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NANOCARRIERS FOR SKIN DELIVERY OF COSMETIC ANTIOXIDANTS

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Escola de Ciências e Tecnologias da Saúde

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Abstract

Ethosomes are composed by phospholipids, ethanol and water. Ethanol is an efficient permeation enhancer that affects the intercellular region of the stratum corneum.

Their application on cosmetic products aims to increase the stability of the cosmetic chemicals, to decrease skin irritation from other cosmetic compounds and improve skin permeation.

In this study, we compared different well known antioxidants in the cosmetic industry, such as ferulic acid and Rutin. The concentrations studied were 0.03%, 0.05%, 0.075% and 0.1% (w / v) for Rutin and 0.03%, 0.05% and 0.075% (w / v) for ferulic acid.

The colloidal dispersions were analyzed for size, zeta potential, PDI, pH and antioxidant activity. Emulsions with ethosomes and with methanolic and aqueous solutions were also analyzed for their organoleptic properties, pH and antioxidant activity.

Results showed that the antioxidant activity of Rutin and ferulic acid was higher when encapsulated in ethosomes. In addition, the association of ionic liquids to these vesicular systems increases the solubility of the active substances, increasing their antioxidant activity. It is still possible to conclude that the size of ethosomes encapsulated with Rutin and ionic liquid decreases dramatically for the ethosomes with ferulic acid. The zeta potential and the polydispersity index showed that the stability and homogeneity of the ethosomes have to be continuously evaluated.

Key-words: Ethosomes; Nanocarriers; Aqueous solution; Methanolic solution; Ferulic Acid; Rutin; Antioxidant Activity; Emulsion.

Resumo

Os etossomas são compostos por fosfolípidos, etanol e água. O etanol é um eficiente potenciador da permeação, perturbação da bicamada lipídica da pele, que afeta a região intercelular do estrato córneo.

A sua aplicação em produtos cosméticos tem em vista aumentar a estabilidade dos produtos químicos cosméticos, diminuir a irritação da pele de outros compostos cosméticos e melhorar a permeação da pele.

Neste estudo, comparamos diferentes antioxidantes bem conhecidos na indústria de cosméticos, como o Ácido Ferúlico e a Rutina. As concentrações estudadas foram 0,03%, 0,05%, 0,075% e 0,1% para Rutina e 0,03%, 0,05% e 0,075% (p / v) para ácido ferúlico.

As dispersões coloidais foram analisadas quanto ao tamanho, potencial zeta, PDI, pH e atividade antioxidante. As emulsões com etossomas e com soluções metanólicas e aquosas também foram analisadas quanto às propriedades organolépticas, pH e atividade antioxidante.

Os resultados mostraram que a atividade antioxidante da Rutina e do ácido ferúlico foi maior quando encapsulada em etossomas. Além disso, a associação de líquidos iónicos a esses sistemas vesiculares aumenta a solubilidade das substâncias ativas, aumentando a sua atividade antioxidante. Ainda é possível concluir que o tamanho dos etossomas encapsulados com Rutina e líquido iónico diminui drasticamente em relação aos etossomas com Ácido Ferúlico. O potencial zeta e o índice de polidispersidade mostraram que a estabilidade e homogeneidade dos etossomas deve ser avaliada continuamente.

Palavras-chave: Etossomas; Sistemas de veiculação de fármacos; Solução aquosa; Solução metanólica; Ácido ferúlico; Rutina; Atividade antioxidante; Emulsão.

Abbreviations

BTH – Butylated hydroxytoluene

DPPH – 2,2-diphenyl-1-picrylhydrazil

ET – Ethosomes

FA – Ferulic Acid

FRS – Free Radical Scavenging

IL – Ionic Liquids

Met. - Methanolic

PDI – Polydispersity index

PEG - Polyethylene glycol

ROS – Reactive Oxygen species

RT – Room Temperature

Rut - Rutin

SC – Stratum Corneum

Sol. – Solutions

UV – Ultraviolet

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Introduction

Skin

Skin is the largest organ of the human body. It is not just a layer that covers the body, it fulfills several functions, such as defensive, thermoregulatory, metabolic, sensory, resorptive and excreting (Darlenski, Kazandjieva, & Tsankov, 2011). Of these functions, the most critical is the permeability barrier that slows down the transepidermic loss of water (Elias, 2008).

The skin has three main layers with very different structures, the epidermis, the dermis and hypodermis.

The bottom layer of the skin is hypodermis, subcutaneous fat. This layer helps the blood vessels and nerve cells and it also stores fat to be transformed into energy as needed (Kolarsick, Kolarsick, & Goodwin, 2008).

The dermis is the middle layer, it provides flexibility, elasticity and tensile strength. It is made of a fibrillar structure protein better known as collagen. The thickness of this layer depends on the location in the body. Collagen fibers are resistant to deformation and mechanical stress on the skin (Kolarsick et al., 2008; Wickett & Visscher, 2006).

The outer layer of the skin is epidermis, which it is composed of two types of cells, the keratinocytes and dendritic cells. It consists of several layers such as basal stratum, spinosum layer, granulosum layer, lucidum stratum and stratum corneum, SC, (Bouwstra & Ponec, 2006). The basal layer, stratum germinativum, contains keratinocytes that are attached to the dermis. The basal cells form a single layer that join together through the desmosomes junctions. This layer has melanin pigments and is the primary location of mitotically active cells (Kolarsick et al., 2008). The stratum spinosum is composed of different cells with different chaps, structures and subcellular properties, depending on where it is located. In this layer the bottom cells are polyhedral in shape and have a rounded nucleus however the upper cells are bigger in size and are flettend as they are pushed into the surface of the skin (Kolarsick et al., 2008). The granulosum layer isn't the outer layer of the skin but it is the last layer with living cells, and it is composed by flattened cells that hold keratohyalin granules on their cytoplasm. This layer varies in thickness and in proportion to the over laying horny cells layer. The keratohyalin granules are basophilic and irregular in shape and size (Kolarsick et al., 2008). The lucidum stratum is located between granulosum layer and SC. The cells are tightly compressed, flattened and indistinguishable from one another (Wickett & Visscher, 2006).

The SC in addition to protecting against water loss is also a barrier against the entry of microorganisms, has an acidic pH in the range of 4 to 6.5 (Yosipovitch et al., 1998). It

serves many purposes including: helping the native antioxidant system, starting the native immune responses of the host and as previously stated, being a barrier against external threats such as ultraviolet radiation, toxins, allergens, and pathogens (Prescott et al., 2017). It is a heterogeneous layer consisting of terminally differentiated keratinocytes, corneocytes. Every actively proliferating keratinocyte is the root of a corneocyte (Rawlings & Harding, 2004). As mentioned before the keratinocytes are found in the epidermis and arranged in layers. These cells are formed in the basal layer, which is the closest to the dermis, as they start to divide and proliferate that layer, they begin to differentiate. This process is called keratinization and involves the production of specialized structural proteins, secretion of lipids, and the formation of a cellular envelope of crosslinked proteins. During this process, virtually all of the subcellular organelles disappear, including the nucleus. In this extrusion process their content is pressed into the intercellular space. There, the polar lipid precursors are enzymatically converted into nonpolar products and assembled into lamellar structures surrounding the corneocytes. Therefore, the point of contact of the epidermis with the environment is composed of flattened metabolically dead cells (Bouwstra & Ponec, 2006; Pullar, Carr, & Vissers, 2017).

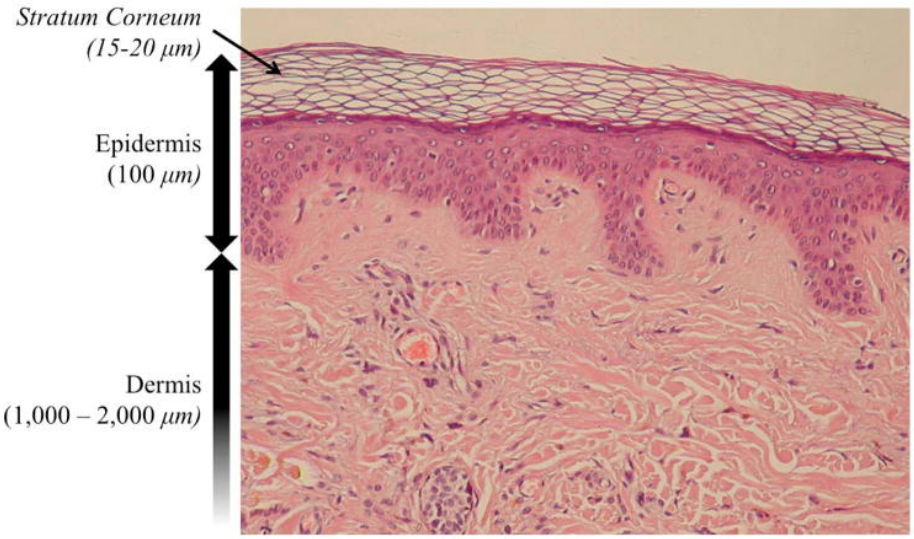

Figure 1 Histological cross-section of the skin (Schoellhammer, Blankschtein, & Langer, 2014).

The SC is not totally impermeable to substances applied directly to the surface of the skin, however it hinders the flux of substances through skin (Barry, 2001; Darlenski et al., 2011).

Antioxidants and Aging

Nowadays more and more cosmetics are used, since people have more concerns about aesthetics (Iohani & Verma, 2017).

There are various factors that help the process of skin aging or commonly known as photoaging such as genetics, hormones and environment. Intrinsic aging or natural aging and extrinsic aging or aging caused by external factors have visible effects on skin but only extrinsic aging can be fought. The skin natural aging usually starts in the 20's with slight slowed production of collagen and elastin, decreased cellular regeneration and thinning as sagging skin. These effects become more noticeable as years go by (Iohani & Verma, 2017).

Antioxidants help to protect the skin from oxidative stress by helping to minimize damage from free radicals and other agents. Although antioxidants can reduce free radicals damage and inhibit inflammation, protection against photo damage and skin cancer, there is no agent that works well enough on humans. Most of the studies made with antioxidants have incomprehensive or inconclusive results (Oroian & Escriche, 2015).

The topical delivery of antioxidant main problem is its instability in the formulations and ineffective penetration into the skin.

There has been reports that say that vesicular systems have an important role in maintaining the stability of the active ingredient through the layers of skin right to the cellular level where they are more effective (Du, Anderson, Lortie, Parsons, & Bodnar, 2014).

Due to the increasing number of users of this type of products, there is an increasing need to improve their characteristics (Iohani & Verma, 2017).

Lipid nanocarriers for topical delivery

Although the skin is a route for drug administration, efficient release of drugs by topical application remains a challenge. As previously stated, the natural barrier of the skin hinders the permeation of substances or drugs through the skin. To overcome the disadvantages of this system, it was resorted to liposomes, ethosomes, transferosomes and niosomes (Elsayed, Abdallah, Naggar, & Khalafallah, 2007b).

These systems have a number of advantages, such as acting as carriers of molecules, acting as penetration enhancers, and, finally, are biodegradable, non-toxic and relatively immunogenic vesicles (Iohani & Verma, 2017).

On the down side there is lack of well-established regulatory guidance for skin delivery, high cost of lipids and lack of longue term stability during storage (Jain, Patel, Shah, Khatri, & Vora, 2016).

Liposomal vesicles

In the last few years, topical delivery of drugs by liposomal formulations has gained a considerable interest (Elsayed, Abdallah, Naggar, & Khalafallah, 2007a).

Liposomes were discovered by Alec D Bangham in the 60's. The first liposome formulation was entirely composed of natural lipids. Nowadays it is possible to create liposomes as single vesicles with one or more lipid bilayers composed of lipid molecules such as natural or synthetic phospholipids, with and without cholesterol and aqueous medium. Additional modifications can be added for different reasons.

In aqueous solutions phospholipids have a strong tendency to form membranes due to their amphipathic nature. Hydrophobic drugs can be trapped in between membranes, while hydrophilic drugs can be encased inside central compartment (Wang & Chao, 2017).

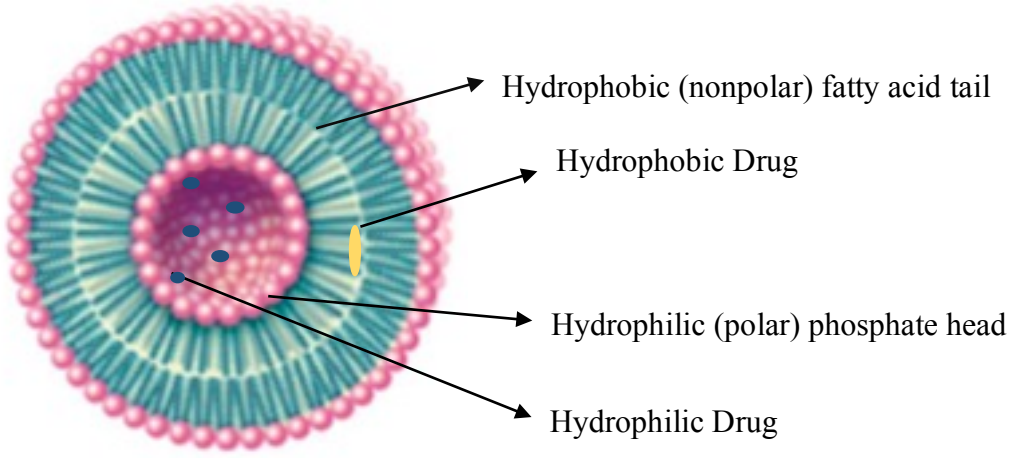

Figure 2 Adapted image of a liposome containing hydrophilic and hydrophobic drugs (Cogoi, Jakobsen, Pedersen, Vogel, & Xodo, 2016).

Liposomes can vary from a few nanometers to several micrometers and may have one or more lipid bilayers (Manaia et al., 2017; Alexander, Patel, Saraf, & Saraf, 2016; Jain et al., 2016).

Due to their biocompatibility and biodegradability, liposomes are considered safe drug delivery systems. Both diagnostic and therapeutic agents can be encapsulated into liposomes, since they protect them from enzymatic degradation and immunologic inactivation, therefore improving their therapeutic activity. The encapsulation also minimizes exposure of healthy tissue to drugs, reducing the systemic toxicity when compared with free drugs (He & Tang, 2017; Cortesi et al., 2010).

The potential value of liposomes for topical therapy was first introduced by Mezei and Gulasekharam in 1980 (Elsayed et al., 2007b). In this study the authors compared a conventional formulation with a liposomal lotion with the same drug concentration. The

results where four to five times more concentration on the epidermis and dermis when using liposomes. The drug of test was triamcinolone acetonide.

However, several subsequent studies have shown that liposomes provide high drug deposition into the upper layers of the skin and low penetration into deep layers (Ghanbarzadeh & Arami, 2013), thus have low efficiency as a carrier in transdermal drug delivery and do not provide percutaneous absorption or permeation. Due to this problem, the use of conventional liposomes for topical delivery was very limited and research was developed to improve the application of these systems to skin. By this way, ethosomes were developed as a better topical delivery system. (Jain et al., 2016).

The main advantages of liposomes are protection of the drug, low toxicity, easily soluble and immunogenicity of the drug delivered. As disadvantages, liposomes are poorly efficient in drug delivery to the phospholipid bilayer. It can occur degradation by hydrolysis, oxidation, sedimentation and also fusion during storage (Cortesi et al., 2010).

Ethosomes

Ethosomes are an efficient skin drug delivery system (Ainbinder, Paolino, Fresta, & Touitou, 2010), since they stimulate the transdermal and dermal administration, to increase the effectiveness of products application on the skin.

Ethosomes are non-toxic and biodegradable (Maheshwari & Al, 2012), consisting in microscopic spheres of phospholipids (Ghanbarzadeh & Arami, 2013) that have a soft and malleable structure (Waleed, M, & M, 2016; Elka Touitou, Godin, & Weiss, 2000). They are mainly composed by phospholipids, ethanol and water (Marto et al., 2016), and they increase skin permeation since they incorporate a high concentration of ethanol, 20-45%, which causes an increase in the fluidity of the membranes (Rakesh & Anoop, 2012; Esposito et al., 2016). Its size is small in comparison to liposomes, and this is explained by the incorporation of high concentrations of ethanol (Rakesh & Anoop, 2012). Ethanol imparts a negative net surface charge to the ethosomes, which causes the size of the vesicles to diminish, and to prevent their aggregation due to the electrostatic repulsion (Jain et al., 2016). They also have higher encapsulation efficiency and better stability than liposomes (Dayan & Touitou, 2000; L. Yang et al., 2017). The size of the ethosomes can range from tens of nanometers to a few microns, depending on the method of preparation thereof (Rakesh & Anoop, 2012).

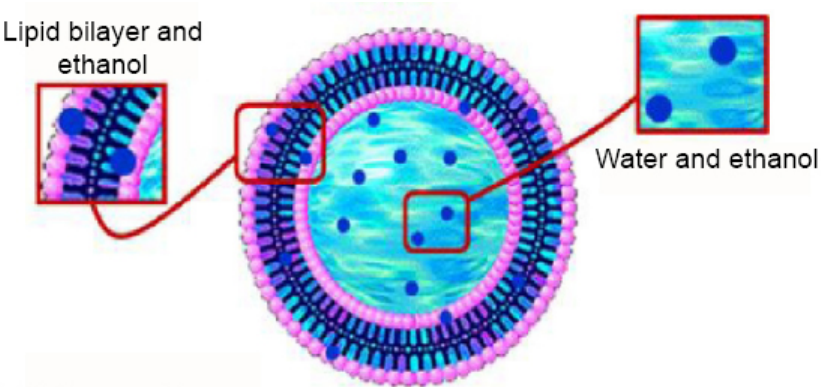

Figure 3 Adapted image of the structure of an ethosome (Ascenso et al., 2015).

Ethanol is known as an efficient permeation enhancer (E Touitou, 2000). It was thought that high concentrations of ethanol in lipid vesicular systems were destructive due to the interdigitation effect (E Touitou, 2000; Elsayed et al., 2007b). However, in recent studies (E Touitou, 2000) the coexistence of phospholipids and ethanol in high concentrations improved the nanocarrier system. Ethanol acts to cause disturbances in the lipid bilayer of the skin and therefore increases the ability of the ethosomes to penetrate the stratum corneum (Waleed et al., 2016).

The main advantages of ethosomes are (Rakesh & Anoop, 2012; Verma & Pathak, 2010):

- Increases the permeation of drugs through the skin;
- Ability to deliver several molecules with different physico-chemical properties, such as hydrophilic, lipophilic molecules and macromolecules;
- The components that are part of its structure are approved for pharmaceutical and cosmetic use;
- Its toxicological profile is well studied, and therefore does not present risks;
- High compliance with the user, since it is administered in the form of gel or cream, that is, it is not invasive.

One of the biggest disadvantages is that they retain part of the active component encapsulated inside. Others disadvantages may include the high cost of lipids, process scalability challenges for commercial application along with risk of residual organic solvent in the drug product, lack of long-term structural and chemical stability data during storage, challenge in optimizing lipid and ethanol concentration to improve physicochemical properties without compromising the stability and finally lack of well-established regulatory guidance for skin delivery (Jain et al., 2016). The interdigitation effect of alcohol on lipid bilayers is also a major disadvantage, because of the damage done by the ethanolic

atmosphere to the vesicular structure and the possibility of vesicles inability to exist side-by-side with a high concentration of alcohol.

Another disadvantage is related to oxidative reactions. Phospholipids containing unsaturated fatty acids are known to oxidize. The by-products of this reaction can change the permeability of the ethosomes bilayers. It is possible to minimize the oxidative degradation of lipids by protecting the solution from light and adding antioxidants such as a-tocopherol (Pandey, Golhani, & Shukla, 2014).

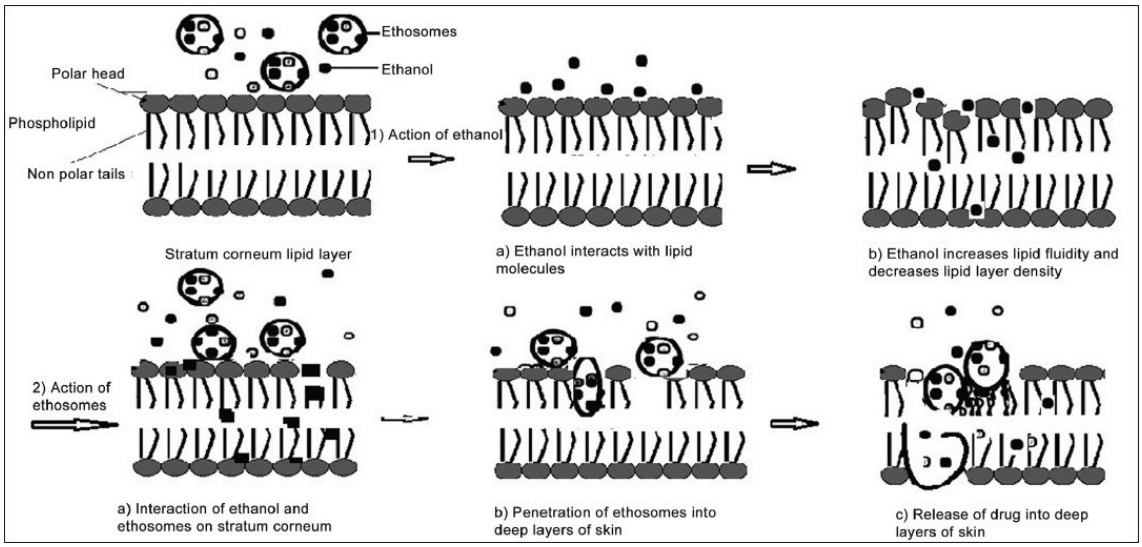

Figure 4 System of penetration of ethosomes through the skin (Verma & Pathak, 2010).

The interaction between ethanol and the lipid molecules in the polar head reduces the transition temperature of the lipids in the SC. This reaction causes the lipid layer to become more fluid and less dense. This reaction is called the ethanol effect.

The ethosome effect is the penetration and permeation by opening of new pathways due to the malleability. This effect also includes the fusion of ethosomes with skin lipids, which in term, releases the drug into deeper layers of skin. The release of drugs is made along with the penetration pathway (Verma & Pathak, 2010; Rakesh & Anoop, 2012).

Ethanol also helps vesicles to become more flexible which allow them to penetrate more and more easily into the deeper layers of the skin.

Ethosomes can be prepared in a number of ways, as will be exemplified below.

- The classical cold method:

Here the organic phase is obtained upon dissolution of the phospholipids in ethanol or a mixture of solvents such as ethanol and propylene glycol, at room temperature or at 30 °C. In the aqueous phase, water, buffer solution or a saline solution is used. The aqueous phase is slowly added to the organic phase and this mixture has to be stirred at a speed between 700 and 2000 revolutions per minute, rpm. The mixture can thus be up to 30

minutes, until the colloidal suspension is obtained. The drug can be added either to the aqueous phase or to the organic phase, depending on its physicochemical properties.

This is a simple method and also the most used in the preparation of ethosomes. Introduced by Touitou in 1996, where the aqueous phase and the organic phase are prepared separately.

- The ethanol injection-sonication method:

The organic phase has the phospholipid dissolved in ethanol, and this is injected into the aqueous phase using a syringe system. Subsequently the mixture is homogenized with an ultrasonic probe for 5 minutes.

- The hot method:

The phospholipid is dispersed in water and then placed in a water bath at 40 °C until a colloidal suspension is obtained. In another container the ethanol is heated to 40 °C. The solution is then added to the phospholipid solution with water using a magnetic stirrer. Here, the drug can also be dissolved in both the aqueous phase and the organic phase.

- The thin-film hydration method:

This method is basically the same as the conventional method of preparation of ethosomes, with the difference that the lipid film is hydrated by a hydro alcoholic solution. The phospholipid is dissolved in clear form or in a mixture of chloroform and ethanol in a round bottom flask. It is then placed in a rotary evaporator where the organic solvents are removed. The lipid film is then hydrated with a solution of ethanol and water or a buffered saline and ethanol and this process is carried out at the desired temperature for 30 minutes, or 1 hour or 6 hours.

- The reverse-phase evaporation method:

In the organic phase the phospholipid is dissolved in diethyl ether and then mixed in the aqueous phase in a 3:1 preparation for 5 minutes in an ultrasonic bath without temperature. Subsequently the organic solvent is removed under reduced pressure to gel. This method is the least used, used to produce unilamellar vesicles.

- Transmembrane pH gradient method:

This method is only used for water-soluble drugs having protonatable amines. It is a method that involves 3 steps: blank ethosome preparation, active encapsulation and incubation. It is necessary to take into account that the active is actively loaded, based on the difference of pH gradient between the interior acid of the internal phase and the basic exterior of the external phase of the ethosome (Abdulbaqi, Darwis, Khan, Assi, & Khan, 2016).

As previously stated, the ethosomes have better stability than liposomes, and the surface charge is essential for this. The zeta potential is an analytical measure which

characterizes the vesicles surface charge and is dependent on a number of factors, such as temperature, pH, conductivity and solvent. Any change in one of these parameters can affect the zeta potential value (Smith, Crist, Clogston, & Mcneil, 2017).

The application of ethosomes to cosmetics aroused from the need to increase the stability of the chemicals and to enhance the transdermal permeation (E Touitou, 2000).

Transfersomes

Transferosomes also have been developed to combat some of the drawbacks of classical liposomes. They are elastic, or highly deformable liposomes. Unlike the classic liposomes, the transfersomes penetrate the skin intact (Elsayed et al., 2007b; Jain et al., 2016). These were first introduced in 1992 by Cevc and Blume, and consist of phospholipids and edge activators, which are mostly single chain surfactants, causing a destabilization of the vesicle lipid bilayer, and as a consequence a deformability of the bilayer, affecting the interfacial tension of the vesicles (Elsayed et al., 2007b; Hussain et al., 2017).

The type and concentration of edge activators may affect the physicochemical properties of the vesicles, such as their size, zeta potential, fluidity and also the entrapment efficiency of the drug. Being the edge activators most used for the preparation of transfersomes, Span and Tween (Elsayed et al., 2007b).

Niosomes

The niosomes also appear to overcome the disadvantages of classical liposomes. These have a structure similar to the classic liposomes, composed of phospholipids and nonionic surfactants with cholesterol (Hussain et al., 2017; Alexander et al., 2016). Like liposomes, the niosomes can be unilamellar or multilamellar, and may carry both lipophilic and hydrophilic drugs, and be capable of delivering the drug at the target site. Surfactants are readily derivatized and provide greater versatility to the vesicles, increasing permeation, and as a consequence increasing the efficacy of the drug upon administration (Coviello et al., 2015; Marianecci et al., 2014). Niosomes are non-toxic, have a lower cost of production and are stable for a longer period of time, greater stability for oxidative degradation, when compared to the liposomes (Marianecci et al., 2014).

Table 1 Summary table of lipid nanocarries (Iohani & Verma, 2017).

Lipid nanocarrier	Composition	Mechanism of Skin Penetration
Liposomes	• Spherical vesicles • 30-100 nm. • Active molecules encapsulated in aqueous space or intercalated into the lipid bilayers	The drug release from the liposome followed by permeation through the skin. Liposome causes ultrastructrual changes in the SC, suggesting penetration enhancement effect. They may adsorb to the SC or fuse with the SC lipid matrix with subsequent discharge of the enclosed drug to the skin.
Ethosomes	• Soft vesicles ranges from • 30 nm to a few microns • Composition: lipids, ethanol, propylene glycol and water.	Ethanol causes SC lipid disruption thereby permitting entry of ethosomes and their associated payload to the deeper skin layers. Fluidizing effect of ethanol on phospholipid bilayers creates soft deformable vesicles and enhances transport of active ingredient deep in to the skin via a variety of mechanism including an action as true 'carrier', SC lipid disruption, osmotic gradients and so on.
Transfersomes	• Elastic, deformable vesicles with phospholipids, cholesterol, and sodium cholate as a biocompatible edge activator. • Complex vesicular aggregates optimized to attain extremely flexible and self-regulating membranes	Water evaporates from the skin surface and the vesicles start to dry out. Owing to strong hydrophilicity, vesicles are attracted to the areas of high water content in narrow gaps between adjoining cells in the skin. Their flexibility and deformability enable them to temporarily open the pores between the cells. Such intercellular passages maintain the integrity of vesicles but change their shape to fit the channel.
Niosomes	• Mainly formed by self-assembly of synthetic non-ionic surfactants with the optional combination of cholesterol to increase stability of vesicle and reduce leakage of active ingredient from them. • Presence of non-ionic surfactants acts as a permeation enhancer.	Niosomes diffuses from SC as a whole or may modify the structure of SC, which loosens the lipid barrier, and make it permeable for the active molecule. They interact with SC with fusion, aggregation and adhesion to the cell surface which results in higher thermodynamic activity gradient of active ingredient at the vesicle-SC surface, which act as a driving force for the penetration of lipophilic drug.

Free radicals and Reactive Oxygen Species

According to Lavoisier, the oxygen molecule has been considered to have a dual role (Kohen & Nyska, 2002), both essential for the life of a living organism, and can be considered a toxic substance. Oxygen is essential for the production of energy in living organisms, but it is also an indicator of the generation of free radicals, which can damage the living organism in different ways. In this way, antioxidants serve to protect organisms against the toxicity of free radicals (Kohen & Nyska, 2002; Thanan, Oikawa, Hiraku, Ohnishi, & Ma, 2015).

Free radicals are produced naturally or via some biological dysfunction. Free radicals that have an unpaired electrode are centered on oxygen or nitrogen atoms and are therefore called reactive oxygen species, ROS, and reactive nitrogen species (Barreiros & David, 2006). These are involved in energy processes, phagocytosis, regulation of cell growth, intercellular signaling and synthesis of biological substances (Barreiros & David, 2006).

Oxidative stress has a role in the aging process of skin (Richter, 2015), because reactive oxygen species, ROS, molecules have the capacity to induce the lipid peroxidation process (Kohen & Nyska, 2002). There are several factors that contribute to ROS production on skin, endogenous and exogenous factors, such as UV irradiation and enzymatic activity (Richter, 2015; Petruk et al., 2017).

Oxidative stress causes a high production of oxidants and a low production of antioxidants, which results in an imbalance between the formation of free radicals and the presence of antioxidants in the body (Sies, 2015).

The reactive oxygen species can be divided into two groups, the radicals and the non-radicals. Within the radicals the hydroxyl, superoxide, peroxyl and alkoxy radicals are inserted. Within the non-radicals include oxygen, hydrogen peroxide and hypochlorous acid. Relative nitrogen reactive species are nitric oxide, nitrous oxide, nitrous acid, nitrites, nitrates and peroxynitrite (Barreiros & David, 2006; Han, Zhang, & Skibsted, 2012).

In the past years, numerous synthetic ROS scavengers and antioxidants have been studied to suppress the resultant of attack of ROS.

An antioxidant should have some characteristics to combat oxidative stress in organisms, such as: depletion of oxygen, quenching of singlet oxygen, chelation of metal ions, scavenging of reactive oxygen species and repair of oxidative damage (Han et al., 2012).

Phenolic compounds

Phenolic compounds are known for their properties on oxygen radicals, they are secondary metabolites produced by plants for their growth, reproduction and defense against pathogens. These are a very important class of natural antioxidants (Guo, Guo, Li, Fu, & Liu, 2016; Vaisali, Belur, & Iyyaswami, 2017).

There are several ways to classify phenolic compounds because of their constitution, which can range from simple molecules to highly polarized compounds. Another way of being classified can be by its solubility or by its carbon chain. Phenolic compounds may have a wide variety of physiological properties, for example antiallergics, anti-inflammatories, antimicrobials, antioxidants, antithrombotics and vasodilators (Lourdes & Giada, 2013; Balasundram, 2006).

Rutin

Rutin, is a natural flavonoid (J. Yang, Guo, & Yuan, 2008), abundantly found in plants, such as passion flower, buckwheat, tea, and apple. Buckweat is rich in vitamin B1 and B2, having a balanced composition in amino acids and rich in lysine (Holasova et al., 2002). This compound shows anti-inflammatory, antibacterial properties and antioxidant activity (J. Yang et al., 2008). Rutin, quercetin-3-rutinoside, is a nontoxic, non-oxidative molecule that catalyzes the production of oxygen radical (Alam et al., 2017), it is a glycoside comprising of flavonolic aglycone quercetin along with disaccharide rutinose (Ganeshpurkar & Saluja, 2017).

Rutin has antioxidant activity, and therefore can be used to prevent damage caused by UV radiation. It has a molecular weight of 610.521 g/mol, melting point 125 °C and a solubility in water of 0.125 mg/ml (PubChem, n.d.-b). This latter parameter hinders the permeability of Rutin in biological membranes.

Several studies have already been carried out between nanoparticles and Rutin. An example of this is Yang et al 2008, which encapsulated Rutin, ascorbic acid and BHT at different concentrations, and compared them. The results showed that ascorbic acid had higher antioxidant power than Rutin, however Rutin had higher antioxidant power than BHT. And they came to the conclusion that the free radical scavenging activity increases with increasing concentration (L. Yang et al., 2017).

Kerdudo et. Al 2014, incorporated Rutin and naringenin into multilamellar lipid vesicles. Rutin showed that it had high encapsulation efficiency, and that lipid vesicles protected the Rutin from degradation. Only free and adsorbed Rutin had antioxidant activity.

Ferulic Acid

Ferulic acid, FA 4-hydroxy-3-methoxycinnamic, is a ubiquitous natural phenolic phytochemical present in seeds, leaves, both in its free form and covalently conjugated to the plant cell wall polysaccharides, glycoproteins, polyamines, lignin and hydroxy fatty acids, and arises from the metabolism of phenylalanine and tyrosine (Srinivasan, Sudheer, & Menon, 2007).

FA generally exists as the trans isomer, has a molecular weight of 194.186 g/mol, and a melting point of 168-171 ºC (PubChem, n.d.-a) being soluble in ethanol, ethyl acetate and hot water, among others (Ggaf, 1992).

When in the free form, FA is considered a good antioxidant, since it forms a phenoxy radical stabilized by resonance. This shows high activity of elimination of hydrogen peroxide, superoxide, hydroxyl radical and free radicals of nitrogen dioxide. AF does not absorb free radicals, however it increases the activity of enzymes that are responsible for the elimination of free radicals and also inhibits enzymes that catalyze the production of free radicals (Ou & Kwok, 2004).

It shows a wide range of therapeutic properties, like antiageing, radioprotective, and others. This is attributed to the capacity of phenolic nucleus and extended side chain conjugation (Srinivasan et al., 2007).

FA was synthesized in 1925, and showed the presence of an unsaturated side chain and also the existence of cis and trans isomeric forms.(Kumar & Pruthi, 2014)

FA was proposed as a new antioxidant compound with a strong cytoprotective activity due to the ability to eliminate free radicals and to activate the cellular stress response, due to the double bond present in the side chain, subjected to cis-trans isomerization, and the phenoxy radical stabilized by resonance explains the effective antioxidant activity, this activity in response to free radicals through the donation of a hydrogen atom of its phenolic hydroxyl group, showing anti-inflammatory activity (Kumar & Pruthi, 2014; Mancuso & Santangelo, 2014). However, objectionable pharmacokinetics, which reduce the bioavailability of FA after ingestion and the limited number of clinical studies performed to prove the efficacy and safety of FA, have limited the evidence regarding the potential interest of this phenolic acid in humans (Kumar & Pruthi, 2014; Mancuso & Santangelo, 2014).

Although FAs show poor bioavailability when ingested, it is well absorbed by the skin through dermal administration, both at acidic and neutral pH, which demonstrates that the molecule can be absorbed by the skin in dissociated and non-dissociated forms.

Ionic Liquids

Ionic liquids, ILs, are organic salts that are liquids at room temperature, RT, or below 100 ºC with very attractive characteristics, such as high thermal and chemical stability, low vapor pressure, non-flammability, non-volatility, possibility of recycling and solubility in a large spectrum of solvents (Dobler, Schmidts, Klingenhöfer, & Runkel, 2012), and some of them show bactericidal activity, toxicity and cytotoxicity (Kubota, Shibata, & Yamaguchi, 2016; Mccrary et al., 2013). These salts have been used for various applications, such as reaction media for many organic, in separations and extractions, as lubricants and also in nanotechnology, among others (Almeida et al., 2017). They are composed by large asymmetric organic cations and inorganic or organic anions. ILs are advantageous to solubilization of poorly soluble drugs and to enhancement the efficacy of topical drug delivery (Dobler et al., 2012).

Since the aqueous solubility of the Rutin and FA is very low (Zi, Peng, & Yan, 2007; Mota, Queimada, & Pinho, 2008), the incorporation of ILs may be an advantageous, since these compounds are solubility promoters. In terms of emulsion / topical application, ILs may also, together with the ethosomes, be an advantage since they are permeation promoters, because they may be placed in several delivery systems.

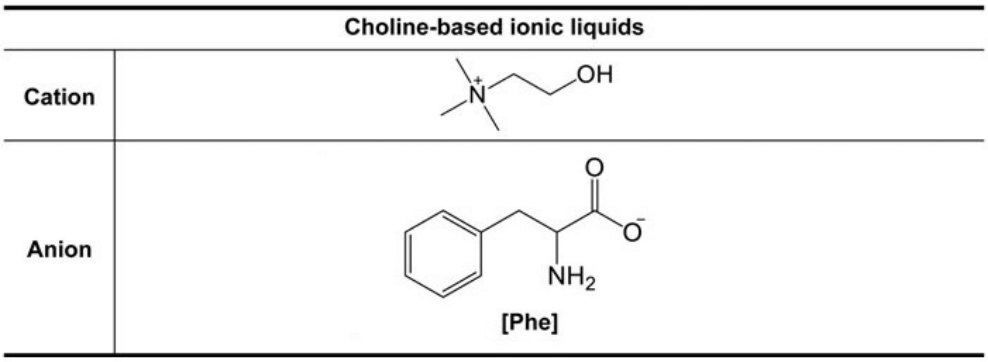

Figure 5 Adapted image of structure of the choline-Phe ionic liquids (Almeida et al., 2017).

ILs are currently used by pharmacists for the possibility that they have in changing their physicochemical properties in order to achieve a specific property.

One of the most important properties is the ability to solubilize hydrophobic and hydrophilic drugs when the drugs in question are poorly soluble. In addition, they may be placed in aqueous, oily or hydroalcoholic solutions. This latter characteristic makes ILs an alternative with less toxicity to other organic solutions when used as excipients in topical emulsions. In the case of the cosmetics industry ILs have already been used in the development of personal care products. As an example of some advantages, choline-based

ILs have been found to exhibit low toxicity to humans and the environment (Almeida et al., 2017).

Some recent studies demonstrate the successful incorporation of ILs into emulsions and especially into microemulsions acting as oil or water substitutes, additives or surfactants. ILs can provide chemical protection and improve physicochemical properties through intermolecular interactions. They also improve the stability of nanoparticles for different applications. The interactions between ILs and NPs act together to organize materials with different structures. These liquids may be covalently bonded to the surface of the NP's, the NPs may also be incorporated into preformed IL-based structures or emulsions. The size of the NPs must also be taken into account since it influences their performance, the use of ILs has been shown to be important in controlling the size of NPs (Almeida et al., 2017).

The main objective of this work is the development and physicochemical characterization of ethosomal delivery systems for antioxidants, such Rutin and FA. The choice of etosomes was because they increase the permeability of the active substances through the skin, allowing them to penetrate more deeply, but also to increase the antioxidant activity of the actives. We added IL to the formulations, since the subtances we are using, Rutin and Ferulic Acid have extremely low aqueous solubility, and it is proven that they increase the solubility of the active substances.

Materials and Methods

Materials

All equipment used in this work, are represented in **Table 2**:

Table 2 Equipment used in this work.

Equipment	**Supplier**
Analytical Balance	Startorius®
Hot plate	Stuart®
Mechanical mixer	KIKA Labortechnik® mixer
Water bath with thermostat control	Memmert®
Centrifuge	Hitachi® RX2
pH Meter	Metrohm® 827 pH labor
Inverted Microscope	OLYMPUS® CKX41
Camera	OLYMPUS® SC20
Spectrophotometer UV - visible	Evolution® 300 UV- visible – Thermo Scientific
Rotary Evaporator	Bath IKA Labortechnik HB4 basic® and Elevator IKA WERKE RV06-ML®
Size, zeta potencial and PDI	DelsaTM NanoC

All materials used to ethosomes and emulsions development, are represented in **Table 3**:

Table 3 Materials used to ethosomes and emulsions development.

Material	**Supplier**
Rutin	DEG, São Paulo, Brasil
Ferulic Acid	DEG, São Paulo, Brasil
Ionic Liquid [Cho][Phe]	Synthesized in laboratory
Chloroform	Sigma Aldrich®
Absolute etanol	Sigma Aldrich®
Double distilled water	Made in laboratory
Phosphatidylcholine	Phospholipon® 90 G Lipoid
Crodafos	Croda Inc CES®
BHT	Mapric®
Isopropyl Myristate	Pharma Special®
Disodium EDTA	Fagron®
PEG 400	Dow®
Propylene glycol	Mapric®
Parabens concentrate	Made in laboratory
DPPH	Sigma Aldrich®
Methanol	VWR®

Methods

Preparation of Solutions Containing Rutin and FA

Aqueous and methanolic solutions were prepared with Rutin at 0.03%, 0.05 % and 0.075%, FA at 0.03 %, 0.05 % and 0.075 %, with and without IL ((2-Hydroxyethyl)trimethylammonium-L-phenylalaninate) at 0.2 %.

Development of Ethosomes

Ethosomes were prepared with 0.03%, 0.05% and 0.075% (w/v) of FA and 0.03%, 0.05%, 0.075% and 0.1% (w/v) of Rutin. Ethosomes were prepared by mixing phosphatidylcholin 2%, antioxidants, Rutin or FA, and chloroform.

After total dissolution and subsequent evaporation, Rotary Evaporator with bath IKA Labortechnik HB4 basic® and Elevator IKA WERKE RV06-ML®, the lipidic film is hydrated with ethanol at 20%, ethanol:water – 20:80, by stirring during 1h at 600 rpm and 30 °C. The suspension is then exposed to an ultrasonic bath during 30 minutes, without heating. Ethosomes without FA and without Rutin were also prepared. In addition, ethosomes containing IL at a concentration of 0.2% were prepared.

Size, Zeta Potential and Polydispersity Index (PDI)

The mean particle size, zeta potential and polydispersity index of unloaded and loaded ethosomes were measured in DelsaTM NanoC by photon correlation spectroscopy and electrophoretic mobility, respectively. Each analysis was carried out at 25 ºC. The analyses were performed in triplicate.

In Vitro Antioxidant Activity

The spectrophotometric readings were obtained using methanolic solutions of samples and a 100.0 µM solution of DPPH at a ratio of 1:40, after 1 hour of reaction at RT, 24.0 ± 2.0 °C, in the dark. All samples were prepared and analyzed in triplicate. The absorbance values were measured at 515 nm and converted into percentage of free radical scavenging, % FRS, using **Equation 1**.

Equation 1 Percentage of free radical scavenging. Legend: % FRS: Percentage of Free Radical Scavenging; ABS control: Absorbance of negative control; ABS sample: Absorbance of samples.

$$\%\ FRS = \frac{\left(Abs_{control} - Abs_{sample}\right)}{Abs_{control}} \times 100$$

pH Measurement

The pH assay of the solution, ethossome dispersions, solutions and emulsions was measured by the potentiometer 827 pH lab Metrohm and the temperature was controlled to 25.0 ± 2.0 ºC. Measurements were performed in triplicate.

Microscopic Analysis

To analyze microscopic aspect, cover slips were mounted on microscope slides with emulsions. Images acquisition was performed with an OLYMPUS SC20 camera coupled to an OLYMPUS CKX41 inverted microscope with 40x objective.

Preparation of Oil/Water (O/W) Emulsions

In the preparation of the O/W emulsions, the compounds of the aqueous and oily phases were weighed separately. Both phases were placed in a thermostated bath at 65 °C to melt the oily phase, while at the same time the temperature of the two phases was equalized. The aqueous phase was then added to the oil phase, still in the bath and always with vigorous stirring. Finally, the mixture was removed from the bath, continued with stirring until the temperature decreased to room temperature and the emulsion appeared homogeneous.

The composition of the chosen emulsions is shown in **Table 4**.

Table 4 Composition of emulsion (% w/w) used as a base for ethosomes and solutions.

Compounds	**(% w/w)**
Crodafos ES	6
Butylated hydroxytoluene (BHT)	0.1
Isopropyl myristate	2
Polyethylene glycol 400 (PEG 400)	5
Propylene glycol	5
Disodium EDTA	0.1
Parabens concentrate	1
Water	Qs 100

The ethosomes and solutions were incorporated into the bases after they were fully homogenized. It was placed 2 g of ethosome or solution in each 8 g of base, making a total of 10 g of emulsion.

Preliminary Stability

Heat Stress Test

About 4 g of each emulsion were weighed in centrifuge tubes in duplicate. The test was initiated at 40 °C and ended at 80 °C with the temperature elevated by 10 °C every 30 minutes. After the final incubation, the replicas were taken from this condition and evaluated (Baby et al., 2008).

Centrifuge Test

The emulsions were subjected to a centrifugation test. Approximately 4 g of each emulsion was weighed in centrifuge tubes in duplicate. The study was carried out at room temperature, rotation speed 3000 rpm, 210 g, for 30 minutes.

Statistical analysis

Statistical analysis was performed using Prism GraphPad 7.0 software, with a significance level of 5.0% with $\alpha = 0.05$). Data were treated using one-way ANOVA followed by the Tukey test for multiple comparisons, the 2-sample t-test, and paired t-test. Values

where expressed as mean +/- standard deviation, Sd. The differences between individual means where significant at * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

Results and Discussion

Rutin

Solutions, colloidal dispersions and emulsions containing Rutin with different concentrations were developed. **Figure 6** shows a) Rutin aqueous and methanolic solutions, b) colloidal dispersions, Ethosomes, and c) emulsions.

a) b) c)

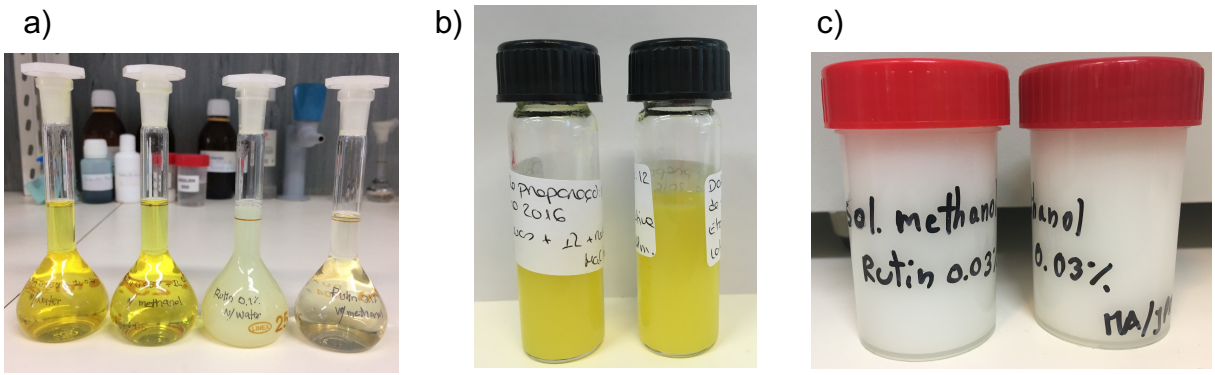

Figure 6 Solution, ethosomes and emultions containing rutin, developed in laboratory

Size, Zeta Potential and Polydispersity Index (PDI)

Ethosomes physical characterization was performed by measuring the particle size and zeta potential, **Table 5**.

With respect to the particle size of ethosomes, results show that blank ethosomes and blank ethosomes with Ionic Liquid at a concentration of 0.2%, because it is the percentage that it has tissue ability, that is, it is not toxic to the body. This show a very small size. In addition, the effect of increasing Rutin loading had contradictory results. For example, the size increases when increasing drug loading from 0.03% to 0.05% and decreases when drug loading was increased to 0.075% and 0.1%. On the contrary, when IL was incorporated in the ethosomes, particle size decreases, irrespective of Rutin loading.

Zeta potential is an indicative of the surface potential of the particles, besides of being used to control stability of colloidal dispersions; it is an important factor to understand the dispersion and aggregation phenomena. Most of the suspended particles have a surface charge, due to the ionization of functional groups, or even, by the adsorption of ions on their surface (Siqueira, 2008). In general, colloidal suspensions, which have higher values than 30.0 mV, in module, exhibit good physical stability. As can be seen in **Table 5**, results do not show any consistency and therefore further studies should be performed.

PDI values can be between 0-1 and it is equivalent to the distribution variance (Zwiorek, 2006).

In general, PDI values increased with the presence of Rutin and FA, as seen in **Table 5**. However, in order to consider uniform homogeneity of the formulations, the values must be approximately 0, this does not occur in the ethosomes with Rutin, thus demonstrating that the stability of colloidal dispersions may be compromised, polydispersity index >0.2, (Zwiorek, 2006).

Table 5 Ethosomes containing Rutin particle size, zeta potential and PDI.

Ethosomes	**Mean size ± SD (nm)**	**Mean zeta potential (mV)**	**Mean PDI (mV)**
Blank	71 ± 73.6	- 0.31	0.180
IL	92.9 ± 24.8	- 0.88	0.321
Rut. 0.03 %	532.5 ± 158.8	- 0.27	0.366
Rut. 0.03 % + IL 0.2 %	93.5 ± 23.3	-2.74	0.471
Rut. 0.05 %	1,048.5 ± 273.5	-0.47	0.261
Rut. 0.05% + IL 0.2%	173.4 ± 50.2	-10.98	0.246
Rut. 0.075%	68.3 ± 18.6	10.41	0.266
Rut. 0.075% + IL 0-2%	49.9 ± 12.9	9.16	0.245
Rut. 0.1%	249.2 ± 165.1	-9.87	0.409
Rut. 0.1% + IL 0.2%	172.9 ± 136.3	45.06	0.236

pH Assay

All pH values were acid, with the exception of the formulations with IL, which presents a basic pH which is in accordance to its physicochemical nature, **Table 6**

Table 6 pH values of aqueous, methanolic solutions and colloidal dispersions with and without Rutin and IL.

Formulation	pH
Aqueous Solution IL 0.2%	9.62
Methanolic Solution IL 0.2%	9.57
Methanolic Solution Rutin 0.03% + IL 0.2%	9.55
Aqueous Solution Rutin 0.03% + IL 0.2%	9.44
Methanolic Solution Rutin 0.03%	5.56
Aqueous Solution Rutin 0.03%	5.36
Methanolic Solution Rutin 0.075% + IL 0.2%	9.66
Aqueous Solution Rutin 0.075% + IL 0.2%	9.51
Methanolic Solution Rutin 0.075%	5.33
Aqueous Solution Rutin 0.075%	5.17
Ethosomes Blank	4.38
Ethosomes IL 0.2%	9.91
Ethosomes Rutin 0.03%	4.55
Ethosomes Rutin 0.03% + IL 0.2%	9.56
Ethosomes Rutin 0.05%	4.67
Ethosomes Rutin 0.05% + IL 0.2%	8.48
Ethosomes Rutin 0.075%	4.33
Ethosomes Rutin 0.075% + IL 0.2%	8.05
Ethosomes Rutin 0.1%	4.41
Ethosomes Rutin 0.1% + IL 0.2%	8.27

After measuring the pH in solution and in the colloidal dispersion, it was decided to measure the pH of the emulsions.

pH values in emulsions with different compositions were all acidic, **Table 7**. This is due to the acid nature of the Crodrafos CES ingredient (Cetearyl Alcohol (and) Dicetyl Phosphate (and) Ceteth-10 Phosphate) (Inc., n.d.).

Table 7 Values of pH in emulsions.

Emulsions	**pH**
Aqueous solution with. IL 0.2 %	2.93
Methanolic solution with. IL 0.2 %	3.01
Methanolic solution with. Rutin 0.03 % + IL 0.2 %	3.08
Aqueous solution with. Rutin 0.03 % + IL 0.2 %	2.88
Methanolic solution with. Rutin 0.03 %	3.04
Aqueous solution with. Rutin 0.03 %	2.95
Ehosomes witht. IL 0.2 %	3.18
Ethosomes. Blank	2.98
Ethosomes. with Rutin 0.03 %	2.98
Ethosomes with Rutin 0.03 % + IL 0.2 %	3.14

In Vitro Antioxidant Activity

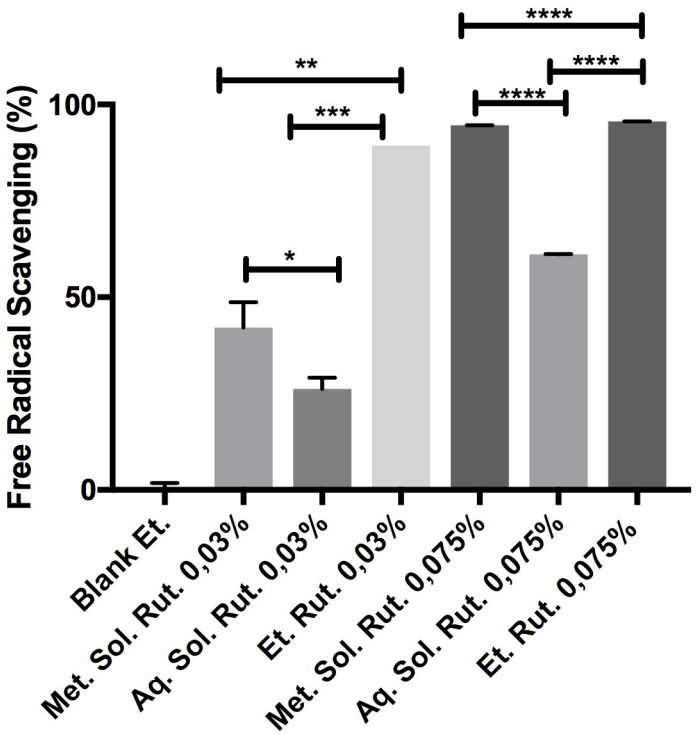

Figure 7 DPPH radical scavenging (%) for non-encapsulated and encapsulated formulations containing Rutin 0.03% and Rutin 0.075%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

By comparing the formulations with 0.03% Rutin, ethosomes significanty increase antioxidant activity, **Figure 7**. With 0.075% of Rutin, methanolic solution and ethosomes present similar antioxidant activity.

The lowest antioxidant activity was found to be the aqueous solution at both concentrations, which can be attributed to the fact that this active substance has very low solubility in water (Zi et al., 2007). The solubility of Rutin in water, at a temperature of 25 ºC is 0.02% (Caparica, 2017), so both loadings are above the solubility of Rutin in water.

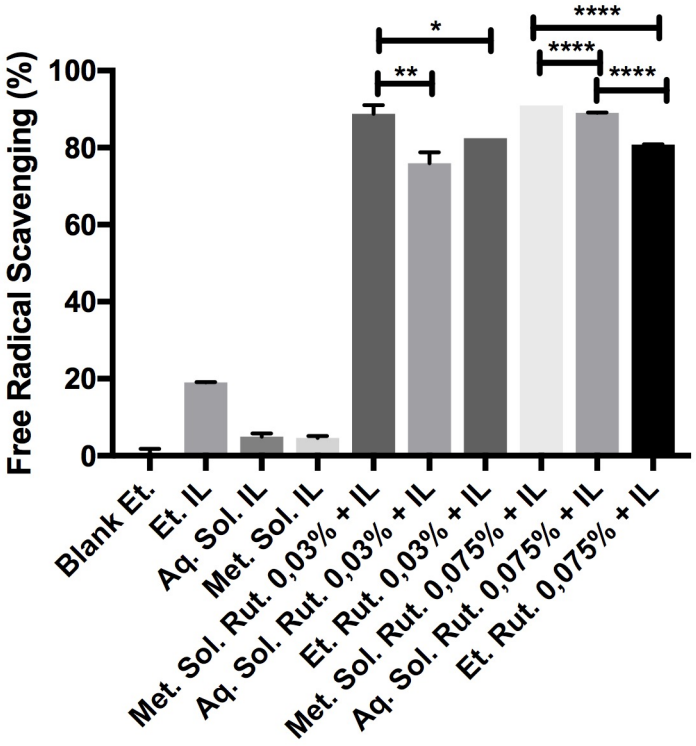

Figure 8 DPPH radical scavenging (%) for non-encapsulated and encapsulated formulations containing Rutin 0.03 % + IL 0.2 % and Rutin 0.075% +IL 0.2%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

In the presence of IL, **Figure 8**, the results are more difficult to explain, since it is the methanolic solution that presents a greater antioxidant activity when compared with the aqueous solution and with the ethosomes. This may occur due some phospholipid bilayer destabilization performed by the IL.

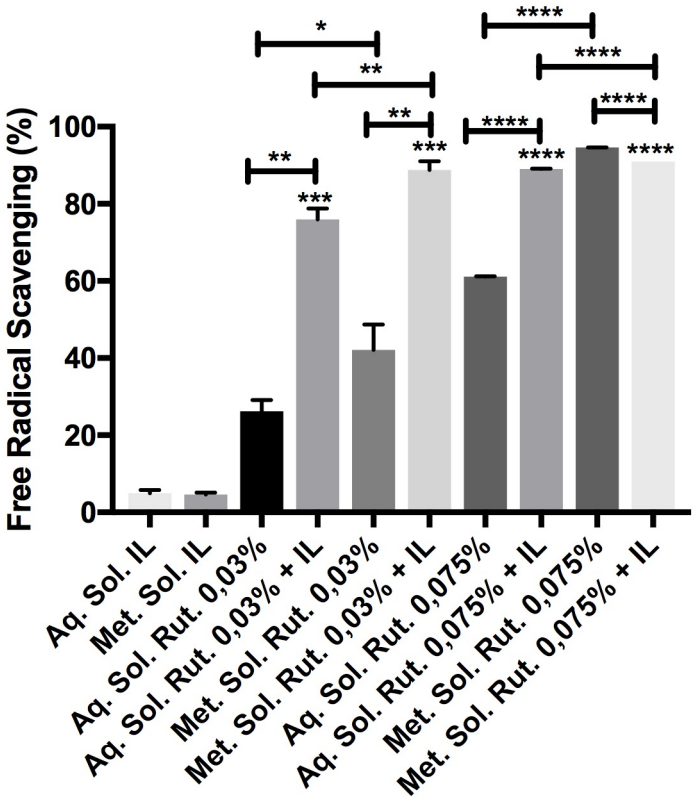

Figure 9 DPPH radical scavenging (%) for non-encapsulated formulations, with and without IL, containing Rutin 0.03% and 0.075%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

The same cannot be said for solutions containing Rutin, since solutions containing IL in their composition have a higher antioxidant activity than those which do not. Again, this demonstrates that IL may be compromising the integrity of the ethosomes, since in solutions the results are not in accordance, **Figure 9.**

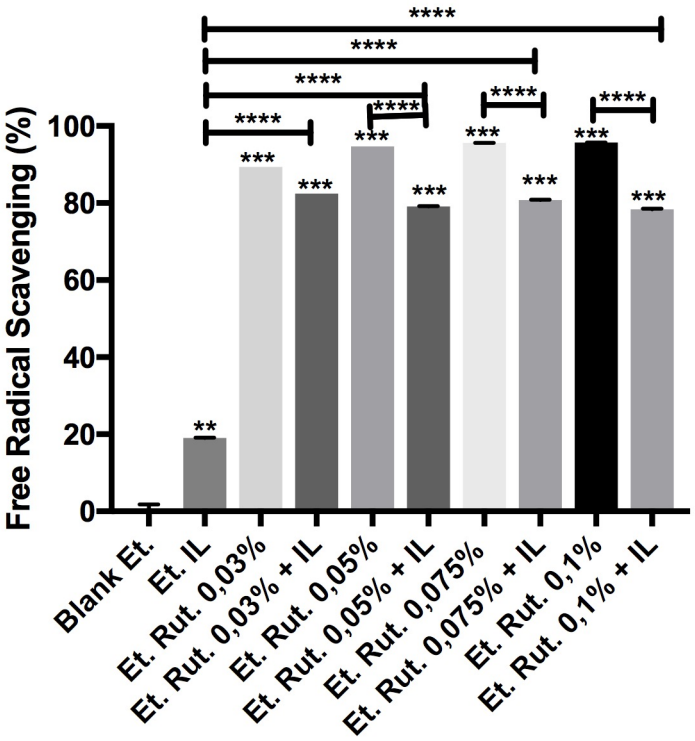

Figure 10 DPPH radical scavenging (%) for ethosomes containing Rutin with and without IL at 0.03%, 0.05%, 0.075% and 0.1%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

In **Figure 10**, it can be seen that ethosomes with and without IL are presented at different Rutin concentrations. The presence of IL decreases antioxidant activity for all Rutin loadings. As previously mentioned this may be due to the fact that IL destabilizes the phospholipid bilayer of the ethosomes, consequently reducing its antioxidant activity.

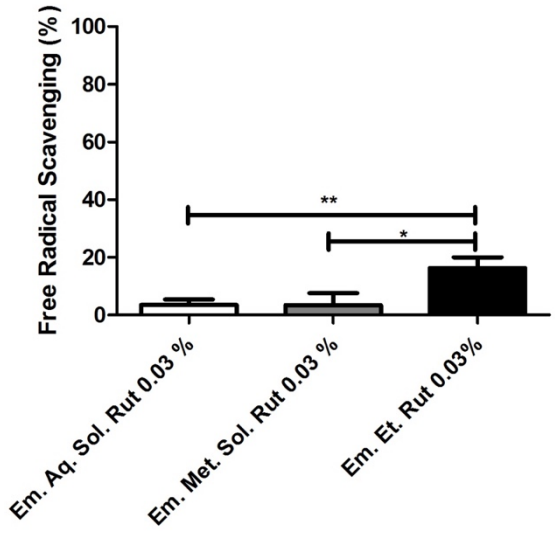

Figure 11 Comparison of free radical scavenging between emulsions with non-encapsulated and encapsulated Rutin 0.03%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

When 0.03% Rutin was incorporated in emulsions, ethosomes also present higher antioxidant activity than aqueous or methanolic solutions, **Figure 11**.

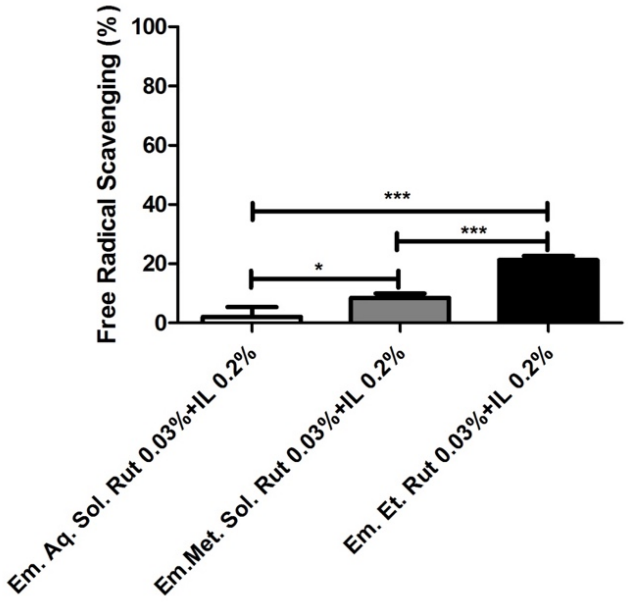

Figure 12 Comparison of free radical scavenging between emulsions with non-encapsulated and encapsulated Rutin 0.03% + IL 0.2%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

In the presence of ILs, same tendency was observed, where ethosomes also present higher antioxidant activity than aqueous or methanolic solutions, **Figure 12**.

Microscopic Analysis

Microscopic analysis of emulsions showed that all formulations were homogeneous **Figure 13**.

Emulsions containing 0.03 % Rutin showed crystals in the three emulsions, both in ethosomes, as in aqueous solutions as well as in methanolic solution, that may be occur due to non complete dissolution of the Rutin in the formulations and/or due to some evaporation of the solvent during microscopic analysis.

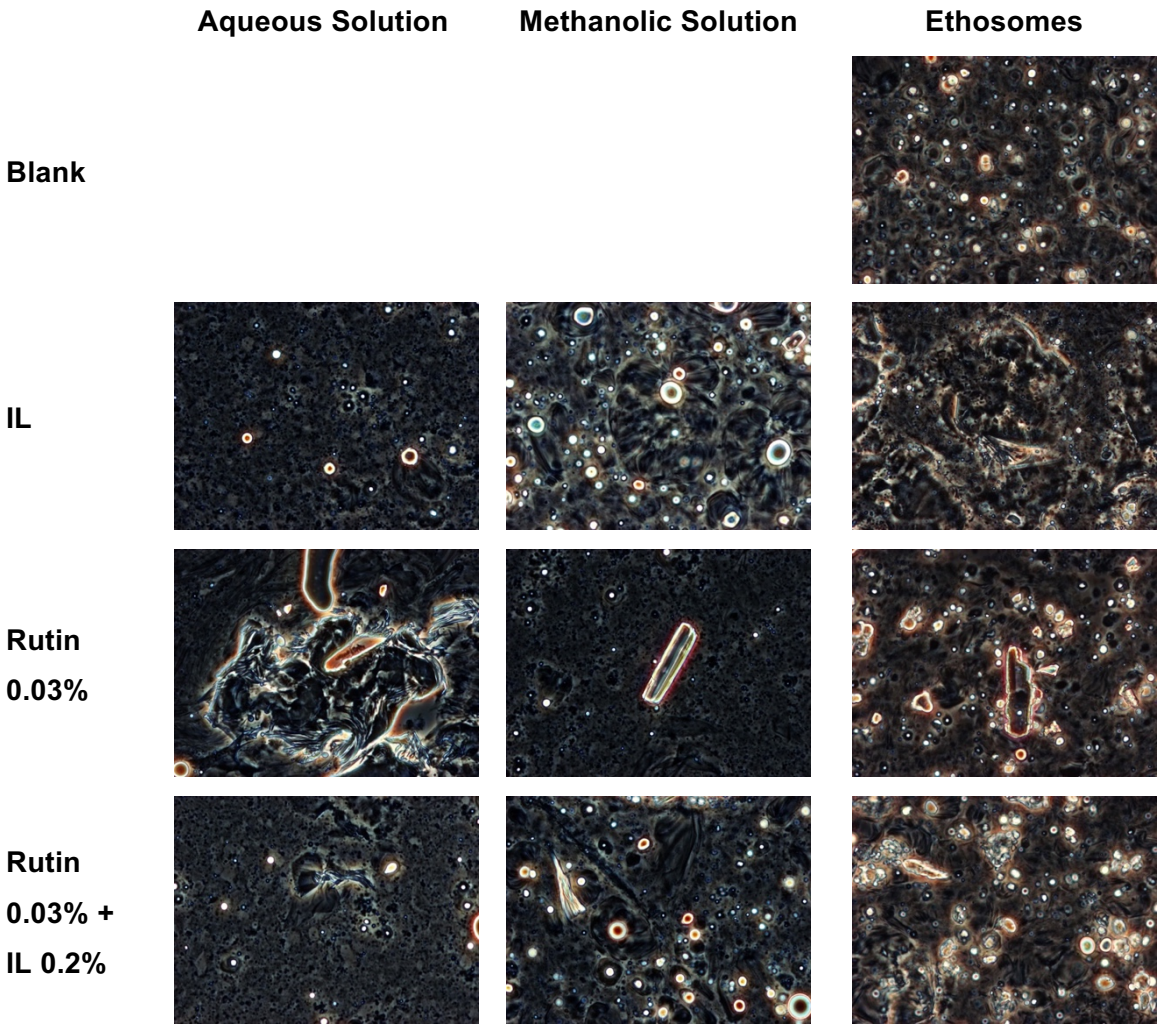

Figure 13 Microscopic photos of emulsions with aqueous solutions, methanolic solutions and ethosomes containing Rutin 0.03%, with and without IL.

Preliminary Stability

All emulsions showed to be stable after preliminary stability studies, since no signs of flocculation and/or phase separation were observed, results not shown.

Ferulic acid

Solutions, colloidal suspensions, ethosomes, and emulsions containing ferulic acid with different concentrations. **Figure 14** shows a) ferulic aqueous and methanolic solutions, b) colloidal dispersions, ethosomes, and c) emulsions.

a) b) c)

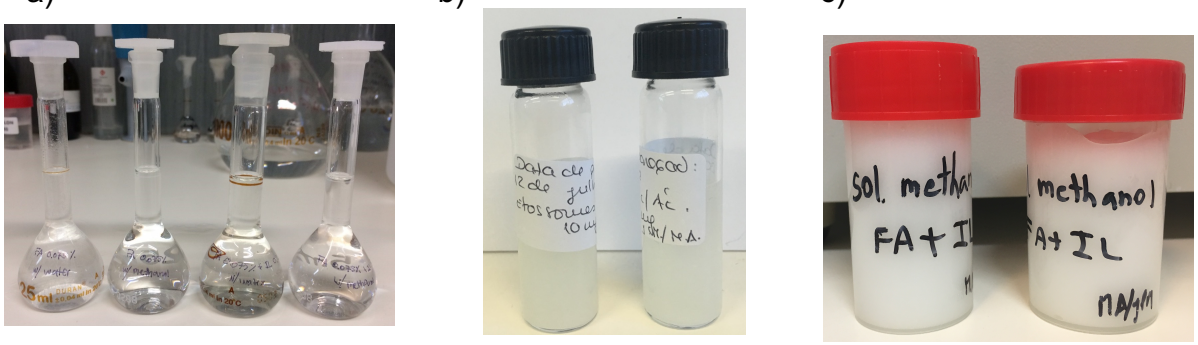

Figure 14 Solution, ethosomes and emultions containing ferulic acid, developed in laboratory.

Size, Zeta Potential and Polydispersity Index (PDI)

With regard to the size of ethosomes containing FA, results are not clear, since whenever IL is added, the particle size does not always decrease, and there is also no relationship between FA concentration and particle size, as can be seen in **Table 8**. Thus, these results should be confirmed.

As well as in the zeta potential of the ethosomes that contain Rutin, in those containing FA, the results are also not coherent.

Regarding the PDI of the FA ethosomes, the values also demonstrate that more studies are needed to evaluate the homogeneity of the colloidal dispersion.

Table 8 Ethosomes containing FA particle size, zeta potential and PDI.

Ethosomes	Mean size ± SD (nm)	Mean zeta potential (mV)	Mean PDI (mV)
Blank	71 ± 73.6	- 0.31	0.180
IL	92.9 ± 24.8	- 0.88	0.321
FA 0.03 %	1,025.9 ± 288.1	-1.33	0.782
FA 0.05 %	125 ± 306.0	-0.50	1.134
FA 0.05 % + IL 0,2 %	200.4 ± 76.2	-0.43	0.313
FA 0.075 %	361.1 ± 1,667.2	-0.02	0.634
FA 0.075% + IL 0.2%	107.2 ± 35.9	12.13	0.327

pH Assay

All pH values were acid, with the exception of the formulation with IL, which presents a basic pH, **Table 9**, which is in accordance to its physicochemical nature.

Table 9 pH values of aqueous, methanolic solutions and colloidal dispersions with and without FA and IL.

Ethosomes and Solutions	pH
Aqueous Solution IL 0.2%	9.62
Methanolic Solution IL 0.2%	9.57
Aqueous Solution FA 0.075%	2.74
Methanolic Solution FA 0.075%	5.93
Aqueous Solution FA 0.075% + IL 0.2%	8.03
Methanolic Solution FA 0.075% + IL 0.2%	9.02
Aqueous Solution FA 0.05%	3.62
Methanolic Solution FA 0.05%	5.09
Aqueous Solution FA 0.05% + IL 0.2%	8.69
Methanolic Solution FA 0.05% + IL 0.2%	8.97
Ethosomes Blank	4.38
Ethosomes IL 0.2%	9.91
Ethosomes FA 0.03%	3.99
Ethosomes FA 0.05%	3.78
Ethosomes FA 0.05% + IL 0.2%	9.74
Ethosomes FA 0.075%	3.68
Ethosomes FA 0.075% + IL 0.2%	8.40

After measuring the pH in solution and in the ethosomes, it was decided to measure the pH of the emulsions.

pH values in emulsion with different composition were all acidic, **Table 10**. This is due to the acid nature of the Crodrafos CES ingredient (Cetearyl Alcohol (and) Dicetyl Phosphate (and) Ceteth-10 Phosphate) (Inc., n.d.).

Table 10 Values of pH in emulsions.

Emulsions	**pH**
Aqueous solution with. FA 0.05 %	2.94
Methanolic. solution with. FA 0.05 %	3.09
Aqueous solution with. FA 0.05 % + IL 0.2 %	2.84
Methanolic solution with. FA 0.05 % + IL 0.2 %	2.79
Aqueous solution with. IL 0.2 %	2.93
Methanolic solution with. IL 0.2 %	3.01
Ethosomes with. FA 0.05 %	2.92
Ehosomes witht. IL 0.2 %	3.18
Ethosomes with. FA 0.05 % + IL 0.2 %	3.09
Ethosomes. Blank	2.98
Ethosomes with. FA 0.03 %	3.19

In Vitro Antioxidant Activity

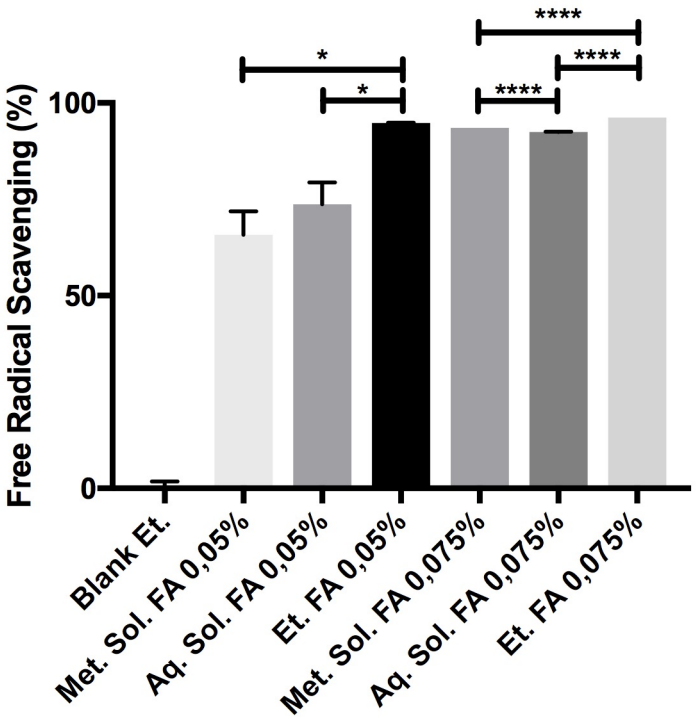

Figure 15 DPPH radical scavenging (%) for non-encapsulated and encapsulated formulations containing FA 0.05 % and 0.075%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

Fugure 15 shows that antioxidant activity of both 0.05% and 0.075% FA in ethosomes is higher than aqueous and methanolic solutions with the same concentration.

Methanolic and aqueous solutions present similar antioxidant activity, which is not in accordance with Rutin results, where methanolic solutions presented higher antioxidant activity. The results may be related with the solubility of both actives in aqueous and methanolic solutions. The solubility of FA in water is 0.06%, at a temperature of 25 ºC (Caparica, 2017).

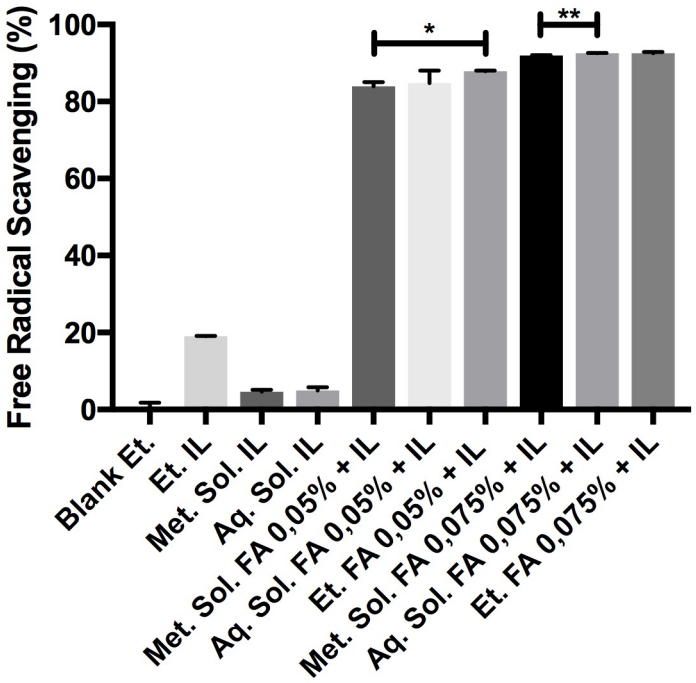

Figure 16 DPPH radical scavenging (%) for non-encapsulated and encapsulated formulations containing FA 0.05 % + IL 0.2 % and FA 0.075% + IL 0.2%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

The same solutions and ethosomes at exactly the same concentrations were prepared with IL. FA in ethosomes continue to have higher antioxidant activity when compared with the solutions **Figure 16.**

Regarding the solutions with IL, the aqueous ones are those that present greater antioxidant activity, for the two concentrations studied.

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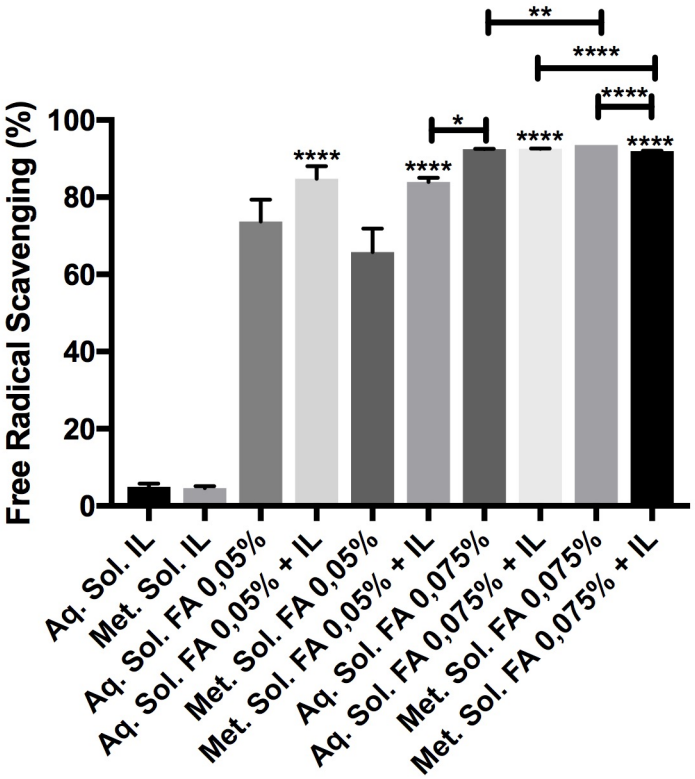

Figure 17 DPPH radical scavenging (%) for non-encapsulated formulations, with and without IL, containing FA at 0.05%, 0.075%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

In this **Figure 17** all non-encapsulated formulations of FA are presented at different concentrations with and without IL. Almost all solutions containing IL in their composition have a higher antioxidant activity than without IL. This happens because IL increases the solubility of the active substances, which increases its antioxidant activity, as can be seen in this Figure.

. Being that the aqueous solution with IL that presents greater % of free radical scavenging.

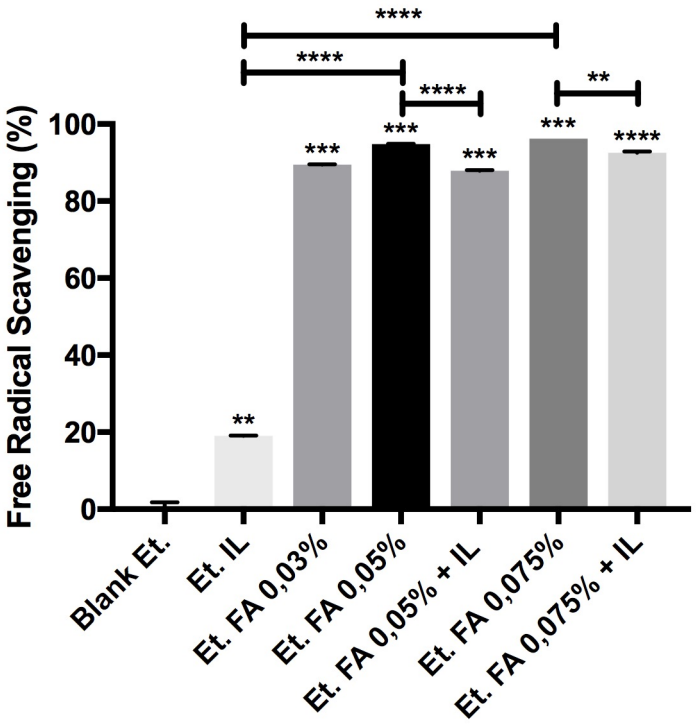

Figure 18 DPPH radical scavenging (%) for ethosomes containing FA with and without IL at 0.03%, 0.05% and 0.075%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

As expected, in **Figure 18**, blank ethosomes do not have antioxidant activity, since they have no antioxidant properties in their composition. The same cannot be said for ethosomes that contain only IL, since they have an antioxidant activity of approximately 19.05%. This result shows that IL can contribute to the some antioxidant activity alone. In contrast, when FA is encapsulated in the presence of IL, antioxidant activity is slightly lower when compared with FA ethosomes without IL. Thus, the results show to be contradictory, and have to be further investigated.

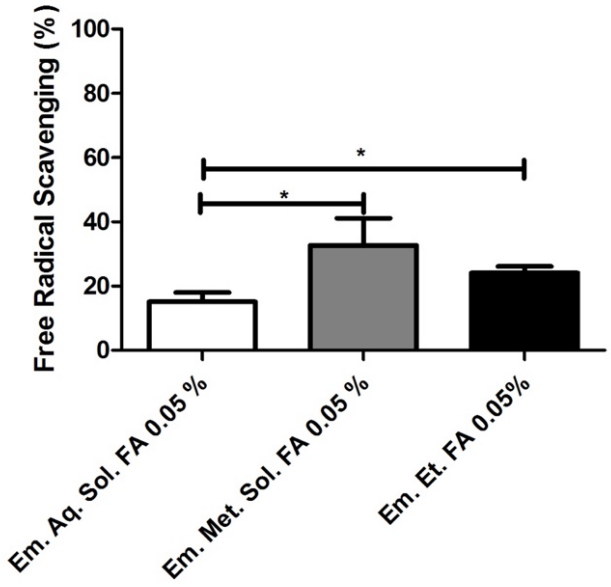

Figure 19 Comparison of free radical scavenging between emulsions with non-encapsulated and encapsulated FA 0.05%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

Emulsions containing methanolic solution with FA presents higher antioxidant activity, whereas the emulsion containing aqueous solution with FA presents the lowest antioxidant activity, **Figure 19**. These results are not in agreement with the values presented in solutions or in ethosomes alone, **Figure 15**, in which the ethosome with FA presents a greater antioxidant activity.

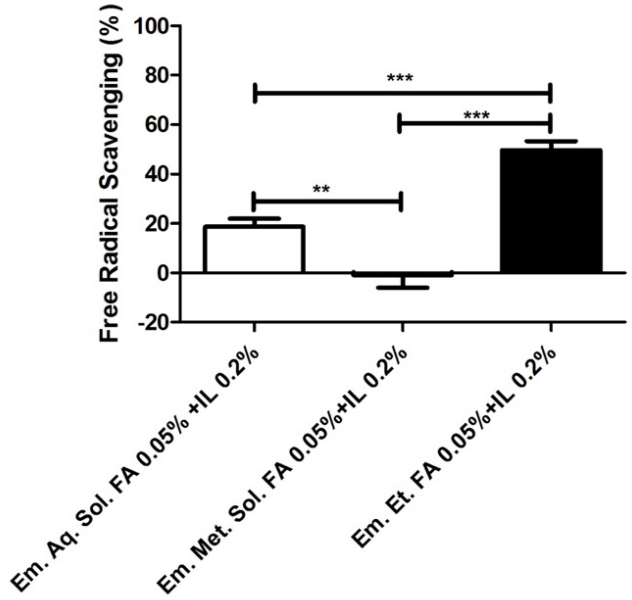

Figure 20 Comparison of free radical scavenging between emulsions with non-encapsulated and encapsulated FA 0.05% + IL 0.2%. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

For the emulsions containing FA 0.05% and IL 0.2%, the results are more in agreement with the expectations. When we compare the solution and ethosomes in emulsions with them when they are alone, **Figure 16**, we find that there is an increasing order of antioxidant activity, being the emulsion with ethosomes with FA + IL, then the emulsion with aqueous solution containing FA + IL, and finally the emulsion with methanolic solution with FA + IL, **Figure 20**.

Microscopic Analysis

Microscopic analysis of emulsions showed that all formulations homogeneous **Figure 21**.

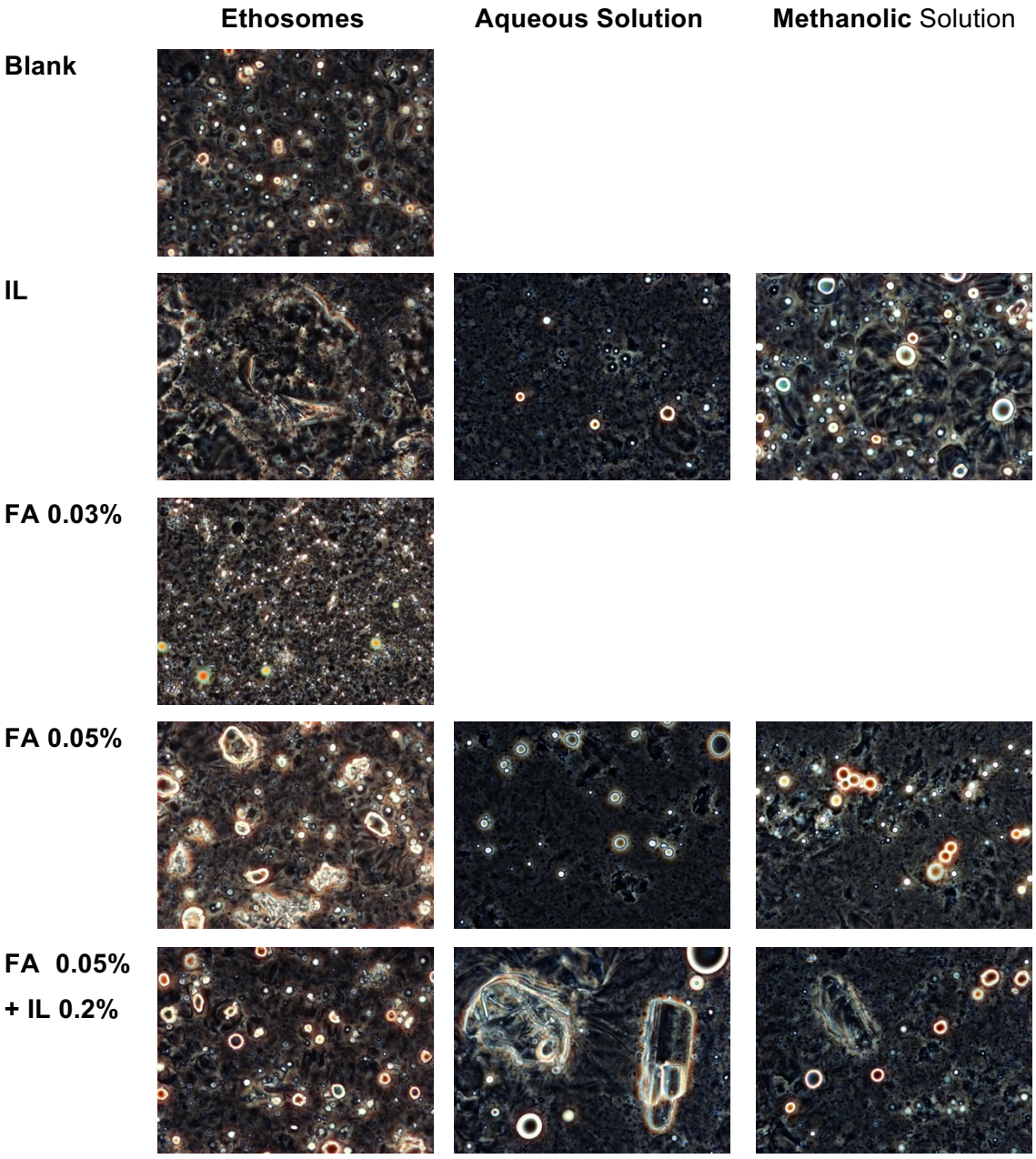

Figure 21 Microscope photos of emulsions with ethosomes, aqueous solutions and methanolic solutions with FA 0.03 and 0.05, with and without IL.

Emulsions containing FA 0.05 % + IL 0.2 % showed crystals, which may be attributed to some FA crystallization during formulation or microscope examination due to water evaporation.

Preliminary Stability

All emulsions showed to be stable after preliminary stability studies, since no signs of flocculation and/or phase separation were observed, results not shown.

Rutin VS Ferulic acid

In vitro antioxidant activity

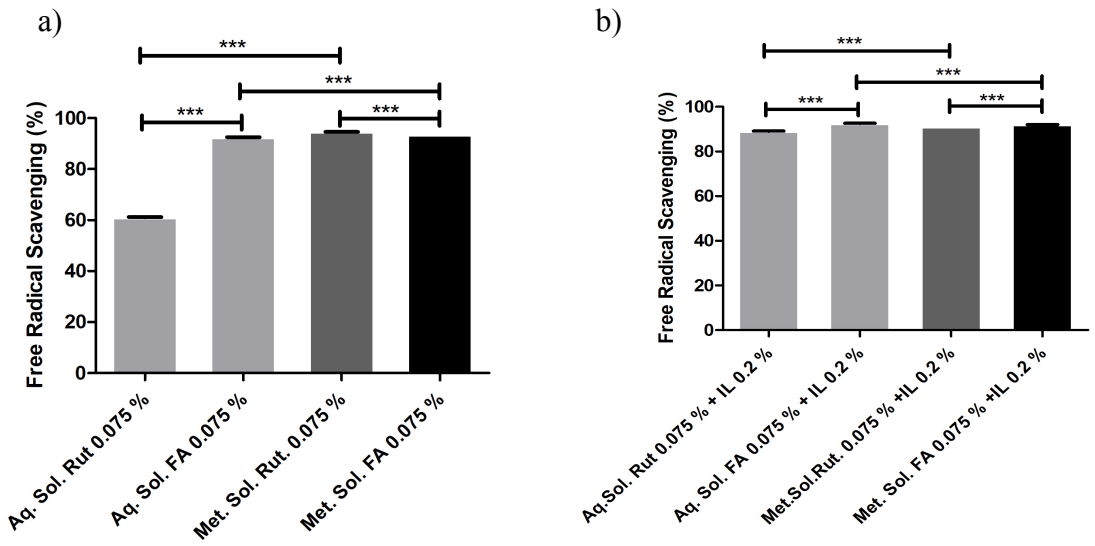

Figure 22 DPPH radical scavenging (%) for non-encapsulated formulations containing FA 0,075% and Rutin 0,075% a) Without IL b) With IL. n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

Rutin has a higher antioxidant activity in methanol, while FA has a higher antioxidant activity in water. These results clearly demonstrate that the solubility is playing an important role in the antioxidant activity, where FA solubility in water is greater than Rutin, 0.6% vs. 0.2%. These results are in agreement with the literature studies (Zi et al., 2007; Alexandra et al., 2017).

In the **Figure 22** it can be seen that by adding IL to the formulation, all solutions have a higher antioxidant activity when compared to solutions without IL. However, when we compare the aqueous solution with the methanolic solution of Rutin or FA, the differences between them are much smaller.

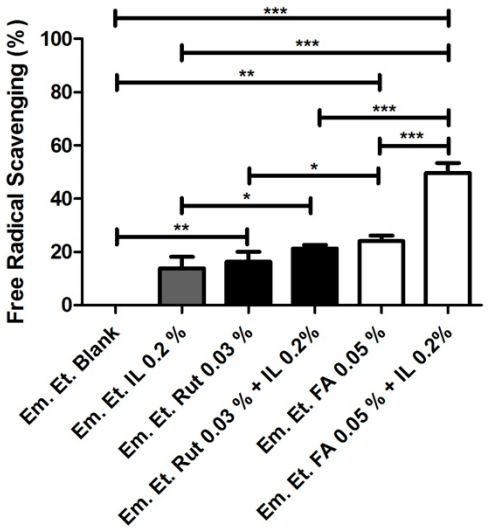

Figure 23 Comparison of free radical scavenging between emulsions with ethosomes containing Rutin 0.03% and FA 0.05%, with and without IL n=3, mean ± SD and * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA, Tukey's test).

For emulsions containing ethosomes, **Figure 23**, we can say that the emulsion with ethosomes containing IL has antioxidant activity, however as it would be expected, the lowest of all the emulsions, after the emulsion with blank ethosomes.

By comparing Rutin alone with Rutin with IL, the latter has higher antioxidant activity, as does FA. This result is not in agreement with previous results where it could be seen that the addition of IL to Rutin and FA loaded ethosomes decreases antioxidant activity, probably attributable to some destabilization of the phospholipid membrane, **Figure 9 and 17**.

However, the addition of IL to FA is more beneficial that for Rutin, since its antioxidant activity is more potent. These results need more studies to be done to confirm this phenomenon.

We can also say that different actives have an influence on the way in which IL behaves in the ethosomes, since in the emulsion with ethosome of FA + IL, this increases much more than when compared to the emulsion containing Rutin.

Conclusion

Ethosomes containing Rutin and FA were successfully developed. Rutin loaded ethosomes were smaller when IL was incorporated. For FA, size results were inconclusive.

Additionally, the zeta potential values were close to zero, and all the PDI values are above 0.2. For that reason, stability should be carefully analyzed.

Both ethosomes with Rutin and FA have higher antioxidant activity than aqueous and methanolic solutions. For most of the ethosomes the presence of ILs decreased the antioxidant activity for Rutin and FA. These results do not make much sense, since the ethosomes only with IL, present antioxidant activity. With this, we can conclude that there may be an interaction between the IL and the phospholipid bilayer of the ethosomes, which may destabilize this ethosomes, causing the value of the antioxidant activity to decrease.

Rutin and FA solutions were also prepared to compare with colloidal dispersions. Both Rutin and FA solutions present higher antioxidant activity when IL is incorporated, since the aqueous and methanolic solution with IL alone (without any loading) have antioxidant activity. With this we can conclude that IL has a contribution for the antioxidant activity and/or increases the solubility of the actives.

Stable emulsions were also prepared by incorporating Rutin and FA colloidal dispersions.

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