Physical characterization and permeability of lupeol by use of organogel-based emulsions (o/w)

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ABSTRACT

Lupeol, is naturally occurring compound present in plants, with some beneficial health effects. In the present study, organogel-based emulsions (o/w) were obtained from canola oil. Particle size analysis, rheology test, optical microscopy, and permeability of nutraceuticals by cell culture and UPLC-MS/MS were done. Particle hydrodynamic diameters obtained in organogel-based emulsions loaded with nutraceuticals were <200nm, Polydispersity Index (PdI) was around 0.25 - 0.30, and Zeta Potential (ZP) was about -19 to -25mV. Droplets in emulsions presented spherical shapes and adjusted to the Herschel-Bulkley model. Interestingly, permeability of lupeol was increased compared with its crystalline; therefore organogel-based emulsions loaded with lupeol have potential for controlled delivery of this nutraceutical.

Keywords: Lupeol; organogel-based emulsion; permeability.

1. INTRODUCTION

According to the World Health Organization (WHO), chronic diseases cause about 38 million people deaths annually, being cardiovascular disease, cancer, respiratory diseases and diabetes responsible for 82% of non-communicable diseases related deaths [1]. Bad lifestyle habits, such as consumption of addictive substances (e.g., alcohol and tobacco), as well as bad eating habits, are some of the main risk factors that contribute to these ailments. A diet rich in plants-derived foods, in addition to usual macronutrients and nourish, promotes health benefits because these foods contain phytochemicals.

Nutraceuticals are a class of phytochemicals recognized by their therapeutic importance. Nutraceuticals have been recognized as potential tools for prophylaxis and treatment for multiple diseases. Most nutraceuticals are safe, less toxic and with fewer side effects than conventional drugs [2]. Nutraceuticals such as polyphenols or triterpenoids are known to have therapeutic activities, such as hepatoprotective, anti-microbial, anti-inflammatory, anti-cancer, and chemopreventive [3, 4, 5].

Oral route is the most common way for the administration of nutraceuticals, because of its convenience and cost efficiency. However, in drug discovery, approximately 70% of candidates show limited aqueous solubility [6]. Lupeol, have poor systemic bioavailability once they have been subjected to digestive process because it has poor aqueous solubility. An alternative is topical route.

Methods for increasing bioavailability of nutraceuticals are focused on three main factors: increasing dissolution rate, improving absorption, and minimizing metabolism [7]. In the simplest process (dissolution), nutraceuticals are regularly in crystalline form, which is the lower thermodynamic state that requires a large amount of energy to be dissolved in an aqueous solution. Some methods for increasing bioavailability include self-dispersing lipid formulations, solid lipid nanoparticles, colloidal lipid carriers (e.g. liposomes), emulsions, micro/nano-emulsions, polymeric nanoparticles, among others. Particularly, emulsions with r<100 nm offer better stability against aggregation of particles. These emulsions contain particles with weak light scattering and are optically clear or slightly turbid; according to [8] an emulsion is transparent when the oil droplet radius is equal or below to 30 nm. They can also present rheological characteristics as high viscosity or gel-like characteristics and may increase the bioavailability of lipophilic compounds increasing aqueous solubility, and permeability enhancement [9].

Emulsified organogels, nano or micro emulsions are considered as a novel vehicle for bioactive compounds with low aqueous solubility. They show the ability to solubilize lipophilic compounds within their lipid domains, mainly due to their hydrophilic-lipophilic nature. It is common to use edible oils composed of medium and long chain triglycerides (TGs) for preparing lipid-based formulations such as emulsions. Triacylglyceride-based formulations improve bioavailability and show no toxicity, turning these formulations as suitable nanocarriers approved by the Food and Drug Administration (FDA) [10]. It has been reported the use of organogels-based emulsions (OBEs) of water in oil type (w/o) [11], however, obtained results were unsatisfactory for several nutraceuticals compounds Therefore, the aim of this research was to develop and to evaluate organogel-based emulsions (OBEs) (o/w) as carrier for lupeol delivery system.
2. MATERIALS AND METHODS

Lupeol (PubChem CID: 259846) was obtained from Sigma-Aldrich (St. Louis, Mo., USA). Canola oil was purchased from a local supermarket. Myverol 18 -04 k, which is a mixture of saturated monoglycerides (MAGs) with roughly a 60:40 ratio, between monopalmmitin and monostearin was obtained from Kerry Mexico (Irapuato, Gto, Mexico). Emulsifiers Tween 20 (Polyoxyethylene-20-sorbitan monolaureate) was purchased from Fisher Scientific (Hampton, NH, USA) and Tween 80 (Polyoxyethylene-20-sorbitan monooleate) was obtained from FagaLab (Mocorito, Sin, Mex).

2.1. Organogel preparation.

Concisely, canola oil was heated to 80°C for 10 min. Myverol was dissolved in oil in 5% w/v proportion. There are several organogelators, such as MAGs, which are rare systems capable of self-assembling both aqueous and organic media [12]. Also, there are more efficient organogelators as waxes [13] or fatty alcohols [14]; the availability, cost and after use in emulsified systems are important factors to election of MAGs as organogelators. The mixture was then stirred to dissolve the crystalline material. The mixture was allowed to cool and stabilize during 24 h at room temperature (~25°C). Lupeol was loaded at the following concentration: 2 mg/mL of canola oil. Organogel samples were maintained at room temperature for one month, after this time organogels were renewed to ensure their stability.

2.2. Nutraceuticals oil solubility.

Nutraceuticals oil solubility was obtained following the method reported by [15]. Briefly, lupeol was weighted (40 mg) to reach a final concentration of 2 mg/mL in canola oil. After a pre-warming of oils at 80°C during 10 min, it was added in a beaker and stirred during 10, 60, 120 and 180 min at 80°C to evaluate their dissolution. Samples did not show visual changes after two weeks of storage at room temperature. This experiment was carried out in triplicate.

2.3. Preparation of organogel-based emulsions and canola organogel-based emulsion loaded with lupeol.

For unloaded organogel-based emulsions a mixture was prepared to contain 5 or 10% of canola organogel, 10% of Tween 20 or Tween 80, plus 85 or 80% of deionized water, respectively. Samples were placed in a Branson ultrasound bath (Richmond, VA, USA) for 30 min; subsequently, it was used an ultrasonic homogenizer Hielscher UP200Ht (Ringwood, NJ, USA) at 200 W and amplitude 90% coupled to a water bath at temperature control (25 °C) during 2 min to complete homogenization and the emulsion formation.

2.4. Particle Size (PS), polydispersity index (PdI) and zeta potential determinations.

Particle size, polydispersity index and zeta potential were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, WR, UK) at a fixed scattering angle of 90° at room temperature. Samples were diluted 10 times in deionized water. All measurements were performed on freshly prepared organogel-based emulsions. For each sample, the mean diameter [4,3] and the standard deviation were calculated (n=3).

2.5. Rheological characterization of organogel-based emulsions.

Rheological properties of emulsions were measured in a rheometer Discovery HR-3 (TA Instruments, New Castle, DE, USA), using a sandblasted parallel plate geometry (40 mm). The gap was adjusted to 700 μm and sample volume used was 900 μL for every single test. Due to emulsions nature, only single shear tests were performed. Operation conditions for rheometer were as follows: sample conditioning at 25°C for 10 s; then a flow ramp was set at 25°C during 180 s and a shear rate of 100/s up to 800/s (Range obtained in previous experimental work). After this, a flow ramp was performed in reverse direction starting at 800/s up to 100/s, in order to determine if the evaluated material was thixotropic. A temperature flow ramp test was performed from 25 to 55°C at a shear rate of 600/s, in order to calculate the activation energy by using Arrhenius equation. Rheological measurements were carried out by duplicate.

In vitro permeability. Caco-2 cells were obtained from the American Type Culture Collection (ATCC) (Rockville MD, USA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis MO, USA) supplemented with 10% fetal bovine serum (FBS, Corning, Tewksbury MA, USA), 2.2 g/L of sodium bicarbonate, 100 U/mL penicillin and 100 μg/mL streptomycin at a final pH 7.2–7.4 and maintained at 37°C under a 5% CO2 and 95% air atmosphere at constant humidity.

Caco-2 cell monolayers (passages 13-18) were grown to confluence and allowed to differentiate until day 21. At days 1, 7, 10, 14, 17 and 21, cells were washed twice with phosphate buffered saline (PBS) (1X), fixed with ethanol and incubated with 5-bromo-4-chloro-3-indoly1-phosphate/nitro blue tetrazolium (BCIP®/NBT) for 2h. Blue color presence indicates if alkaline phosphatase is present. Alkaline phosphatase was used as marker of differentiation of Caco-2 to enterocytes.

Controls of free compounds and organogel-based emulsions were used at 1 μg/mL. Samples (250 μL) were added to the apical compartment dissolved in Hank's balanced salt solution (HBSS, pH 7.3±0.1) in 1:1 relation, while the basolateral compartment contained no treatments (HBSS+4% bovine serum albumin (BSA), pH 7.4). After different time intervals (60 and 120 min), liquids on the apical and basolateral chamber were recovered and stored at -80°C until analysis.

Permeability was calculated using the next equation:

\[
P_{app} = \left(\frac{dQ}{dt}\right) \frac{1}{A_{c0}}
\]

Where the Papp is the apparent permeation rate; dQ/dt is the mass transport rate, A the surface area of the insert and C0 will be the concentration of compound on the apical side.

2.6. Liquid-liquid micro-extraction (LLME).

Samples from apical (0.5 mL) or basolateral fractions (0.5 mL) were taken and subjected to LLME. Briefly, 500 μL of ethyl acetate was added to fraction and this mixture was vortexed for 1 min. After this, samples were centrifuged at 10,000g and 4°C for 5 min. Organic phase was separated and ethyl acetate (500 μL) was added again to the non-recovered sample and the procedure was repeated in the same manner. Ethyl acetate fraction (1 mL) was then dried using a CentriVap Labconco (Kansas City, MO, USA).
at a temperature operation of 37°C for 4 h until dryness. Later, the samples were suspended in 100 μL of methanol and further centrifuged at 10000g and 4°C for 10 min. The supernatants were recovered and analyzed by UPLC-APCI-QqQ.

2.7. UPLC-APCI-QqQ analysis

The chemical analysis of reference compound (lupeol) in samples was performed in an Acquity UPLC system (Agua Corp. Milford, MA, USA) coupled to a Xevo tandem mass spectrometer TQ-S triple quadruple (QqQ) (Waters Corp) using positive ionization module (APCI+). Chromatographic separations were performed on a Acquity UPLC BEH C18 column (Waters) (50 x 2.1 mm, particle size 1.7 μm), using water/formic acid 7.5 mM (A) and methanol (B) as mobile phases at 200 μL/min and sample volumes of 5 μL. A solvent gradient was used starting with 85% B to reach 90% B at 2.0 min, 100% B at 8.0 min and again 85% B at 9.0 min. This last condition was maintained for 3.0 min.

Determination of droplet size distribution usually requires dilution of emulsions (≤1% v/v), which may itself alter their size distribution, especially in highly concentrated, polydisperse or marginally stable systems [16]. PS of polydisperse emulsions is characterized by the measurement of both the mean droplet size and the size of their distribution [17]. For OBEs obtained, it was observed that polydispersity was affected by the surfactant (p<0.05). In general, OBEs made with Tween 80 showed lower PdI values (Table 2). Higher PdI values can contribute to a more unstable emulsion, making it more susceptible to a destabilization phenomenon such as flocculation or coalescence. Another important parameter is the particle charge, usually measured as ZP.

The ZP will determine if the particles inside the liquid tend toward flocculation or not, therefore, knowing the ZP is useful for stability assessment [17].

The values obtained from the characterization of OBEs showed that ZP values ranged from -10 to -16 mV (Table 2). These ZP values mean a certain degree of stability by emulsions. Theoretically, a higher value of ZP indicates major stability. Results showed that more negative values of ZP were observed at 5% organogel, 10% Tween 80 and 85% deionized water. This condition was chosen to perform the loading of lipophilic bioactive compounds in function of their results on particle charge (ZP).

Unloaded OBEs at all experimental conditions, behave as time independent non-Newtonian fluids, i.e., the shear stress depends only on the shear rate (γ) and fits the Herschel-Bulkley model (Table 3). Fluids that fit into Herschel-Bulkley model are pseudo-plastic and exhibit a not solid, but a solid-like behavior, while the shear stress does not exceed a yield stress value τ₀, once this value is exceeded they can adopt a Bingham behavior.

Activation energy of OBEs (Table 3) was calculated from Arrhenius equation; whereas the activation energy increases, emulsion becomes more stable. A similar tendency was found in activation energy and viscosity; thus, this behavior seems to be organogel concentration-dependent.

3.1. Characterization of organogel-based emulsions with loaded nutraceuticals

Prior to organogels preparation loaded with nutraceuticals to subsequently obtain the emulsions (OBEs), the solubility test of lupeol was made to measure their dissolution in canola. Lupeol has shown good solubility at 2 mg/mL in oil after 10 min of stirring at 80°C. There are no reports for lupeol solubility in oils; however, betulin (member of lupane-type triterpenes like lupeol) has a solubility of ~3 mg/mL in sunflower oil [18].

### Table 1. Analytical parameters for the identification and quantification of nutraceutical compounds.

| Compound | Time (min) | m/z | MRM transitions | Cone voltage | Collision energy | Linear range (ng) | Slope (r²) |
|----------|------------|-----|----------------|--------------|-----------------|------------------|------------|
| Lupeol   | 8.74       | 427 | 109–95         | 16.0         | 28, 36          | 696.11 ± 8.20   | 0.984      |

3. RESULTS

Choice of oil concentration, surface agent and physical characterization for organogel-based emulsions

Obtained ultrasound homogenization and organogel-based emulsions (OBEs) showed that organic phase concentration and surfactant, influence the size of constituent particles in the emulsion. Smaller particle sizes were obtained with tween 80 and a lower concentration of lipids (5%) (Table 2).

Oil concentration is important for particle size. Concentrations of oil in systems stabilized by surfactants. Although PS is important for emulsion stability, it is also important to determine the particle size distribution (polydispersity).

Determination of droplet size distribution usually requires dilution of emulsions (≤1% v/v), which may itself alter their size distribution, especially in highly concentrated, polydisperse or marginally stable systems [16]. PS of polydisperse emulsions is characterized by the measurement of both the mean droplet size and the size of their distribution [17]. For OBEs obtained, it was observed that polydispersity was affected by the surfactant (p<0.05). In general, OBEs made with Tween 80 showed lower PdI values (Table 2). Higher PdI values can contribute to a more unstable emulsion, making it more susceptible to a destabilization phenomenon such as flocculation or coalescence. Another important parameter is the particle charge, usually measured as ZP.

ZP represents a measure of electrostatic repulsion between the particles present in the emulsion and becomes important when the droplets approach each other and the double layer begins to interfere. Energy is required to overcome this electrostatic repulsion and to force the bond between particles; therefore, ZP is a measure of this energy. A high ZP value does not necessarily lead to emulsion stability because the rate of ZP reduction is in fact what controls the emulsion stability. This indicates that the stability of emulsion is governed by the rate at which desorption of the emulsifier from the interfacial film takes place during aging.

Flow at 150 L/h, probe and source temperatures were 300 and 150°C, respectively; nebulizer gas (6.0 Bar) and collision gas flows were set at 0.12 mL/min. Multiple Reactions Monitoring (MRM) transitions were determined from the MS/MS spectra of existing standards.

Data acquisition and processing were performed using MassLinx (Waters Corp.) software. Peak identification was based on a comparison of their retention times and MRM transitions with pure standard. Quantitative determinations of compounds were carried out using standard calibration curves of the available standards (range 4 to 32 ng). Validation parameters are summarized in Table 1.

2.8. Statistical Analysis

All data were expressed as mean ± SD. Experimental data were analyzed by ANOVA and Tukey test with a confidence level of 95% by the use of Software Statistica v 7.2 (StatSoft, Tulsa, OK, USA).
Unloaded OBEs were used as controls for comparison against OBEs loaded with lupeol. Particles in unloaded OBEs (Table 3) had an average diameter of 153.91±3.25 nm, while the particle size for loaded lupeol OBEs was 153.55±0.95 nm. OBEs loaded with lupeol did not show significant differences regarding unloaded OBEs (p≥0.05).

PdI (Table 3) of drops in the OBEs ranged from 0.41±0.018, 0.28±0.0101, for unloaded and lupeol-loaded, respectively. It is remarkable that the load of the nutraceutical compounds significantly decreased the PdI, which may be beneficial for emulsion stability, because at smaller PdI, less possibility of having destabilization phenomena.

ZP values from OBEs were the determinant parameter for choosing the most stable condition. For most stable condition, ZP values for unloaded emulsions were around -19.0±0.391 mV; and for lupeol-loaded, 21.83±0.075 mV (Table 3).

The Herschel-Bulkley model can fit more or less all the flow curves independently on the tested material. Yield stress for the loaded OBEs (Table 3) did not show significant differences from unloaded OBEs. Also for apparent viscosity (Table 3), no significant differences (α ≥ 0.05) were found between the OBEs. Regarding activation energy (Table 3), loading of lupeol did not disturb values obtained for the emulsions compared with the control OBEs (α ≥ 0.05). All parameters remained constant (PS, PdI, ZP, yield stress, viscosity and activation energy), which implicated that lupeol did not alter by themselves the physicochemical properties of emulsions.

### 3.2. In vitro permeability.

Caco-2 monolayers can be used to predict drug transport through the epithelium. Papp is a measure of the ability of a compound to cross the epithelium barrier, and values <1x10^-6, 1-10x10^-6, and >10x10^-6 cm/s, are considered as poor (0-20%), moderate (20-70%) and well absorbed (70-100%) in humans, respectively [19]. For pure compounds values obtained were 5.72x10^-5±3.35x10^-6 cm/s for lupeol.

### Table 2. Particle size, polydispersity index, zeta – potential of unloaded organogel-based emulsions (OBEs).

| Lipid phase | Surfactant | Zeta potential (mV) | Polydispersity index (PDI) | Particle size (nm) |
|-------------|------------|---------------------|----------------------------|-------------------|
| 5%          | Tween 20   | -17±0.4             | .38±0.1                    | 210±5.8           |
| 10%         | Tween 20   | -16±0.3             | .37±0.1                    | 230±4.9           |
| 5%          | Tween 80   | -19±0.3             | .41±0.1                    | 154±3.3           |
| 10%         | Tween 80   | -18±0.4             | .41±0.1                    | 230±4.7           |

Data Shown are the mean ± sd

### Table 3. Particle size, polydispersity index, zeta – potential, viscosity, yield stress, activation energy, and apparent permeability of unloaded organogel-based emulsions (OBEs), and lupeol-loaded OBEs.

| Treatment                  | Particle size (nm) | Polydispersity index | Z-Potential (mV) | Viscosity (Pa.s) | Yield stress (Pa) | Activation Energy (Ea) (KJ/K) | Apparent permeability (Papp, cm/s) |
|----------------------------|--------------------|----------------------|------------------|------------------|-------------------|-------------------------------|-----------------------------------|
| Unloaded OBEs              | 154±3.3            | 0.41±0.01            | -19.0±0.4        | 7.92x10^-6 x 10^0 | 0.41±0.01         | 16.24±0.43                    | 4.80 x10^-8 ± 3.34 x10^-9         |
| OBEs loaded with lupeol    | 153.5±0.9          | 0.29±0.01            | -21.4±0.8        | 8.11 x10^-8 x 10^4 | 0.39±0.01         | 15.45±0.34                    |                                  |

Data shown mean ± standard deviation

### 4. CONCLUSIONS

In this study, organogel based emulsions were successfully obtained to protect lupeol and increase their bioaccessibility. Though it was possible to obtain the emulsion with long chain (canola) oils, particle size characteristics were better when using Tween 80 as surfactant. The condition with the highest physical stability (coconut organogel 5%, 10% Tween 80, and 85% deionized water) was loaded with lupeol, and showed no alterations in physical variables such as PS, PdI, ZP, or rheological parameters, suggesting that lupeol does not alter the stability of emulsions.

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