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IONIC LIQUIDS: SYNTHESIS AND APPLICABILITY TO IMPROVE THE DELIVERY OF FERULIC ACID

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Escola de Ciências e Tecnologias da Saúde

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Abstract

The applicability of ionic liquids (ILs) in the pharmaceutical field has been a topic of growing interest due to the valuable properties of these salts, namely, to improve drug solubility, permeation and stability. This strategy has proven to be useful since ILs may improve the delivery of bioactive compounds with very low aqueous solubility, which otherwise would not be as accessible.

Additionally, ILs may have different properties according to their structure and combining ILs with different properties may also be an interesting approach to improve the efficiency of drug delivery systems.

Hence, herein two ILs, of different classes were synthesized, the choline-based [Cho][Phe] and the imidazole-based [Emim][Br], and the impact of their combination on the solubility of the poorly water soluble ferulic acid was assessed.

Results showed that the studied ILs increase the solubility of the studied compound and further display that their combination allows an even higher solubility enhancement.

Furthermore, due the possible topical applicability of ferulic acid this compound was incorporated in gel formulations and IL-IL-nanoparticles hybrid systems. All prepared formulations were stable and the presence of ILs allowed a higher ferulic acid loading.

In summary, results revealed that ILs, either alone or combined, may be a powerful strategy to increase the drug delivery of poorly soluble drugs, such as ferulic acid.

Keywords: Ionic liquids; Synthesis; Ferulic acid; Gels; IL-IL-nanoparticles hybrid systems; Higher drug solubility/loading.

Resumo

A aplicabilidade dos líquidos iónicos (LIs) na área farmacêutica tem sido um tópico de crescente interesse devido às propriedades destes sais, como por exemplo, poderem melhorar a solubilidade, permeação e estabilidade do fármaco. Esta estratégia provou ser útil, uma vez que os LIs podem melhorar a distribuição de compostos bioativos com solubilidade aquosa muito baixa, o que de outra forma não seria tão acessível.

Além disso, os LIs podem ter propriedades diferentes de acordo com sua estrutura, e a combinação de LIs com propriedades diferentes também pode ser uma abordagem interessante para melhorar a eficiência dos sistemas de administração de fármacos.

Assim, foram sintetizados dois LIs de diferentes classes, o [Cho][Phe] à base de colina e o [Emim][Br] à base de imidazólio, e foi avaliado o impacto da sua combinação na solubilidade do ácido ferúlico, que é pouco solúvel em água.

Os resultados mostraram que os LIs estudados aumentam a solubilidade do composto estudado e indicam ainda que a sua combinação permite um aumento ainda maior da solubilidade.

Além disso, devido à possível aplicabilidade tópica do ácido ferúlico, este composto foi incorporado em formulações em gel e sistemas híbridos de LI-LI-nanopartículas. Todas as formulações preparadas foram estáveis e a presença de LIs permitiu uma maior encapsulação de ácido ferúlico.

Em resumo, os resultados revelaram que os LIs, isoladamente ou combinados, podem ser uma estratégia poderosa para aumentar a veiculação de compostos pouco solúveis, como o ácido ferúlico.

Palavra-Chave: Líquidos iónicos; Ácido ferúlico; Geles; Sistemas híbridos LI-LI-nanopartículas; Aumento da solubilidade/incorporação de ativos.

List of abbreviations and acronyms

[Cho][Phe]	(2-hydroxyethyl) trimethylammonium-L-phenylalaninate
[Emim][Br]	1-ethyl-3-methylimidazolium bromide
AE	Association efficiency
BODIPY	4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid saccinimidyl ester
COX	Cyclooxygenase
FA	Ferulic Acid
i-NOS	Nitric oxide synthase
IL-6	Interleukin-6
IL-8	Interleukin-8
IL	Ionic liquid
IUPAC	International Union of Pure and Applied Chemistry
LC	Loading capacity
LDL	Low-density lipoprotein
O/W	Oil in water
PBS	Phosphate buffer saline
PdI	Polydispersity index
PLGA	Poly (lactic-co-glycolic Acid)
PVA	Polyvinyl alcohol
RTILs	Room temperature ionic liquids
SPF	Sun protection factor
UV-visible	Ultraviolet visible
UVA-PF	Ultraviolet A - protection factor
W/O	Water in oil
W/O/W	Water in oil in water
ZP	Zeta Potential

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1. Introduction and relevance of the study

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The Pharmaceutical Industry has been widely concerned about the development of new or more effective delivery systems. However, in this context there are many challenges to surpass, such as the poor drug solubility, permeation, as well as, the low stability of some drugs and of the delivery systems. In fact, these problems may even impair the applicability of some compounds with potential therapeutic use, but with limited water solubility. In consequence, several studies have been performed to overcome these weaknesses.

Strategies such as finding new functional excipients, that lead to more efficient delivery systems, are promising tactics, and in this context, ionic liquids (ILs) have proven to be a valuable approach (Tânia Santos de Almeida, Júlio, Mota, & Pinto Reis, 2017; Caparica et al., 2018).

More recently, the combination of ILs with nanosystems has also been studied and disclosed as a useful tactic. In fact, nanosystems which are also considered a useful approach that leads to several advantages in drug delivery (Qiu & Texter, 2008; He & Alexandridis, 2015; Martinis, Grijalba, Pérez, Llaver, & Wuilloud, 2017; Tânia Santos de Almeida, Júlio, Mota, & Pinto Reis, 2017). Hence, their combination with ILs may be a step forward in improving these characteristics. This type of systems offers many advantages, such as allowing a more controlled delivery to the target site, improving the therapeutic effects and reducing cytotoxicity in normal cells, by leading to less accumulation in the healthy body sites (Mora-Huertas, Fessi, & Elaissari, 2010; Ghosh, Saha, & Sur, 2015; Fonte, Reis, & Sarmento, 2016; Lim, Chung, & Chung, 2016; Gaul, Ramsey, Heise, Cryan, & Greene, 2018; Hua, de Matos, Metselaar, & Storm, 2018; Sharma et al., 2018; Sousa, Ferreira, Rodrigues, & Rodrigues, 2019).

Concerning ILs, the growing interest in their use in drug delivery, arises from their many valuable properties, such as the fact that some have been considered as possible substitutes for toxic volatile organic solvents (Earle et al., 2006; Marrucho, Branco, & Rebelo, 2014; Tânia Santos de Almeida, Júlio, Mota, et al., 2017). They also have high thermal and chemical stability, they are soluble in several solvents and may improve the solubility and stability of many different compounds (Qiu & Texter, 2008; Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

Thus, the number of studies that incorporate ILs in different delivery systems, such as O/W emulsions, gels (Tânia Santos de Almeida, Júlio, Mota, et al., 2017) and nanodelivery systems (Júlio, Caparica, et al., 2019; Caparica et al., 2020) to improve the efficiency of these systems, has increased considerably.

Hence, it is quite relevant to continue to study the possible benefits of using ILs as functional excipients, particularly in drug delivery, by incorporating them in different formulations. In fact, these strategies may lead to the development of innovative and more efficient drug delivery systems.

Bearing this in mind and knowing that some ILs may be more suited as solubility enhancers and others as permeation promoters, herein two ILs from different classes were synthesised and combined to assess if their combination could improve the delivery of the poorly soluble ferulic acid.

2. Ferulic acid

2. Ferulic acid

Ferulic acid (FA) is a phenolic compound with the International Union of Pure and Applied Chemistry (IUPAC) name of (E)-3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid (**Figure 1**). Hence, this compound is a phenolic acid and it present at relatively high concentrations in the tissues and in cell walls of several plants, therefore, it constitutes a bioactive ingredient of many foods, such as vegetables, fruits, some beverages such as coffee and beer and in some Chinese medicinal herbs such as *Cimicifuga racemose*, *Angelica sinensis*, and *Ligusticum chuangxiong* (Mathew & Abraham, 2004; Zhao & Moghadasian, 2008; Mancuso & Santangelo, 2014; Oliveira Silva & Batista, 2017).

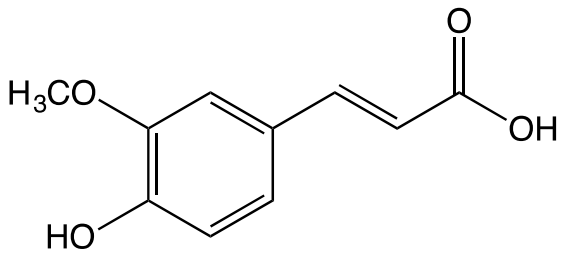

Figure 1: Chemical structure of ferulic acid.

Phenolic compounds, such as ferulic acid, are comprised of a heterogeneous class of natural products, which are present in plant-based foods (Soto-Vaca, Gutierrez, Losso, Xu, & Finley, 2012; Oliveira Silva & Batista, 2017). These compounds are secondary metabolites of plants, with different structures and with a broad phylogenetic distribution and most of these compounds come from a common origin: the amino acids phenylalanine or tyrosine (Chirinos et al., 2009; Pereira, Valentão, Pereira, & Andrade, 2009; Khoddami, Wilkes, & Roberts, 2013). They include a very broad spectrum of molecules that contain an aromatic group and one or more hydroxyl groups on the aromatic ring (Soto-Vaca et al., 2012; Oliveira Silva & Batista, 2017). Thus, based on the number of phenolic rings they contain and based on the structural elements that link these rings together, they can be classified into different groups (Chirinos et al., 2009).

These compounds are generally divided into phenolic acids, were FA is included, polyphenols, flavonoids, tannins and lignans (Oliveira Silva & Batista, 2017). Two families of phenolic acids are hydroxybenzoic acids and hydroxycinnamic acids. The latter includes ferulic

acid, the compound of interest for the present work, as well as the coumaric, caffeic, and sinapic acids which have many health benefits such as antioxidant, anti-inflammatory, antibacterial, anticarcinogenic activities, among others (Shahidi & Chandrasekara, 2010).

Ferulic acid, is one of the metabolites of the biosynthesis of lignin from phenylalanine and tyrosine in plants and it can be found in the free form and in the conjugated form in the plant tissues, indicating the sum of the two, the total FA (Zhao & Moghadasian, 2008). They are intermediates in the biosynthesis of some important natural products, such as, chlorogenic acid, sinapic acid, diferulic acids, curcumin, *p*-coumaryl alcohol, coniferyl alcohol, and vanillin, among others (Shahidi & Chandrasekara, 2010).

Ferulic acid, has been associated with many valuable properties of interest that justifies its choice for this study. Among these are its antioxidant, antimicrobial, anti-inflammatory, antidiabetic, anticarcinogenic, antiallergic, antiviral, hepatoprotective, antithrombotic and vasodilatory properties (Kumar & Pruthi, 2014; Mancuso & Santangelo, 2014; Oliveira Silva & Batista, 2017; Caparica et al., 2018) and they will be further discussed next.

2.1. Biological Activities

One of the best described biological activities of FA is its antioxidant properties with a strong cytoprotective activity. This is strongly associated with its phenolic hydroxyl group and an extended side chain, and the ability to eliminate free radicals, via a radical-scavenging mechanism, and activate the response to cellular stress (Zhao & Moghadasian, 2008; Shahidi & Chandrasekara, 2010; Mancuso & Santangelo, 2014; Oliveira Silva & Batista, 2017), since, an antioxidant has the ability to reduce the formation of free radicals and scavenge ROS, which will inhibit or repair damage caused by the oxidation and degradation of other molecules (Shahidi & Chandrasekara, 2010; Oliveira Silva & Batista, 2017).

Several *in vitro* and *in vivo* studies have demonstrated many benefits of FA, as shown in **Table 1**, and its antioxidant activity is one of the most appraised ones, to combat diseases associated with oxidative damage (Shahidi & Chandrasekara, 2010; Oliveira Silva & Batista, 2017). For instance, Castelluccio et al. (1996) showed that FA is an effective antioxidant against oxidation of low-density lipoprotein (LDL) compared to ascorbic acid (Castelluccio, Bolwell, Gerrish, & Rice-Evans, 1996). Another study showed that FA decreased oxidative stress in neuronal cell systems (Kanski, Aksenova, Stoyanova, & Butterfield, 2002). Furthermore, Itagaki et al. (2009) showed that Ferulic acid also significantly inhibited 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a,-diaza-s-indacene-3-propionic acid saccinimidyl ester (BODIPY) oxidation at a concentration of 1 mM (Itagaki et al., 2009).

Regarding the antimicrobial activity of ferulic acid, it has been shown that FA inhibits the growth of human microorganisms, such as the yeast *Dekkera bruxellensis* (Harris, Ford, Jiranek, & Grbin, 2008) and also of *Cronobacter sakazakii*, which is an opportunistic pathogen transmitted by food (Shi et al., 2016).

Concerning the anti-inflammatory activity, a study portrayed that the intraperitoneal and topical administration of FA promoted apoptosis in the mice skin cells, when chronically irradiated by ultraviolet B, decreasing the oxidative imbalance and, also, angiogenic and inflammatory signalling (Ambothi, Prasad, & Balupillai, 2015). These facts were due to the concomitant decrease in the expression of the mutated *p53* and the anti-apoptotic protein *Bcl-2*. Thus, this impeded the progress of photocarcinogenesis (Ambothi et al., 2015). Still, other studies have demonstrated the ability of FA together with other antioxidants, to inhibit the function or expression of some inflammatory mediators, such as cyclooxygenase (COX),

caspase-1 and nitric oxide synthase (i-NOS) (Doss, Dey, Sudandiradoss, & Rasool, 2016; Nile, Ko, Kim, & Keum, 2016). Others refer that FA decreases the level of some chemokines such as murine interleukin-6 (IL-6) and interleukin-8 (IL-8), causing them to migrate toward the site of infection (Lampiasi & Montana, 2016).

The antidiabetic activity of FA as also been considered and studies have shown that in diabetic animals FA decreases the blood glucose levels, increases the activities of glutathione peroxidase, superoxide dismutase catalase and the expansion of pancreatic islets. This means that there has been an increase in antioxidant activity, neutralizing free radicals and reducing the intensity of this disease in the studied animals (Balasubashini, Rukkumani, Viswanathan, & Menon, 2004). In addition, other articles demonstrated that ferulic acid may modulate insulin signalling molecules in the liver (Narasimhan, Chinnaiyan, & Karundevi, 2009) and ultimately improve the capacity for oxidative stress of hepatocytes and cardiomyocytes when exposed to high concentrations of glucose (Song et al., 2016).

Finally, regarding the anticancer activity of ferulic acid, studies revealed cytotoxicity of this molecule in relation to histiocytic lymphoma U937 (Chiang, Chiang, Chang, Ng, & Lin, 2003), and melanoma cancer cell lines, leading to the suppression of melanoma growth by targeting the fibroblast growth factor receptor 1-mediated PI3K-Akt-signaling pathway (Yang, Jiang, & Lu, 2015) and human cervical ME-180 (Panwar, Sharma, Kaloti, Dutt, & Pruthi, 2016). Another *in vitro* study demonstrated the anticancer activity of some phenolic compounds, including ferulic acid, on the human breast cancer T47D cell line, showing an additional protective effect on hormone-dependent breast tumours (Kampa et al., 2004).

Finally, an *in vivo* study demonstrated that ferulic acid reinforces the spectrum of photoprotective formulations, increasing the sun protection factor (SPF) and also the ultraviolet A - protection factor (UVA-PF) of these sunscreens, concluding that this compound may be useful in this type of topical formulations (Peres, Sarruf, de Oliveira, Velasco, & Baby, 2018).

Table 1: Overview on some of Ferulic Acid activities.

Ferulic Acid Activity		References
Antioxidant	Neuronal cell systems	Kanski et al 2002
	LDL	Castelluccio et al 1996
	BODIPY	Itagaki et al, 2009
Antimicrobial	*Cronobacter sakazakii*	Shi et al, 2016
	Dekkera bruxellensis	Harris et al., 2008
Anti-inflammatory	COX; caspase-1; i-NOS	Lampiasi & Montana 2016
	IL-6; IL-8	
	p53, Bcl-2	Ambothi et al., 2015
Antidiabetic	Glucose; glutathione peroxidase; superoxide dismutase	Balasubashini et al 2004
	Insulin	Narasimhan et al., 2009
	Hepatocytes; Cardiomyocytes	Song et al., 2016
Anticarcinogenic	Histiocytic lymphoma U937	Chiang et al., 2003
	Human cervical ME-180	Panwar et al., 2016
	Fibroblast growth factor receptor	Yang et al., 2015
	T47D cell line	Kampa et al., 2004
Photoprection	SPF; UVA-PF	Peres et al., 2018

In addition, some studies have also shown that the use of this compound in topical delivery systems can be of great interest. As an example of this, the combination of FA with ascorbic acid and tocopherol prevents the appearance of wrinkles, erythema and hyperpigmentation (Lin et al., 2005; Kumar & Pruthi, 2014; Caparica et al., 2018) .

Hence, it is quite clear that there are already several studies that show the promising properties that could make FA a compound with potential interest in the pharmaceutical and also in the cosmetic area. However, this phenolic acid has a low solubility in water, which limits its delivery and bioavailability. Thus to ensure the applicability of FA, this problem must be overcome to ensure that its described bioactivities may have a potential application in drug delivery, particularly in topical delivery (Mota, Queimada, Pinho, & Macedo, 2008; Caparica et al., 2018).

2.2. Topical Applications

Topical drug delivery is defined as the application of a formulation containing drug on the skin with the aim of treating skin disorders or cosmetic applications. Thus, it is a route of administration that can allow a more efficient administration according to the intended objective and is an alternative that has been increasingly explored (Brown, Martin, Jones, & Akomeah, 2006; Joshi, Butola, & Saha, 2014; Singh Malik, Mital, & Kaur, 2016; Caparica et al., 2018).

This type of drug delivery system has several advantages, such as:

- Being: painless and non-invasive,
  easy to finish the medication whenever needed
  easy route of administration
- Allowing: direct access to the target site,
  an improved patient compliance and acceptance
  high efficiency using very low total dosage
- Avoiding: hepatic first pass metabolism,
  the gastric pH variations,
  gastro-intestinal incompatibility,
  the risk and issues of intravenous medication.

However, there are also a few disadvantages, such as, the possibility of occurring allergic reactions, difficulty in drug permeation and in drug loading into the topical delivery systems, appearance of possible contact dermatitis or irritation and lastly drug denaturation due to the presence of enzymes in the epidermal layer of the skin (Joshi et al., 2014; Caparica et al., 2018). Nonetheless, there are much more advantages than disadvantages, in the use of this type of delivery systems, particularly if the possible downsides are taken into account and avoided during formulation.

Bearing all of this in mind, as well as the previously described characteristics of ferulic acid, that indicated that the use of this compound in topical delivery systems can bring advantages, it may be quite relevant to further study the topical delivery of FA.

Nonetheless, even though ferulic acid, and many other phenolic compounds, may present several promising properties that could be useful in the Health area, its low solubility limits its applicability. In this context, Ionic Liquids may be an interesting strategy to surpass this obstacle and this strategy will be studied herein.

3. Ionic Liquids

3. Ionic Liquids

3.1. Definition and characteristics

Ionic liquids are low-melting salts (liquid at temperatures below 100 °C) which are composed of ions, organic cations and organic or inorganic anions. They are usually viscous liquids with a good capacity to dissolve inorganic and organic substances (Qiu & Texter, 2008; Tânia Santos de Almeida, Júlio, Mota, et al., 2017). ILs have many prised properties (**Figure 2**). To name a few, ILs have been used as solvents, catalysts for the synthesis of reactions, biocatalysts, in electrochemistry (Hapiot & Lagrost, 2008; Macfarlane et al., 2007), as active solubilizers, thermal fluids, as reaction media for many reactions (Miao & Tak, 2006), as colloidal stabilizers for nanoparticles (Tânia Santos de Almeida, Júlio, Mota, et al., 2017) and can be used in separations (Armstrong & Welch, 2007) and extractions (Huddleston, Willauer, Swatloski, Visser, & Rogers, 1998).

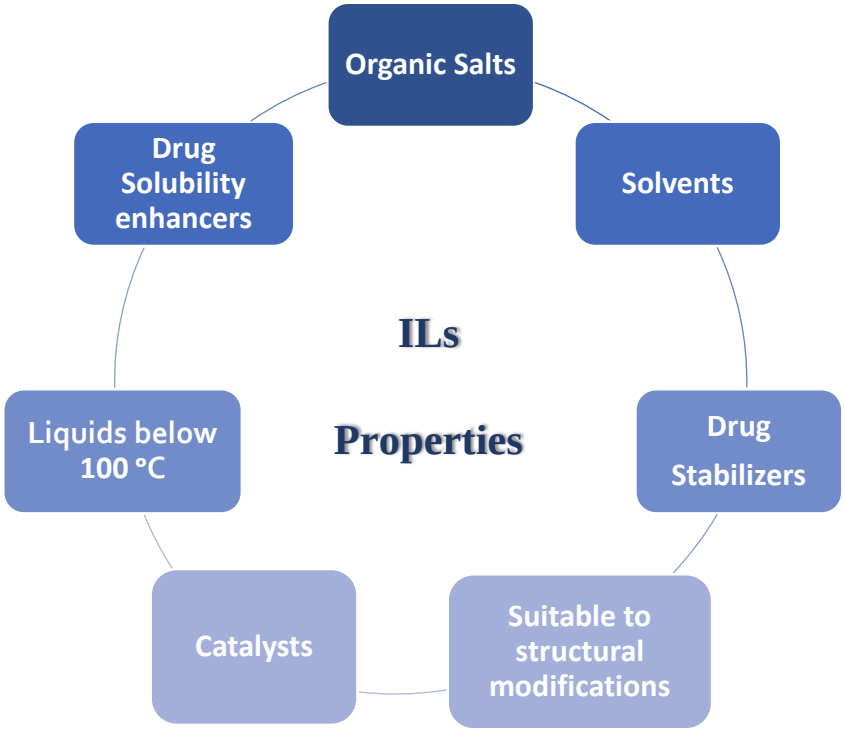

Figure 2: General properties of ionic liquids.

Furthermore, these organic salts may be liquid at room temperature, which can be a quite useful property when trying to incorporate them in delivery systems, and in this case, they

are named Room Temperature Ionic liquids (RTILs). Furthermore, they also have other singular characteristics, such as good thermal and chemical stability, high ionic conductivity, nonflammability and non-volatility, low vapour pressure and, in some cases, they may be recycled. Also, these salts have high solubility in different types of solvents, which is a useful characteristic when considering their application in drug delivery. They also present high suitability for structural modifications, which allows the adjustment of their structures, and consequently the adaptation of their properties and functions, accordingly to a desired application (Qiu & Texter, 2008; Zech & Kunz, 2011; Gouveia et al., 2014; He & Alexandridis, 2017; Martinis et al., 2017; Tânia Santos de Almeida, Júlio, Mota, et al., 2017; Tânia Santos de Almeida, Júlio, Saraiva, et al., 2017).

So, considering all that was mentioned above and depending on these praised properties, ILs may be incorporated in water, oils or hydroalcoholic solutions, facilitating their incorporation into different formulations, to improve drug solubility, loading and delivery (Moniruzzaman, Tamura, Tahara, Kamiya, & Goto, 2010; Dobler, Schmidts, Klingenhöfer, & Runkel, 2013; T. Santos de Almeida et al., 2015; Tânia Santos de Almeida, Júlio, Mota, et al., 2017) .

ILs have been considered either protic ILs or aprotic ILs, regarding their synthesis and their structure. Protic ILs are formed through proton transfer from a Brönsted acid to a Brönsted base and aprotic ILs require strategies different from these acid-base reactions (Tânia Santos de Almeida, Júlio, Mota, et al., 2017) Also, depending on the cation or anion present, the ILs properties will differ from each other, and in this way, the different ILs will offer different applicability's.

Considering the cations present, ILs have also been classified into four main categories. The considered cations are: *N*-alkyl-pyridinium cation, a dialkylimidazolium cation, a phosphonium cation and alkylammonium cation (Ghandi, 2014; Ja Hye Myung, Sin-jung Park, Andrew Z. Wang, 2017; Tânia Santos de Almeida, Júlio, Mota, et al., 2017). It should be noted herein, considering the aim of the present study, that amongst these cations, the imidazole derivatives consist of the most studied class, and the ones based on the alkylammonium cation, particularly those derived from choline cation, have been referenced as the less toxic and thus more suited for pharmaceutical application (Qiu & Texter, 2008; Zech & Kunz, 2011; Gouveia et al., 2014; He & Alexandridis, 2017; Martinis et al., 2017; Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

3.2. Overview on the advantages and disadvantages

Due to the attractive characteristics of ILs, there has been an emergent interest in their study and some of the advantages of these salts have already been stated in this work, such as their high thermal and chemical stabilities and the fact that they are soluble in many solvents (Gouveia et al., 2014; Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

Moreover, the use of ILs as reaction media, in the synthesis of active pharmaceutical ingredients, as also proven to bring more advantages than in the presence of the usual organic solvents (Mizuuchi, Jaitely, Murdan, & Florence, 2008; He & Alexandridis, 2017; Tânia Santos de Almeida, Júlio, Mota, et al., 2017). Also, ILs have a very low vapour pressure, nonetheless this may not always be considered an advantage, particularly if it is needed to separate the IL from a mixture (Zech & Kunz, 2011).

So, each property, and its associated advantages or disadvantages, need to be balanced according to the purpose of their use, and other examples corroborating this consideration may be given. For instance, the fact that ILs may be structurally modified towards a specific application has also been previously referred, in the literature and this work, as an advantage. Nonetheless, this characteristic may have a less positive feature, arising from their reactivity, since ILs may not always be considered as inert solvents, as in certain cases they react with other reactants (Zech & Kunz, 2011; Ghandi, 2014; Gouveia et al., 2014; Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

Furthermore, the fact that some ILs may possibly substitute volatile organic solvents, that are usually more toxic, has been the justification for considering these salts green materials and mentioned as a clear advantage (Earle et al., 2006; Qiu & Texter, 2008; Ghandi, 2014; Gouveia et al., 2014; Kubota, Shibata, & Yamaguchi, 2016; He & Alexandridis, 2017; Martinis et al., 2017; Tânia Santos de Almeida, Júlio, Mota, et al., 2017) Nonetheless, it is vital not to forget that some ionic liquids may be quite toxic, depending on the cation and/or anion present, and their use may be far from what is considered “green”. In fact, for example, imidazole-based ILs with a cation including long alkyl sidechains, have been considered quite toxic (Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

Hence, when classifying ionic liquids as “green solvents”, which represents a huge advantage, caution needs to be taken since not all ILs may be included in this classification. In

this context, ILs containing ions derived from biomaterials such as amino acids, have been described as less toxic and more biodegradable and thus more advantageous for pharmaceutical applications (Plechkova & Seddon, 2008; Gouveia et al., 2014; Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

3.3. Applicability in the pharmaceutical field

There has been a growing interest in the applicability of ILs in the pharmaceutical field, particularly as promotors of drug solubility, permeation and stability. Also, as already mentioned, ILs may be soluble in different types of solutions, and hence they may be applied as excipients and incorporated into different delivery systems becoming a functional alternative to other more toxic organic solvents (Tânia Santos de Almeida, Júlio, Mota, et al., 2017). Moreover, other studies have also shown that some ILs may present antimicrobial activity, like antibacterial and antifungal activities, which reinforces their possible functionality in delivery systems, viz. as preservatives (Obando et al., 2009; Busetti et al., 2010; Czekański, Santos de Almeida, P. Mota, Rijo, & M. Araújo, 2014; Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

To go even further, from a cosmetic point of view, these salts have also been used for the development of antiperspirants/deodorants and perfumes (Mohamed, 2015; Tânia Santos de Almeida, Júlio, Mota, et al., 2017) Also, and returning to the pharmaceutical area, they have been used to overcome challenges such as poor drug solubility, polymorphism and low bioavailability, and have also been considered through the use of active pharmaceutical ingredients to produce ILs with biological activity (Ferraz, Branco, Prudêncio, Noronha, & Petrovski, 2011; Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

More recently, it has also been described that the association of ILs with nanosystems may be relevant due to the ILs advantages that may improve the performance of these resulting combined systems. In fact, the use of ILs contributes to the stabilization of nanoparticles and may be quite relevant in controlling their size (He & Alexandridis, 2017; Martinis et al., 2017; Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

ILs have also been used in other delivery systems, such as in emulsions or microemulsions, as oil or water substitutes, as surfactants or as additives (Qiu & Texter, 2008; Zech & Kunz, 2011 Dobler et al., 2013; Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

Moreover, it has already been demonstrated that large amounts of water in a hydrophilic IL lead to hydrolysis reactions (Taubert & Li, 2006). Thus, as an example, several studies indicate an increasing interest in producing nonaqueous microemulsions, where the water was replaced by polar solvents, being useful in formulations where it is intended to avoid contact with water. So, the use of ILs to replace water and forming nonaqueous formulations has also

been a topic of increasing interest (Gao et al., 2006; Qiu & Texter, 2008). Furthermore, ILs have also been used as water substitutes not only in emulsions, but also for other functionalities such as in Diels-Alder cycloaddition reactions (Thevenin, Grossiord, & Poelman, 1996; Gao et al., 2006; Qiu & Texter, 2008)

Therefore, as described throughout this work, ILs have properties of great interest and particularly relevant in drug delivery, such as the fact that they may be used as water substitutes, they may be able to stabilize formulations, they may enhance drug solubility and loading and, in some cases, they may be less toxic than commonly used organic solvents. Nonetheless, the toxicity topic should always be considered with caution.

3.4. Toxicity considerations

The toxicity of ILs depends on several factors. Amongst them, the most referred ones are the nature of the cation and of the anion contained in the IL, the interactions between both ions, the type of functional groups present in the cation, and also the length of the alkyl side chain in the cation (Egorova & Ananikov, 2014; Egorova, Gordeev, & Ananikov, 2017).

Although some ILs may show relatively low toxicity, this is not true for all ILs and this needs to be pondered for each particular application. In fact, the higher solubility of ILs in water, may lead to a greater introduction of the water-soluble chemicals in the ecosystems and thus be responsible for a greater environmental risk (Kurnia et al., 2014; T. Santos de Almeida et al., 2015; Egorova et al., 2017). Also, toxicological studies on fungi, bacteria and cell cultures have revealed high antimicrobial and cytotoxic activity of many ILs. This may be due to interactions between cation alkyl side chains and cell membranes, which can create instability, leading to the entrance in the phospholipid bilayer causing damage (Walkiewicz et al., 2010; McLaughlin et al., 2011; Gal et al., 2012; O'Toole et al., 2012; Jing, Lan, Qiu, & Zhu, 2016; Egorova et al., 2017) .

Hence, it is fundamental to always perform cytotoxicity studies when considering the functionality and applicability of ILs. In fact, although evidence shows that certain ILs present toxicity and cannot be considered ecologically safe, they also have specific and nonspecific mechanisms of IL biological action which suggests interest in their use towards the development of pharmaceutical and biochemical agents (Ranke et al., 2007; Walkiewicz et al., 2010; Egorova & Ananikov, 2014; Kurnia et al., 2014; Brunel et al., 2016). Also, not all ILs are considered toxic, and some ILs, such as choline-amino acid ILs have been considered less toxic and thus more suited to be used in drug delivery studies. Actually, recently there has been a growing interest in using these choline-amino acid ILs in the development of new delivery systems, as solubility and loading enhancers in W/O (water in oil) emulsions and gels (Caparica et al., 2018) and even in ILs-nanoparticles hybrid systems (Dadhania, Raval, & Dadhania, 2015; Giacalone & Gruttadauria, 2016; He & Alexandridis, 2017; Júlio, Caparica, et al., 2019; Júlio, Costa Lima, Reis, Santos de Almeida, & Fonte, 2019). In fact, in the case of nanosystems, it is already known, and previously summarized in this work, that this type of formulations presents several advantages useful in drug delivery and their recent combination with ILs has shown that ILs may improve the benefits arising from these systems.

3.5. Combination of ionic liquids and nanodelivery systems

It has been stablished herein that ionic liquids present many valuable properties that may be quite valuable in drug delivery. Moreover, their combination with nanoparticles may lead to numerous adicional advantages and in consequence this combination has raised recent interest.

When considering the application of nanotechnology to health, this subject has also been quite studied in the pharmaceutical area (Jain, 2010; Satalkar, Elger, & Shaw, 2016), since this type of systems lead to many advantages, such as drug protection from degradation factors, targeted drug delivery (enhancing the therapeutic effect and reducing adverse effects) and drug administration with prolonged release, to reduce dosing frequency. They also may allow a higher cell permeation, due to their reduced size, and may improve the drug's solubility, stability, bioavailability and absorption (Mora-Huertas et al., 2010; Ghosh et al., 2015; Lim et al., 2016; Hua et al., 2018; Gaul et al., 2018; Sharma et al., 2018; Sousa et al., 2019).

Nanoparticles are nanospheres, are comprised of a homogeneous matrix system, where the drug can be adsorbed on the surface of the sphere or encapsulated within the particle, or the nanocapsules. These drug-delivery systems may be based on biomaterials, or in natural, semi-synthetically or synthetically produced materials in the nanometer (Pinto Reis, Neufeld, Ribeiro, & Veiga, 2006; Vrignaud, Benoit, & Saulnier, 2011; Fonte et al., 2016; Tânia Santos de Almeida, Júlio, Mota, et al., 2017). Furthermore, by changing the formulation process, characteristics such as particle size, which generally ranges between 100-1000 nm, surface charge and drug loading, may be modulated. In this context, ILs may be quite useful to improve and allow the modulation of some of these properties and some studies have already shown that this strategic combination may be relevant to promote drug delivery (Ueno & Watanabe, 2011; Prabhu Charan et al., 2014; He & Alexandridis, 2017; Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

ILs form a protective layer around the nanoparticles and thus they may confer chemical protection to these systems and improve their thermal and chemical stability, since they may act as electrostatic stabilizing agents (He & Alexandridis, 2017).

Thus, the use of ILs combined with nanoparticles has been studied for different applications and not only in health. Studies have shown that the synthesis of metal nanoparticles in the presence of ILs leads to a lower agglomeration predisposition (Schrekker et al., 2008;

Vollmer & Janiak, 2011; He & Alexandridis, 2015) and in general the combination of nanoparticles with ILs may lead to synthesis with high yields and allow better recycling properties (Itoh, Naka, & Chujo, 2004; Zahmakiran & Özkar, 2011; He & Alexandridis, 2015; Huang, Chen, Li, & Yu, 2016; Tânia Santos de Almeida, Júlio, Mota, et al., 2017). Moreover, ILs containing the imidazole cation, particularly the 1-Alkyl-3-alkyl-imidazolium IL, have shown to be useful as reaction medium, for the production of metal nanoparticles, and also as catalysts and stabilizers (He & Alexandridis, 2017; Wegner & Janiak, 2017). Additionally, since ILs may act as surfactants they may have the capacity to prevent the aggregation of nanoparticles (Dupont & Scholten, 2010; He & Alexandridis, 2015, 2017).

Thus, ILs have proven to be useful to increase the functionality of nanoparticles, namely by refining their physicochemical properties, by helping to control the particle size, by increasing their selectivity (Selvam, MacHoke, & Schwieger, 2012; Prabhu Charan et al., 2014; He & Alexandridis, 2017; Tânia Santos de Almeida, Júlio, Mota, et al., 2017;) and even by allowing a higher drug solubility and loading (Tânia Santos de Almeida, Júlio, Mota, et al., 2017; Caparica et al., 2018).

From all that has been mentioned herein, it becomes clear that it is quite relevant to continue to evaluate the applicability of ionic liquids to improve the efficiency of drug delivery in different formulations. Consequently, the aim of this study was to synthesise and further evaluate the applicability of combining two ionic liquids, with different characteristics, to improve the delivery of the poorly soluble ferulic acid.

4. Materials and Methods

4. Materials and methods

4.1. Materials

The studied choline-based ionic liquid was the (2-hydroxyethyl) trimethylammonium-L-phenylalaninate [Cho][Phe], while the imidazole-based IL was the imidazolium 1-ethyl-3-methylimidazolium bromide [Emim][Br]. Both ILs were prepared within the scope of this study.

For the synthesis of [Cho][Phe] it was used choline hydroxide in methanol [Cho][OH]/MeOH 45%, from Sigma-Aldrich (St. Louis, MO, USA) as well as acetonitrile, from VWR (Fontenay-sous-Bois, France), methanol, from Sigma-Aldrich Chemie Gmbh (Munich, Germany) and L-phenylalanine, from PanReac AppliChem® ITW Reagents (Barcelona, Spain). On the other hand, to synthesise [Emim][Br] was used bromoethane and tetrahydrofuran (THF), both from Sigma-Aldrich (St. Louis, MO, USA) and 1-methylimidazolium, from Alfa Aesar Chemicals (Haverhill, MA, USA). Bidistilled water was prepared "in house" and used in all the performed techniques.

The poorly soluble drug studied was ferulic acid, which was purchased by Henrifarma, São Paulo, Brazil.

Towards the development of the gel formulations, it was used Carbopol® 940, a crosslinked polyacrylic acid polymer, and triethanolamine both from José Vaz Pereira (Benavente, Portugal). Propylene glycol, from Fagron (St. Paul, MN, USA), and methylparaben and propylparaben, both from Sigma-Aldrich (St. Louis, MO, USA), were also used.

In the production of IL_1-IL_2-nanoparticles hybrid systems, the organic solvent, dichloromethane, from Fisher Chemical (Loughborough, United Kingdom), was used, as well as the surfactant, polyvinyl alcohol (PVA), from Sigma-Aldrich (St. Louis, MO, USA). The matrix of the IL-IL-nanosystem was produced using poly(lactic-co-glycolic acid) (PLGA) 50:50, Purasorb® PDLG 5002ª from Corbion Purac (Amsterdam, the Netherlands).

4.2. Methods

Synthesis and characterization of Ionic Liquids

The amino acid derived IL [Cho][Phe] was prepared according to the procedure described in the literature (Tânia Santos de Almeida, Júlio, Mota, et al., 2017).

Briefly, 15.6 mL (57.76 mmol) of a solution of [Cho][OH]/MeOH 45%, and to remove methanol the mixture was evaporated under vacuum at 50 °C. Next, an equimolar quantity (57.76 mmol) of the phenylaniline amino acid was dissolved in the minimum volume of water capable of solubilizing this amino acid. This mixture was maintained under stirring and cooled in an ice bath overnight. Then the water was removed under vacuum at 60 °C. Following this, a mixture of acetonitrile and methanol (9:1) was added to the reaction mixture, to precipitate all the unreacted amino acid, and the mixture was maintained under vigorous stirring. Next, this blend was centrifuged at 10 080 xg for 20 min and filtered. Finally, the solvents were evaporated under vacuum at 60 °C.

The halogenated IL [Emim][Br], was prepared according to the procedure described in literature (Gouveia et al., 2014). In summary, 5.9 mL (0.08 mol) of bromoethane were slowly added to 0.58 mL (0.0073 mol) of 1-methylimidazole and then the mixture was stirred during 1 h. This combination was maintained under stirring overnight at 70 °C. After that, the mixture was evaporated under vacuum and washed 3 times with THF.

Both prepared ILs were stored under moisture-free conditions until use and they were characterized by ^{1}H-NMR and ^{13}C-NMR spectra, using a Brucker Avance 400® apparatus (Billerica, MA, USA) at 400 MHz, using D_2O.

Solubility studies

The solubility studies were performed by preparing several saturated solutions of ferulic acid in water, in water:IL mixtures (99.9:0.1) and in water:**IL$_1$**:**IL$_2$** mixtures (99.8:0.1:0.1). From here on, **IL$_1$** refers to [Cho][Phe] and **IL$_2$** refers to [Emim][Br]. Following this, all saturated solutions were placed, for 72 h and at 25 ºC, on a horizontal shaker (IKA VIBRAX VXR®, LTF Labortechnik GmbH & Co., Bodensee, Germany). All the mixtures were prepared

in triplicate. After this the mixtures were filtrated and the solute in excess was removed. All the samples were then analysed, at the maximum absorption wavelength of ferulic acid (313 nm), using a UV-visible spectrophotometry (Evolution® 300, Thermo Scientific, Hertfordshire, England).

Development of gel formulations

Preparation of the gels

The formulations were prepared by weighing all the compounds (**Table 2**). The ferulic acid was added, under stirring and until total solubilization, either in bidestilled water, or in each of the following mixtures, water:**IL$_1$**, water:**IL$_2$** or water:**IL$_1$**:**IL$_2$**. Then the other liquid compounds were blended. Finally, Carbopol® 940 was poured until total viscosity elevation.

Table 2: Qualitative and quantitative composition (%, w/w) of the prepared gels with and without ferulic acid (FA) and/or IL.

Composition	Control	With FA	With IL			With FA and IL		
			IL$_1$	**IL$_2$**	**IL$_1$:IL$_2$**	**IL$_1$**	**IL$_2$**	**IL$_1$:IL$_2$**
Carbopol® 940	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Propylene glycol	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Parabens solution	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Ferulic acid	-	0.6	-	-	-	1.0	0.7	2.7
[Cho][Phe] (**IL$_1$**)	-	-	0.1	-	0.1	0.1	-	0.1
[Emim][Br] (**IL$_2$**)	-	-	-	0.1	0.1	-	0.1	0.1
Triethanolamine	q.s. to pH=5							
Bidestilled water	q.s. 100							

Preliminary stability studies

The organoleptic properties, the viscosity and the preliminary stability studies (centrifuge test) were evaluated for all the developed formulations. To perform the centrifuging test, 5 g of each formulation (n=2) was heated at 45 °C, for 30 mins, and then centrifuged at 7 200×*g* during 30 mins using a Hermle LaborTechnik GmbH Centrifuge (Wehingen, Germany).

Production of the IL_1-IL_2-nanoparticles hybrid system

The IL-IL-nanoparticles hybrid systems were produced by a water in oil in water (W/O/W) double emulsion technique (Júlio, Caparica, et al., 2019). Briefly, 200 mg of PLGA 50:50 was dissolved in 2 mL of dichloromethane, after that 200 µL of a solution of ferulic acid was added. This solution was prepared in different combinations of solvents, namely, in water, in water:**IL_1**(99.9:0.1), in water:**IL_2** (99.9:0.1) and in water:**IL_1**:**IL_2** (99.8:0.1:0.1), at the maximum solubility of ferulic acid in each mixture, according to the results obtained in the solubility studies. All prepared mixtures were sonicated for 30 seconds at 70 % amplitude using the Q125 Sonicator, from QSonica Sonicators (Newtown, CT, USA). Then, the first emulsion was blended with 23 mL of a PVA 2 %(w/v) aqueous solution and sonicated under the previous conditions. At the end, the **IL_1-IL_2**-nanoparticles were stirred during 3 h. All formulations were performed in triplicate.

Physicochemical properties

The **IL_1-IL_2**-nanoparticles were characterized according to their diameter, polydispersion index (PdI) and zeta potential (ZP). All properties were obtained in a Delsa^{TM} Nano C from Beckman Coulter, Inc. (Brea, California, USA), using dynamic light scattering, for diameter and PdI, and through electrophoretic mobility technique, for ZP. The samples were analysed in triplicate and at room temperature (25 ± 2 ºC), after being previously diluted with bidistilled water.

Association Efficiency (AE) and Loading Capacity (LC)

To evaluate the **AE** of ferulic acid (FA), the amount of drug associated with the nanoparticles, and the **LC**, the drug transport capacity of the nanoparticles, the following equations **(1)** and **(2)** were used, respectively:

$$\boldsymbol{AE} = \frac{Total\ amount\ of\ FA - Free\ FA\ in\ supernatant}{Total\ amount\ of\ FA} \times 100 \qquad \textbf{(1)}$$

$$\boldsymbol{LC} = \frac{Total\ amount\ of\ FA - Free\ FA\ in\ supernatant}{Total\ dry\ weight\ of\ nanoparticles} \times 100 \qquad \textbf{(2)}$$

For both parameters, the **IL_1-IL_2**-nanocarriers were centrifuged at 16,350 ×g, for 15 minutes at 4 °C, in a centrifuge Hermle LaborTechnik GmbH (Wehingen, Germany), and the ferulic acid present in the supernatant was determined by UV-visible spectroscopy, at the maximum absorption wavelength of this phenolic acid (313 nm). This was performed using a UV-Vis Spectrophotometer Evolution® 300, from Thermo Scientific (Hertfordshire, England). The total dry weight of nanoparticles was determined through the difference in mass of the vials, before and after a freeze-drying process.

The freeze-drying of the formulations was performed after their centrifugation at the previous described conditions. The supernatant was removed, and the **IL_1-IL_2**-nanoparticles were re-dispersed in bidistilled water. Next, they were frozen at -80 °C during 12 h and freeze-dryied for 24 h, at a surface condenser temperature of -50 °C and at 400 mTorr, in a Labconco FreeZone 25® (Kansas City, MO, USA).

Ferulic acid *in vitro* release study

To evaluate the ferulic acid release profile, the formulation was centrifuged at 12,600x*g* for 20 minutes at 4 °C. The supernatant was discarded, and the pellet was resuspended in 10.0 mL of pH 7.4 phosphate buffer saline (PBS) solution. The solutions were incubated at 32 °C with stirring at 100 rpm, in an Heidolph® 1000 incubator with a motor Heidolph® Unimax 1010 (Schwabach, Germany).

At predetermined time intervals (30 minutes, 1, 2, 4, 6, 8, 12, 24, 48, 72, 120 and 144 hours), aliquots of 1.0 mL of the samples were taken and replaced with the same volume of pH 7.4 PBS solution. The samples were once again centrifuged at the same conditions and the ferulic acid was quantified by UV-visible spectroscopy at 313 nm (maximum absorption wavelength).

All centrifugations were performed in a Hermle Z 32 HK centrifuge.

Statistical analysis

Statistical analysis was performed using Origin® 2018 by OriginLab Corporation (Northampton, MA, USA). The differences in the mean values were assessed using a one-way analysis of variance, ANOVA and then followed by Tukey's multiple comparison test. All obtained values were presented as mean ± standard deviation, SD. Observed differences between individual means were significant with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

5. Results and discussion

5. Results and discussion

The main goal of the present study was two synthesise and characterize two ILs, from different classes, and evaluate the impact of combining these ionic liquids, on the delivery of the poorly soluble ferulic acid, considering a topical application.

Concerning this combination, it is already described in the literature that the combination of ionic compounds with more than two types of salts, such as IL-IL mixtures, are called double salts (Chatel, Pereira, Debbeti, Wang, & Rogers, 2014; Cha & Kim, 2015). Thus, they can have more than one anion and/or cation acquiring different properties from those that they had individually, which means that its behaviour may or may not be intended (Chatel et al., 2014) or desired and needs to be evaluated. Therefore, physical properties such as viscosity, density and solubility can be modified because they depend on chemical associations and chemical reactions between ions (Balarew & Tepavitcharova, 2003; Chatel et al., 2014) and these changes may be positive, if well evaluated. Hence, in this work, we studied the influence of combining two ILs on the aqueous solubility and on the loading of ferulic acid, into the prepared delivery systems.

The two prepared ILs belong to different classes, a choline-based IL [Cho][Phe] (**IL_1**), known to be a good solubility promoter (Caparica et al., 2018), and an imidazole-based IL [Emim][Br] (**IL_2**), recognized to be better as permeation enhancer than as solubility promotor (Tânia Santos de Almeida, Júlio, Mota, et al., 2017). Hence, their combination could lead to an increase in their functionality. In consequence, one of the goals of this study was to include these ILs into different delivery systems, to evaluate if this strategy could improve the incorporation of ferulic acid in the developed systems.

5.1. Synthesis and characterization of Ionic Liquids

The two ionic liquids, [Cho][Phe] and [Emim][Br], were prepared according to the literature (Tânia Santos de Almeida, Júlio, Mota, et al., 2017). Considering their organoleptic properties, the [Cho][Phe] IL is a yellow viscous liquid at room temperature, while the [Emim][Br] is a white solid, as expected. The spectroscopic data for both ILs is in agreement with previously described results (Tânia Santos de Almeida, Júlio, Mota, et al., 2017). The obtained yields for both ILs were 73 %, for [Cho][Phe] and 62 %, for [Emim][Br].

5.2. Solubility studies

The influence of the two studied ILs on the solubility of the poorly soluble ferulic acid, was assessed in water, in water:IL (99.9:0.1) and in water:**IL_1**:**IL_2** (99.8:0.1:0.1) mixtures. Since a topical application is being considered in this study, the concentrations of each IL were chosen according to the cell viability studies, performed in HaCat cells, already described in other projects of our group (Tânia Santos de Almeida, Júlio, Mota, et al., 2017; Caparica et al., 2018).

Results showed that the solubility of ferulic acid in water alone, at 25 ºC, was 0.68 ± 0.02 mg/mL (**Figure 3),** which is in agreement with previously published data of 0.64 mg/mL (Caparica et al., 2018).

In addition, the two ILs seem to portray an enhancement in the solubility of ferulic acid. Nonetheless, only the enhancement with [Cho][Phe] shows a statistically significant increase (to 1 ± 0.01 mg/mL), showing that the choline-based IL is a better solubility promoter, as expected, when compared to the solubility in the presence of [Emim][Br] (0.76 ± 0.01 mg/mL) (**Figure 3**).

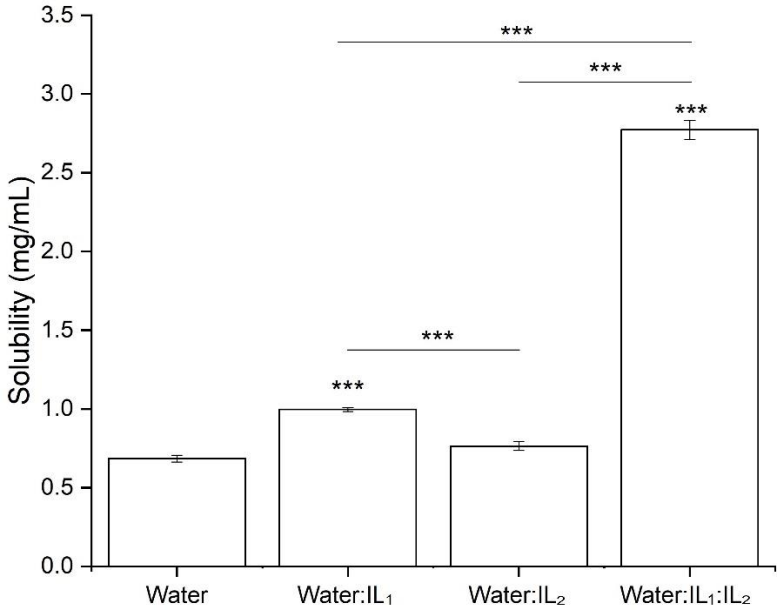

Figure 3: Ferulic acid solubility in water or mixtures water:IL_1 (99.9:0.1), water:IL_2 (99.9:0.1) and water:IL_1:IL_2 (99.8:0.1:0.1), where IL_1 and IL_2 correspond to [Cho][Phe] and [Emim][Br], respectively, at 25 ºC. n=3, mean ± SD and ***p<0.001 (ANOVA, Tukey's test).

More importantly, when we evaluate the impact of combining both ILs on the solubility of ferulic acid, we can observe what seems to be a synergistic effect. Results clearly showed that this combination leads to a considerable increase in solubility, allowing a drug

solubility 4 times higher (2.77 ± 0.06 mg/mL). Moreover, this enhancement in solubility is even higher than the observed increase in the presence of 0.2 % of [Cho][Phe] (which only allowed a 2-fold increase to 1.30 mg/mL) previously described by our group (Caparica et al., 2018). Thus, these results were the first indicator that the combination of ILs from different classes, may be a way to improve the delivery of drugs with low solubility, as it is the case of ferulic acid.

Taking these results into account, the incorporation of ferulic acid in topical delivery systems was also evaluated. In this study, the choice of the compound used and of the type of formulations developed raised from the fact that FA is described as having properties of potential topical interest. Namely, as antioxidant, as photoprotective excipient in sunscreens and skin lotions, to improve the stability of topical formulations and enhance the photoprotection of the skin (Lin et al., 2005; Kumar & Pruthi, 2014; Peres et al., 2018) and also with anti-inflammatory activity, where it is described that, the topical delivery of ferulic acid decreases the oxidative imbalance and, also, angiogenic and inflammatory signalling, impeding the progress of photocarcinogenesis (Ambothi et al., 2015).

Furthermore, the development of these topical systems was mainly performed to evaluate if the combination of the studied ILs also allows a higher incorporation of the studied phenolic compound into the developed formulations. To achieve this, several gel formulations, in the presence or absence of ferulic acid and of the studied ILs, were prepared and their stability was evaluated.

5.3. Development of gel formulations

To evaluate the impact of the studied ILs on the incorporation of ferulic acid, several gel formulations in the presence or absence of ferulic acid and/or the ILs were developed. All gel formulations were prepared according to **Table 2** and to their organoleptic properties. Ferulic acid was incorporated into the delivery systems at the maximum percentage of its solubility in the aqueous phase of each formulation (**Table 3**). All formulations without ferulic acid were transparent while the formulations containing this phenolic acid were white. Furthermore, all formulations with ferulic acid presented this active compound in a solubilized form. Finally, and more importantly, results showed that in the presence of the mixture **IL_1:IL_2** it was possible to incorporate a higher amount of ferulic acid.

Table 3: Colour, pH, viscosity and centrifuge test results of the gels with and without ferulic acid (FA) or [Cho][Phe] (IL_1) or [Emim][Br] (IL_2).

Formulation		Colour	pH	Viscosity (mPas)	Centrifuge test
Control		Transparent	5.00 ± 0.01	1692.3 ± 18.8	Stable
With FA		Light white	4.99 ± 0.01	1566.3 ± 16.5	
With IL and without FA	IL_1	Transparent	5.01 ± 0.01	10686.7 ± 430.2	
	IL_2		5.00 ± 0.01	849.7 ± 101.1	
	IL_1:IL_2		5.00 ± 0.01	3494.3 ± 76.8	
With FA and IL	IL_1	White	5.01 ± 0.01	16059.7 ± 53.5	
	IL_2		4.99 ± 0.01	1058.0 ± 50.4	
	IL_1:IL_2		5.00 ± 0.01	7683.0 ± 92.7	

Moreover, after the performed preliminary stability studies, namely the centrifuge test, all formulations were stable in terms of pH value, viscosity and organoleptic properties (**Table 3**). The formulations containing the [Cho][Phe] IL_1 proved to be more viscous when compared to [Emim][Br] IL_2 and the formulations with the two ILs showed an intermediate viscosity, portraying a combined contribution from both ILs.

In sequence of these results it was also studied, in an exploratory point of view, the impact of combining both ILs with nanoparticles to further access the potential of this combination to increase the loading of ferulic acid into a controlled delivery system.

5.4. Production of the IL_1-IL_2-nanoparticles hybrid system

All hybrid systems developed in this study were evaluated in terms of their physical chemical properties and proved to be stable. Thus, the particle size, the Zeta Potential, the AE, and LC of all formulations were assessed (**Figures 4 to 5 and Table 4**). Results showed that the diameter of all the unloaded nanoparticles is between 280-225 nm (**Figure 4-Upper part**), while the loaded nanoparticles range between 250-300 nm (**Figure 5-Upper part**). Although the loaded nanoparticles seem to show a moderately higher size, which is expectable, the differences in size between the loaded and unloaded nanoparticles is not statistically significant.

Regarding the zeta potential, results indicate that the loaded nanoparticles are more stable when the particles are combined with the ILs, either alone (**IL_1**:-28.1 ± 2.9 mV; **IL_2**:-26.2 ± 1.9 mV) and even more stable in combination (**IL_1:IL_2**: -32.6 ± 1.8 mV), then

compared to the loaded nanoparticles without ILs (-11.7 ± 3.6 mV) (**Figure 5-Down part**). These differences are not visible on the unloaded systems (**Figure 4-Down part**).

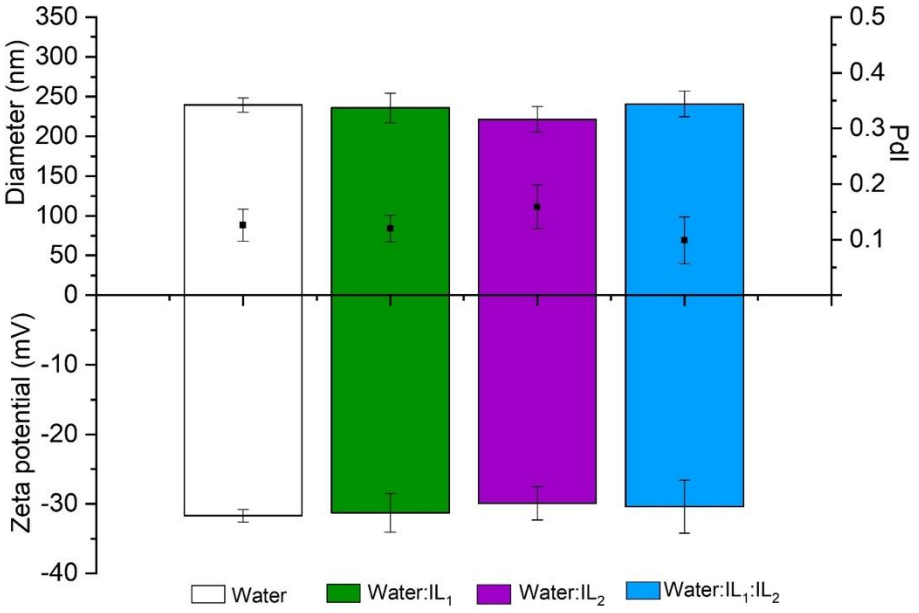

Figure 4: Physical chemical properties of unloaded nanoparticles, unloaded IL-nanoparticles hybrid systems and unloaded IL_1-IL_2-nanoparticles hybrid systems. **Upper part:** Diameter (nm) (top bars), PdI (black squares). **Down part:** Zeta Potential (mV) (down bars). IL_1 is [Cho][Phe] and IL_2 is [Emim][Br]. n=3, mean ± SD.

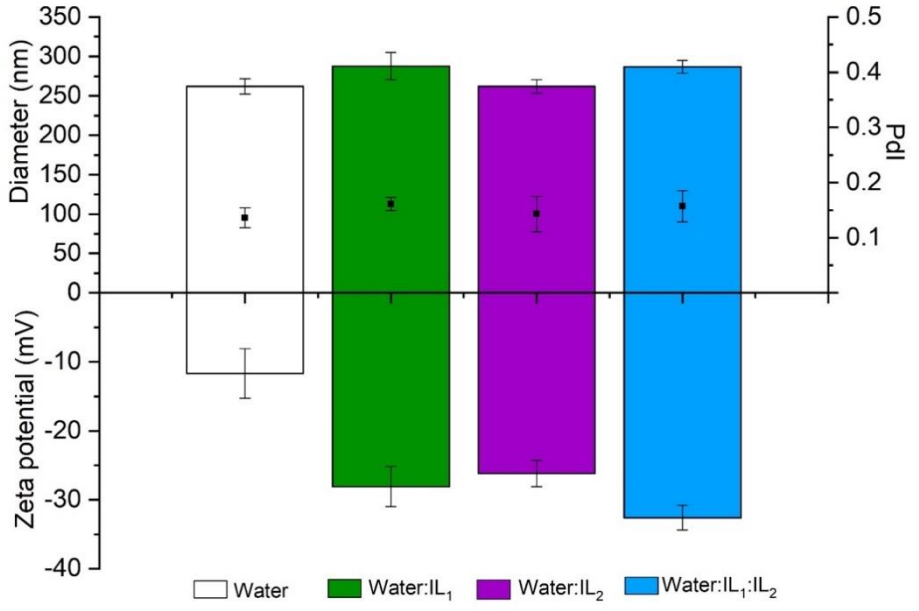

Figure 5: Physical chemical properties of loaded nanoparticles, loaded IL-nanoparticles hybrid systems and loaded IL_1-IL_2-nanoparticles hybrid systems. **Upper part:** Diameter (nm) (top bars) and PdI (black squares). **Down part:** Zeta Potential (mV) (down bars). IL_1 is [Cho][Phe] and IL_2 is [Emim][Br]. n=3, mean ± SD.

Furthermore, results also suggest that the LC (**Table 4**) of all the developed systems is quite similar, indicating that the presence of the ILs does not significantly impacts this particular property, showing that the loading capacity of this compound is maintained.

Table 4: Loading capacity (LC) (%) of ferulic acid loaded nanoparticles, IL-nanoparticles hybrid systems and IL-IL-nanoparticles hybrid systems, where IL_1 and IL_2 correspond to [Cho][Phe] and [Emim][Br], respectively. n=3, mean ± SD and *p<0.05, ***p<0.01 and ***p<0.001 (ANOVA, Tukey's test).

Formulations with	LC (%)
Water	1.1 ± 0.1
Water:IL_1	1.3 ± 0.2
Water:IL_2	0.8 ± 0.1
Water:IL_1:IL_2	1.1 ± 0.2

Interestingly, our study showed that by blending the two studied ILs it was possible to incorporate a higher amount of ferulic acid, as a result of this combination, compared to the nanoparticles without IL, the **IL_1**-nanoparticles or the **IL_2**-nanoparticles. Nonetheless, it should be noticed that the **IL_1**, namely [Cho][Phe], allows a higher AE then in the presence of **IL_2**, [Emim][Br] or the combination of both ILs (**Figure 6**). This indicates that the choline-based IL seems to have a higher impact on the association of the drug to the hybrid nanocarriers.

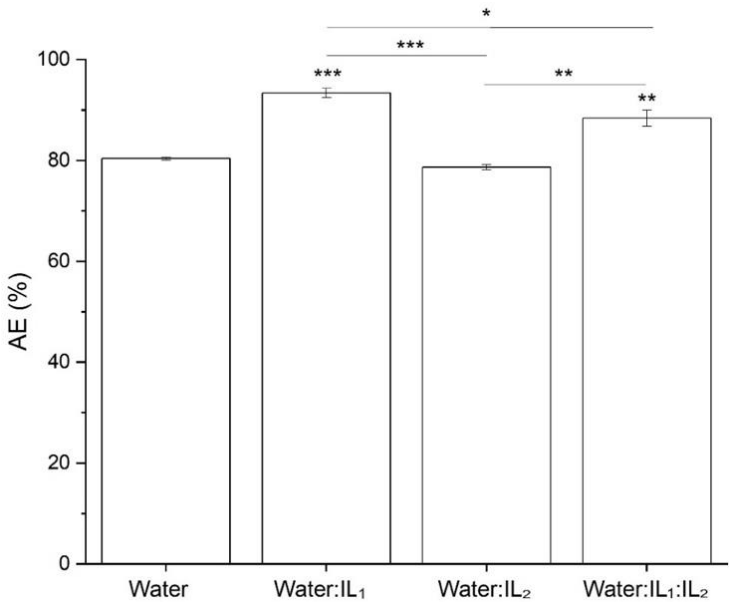

Figure 6: Association efficiency (AE) (%) of ferulic acid loaded nanoparticles, loaded IL-nanoparticles hybrid systems and loaded IL_1-IL_2-nanoparticles hybrid systems. systems, where IL_1 and IL_2 correspond to [Cho][Phe] and [Emim][Br], respectively. n=3, mean ± SD and *p<0.05, ***p<0.01 and ***p<0.001 (ANOVA, Tukey's test).

Finally, concerning the release profile of ferulic acid all the prepared formulations showed a controlled release (**Figure 7**), ranging from 90-100 % within 144 h. This profile is characteristic of PLGA nanoparticles (Júlio, Caparica, et al., 2019). This profile is characterized by an initial burst release, which herein is observable, for all the formulations, in the first 8 h. The detected burst is due to the adsorption of the drug to the surface of the nanoparticles.

All the formulations exhibited a sustained release, as desired, which shows that the ILs allow a higher drug loading without interfering with the desired controlled release. It could also be mentioned that the obtained profile for the **IL_1-IL_2**-nanoparticles hybrid systems seems to contribute to a more controlled release over time, since its plateau was only obtained at 120 h, compared to the other systems were the plateau was reached at 48 h.

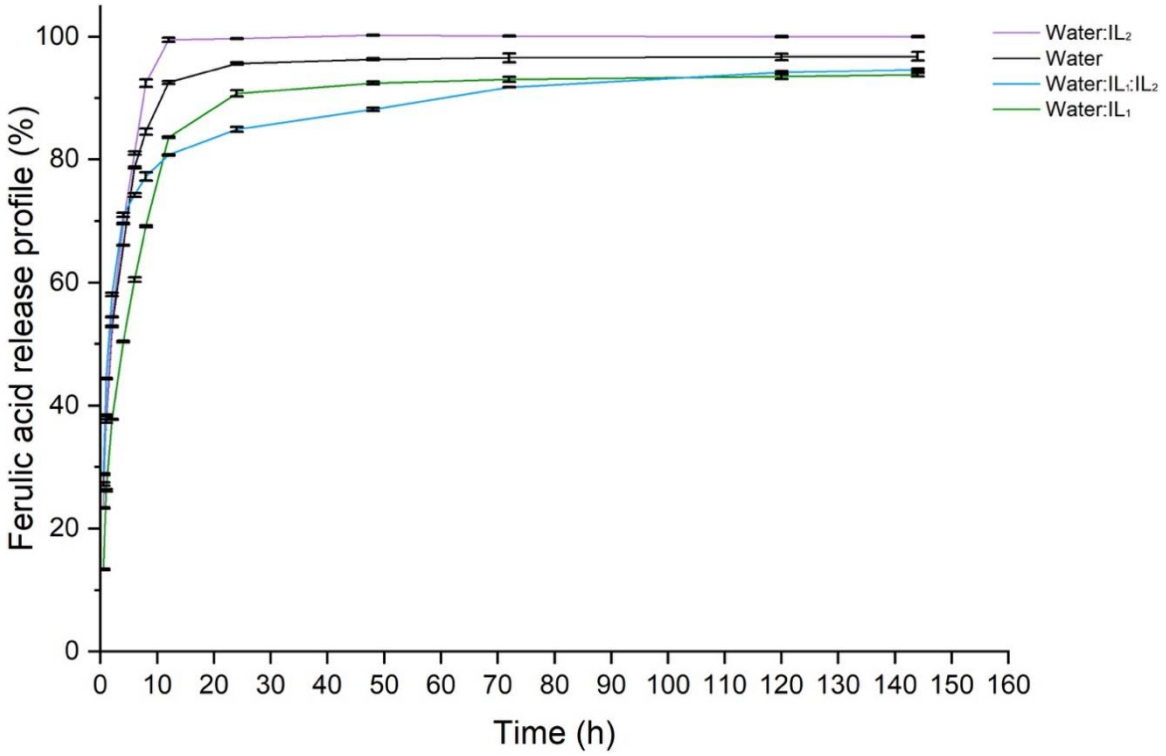

Figure 7: Release profile of ferulic acid loaded nanoparticles, loaded IL-nanoparticles hybrid systems and loaded IL_1-IL_2-nanoparticles hybrid systems during 144 h in phosphate buffer saline at pH 7.4. IL_1 is [Cho][Phe] and IL_2 is [Emim][Br]. n=3, mean ± SD.

Finally, all the results obtained, clearly showed that ILs alone or in combination may be a valuable strategy for improving the delivery of drugs that are poorly soluble in water, such as the ferulic acid studied here, in this case in topical delivery systems.

6. Conclusions and future perspectives

The main goal of this study was to further evaluate the effect of using ILs as functional excipients in drug delivery systems to improve their efficiency. Moreover, with this study it was assessed how the combination of two ILs, from different classes and with different properties, a choline-based IL and an imidazole-based IL, could impact the drug delivery of the sparingly soluble ferulic acid.

Solubility studies were performed in water and in the presence of water:IL mixtures and the results showed that each studied ILs, [Cho][Phe] and [Emim][Br], seem to have some impact on the solubility of ferulic acid, although only [Cho][Phe] showed a significant increase, improving drug solubility by 1.5 times. More importantly, the combination of both ILs clearly has a much significant impact, allowing a 4-fold enhancement in the solubility.

Considering these interesting results, ferulic acid was incorporated into gel and nanoparticles formulations, both in the absence or presence of ILs. Firstly, results showed that all the prepared formulations proved to be stable, displaying that the ILs do not destabilized the developed systems. Additionally, it was also proven that the presence of ILs allows a higher drug loading, with the combination of ILs being the strategy that allows a higher drug incorporation. Concerning only the hybrid nanosystems, it was also possible to conclude, that even though the combination of both ILs allows the higher drug loading, compared to each IL alone, the AE for [Cho][Phe] isolated is greater, then in combination with [Emim][Br]. Furthermore, this study also showed that the combination of IL_1 with IL_2 allows a more controlled release, with the plateau being reached later.

Hence, as intended this study showed that using ILs and combining different ILs may be an interesting tactic to further invest in drug delivery, and thus, it would be interesting in the future to evaluate other ILs and other combinations of ILs.

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