Ibuprofen solubility and cytotoxic study of deep eutectic solvents formed by xylitol, choline chloride and water

Laura Lomba[a,*], Pilar Garralaga[a], Álvaro Werner[a], Beatriz Giner[a,], Pedro M. Baptista[b,c,d,e,f,], Natalia Sánchez-Romero [a,b,g]

[a]Facultad de Ciencias de la Salud, Universidad San Jorge. Campus Universitario, Autov A23 km 299, 50830. Villanueva de Gállego Zaragoza, Spain

[b]Instituto de Investigación Sanitaria de Aragón (IIS Aragón), Zaragoza, Spain

[c]Centro de Investigación Biomédica en Red en el Área Temática de Enfermedades Hepáticas (CIBERehd), Madrid, Spain

[d]Fundación ARAID, Zaragoza, Spain

[e]Instituto de Investigación Sanitaria de la Fundación Jiménez Díaz, Madrid, Spain

[f]Department of Biomedical and Aerospace Engineering, Universidad Carlos III de Madrid, Madrid, Spain

[g]Cytes Biotechnologies, Barcelona, Spain

*Corresponding author: Laura Lomba, e-mail: llomba@usj.es, phone: 0034976060100

Abstract: Currently, many of the drugs that are discovered or are on the market are not soluble in water and they are classified in categories II and IV of the biopharmaceutical classification system (BCS). All this leads to the search for new solvents or excipients that allow improving the solubility of the active ingredients in aqueous media. One of the new trends is the use of Deep Eutectic Solvents (DES) to solubilize drugs. That is why, in this article, a eutectic mixture formed by xylitol and choline chloride with different proportions of water have been prepared to improve the formulation possibilities of ibuprofen. This pharmaceutical active ingredient has poor solubility in water. In addition to using these mixtures to study the solubility of an active ingredient, the toxicity of the mixtures using cell viability and protein quantification studies has been also checked. The results obtained show that these mixtures improve the solubility of ibuprofen, and they can be classified as non-toxic. These results open the door to the possibility of carrying out other solubility studies with other active ingredients and increasing the biological studies of these mixtures.

Keywords: DES, xylitol:choline chloride, solubility, ibuprofen, cytotoxicity.

1. INTRODUCTION

One of the main problems in the liquid formulation of drugs is the low solubility of some of the active ingredients (API) in aqueous systems. This reduces the amount of drug that reaches the blood circulation (bioavailability) and, therefore, compromises the pharmacokinetic profiles and parameters (Goeke *et al.*, 2018).

There are numerous approaches to increase the solubility of active ingredients. One of the most widespread is the use of co-solvents which, in addition, are usually organic solvents. However, on many occasions the use of this type of substances can generate problems during their use such as flammability, cost, difficulty in purification techniques or even toxicity problems (M. Mokhtarpour *et al.*, 2019). There are also a number of other techniques that can be used to improve the solubility of the APS such as the use of surfactants or nanosuspension technology, the diminution of particle size, solid dispersion, salt and esters formation, modification of pH, hydrotropy, cocrystal, amorphous compound formation or even though inclusion complexes (Ainurofiq *et al.*, 2021; Barrett *et al.*, 2022).

One of the new trends that is being used to improve the solubility of drugs is the use of Deep Eutectic Solvents (DES). Deep eutectic solvents are liquid mixtures at room temperature whose melting points are low due to the low lattice energy formed by the large and asymmetric ions that form them. Usually, DES are formed by combining a substance called hydrogen bond donor (HBD) with a hydrogen bond acceptor (HBA), so that hydrogen bonds are formed by delocalization of load. The result is a mixture whose melting point is lower than that of its separate components. The typical components used in these mixtures are quaternary ammonium salts (choline chloride) acting as HBA and alcohols, natural metabolites, carboxylic acids, urea and sugars, among others (Nystedt *et al.*, 2021) acting as HBD. Thanks to the sustainable source of the components that form them and low vapor pressure of the resulting mixtures, the DES have been considered by some authors as green solvents and an alternative to some toxic and unsustainable solvents such as ionic liquids or Volatile Organic Compounds (VOC) (Castro *et al.*, 2018; Liu *et al.*, 2018; Mulia *et al.* 2016). As dictated by Green Chemistry (Anastas & Eghbali, 2010) and some authors (Kudlak *et al.*, 2015), green solvents have to show a series of characteristics such as such as high versatility, ease of obtaining, sustainable origin of raw materials, low or no toxicity or high biodegradability throughout its entire life cycle (synthesis, uses and applications, recycling and recovery, final destination/environmental

disposal). It is true that DES have been shown to deserve this label, since some of them have shown low (eco)toxicity (Ahmadi *et al.*, 2018; Ferreira *et al.*, 2022; Hayyan *et al.*, 2016; Macario *et al.*, 2019) and good biodegradability (Lapena *et al.*, 2021), although it is necessary to continue studying these solvents from the beginning point of view of Green Chemistry.

In several works the ability of DES to improve the solubility of certain active ingredients has been demonstrated (Abdkarimi & Haghtalab, 2021; Asgari *et al.*, 2021; Barzegar-Jalali *et al.*, 2022; Daadoue *et al.*, 2022; Masumeh Mokhtarpour *et al.*, 2019; M. Mokhtarpour *et al.*, 2019; Pedro *et al.*, 2019; Tajmir & Roosta, 2020). For instance, Asgari *et al.* used DES formed by choline chloride and ethylene glycol at different temperatures to analyze de solubility of carvedilol. They observed that the solubility of this drug in increase as the higher is the value of mass fraction of DES in aqueous solution (Asgari *et al.*, 2021). Daadoue et *al.* prepared five DES formed by capric acid and menthol in different proportions (7:3. 6:4. 5:5. 4:6 and 3:7) and studied the solubility of risperidone in these mixtures. They observed that the system formed by 7:3 capric acid and menthol increases the solubility of this drug 73000 folds compared to water (Daadoue *et al.*, 2022). Golgoun *et al.* prepared DES formed by choline chloride and urea, ethylenglycol and glycerol and used them to solubilize betamethasone, meloxicam and piroxicam. Results showed that the mixture which present higher solubility values was choline chloride and urea for betamethasone and piroxicam (Golgoun *et al.*, 2021).

Additionally, there are other type of DES called Therapeutic Deep Eutectic Solvent (THEDES), in which one of the components is an API (Al-Akayleh *et al.*, 2021; Aroso *et al.*, 2015; Duarte *et al.*, 2017; Li *et al.*, 2021; Santos *et al.*, 2019; E. Silva *et al.*, 2020; J. M. Silva *et al.*, 2018). Yin *et al.* prepared THEDES based on osthole and paeonol and analyzed the solubility of these mixtures compared to the pure API in water(Yin *et al.*, 2022). Al-Akayleh *et al.* designed several systems of risperidone with fatty acids (lauric acid, capric acid and myristic acid) and observed that solubility of risperidone was up to 70000 fold if it is compared to water (Al-Akayleh *et al.*, 2021). Sarraguca *et al.* prepared several THEDES formed by chlorpropamide and tolbutamide with arginine, tryptophan, citric acid, malic acid, ascorbic acid and p-aminobenzoic acid in different molar ratios. Results showed that solubility increase for chlorpropamide was 188 times and 120 times for tolbutamide (Sarraguca *et al.*, 2022).

Ibuprofen is a Non-Steroidal Anti-Inflammatory Drug (NSAID) widely used today to control and treat mild to moderate pain, fever or inflammatory diseases (Vicent

Trung H. Ngo, 2022). The mechanism of ibuprofen consists of the inhibition of prostaglandin synthesis through the inhibition of cyclooxygenase enzymes (COX-1 and COX-2). The COX-1 plays an important role in physiological processes and regulates different functions such as vascular homeostasis, renal hemodynamics, platelet function and gastrointestinal protection while COX-2 is related to inflammatory processes (Pedro *et al.*, 2022). When both are inhibited, anti-inflammatory and analgesic effects can be achieved. Simultaneously, side effects such as gastrointestinal ulceration or renal toxicity can appear. For this reason, sometimes NSAIDs are administered together with gastroprotective drugs (Harirforoosh *et al.*, 2013; Juan A García Meijide 2000). Ibuprofen is usually formulated as oral tablets, capsules, chewable, suspension, intravenous solution, or topical gel. The solubility of ibuprofen in water is poor, therefore the making the formulation in liquid solution difficult. Thus, we can find suspensions but not liquid solutions in market. In addition, may times ibuprofen suspensions are formulated with excipients that cannot be used in specific populations, such as fructosemics or diabetics patients.

A revision of literature reveals that the solubility of ibuprofen and the use of different DES have been studied before. For example, Pedro *et al.* prepared several DES formed by arginine and glycerol that increased the solubility of ibuprofen 7917-fold and used it to develop liquid formulations. The stability of the formulations and the biological activity in macrophages was also checked. Furthermore, several alginate hydrogels were also prepared and a dissolution test, skin and permeation test were carried out. (Pedro *et al.*, 2022). Phaechamud et *al.* prepared a mixture formed by menthol:camphor (1:1) as solvent and co-solvent for delivery systems. The solubility of ibuprofen in this mixture was analysed and it was observed an increase of 4-folds (Phaechamud *et al.*, 2016). Other study described by Lu *et al.* showed a solubility enhancement of ibuprofen when different DES were used: choline chloride: 1,2-propanediol (1:5) or choline chloride:levulinic acid (1:2) which 3810-fold and 4000 fold respectively (Lu *et al.*, 2016). Other studies described are related to solubility of THEDES formed by an API, in this case, ibuprofen. One of these works was described by Pereira *et al.* where they prepared THEDES formed by ibuprofen, safranal and menthol. They could observe that the solubility in the THEDES was higher than ibuprofen.

In this work, we have prepared, characterized and tested a DES formed by the sugar xylitol, and choline chloride. Xylitol is a polyol derived from xylose and found in some vegetal materials (vegetables or fruits). Thanks to its versatility, xylitol can be used

in several applications such as sugar substitute, nutraceuticals, for the treatment of glucose 6-phosphate deficiency-associated to hemolytic anemia and in oral hygiene as an active compound, among others. In the case of diabetic's patients, xylitol can be used as a sweeting agent or excipient but also as a better substance with anticatabolic action (insulin resistance) (Ahuja *et al.*, 2020; Ruiz, 2020). The DES has been mixed with water at different proportions and some physicochemical properties such as density and viscosity have been measured to characterize the moieties depending on the water content (Lapena *et al.*, 2020; Mokhtarpour *et al.*, 2020). Additionally, the toxicity of these mixtures has been analyzed due to the importance of knowing if these substances are toxic or not and if (Ferreira *et al.*, 2022), in the future, they could be used in the liquid formulation of drugs. To carried out the toxicological analysis, a cytotoxicity study (Prestoblue, BCA protein and violet crystal assay) has been measured. Finally, the solubility of ibuprofen has been analyzed in these mixtures.

2. MATERIALS AND METHODS

2.1 Chemicals

All the chemicals have been dried under vacuum for 24 h prior to use. Choline chloride (Ref. C1879) and xylitol (Ref. X3375) have been purchased by Sigma-Aldrich with 98% and 99% of purity, respectively. R-ibuprofen has been obtained from Fagron with 98.5% of purity.

2.2 Preparation of DES

In this study, a DES formed by xylitol and choline chloride with different proportions of water has been prepared (Table 1). Mixtures have been obtained by mixing the components at their corresponding compositions (using a Sartorius Entris 5201-1S) with magnetic stirring and gentle heating (below 70ºC) until a homogeneous, transparent and colourless liquid has been formed. The uncertainty of the mass determination has been $\pm 10^{-4}$ g. Mixtures with water have been prepared considering the previous amount of water in the mixtures with Milli-Q water (resistivity less than 18.2 MΩ cm). Mixtures have been stored in darkness until use.

Table 1. Studied mixtures: composition and abbreviation

HBD	HBA	Add-on	Molar ratio	Abbreviation
Xylitol	Chloline chloride	Water	1:2:4	XChCl4
			1:2:10	XChCl10
			1:2:35	XChCl35
			1:2:50	XChCl50
			1:2:75	XChCl75
			1:2:90	XChCl90

2.3 Physicochemical properties

Kinematic viscosity, *v,* has been determined with a Schoot-Geräte AVS-440 automatic measurement unit. Several Ubbelohde capillary viscosimeter have been used to measure the kinematic viscosity. The thermostat, Schoot-Geräte CT 11502 has been used in order to control the temperature of the measurements at 25ºC ±0.01 K. The dynamic viscosity, η, can be calculated by multiplying density and kinematic viscosity. The uncertainty of viscosity determinations is ± 1%.

Density, ρ, has been measured with Anton Paar DSA 5000 densimeter. The temperature is internally controlled at ±0.005 K and, the densimeter corrects the effect of viscosity. The uncertainty of density determinations is ± 0.1 $kg \cdot m^{-3}$.

Additionally, apparent density of ibuprofen has been obtained using European Pharmacopeia method. The solid has been sieved using a hole of 1 mm to break up agglomerates. In a graduated cylinder (250 ml), 100 g of sieved ibuprofen has been introduced carefully, without compacting. The volume has been read and the bulk density has been obtained using the formula m/V_0 where m is the mass and V_0 the unsettled apparent volume. The uncertain for this method is ± 0.8% (PhEur(10), 2022).

2.4 Cytotoxicity study

2.4.1 Cell culture

HaCat and HepG2 cells have been cultured in Advanced-DMEMF12 medium supplemented with 10% FBS, 1% glutamine 2mM, 1% penicillin and streptomycin. Cells were seeded at a density of 6000 cells/cm^2 in a T25 flask at 37ºC, in a 5% CO_2 atmosphere, until reach 90% of confluence and proceed with a cellular passage. All reagents have been purchased by Fisher-scientific.

2.4.2 Concentrations

Different solutions of xylitol and choline chloride have been prepared by dissolving the chemical in culture medium (concentrations 100000, 50000, 25000, 10000, 1000 and 100 mg/L). Mixtures have been prepared using MEM10X medium supplemented with 10% FBS, 1% penicillin and streptomycin, 1X HEPES, and ultrapure water. The concentrations have been 50000, 25000, 17500, 10000, 1000 and 100 mg/L for XChCl4 and 100000, 50000, 25000, 10000, 1000 and 1000 mg/L for the rest of mixtures. After that, pH was adjusted (at pH=7) and stock solutions were filtered by using a 0.22 μm filter.

2.4.3 Cell viability assay

Two experiments related to cell viability have been carried out: Prestoblue cell viability and crystal violet assay. Both experiments have been conducted in triplicate using 3 wells plates for each concentration and 6 wells plates in the case of controls.

2.4.3.1 Prestoblue assay

Cell viability experiments have been performed by using PrestoBlue cell viability reagent.

Cells have been seeded at a density of 6000 cells/well in 96-well plates and incubated at 37ºC and 5% of CO_2 for 4 days to reach the confluence. At that moment, cells have been exposed to the studied mixtures at different concentrations. After 24 and 72 hours of incubation, cells have been washed twice with phosphate-buffered saline (PBS) and then, Prestoblue reagent has been added in each well by diluting the reagent in the cell culture medium (1/10) and it has been incubated for 1 hour. At 37ºC. The results have been determined thought fluorescence by using a microplate reader (Bio-Tek. Synergy H1, 1608177) at ex.530 nm / emm.590 nm

2.4.3.2 Crystal Violet assay

After 72 hours of incubation with the tested mixtures, the cells have been washed twice with PBS and fixed with paraformaldehyde 4% incubated for 15 min at room temperature. Then, well plates have been washed with PBS and crystal violet 0.1% (in PBS) has been added and incubated during 30 min at room temperature. Next, the excess dye has been removed with tap water and photos have been taken using an inverted microscope. Finally, for quantitative measurements, formed crystals have been dissolved using 10% of acetic acid and crystal violet concentrations has been measured in a plate reader at 590 nm.

2.4.4 BCA Protein Assay Kit

After finalizing the cell viability assay at 72h after the exposure to the studies compounds, total protein amount per well has been determined by BCA method.

Cells were washed twice with PBS and lysed by adding to each well NaOH 0.1M incubated during 20 min at room temperature with agitation. A duplicated BCA protein standard was prepared (0, 50, 100, 400, 600 y 800 μg/mL of albumin) and then, 200 μL/well of the BCA protein assay kit working reagent were added and incubated at 37ºC

for 30 minutes. The results have been measured in a plate reader at 562 nm. The experiments have been carried out in triplicate and using 3 well plates for each tested mixture concentration and 6 wells plates in the case of controls, to ensure the reproducibility.

2.5 Solubility measurements

The solubility of ibuprofen in the studied mixtures has been obtained using the modified shake-flask method (Kalepu & Nekkanti, 2015) described as follows:

To quantify the solubility of ibuprofen, a spectrum scan has been obtained at different wavelengths to select the maximum absorbance. For this, a methanol solution of ibuprofen has been used, being the maximum wavelength 222 nm. The concentrations of the solutions prepared for the obtention of calibration curve of ibuprofen have been 1, 10, 30, 50, 70 and 100 mg/L. These solutions have been prepared in distilled water.

Then, supersaturated solutions have been prepared following the general rules for solutions preparation and, supersaturation was checked visually. These solutions have been stirred during 24 hours at controlled temperature (25ºC). Afterwards, samples have been protected from light using aluminium foil and rested for another during 24 h at 25ºC. After this, supersaturation of mixtures was checked visually again. This study has been carried out using a J.P. Select heater.

Then, the samples have been centrifuged using Biofuge Primo R centrifuge for 5 min at 5000 rpm. Supernatants have been filtered using PES syringe filter of 0.22 μm and subsequently, the concentration of ibuprofen has been measured by High Performance Liquid Chromatography with diode array detection (HPLC-DAD) (1220 DAD of Agilent) and with C18 reversed-phase column Liquid Purple (ODS 5μmx250x4) from Analysis Vinílicos®. The mobile phase consisted of 20% (v/v) of phosphate buffer (at pH=3) and 80% (v/v) of methanol during 5 min and after that, 30% (v/v) of phosphate buffer (at pH=3) and 70% (v/v) of methanol. The separation has been conducted using an injection volume of 20 μL at a flow rate of 1.0 mL/min. Under the described conditions, ibuprofen presented a retention time of 12.5 min.

2.6 Statistical analysis

Statistical analysis has been carried out using GraphPad Prism 9.0 program. T-student test has been used in order to analyze the differences. In the null hypothesis (H_0), it is considered that there are no statistically significant differences between the moments

and therefore they are equal, and in the alternative hypothesis (H_1) it is assumed that there are differences between groups. A 95% confidence interval is chosen, so if $p < 0.05$, the null hypothesis is rejected, and the alternative is accepted.

3. RESULTS AND DISCUSSION

3.1 Preparation and physicochemical characterization

All the studied mixtures are liquid, transparent and stable at room temperature. In Table 2, the physicochemical properties of these moieties are gathered. The average molecular weight of each moiety has been calculated according to the following equation (Ahmadi *et al.*, 2018):

$$MW_{MIX} = X_{xylitol} \cdot MW_{xylitol} + X_{chloline\ chloride} \cdot MW_{chloline\ chloride} + X_{water} \cdot MW_{water.} \quad \text{(Eq. 1)}$$

where X is the mole fraction and MW the molecular weight of the component.

It is important to note that the molecular weight of water is lower than xylitol and choline chloride; that means that as the amount of water in the system increases the molecular weight decreases.

Density and viscosity are important properties not only from the engineering point of view but also for explaining some toxicity processes (Hayyan *et al.*, 2016; T. Liu *et al.*, 2014). Density provides information about the intermolecular interactions in a DES and viscosity can describe the resistance of a fluid in response to a deformation at a given shear rate. This information is also important to understanding at molecular level the interaction in liquid phases.

Results indicate that both properties, density and viscosity decreases as the content of water increases (Hansen *et al.*, 2021). This is related to intermolecular interactions; in this case is due the formation of hydrogen bonds between xylitol and choline chloride. The reduction of density is not as pronounced as the case of viscosity; the amount of water does that the viscosity of mixture diminished considerably. This trend has been reported before for some physicochemical properties (Lapena *et al.*, 2020; Lapena *et al.*, 2019a, 2019b).

Table 2. Molecular weight, density and viscosity values at 25ºC.

Components/Mixtures	Molecular weight (g/mol)	Density (g/mL)	Viscosity (mPa·s)
Choline chloride	139.62	-	-
Xylitol	152.15	-	-
XChCl4	71.91	1.177363	106.3
XChCl10	47.03	1.143159	17.26
XChCl35	27.93	1.108058	2.808
XChCl50	25.12	1.062445	1.897
XChCl75	22.83	1.045364	1.485
XChCl90	22.05	1.038730	1.370

In the case of ibuprofen, the values obtained have been 0.515 g/mL for bulk density. Several values of this property are gathered in bibliography. For instance, Nokhodchi *et al.* designed several ibuprofen crystals using two disintegrants (starch and sodium starch glycolate) in order to improve ibuprofen´s flow. Its compatibility and dissolution behaviour. Value of bulk density for pure ibuprofen was 0.22 g/mL (Nokhodchi *et al.*, 2015). Garekani *et al.* analyzed and observed that the bulk density of ibuprofen was modified depending on the solvents used for crystallization. Values for this property was 0.45 g/mL for methanol and ethanol, 0.42 g/mL for isopropanol and 0.29 g/mL in the case of hexane. These differences were because of their various crystal habits, which make different contact points, cohesive and frictional forces between crystals(Garekani *et al.*, 2001).

Han *et al.* used micronization of ibuprofen in a fluid energy mill (FEM) along with dry coating to observe if the dissolution process produced and increase in the bulk density and flowability of ibuprofen compared to the uncoated micronized ibuprofen powder. The obtained bulk density for pure ibuprofen was 0.45 g/ml (Han *et al.*, 2011).

As we can see in the different studies, there is a great variability in the values of bulk density, this may be due to the supplier or even in the method used to measure the bulk density.

3.2 Cytotoxicity study

This cytotoxicity study has been carried out on two cell lines to evaluate the security of these mixtures to be used in pharmaceutical formulations. The selected cell lines have been HaCat (keratinocyte line) can give a first idea to know if these mixtures

(xylitol and choline chloride) could be used for drugs to be administered topically and the hepatic line HepG2, very useful because most of the drugs are metabolized by the hepatic pathway.

3.3 Cell viability assay

In this work, two tests have been performed to analyze cell viability, using the Prestoblue assay and the crystal violet assay.

3.3.1 Prestoblue assay

Prestoblue reagent is a resazurin solution that acts as an indicator of cell viability that makes use of the reducing power of living cells. Resazurin is a non-toxic, cell-permeable (blue) and virtually non-fluorescent compound. When added to cells, resazurin is converted to resafurin by the reducing environment of the living cell and becomes red and fluorescent. Viable cells continuously convert resazurin to resafurin, resulting in a quantitative measure of viability and cytotoxicity (Lall *et al.*, 2013). This test gives information on the cells ability to metabolize a salt into a different product. The higher the salt conversion, the higher the metabolism and thus the higher cell quantity.

Xylitol can be metabolized to D-xylulose by an unspecific cytoplasmatic NAD-linked polyol dehydrogenase and other specific NADP-linked xylitol dehydrogenase. Through phosphorylation, glucuronidation and pentose phosphate pathway, xylitol is finally, converted to glyceraldehyde 3-phosphate and fructose 6-phosphate. These compounds are glycolytic and gluconeogenic intermediates(Ahuja *et al.*, 2020). Xylitol is, fundamentally metabolized in liver (50-80%) although it can be transformed in other organs such as kidney or lungs and even in erythrocytes or adipose tissue. Metabolized compounds can be converted into CO_2 and H_2O carbohydrate pathways (Ur-Rehman *et al.*, 2015).

In the case of choline chloride, this chemical is not completed absorbed because it is metabolized by intestinal bacteria to trimethylamine (Laurence Brunton 2022). It can be used as precursor of several biological compounds. In the liver, it participates in some metabolic reactions, such as the formation of CDP-choline for the obtention of phosphatidylcholine. Using oxidation reactions, it forms the methyl donor betaine, which is essential in the sustaining methylation capacity in the organisms (Mehedint & Zeisel, 2013).

Figures 1 and 2 show the dose-response curves for xylitol, choline chloride and the studied mixtures in both cell lines (HaCat and HepG2). Additionally, in Table 3 values of EC_{50} are shown. For all cases, a dependence between concentration and cell viability has been observed. The cell viability decreases as the concentration of toxic increases. In the case of the studied mixtures, the toxicity of these mixtures decreases as the amount of water in the system increases being the less toxic compound XChCl90 and the highest XChCl4.

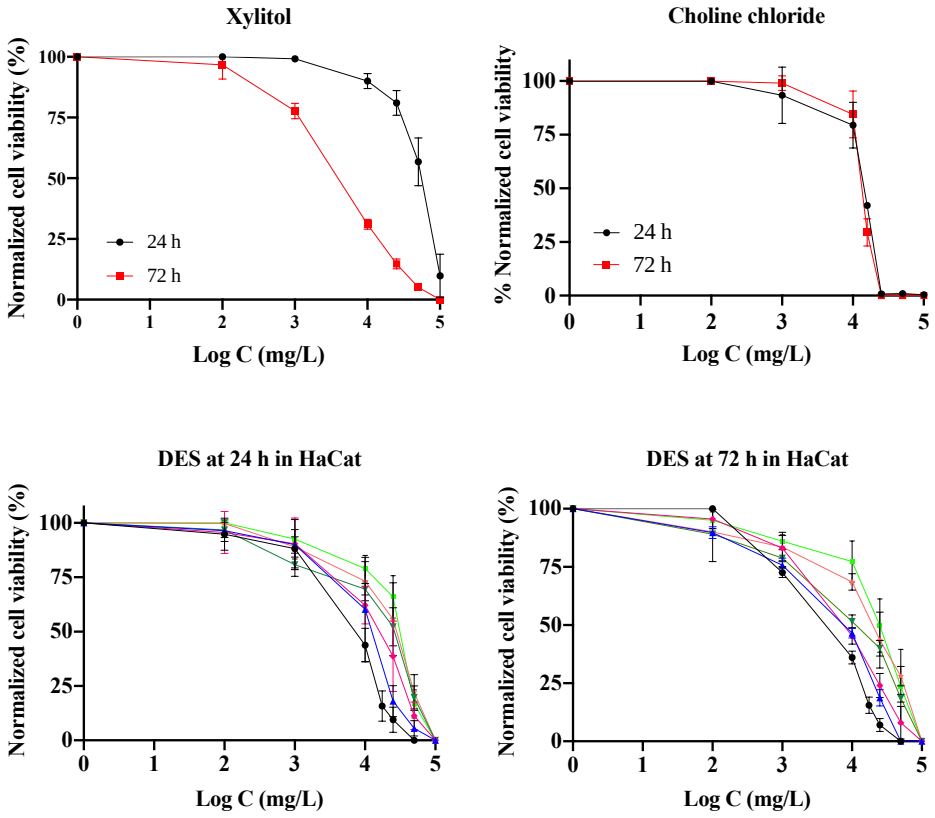

Figure 1. Dose-response curves in HaCat cell line for: xylitol, choline chloride and studied mixtures: XChCl4 (●); XChCl10 (▲); XChCl35 (◆); XChCl50 (▼); XChCl75 (★); XChCl90 (■) at 24h and 72 h.

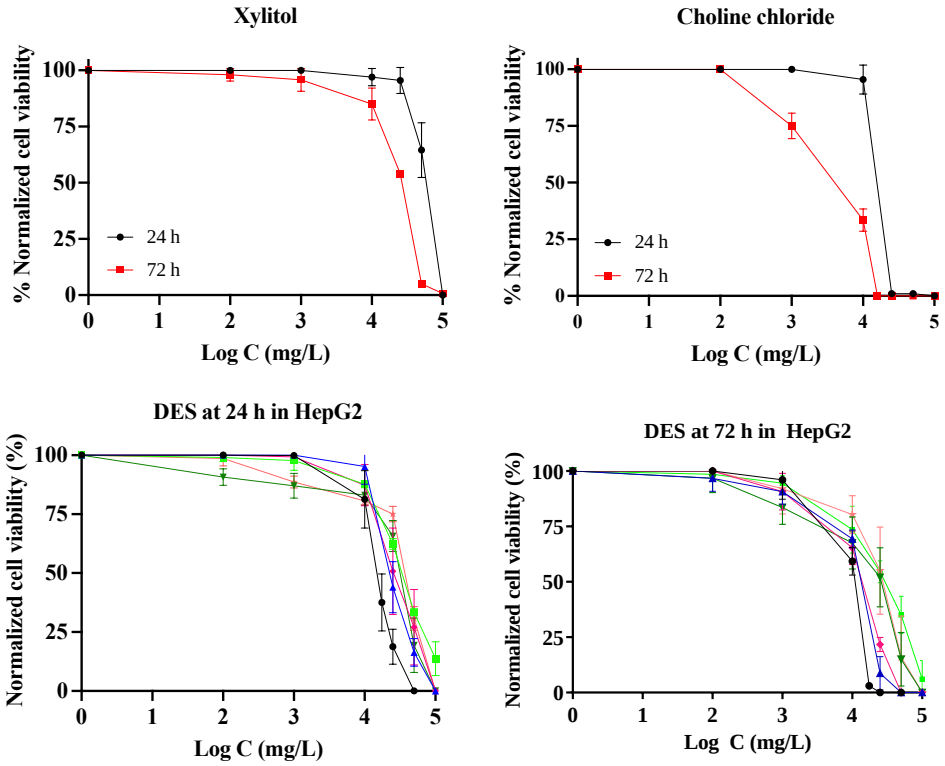

Figure 2. Dose-response curves in HepG2 cell line for: xylitol, choline chloride and studied mixtures: XChCl4 (●); XChCl10 (▲); XChCl35 (◆); XChCl50 (▼); XChCl75 (★); XChCl90 (■) at 24h and 72 h.

When results are analyzed at 24 and 72 h, for pure components, it is observed that, in the case of xylitol the toxicity drastically decreases from 24h to 72 h. This trend is more pronounced in the case of HaCat cells; however, in the case of choline chloride the trend is different, with the EC_{50} value remains almost stable at 24h and 72 h for the HaCaT line and dramatically decreases in the case of HepG2. If the results obtained for the DES with different water proportions are analyzed, it can be observed that there are differences between toxicity at 24h and 72 h being more toxic in the case of 72 h and for HaCat cell line.

Ahmadi *et al.* analyzed the cytotoxicity of some DES formed by choline chloride and some sugars in HEK-293 cells. One of the studied DES was choline chloride and xylitol in a molar ratio 5:2. Results obtained in that case revealed that IC_{50} was 8.55 mM (IC=1714 mg/L); additionally, IC_{50} for choline chloride was 62.88 mM (8339 mg/L) and xylitol was considered as nontoxic because it IC_{50} was higher than 100 mM (Ahmadi *et al.*, 2018). Although these values were measured for other cell line, it is worth mentioning that the same trend is the same as the obtained in this work.

389 Table 3. Vales of EC_{50} at 24 and 72 h in HaCat and HepG2 cell lines.

	HaCat (mg/L)		HepG2 (mg/L)	
Compound	24 h	72 h	24 h	72 h
Xylitol	50829 ± 4701	3923 ± 509	55085 ± 4823	30724 ± 2523
ChCl	13554 ± 457	13488 ± 1747	14450 ± 895	2483 ± 212
XChCl4	8350 ± 400	3423 ± 450	11936 ± 256	10660 ± 460
XChCl10	12076 ± 658	4462 ± 890	21666 ± 228	12638 ± 287
XChCl35	14950 ± 543	6963 ± 1371	25435 ± 831	13349 ± 569
XChCl50	20505 ± 492	8670 ± 827	29663 ± 404	20230 ± 680
XChCl75	23562 ± 606	18412 ± 41	30311 ± 361	25049 ± 259
XChCl90	28645 ± 379	23117 ± 2535	35727 ± 641	26157 ± 474

390

391 The statistical analysis carried out is presented in Table 4. As it can be shown, in
392 general, there are significant differences between the toxicity values at 24 h and 72 h for
393 all the compounds analyzed except in the case of choline chloride in the HaCat cell line.

394

395 Table 4. *p* values for the Prestoblue assay in HaCat and HepG2 cell lines.

HaCat cell line								
					72 h			
24 h	Xylitol	ChCl	XChCl4	XChCl10	XChCl35	XChCl50	XChCl75	XChCl90
Xylitol	< 0.001	-	-	-	-	-	-	-
ChCl	-	0.940	-	-	-	-	-	-
XChCl4	-	-	< 0.001	-	-	-	-	-
XChCl10		-	-	< 0.001	-	-	-	-
XChCl35	-	-	-	-	< 0.001	-	-	-
XChCl50	-	-	-	-	-	< 0.001	-	-
XChCl75	-	-	-	-	-	-	< 0.001	-
XChCl90	-	-	-	-	-	-	-	< 0.001
HepG2 cell line								
					72 h			
24 h	Xylitol	ChCl	XChCl4	XChCl10	XChCl35	XChCl50	XChCl75	XChCl90
Xylitol	<0.001	-	-	-	-	-	-	-
ChCl	-	<0.001	-	-	-	-	-	-
XChCl4	-	-	< 0.001	-	-	-	-	-
XChCl10		-	-	< 0.001	-	-	-	-
XChCl35	-	-	-	-	< 0.001	-	-	-
XChCl50	-	-	-	-	-	< 0.001	-	-
XChCl75	-	-	-	-	-	-	< 0.001	-
XChCl90	-	-	-	-	-	-	-	< 0.001

3.3.1 Crystal violet assay

Crystal violet assay is a rapid and versatile test that allows the analysis of cell viability when cells are placed in contact with a specific stimulus such as chemicals or toxicants. Adherent cells, during cell death, detach from the culture plates. This feature can be used to, indirectly, measure cell death in a culture and, in addition, to analyze and determine differences in the proliferation of cultures after contact with a specific stimulus. Staining of the cells with crystal violet dye because it can bind to proteins and DNA. When cells undergo cell death, they detach from the plate and a decrease in the staining of the plate is observed (Feoktistova *et al.*, 2016).

This assay has been carried out to test the toxicity of the studied mixtures in both qualitative and quantitative way. In the first case, several photos have been taken under the light microscope to see how the cells are after contact with pure substances and the studied solvents. As an example, in Figures 3 and Figure 4 several photos are shown, taken at 10X magnification, for the sample control and 10000 mg/L concentration samples of pure and mixtures for HaCat and HepG2 cell line after 72 h of exposition. It is observed that, in all cases, the cell viability decreases as the amount of water does in DES mixtures. In this case, the photos obtained show that there is a correlation between the cell concentration in the culture plates and the amount of toxicant used.

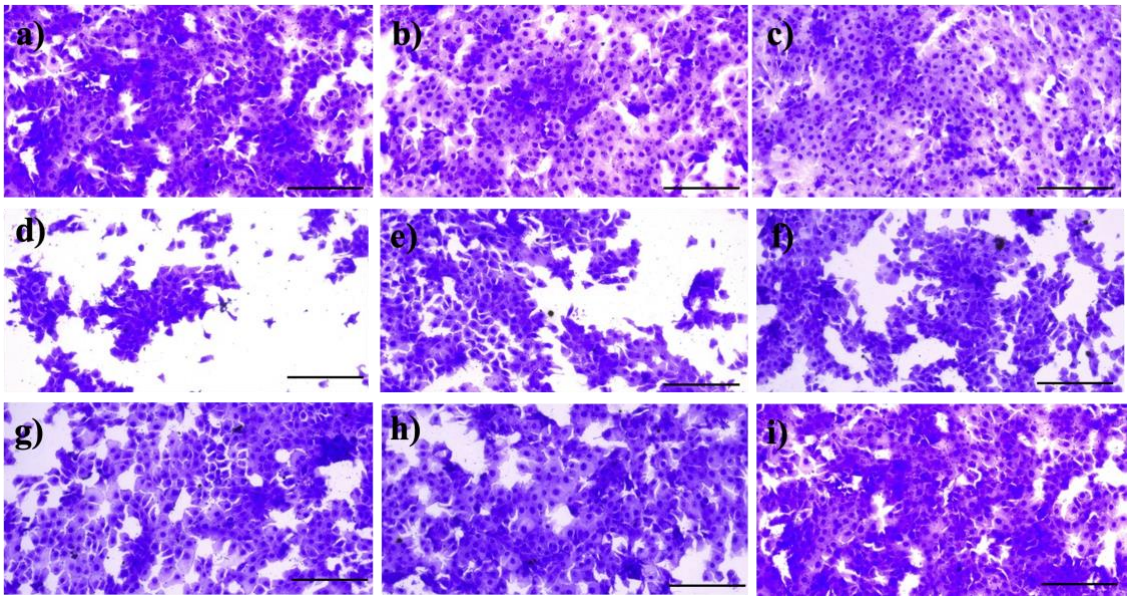

Figure 3. Crystal violet assay at 72h after the exposure of 10000 mg/L of the studied mixtures with the HaCat cell line. a) control, b) xylitol, c) choline chloride, d) XChCl4, e) XChCl10, f) XChCl35, g) XChCl50, h) XChCl75 and XChCl90. Photos are shown at 10X magnification, the scale line used in this experiment is 100 μm.

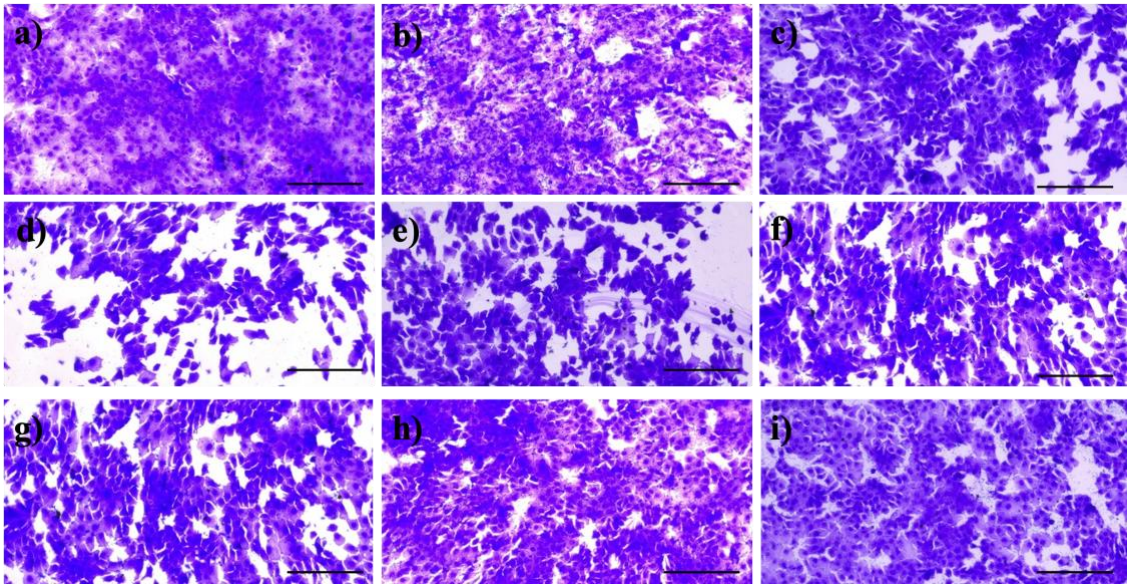

Figure 4. Crystal violet assay at 72h after the exposure of 10000 mg/L of the studied mixtures with the HepG2 cell line. a) control, b) xylitol, c) choline chloride, d) XChCl4, e) XChCl10, f) XChCl35, g) XChCl50, h) XChCl75 and XChCl90. Photos are shown at 10X magnification, the scale line used in this experiment is 100 μm.

In Figures 5 and 6, the dose-response curves in HaCat and HepG2 at 72h are shown. The qualitative trend is the same as the quantitative one and in all cases, the viability decreases as the concentration of water decreases being more toxic XChCl4 and the lowest XChCl90.

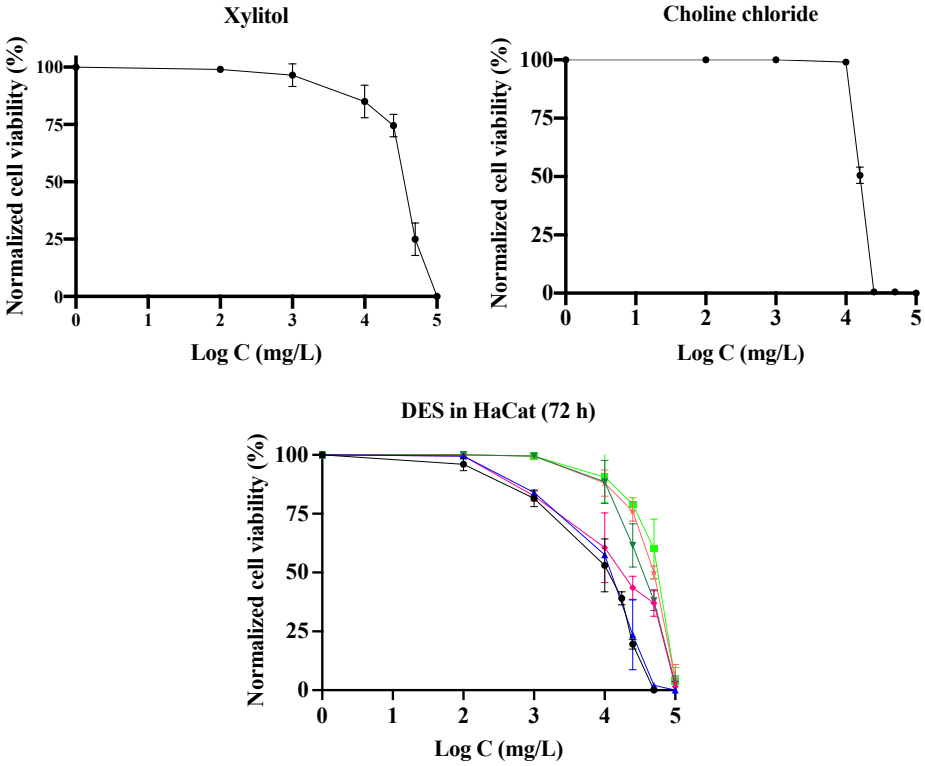

Figure 5. Dose-response curves in HaCat cell line for crystal violet assay: xylitol, choline chloride and studied mixtures: XChCl4 (●); XChCl10 (▲); XChCl35 (◆); XChCl50 (▼); XChCl75 (★); XChCl90 (■) at 72 h.

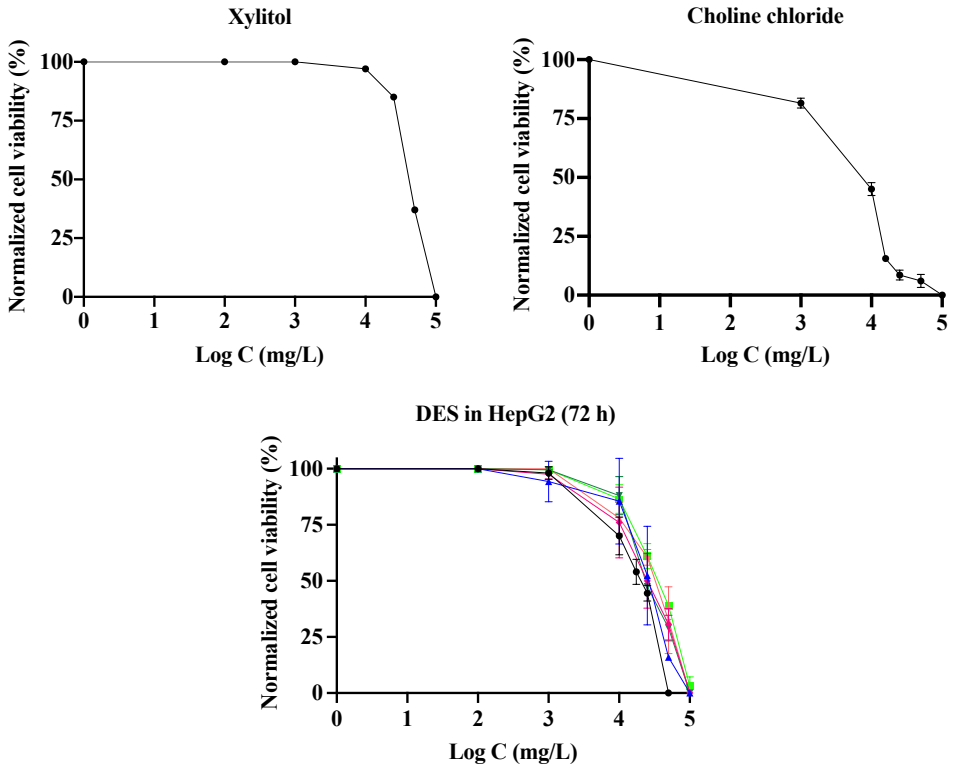

Figure 6. Dose-response curves in HepG2 cell line for crystal violet assay: xylitol, choline chloride and studied mixtures: XChCl4 (●); XChCl10 (▲); XChCl35 (◆); XChCl50 (▼); XChCl75 (★); XChCl90 (■) at 72 h.

3.4 BCA Protein Assay Kit

The colorimetric protein quantitative bicinchoninic acid (BCA) test is a common tool used for the determination of protein concentration. This simple method can tolerance some detergents and used in microplate format (Brown *et al.*, 1989; Rogatsky, 2021; Smith *et al.*, 1985). In this assay, the color formation results by two consecutive reactions. Firstly, Cu^{2+} is reduced to Cu^{+} by a protein in alkaline medium using the biuret reaction. Next, BCA reacts with Cu^{+} forming a purple-colored complex. This complex can be measured at 562 nm (Smith *et al.*, 1985).

In Figures 7 and 8, the cytotoxic effects caused by the mixtures are shown. Results indicate that the effects are dose-dependent and are consistent with the previous results obtained in the PrestoBlue test. For both the pure chemicals and the solvents, the normalized amount of protein decreases as the concentration of chemicals increases. The graphs for the evaluation of cell viability and protein concentration show a similar behavior, the reduction in the normalized fluorescence for the Prestoblue test correlates with the normalized absorbance for BCA.

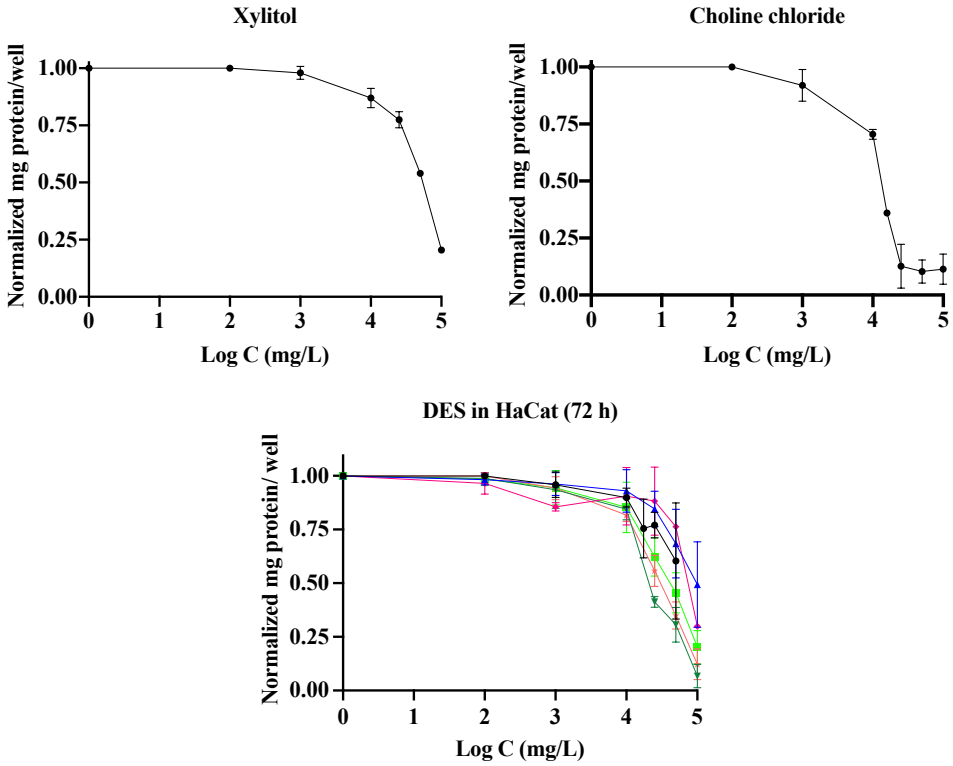

Figure 7. Dose-response curves in HaCat cell line for BCA assay: xylitol, choline chloride and studied mixtures: XChCl4 (●); XChCl10 (▲); XChCl35 (◆); XChCl50 (▼); XChCl75 (★); XChCl90 (■) at 72 h.

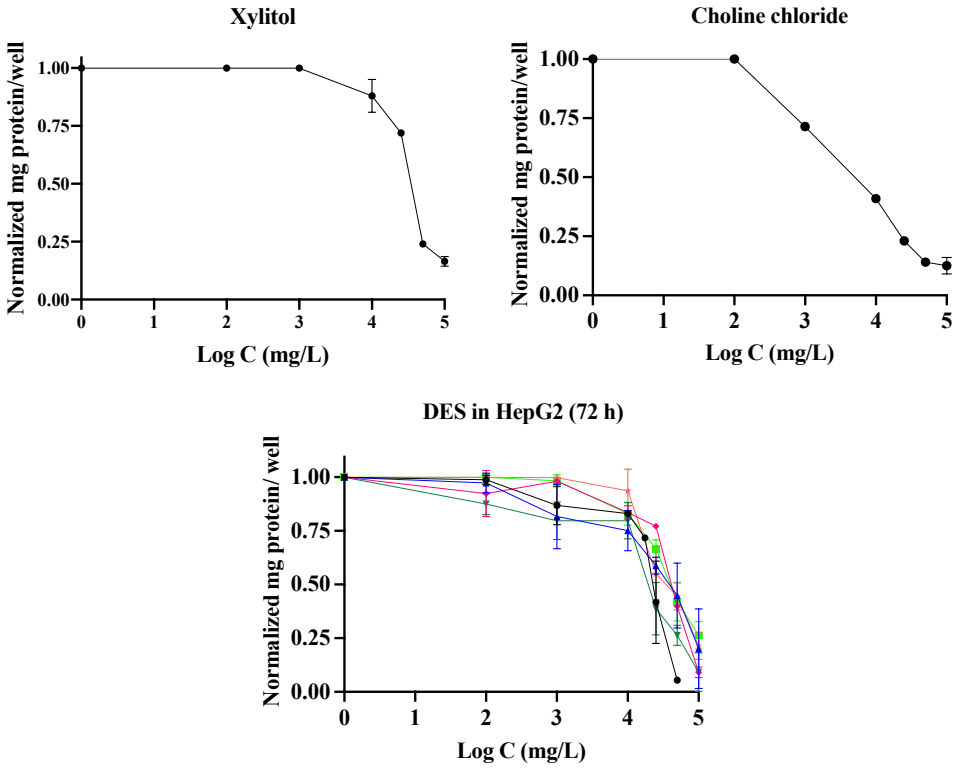

Figure 8. Dose-response curves in HepG2 cell line for BCA assay: xylitol, choline chloride and studied mixtures: XChCl4 (●); XChCl10 (▲); XChCl35 (◆); XChCl50 (▼); XChCl75 (★); XChCl90 (■) at 72 h.

3.5 Ibuprofen solubility in studied mixtures

Ibuprofen is a NSAID which presents very low values of solubility in water at room temperature (Pedro *et al.*, 2022; Watkinson *et al.*, 2009). Several previous studies have explored the increment of with temperature and the addition of some organic co-solvents (Heyneman *et al.*, 2000; Rhee *et al.*, 2008). However, the liquid formulations of this drug are very limited, so it is important the search in depth of new excipients. Moreover, the use of these excipients should be compatible with some conditions like diabetics, fructosemics or even children population. For these reasons, the use of xylitol for the development of oral pharmaceutical formulas is considered a good option when these aspects are considered. Next the results obtained in the solubility study of ibuprofen using the studied mixtures are shown.

In Figure 9, the prepared ibuprofen solutions in each of the moieties are presented: 1a) corresponds to the saturated solutions just prepared; 1b) the ibuprofen solutions after 24 h of shaking at controlled temperature (25ºC) are shown and, finally, 1c) the solutions

after 24 h stopping the shaking. The latter are the solutions used to determine the amount of drug solubilized in each of the mixtures.

Note that 1) the density of the ibuprofen powder is lower than any of the studied eutectic mixtures 2) the density and viscosity of the mixtures decreases with the water content of the mixtures (Table 2). The excess of non-solubilized ibuprofen powder is placed at the bottom of the test tubes, in the case of the mixture with higher water content. For samples with a lower proportion of water, the non-solubilized ibuprofen powder sits on top of the inhomogeneous solution (Figure 9) probably due to the high viscosity of the mixtures. For this reason, it is necessary that the solubility measurement be carried out in flat bottom test tubes, making it easier for the contact surface between solute and solvent to be as similar as possible in all cases, regardless of the density difference between solute and solvent.

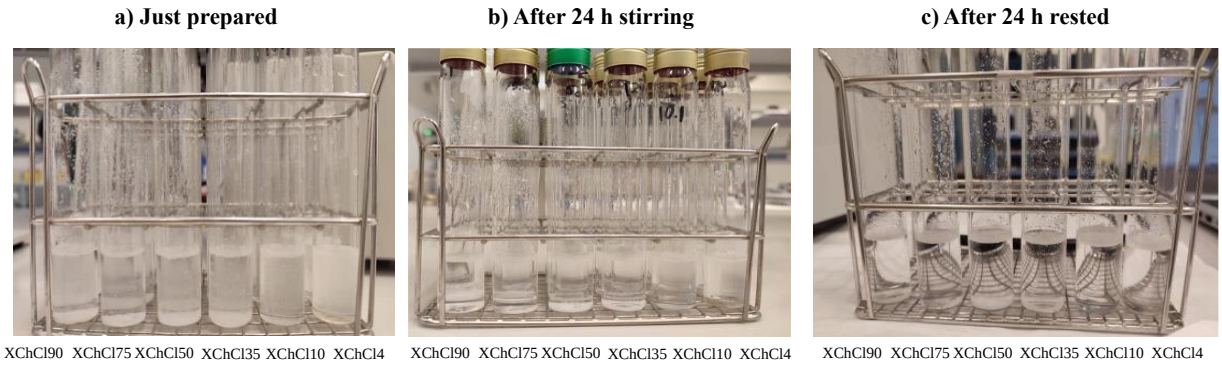

Figure 9. Solutions of ibuprofen in the studied mixtures. a) Just prepared, b) solutions stirred during 24 h at controlled temperature, c) rested solution during 24 h.

In Table 5, the results obtained for the calibration curve are shown. These results include validation parameters such as coefficient of determination, R^2, limit of detection, *LD*, and limit of quantification, *LQ*. Methanol has not shown interference in the spectra.

Table 5. Calibration equation ibuprofen in ethanol. Wavelength of maximum absorbance (Abs), $\lambda\, \boldsymbol{Abs_{max}}$, and validation parameters: coefficient of determination, R^2, limit of detection, *LD*, and limit of quantification, *LQ*. [a] $LD = \frac{x+3S}{m}$; [b] $LQ = \frac{x+10S}{m}$ being m the slope and the intercept equals to 0, and x and S , the average and the deviation of the blank.

API	Slope calibration line c in c (mg/l)	λ (Abs_{max}) (nm)	R^2	LD[a] (mg/l)	LQ[b] (mg/l)
Ibuprofen	40.608	222	0.999	0.05	0.15

The solubility of ibuprofen has been obtained at room temperature and values of the mean of solubility and the standard deviation are shown in Table 6. The solubility of ibuprofen in water was measured, however, the value obtained was below the LD of the method, therefore, it was decided to take the value from the literature in order to calculate the S/So ratio and be able to make the comparison. In this ratio, S is solubility of API in DES and S_0 the solubility of ibuprofen in water.

Table 6. Solubility, *s* (mg/L) with their corresponding standard deviation and the solubility ratio for ibuprofen in studied mixtures.

Solvent	*s* (mg/l)	ratio (S/S_0)
XChCl4	442 ± 15.0	21.0
XChCl10	107 ± 1.96	5.1
XChCl35	53.2 ± 2.63	2.5
XChCl50	46.8 ± 0.63	2.2
XChCl75	30.7 ± 1.36	1.5
XChCl90	41.3 ± 1.50	2.0
Water	21*	-

* Hazardous Substances Data Bank (HSDB). Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Ibuprofen#section=Solubility

The analysis of the molecular structure and the hydrogen bonds that can be formed between drugs and the active ingredients of DES is fundamental when it comes to explaining the increased solubility (Abdkarimi & Haghtalab, 2021). As shown in Table 6, it can be observed that, as a general trend, the solubility of the drug increases in mixtures with a lower proportion of water, being 21-fold increase in the case of XChCl4.

This may be due to the hydrophobic nature of the compound, and thus, the solubility decreases as the water concentration increases. However, the behavior observed in the mixtures with higher water content is slightly different: solubility of XChCl90 is greater than that of XChCl75; a priori, this is an anomalous behavior and could perhaps be explained due to the intermolecular interactions that occur between xylitol, choline chloride and water. In addition, it is possible that this phenomenon is a salting in/salting out process type: when the concentration of water in the DES increases, the water molecules penetrate the DES network more effectively, being in the middle with less "effective" water molecules in solution and thus, the mixture behaves as if there is less water content.

Ibuprofen can be currently formulated as a syrup. However, on many occasions the excipients used for developing the liquid oral formulation include some sugars sucha as fructose that can affect different special populations. In this case, intolerance fructose patients, who have a deficiency of the enzyme fructose 1,6-biphosphatase and are not capable of metabolizing fructose (Maiorana *et al.*, 2020). Another important point is the case of diabetic patients: xylitol can be used as an alternative sugar since it does not raise blood glucose or insulin levels. Additionally, this sweetener presents a reduced caloric value, and this is important in the weight control (Nontokozo Z. Msomi *et al.*, 2021; Nontokozo Zimbili Msomi *et al.*, 2022).

Although as a general rule, the use of sweeteners in the pediatric population should be avoided; it is worth noting that, on the market, there are different preparations with xylitol in the form of gummies, chewing gum, pills, etc. (Le *et al.*, 2022) that are widely used for the treatment of dental caries (Wu *et al.*, 2022). In fact, (Tagami *et al.* developed gums and chewing gums made up of xylitol and ibuprofen and the results provided information about the preparation of gum in clinical settings (Tagami *et al.*, 2021). Additionally, other study published by Miyazaki et *al.* studied how polyhydric alcohols (xylitol, sorbitol) can modify the rheological properties of gelling pectin formulations of paracetamol and ambroxol (Miyazaki *et al.*, 2005). The main problem of xylitol is its effect as laxative generating gastrointestinal adverse effects such as excess gas, diarrhea, soft stools, especially in the case of the little ones (Bueno-Hernandez *et al.*, 2019; Sylvetsky *et al.*, 2011; Vernacchio *et al.*, 2007). The study we present here could be a starting point for the liquid oral formulation. However, more studies must be carried out to ensure the possible effects of these excipients in different populations.

4. CONCLUSIONS

In this work, a DES formed by xylitol and choline chloride have been prepared and mixed with different proportions of water. Mixtures remained liquid at room temperature. Density and viscosity for the mixtures were also measured at 25ºC. The toxicity study showed that these mixtures are not toxic, although the mixtures formed by xylitol and choline chloride present higher toxicity than xylitol itself. It was also checked that the HepG2 cell line is less sensitive to the mixtures analyzed than the HaCat line. In addition, the solubility of ibuprofen in the mixtures has been also analyzed. It has been observed that the solubility of the drug increases up to 21-folds with respect to the solubility of the drug in water, which is very slightly soluble. It has also been seen that as the proportion of water in the DES increases, the solubility of ibuprofen decreases, being the best mixture to solubilize the drug, XChCl4.

Considering the obtained the results, it could be said that the studied mixtures could be a starting point in the formulation of drugs in liquid formulation; however, more studies would have to be carried out in order to develop drugs using them. This is an open door to the use of new oral liquid formulations in the field of ibuprofen for special populations, such as diabetics or fructosemics patients. Xylitol formulations helps to increase the solubility of drugs but, additionally, acts as an energy supplier with enhanced anti-diabetic activity.

Acknowledgements

We want to thank Universidad San Jorge for financial supporting (call for internal projects 2022). Authors want to thank Angela Domingo because of her collaboration.

REFERENCES

Abdkarimi, F., & Haghtalab, A. (2021). Solubility measurement and thermodynamic modeling of sertraline hydrochloride and clopidogrel bisulfate in deep eutectic solvent of choline chloride and malonic acid. *Journal of Molecular Liquids*, 344, 117940

Ahmadi, R., Hemmateenejad, B., Safavi, A., Shojaeifard, Z., Mohabbati, M., & Firuzi, O. (2018). Assessment of cytotoxicity of choline chloride-based natural deep eutectic solvents against human HEK-293 cells: A QSAR analysis. *Chemosphere*, 209, 831-838.

Ahuja, V., Macho, M., Ewe, D., Singh, M., Saha, S., & Saurav, K. (2020). Biological and pharmacological potential of xylitol: A molecular insight of unique metabolism. *Foods*, 9 (11), 1592.

Ainurofiq, A., Putro, D. S., Ramadhani, D. A., Putra, G. M., & Santo, L. D. D. (2021). A review on solubility enhancement methods for poorly water-soluble drugs. *Journal of Reports in Pharmaceutical Sciences*, 10, 137-147.

Al-Akayleh, F., Adwan, S., Khanfer, M., Idkaidek, N., & Al-Remawi, M. (2021). A novel eutectic-based transdermal delivery system for risperidone. *Aaps Pharmscitech*, 22.

Anastas, P., & Eghbali, N. (2010). Green chemistry: Principles and practice. *Chemical Society Reviews*, 39, 301-312.

Aroso, I. M., Craveiro, R., Rocha, A., Dionisio, M., Barreiros, S., Reis, R. L., et al. (2015). Design of controlled release systems for thedes-therapeutic deep eutectic solvents, using supercritical fluid technology. *International Journal of Pharmaceutics*, 492, 73-79.

Asgari, S. P., Behroozi, M., Jouyban, A., & Rahimpour, E. (2021). Solubility of carvedilol in aqueous mixtures of a deep eutectic solvent at different temperatures. *Physics and Chemistry of Liquids*, 342, 117537.

Barrett, J. A., Yang, W., Skolnik, S. M., Belliveau, L. M., & Patros, K. M. (2022). Discovery solubility measurement and assessment of small molecules with drug development in mind. *Drug Discovery Today*, 27, 1315-1325.

Barzegar-Jalali, M., Jafari, P., & Jouyban, A. (2022). Acetaminophen solubility in aqueous solutions of betaine-propylene glycol natural deep eutectic solvent at different temperatures. *Journal of Molecular Liquids*, 349, 118199.

Brown, R. E., Jarvis, K. L., & Hyland, K. J. (1989). Protein measurement using bicinchoninic acid - elimination of interfering substances. *Analytical Biochemistry*, 180, 136-139.

Bueno-Hernandez, N., Vazquez-Frias, R., Abreu y Abreu, A. T., Almeda-Valdes, P., Barajas-Nava, L. A., Carmona-Sanchez, R. I., et al. (2019). Review of the scientific evidence and technical opinion on noncaloric sweetener consumption in gastrointestinal diseases. *Revista De Gastroenterologia De Mexico*, 84, 492-510.

Castro, V. I. B., Craveiro, R., Silva, J. M., Reis, R. L., Paiva, A., & Duarte, A. R. C. (2018). Natural deep eutectic systems as alternative nontoxic cryoprotective agents. *Cryobiology*, 83, 15-26.

Daadoue, S., Al-Remawi, M., Al-Mawla, L., Idkaidek, N., Khalid, R. M., & Al-Akayleh, F. (2022). Deep eutectic liquid as transdermal delivery vehicle of risperidone. *Journal of Molecular Liquids*, 345.

Duarte, A. R. C., Ferreira, A. S. D., Barreiros, S., Cabrita, E., Reis, R. L., & Paiva, A. (2017). A comparison between pure active pharmaceutical ingredients and therapeutic deep eutectic solvents: Solubility and permeability studies. *European Journal of Pharmaceutics and Biopharmaceutics*, 114, 296-304.

European pharmacopoeia 10, 2022. Bulk density and tapped density of powders (2.9.34). London: European medicines agency. Science medicines health.

Feoktistova, M., Geserick, P., & Leverkus, M. (2016). Crystal violet assay for determining viability of cultured cells. *Cold Spring Harbor protocols*, 2016, pdb.prot087379-pdb.prot087379.

Ferreira, I. J., Meneses, L., Paiva, A., Diniz, M., & Duarte, A. R. C. (2022). Assessment of deep eutectic solvents toxicity in zebrafish (danio rerio). *Chemosphere*, 299,134415.

Garekani, H. A., Sadeghi, F., Badiee, A., Mostafa, S. A., & Rajabi-Siahboomi, A. R. (2001). Crystal habit modifications of ibuprofen and their physicomechanical characteristics. *Drug Development and Industrial Pharmacy*, 27, 803-809.

Goeke, K., Lorenz, T., Repanas, A., Schneider, F., Steiner, D., Baumann, K., et al. (2018). Novel strategies for the formulation and processing of poorly water-soluble drugs. *European Journal of Pharmaceutics and Biopharmaceutics*, 126, 40-56.

Golgoun, S., Mokhtarpour, M., & Shekaari, H. (2021). Solubility enhancement of betamethasone, meloxicam and piroxicam by use of choline-based deep eutectic solvents. *Pharmaceutical Sciences*, 27, 86-101.

Han, X., Ghoroi, C., To, D., Chen, Y., & Dave, R. (2011). Simultaneous micronization and surface modification for improvement of flow and dissolution of drug particles. *International Journal of Pharmaceutics*, 415, 185-195.

Hansen, B. B., Spittle, S., Chen, B., Poe, D., Zhang, Y., Klein, J. M., et al. (2021). Deep eutectic solvents: A review of fundamentals and applications. *Chemical Reviews*, 121, 1232-1285.

Harirforoosh, S., Asghar, W., & Jamali, F. (2013). Adverse effects of nonsteroidal antiinflammatory drugs: An update of gastrointestinal, cardiovascular and renal complications. *Journal of Pharmacy and Pharmaceutical Sciences*, 16, 821-847.

Hayyan, M., Mbous, Y. P., Looi, C. Y., Wong, W. F., Hayyan, A., Salleh, Z., et al. (2016). Natural deep eutectic solvents: Cytotoxic profile. *Springerplus*, 5, 913.

Heyneman, C. A., Lawless-Liday, C., & Wall, G. C. (2000). Oral versus topical nsaids in rheumatic diseases - a comparison. *Drugs*, 60, 555-574.

Juan A García Meijide , J. J. G.-R. C. (2000). The physiopathology of cyclooxygenase-1 and cyclooxygenase-2. *Revista Española de Reumatología*, 27, 33-35.

Kalepu, S., & Nekkanti, V. (2015). Insoluble drug delivery strategies: Review of recent advances and business prospects. *Acta Pharmaceutica Sinica B*, 5, 442-453.

Kudlak, B., Owczarek, K., & Namiesnik, J. (2015). Selected issues related to the toxicity of ionic liquids and deep eutectic solvents-a review. *Environmental Science and Pollution Research*, 22, 11975-11992.

Lall, N., Henley-Smith, C. J., De Canha, M. N., Oosthuizen, C. B., & Berrington, D. (2013). Viability reagent, prestoblue, in comparison with other available reagents, utilized in cytotoxicity and antimicrobial assays. *International journal of microbiology*, 2013, 420601.

Lapena, D., Bergua, F., Lomba, L., Giner, B., & Lafuente, C. (2020). A comprehensive study of the thermophysical properties of reline and hydrated reline. *Journal of Molecular Liquids*, 303, 112679.

Lapena, D., Errazquin, D., Lomba, L., Lafuente, C., & Giner, B. (2021). Ecotoxicity and biodegradability of pure and aqueous mixtures of deep eutectic solvents: Glyceline, ethaline, and reline. *Environmental Science and Pollution Research*, 28, 8812-8821.

Lapena, D., Lomba, L., Artal, M., Lafuente, C., & Giner, B. (2019a). The nades glyceline as a potential green solvent: A comprehensive study of its thermophysical properties and effect of water inclusion. *Journal of Chemical Thermodynamics*, 128, 164-172.

Lapena, D., Lomba, L., Artal, M., Lafuente, C., & Giner, B. (2019b). Thermophysical characterization of the deep eutectic solvent choline chloride:Ethylene glycol and one of its mixtures with water. *Fluid Phase Equilibria*, 492, 1-9.

Laurence Brunton , B. K. (2022). *Goodman and Gilman's the pharmacological basis of therapeutics*: McGraw Hill / Medical.

Le, H., Wang, X., Wei, Y., Zhao, Y., Zhang, J., & Zhang, L. (2022). Making polyol gummies by 3d printing: Effect of polyols on 3d printing characteristics. *Foods*, 11.

Li, S., Culkin, A., Jones, D. S., & Andrews, G. P. (2021). Development of polycaprolactone-based metronidazole matrices for intravaginal extended drug delivery using a mechanochemically prepared therapeutic deep eutectic system. *International Journal of Pharmaceutics*, 593.

Liu, T., Liu, X., Spring, D. R., Qian, X., Cui, J., & Xu, Z. (2014). Quantitatively mapping cellular viscosity with detailed organelle information via a designed pet fluorescent probe. *Scientific Reports*, 4.

Liu, X., Ahlgren, S., Korthout, H. A. A. J., Salome-Abarca, L. F., Bayona, L. M., Verpoorte, R., et al. (2018). Broad range range chemical profiling of natural deep eutectic solvent extracts using a high performance thin layer chromatography-based method. *Journal of Chromatography A*, 1532, 198-207.

Lu, C., Cao, J., Wang, N., & Su, E. Z. (2016). Significantly improving the solubility of non-steroidal anti-inflammatory drugs in deep eutectic solvents for potential non-aqueous liquid administration. *Medchemcomm*, 7, 955-959.

Macario, I. P. E., Oliveira, H., Menezes, A. C., Ventura, S. P. M., Pereira, J. L., Goncalves, A. M. M., et al. (2019). Cytotoxicity profiling of deep eutectic solvents to human skin cells. *Scientific Reports*, 9.

Maiorana, A., Sabia, A., Corsetti, T., & Dionisi-Vici, C. (2020). Safety of vaccines administration in hereditary fructose intolerance. *Orphanet Journal of Rare Diseases*, 15(1),274.

Mehedint, M. G., & Zeisel, S. H. (2013). Choline's role in maintaining liver function: New evidence for epigenetic mechanisms. *Current Opinion in Clinical Nutrition and Metabolic Care*, 16, 339-345.

Miyazaki, S., Kubo, W., Itoh, K., Konno, Y., Fujiwara, M., Dairaku, M., et al. (2005). The effect of taste masking agents on in situ gelling pectin formulations for oral sustained delivery of paracetamol and ambroxol. *International Journal of Pharmaceutics*, 297, 38-49.

Mokhtarpour, M., Shekaari, H., Martinez, F., & Zafarani-Moattar, M. T. (2019). Performance of local composition models to correlate the aqueous solubility of naproxen in some choline based deep eutectic solvents at t = (298.15-313.15) k. *Pharmaceutical Sciences*, 25, 244-253.

Mokhtarpour, M., Shekaari, H., Martinez, F., & Zafarani-Moattar, M. T. (2019). Study of naproxen in some aqueous solutions of choline-based deep eutectic solvents: Solubility measurements, volumetric and compressibility properties. *International Journal of Pharmaceutics*, 564, 197-206.

Mokhtarpour, M., Shekaari, H., & Shayanfar, A. (2020). Design and characterization of ascorbic acid based therapeutic deep eutectic solvent as a new ion-gel for delivery of sunitinib malate. *Journal of Drug Delivery Science and Technology*, 56 (A), 101512.

Msomi, N. Z., Erukainure, O. L., & Islam, M. S. (2021). Suitability of sugar alcohols as antidiabetic supplements: A review. *Journal of Food and Drug Analysis*, 29, 1-14.

Msomi, N. Z., Erukainure, O. L., Salau, V. F., Olofinsan, K. A., & Islam, M. S. (2022). Xylitol improves antioxidant, purinergic and cholinergic dysfunction, and lipid metabolic homeostasis in hepatic injury in type 2 diabetic rats. *Journal of Food Biochemistry*, 46, (4), e14040.

Mulia, K., Krisanti, E., Fauzia, F., Putri, S. (2016). Dio-based natural deep eutectic solvent extraction of alpha-mangostin from the pericarpt of Garccinia mangostona. 9th Joint Meeting of AFERP, ASP, GA, JSP, PSE and SIF, 82(1), P762.

Nokhodchi, A., Homayouni, A., Araya, R., Kaialy, W., Obeidat, W., & Asare-Addo, K. (2015). Crystal engineering of ibuprofen using starch derivatives in crystallization medium to produce promising ibuprofen with improved pharmaceutical performance. *Rsc Advances*, 5, 46119-46131.

Nystedt, H. L., Gronlien, K. G., & Tonnesen, H. H. (2021). Interactions of natural deep eutectic solvents (nades) with artificial and natural membranes. *Journal of Molecular Liquids*, 328, 115452.

Pedro, S. N., Freire, M. G., Freire, C. S. R., & Silvestre, A. J. D. (2019). Deep eutectic solvents comprising active pharmaceutical ingredients in the development of drug delivery systems. *Expert Opinion on Drug Delivery*, 16, 497-506.

Pedro, S. N., Mendes, M. S. M., Neves, B. M., Almeida, I. F., Costa, P., Correia-Sa, I., et al. (2022). Deep eutectic solvent formulations and alginate-based hydrogels as a new partnership for the transdermal administration of anti-inflammatory drugs. *Pharmaceutics*, 14, (4), 827.

Phaechamud, T., Tuntarawongsa, S., & Charoensuksai, P. (2016). Evaporation behavior and characterization of eutectic solvent and ibuprofen eutectic solution. *Aaps Pharmscitech*, 17, 1213-1220.

Rhee, Y.-S., Chang, S.-Y., Park, C.-W., Chi, S.-C., & Park, E.-S. (2008). Optimization of ibuprofen gel formulations using experimental design technique for enhanced transdermal penetration. *International Journal of Pharmaceutics*, 364, 14-20.

Rogatsky, E. (2021). Pandora box of bca assay. Investigation of the accuracy and linearity of the microplate bicinchoninic protein assay: Analytical challenges and method modifications to minimize systematic errors. *Analytical Biochemistry*, 631, 114321.

Ruiz, O. (2020). Effects of sweeteners on the gut microbiota: A review of experimental studies and clinical trials (vol 10, pg s31, 2019). *Advances in Nutrition*, 11, 468-468.

Santos, F., Leitao, M. I. P. S., & Duarte, A. R. C. (2019). Properties of therapeutic deep eutectic solvents of l-arginine and ethambutol for tuberculosis treatment. *Molecules*, 24(1), 55.

Sarraguca, M. C., Ribeiro, P. R. S., Nunes, C., & Seabra, C. L. (2022). Solids turn into liquids-liquid eutectic systems of pharmaceutics to improve drug solubility. *Pharmaceuticals*, 15 (3), 279.

Silva, E., Oliveira, F., Silva, J. M., Matias, A., Reis, R. L., & Duarte, A. R. C. (2020). Optimal design of thedes based on perillyl alcohol and ibuprofen. *Pharmaceutics*, 12(11), 1121.

Silva, J. M., Reis, R. L., Paiva, A., & Duarte, A. R. C. (2018). Design of functional therapeutic deep eutectic solvents based on choline chloride and ascorbic acid. *Acs Sustainable Chemistry & Engineering*, 6, 10355-10363.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., et al. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, 150, 76-85.

Sylvetsky, A., Rother, K. I., & Brown, R. (2011). Artificial sweetener use among children: Epidemiology, recommendations, metabolic outcomes, and future directions. *Pediatric Clinics of North America*, 58, 1467.

Tagami, T., Kuwata, E., & Ozeki, T. (2021). Confectionery xylitol gum-containing tablets for medical application and the sintering effect on gum tablets. *Biological & Pharmaceutical Bulletin*, 44, 1309-1315.

Tajmir, F., & Roosta, A. (2020). Solubility of cefixime in aqueous mixtures of deep eutectic solvents from experimental study and modeling. *Journal of Molecular Liquids*, 303, 112636.

Ur-Rehman, S., Mushtaq, Z., Zahoor, T., Jamil, A., & Murtaza, M. A. (2015). Xylitol: A review on bioproduction, application, health benefits, and related safety issues. *Critical Reviews in Food Science and Nutrition*, 55, 1514-1528.

Vernacchio, L., Vezina, R. M., & Mitchell, A. A. (2007). Tolerability of oral xylitol solution in young children: Implications for otitis media prophylaxis. *International Journal of Pediatric Otorhinolaryngology*, 71, 89-94.

Vicent Trung H. Ngo, T. B. (2022). *Ibuprofen*: Treasure Island (FL): StatPearls Publishing.

Watkinson, R. M., Herkenne, C., Guy, R. H., Hadgraft, J., Oliveira, G., & Lane, M. E. (2009). Influence of ethanol on the solubility, ionization and permeation characteristics of ibuprofen in silicone and human skin. *Skin Pharmacology and Physiology*, 22, 15-21.

Wu, Y.-F., Salamanca, E., Chen, I. W., Su, J.-N., Chen, Y.-C., Wang, S. Y., et al. (2022). Xylitol-containing chewing gum reduces cariogenic and periodontopathic bacteria in dental plaque-microbiome investigation. *Frontiers in Nutrition*, 9, 882636.

Yin, T., Wu, J., Yuan, J., & Wang, X. (2022). Therapeutic deep eutectic solvent based on osthole and paeonol: Preparation, characterization, and permeation behavior. *Journal of Molecular Liquids*, 346, 117133.