, natural preservatives (Burlando *et al*., 2010). Moreover, they are hypoallergenic compared with synthetic products (Kumar *et al*., 2016), which have been demonstrate that may cause serious skin problems besides being environmentally hostile (Chen, 2009). In fact, several chemical compounds widely used in cosmetic including thickeners agents such as polyethyleneglycols (PEG´s), phthalates, mineral oils and parabens have been found to be toxic, causing several side effects such as allergies and irritations that can lead to numerous diseases, as well as cancer (Okereke *et al*., 2015). Furthermore, it seems that cosmetic chemicals such as UV screens, musk fragrances are relevant environmental contaminants that can disturb our eco-system (Schlumpf *et al*., 2001)

Several studies have also showed that some ingredients used in cosmetics may increase breast cancer risk, acting as endocrine disrupting chemicals (EDCs), which mimic estrogens. Parabens, which are used worldwide as preservatives in shampoos, face creams, and body lotions, are known EDCs and have been linked to breast cancer. Parabens, which are absorbed through the skin without being degraded, have an estrogen-like structure leading to a potential increase in female breast cancer incidence (Garner *et al*., 2014; Okereke *et al*., 2015; Prusakiewicz *et al*., 2007). In the literature, it is possible to find many examples of synthetic compounds that can present adverse effects to human health. For instance, triclosan, which can be used in soaps, deodorants, and toothpastes as a preservative is reported as an agent that can interfere with muscle function, besides altering hormone regulation having oestrogenic and androgenic activity acting therefore as EDCs (Gee *et al*., 2008; Wang & Tian, 2015) as well as synthetic musks, such as galaxolide and tonalide, which are used as fragrances in perfumes, cosmetics and after shave, have been also reported as EDCs besides their pollutant effect (Taylor, Weisskopf, & Shine, 2014). Also, sunscreen agents acting as UV filters such as benzophenones are reported as irritant, allergic, phototoxic or photo-allergic agents (Okereke *et al*., 2015; Schlumpf *et al*., 2001).

Following the controversies about synthetic compounds, the European Commission has completely banned the use of certain compounds, and limited the use of others listed in Annex II and III of regulation (EC) (European Commission, 2009). Indeed, the European Commission banned some fragrance ingredients, including several synthetic nitro musk due to their toxicity, tendency of bioaccumulation, and environmental impact (Walters, Santillo & Johnston, 2005). In 2012, the Committee for Risk Assessment (RAS) banned the use of some fragrances, including phthalates in cosmetics and children's toys, following their classification as reproductive toxicants (SCCP, 2007). In 2014, the European Commission limited the permissible concentrations of butyl and propylparabens in most cosmetic products to 0.19 %, and imposed their ban in nappy creams. Whereas, the SCCS, 2013 confirmed that methylparaben and ethylparaben are safe at the maximum authorized concentrations (0.4 % for single paraben and 0.8 % for mixtures of parabens). Also, the use of formaldehyde, which is used as a nail hardener, has been restricted to a maximum concentration of 5.0% (SCCS, 2014).

Therefore, natural products, including bee products and extracts from plants, are becoming particularly appreciated by consumers as an alternative solution for synthetic chemical compounds. Moreover, natural products are gaining increased popularity as cosmetic ingredients due to the various roles they can play in a single cosmetic formula, acting as moisturizers, fragrances, surfactants, and preservatives (Kerdudo *et al*., 2016), which is creating a novel trend in biotechnological industries, particularly in cosmetic industries. Cosmetics with incorporated herbal and/or hive products are a fast growing segment with a wide range of formulations produced in the last years.

### ***3.2.Properties of bee pollen and thyme oil incorporated in cosmetic formulation***

#### **3.2.1. Bee pollen**

Bees are a symbol of laborious activity, with socially organized beehives, that are very hard working and always seem to have endless energy. They dedicate their whole life to the manufacture and storage of natural healthy products, such as bee pollen, royal jelly, honey, among others. Their complex composition and richness in bioactive compounds are at the origin of many interesting properties that make them very useful as food, medicine, and cosmetic ingredients since ancient time. Moreover, their use has grown intensively in recent years thanks to many scientific studies that determined their complex composition and

highlighted their functional and biological properties, including antibacterial, antifungal, and antiviral actions, antioxidant and anti-inflammatory properties among others (Bogdanov, 2011).

Bee pollen (BP) is often referred to as nature's most complete food, being consumed by humans since ancient times, go backs to thousands of years, it has been mentioned for its medicinal and health-promoting properties. Historically, the consumption of BP was revered in the Bible, Genesis. It formed a diet of ancient notable Chinese and Egyptian populations. Ancient medical texts from Greece and Rome mentioned that Aristotle, Hippocrates, Pythagoras, or Pliny the Elder respected pollen as part of a healthful diet. Information of sedative pollen properties and its beneficial effect on gastric and cardiovascular disorders come from the middle Ages. Pollen has been produced for use in folk medicine around the world (Denisowa, B & Denisow-Pietrzyk, M, 2016).

In new times, BP began to be used for human nutrition only after the World War II, when pollen traps were developed (Bogdanov, 2016; Denisowa, B & Denisow-Pietrzyk, M, 2016).

#### 3.2.1.1.Definition of BP

Pollen is a fine, powder-like material produced by flowering plants and gathered by bees containing the male gametophyte, packed by worker honeybees into granules with added salivary or nectar (LeBlanc *et al*., 2009) and placed in specific baskets (corbiculae), which are situated on the tibia of their hind legs (**Figure 1**). Subsequently, these pollen loads are transferred back to the hive, mixed with saliva, fragmented by flightless bees, and packed in honeycomb cells. Next, the surface of the collected pollen is covered with a thin layer of honey and wax and a part of bee pollen is transformed by fermentation into bee bread, used as food for young bees (Graikou *et al*. 2011; LeBlanc *et al*. 2009; Komosinska-Vassev *et al*. 2015).

**Figure1:** Worker bee carrying pollen in her pollen basket.
(Source : https://honeybeesuite.com/the-role-of-pollen-in-honey-bee-nutrition/).

### 3.2.1.2.Morphological characteristics of bee pollen

The bee pollen occurs in the anthers of seed plants in the form of 2.5–250 $\mu$m grains. The pollen grain (**Figure 2**) is surrounded by a double-layered cell wall composed by an internal thin delicate wall of unaltered cellulose called intine, and a tough resistant outer cuticularized wall composed largely of sporopollenin called exine. Furthermore, numerous pores and furrows, as well as a layer of balsam, can be found in its surface, making easier the gluing of pollen to bees abdomens.
(Komosinska-Vassev *et al*., 2015; https://fr.wikipedia.org/wiki/Pollen).

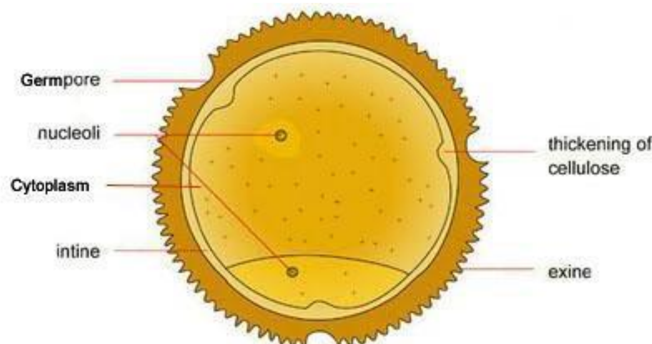



**Figure 2:** Pollen grain structure.

(Source: https://www.studyblue.com/notes/note/n/lecture-5-biology-ofpollen/deck/13973131)

Depending on the plant species, pollen grains differ in shape, which can be round, cylindrical, bell-shaped, triangular, or thorny (**Figure 3**). Also, the grains of pollen can exhibit different colors (**Figure** 4), ranging from bright yellow to black (Komosinska-Vassev *et al*.,

2015; Shubharani, Roopa, P., & Sivaram, 2013). The different colors are due to the presence of different pigments, including flavonoids and carotenoids (Mărgăoan *et al*., 2010). The majority of pollens consist of single grains that are sometimes joined with two or more grains, presenting weights that can be equal to a dozen or several dozens of micrograms.

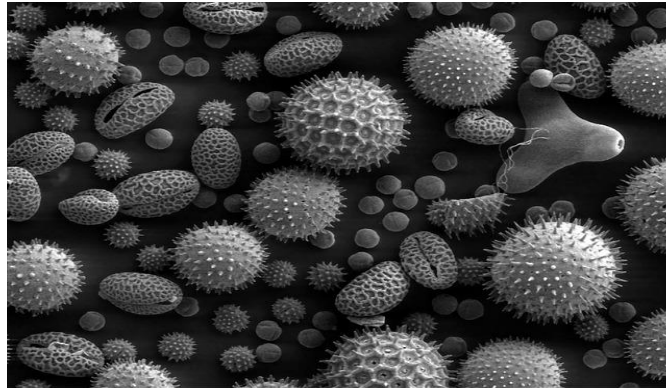


**Figure 3:** Pollen grains presenting different shapes.

(Source: http://honeypedia.info/what-is-pollen-what-role-plays-palynology)

**Figure 4:** Different colored pollen grains collected by honeybees.

(Photo courtesy of F. Intoppa)

### 3.2.1.3.Chemical composition

Pollen is a plant product with a complex chemical composition, rich in biologically active substances, and that is strongly influenced by, the plant species visited by the bees, environmental and storage conditions (Komosinska-Vassev *et al*., 2015; Regina da Silva *et al*., 2014).

The main components of bee pollen are carbohydrates and proteins, in addition to lipids and fibers, present in smaller proportion as mentioned in **Table 2**.

Carbohydrates may comprise starch, fructose, glucose, and sucrose. Several essential amino acids can be found in pollen such as methionine, lysine, threonine, histidine, among others. Moreover, lipids including phospholipids, and essential free fatty acids, such as linoleic, $\gamma$-linolenic and archaic (hydrosulfurous acid), are also present in pollen (Komosinska-Vassev *et al*., 2015; Regina da Silva *et al*., 2014).

**Table 2:** Main components of bee pollen (source: Feas et al., 2012).

| MainComponents | Content (g/100g dry weight) |
|---|---|
| Proteins | 10-40 |
| Lipids | 1-10 |
| Carbohydrates | 13-55 |
| Dietary fiber | 0,3-20 |

Amongst the minor components there are minerals, and vitamins (**Table 3**). Pollen contains significant amounts of mineral elements, such as potassium, calcium, magnesium, and phosphorous., besides being an excellent source of hydro-soluble vitamins, such as vitamin C and complex B vitamins B, and liposoluble vitamins, including vitamin E and β-carotene (Regina da Silva *et al*., 2014). Overall, the chemical composition of pollen makes it a food product with an interesting nutritive value.

**Table 3:** Minor components of bee pollen ( Bogdanov, 2012)

| Minerals | Content (mg/Kg dry weight) | Vitamins | Content (mg/Kg dry weight) |
|---|---|---|---|
| Potassium | 4000-20000 | Thiamin ($B_1$) | 6-13 |
| Magnesium | 200-3000 | β-Carotene | 10-200 |
| Calcium | 200-3000 | Niacin($B_3$) | 40-110 |
| Phosphorus | 800-6000 | Pantothenic acid ($B_5$) | 5-20 |
| Iron | 11-170 | Pyridoxin ($B_6$) | 2-7 |
| Zink | 30-250 | Ascorbic acid (C) | 70-560 |
| Copper | 2-16 | Biotin (H) | 0.5-0.7 |
| Manganese | 20-110 | Folic acid | 3-10 |
| | | Tochopherol (E) | 40-320 |

#### 3.2.1.4.Functional properties of BP

Besides the nutritional role of bee pollen, biological and therapeutic actions were also attributed to pollen, namely related to the flavonoids, which are polyphenolic compounds with an intrinsic capacity to reduce reactive oxygen species, making them compounds with important physiological and pharmacological activities (Campos *et al*., 2010). In fact, different studies attributed important properties to bee pollen, such as antimicrobial and antioxidant activities.

**a. Antimicrobial activity**

Several studies demonstrated significant antimicrobial activities of bee pollen attributed to phenolic acids and flavonoids. The mechanism of action seems to involve degradation of the cytoplasm membrane, which leads to loss of potassium ions and initiation of cell autolysis (Erkmen & Özcan, 2008; Kacániová *et al*., 2012; Morais*et al*., 2011). Kacániová *et al*., 2012 studied the antimicrobial activity of bee pollen and found that bee pollen extracts were active against different Gram-positive and Gram-negative pathogenic bacteria, including *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli*, and also against microscopic fungi, such as *Aspergillus, Candida* species, and other yeasts.

**b. Antioxidant activity of BP**

Bee pollen appears to provide a strong antioxidant activity mediated by phenolic as flavonoids, especially against reactive oxygen species mainly $H_2O_2$and $O_2-$, although its effects were only one-tenth as powerful as those of propolis (Nakajima *et al*., 2009), which is another hive product that is traditionally used as an anti-aging food. The antioxidant activity of BP has been expressed as free radical scavenging activity and as lipid peroxidation inhibition (Carpes *et al*., 2013; Čeksteryte *et al*., 2016). Therefore, the wealth of bee pollen in flavonoids and vitamins allowed its use as antioxidant substance in cosmetic products which support our work.

#### 3.2.1.5.Field of application

- Health use

Due to some of the properties previously reported in this document, BP widely consumed and commercially available as a dietary supplement:

- ✓ nutrient richness of BP sustains and enhances quality performance, which explains its use by athletes as a dietary supplement;
- ✓ BP is used as a natural medicine against many diseases, among which metabolic troubles, arterial problems, neurovegetative illness, prostate problems, and women's climacterial troubles during the menopause (Bogdanov, 2016).
- ✓ dry pollen, loads or pollen in the form of capsules or pills are also taken as a remedy for hay fever, asthma and colds (Mărgăoan *et* al., 2010).

- ➢ Cosmetic use

The presence of high levels of antioxidants and vitamins makes BP very used, in a range of natural supplements and skincare products, as an antibiotic, anti-itch, and anti-inflammatory agent. It has been reported BP not only prevents skin-aging and stimulates growth of new skin tissue, but also repairs damaged skin cells and smoothes wrinkles away, thus skin regeneration (Anita Bénech, 2006; Tsutsumi & Oishi, 2010). Therefore, pollen extract is highly recommendable to formulate cosmetic products with moisturizing, smoothing effects and protection of skin against oxidative processes. Also BP is used in cosmetic formulations as pigment agent.

Depending on its bioactive compound, BP can be applied in cosmetic as follows (**Table 4**).

**Table 4:** Main application of bee pollen in cosmetic.

(Source: http://www.centerchem.com/Products/DownloadFile.aspx?FileID=6982)

| Action | Active compound | Cosmetic application |
|---|---|---|
| Skin conditioning | Aminoacids/ Proteins | Moisturizing Soothing |
| Skin regeneration | Aminoacids/ Proteins | Stimulation of cell metabolism Epithelizing |
| Antioxidant | Flavonoids Vitamins | Anti-ageing |
| Vitamin and mineral Replenishing | Vitamins Minerals | Revitalizing |
| Antimicrobial | Carbohydrates Enzymes | Purifying Antiseptic |

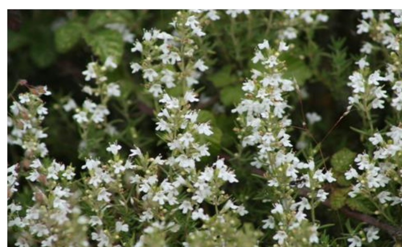
#### 3.2.1.6.Side effects

Bee-pollen is normally well tolerated, but the presence of allergens cannot be excluded. Pollen allergy like hayfever, concerns mainly allergy against airborne pollen, while allergies to ingested pollen are relatively rare (Campos *et al*., 2010). Some studies have reported acute allergic reactions to ingested pollen, including anaphylaxis, but these reaction are mainly attributed to the presence of airborne pollen in the ingested bee pollen (Choi *et al*., 2015; Jagdis & Sussman, 2012). Nevertheless, patients with pollen allergies are at risk for serious allergic reactions including itching, swelling, and shortness of breath (Ulbricht *et al*., 2009). Thus, it is recommended that people who are susceptible to allergies or asthma should avoid bee pollen.

### 3.2.2. Thyme essential oil

Thyme plant synthesizes volatile compounds as secondary metabolites, playing defense role in some part of a plant (leaves, flowers, buds, rods) known as essential oils which can present distinct odors and flavors that are governed by the types and amount of chemical constituents present in the oil. They are characterized by strong characteristic odors, rarely colored, and present lower density than that of water (Miguel, 2010), besides, Thyme essential oils exhibit several biological activities, namely antibacterial, antifungal, antiviral, and antioxidant activities (Hazzit *et al*., 2009; Mkaddem *et al*., *2010*; Stahl-Biskup, 1991; Elisabeth Stahl-Biskup & Sáez, 2002).

Two different essential oil chemotypes were studied in this work from different thyme species (**Figure5**) namely the subspecies *Thymus zygis zygis* which is collected from Rebordãos, Bragança, Portugal and the species *Thymus capitatus* that grows in Mediterranean territory commonly named ‘‘Zaâtr’’, located in Tunisia.



*Thyus zygis subp zygis*

*Thymus capitatus*

**Figure 5:** two different Thyme species used in the present work

### 3.2.2.1.Chemical composition of Thyme oil

The essential oils have a complex composition, containing several volatiles, such as terpenes (monoterpenes and sesquiterpenes), aldehydes, alcohols, phenols, and terpenoids (Tongnuanchan & Benjakul, 2014). The essential oil chemical composition varies depending on the species and chemotype considered and is influenced by environmental conditions (El Abed *et al.*, 2014). The genus *Thymus* comprises different chemotypes (refers to chemical variation in the same species), depending on the major component of the essential oil being thymol, carvacrol, linalool, geraniol, thujanol, α-terpineol, borneol, or p-cymene cited in **Figure 6**.

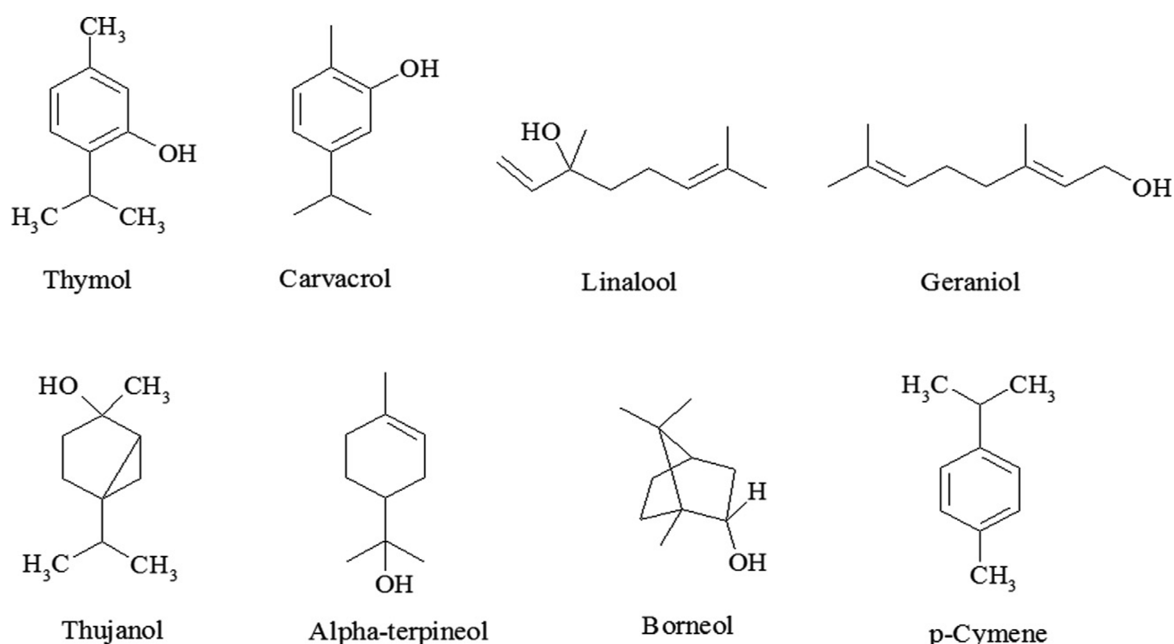



**Figure 6:** Chemical structure of the main chemical compounds occurring in genus *Thymus* plants (Nabavi *et al.*, 2015).

Thymol (39.6%) and p-cymene (21.2%) have been reported as the major chemical compounds present in the essential oil of *T. zygis* (Pina-Vaz et al., 2004) while carvacrol was reported as the main component present in *T. capitatus* essential oils (Kreck *et al*. 2002; Nabavi *et al*., 2015).

### 3.2.2.2.Biological activities

As stated in the previous section, carvacrol, thymol, *p*-cymene, and α-terpineol have been reported to be present in the essential oils extracted from plants of the *Thymus* genus. Several

studies have described a variety of biological activities associated with the presence of these compounds (El Abed *et al*., 2014; Mkaddem *et al*., 2010). However, the biological activities of an essential oil are not mediated only by the main components, but by all the compounds present in the oil, which interact with each other, producing a synergetic effect (Bounatirou *et al*., 2007; Mkaddem *et al*., 2010).

**a. Antibacterial activity**

Thyme oil was reported to possess important antimicrobial activityattributed to the main compounds of *Thymus* genus, namely thymol, carvacrol, γ-terpinene, and *p*-cymene. Literature data has reported that *Thymus capitatus* essential oil was active against skin microorganisms, such as *Staphylococcus epidermidis* and *Propionibacterium acnes* (Nabavi *et al*., 2015).This activity was attributed to the high amount of carvacrol, which has been considered biocidal, acting in synergy with its precurs or p-cymene and causing destabilization of the bacterial membrane (Ultee, Bennik, & Moezelaar, 2002).

**b. Antifungal activity**

Thyme essential oils or some of their constituents are indeed effective against a large variety of fungi, particularly the oils with high amounts of thymol and/or carvacrol, such as those from *T. zygis* subsp *zygis* (ct. thymol), and *T. capitatus* (ct. carvacrol). The essential oil of *T. zygis zygis* have been reported to possess a potent fungicidal activity against *Candida albicans*, resulting from direct damaging of the cytoplasmic membrane, attributed to synergistic antifungal effect between thymol and p-cymene, which is the precursor of carvacrol (Pina-Vaz *et al*., 2004).

**c. Antioxidant activity**

Several studies have demonstrated the great antioxidant potential of several Thymus species oils, attributed to phenolic compounds (Faleiro *et al*., 2005; Miguel *et al*., 2004), either by preventing lipid peroxidation or by scavenging free radicals (Bounatirou *et al*., 2007; Hazzit *et al*., 2006). The highest antioxidant ability has been revealed in the oils rich in carvacrol, thymol or *p*-cymene, including those from T. zygis subsp *zygis* (ct. thymol), which possess the best antioxidant capacity by preventing lipid peroxidation with $IC_{50}$ = 0.030 ± 0.003 mg/mL) (Dandlen *et al*., 2010), and those from *T. capitatus* (Mkaddem *et al*., 2010).

**3.2.2.3.Field of application**

Thyme oil is a natural mixture with high added value, used in many industries:

- ✓ Food industries, used as food flavors and preservative agents thanks to their antimicrobial and antioxidant properties, being actually ranked among the top 10 essential oils of the world also used as a food preservatives (Ehivet *et al*., 2011; Elisabeth Stahl-Biskup & Sáez, 2002);
- ✓ Pharmaceutical industries for their functional properties (as an active ingredient);
- ✓ Cosmetic industries. In fact, a large number of essential oils and their constituents are used in the production of the majority of perfumes and cosmetics as preservatives in order to prevent microbial spoilage and therefore to extend the shelf life of the products because of their antimicrobial activities while ensuring their pleasant odor. Thyme oil, generally, *T. vulgaris* oil, is often incorporated into hygiene and skin care products, such as soaps, toothpastes, shower gels, shampoos, deodorants and body lotions because of its purifying and tonic properties (Varvaresou *et al*., 2009). Regards to its powerful antioxidant properties, thyme extracts are also used in anti-aging care.

#### 3.2.2.4.Toxicity effect

EOs are highly recommended as promising candidates to replace synthetic preservatives, since they are considered as safe due to their natural origin. Despite the various advantages of using EOs as antimicrobial additives in the cosmetic industry, some of them such as thyme oil may be toxic and can show adverse reactions to skin such as irritation and allergic reactions (Dreger & Wielgus, 2013; Nathalie *et al*., 2006). Thus, they should be evaluated for their toxicity in order to ensure their safety in cosmetics.

Studies concerning the toxic effects of thyme oil on mammals presented an oral acute toxicity, expressed as the $LD_{50}$ value of 4.7 g/Kg rat. This was attributed to the presence of thymol and /or carvacrol, which also caused severe irritation of mouse and rabbit skin when exposed to undiluted thyme oil (Stahl-Biskup & Venskutonis, 2012).

Take into consideration those controversies; the main challenge is the use of optimal concentration for effective antimicrobial activity showing relatively low toxicity of essential oils at the same time.

As reported in previous sections, due to their safety and their richness in bioactive compounds which have the ability to calm or smooth the skin but also to restore actively, heal and protect the skin, natural products are promoting candidates to replace chemicals; in fact

their cosmetic use is expanded nowadays. However, phytocosmetics should be assessed for their stability since any change occurring in formulation cause unpleasant discoloration and odor, and can degrade active compounds in products, leading to the instability of finished product therefore affected product quality, efficacy and safety (Baby *et al*., 2007).

## 4. Cosmetics stability

The characteristics of cosmetic products can be affected by environmental factors, such as temperature, pH, light, air and humidity, which affect their stability contributing to severe damages on the constituents of the product (Baby *et al*., 2007), which can lead to phase separation, odor, color and viscosity changes, loss of activity and microbial growth. Thus, evaluation of cosmetics stability is necessary to ensure the quality, safety, and efficacy of products with potential for commercial success.

Because of the wide variety of cosmetic products and their inherent complexity, it is hard to find standard stability tests that can be applied to a vast range of products. However, the stability tests most widely used comprise assessment of:

- ✓ Stability and physical integrity of cosmetic products under appropriate conditions of storage, transport and use;
- ✓ Chemical stability;
- ✓ Microbiological stability;
- ✓ The compatibility between the product and packaging employed (Marx, 2004);

The physical stability of the product should be established in order to determine whether or not shipping movements may damage the cosmetic and/or its packaging causing coalescence of emulsions, phase separation, crystallization or precipitation of ingredients, color changes, occurring during transport, storage or handling of the product. The centrifuge and vibration tests are widely used for the evaluation of the physical stability of cosmetic products.

Both formulas and packaging exposed to light can be sensitive to the UV radiation which with the oxygen leads to the formation of free radicals and therefore oxidation of formulation. Therefore, it is important to evaluate the stability of formulation, towards light exposure, also this test give us an idea about the effectiveness of antioxidant substances added to the formulation (ANVISA, 2005).

Chemical stability assays, such as long-term and accelerated tests, are used to characteristics of cosmetic products, including color, odor, viscosity, and texture which may be affected by high or very low temperatures (ANVISA, 2005). Whereas, accelerated stability test recognized as appropriately predicting product shelf life in many industries (Marx,2004), therefore, help and guide the choice of formulations (ANVISA, 2005). The formulations under test are subjected to stress conditions aimed at accelerating the appearance of signs of possible instability. Generally the samples are submitted to heating in ovens, cooling in refrigerators and to alternated cooling and heating cycles (ANVISA, 2005). Whereas, Long-term stability test is aimed to validate the stability limits of the product and to test the expiry date estimated using the accelerated stability test (ANVISA, 2005).

Microbial contamination may occur during production and filling of cosmetic products or during their use by the consumer, causing undesired changes in the composition, odor, or color of the product, besides putting the consumers health at risk (Lundov *et al*., 2009). Therefore, microbiological stability is very important to ensure the microbial safety of cosmetics for the consumer, and the maintenance of the product quality. In addition, the microbiological assessment determines the adequacy of the choice of the preservative agent or whether the interactions between the compounds of the formulation may impair its effectiveness (ANVISA, 2005).

Additionally, stability testing should include packaging since it can directly affect the stability of the finished product, due to interactions that can occur between the product, the package, and the environment, namely adsorption of product constituents into the container (Marx, 2004).

## 5. Objectives

The main aim of this work was to formulate and subsequently evaluate the stability of an anti-aging gel based on natural products, namely bee pollen and essential oils from two varieties of Thymus, applied as natural preservatives: Portuguese species *Thymus zygis zygis* and Tunisian species *Thymus capitatus*.

In order to attain this objective, during this work several tasks were performed:

- ✓ Extraction of volatiles (essential oils) from 2 genus of Thymus (Portuguese variety *Thymus zygis zygis* and Tunisian variety *Thymus capitatus*);
- ✓ Identification and concentration of volatile compounds from the selected plants;
- ✓ Evaluation of accelerated stability using physicochemical assays;
- ✓ Assessment of the acute toxicity of the compounds incorporated (bee pollen, thyme oil) in the cosmetic formulation using the *Artemia salina* assay;
- ✓ Evaluation of the antimicrobial activity of cosmetic formulation and its microbial stability against various microorganisms (bacteria, yeast);
- ✓ Evaluation of ocular irritability using "HET-CAM" test;

# CHAPTER 2. MATERIALS AND METHODS

## 1. Plant materials

Fresh Thyme (*Thymus capitatus*) was collected in May 2017 at flowering stage from Hamada of Sidialouane Mahdia (center of Tunisia). Botanical identity of the samples was confirmed by Professor El Ayeb Naceur from the Higher Institute of Applied Sciences and Technology of Mahdia University of Monastir. After harvest, samples were dried in the dark at room temperature before being transferred to IPB biological laboratory Bragança, Portugal. Also, *T. zygis zygis* variety was collected from Rebordãos, Bragança, Portugal, at flowering stage, dry and used for extraction, of essential oil. A voucher of this species was kept in BRESA de Escola Superior Agrária de Bragança herbarium. The essential oils were subsequently incorporated in cosmetic formulation.

Commercial bee multiflora pollen from the region of Bragança was purchased then incorporated in cosmetic formulation in this study.

## 2. Standard reagents

The selected chemicals used in the present work are:

Methylcellulose (Pharmachemica), glycerin, almond oil, and methylparaben were purchased from CMD chemicals, dimethylsulfoxide (DMSO), sodium chloride (NaCl), sodium hydroxide (NaOH), magnesium sulfate ($MgSO_4$), calciumchloride ($CaCl_2$), potassium chloride (KCl), and sodium bicarbonate ($NaHCO_3$) were purchased from Merck. Magnesium chloride ($MgCl_2$) was obtained from Panreac, meat extract (Difco laboratories), agar (Prona agar), glucose (Hi media laboratories), yeast extract and peptone were purchased from Ultimed.

Two antibiotic discs were used in microbial stability assay which are Chloroamphemical C30 (Biolab) and Flucanosole F100 (Liofil chem).

## 3. Essential oils extraction

The essential oils (EOs) were extracted using a Clevenger-type apparatus (**Figure 7**), according to the method described in the European Pharmacopoeia (Dandlen *et al*., 2011).

Briefly, approximately 80-90 g of dried aerial parts (stems, leaves, and flowers), were cut and placed in 800 ml of ultra-pure water and subjected to hydrodistillation for 3 hours. EOs were stored in a amber vial at -20°C in the dark until analysis. The yield of EOs was determined, and expressed as ml of essential oil/g of dry matter.

**Figure 7**: Extraction of EO by Clevenger apparatus.

## 4. Chemical analyses of essential oils

*Thyme* oils were analyzed by gas chromatography (GC) and the components were identified by GC-MS (gas chromatography-mass spectrometry) and by comparing the retention times of GC peaks with those of standards (Dandlen *et al*., 2011)

### *4.1.Gas chromatography (GC)*

GC analyses were performed using a Perkin Elmer Autosystem XL (Perkin Elmer, Shelton, Connecticut, USA) gas chromatograph equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m x 0.25 mm i. d., film thickness 0.25 µm) (J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30m x 0.25mm i. d., film thickness 0.15 µm) (J & W Scientific Inc.). Oven temperature was programmed, 45-175°C, at 3°C/min, subsequently at 15°C/min up to 300°C,

and then held isothermal for 10 min; injector and detector temperatures, 280°C and 300°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using split sampling technique, ratio 1:50. The volume of injection was 0.2 µl of a pentane-oil solution. The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as mean values of two injections from each oil, without using correction factors.

### *4.2.Gas chromatography-mass spectrometry (GC-MS)*

The GC-MS unit consisted of a Perkin Elmer Autosystem XL (Perkin Elmer, Shelton, Connecticut, USA) gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm) (J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1, Perkin Elmer, Shelton, Connecticut, USA). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion trap temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 µA; scan range, 40-300 amu; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C9-C19n-alkane indices and GC-MS spectra from a homemade library, constructed based on the analyses of reference oils, laboratory synthesized components and commercial available standards.

## 5. Preparation of gel formulation

The gel formulations with incorporated natural products namely bee pollen and thyme oils (**Table 5**) were prepared according to the method described by Nasatto *et al.* (2015): methylcellulose powder was dispersed firstly in 60 ml of sterile hot ultra-pure water (~75°C), and then cooled down to approximately 5°C under continuous stirring to provide faster dissolution of the particles resulting in a homogeneous solution. Bee pollen was added to the gel, after previous dissolution in glycerin, and then adequate amount of almond oil was added to the cosmetic formulation. Finally, drops of thyme oil were added to the formulation and all the ingredients were mixed properly to form a homogenous gel. Various formulations were prepared using combinations of various percentages of methylparaben (0.02% and 0.4%), and thyme oil (0.02% and 0.1%). Additionally, a control formulation was prepared without bee pollen, thyme oil, and methylparaben. A total of 16 formulations (a code attributed to each formulation see in Annex I) were prepared and subsequently were evaluated for their stability and safety.

**Table 5:** Formula for gel preparation.

| Ingredients | Function | Percentage (w/w) |
|---|---|---|
| Methylcellulose | Gelling agent | 3 % |
| Glycerin | Humectant | 20 % |
| Almond oil | Emollient | 20 % |
| Bee pollen | Biological product/natural active substance | 1% |
| Methylparaben | Preservative | 0.02% / 0.4% |
| Thymeoils (*T. capitatus* and *T. zygis zygis*) | Preservative/fragrance | 0.02%/0.1% |
| Water | Solvent | Up to 100 ml |

## 6. Stability assays

Organoleptic characteristics of the cosmetic preparations, such as color, smell, texture, and consistency, were evaluated by visual inspection. Additionally, several physicochemical analysis were performed, such as centrifugation, mechanical vibration, and light tests, pH, density, and viscosity determination, spectrophotometric assays, besides accelerated and microbial stability tests.

### *6.1. Centrifugation test*

To perform the centrifugation test, 5 g of sample was subjected to a cycle of 3000 rpm for 30 minutes at room temperature. At the end of the centrifugation period, the cosmetic formulations were examined for phase separation which is an indication of cosmetic formulation instability.

### *6.2. Mechanical Vibration test*

This test evaluates the stability of the cosmetic formulation when submitted to mechanical vibration movement, which may cause instability detected as phase separation. Briefly, 5g of sample were subjected to vibration on a vortex shaker (Haidalph) for 10 seconds (**Figure 8**).

**Figure 8:** Vibration test with the vortex equipment.

### *6.3. Light test*

The cosmetic formulations were placed in transparent plastic containers and subsequently exposed to extreme light source for 15 days, using a daylight bulb with photoperiodicity system (16hours light and8 hours dark). At the end of the exposure period, the samples were examined for any changes in physical properties, such as appearance, clarity, or color, liquefaction. Any phase separation or change in color observed is considered as indicative of product instability.

### *6.4. pH determination*

The pH value of cosmetic formulations stored at different conditions was determined using a digital pH Meter (Mettler Toledo), as follows: 0.5 g of gel was dissolved in 50 mL ultra-pure water and stored for two hours. The measurement of pH (**Figure 9**) of each formulation was done in triplicate and average values were calculated.

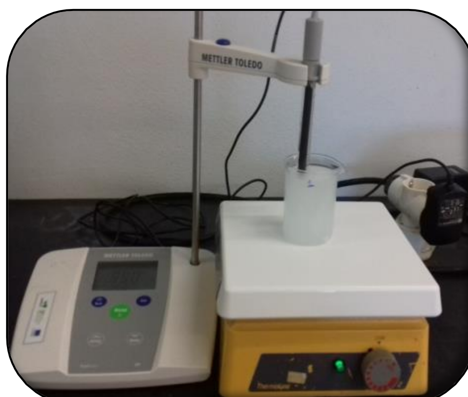



**Figure 9:** pH test with a potentiometer apparatus.

### *6.5. Spectrophotometric test*

The cosmetic formulations were diluted in ultra-pure water at ratio of 1/100 (m/v) and then submitted to scanning analysis by spectrophotometry in the UV-VIS region (210 nm - 600 nm), and its spectrum is compared to the reference spectrum corresponding to the control formulation. Variations in the intensity or wavelength of the absorption bands indicate alterations in the intensity of the color or even modification of the coloring material which is considered as formulation instability (ANVISA, 2005).

### *6.6. Accelerated Stability studies*

All the cosmetic formulations were subjected to accelerated stability testing for 2 weeks at a temperature of 40º ± 2°C and 25±2°C, using two relative humidity conditions: 75±5% RH and 60±5% RH respectively. After 8 days, the cosmetic formulations were inspected for their organoleptic characteristics (color, smell, phase separation, texture, and consistency), and their pH value was determined. This procedure was repeated at the end of the storage period.

### *6.7. Viscosity test*

The evaluation of the viscosity determine the adequate stability of formulation in terms of consistency and thus indicates the product behavior over a period of time (ANVISA, 2005).
The viscosity measurement of the gel (**Figure 10**) was performed at a controlled temperature of 25±2°C with a Viscometer (Mylr), using an adequate splindle (L4) with different rotation speeds (1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 rpm) and were done in triplicate.

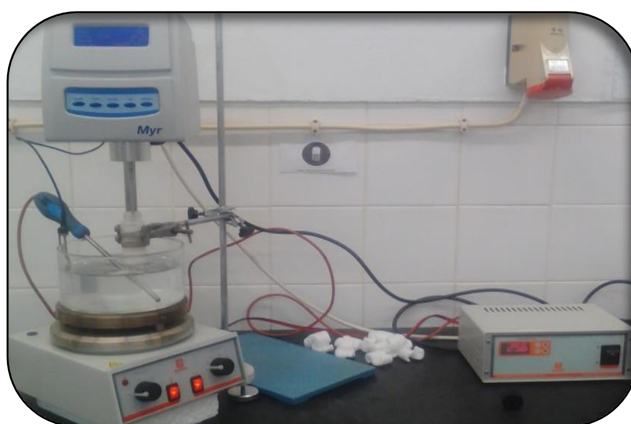



**Figure 10:** Viscosity measurement with the viscometer.

### *6.8. Density*

This is the ratio between the mass of a substance and the volume that it occupies. In the case of liquids or semi-solids this parameter can indicate the incorporation of air or the loss of volatile ingredients (ANVISA, 2005). For determining the (apparent) density of the cosmetic formulations, a graduated cylinder and a balance were used; the test was done in triplicate using 10 mL of each formulation then an average was calculated. The apparent density is related to the capacity of the recipient.

### *6.9. Microbial stability*

The microbial stability of the cosmetic formulations was evaluated through the microbial contamination test. After being prepared (**Table 6**), the culture media were autoclaved at 125°C for 20 minutes and then 20 mL of the culture medium was poured into a sterile petri dish. Then 0.2g of each formulation was placed in the center of each petri dish, and then the plates were incubated at 37°C or at 25°C for 3 days according to the inoculated microorganisms. After the incubation period, plates were taken out and checked for microbial growth, which is an indication of contamination.

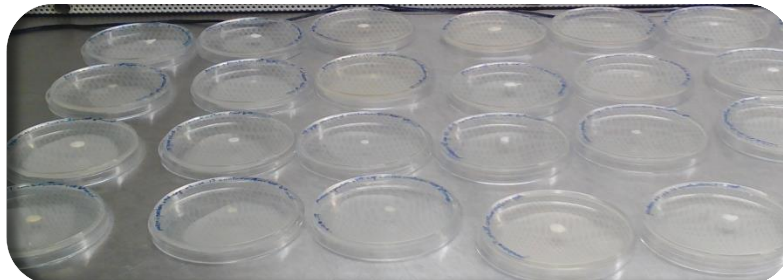


**Figure 11:** Formulations subjected to contamination test.

## 7. Antimicrobial activity

The antimicrobial activity of the cosmetic formulations was evaluated through agar diffusion assays using cultures of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Bacillus subtilis* and *Candida albicans.* Briefly, 100µL of sterile liquefied medium was inoculated with bacterial and yeast suspensions, placed in the plates and spreaded using an adequate spreader. After complete solidification of the liquefied inoculated medium, 0.2g of the cosmetic formulation was placed in each plate. Two different antibiotics: Chloramphenicol (disc C30) against bacteria, and Fluconazole (disc F100) against yeast were placed in the center of each plate, serving as a positive control.

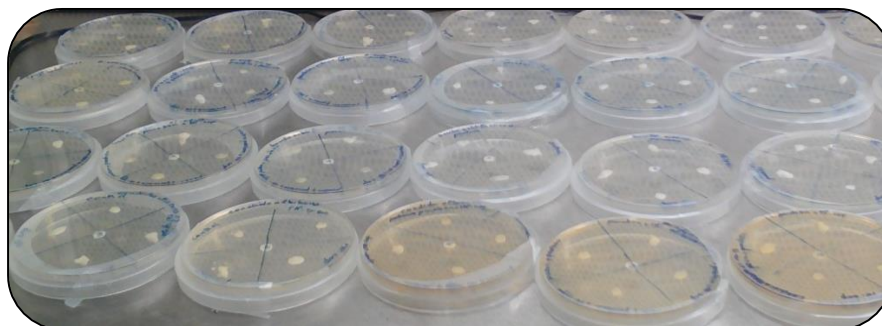


**Figure 12:** Formulations subjected to antimicrobial test.

The plates were incubated for 3 days at 37ºC, or at 25ºC, for bacteria, and fungi, respectively. After the incubation period, the zone of inhibition was measured, which allowed to observe if inhibition of microbial growth by the cosmetic formulations occurred.

**Table 6:** Culture medium of bacteria and yeast.

| Bacteria culture media | Quantity (g/L) | Yeast culture media | Quantity (g/L) |
|---|---|---|---|
| Meat extract | 3 | Glucose | 20 |
| Peptone | 5 | Yeast extract | 5 |
| Agar | 15 | Peptone | 10 |
| | | Agar | 20 |

200 mL of the same respective medium, but without agar, used for corresponding microorganism inoculation.

**8. *In vitro* citotoxicity assay using brine shrimp (*Artemia salina*)**

*Artemia salina* eggs were hatched in artificial seawater prepared (**Table 6**), as described by Sorgeloos *et al*. (1986). Brine shrimp eggs were added to artificial seawater in an Erlenmeyer and kept under constant aeration and light source after 48h incubation at room temperature (25-30°C), the larvae (*nauplii*) were attracted to one side of erlenmeyer using a light source and collected with a pipette.

**Table7:** Artificial seawater used in Artemia hatching.

| Medium composition | Amount (g/L) |
| --- | --- |
| NaCl | 5.0 |
| $MgSO_4$ | 1.3 |
| $MgCl_2$ | 1.0 |
| $CaCl_2$ | 0.3 |
| KCl | 0.2 |
| $NaHCO_3$ | 2.0 |

The bioactivity of bee pollen and thyme oil (*T. capitatus* oil and *T. zygis zygis* oil) was monitored by the brine shrimp lethality assay to predict the presence of cytotoxic activity in the compound. Therefore, the samples were dissolved in 1.6 mL of DMSO and diluted with artificial see water.

A series of concentration of bee pollen (0.5%, 1%, 1.5%, 2%) and EOs (0.01%, 0.02%, 0.05%, 0.1%) were prepared and the final volume was adjusted to 40 ml by artificial sea water prepared before. Afterwards, 10 mL of each concentration was poured into 4 wells of microplate, and then 8 *nauplii* were selected and transferred into each sample well. After 24 hours, microplates were then examined under a magnifying glass and the number of survivors and dead *nauplii* were checked and counted in each well. Larvae were considered dead if they did not exhibit any external movement during several seconds of observation. Experiments were conducted with 2 controls (Artificial sea water and DMSO in seawater) and different concentrations of the test substances in a set of 4 wells per dose.

The percentage of mortality (M %) was calculated using the following formula proposed bypprevious studies (Naidu, Ismail, & Sasidharan, 2014; Otang *et al*., 2013):

M% = Percentage of survival in the blank control - percentage of survival in the treatment or positive control.

The concentration that caused 50% lethality to the *nauplii* ($LC_{50}$) was determined from the best-fit line obtained by linear regression analysis of the percentage lethality *versus* the concentration (Otang*et al*., 2013)

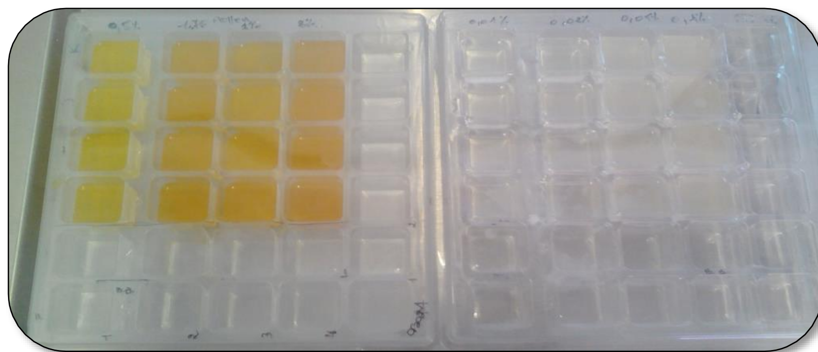


**Figure 13:** Microplates used for Toxicity test.

## 9. Evaluation of ocular irritancy using HET-CAM test

The HET-CAM was made according to the procedure used by Steiling *et al* (1999) and Barile (2010) that is described herein. Fresh and fertilized eggs were incubated during 9 days in an automatic rotating device, at 37°C±1°C and 62.5% of relative humidity. On the 10th day, each egg was tested with candling light to ensure that all were viable. The eggs that did not showed an emergent embryonic vascular system, which normally develops during embryogenesis, were discarded. Therefore, after selecting the viable eggs, the air chamber of the egg was located by exposing the eggs to light and a portion of the shell was broken, and the inner membrane was removed without injuring blood vessels, using tapered forceps. Subsequently, 0.3 g of the cosmetic formulation was applied directly onto CAM of each egg and alterations of the membrane and of its blood vessel network were observed over a period of 0.5 seconds, 1 minute and 5 minutes. The tests were done in duplicate, for 7 formulations (G2: gel contains only *T. zygis zygis* essential oil, G3: gel contains only *T. capitatus* essential oil, G4: gel contains a mixture of essential oil, G5: gel contains only pollen, G6: gels contains pollen and *T. zygis zygis* essential oil and G6: gel contains bee pollen and *T. capitatus* essential oil) and two controls were used: 0.9% NaCl and 10% NaOH as negative and positive controls, respectively.

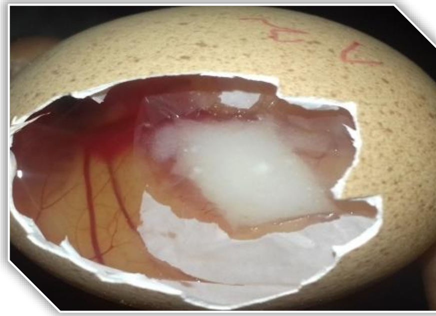


**Figure 14:** Gel applied into the surface of CAM.

These are the alterations that might occur:

- ✓ Hemorrhage (bleeding from the vessels).
- ✓ Vascular lysis (Blood vessel disintegration).
- ✓ Coagulation (intra and/or extra-vascular protein denaturation).

# CHAPTER3. RESULTS AND DISCUSSION

## 1. Essential oil yields and chemical composition

The yields of essential oils of *Thymus capitatus* and *Thymus zygis zygis*, based on the dry weight of the plants, were as follows: 2.06 % and 1.14 %, respectively. The great yield obtained for *T. capitatus* was similar to the maximum yield obtained at flowering stage ranging from 1.08 –2% reported by Akrout *et al* (2010).

*T. capitatus* oil composition was characterized by 32 constituents, which accounted for 99.3% of the total oil (**Table 8**). The main compound was carvacrol (59.2%) followed by *p*-cymene (15.3%). These results are in accordance with previous studies *T. capitatus* essential oil with carvacrol as a major component, however, they also reported that *T. capitatus* show an important variation in essential oil composition particularly in the main compound which can be Thymol or carvacrol due to geographical and bioclimatic factors conditions (El Abed *et al*., 2014; Mkaddem *et al*., 2010; Nabavi *et al*., 2015).

GC–MS analysis of essential oil from *T. zygis zygis* oil showed the presence of 27 compounds representing 99.1% of the total volatile. Carvacrol was the major one with 43,6% followed by cymene (24,10%) and trans-sabinene hydrate (15.8%) as showed in **Table 8**. This result is consistent with the study of Figueiredo *et al*., 2008.

The chemical composition of *T. capitatus* and *T. zygis zygis* showed that it is rich in oxygen containing monoterpenes (64.4% and 49.8% respectively), followed by monoterpene hydrocarbons (31.2% and 49.6% respectively), while, both sesquiterpene and oxygen containing sesquiterpene were present in minor proportion. This wealth of oxygen-containing monoterpenes (OM), especially carvacrol, can enhance the value of those EOs as an active natural product (El Abed *et al*., 2014). The major compound carvacrol was described as a strong antibacterial molecule (Bounatirou *et al*., 2007; Figueiredo *et al*., 2008; Nabavi *et al*., 2015) and it is now considered one of the products most frequently selected for their pharmacological effects. Therefore, the results obtained support the selection of these thyme oils as preservative agents in our cosmetic formulations.

**Table 8:** Yields and percentage composition of the essential oils isolated from the aerial parts of T. capitatus and T. zygis zygis collected during flowering stage.

| Components | RI | *T. capitaus* | Components | RI | *T. zygis zygis* |
|---|---|---|---|---|---|
| α-Thujene | 924 | 2,3 | Tricyclene | 921 | T |
| α-Pinene | 930 | 1,6 | α-Thujene | 924 | 1.6 |
| Camphene | 938 | 0,7 | α-Pinene | 930 | 0.8 |
| Sabinene | 958 | 0,0 | Camphene | 938 | 1.0 |
| 1-Octen-3-ol | 961 | 0,7 | Thuja-2.4(10)-diene | 940 | - |
| β-Pinene | 963 | 0,3 | Sabinene | 958 | - |
| β-Mirceno | 975 | 3,1 | β-Pinene | 963 | - |
| α-Phellandrene | 995 | 0,2 | 3-Octanol | 974 | 1,00 |
| Δ3-Carene | 1000 | 0,2 | β-Myrcene | 975 | 1,00 |
| α-Terpinene | 1002 | 1,4 | α-Terpinene | 1002 | 1,40 |
| *p*-Cymene | 1003 | 15,3 | p-Cymene | 1003 | 24,10 |
| β-Phellandrene | 1005 | 0,4 | β-Phellandrene | 1005 | T |
| Limonene | 1009 | 0,5 | 1,8-Cineole | 1005 | T |
| *trans*-β-Ocimene | 1027 | 0,0 | Limonene | 1009 | 0,20 |
| γ-Terpinene | 1035 | 5,0 | trans-β-Ocimen | 1027 | 1,10 |
| *trans*-Sabinene hydrate | 1037 | 0,6 | γ-Terpinene | 1035 | - |
| Terpinolene | 1064 | 0,2 | trans-Sabinene hydrate | 1037 | 15,80 |
| *cis*-Sabineno hydrate | 1066 | 0,2 | cis-Linalool oxide | 1045 | 0,60 |
| Linalool | 1074 | 1,7 | trans-Linalool oxide | 1059 | T |
| Borneol | 1134 | 0,9 | Terpinolene | 1064 | T |
| Terpinen-4-ol | 1148 | 0,6 | cis-Sabinene hydrate | 1066 | 0,10 |
| α-Terpineol | 1159 | 0,1 | Linalool | 1074 | T |
| *p*-Cimen-7-ol | 1265 | 0,1 | Camphor | 1095 | 3,20 |
| Thymol | 1275 | 0,4 | trans-Verbenol | 1114 | - |
| Carvacrol | 1286 | 59,5 | Borneol | 1134 | 1,20 |
| Eugenol | 1327 | 0,1 | Terpinen-4-ol | 1148 | 0,60 |
| Carvacrol acetate | 1348 | 0,2 | α-Terpineol | 1159 | 0,10 |
| *trans*-β-Caryophyllene | 1414 | 2,2 | Linalyl acetate | 1245 | - |
| Aromadendrene | 1469 | 0,1 | Bornyl acetate | 1265 | T |
| α-Humulene | 1487 | 0,1 | Thymol | 1275 | 0,30 |
| β-Bisabolene | 1500 | 0,2 | Carvacrol | 1286 | 43,60 |
| *trans*-α-Bisabolene | 1536 | 0,1 | Thymyl acetate | 1330 | - |
| β-Caryophyllene oxide | 1561 | 0,3 | | | |
| Identified components (%) | 99.3 | | | | 99.1 |
| Yields (v/w) | | 2.06 | | | 1.14 |

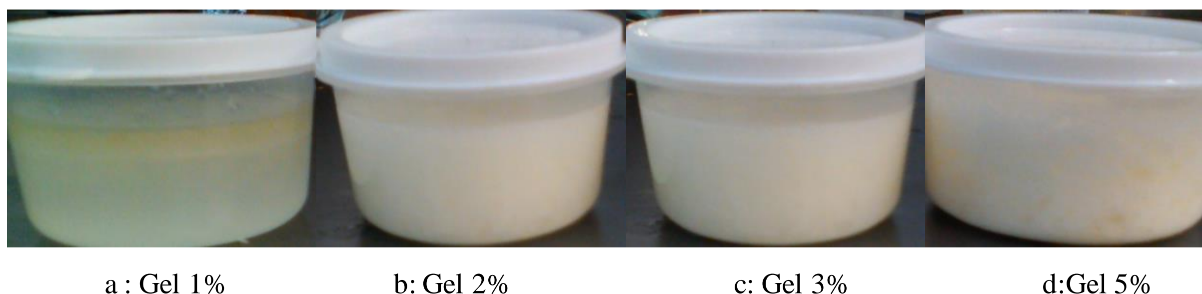
**Table 8 (continued):** Yields and percentage composition of the essential oils isolated from the aerial parts of T. capitatus and T. zygis zygis collected during flowering stage.

| Grouped Components | *T. capitatus* | *T. zygis zygis* |
|---|---|---|
| Monoterpene hydrocarbons | 31,2 | 49.6 |
| Oxygen-containing monoterpenes | 64,4 | 49.8 |
| Sesquiterpene hydrocarbons | 2,7 | 1.1 |
| Oxygen-containing sesquiterpenes | 0,3 | 0.3 |
| Others | 0,7 | 1 |

## 2. Choice and preparation of cosmetic formulation

We chose to prepare a natural hydrogel with antiaging properties for skin application because it provides a better application property in particular higher water absorption and better stability in comparison to cream and ointment (Kaur Loveleen Preet, 2013). In this context, different concentrations of bee pollen (0.5%, 1.0%, and 1.5%) were tried with different percentages of methylcellulose (1%, 2%, 3%, and 5%) in order to choose the combination that originated the gel formulation with the desired characteristics.

The gel with 1 % of methylcellulose (**Figure 15a**) was non homogeneous, too much liquid, the oil phase was not miscible in water phase then floating on the surface forming 2 phases, therefore this concentration was not adequate for elaboration of gel. In the case of concentration of 5% (**Figure 15d**), the gel was almost solid, highly viscous and not easy to spread. Regarding to the concentrations of 2% and 3% (**Figure 15b** and **15c**, respectively), gels showed good features regarding texture, consistency and spreadability, however gel 2 % evidenced a lower viscosity. Therefore 3% of methylcellulose was chosen as the optimum concentration of gelling agent.



a : Gel 1% b: Gel 2% c: Gel 3% d:Gel 5%

**Figure 15:** Different concentration of methylcellulose tested for gel preparation (a;b;c;d).

Later, different concentration (0.5%, 1%, 1.5%, 2%) of bee pollen were incorporated into the gel 3% in order to choose the optimum concentration with good features of the formulation. Afterwards, the characteristics of gel were cheeked by visual inspection. The gel with 0.5% of bee pollen was liquid inapt to use. For first visual inspection, gel with 1.5 % pollen showed a good features, however we noticed gel liquefaction after 3 days of its elaboration. The concentration of 1% of pollen was confirmed as the best concentration to use comparing to the concentration of 1.5 % since the viscosity remained stable over the time.

Based on the results obtained, the best combinations were the 1% of pollen in 3% of methylcellulose as gelling agent. These concentrations were used for preparation of the gel, which was subsequently subjected to stability analysis.

## 3. Stability evaluation of gel formulation.

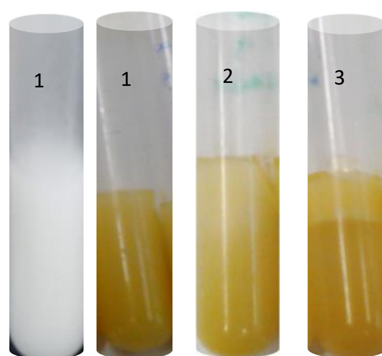
Stability studies of cosmetic formulations are essential to ensure product quality, safety, and efficacy. Thus, these studies contribute to the development and improvement of formulations, establishing the validity, and monitoring physical, chemical, and microbiological characteristics (Deuschle *et al*., 2015).

Stability can be affected by environmental factors such as temperature, pH, light, air, and movements which can provoke severe damages on the constituents of the product (Baby *et al*., 2007). For that, the gels elaborated are subjected to several tests in order to evaluate their stability in different conditions.

### *3.1.Centrifugation test*

The centrifugation test produces stress in the sample, simulating an increase in the force of gravity and increasing the mobility of the particles, thus anticipating possible instabilities. These changes may appear in the form of precipitation, separation of phases, caking, or coalescence among others (ANVISA, 2005).

The formulations were centrifuged at 25°C during 30 minutes. Afterwards, they were visually evaluated and a score was attributed for each formulation depending on its aspect as shown in **Figure 16**.




1: No phase separation, 2: Slight phase separation
3: Complete phase separation

**Figure 16:** Physical evaluation (centrifugation) of aspect formulations.

A score equal to 1 was attributed to the formulations that did not show any phase separation, such as the case of all the formulations tested under a concentration of 0.02% of preservatives and also for all the formulations with 0.4% methylparaben and 0.1% Eos except the formulations based on bee pollen and methylparaben (G13, G14, G15, G16) were attributed to them a score equal to 3 since they showed a clear, complete phase separation. It seems that those formulations were affected gravelly by the force.

Based on these results, we can point out that both bee pollen and methylparaben at this concentration could not be used together in cosmetic formulation, fortunately, we revealed a good stability with essential oils therefore, they can substitute methylparaben.

### *3.2. Vibration test*

Vibration during transportation may affect the stability of the formulations, causing a separation of the phases of emulsions, solidification of suspensions, alteration of viscosity, among others (ANVISA, 2005).

After being submitted to the vibration test, none of the cosmetic formulations exhibited phase separation, which evidenced their physical stability.

### *3.3. Light test*

Ultraviolet radiation along with oxygen leads to the formation of free radicals, which can result in the oxidation of formulations that can cause changes in color and odor of the product, and also lead to the degradation of formulation ingredients (ANVISA, 2005).

In order to evaluate the behavior of formulations towards light, they were exposed to day light source with photoperiodicity (16 hours of day and 8 hours of dark) during 2 weeks. After the end of the period of testing, they were visually inspected and no change occurred for all the formulations with higher concentration of preservatives. The same results obtained in the formulations with 0.02% of preservatives except the formulations based on bee pollen, we were not able to evaluate their stability because of the contamination (**Figure 17**). Therefore, the formulations remain stable during the test.

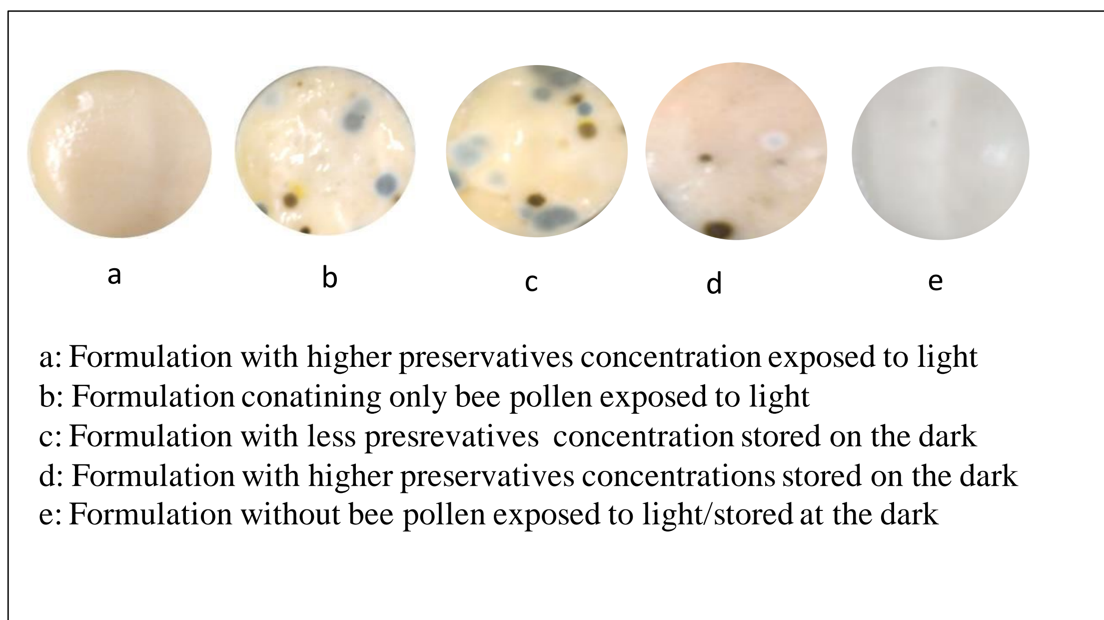



**Figure 17:** Visual aspect of the cosmetic formulations after exposure day light source with photoperiodicity (16 h of day and 8 h of dark), during 2 weeks.

### *3.4. Viscosity test*

The viscosity of a formulation depends on its physicochemical characteristics, and on the temperature conditions to which it is subjected. Evaluation of this parameter permits to determine the appropriate consistency and fluidity, and by indicating the performance of the product over time (Deuschle *et al*., 2015).

For all gels elaborated, we observed a decrease in viscosity with increasing rate of shear, evidencing non-Newtonian flow (shear thinning), as shown in **Figure 18**. This behavior is frequently observed in cosmetic formulations and suggests an interesting spreadability behavior due to the decrease in viscosity when applying certain force. The lower viscosity was revealed in the control (G1) formulation without incorporation of natural products or synthetic

preservatives, and also in the formulation that only contained methylparaben (G9). The formulations with incorporated natural products (G2, G3, and G5) showed a good stability.

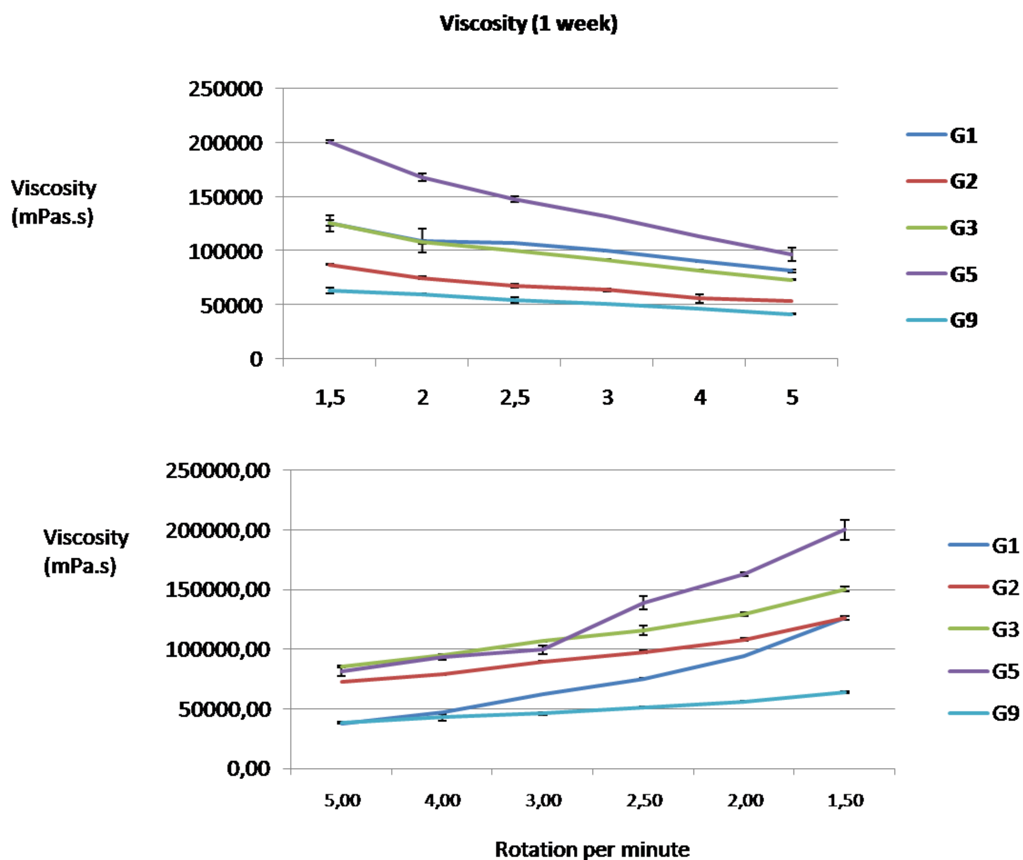



**Figure 18:** Evaluation of viscosity of gels during the first week of testing.

The viscosity of the cosmetic formulations remained stable over the period of testing (2 weeks), as shown in **Figure 19,** and all the formulations exhibited the shear thinning behaviour that was registered in the first week. The formulations based on bee pollen were not tested during the second week because of the contamination.

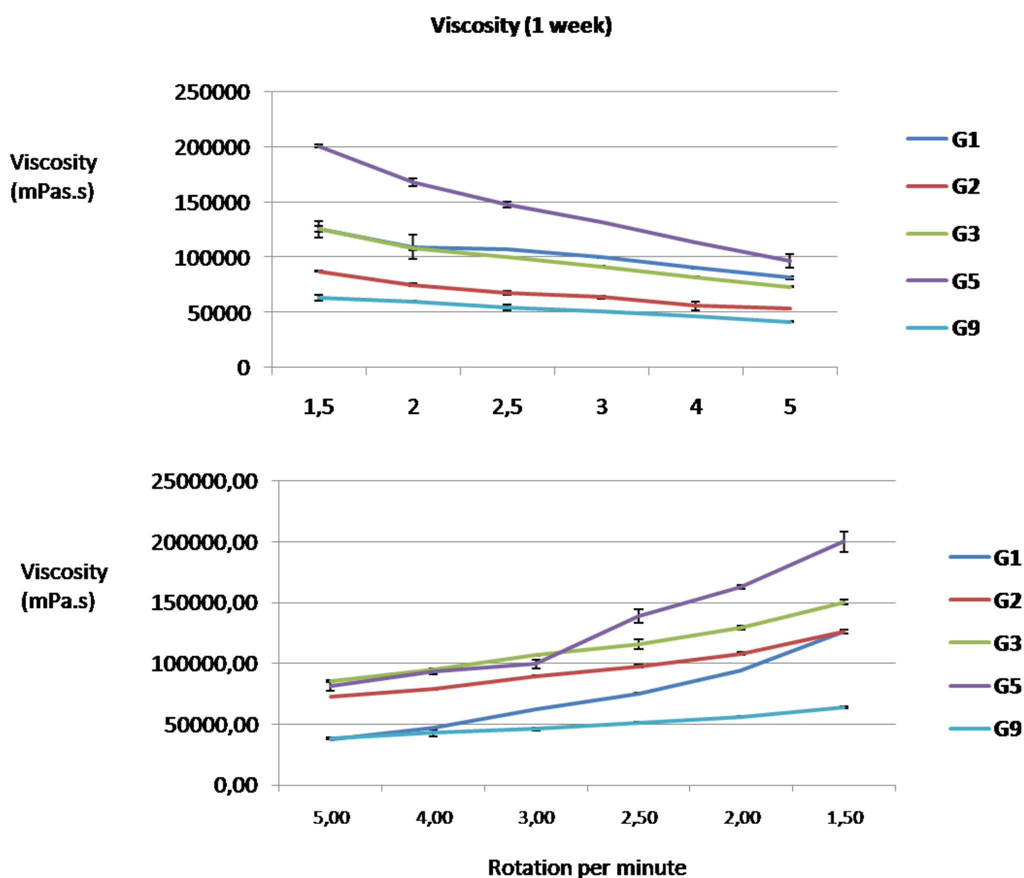



**Figure 19:** Evaluation of viscosity of gels during the second week.

### *3.5.pH determination*

The pH values of all the prepared formulations ranged from 4,78 ± 0,01 to 5,42 ±0,08 which is considered acceptable to avoid the risk of irritation upon application to the skin since it is closer to the human skin pH which normally range from 4.5 to 6 (Lucero, Vigo, & Lecin, 1994). The pH values of gel also remained stable during storage although there was a pH variation over the test period (9 days) as shown in **Figure 20** but it is considered as non significant change since it did not exceed the pH range of skin. Hence, the prepared gel is suitable for topical application.

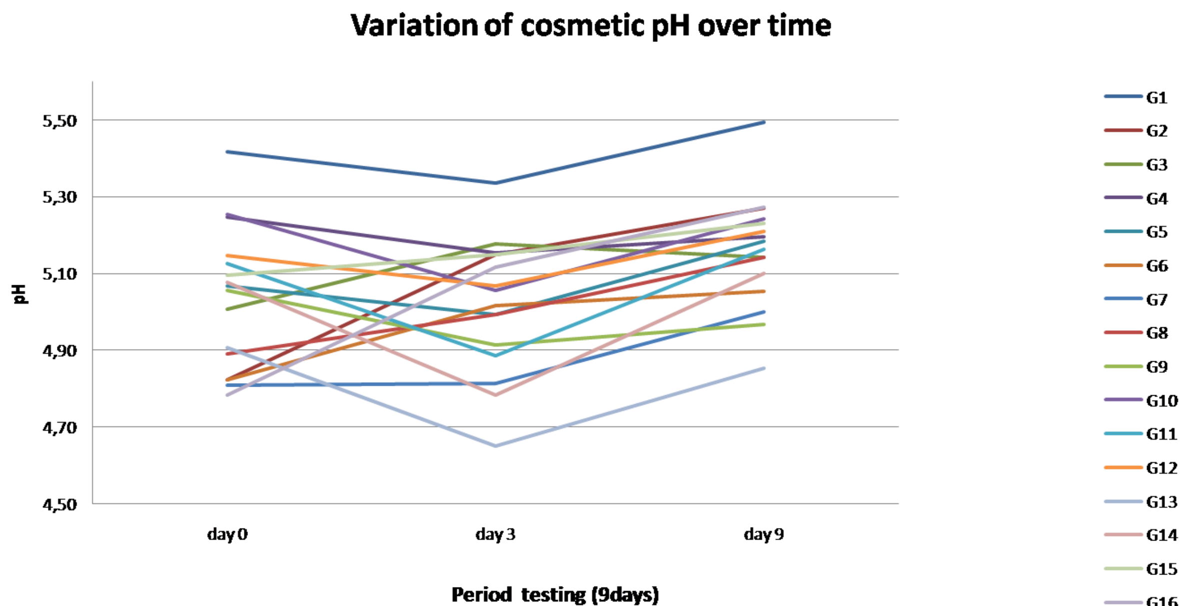



**Figure 20:** Variation of pH over time.

### *3.6.Density*

Density can indicate the incorporation of air or the loss of volatile ingredients in the case of liquids or semi-solids (ANVISA, 2005). The apparent density was determined by calculating the ration between the mass of the formulation and the volume that it occupies. Results obtained are listed on **Table 9**. The basic formulation (G1), which served as the control, presented a density value of 1.03 g/mL, which is similar to the values registered for the remaining formulations that ranged from 1.0 to 1.2 g/mL. Thus, we can point out that the compounds (bee pollen, thyme oil and methylparaben) incorporated into the cosmetic formulations did not affect the density of the basic formulation.

**Table 9:** Density values for all the gels elaborated.

| Gels code | Density (g/mL) |
|---|---|
| G1 | 1.02±0.03 |
| G2 | 1.11±0.01 |
| G3 | 1.2±0.05 |
| G4 | 1.1±0.01 |
| G5 | 1.08±0.03 |
| G6 | 1.09±0.02 |
| G7 | 1.04±0.02 |
| G8 | 1±0.03 |
| G9 | 1.11±0.01 |
| G10 | 1.12±0.01 |
| G11 | 1.0 ±.01 |
| G12 | 1.04±0.03 |
| G13 | 1±0.01 |
| G14 | 1.01±0.02 |
| G15 | 1.11±0.02 |
| G16 | 1.12±0.03 |

### *3.7.Spectrophotometry test*

Ultraviolet/Visible (UV/Vis) spectrophotometric method was used to check the chemical stability of cosmetic formulations. For that the formulations were subjected to spectrophotometric analysis in the region of UV-VIS (210 to 600 nm), and the results obtained are presented in **Figures 21** and **22**.

Regards to the formulations tested under 0.02% of preservatives, the spectrum were similar to the spectrum obtained from the control during the two weeks except the one of methylparaben (G9) is different comparing to the control during the second week with maximum of absorbance at 264 nm. Whereas, for the formulations with 0.1% and 0.4 % of thyme oil and methylparaben preservatives, different profile were obtained. Whereas, the formulations with bee pollen and/or thyme oil showed almost the same profile than the control. Natural products seem that did not affect the chemical behavior of the basic formulations.

The formulations based on methylparaben with incorporated natural products behave differently comparing to the control. Methylparaben seems that change chemical properties of basic formulations comparing to the natural products. The same behavior was observed during the second week, which confirms our result analysis.

The formulations with bee pollen were not tested in the second week because of the contamination.

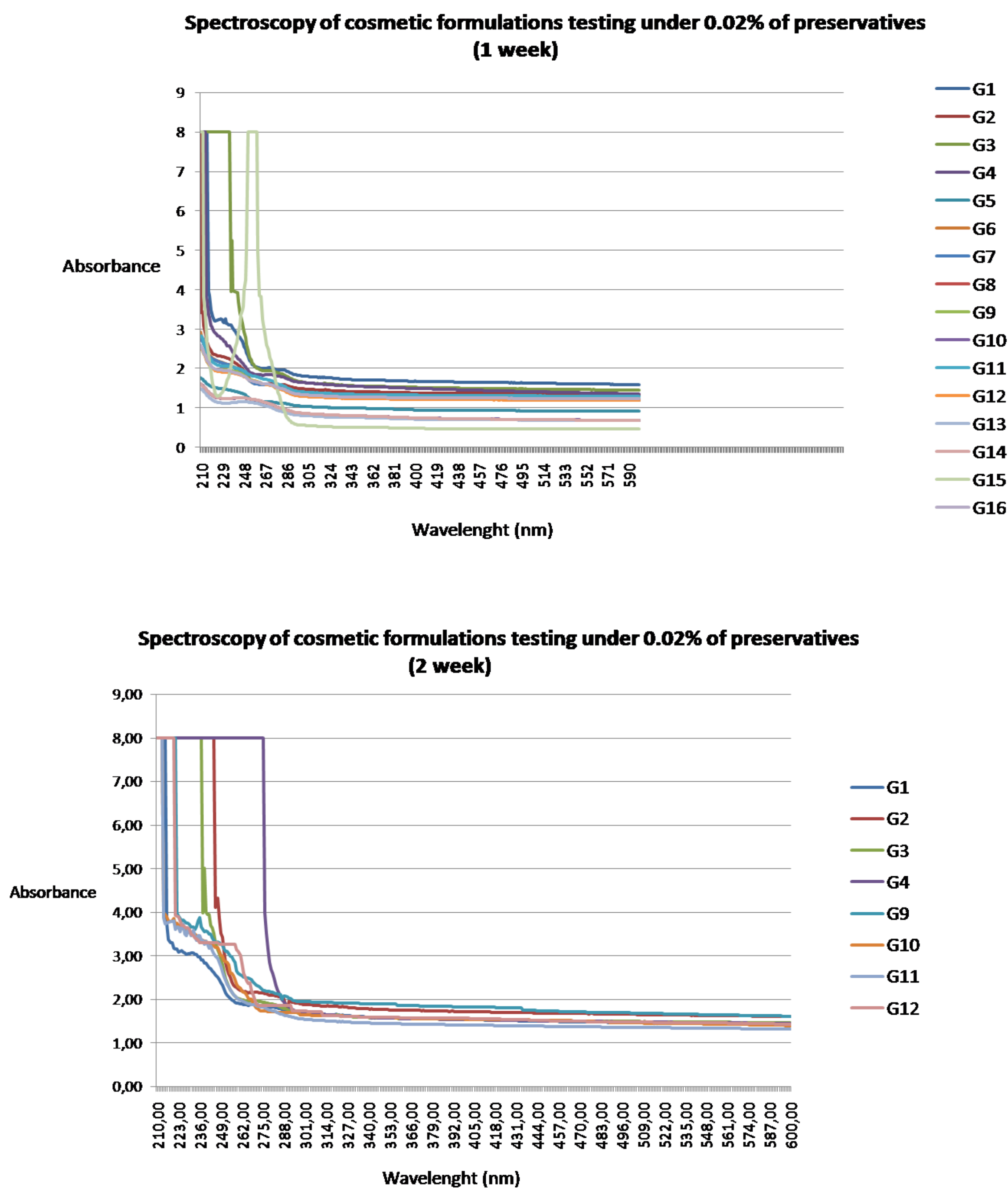
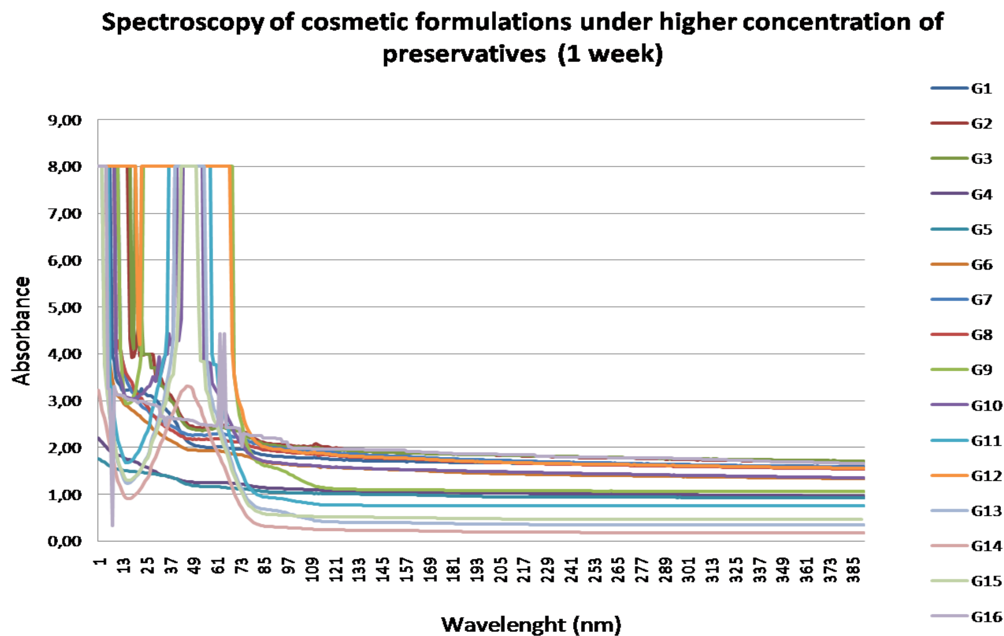



**Figure 21:** Spectrophotometric analysis of formulations tested under 0.01% of preservatives during 2 weeks of testing.



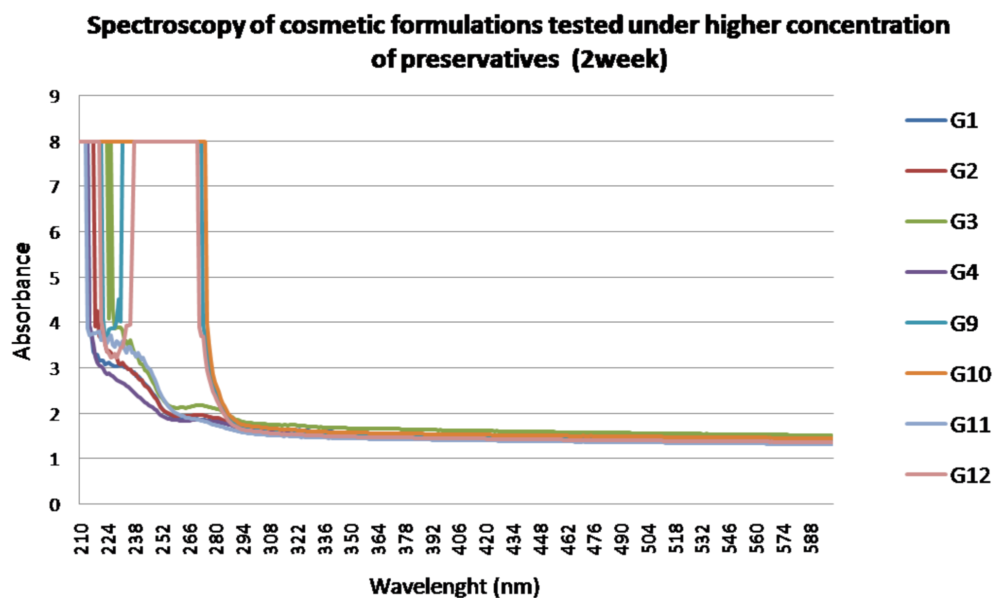




**Figure 22:** Spectrophotometric analysis of cosmetic formulations with higher concentration of preservative during 2 weeks.

### *3.8.Accelerated stability*

All formulations were stored at 25±2°C at 60 ± 5% RH, and at 40±2°C at 75% ± 5% RH for 2 weeks and their physicochemical stability (organoleptic characteristics and pH) was evaluated each week. Based on results obtained listed in **Tables 10** and **11**, no particular changes in the evaluated characteristics were registered, although we noticed a slight change observed visually as decrease in viscosity for G14 and G16 during the second week of testing. However, we were not able to evaluate the effect of temperature on organoleptic characteristics of G5, G8 because of the contamination that occur during the test.

The characteristics of the various formulations did not seem to be affected by the type (natural and synthetic) or concentration (0.02%, 0.1%, and 0.4%) of the preservatives incorporated in the formulations. However we were not able to evaluate the features of all the formulations containing bee pollen (G5, G6, G8, G13, G14, G15, G16) because of the contamination that occur at the end of first week of testing (**Figure 23**).

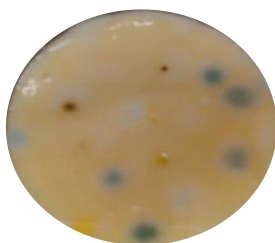


**Figure 23:** Formulation based on bee pollen presenting a contamination during the first week of stability assay.

**Table 10:** Results from the accelerated stability assays performed on the various formulations during the first week.

| Gels code | pH | | Color | | Smell | | Texture | | Consistency | | Phase separation | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** |
| G1 | 4.83±0.01 | 5.03±0.01 | W | W | N | N | S | S | V | V | - | - |
| G2 | 5.35±0.01 | 5.21±0.01 | W | W | SS | SS | S | S | V | V | - | - |
| G3 | 5.15±0.01 | 4.93±0.01 | W | W | SS | SS | S | S | V | V | - | - |
| G4 | 5.52±0.01 | 5.15±0.01 | Y | Y | N | N | S | S | V | V | - | - |
| G5 | 4.77±0.01 | 4.85±0.01 | Y | Y | N | N | S | S | V | V | - | - |
| G6 | 5.16±0.01 | 5.07±0.01 | Y | Y | SS | SS | S | S | V | V | - | - |
| G7 | 5.06±0.01 | 4.98±0.01 | Y | Y | SS | SS | S | S | V | V | - | - |
| G8 | 5.21±0.01 | 5.18±0.01 | Y | Y | SS | SS | S | S | V | V | - | - |
| G9 | 5.06±0.01 | 5.21±0.01 | W | W | N | N | S | S | V | V | - | - |
| G10 | 5.36±0.01 | 5.37±0.01 | W | W | SS | SS | S | S | V | V | - | - |
| G11 | 5.20±0.01 | 5.52±0.01 | W | W | SS | SS | S | S | V | V | - | - |
| G12 | 4.94±0.01 | 5.21±0.01 | W | W | SS | SS | S | S | V | V | - | - |
| G13 | 4.98±0.01 | 5.04±0.01 | Y | Y | N | N | S | S | V | V | - | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G14 | 5.2 ±0.01 | 5.18±0.01 | Y | Y | SS | SS | S | S | V | V | - | - |
| G15 | 5.25±0.01 | 5.21±0.01 | Y | Y | SS | SS | S | S | V | V | - | - |
| G16 | 5.32±0.01 | 5.53±0.01 | Y | Y | SS | SS | S | S | V | V | - | - |

The gels codes corresponding to the formulations containing:

G1 : Control (methylcellulose + glycerin + almond oil), G2: *T. zygis zygis*, G3: *T. capitatus*, G4: Mixture of EOs, G5: Pollen, G6: Pollen + *T. zygis zygis*, G7: Pollen + *T. capitatus*, G8: Pollen + 2 EOs, G9: Methylparaben, G10: Methylparaben + *T. zygis zygis*, G11: Methylparaben + *T. capitatus*, G12: Methylparaben + 2 EOs, G13: Methylparaben + Pollen, G14: Methylparaben + pollen + *T. zygis zygis*, G15: Methylparaben + pollen + *T. capitatus*, G16: Methylparaben+Pollen+2EOs.

W: White, Y: Yellow, N: Noticeable, SS: Strong smell, S: Smooth, V: Viscous, +: Slight change in viscosity,

-: No phase separation, +: phase separation

**Table 11:** Results from the accelerated stability assays performed on the variousformulations during the second week.

| Gels code | pH | | Color | | Smell | | Texture | | Consistency | | Phase separation | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** | **40±2°C 75±5 %RH** | **25±2°C 60±5 %RH** |
| G1 | 5.05 0.01 | 4.57±0.01 | W | W | N | N | S | S | V | V | - | - |
| G2 | 5.53±0.02 | 5.15±0.01 | W | W | SS | SS | S | S | V | V | - | - |
| G3 | 5.61±0.01 | 5.43±0.01 | W | W | SS | SS | S | S | V | V | - | - |
| G4 | 5.54±0.01 | 5.34±0.01 | Y | Y | N | N | S | S | V | V | - | - |
| G5 | - | - | - | - | - | - | - | - | - | - | - | - |
| G6 | 5.26±0.01 | 5.32±0.03 | Y | Y | SS | SS | S | S | + | V | - | - |
| G7 | 5.15±0.01 | 5.25±0.01 | Y | Y | SS | S | | | | | - | - |
| G8 | - | - | - | - | - | - | - | - | - | - | - | - |
| G9 | 4.94±0.01 | 4.94±0.01 | W | W | N | N | S | S | V | V | - | - |
| G10 | 5.±0.01 | 5.30±0.03 | W | W | SS | SS | S | S | V | V | - | - |
| G11 | 5.20±0.01 | 5.52±0.01 | W | W | SS | SS | S | S | V | V | - | - |
| G12 | 5.34±0.01 | 5.21±0.01 | W | W | SS | SS | S | S | V | V | - | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **G13** | 5.40±0.01 | 5.62±0.01 | Y | Y | N | N | S | S | V | V | NO | NO |
| **G14** | 5.19±0.01 | 5.01±0.01 | Y | Y | SS | SS | S | S | + | + | NO | NO |
| **G15** | 5.25±0.01 | 5.21±0.01 | Y | Y | SS | SS | S | S | V | V | NO | NO |
| **G16** | 5.32±0.01 | 5.53±0.01 | Y | Y | SS | SS | S | S | + | + | NO | NO |

The gels codes corresponding to the formulations containing:

G1 : Control (methylcellulose + glycerin + almond oil), G2: *T. zygis zygis*, G3: *T. capitatus*, G4: Mixture of EOs, G5: Pollen, G6: Pollen+ *T. zygis zygis*, G7: Pollen + *T. capitatus*, G8: Pollen + 2 EOs, G9: Methylparaben, G10: Methylparaben + *T. zygis zygis*, G11: Methylparaben + *T. capitatus*, G12: Methylparaben + 2 EOs, G13: Methylparaben + Pollen, G14: Methylparaben + pollen + *T. zygis zygis*, G15: Methylparaben + pollen + *T. capitatus*, G16: Parabren + Pollen + 2 EOs.

W: White, Y: Yellow, N: Noticeable, SS: Strong smell, S: Smooth, V: Viscous, +: Slight change in viscosity.

-: No phase separation, +: Phase separation.

### *3.9.Contamination test*

Microbial contamination constitutes a threat to consumer safety and to the marketing image of cosmetics. Therefore, it is crucial to evaluate the microbial stability of products to ensure its safety. For that, the microbial stability of formulations was evaluated through contamination test using a variety of microorganisms namely *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeurigonosa, Bacillus subtilis* and *Candida albicans.* After the incubation period, the plates were taken out and checked for microbial growth by comparing it with the control. No microbial growth was observed for all the formulations incorporated with both concentrations of natural preservatives (0.02% and 0.1%) and also for those incorporated with the synthetic one (0.02% and 0.4%), as shown in **Figure 24**. The obtained results had confirmed the microbial stability of our formulations.

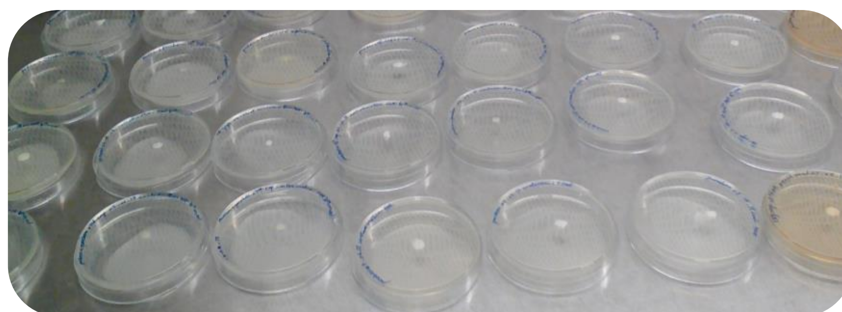


**Figure 24:** Microbial stability evaluation of the various formulations.

## 4. Antimicrobial activity

The different formulations were also assayed regarding their antimicrobial activity towards various microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. The formulations that contained 0.02% of preservatives (both natural and synthetic) did not reveal any significant antimicrobial activity against all the strains tested although the slight antimicrobial activity registered against *S. aureus, C. albicans* and *P. aeruginosa.*

The antimicrobial activity was also evaluated when higher concentrations of preservatives were incorporated in the cosmetic formulations. It was observed a significant and promising activity against *E. coli*, *S. aureus* and *B. subtilis* and *C. albicans* with lower sensitivity comparing with the standard antibiotics (C30 for bacteria and F100 for fungi).The formulations showed a bacteriostatic effect against *E. coli, B. subtilis* and *C. albicans*, whereas *S. aureus* was the most sensitive microorganism (**Figure 26**). It was also observed

that none of the formulations exhibited microbial activity towards *P. aeruginosa*. The blank formulation (G1) was not active against any of the strains tested.

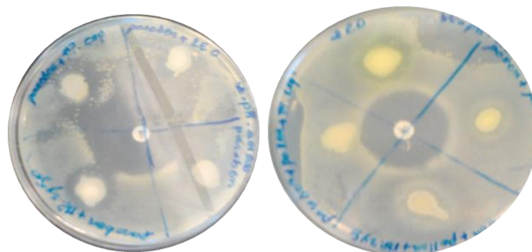


**Figure 25:** Antimicrobial activity against *S. aureus*.

The gels G2 and G3 contained only one essential oil, while in the gel G4 two essential oils were incorporated. As shown in **Table 12**, it was observed that the gels G2 and G3 caused a smaller inhibition zone for *B. subtilis and S. aureus* in comparison with G4. This shows a possible synergistic effect resulting from the mixture of the essential oils from *T. zygis zygis* and *T. capitatus*. The gel G9, which contained the synthetic preservative methylparaben, presented a higher inhibition zone for *B. subtilis and S. aureus* in comparison with G2, G3 and G4.

The gel (G4) contains a mixture of essential oils showed a possible synergistic effect against *S. aureus* and *B. subtilis* compared to the gels containing only one essential oil (G2, G3), as shown in **Table 12** but less than the formulations containing only methylparaben (G9) which is normal because it is known that methylparaben had a stronger antimicrobial activity than essential oil, moreover the antimicrobial activity of essential oils is dose-dependent with greater activity seen at the higher oil concentrations. Maybe if we increase more the concentration of thyme oils, we will have a better result comparing with those of methylparaben. However, according to this results Thyme oil can be used as natural preservative.

The highest antimicrobial activity was observed against *S. aureus* in the formulation G16, which contained all the raw materials, possibly due to the synergetic effect between the natural products incorporated and synthetic preservative methylparaben.

**Table 12:** Inhibition zone.

| Inhibition zone | | | | |
|---|---|---|---|---|
| | *E. coli* | *B. subtilis* | *S. aureus* | *C. albicans* |
| G1 | - | - | - | - |
| G2 | - | 15 mm | 12 mm | - |
| G3 | - | 14 mm | 12 mm | - |
| G4 | - | 17 mm | 17 mm | - |
| G5 | - | 14 mm | 10 mm | - |
| G6 | 10 mm | 10 mm | 10 mm | - |
| G7 | 10 mm | 10 mm | 16 mm | - |
| G8 | 20 mm | 18 mm | 18 mm | 10 mm |
| G9 | 15 mm | 20 mm | 20 mm | - |
| G10 | 15 mm | 12 mm | 20 mm | - |
| G11 | 10 mm | 12 mm | 20 mm | - |
| G12 | 10 mm | 25 mm | 25 mm | - |
| G13 | 10 mm | 15 mm | 15 mm | - |
| G14 | 10 mm | 8 mm | 16 mm | 10 mm |
| G15 | 12 mm | 8 mm | 18 mm | 10 mm |
| G16 | 15 mm | 18 mm | 25 mm | 18 mm |
| ATB C30 | 35 mm | 35 mm | 26 mm | - |
| ATB F100 | - | - | - | 25 mm |

## 5. Discussion of the contamination occurred in formulations based on bee pollen

During this work various formulations containing bee pollen in its composition (G5, G6, G7, G8, G13, G14, G15, and G16) were prepared using different experimental methodologies, including aseptic conditions. However, fungi contaminations were detected in all of them, which might be attributed to the chemical composition of bee pollen, which includes several important nutrients that may provide a suitable substrate for the fungi growth. Moreover, the bee pollen is a highly hygroscopic material that exhibits a high water activity ($a_w$), which is also essential for microorganism growth. Several authors have reported pollen contamination during collection, treatment or storage of bee pollen (Gonzalez *et al*., 2005; Kačániová *et al*., 2011; Petrovic *et al*., 2014). In addition, Literature reported that a microbial contamination of cosmetic formulation may occur in the course of production, through raw materials,

ingredients, and during handling and can be controlled by sanitary processing and using appropriate and adequate preservatives (Elmorsy & Hafez, 2016).

Take into consideration those informations, some techniques were used in order to avoid the fungi growth, such as freeze-drying and pasteurization of bee pollen at 65°C for 2 hours, besides working in aseptic condition (under flow laminar cabinet), also an increase on preservatives concentration to 0.1% and 0.4% for thyme oil and methylparaben, respectively since 0.02% of preservatives was not efficient and allowed fungi growth. Using 0.1% of thyme oils and 0.4% of methylparaben, we observed decrease in the fungal growth in the case of the formulations based on bee pollen and thyme essential oil or mixture of essential oils during the storage and the stability assays except the formulations based on bee pollen (G8) and mixture of essential oils (*Thymus zygis zygis* essential oil and *T. capitatus* essential oil), this could be explained by reactions that may have occurred between the compounds of the two essential oils, leading to a reduction in the concentration of the active principles, which are responsible for their antifungal activities. Still, it was not possible to assess exactly the mechanism that led to the registered results. Whereas, no contamination occurred in the case of formulations based on bee pollen and methylparaben, or mixture of methylparaben and thyme essential oils, which explained by the potential activity of methylparaben. In addition, none of the formulations that contained bee pollen and were exposed to light presented contaminated by fungi compared to those stored on dark. The light may probably inhibit fungi growth since literature reported that light affect either positively or negatively fungal growth by inducing or inhibiting their sexual development depending on the species, some *Aspergillus* species may be inhibited by light such as *Aspergillus orizae* (Murthy*et al*., 2015). This hypothesis should be considered for possible future research in order to understand clearly what was observed throughout this work.

## 6. Safety assessement of formulations

### *6.1.Ocular irritancy*

Several tests are used to evaluate the ocular irritancy of cosmetic products including the bovine corneal opacity and permeability (BCOP) assay, the isolated chicken eye test method (ICE), the isolated rabbit eye (IRE) assay, and the hen's egg test–chorioallantoic membrane (HET-CAM) assay (Barile, 2010),which was used in this present work.

The assayed formulations were placed into the CAM and afterwards irritant reaction was evaluated as hemorrhage, vascular lysis and coagulation during 5 minutes. The formulations presented the following composition: G2 contained only *T. zygis zygis* essential oil, G3 contained only *T. capitatus* essential oil, G4contained a mixture of essential oils, G5 contained only pollen, G6 contained pollen and *T. zygis zygis* essential oil, and G7 contained bee pollen and *T. capitatus* essential oil, with concentration of 0.1%. After 5 minutes, and in comparison with 10% of the positive control (NaOH) no alterations on CAM were registered for all the formulations tested as shown in **Figure 26**, evidencing that the formulations did not had any irritant potential. Therefore, we can point out that the cosmetic formulations elaborated with incorporated natural products under 0.1% of essential oils can be assessed as safe to be applied on the skin near the eyes.

The formulations that contained methylparaben were not tested, since parabens were considered as safe regarding the ocular irritancy since it is widely used in a variety of cosmetic product however there is a big concern regarding some health risks such as breast cancer inducing by this compound and they are substitute in various products by natural preservatives.

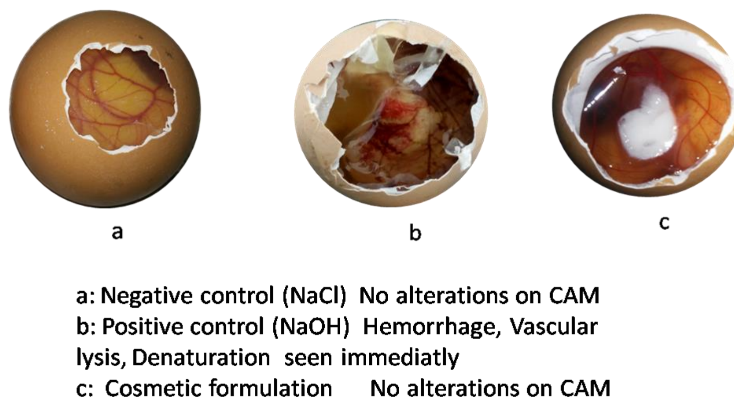



**Figure 26:** Evaluation of ocular irritancy of cosmetic formulation.

### 6.2. Acute toxicity

The safety of a cosmetic product is based on the safety of its ingredients. Therefore it is crucial to evaluate the toxicity of raw materials used in cosmetic formulations. In the present study, the determination of raw materials toxicity in the formulation concerned only the following natural products bee pollen and thyme oils from 2 different species (*T. zygis zygis* and *T. capitatus*). Methylparaben was not tested for its toxicity since it is already used in cosmetic and is, regarded as safe.

Bee pollen and thyme oils were subjected to brine shrimp lethality bioassay at different concentrations to determine its toxicity range and thus ascertain whether or not its possible toxicity could reduce its cosmetic use. The results of brine shrimp lethality assay were interpreted as follows: in the case of $LC_{50}$ values > 1000 µg /mL , compound is considered as non toxic; for $LC_{50}$ ranged between 500µg/mL and 1000µg/mL, compound sowed a weak toxicity; whereas $LC_{50}$ <1000 µg/mL compound regarded as toxic as reported by Bastos *et al* (2009).

The concentration of compounds that kills 50 % of *Artemia* was determinedthrough the equation of linear regression line of each compound (Y=25.6x, Y=25.83 x and Y=2.812x for *T. zygis zygis* essential oil*, T. capitatus* essential oil and bee pollen respectively) as shown in **Figure 27**. The LC50 values for the percentage of mortality brine shrimp treated by the natural products were found pollen were found to be 1.88g/mL, 1.94 g/mL and 17.78 g/mL for *T. zygis zygis*, *T. capitatus*, and bee pollen.

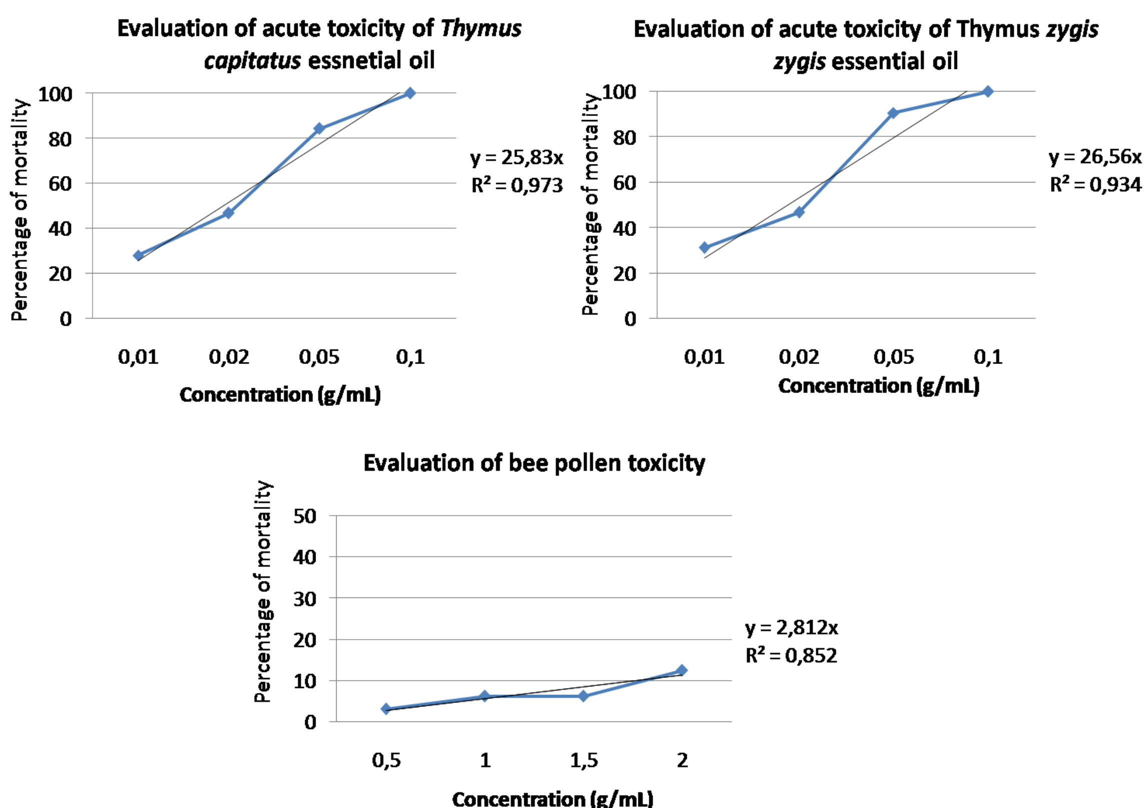



**Figure 27:** Evaluation of acute toxicity of compounds incorporated in cosmetic formulation.

The natural products tested presented $LC_{50}$ values higher than 1g/mL, and can therefore be considered as safe.

A direct proportional correlation was observed between the concentration of the thyme oil used and the percentage of mortality value: it was found to be increasing with increase in the concentration for the both plants. This is shown by the fact that maximum mortalities of *Artemia* occurred at the highest thyme oil concentrations of 0.1 mg/mL. Similarly, least mortalities were observed at the lowest extract concentration of 0.01 mg/mL (**Figure 27**). The both thyme oils showed almost the same profile and very similar $LC_{50}$ values. Whereas, the lowest toxicity value was registered for the bee pollen, which is in accordance with its safe composition that allowed it to be considered as safe product (Bogdanov, 2016; Campos, 2003).

Our findings may lend some support to the cosmetic use of essential oils and bee pollen under the $LC_{50}$without apparent adverse effect. Whereas, we point out that the formulations were assessed in duplicate regarding the HET-CAM test. This may not be sufficient to guarantee the safety of the formulations. Hence, the formulations should be evaluated several times for more rigorous results. For that, the natural products must be tested more than 4 times for accurate results to be surer concerning the compounds safety. Also, it should be mentioned that the *Artemia salina* bioassay provides preliminary screening data that can be backed up by more specific bioassays.

# CHAPTER 4. CONCLUSIONS AND PERSPECTIVES

The findings presented in the current study indicate that the formulations with 3% of methylcellulose and 1% of bee pollen showed a good physicochemical and microbiological stability, thus providing a safe and stable gel delivery system. The formulation and subsequent evaluation of the gel presented here showed no phase separation at different storage conditions for a long period. The phase separation on centrifugation was noted, only in formulations containing both bee pollen and methylparaben. The gels showed a non-Newtonian flow behavior (shear thinning), and also pH values that are adequate for cosmetic formulations for topic application. In addition, the rheological behavior and organoleptic properties were stable under 25°C±2 with 65±5 % RH and 40°C±2 with 75 ±5% RH during the accelerated stability test.

The antimicrobial activity of the gels was improved using0.1 % and 0.4% of natural and synthetic preservatives, respectively. The formulations showed a bacteriostatic effect against all the strains except *P. aeruginosa*, whereas *S. aureus* was the most sensitive bacteria. Thyme essential oils showed remarkable preservative capabilities under 0.1%and could therefore be considered as alternative preservatives.

The safety of the formulations incorporated with natural products was determined through HET-CAM assay, and no irritant reaction on the chorioallantoic membrane of chicken embryo was observed. Also, the natural products incorporated in the gel did not show a toxic effect through *Artemia salina* assay with $LC_{50}$ values higher than 1 mg/mL (1.88g/mL, 1.94g/mL, and 17.78 g/mL for *Thymus zygis zygis* essential oil, *Thymus capitatus* essential oil, and for bee pollen, respectively).

The cosmetic formulations based on bee pollen that were prepared throughout this work presented fungal contamination. In order to overcome this contamination issue and preserve our formulations, procedures such as pasteurization of bee pollen at 65°C for 2 hours, freeze-drying, and working in aseptic conditions during the preparation of the formulations were tested. However, the fungal contamination remained in the formulations stored at room temperature. Only when the concentration of methylparaben and thyme essential oils was increased; an improvement on formulation resistance against fungi growth was observed. This effect was particularly evident for the formulations containing methylparaben, where no

contamination occurred in all the formulations stored for a long time at room temperature or during the elaboration of tests, whereas a decrease of contamination in formulations containing thyme essential oil as a natural preservative was registered although the contamination remained during the experiment.

Therefore, according to these results, it was evident that a preservative concentration of 0.02% is not enough for the preservation of our formulations, particularly those containing bee pollen, and that 0.4% of methylparaben is an adequate concentration for preservation. For thyme oil, a concentration of 0.1% permitted the reduction of contamination but did not completely eliminated it, suggesting that further studies need to be carried out to overcome the contamination problem and also to evaluate the long term stability of the formulations in order to determine its shelf life. To overcome the problem of contamination we suggest the use of higher concentration of essential oils but under the $LC_{50}$ value obtained because the essential oils are active at higher concentrations, also a sterilization of bee pollen through UV light because the microorganisms are killed in UV light, or irradiation with δ-rays (gamma).

We can also proposed a preparation of other type of formulation with lower water content because maybe the higher water content on the gel, besides the wealth of bee pollen on nutrient are the suitable medium for microorganisms growth. This formulation can be used as reference to verify the real cause of contamination if either from bee pollen or the type of formulation.

Despite the considerable amount of experimental work performed during this study, these formulations should be tested in animal cellular lines to evaluate their toxicity and dermatologic characteristics. In the future, the formulations will be tested with complementary assays for inflammatory, toxicity and bioactivity in skin cell lines. It is very important to understand how the natural products behave in this type of formulation, in order to be able to use them as alternative for synthetic ones or even as complementary used in more “natural” formulation, as commercial public demands, more and more.

Although this work is just the beginning of these studies, we tried to contribute to a better understanding of the potentialities in cosmetics of the natural products, particularly products natives from our regions, with all the specificities, advantages and guarantees that these spontaneous, or organically produced, products have.

# References

Akrout Ahmed, El jani Hajer, Amouri Sondes, N. M. (2010). Screening of antiradical and antibacterial activities of essential oils of Artemisia campestris L ., Artemisia herba-alba Asso . and ... O ILS OF A RTEMISIA CAMPESTRIS L ., A RTEMISIA H ERBA A LBA A SSO , &. *Recent Research in Science and Technology*, *2(1)*(January), 29–39.

Altuntaş, E., & Yener, G. (2015). Anti-aging potential of a cream containing herbal oils and honey: Formulation and in vivo evaluation of effectiveness using non- invasive biophysical techniques. *IOSR Journal of Pharmacy and Biological Sciences Ver. I*, *10*(6), 2319–7676. https://doi.org/10.9790/3008-10615160

Anita Bénech. (2006). *Wellness from the beehive... ...bee pollen, propolis, royal jelly, honey*. *Wellness Foods Europe*.

ANVISA. (2005). Cosmetics Products Stability Guide.

Arcanjo, D., Albuquerque, A., Melo-Neto, B., Santana, L., Medeiros, M., & Citó, A. (2012). Bioactivity evaluation against Artemia salina Leach of medicinal plants used in Brazilian Northeastern folk medicine. *Brazilian Journal of Biology*, *72*(3), 505–509. https://doi.org/10.1590/S1519-69842012000300013

Azwanida, N. N., Hui, M. S., Afandi, A., Mohamed, S., Zulhisyam, A. K., Ayob, A., … Mohamed, M. (2015). Color Stability Evaluation of Pigment Extracted from Hylocereus polyrhizus , Clitorea ternatae and Pandanus amaryllfolius as Cosmetic Colorants and Premarket Survey on Customer Acceptance on Natural Cosmetic Product. *Jounal of Tropical Resources and Sustaintability Sciences*, *3*(May), 61–67. Retrieved from http://www.jtrss.org/JTRSS/volume3/UN-26/3-1-61-67.pdf

Baby, A. R., Migliato, K. F., Maciel, C. P. M., Zague, V., Pinto, C. A. S. de O., Salgado, H. R. N., … Velasco, M. V. R. (2007). Accelerated chemical stability data of O/W fluid emulsions containing the extract of Trichilia catigua Adr. Juss (and) Ptychopetalum olacoides Bentham. *Revista Brasileira de Ciências Farmacêuticas*, *43*(3). https://doi.org/10.1590/S1516-93322007000300009

Barile, F. A. (2010). Validating and troubleshooting ocular in vitro toxicology tests. *Journal of Pharmacological and Toxicological Methods*, *61*(2), 136–145. https://doi.org/10.1016/j.vascn.2010.01.001

Bastos M. L. A., Lima M. R. F., Conserva L. M., Andrade V. S., Rocha E. M. M., L. R., & L., P. (2009). Studies on the antimicrobial activity and brine shrimp toxicity of Zeyheria

tuberculosa ( Vell .) Bur . ( Bignoniaceae ) extracts and their main constituents. *Annals of Clinical Microbiology and Antimicrobials*, *8*(16). https://doi.org/10.1186/1476-0711-8-16

Bogdanov, S. (2011). Functional and Biological Properties of the Bee Products - a Review. *Bee Products Science*, (February), 1–12. Retrieved from www.bee-hexagon.net

Bogdanov, S. (2012). The Bee Pollen Book. In *The Bee Pollen Book* (pp. 1–13). Retrieved from http://www.bee-hexagon.net/en/pollen.htm

Bogdanov, S. (2016). Pollen : Production , Nutrition and Health : A Review, (April), 1–36.

Bounatirou, S., Smiti, S., Miguel, M. G., Faleiro, L., Rejeb, M. N., Neffati, M., … Pedro, L. G. (2007). Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian Thymus capitatus Hoff. et Link. *Food Chemistry*, *105*(1), 146–155. https://doi.org/10.1016/j.foodchem.2007.03.059

Burlando, B., Verotta, L., Laura;, C., & Botiinini-Massa, E. (2010). *Herbal principles in cosmetics: Properties and mechanisms of action*. CRC Press.

Campos, M. G. R., Frigerio, C., Lopes, J., & Bogdanov, S. (2010). What is the future of Bee-Pollen? *Journal of Apiproduct and Apimedical Science*, *2*(4), 131–144. https://doi.org/10.3896/IBRA.4.02.4.01

Campos, M. G., Webby, R. F., Markham, K. R., Mitchell, K. A., & Da Cunha, A. P. (2003). Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. *Journal of Agricultural and Food Chemistry*, *51*(3), 742–745. https://doi.org/10.1021/jf0206466

Carpes, S. T., de Alencar, S. M., Cabral, I. S. R., Oldoni, T. L. C., Mourão, G. B., Haminiuk, C. W. I., … Masson, M. L. (2013). Polyphenols and palynological origin of bee pollen of *Apis mellifera* L. from Brazil. Characterization of polyphenols of bee pollen. *CyTA - Journal of Food*, *11*(2), 150–161. https://doi.org/10.1080/19476337.2012.711776

Čeksteryte, V., Kurtinaitiene, B., Venskutonis, P. R., Pukalskas, A., Kazernavičiute, R., & Balžekas, J. (2016). Evaluation of antioxidant activity and flavonoid composition in differently preserved bee products. *Czech Journal of Food Sciences*, *34*(2), 133–142. https://doi.org/10.17221/312/2015-CJFS

Chen, Q. (2009). Evaluate the Effectiveness of the Natural Cosmetic Product Compared to Chemical-Based Products. *International Journal of Chemistry*, *1*(Figure 2), 57–59.

Choi, J.-H., Jang, Y.-S., Oh, J.-W., Kim, C.-H., & Hyun, I.-G. (2015). Bee Pollen-Induced Anaphylaxis: A Case Report and Literature Review. *Allergy Asthma & Immunology Research*, *7*(5), 513–517. https://doi.org/10.4168/aair.20153.5.513

Committee, S., & Sccp, C. P. (2007). *Opinion of the SCCP on phthalates in cosmetic products.Sccp/1016/06*.

Retrieved from ec.europa.eu/health/ph_risk/committees/04_sccp/.../sccp_o_106.pdf

Committee for Risk Assessment RAC. (2012). RAC opinion on an Annex XV dossier proposing restrictions on four phthalate, (June). Retrieved from file:///C:/Users/Jana/Downloads/rac_opinion_phthalates_restriction_en.pdf

Dandlen, S. A., Miguel, M. G., Duarte, J., Faleiro, M. L., Sousa, M. J., Lima, A. S., … Pedro, L. G. (2011). Acetylcholinesterase Inhibition Activity of Portuguese Thymus Species Essential Oils. *Journal of Essential Oil Bearing Plants*, *14*(2), 140–150. https://doi.org/10.1080/0972060X.2011.10643914

Denisowa Bozena, & Denisow-Pietrzyk Marta. (2016). Biological and therapeutic properties of bee pollen: a review. *Journal of the Science of Food and Agriculture*, *96(13)*(March), 4303–4309. https://doi.org/10.1002/jsfa.7729

Deuschle, V. C. K. N., Deuschle, R. A. N., Bortoluzzi, M. R., & Athayde, M. L. (2015). Physical chemistry evaluation of stability, spreadability, in vitro antioxidant, and photo-protective capacities of topical formulations containing Calendula officinalis L. leaf extract Viviane. *Journal of Pharmaceutical Sciences*, *51*(1), 63–75.

Devappa, R. K., Rakshit, S. K., & Dekker, R. F. H. (2015). Forest biorefinery: Potential of poplar phytochemicals as value-added co-products. *Biotechnology Advances*, *33*(6), 681–716. https://doi.org/10.1016/j.biotechadv.2015.02.012

Dreger, M., & Wielgus, K. (2013). Application of essential oils as natural cosmetic preservatives. *Herba Polonica*, *59*(4). https://doi.org/10.2478/hepo-2013-0030

Ediriweera, E. R. H. S. S., & Premarathna, N. Y. S. (2012). Medicinal and cosmetic uses of Bee′s Honey - A review. *AYU (An International Quarterly Journal of Research in Ayurveda)*, *33*(2), 178. https://doi.org/10.4103/0974-8520.105233

Ehivet, F. E., Min, B., Park, M. K., & Oh, J. H. (2011). Characterization and Antimicrobial Activity of Sweetpotato Starch-Based Edible Film Containing Origanum (Thymus capitatus) Oil. *Journal of Food Science*, *76*(1). https://doi.org/10.1111/j.1750-3841.2010.01961.x

El Abed, N., Kaabi, B., Smaali, M. I., Chabbouh, M., Habibi, K., Mejri, M., … Ben Hadj Ahmed, S. (2014). Chemical composition, antioxidant and antimicrobial activities of thymus capitata essential oil with its preservative effect against listeria monocytogenes inoculated in minced beef meat. *Evidence-Based Complementary and Alternative Medicine*, *2014*. https://doi.org/10.1155/2014/152487

Elmorsy, T. H., & Hafez, E. A. (2016). Microbial Contamination of Some Cosmetic Preparations in Egypt. *International Journal of Agricultural Technology, 12*(3), 567–577.

Erkmen, O., & Özcan, M. M. (2008). Antimicrobial Effects of Turkish Propolis, Pollen, and Laurel on Spoilage and Pathogenic Food-Related Microorganisms. *Journal of Medicinal Food*, *11*(3), 587–592. https://doi.org/10.1089/jmf.2007.0038

European Commission. (2009). Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. *Official Journal of the European Union*, (1223), 342–359.

European commission Enterprise Directorate-General Pharmaceuticals and cosmetics. (1999). Cosmetics legislation: Cosmetic products, *1*, 7.

Faleiro, L., Miguel, G., Gomes, S., Costa, L., Venâncio, F., Teixeira, A., … Pedro, L. G. (2005). Antibacterial and antioxidant activities of essential oils isolated from Thymbra capitata L. (Cav.) and Origanum vulgare L. *Journal of Agricultural and Food Chemistry*, *53*(21), 8162–8168. https://doi.org/10.1021/jf0510079

Feas, X., Vazquez-Tato, M. P., Estevinho, L., Seijas, J. A., & Iglesias, A. (2012). Organic bee pollen: Botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. *Molecules*, *17*(7),
8359–8377. https://doi.org/10.3390/molecules17078359

Figueiredo, A. C., Barroso, J. G., Pedro, L. G., Salgueiro, L., Miguel, M. G., & Faleiro, M. L. (2008). Portuguese Thymbra and Thymus species volatiles: chemical composition and biological activities. *Current Pharmaceutical Design*, *14*(29), 3120–3140. https://doi.org/10.2174/138161208786404218

Garner, N., Garner, N., Siol, A., & Eilks, I. (2014). Parabens as preservatives in personal care products Parabens as preservatives in personal care products, (November 2015).

Gee, R. H., Charles, A., Taylor, N., & Darbre, P. D. (2008). Oestrogenic and androgenic activity of triclosan in breast cancer cells. *Journal Of Applied Toxicology*, *28*, 78–91. https://doi.org/10.1002/jat

Gonzalez, G., Hinojo, M. J., Mateo, R., Medina, A., & Jiménez, M. (2005). Ocurrence of mycotoxin producing fungi in bee pollen. *International Journal of Food Microbiology*, *105(1)*(July 2017), 1–9. https://doi.org/10.1016/j.ijfoodmicro.2005.05.001

Graikou, K., Kapeta, S., Aligiannis, N., Sotiroudis, G., Chondrogianni, N., Gonos, E., & Chinou, I. (2011). Chemical analysis of Greek pollen - Antioxidant, antimicrobial and proteasome activation properties. *Chemistry Central Journal*, *5*(1), 33.

https://doi.org/10.1186/1752-153X-5-33

Hazzit, M., Baaliouamer, A., Faleiro, M. L., & Miguel, M. G. (2006). Composition of the essential oils of Thymus and Origanum species from Algeria and their antioxidant and antimicrobial activities. *Journal of Agricultural and Food Chemistry*, *54*(17), 6314–6321. https://doi.org/10.1021/jf0606104

Hazzit, M., Baaliouamer, A., Veríssimo, A. R., Faleiro, M. L., & Miguel, M. G. (2009). Chemical composition and biological activities of Algerian Thymus oils. *Food Chemistry*, *116*(3), 714–721. https://doi.org/10.1016/j.foodchem.2009.03.018

Herman, A., Herman, A. P., Domagalska, B. W., & Młynarczyk, A. (2013). Essential Oils and Herbal Extracts as Antimicrobial Agents in Cosmetic Emulsion. *Indian Journal of Microbiology*, *53*(2), 232–237. https://doi.org/10.1007/s12088-012-0329-0

Jagdis, A., & Sussman, G. (2012). Anaphylaxis from bee pollen supplement. *Canadian Medical Association Journal*, *184*(10), 1167–1169. https://doi.org/10.1503/cmaj.112181

Jain, N., & Chaudhri, S. (2009). History of cosmetics. *Asian Journal of Pharmaceutics*, *3*(3), 164. https://doi.org/10.4103/0973-8398.56292

Joshi LS, & HA, P. (2015). Herbal Cosmetics and Cosmeceuticals: An Overview. *Natural Products Chemistry & Research*, *3*(2), 170. https://doi.org/10.4172/2329-6836.1000170

Kacániová, M., Vuković, N., Chlebo, R., Haščík, P., Rovná, K., Cubon, J., … Pasternakiewicz, A. (2012). The antimicrobial activity of honey, bee pollen loads and beeswax from Slovakia. *Archives of Biological Sciences*, *64*(3), 927–934. https://doi.org/10.2298/ABS1203927K

Kaur, R., Kalia, P., Kumar, N. R., & Harjai, K. (2013). Preliminary studies on different extracts of some honey bee products, (2004), 3–5.

Kaur Loveleen Preet, K. G. T. (2013). Topical Gel: A Recent Approach for Novel Drug delivery. *Asian Journal of Biomedical and Pharmaceutical Sciences, 3*(17), 1–5.

Kerdudo, A., Burger, P., Merck, F., Dingas, A., Rolland, Y., Michel, T., & Fernandez, X. (2016a). Comptes Rendus Chimie Development of a natural ingredient e Natural preservative : A case study eveloppement d â€™ un ingr edient naturel : Etude de cas d â€™ un conservateur naturel. *Comptes Rendus - Chimie*, *19*(9), 1077–1089. https://doi.org/10.1016/j.crci.2016.06.004

Kerdudo, A., Burger, P., Merck, F., Dingas, A., Rolland, Y., Michel, T., & Fernandez, X. (2016b). Development of a natural ingredient – Natural preservative: A case study. *Comptes Rendus Chimie*, *19*(9), 1077–1089. https://doi.org/10.1016/j.crci.2016.06.004

Komosinska-Vassev, K., Olczyk, P., Kazmierczak, J., Mencner, L., & Olczyk, K. (2015). Bee

pollen: chemical composition and therapeutic application. *Evidence-Based Complementary and AlternativeMedicine*, *2015*, 1–6. https://doi.org/http://dx.doi.org/10.1155/2015/297425 Review

Kreck, M., Scharrer, A., Bilke, S., & Mosandl, A. (2002). Variability in essential oil composition of Tunisian Thymus capitatus (L.) Hoffmanns. et Link. *Flavour and Fragrance Journal*, *17*(1), 26–28. https://doi.org/10.1002/ffj.1029

Kumar, D., Rajora, G., Parkash, O., Antil, M., & Kumar, V. (2016). Herbal cosmetics : An overview. *I. J. Pharma Res Review,* *5*(12), 36–41.

LeBlanc, B. W., Davis, O. K., Boue, S., DeLucca, A., & Deeby, T. (2009). Antioxidant activity of Sonoran Desert bee pollen. *Food Chemistry*, *115*(4), 1299–1305. https://doi.org/10.1016/j.foodchem.2009.01.055

Lourith, N., & Kanlayavattanakul, M. (2009). Natural surfactants used in cosmetics: Glycolipids. *International Journal of Cosmetic Science*, *31*(4), 255–261. https://doi.org/10.1111/j.1468-2494.2009.00493.x

Lucero, M. J., Vigo, J., & Lecin, M. J. (1994). A study of shear and compression deformations gels of tretinoin on hydrophilic. *International Journal of Pharmaceutics*, *106*, 125–133.

Lundov, M. D., Moesby, L., Zachariae, C., & Johansen, J. D. (2009). Contamination versus preservation of cosmetics: A review on legislation, usage, infections, and contact allergy. *Contact Dermatitis*, *60*(2), 70–78. https://doi.org/10.1111/j.1600-0536.2008.01501.x

Mărgăoan, R., Mărghitaş, L. A., Dezmirean, D., Mihai, C. M., & Bobiş, O. (2010). Bee Collected Pollen – General Aspects and Chemical Composition. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies*, *67*(January), 1–2.

Marx, S. (2004). *Guidelines on Stability Testing of Cosmetic Products*. *The European Cosmetic Toiletry And Perfumery Asociation Colipa*.

Mclaughlin, J. L., Rogers, L. L., & Anderson, J. E. (1998). THE USE OF BIOLOGICAL ASSAYS TO EVALUATE BOTANICALS. *Drug Information Journal, 32*(2), 513–524.

Miguel, G., Simões, M., Figueiredo, A. C., Barroso, J. G., Pedro, L. G., & Carvalho, L. (2004). Composition and antioxidant activities of the essential oils of Thymus caespititius, Thymus camphoratus and Thymus mastichina. *Food Chemistry*, *86*(2), 183–188. https://doi.org/10.1016/j.foodchem.2003.08.031

Miguel, M. G. (2010). Antioxidant and anti-inflammatory activities of essential oils: A short review. *Molecules*, *15*(12), 9252–9287. https://doi.org/10.3390/molecules15129252

Miroslava Kačániová , Miroslav Juráček , Róbert Chlebo , Vladimíra Kňazovická , Miriam Kadasi-Horáková , Simona Kunová , Jadža Lejková , Peter Haščík, J. M. & M. Š. (2011). Journal of Environmental Science and Health , Part B : Mycobiota and mycotoxins in bee pollen collected from different areas of Slovakia Mycobiota and mycotoxins in bee pollen collected from different areas of Slovakia. *Journal of Environmental Science and Health,, Part B: Pesticides, Food Contaminants, and Agricultural Wastes*, *46(7)*(October 2014), 623–629. https://doi.org/10.1080/03601234.2011.589322

Mitsui T. (1997). 5 Raw materials of cosmetics. In *New cosmetic science* (1st ed., pp. 121–147). Elsevier.

Mizrahi. (1997). Herbal Plants: Used as a cosmetics. *J. Nat. Prod. Plant Resour*, *1*(1), 24–32.

Mkaddem, M. G., Romdhane, M., Ibrahim, H., Ennajar, M., Lebrihi, A., Mathieu, F., & Bouajila, J. (2010). Essential oil of Thymus capitatus Hoff. et Link. from Matmata, Tunisia: gas chromatography-mass spectrometry analysis and antimicrobial and antioxidant activities. *Journal of Medicinal Food*, *13*(6), 1500–1504. https://doi.org/10.1089/jmf.2009.0117

Morais, M., Moreira, L., Feás, X., & Estevinho, L. M. (2011). Honeybee-collected pollen from five Portuguese Natural Parks: Palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food and Chemical Toxicology*, *49*(5), 1096–1101. https://doi.org/10.1016/j.fct.2011.01.020

Murthy, P. S., Suzuki, S., & Kusumoto, K. I. (2015). Effect of Light on the Growth and Acid Protease Production of Aspergillus oryzae. *Food Science and Technology Research*, *21*(4), 631–635. https://doi.org/10.3136/fstr.21.631

Nabavi, S. M., Marchese, A., Izadi, M., Curti, V., Daglia, M., & Nabavi, S. F. (2015). Plants belonging to the genus Thymus as antibacterial agents: From farm to pharmacy. *Food Chemistry*, *173*, 339–347. https://doi.org/10.1016/j.foodchem.2014.10.042

Naidu, J. R., Ismail, R., & Sasidharan, S. (2014). Acute oral toxicity and brine shrimp lethality of methanol extract of Mentha Spicata L (Lamiaceae). *Tropical Journal of Pharmaceutical Research*, *13*(1), 101–107. https://doi.org/10.4314/tjpr.v13i1.15

Nakajima, Y., Tsuruma, K., Shimazawa, M., Mishima, S., & Hara, H. (2009). Comparison of bee products based on assays of antioxidant capacities. *BMC Complementary and Alternative Medicine*, *9*(1), 4. https://doi.org/10.1186/1472-6882-9-4

Nathalie, D., Yannick, G., Caroline, B., Sandrine, D., Claude, F., Corinne, C., & Pierre-Jacques, F. (2006). Assessment of the phototoxic hazard of some essential oils using modified 3T3 neutral red uptake assay. *Toxicology in Vitro*, *20*(4), 480–489.

https://doi.org/10.1016/j.tiv.2005.08.018

Okereke, J. N., Udebuani, A. C., Ezeji, E. U., Obasi, K. O., & Nnoli, M. C. (2015). Possible Health Implications Associated with Cosmetics : A Review. *Science Journal of Public Health, 3*, 58–63. https://doi.org/10.11648/j.sjph.s.2015030501.21

Ota, M., & Yokoyama, M. (2010). Comprehensive Natural Products II. *Comprehensive Natural Products II*, 317–349. https://doi.org/10.1016/B978-008045382-8.00728-0

Otang, M. (2013). Assessment of potential toxicity of three South African medicinal plants using the brine shrimp (Artemia salina) assay. *African Journal of Pharmacy and Pharmacology*, *7*(20), 1272–1279. https://doi.org/10.5897/AJPP12.264

Pandey, D., & Wasule, D. (2017). Ejpmr Evaluation of Sugar Alcohol : Humectant in Skin Care Cosmetic, *4*(2), 715–718.

Pauwels, M., & Rogiers, V. (2010). Human health safety evaluation of cosmetics in the EU: a legally imposed challenge to science. *Toxicology and Applied Pharmacology*, *243*(2), 260–274. https://doi.org/10.1016/j.taap.2009.12.007

Petrovic, T., Nedic, N., Paunovic, D., Rajic, J., Matovic, K., Radulovic, Z., & Krnjaja, V. (2014). Natural mycobiota and aflatoxin B1 presence in bee pollen collected in Serbia. *Biotechnology in Animal Husbandry*, *30*(4), 731–741. https://doi.org/10.2298/BAH1404731P

Pham-Huy, L. A., He, H., & Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science : IJBS*, *4*(2), 89–96. https://doi.org/10.1073/pnas.0804252105

Pina-Vaz, C., Rodrigues, A. G., Pinto, E., Costa-de-Oliveira, S., Tavares, C., Salgueiro, L., … Martinez-de-Oliveira, J. (2004). Antifungal activity of Thymus oils and their major compounds. *Journal of the European Academy of Dermatology and Venereology*, *18*(1), 73–78. https://doi.org/10.1111/j.1468-3083.2004.00886.x

Prusakiewicz, J. J., Harville, H. M., Zhang, Y., Ackermann, C., & Voorman, R. L. (2007). Parabens inhibit human skin estrogen sulfotransferase activity: Possible link to paraben estrogenic effects. *Toxicology*, *232*(3), 248–256. https://doi.org/10.1016/j.tox.2007.01.010

Rajabi, S., Ramazani, A., Hamidi, M., & Naji, T. (2015). Artemia salina as a model organism in toxicity assessment of nanoparticles. *DARU Journal of Pharmaceutical Sciences*, *23*(1), 20. https://doi.org/10.1186/s40199-015-0105-x

Regina da Silva, G., Botelho da Natividade, T., Amorim Camara, C., Monica Sarmento da Silva, E., de Assis Ribeiro dos Santos, F., & Maria Sarmento Silva, T. (2014).

Identification of Sugar, Amino Acids and Minerals from the Pollen of Jandaíra Stingless Bees (Melipona subnitida). *Food and Nutrition Sciences*, *5*(5), 1015–1021. https://doi.org/10.4236/fns.2014.511112

SCCS. (2013). *Scientific Committee on Consumer Safety Parabens Updated request for a scientific opinion on propyl- and butylparaben COLIPA n ° P82*. https://doi.org/10.2772/66369

SCCS. (2014). *Scientific Committee on Consumer Safety the safety of the use of formaldehyde in nail hardeners*. https://doi.org/10.2772/51186

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., & Lichtensteiger, W. (2001). In vitro and in vivo estrogenicity of UV screens. *Environmental Health Perspectives*, *109*(3), 239–244. https://doi.org/10.1289/ehp.109-a359

Shivanand, P., Nilam, M., & Viral, D. (2010). Herbs play an important role in the field of cosmetics. *International Journal of PharmTech Research*, *2*(1), 632–639.

Shubharani, R., Roopa, P., & Sivaram, V. (2013). Pollen Morphology of Selected Bee Forage Plants. *Global Journal of Bio Science and Biotechnology*, *2*(1), 82–90.

Stahl-Biskup, E. (1991). The chemical composition of Thymus oils: A review of the literature 1960–1989. *Journal of Essential Oil Research*, *3*(2), 61–82. https://doi.org/10.1080/10412905.1991.9697915

Stahl-Biskup, E., & Sáez, F. (2002). Thyme - The genus Thyme L., *15*, 330. Retrieved from https://books.google.com/books/about/Thyme.html?id=9jnpVP6fvUIC&pgis=1

Stahl-Biskup, E., & Venskutonis, R. P. (2012). Thyme. *Handbook of Herbs and Spices*, 499–525. https://doi.org/10.1533/9780857095671.499

Steiling, W., Bracher, M., Courtellemont, P., & De Silva, O. (1999). The HET-CAM, a useful in vitro assay for assessing the eye irritation properties of cosmetic formulations and ingredients. *Toxicology in Vitro*, *13*(2), 375–384. https://doi.org/10.1016/S0887-2333(98)00091-5

Sumit, K., Vivek, S., Sujata, S., & Ashish, B. (2012). Herbal Cosmetics : Used for Skin and Hair Herbal Cosmetics : Used for Skin and Hair. *Inventi Journals*, (4), 1–7.

Taylor, K. M., Weisskopf, M., & Shine, J. (2014). Human exposure to nitro musks and the evaluation of their potential toxicity : an overview, 1–7.

Tongnuanchan, P., & Benjakul, S. (2014). Essential Oils: Extraction, Bioactivities, and Their Uses for Food Preservation. *Journal of Food Science*, *79*(7), 1231–1249. https://doi.org/10.1111/1750-3841.12492

Tsutsumi, L. H., & Oishi, D. E. (2010). *Farm and Forestry Production and Marketing Profile*

*for Honey for honey bees*. (C. R. Elevitch, Ed.). Specialty Crops for Pacific Island Agroforestry. Permanent Agriculture Resources (PAR), Holualoa,.

Ulbricht, C., Conquer, J., Giese, N., Khalsa, K. P. S., Sklar, J., Weissner, W., & Woods, J. (2009). An evidence-based systematic review of bee pollen by the Natural Standard Research Collaboration. *Journal of Dietary Supplements*, *6*(3), 290–312. https://doi.org/10.1080/19390210903081381

Ultee, A., Bennik, M. H. J., & Moezelaar, R. (2002). The Phenolic Hydroxyl Group of Carvacrol Is Essential for Action against the Food-Borne Pathogen Bacillus cereus The Phenolic Hydroxyl Group of Carvacrol Is Essential for Action against the Food-Borne Pathogen Bacillus cereus. *Applied and Environmental Microbiology*, *68*(4), 1561–1568. https://doi.org/10.1128/AEM.68.4.1561

Varvaresou, A., Papageorgiou, S., Tsirivas, E., Protopapa, E., Kintziou, H., Kefala, V., & Demetzos, C. (2009). Self-preserving cosmetics. *International Journal of Cosmetic Science*, *31*(3), 163–175. https://doi.org/10.1111/j.1468-2494.2009.00492.x

Vernet, P., Gouyon, R. H., & Valdeyron, G. (1986). Genetic control of the oil content in Thymus vulgaris L: a case of polymorphism in a biosynthetic chain. *Genetica*, *69*(3), 227–231. https://doi.org/10.1007/BF00133526

WALTERS, A., SANTILLO, D., & JOHNSTON, . (2005). Environmental and human health concerns relating to synthetic musk compounds. *Technical Note 07/2005. Greenpeace Research Laboratories, Exeter, UK.*, (June), 1–13.

Wang, C., & Tian, Y. (2015). Reproductive endocrine-disrupting effects of triclosan : Population exposure , present evidence and potential mechanisms. *Environmental Pollution*, *206*, 195–201. https://doi.org/10.1016/j.envpol.2015.07.001

## Web sites:

http://www.coolnsmart.com/nature_quotes/

https://news.mongabay.com/2011/04/what-does-nature-give-us-a-special-earth-day-article/

https://honeybeesuite.com/the-role-of-pollen-in-honey-bee-nutrition

www.studyblue.com/notes/note/n/lecture-5-biology-ofpollen/deck/13973131

http://honeypedia.info/what-is-pollen-what-role-plays-palynology

https://fr.wikipedia.org/wiki/Pollen

https://en.wikipedia.org/wiki/

http://www.centerchem.com/Products/DownloadFile.aspx?FileID=6982

# ANNEX I

**Table 13:** Gel code (code attributed to each formulation).

| Gels code | Compound incorporated |
|---|---|
| **G1** | Control |
| **G2** | *Thymus zygis zygis* |
| **G3** | *Thymus capitatus* |
| **G4** | Mixture of the two essential oils |
| **G5** | Pollen |
| **G6** | Pollen+*Thymus zygis zygis* |
| **G7** | Pollen+*Thymus capitatus* |
| **G8** | Pollen+2Eos |
| **G9** | Methylparaben |
| **G10** | Methylparaben+*Thymus zygis zygis* |
| **G11** | Methylparaben+*Thymus capitatus* |
| **G12** | Methylparaben+2Eos |
| **G13** | Methylparaben+pollen |
| **G14** | Methylparaben+pollen+*Thymus zygis zygis* |
| **G15** | Methylparaben+pollen+*Thymys capitatus* |
| **G16** | Methylparaben+pollen+2Eos |

# ANNEX II

## Evaluation of viscosity during testing period

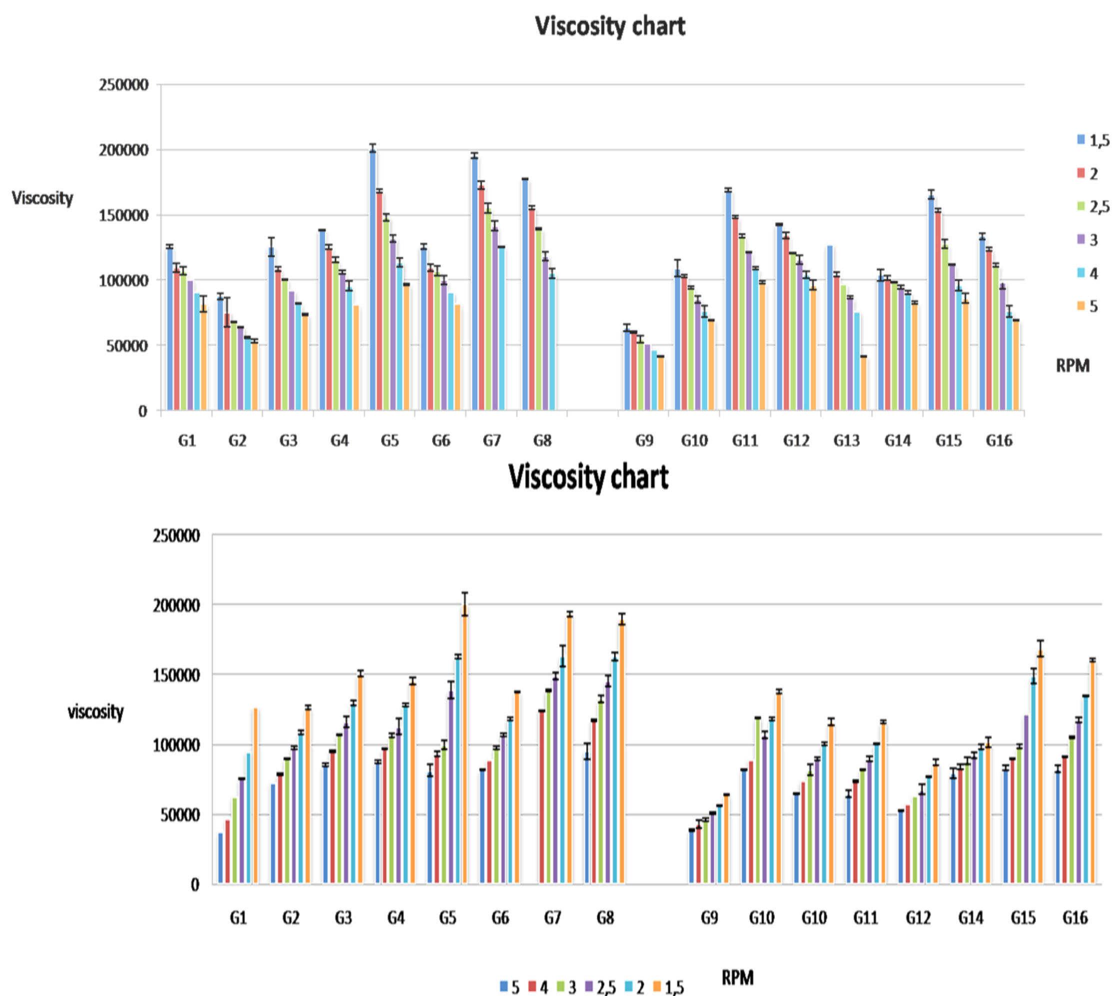



**Figure 28:** Evaluation of viscosity for all cosmetic formulations during the first week.

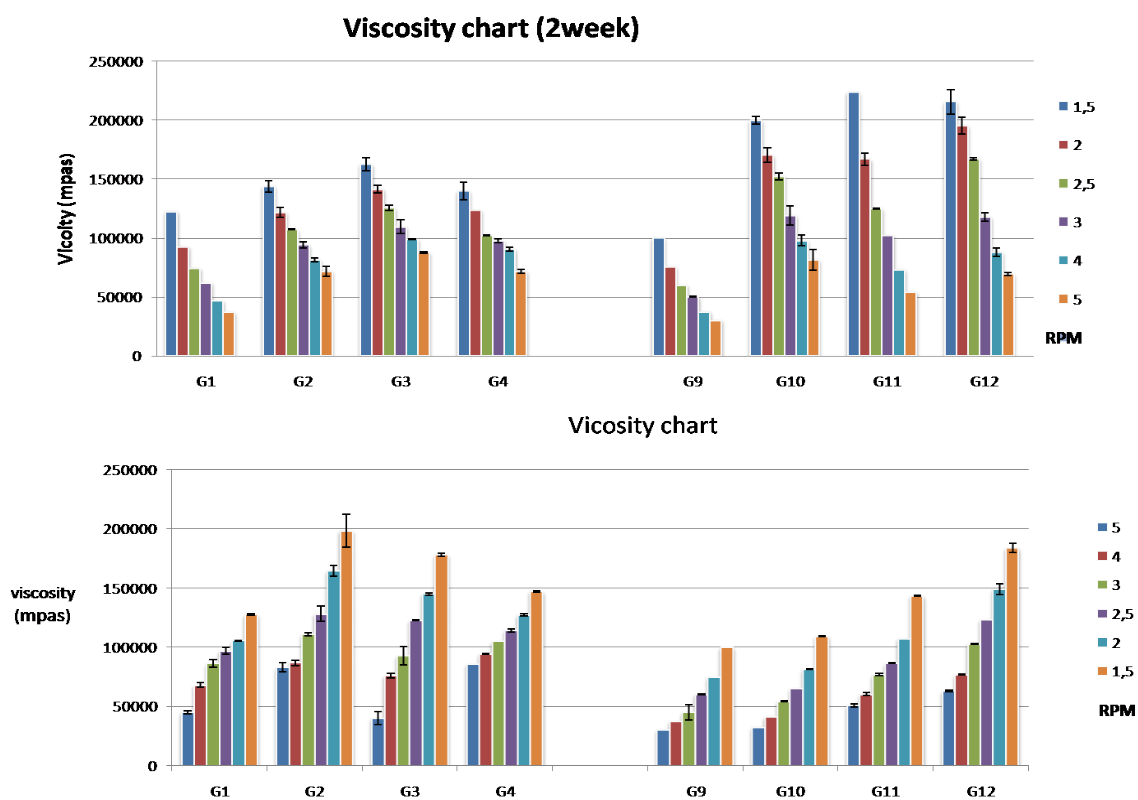



**Figure 29:** Evaluation of viscosity for all cosmetic formulations during the second week.