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# Exploring the biological properties and bioaccessibility of orange peel extracts using deep eutectic systems

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ABSTRACT

The ability to recover bio-compounds from by-products offers a significant chance for their use as bio-additives in the food industry. Carotenoids are known for their properties as antioxidants, antimicrobials, and food colorants. In the present study, the potential of hydrophobic natural deep eutectic systems (HDES) for the revalorization of orange peels was explored. Extracts were obtained, and their biological properties were evaluated *in vitro*, such as cytotoxicity, antiproliferative capacity, antioxidant capacity, and antimicrobial potential against *Staphylococcus aureus* and *Escherichia coli*. To support the potential application of orange peel – HDES extract as a functional ingredient or food additive, INFOGEST *in vitro* static digestion was performed. The results showed that Octanoic acid: Proline (C8:Pro) extract and Menthol: Eucalypthol (Me:Eu) had higher selective index meaning that these HDES have a higher selectivity towards human colorectal adenocarcinoma cell lines. Also, C8:Pro showed a higher carotenoid extraction yield extracting 168 μg/100 g of lutein with ORAC value of 34.8 mM TE. In general, just C8:Pro extract showed inhibition against both bacteria. Bioaccessibility of β-cryptoxanthin was enhanced in C8:Pro extract in 10.1%, indicating high stability during gastrointestinal digestion. These results reveal that orange peel – HDES are promising to be of use in industrial food applications.

## 1. Introduction

The waste and by-products generated by the agri-food sector have a significant impact on the environment (Satari & Karimi, 2018). Implementing circular economy models has the potential to mitigate this negative impact by converting trash and by-products into new raw materials that can be used in a variety of industrial applications such as food, cosmetology and pharmaceutical. For example, the citrus industry is one of the most exploited industries, where the production of orange waste may result in a very high amount of pollution (Murador et al., 2019). Orange peels (OP) are a source of valuable compounds such as vitamins, sugars, antioxidants, pectin and pigments (Gómez-Urios et al., 2022). Among the group of pigments present in orange peels, carotenoids are of great interest to the industry, due to their beneficial effects on health, as well as their coloring, antioxidant, and UV-protection properties that can extend the lifetime of food products (Luzardo-Ocampo et al., 2021; Mercadante et al., 2017).

Organic solvents are commonly used to extract bioactive chemicals from natural matrices, but in recent years the promising properties of Deep Eutectic Systems (DES) have generated interest in studying their potential use as a sustainable alternative to these hazardous solvents (Neuls, Espino, Fernandez, & Silva, 2022; Panić et al., 2019; Viñas-Ospino et al., 2023a). An important feature of DES, since they may be derived from natural resources, is that their components can exhibit a variety of biological properties, including antioxidant, antifungal, antibacterial, antiviral, and anticancer activity (Silva et al., 2021; Viñas-Ospino et al., 2023b). For example, organic acid-based DES have been reported to have antimicrobial and antioxidant activities (Radošević et al., 2018). Similarly, terpenoid-based DES, have been shown to have an antiproliferative effect against cancer cells and to inhibit the effect of reactive oxygen species (ROS) (Rodrigues et al., 2020; Silva et al., 2019). These bioactive properties may result from a

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synergistic effect between the solvent and the natural compound dissolved in it with promising applications (Mišan et al., 2019).

Many biofunctional compounds have a hydrophilic nature and research usually focuses on the use of hydrophilic DES for their extraction; however, numerous lipophilic moieties present in natural substrates (such as carotenoids and tocopherols) should be considered (Cao & Su, 2021). In this context, hydrophobic deep eutectic solvents (HDES) are targets to extract lipophilic compounds and, are formed by combining long-chain quaternary ammonium salts or terpenoids and a long-chain carboxylic acid (Sportiello et al., 2023).

Carotenoids are lipophilic and highly sensitive against environmental stresses, such as elevated temperature, exposure of oxygen and light (Boonlao et al., 2022). These significantly limit their absorption via the gastrointestinal system and solubility in aqueous-based meals, which lowers their bioavailability. In this sense, the search for strategies to stabilize and preserve their properties are of crucial importance. HDES have been shown to have very beneficial effects in terms of antioxidant capacity and improving the stability of the extracted compounds (Gómez-Urios et al., 2023; Pitacco et al., 2022; Van Osch et al., 2020; Viñas-Ospino et al., 2024). Evaluation of the influence of digestion on the bioactive content of OP-HDES extracts is crucial to support its possible use as a functional component or food product (Xia et al., 2023). A helpful method for assessing the bioaccessibility and digestibility of bioactive compounds in food is the *in vitro* simulation of gastrointestinal static digestion (SGD) (Cabezas-Terán et al., 2023).

Based on the above information, this study aims to evaluate the biological properties (cytotoxicity, antiproliferative, antibacterial and antioxidant capacities) of OP extracts obtained by HDES. In addition, an *in vitro* static digestion of the OP-HDES extracts was carried out to investigate their bioaccessibility, which is crucial information for comprehending their biological role. To the best of our knowledge, no evaluation has been done on the impact of OP extracts mediated by HDES in an *in vitro* digesting process. The results presented in this research show the potential of eutectic combinations to improve the biological properties and boost the bioaccessibility of OP extracts, highlighting the capacity of HDES towards increased extraction and stability of bio-compounds.

## 2. Materials and methods

### 2.1. Chemicals and reagents

HDES were prepared using menthol (Me) (purity>98%), dodecanoic acid (C12) (purity>98%), octanoic acid (C8) (purity>99%), L-proline (Pro) and eucalyptol (Eu) (purity>98%) purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol and acetonitrile analysis were purchased from J.T. Baker Chemical Co. Ultrapure water (18.2 MW cm) was used for preparing the mobile phase, and other aqueous solutions were obtained using a Milli-Q water purification system (Millipore, France). Trolox (±)-6-hydroxy-2,5,7,8-tetramethyl chromate-2-carboxylic acid), 2,20-azobis (2-amidinopropane) dihydrochloride (AAPH), and sodium fluorescein were purchased from Sigma-Aldrich (Steinheim, Germany). The standards β-carotene (purity 99.5%), lutein (purity 98.0%), zeaxanthin (purity 99.0%), β-cryptoxanthin (purity 99.0%), Red Nile and DCFH-DA (2′,7′-Dichlorodihydrofluorescein diacetate) were purchased from Sigma-Aldrich (Darmstadt, Germany Chemicals) and enzymes used for the *in vitro* static digestion were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

### 2.2. Sample preparation

Orange (*Citrus sinensis*) samples were obtained from a local agricultural cooperative (Carlet, Spain). The oranges were washed with distilled water and after juice extraction, the OP was processed in an IKA® Tube Mill grinder at 20000 rpm and stored at −20 °C in tightly closed packages until analysis.

### 2.3. Solvent preparation and characterization

For HDES preparation, each component was added in a specific molar ratio (Table 1) and heated at 50 °C, while stirring continuously to form the eutectic mixture, then allowed to cool at room temperature without stirring until use. The Red Nile method was used to characterize the polarity of each HDES and control solvent. According to the method of Jeong et al. (2017). Briefly, a specific $\lambda_{max}$ was obtained from the corresponding UV–Vis spectrum. The molar transition energy ($E_{NR}$) was calculated using the Equation (1). The solvents with higher polarity have the highest shifts of $\lambda_{max}$, i.e. lower $E_{NR}$ values.

$$E_{NR} = 28591/\ \lambda_{max} \qquad \text{Equation 1}$$

### 2.4. Extraction process

Extractions were carried out by combining 1 g of OP with 10 mL of HDES and stirring at 60 rpm. Preliminary tests were performed to determine the extraction conditions (Viñas-Ospino et al., 2023c). Then, extracts were stored at −20 °C in the absence of light until analysis. All experiments were performed in triplicate.

### 2.5. LC- MS/MS QTRAP carotenoid quantification

The acetone and HDES extracts were analyzed in their initial condition and after *in vitro* static digestion (micellar phase). All the samples were chromatographically separated, identified, and quantified following the method from Rivera et al. (2014) using a LC-MS/MS QTRAP 6500 + 8 (AB SCIEX, USA). Separation was carried out on a $C_{18}$ column (1.6 μm, 100 × 2.1 mm) (Phenomenex, USA). A flow rate of 0.3 mL $min^{-1}$ was used, with the following elution binary gradient: 0–10 min, isocratic 15% solvent A: water/formic acid, 99.9/0.1, (v/v), 85% solvent B: methanol/acetonitrile 30/70 (v/v); 10–17 min, 100% B; 17–24 min isocratic 85% B. A. The injection volume was 5 μL. Data was acquired in positive mode, over a mass/charge range of 100–950 m/z, using the following instrument settings: Ion source gas 1, 55 psi and ion source gas 2, 55 psi; curtain gas 1, 30 psi; 550 °C; ion spray voltage, 5500 V. An external calibrant delivery system, which injects a calibration solution before the entry of the sample, was used to carry out automated calibration. Carotenoids were individually quantified using four-point analytical curves ($r^2 = 0.99$). The procedure for carotenoid quantification was assessed based on the validation parameters presented in Table S1. Analyzed samples were diluted in mobile phase B and the results were expressed as μg/100 g of fresh weight.

### 2.6. Oxygen Radical Absorbance Capacity Assay (ORAC)

ORAC was determined using the method described by Zulueta et al. (2009). It was conducted using a Wallac 1420 VICTOR2 multilabel counter equipment (PerkinElmer, USA) equipped with fluorescence filters. The excitation wavelength used was 485 nm and the emission wavelength was 535 nm. The assay was performed in 96-plates (Thermofisher®). The reaction was carried out at 37 °C, in 75 mM phosphate buffer (pH 7.0) The fluorescence measurement was carried out after the addition of the reactants and the measurements were carried out in 5-min cycles until the relative fluorescence intensity decreased by more

**Table 1**
Used solvents, ratios, and polarities.

| Solvent | Abbreviation | Molar ratio | $E_{NR}$(kcal/mol) |
|---|---|---|---|
| Acetone | | – | 53.2 |
| DL-Menthol: Eucalyptol | Me: Eu | 1:1 | 53.2 |
| Octanoic acid: L-Proline | C8:Pro | 4: 1 | 51.8 |
| Dodecanoic Acid: Octanoic Acid | C12:C8 | 1:3 | 52.5 |

than 95% of the initial value. ORAC values expressed in mM Trolox equivalents (mM TE) were calculated by applying Equation (2):

$$\text{ORAC (mM TE)} = \left(\frac{C_{TRX}\ x\ AUC_S - AUC_B}{AUC_{TRX} - AUC_B}\right) x\,k \qquad \text{Equation 2}$$

Where $C_{TRX}$ is the Trolox concentration (mM), k is the sample dilution factor and AUC is the area under the fluorescence decay curve from Sample, Blank, and Trolox calculated by applying Equation (3):

$$\text{AUC} = 0.5 + \left(\frac{f5}{f0} + \frac{f10}{f0} + \ldots + fn + \frac{5}{f0}\right) x\,5 \qquad \text{Equation 3}$$

### 2.7. Cell culture

Two immortalized human colorectal adenocarcinoma cell lines, HT29 and Caco-2, were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). HT29 cells were maintained in Gibco Roswell Park Memorial Institute (RPMI, Corning, USA) 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Corning, USA) and 1% (v/v) penicillin-streptomycin (PS, Corning, USA) for cell maintenance. The Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning, USA) supplemented with red phenol, 10% FBS, and 1% phenol. The cells were grown as a monolayer in 75 $cm^2$ Falcon culture flasks (Corning, USA) as a monolayer in a humidified atmosphere until they reached 80%–90% optical confluence.

#### 2.7.1. Cytotoxicity assessment

To evaluate the cytotoxicity of pure HDES and OP extracts, Caco-2 cells were seeded into 96-well Falcon plates (Corning, USA) at a density of 2 x$10^5$ cells per mL. The cells were allowed to develop for 7 days, and then the media was replaced every 48 h. On day 7, cells were treated with either pure subculture medium (controls) or various concentrations of the samples diluted in the subculture medium. Cell viability was assessed using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (CellTiter 96® AQueous One Solution Cell Proliferation Assay, PROMG3581, Promega, USA) at a 1:10 dilution in assay culture medium (DMEM + 0.5% FBS). After 24 h, the cells were washed twice with PBS (Phosphate buffer saline) (Sigma-Aldrich, USA) pH 7.4. Cell viability was determined at 3 h using a microplate reader (HH35L2019044, Victor Nivo 3S, PerkinElmer, USA). At least three sets of triple replicates were performed. The effective concentration values ($EC_{50}$), or the concentration at which 50% of cell viability is reduced, were then determined.

#### 2.7.2. Antiproliferative assessment

The antiproliferative activity of the pure HDES and OP extracts was evaluated in HT29 cells using the same methods and concentrations as in the cytotoxicity assay. The cells were at a density of 1x$10^5$ cells/mL plated in 96-well culture plates (Corning, USA). After 24 h of culture, cells were exposed to different concentrations of the samples diluted in the subculture medium, as well as to the subculture medium (controls). A 1:10 dilution of MTS (16%) in assay culture medium (RPMI + 0.5% FBS) was added to each well after the cells were incubated for 24 h. The cells were then washed twice with PBS and cell viability was assessed after 3 h using a microplate reader (HH35L2019044, Victor Nivo 3S, PerkinElmer, Massachusetts, USA). At least three triple replicates were performed. The results are presented as a percentage of viable cells compared to the control. To calculate the $EC_{50}$, a dose-response curve was fitted.

#### 2.7.3. Intracellular ROS inhibition

To evaluate the impact of OP extracts and pure HDES on the production of ROS, HT29 cells were seeded at a density of 1.52x$10^5$ cells/mL in a 24-well plate. After 24 h, the cells were exposed to OP extracts and HDES diluted in a subculture medium, or just a subculture medium (control), for 1 h. Then, the culture medium was removed, and the cells were washed twice with PBS. To quantify the presence of ROS, 600 μL of 10 μM DCFH-DA was added to each well and incubated for 1 h. Fluorescence was measured using a microplate reader (HH35L2019044, Victor Nivo 3S, PerkinElmer, USA) with an excitation wavelength of 480 nm and an emission wavelength of 530 nm. At least three replicates were performed in triplicate.

### 2.8. Antibacterial activity

To independently evaluate the antimicrobial capacity of the OP extracts and pure HDES, the agar plate diffusion assay was performed using the reference strains *Escherichia coli* K12 DSM498 (DSMZ, Braunschweig, Germany) and *Staphylococcus aureus* COL MRSA, methicillin-resistant strain (Rockefeller University, USA), as representatives of Gram-negative and Gram-positive species, respectively. The bacterial strains were cultured in Lysogeny broth (LB) medium at 37 °C overnight. The assay was performed using LB solidified with 1.5 % (w/v) agar and Petri dishes were prepared with 20 mL of medium and each plate was inoculated with $10^5$ CFU (colony forming units)/mL of the appropriate bacterial strain and 6 mm of diameter discs (1469 FILTER-LAB, Spain) were placed on at the surface of the plates. Each disc was impregnated with 10 μL of the material to be tested. The plates were then incubated at room temperature for 4 h, followed by an overnight incubation at 37 °C. Positive and negative controls were also tested, the corresponding antibiotics vancomycin (5 μg), oxacillin (1 μg) and ampicillin (25 μg), as well as an empty disc (Table S2). The experiments were performed in duplicate with different microbial cultures and four samples were analyzed for each condition. Images of the plates were taken, and the diameters of the inhibition halos were measured using ImageJ software (version 1.46r, Wayne Rasband, NIH, USA). Each halo was measured 30 times in different orientations.

### 2.9. In vitro static digestion

The bioaccessibility was analyzed by an *in vitro* simulated static digestion model, following the protocol adapted from INFOGEST (Brodkorb et al., 2019). First, an emulsion was prepared by mixing 18% (w/w) of the extracts, 2% (w/w) Tween 80 as a surfactant, and 80% (w/w) distilled water with a Polytron PT 2500 (Fisher Scientific, Spain) hand-held homogenizer operating at 10000 rpm for 5 min, as adapted from Murador et al. (2021). It is crucial to consider that the application of the extracts in an emulsion for the formation of the mixed micelles and, consequently, for the achievement of the results presented. For this reason, before subjecting the extracts to the *in vitro* static digestion, an emulsion was prepared, this was considered because fats and oils affect the bioaccessibility and bioavailability of dietary lipophilic molecules, such as carotenoids (Murador et al., 2021; Saini et al., 2021). The enhancement of bile salt secretion is associated with the availability of lipids, which facilitate the formation of micelles and provide a hydrophobic environment for the released carotenoids (Priyadarshani, 2017).

The INFOGEST protocol criteria was followed for the study of all enzymatic activities and bile salt concentration, with the exception of cholesterol esterase, whose analysis is not covered by the INFOGEST. For the oral phase, 5 mL of SSF (simulated salivary fluid) were mixed and shaken for 1 min. Then, 0.5 mL of α-amylase solution (75 U/mL), 25 μL of 0.3 M calcium chloride and 975 μL of ultrapure water were added to obtain a final volume of 10 mL. The mixture was placed in a shaker bath for 2 min at 37 °C and 95 rpm. To simulate the gastric phase, 7.5 mL SGF (simulated gastric fluid) and 5 μL of 0.3 M calcium chloride were added and mixed, subsequently, 0.5 mL of RGE (rabbit gastric extract) solution was added. RGE provided the pepsin activity, and it was completed with 0.5 mL of pepsin solution to reach a combined activity of 2000 U/mL. The pH of the mixture was adjusted to 3 and ultrapure water was added up to a volume of 20 mL. The mixture was placed in the shaker bath at 37 °C with agitation for 2 h. Finally, to simulate the

intestinal phase, 8.5 mL of SIF (simulated intestinal fluid), 5 mL of pancreatin solution (100 U/mL), 40 μL of 0.3 M calcium chloride, 2.5 mL of bile extract solution (10 mM) and 0.1 mL of cholesterol esterase (1 U/mL) were added. The final mixture was mixed manually for 1 min and adjusted to pH 7, and then ultrapure water was added to a final volume of 40 mL. Subsequently, it was incubated in a shaking bath at 37 °C with agitation for 2 h. Wen et al. (2018) strongly recommended the use of cholesterol esterase to improve the hydrolysis of carotenoid esters in the intestinal phase, in the case of a sample rich in esters as OP.

The aqueous phase containing mixed micelles (supernatant) was separated from the solid portion after centrifugation at 8000×*g* at 4 °C for 60 min and filtered through a 0.22 μm filter, concluding the formation of mixed micelles (Murador et al., 2021). Then carotenoids were exhaustively extracted with diethyl ether (20 mL per extraction) by vortex for 1 min. Ten milliliters of 10% NaCl were added, and the mixture was centrifuged (8000×*g*, 5 min, 4 °C). Organic and aqueous phases were separated until the extract became colorless. The combined organic phase was dried under nitrogen flux and stored in the dark until carotenoid analysis. At least three replicates were performed for each sample and blank. The conventional step of saponification was not applied to enable the analysis of the carotenoid esters present in the extract.

### 2.10. Bioaccessibility calculation

Bioaccessibility was calculated as the ratio between the tested compounds in the digested fraction (after the *in vitro* static digestion) to the original non-digested sample applying Equation (4).

$$\text{Bioaccessibility (\%)} = \frac{Digested}{Non-digested} \text{ x } 100 \qquad \text{Equation 4}$$

Where, digested: carotenoids concentration or antioxidant capacity in the digested fraction; non-digested: carotenoids concentration or antioxidant capacity in the non-digested sample.

### 2.11. Statistical analyses

GraphPad Prism 8.0 (GraphPad Software, USA) was used for the statistical analysis. All assays were determined in three independent experiments. Normality and homoscedasticity were evaluated using Shapiro–Wilk and Levene tests, respectively. Significant differences between samples and control were calculated, and data are expressed as mean ± standard deviation (SD). Statistical significance was attributed to $p$ values below 0.05. One-Way ANOVA was used to compare the data, followed by the Tukey multiple comparison test.

## 3. Results and discussion

### 3.1. Solvents preparation and characterization

In the present study three HDES were used: C8:Pro (4:1), C12:C8 (1:3), and Me:Eu (1:1). These HDES were selected based on previous results (Viñas-Ospino et al., 2023c, 2024). For all subsequent analyses, traditional extraction with acetone was used as a control. The molar ratios and polarities for each HDES, are shown in Table 1. The measurement of polarity was performed as it is expected to affect the extraction of target compounds from a solid matrix (Neuls, Espino, Fernandez, & Silva, 2022).. The solvents exhibited a high degree of non-polarity, with polarities comparable to acetone, except for C8:Pro, which has an $E_{NC}$ value of 51.8 kcal/mol, indicating a higher polarity. These polarities are consistent with those reported for HDES in previous studies (Rebocho et al., 2022; Rodrigues et al., 2020). C8:Pro is slightly more polar than other HDES. This can help in the extraction of polar compounds such as xanthophylls, which belong to the group of polar carotenoids.

### 3.2. Carotenoid content and antioxidant activity

The amount of carotenoids extracted with acetone and with all of the HDES is shown in Table 2. The most abundant carotenoids detected in OP extracts were xanthophylls, which is consistent with the results reported in the literature for orange fruit (El Kantar et al., 2018; Murador et al., 2019). HDES were significantly more efficient ($p < 0.05$) in extracting lutein (168.2 ± 1.2 μg/100 g by C8:Pro) than acetone (96.8 ± 0.5 μg/100 g) and zeaxanthin (40.5 ± 0.6 μg/100 g by Me: Eu) compared to acetone (16.3 ± 0.0 μg/100 g). However, for β-carotene and β-cryptoxanthin, the higher extraction yield was obtained using acetone (37.8 ± 0.3 μg/100g, 40.5 ± 0.5 μg/100 g, respectively). Xanthophylls, such as lutein, which have polar ionone rings, can interact better with carboxyl groups of fatty acids. As a result, fatty acid-based HDES can extract these compounds better than menthol-based HDES (Stupar et al., 2021). No results on OP using HDES have been found in the literature for carotenoid profile. However, similar results have been previously reported before using fatty acid based HDES for carotenoid extraction in tomatoes, specifically octanoic and decanoic acids (Kyriakoudi et al., 2022). Also, Pitacco et al. (2021), demonstrated a high carotenoid extraction of carotenoids from *Haematoccocus pluvialis* using mixture of fatty acids and DL-menthol.

Carotenoids are highly valuable antioxidants, and they are widely used in the production of food, pharmaceuticals, and cosmetics (Yu et al., 2022). This is due the antioxidant activity of the prepared extracts was determined by the ORAC method was also evaluated. The ORAC values are shown in Table 2 and ranged from 26.6 mM TE for the acetone extract to 34.2 mM TE for the C8:Pro extract. It is worth noting that pure HDES have antioxidant activity on their own because their constituents (proline, menthol, fatty acids) also have antioxidant activity (Mitar et al., 2019; Viñas-Ospino et al., 2023a). Moreover, the antioxidant activity levels of the C8:Pro extract was the highest when compared to the acetone extract. The results obtained are consistent with the results obtained for carotenoids, where the extract with the highest concentration of carotenoids was also C8:Pro. In addition, as mentioned above, this HDES extracted more carotenoids from the group of xanthophylls, which are known for their higher antioxidant activity compared to carotenes (Jiao et al., 2019). Considering the promising results of HDES

**Table 2**
Initial carotenoid and antioxidant activity (ORAC) in the extracts obtained from acetone and OP-HDES extracts, bioaccessible amounts obtained in the micellar fraction after *in vitro* static digestion.

| | | Acetone | C8:Pro | C12:C8 | Me:Eu |
|---|---|---|---|---|---|
| Lutein (μg/100 g) | **Extract** | 96.8 ± 0.5$^{b}$ | 168.2 ± 1.2$^{a}$ | 87.4 ± 0.7$^{c}$ | 98.8 ± 2.7$^{b}$ |
| | **BF** | 4.0 ± 1.3$^{B}$ | 6.1 ± 0.7$^{A}$ | 3.3 ± 0.2$^{B}$ | 3.4 ± 1.6$^{B}$ |
| β-carotene (μg/100 g) | **Extract** | 37.8 ± 0.3$^{a}$ | 25.9 ± 0.1$^{b}$ | 19.0 ± 0.1$^{d}$ | 21.4 ± 0.18$^{c}$ |
| | **BF** | 1.7 ± 0.0$^{A}$ | 1.7 ± 0.0$^{A}$ | 1.1 ± 0.7$^{B}$ | 1.6 ± 0.0$^{A}$ |
| Zeaxanthin (μg/100 g) | **Extract** | 16.3 ± 0.0$^{d}$ | 33.4 ± 0.1$^{c}$ | 35.2 ± 0.0$^{b}$ | 40.5 ± 0.6$^{a}$ |
| | **BF** | 1.8 ± 0.0$^{B}$ | 2.6 ± 0.7$^{A}$ | 2.1 ± 0.0$^{A}$ | 1.9 ± 0.1$^{B}$ |
| β-cryptoxanthin (μg/100 g) | **Extract** | 40.5 ± 0.4$^{a}$ | 29.6 ± 0.1$^{d}$ | 33.4 ± 0.1$^{c}$ | 36.1 ± 0.1$^{b}$ |
| | **BF** | 2.0 ± 0.7$^{C}$ | 3.0 ± 0.7$^{A}$ | 2.4 ± 0.0$^{B}$ | 2.5 ± 0.0$^{B}$ |
| ORAC (mM TE) | **Extract** | 26.6 ± 0.5$^{c}$ | 34.2 ± 0.4$^{a}$ | 28.4 ± 0.8$^{b}$ | 29.5 ± 1.5$^{b}$ |
| | **BF** | 6.5 ± 0.3$^{B}$ | 8.2 ± 0.6$^{A}$ | 3.5 ± 0.1$^{D}$ | 4.0 ± 0.2$^{C}$ |

C8:Pro (Octanoic acid: L-Proline), Me: Eu (Menthol: Eucalyptol), C12:C8 (Dodecanoic acid: Octanoic acid), BF: Bioaccessible Fraction. Different lowercase letters (a–d, A-D) indicate statistically significant differences ($p < 0.05$) between solvents.

for the extraction of carotenoids from OP, this suggests the possibility of its further application in the food industry. However, it is important to ensure the non-toxicity and biological properties, which will be discussed in the following sections.

### 3.3. Cytotoxicity and antiproliferative effect

The OP extracts and pure HDES were evaluated *in vitro* considering that HDES possess particular biological properties that may contribute to the extract's biological activity (Panić et al., 2019). In addition, acetone extracts were also evaluated as it was used as a reference solvent for carotenoid extraction. Two different approaches for MTS cell viability assay were used in this study. Caco-2 cells were used to study the cytotoxicity of the OP extracts and pure HDES on the viability of healthy intestinal cells. These cells are regarded as a confluent monolayer in the stationary phase of the cell growth curve, which indicates the safety/toxicity of the extracts. In the antiproliferative assay, HT29 cells were used to evaluate the extracts on cell proliferation considering cells in the log phase and to demonstrate the cytotoxicity of the extracts against cancer cells. Table 3 shows the $EC_{50}$ values for the OP extract and pure HDES for cytotoxicity and antiproliferative activity. Table 3 also shows the Selective Index (SI), which reflects the potential of a treatment to inhibit a specific target without affecting the viability of normal cells (Pereira et al., 2022).

The SI of C8:Pro extract and Me:Eu extract was the highest, meaning that these HDES have a higher selectivity towards cancer cells. C12:C8 extract also shows antiproliferative effects, however, it also exhibits cytotoxicity against the Caco-2 cell line. This indicates that their antiproliferative impact can cause cell death in normal cells without being specific to cancer cells. Cytotoxicity has previously been linked to DES viscosity, concentration, pH, composition, and the interaction of each compound with the numerous functional groups present on the cell surface, as well as the needs of the cell line (Pereira et al., 2022). These results are consistent with those reported previously, where menthol-based HDES had demonstrated antiproliferative effects without affecting normal cells, such as Menthol: Perillyl Alcohol (1:1), Menthol: Eucalyptol (1:1) and Menthol: Myristic Acid (8:1) (Cao & Su, 2021).

To assess whether the antiproliferative and cytotoxic effects of OP extract are related to the HDES or to the extracted compounds, the assays were also performed in pure HDES. As can be observed, the SI increased in the case of Me:Eu extract and C8:Pro extract compared to the pure HDES, which means that the presence of OP extract has a positive effect on inhibiting tumor cells while not affecting normal cells. For example, cryptoxanthin, which was one of the most extracted carotenoids in the previous section, has been previously reported as an anti-tumor promoter due to its antioxidant ability to protect against oxidative stress (Jiao et al., 2019). Finally, looking at the overall results on tumor HT29 cells and normal Caco-2 cells, it can be concluded that OP extracts obtained by HDES may have potential anticancer activity, since they can inhibit the growth of tumor cells and have no effect on healthy cells.

**Table 3**
$EC_{50}$ values for the cytotoxicity and antiproliferative assays, and corresponding selectivity indexes for pure HDES, acetone extract and OP-HDES extracts.

| HDES/ Extract | $EC_{50}$ (μL/mL) | | Selective index |
|---|---|---|---|
| | Cytotoxicity (Caco-2) | Antiproliferative (HT29) | |
| C8:Pro | $19.8 \pm 0.6^{e}$ | $20.0 \pm 2.0^{b}$ | 0.99 |
| Me:Eu | $21.7 \pm 1.2^{d}$ | $20.6 \pm 1.7^{b}$ | 1.05 |
| C12:C8 | $21.3 \pm 0.6^{d}$ | $18.3 \pm 0.4^{c}$ | 1.16 |
| C8:Pro Ext | $27.0 \pm 1.2^{a}$ | $18.1 \pm 0.6^{c}$ | 1.49 |
| Me:Eu Ext | $23.9 \pm 0.8^{b}$ | $19.5 \pm 1.3^{b}$ | 1.22 |
| C12:C8 Ext | $22.1 \pm 0.9^{c}$ | $19.8 \pm 0.4^{b}$ | 1.12 |
| Acetone Ext | $22.9 \pm 0.7^{c}$ | $24.0 \pm 0.4^{a}$ | 0.95 |

Results were obtained from three independent experiments performed in triplicate. Data indicated as mean ± SD. Presented values followed by different lower-case letters (a-e) are significantly different ($p < 0.05$) as measured by Tukey's HSD test. HDES (Hydrophobic deep eutectic solvents), C8:Pro (Octanoic acid: L-Proline), Me: Eu (Menthol: Eucalyptol), C12:C8 (Dodecanoic acid: octanoic acid).

### 3.4. Intracellular ROS inhibition

The evaluation of intracellular ROS was performed in HT29 due to their higher stress levels. Cells were treated with OP extracts and pure HDES at concentration that were non-toxic for HT29 as shown in section 3.3. The results for the % inhibition of ROS is shown in Fig. 1. In Fig. 1a the results for C8:Pro are presented, when the concentration of ROS increases, it indicates that this mechanism plays a role in lowering intracellular ROS generation. There is also a significant difference ($p < 0.05$) between the extract and pure HDES indicating the effect of OP in the ROS inhibition. When 25 μL/mL is applied there is an inhibition of 88% of ROS, suggesting a possible contribution as anti-inflammatory. In Fig. 1b the results for C12:C8 are presented, as can be observed there was a significant difference ($p < 0.05$) between pure HDES and C12:C8 extract only when it was applied at a concentration of 15 μL/mL, and the inhibition was only 26% of ROS. Finally, in Fig. 1c are the results for Me: Eu, and there was a significant difference ($p < 0.05$) between pure solvent and OP extract when the concentrations were 15 μL/mL and 20 μL/mL. The test was also performed in the acetone extracts (Fig. 1d), and comparing the four extracts, it was observed that the HDES extracts have a higher protection against oxidative stress than the acetone extract. It has been previously reported that carotenoids can prevent inflammation and reduce oxidative stress (Jiao et al., 2019; Tiwari et al., 2022). Specifically, β-cryptoxanthin has been associated with the prevention of inflammation, the likely mechanism could be the reduction of oxidative stress by regulating the expression of genes involved in the production of proinflammatory cytokines such as TNF, IL-1, and IL-6 (Jiao et al., 2019). These results are consistent with those presented in previous sections, where C8:Pro also reported higher extraction yields of xanthophylls, especially zeaxanthin, and lutein, and high antioxidant activity, which is related to the anti-inflammatory potential.

### 3.5. Antibacterial effect

To evaluate the antimicrobial capabilities of the OP extracts and pure HDES formulations, the disc diffusion assay was performed against *E. coli* and *S. aureus* which are significant for assessing the potential of preservatives in pharmaceutical, nutraceutical, or cosmetic/personal care stuff (Rodrigues et al., 2020). The corresponding antibiotics used as controls are listed in Table S2, the results obtained are shown in Fig. 2, and representative images of the plates obtained are shown in Table S3. The inhibition halo measurements indicated that only the C8:Pro, both the pure HDES and the OP extract, exhibited antibacterial activity against both microbes tested. There was also a significant difference ($p < 0.05$) between pure HDES and OP extract can be observed in all the cases, demonstrating the antibacterial effect of OP. The acetone extract was also tested, and no inhibitory activity was found. In Fig. 2a, it can be observed that the C8:Pro extract was the only one to show inhibition against *E.coli.* In general, Gram-negative bacteria are more resistant to the effects of hydrophobic substances such as essential oils and their components, due to their complex outer membrane structure composed of lipopolysaccharides, proteins, and phospholipids. Wikene et al. (2017) reported that DES studied in Gram-negative bacteria can dissolve or interact with other membrane components, including divalent cations that are chelated in the membrane. In addition, the C8:Pro extract showed higher extraction yields of carotenoids, which have previously been reported as antimicrobial and preservative agents (Rodrigues et al., 2020).

The inhibition against *S. aureus* in Fig. 2b shows that all the HDES extracts exhibit antibacterial activity. In this context, the antibacterial

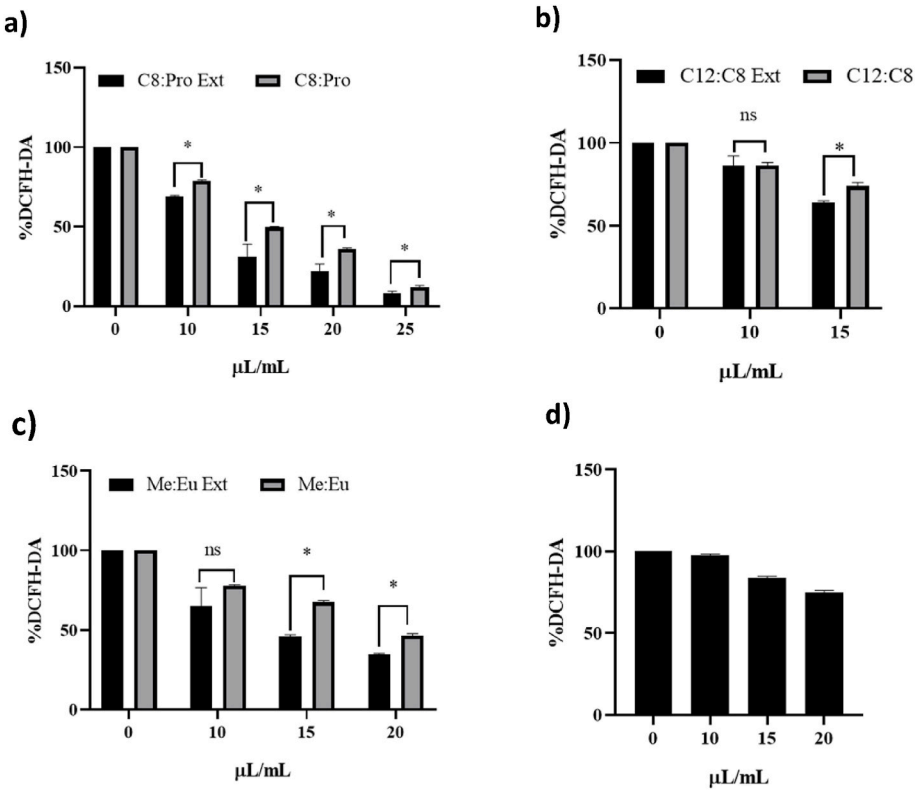


**Fig. 1.** Intra ROS results obtained for the C8:Pro Extract and pure HDES a), C12:C8 Extract and pure HDES b), Me:Eu Extract and pure HDES c), Acetone Extract d). Results were obtained from three independent experiments performed in triplicate. Data indicated as mean ± SD. *$p < 0.05$, as the statistical significance compared between orange peel extract and pure HDES. ROS (Reactive oxygen species), HDES (Hydrophobic deep eutectic solvents), DCFH-DA (2′,7′-Dichlorodihydrofluorescein diacetate), C8:Pro (Octanoic acid: L-Proline), Me:Eu (Menthol: Eucalyptol), C12:C8 (Dodecanoic acid: Octanoic acid).

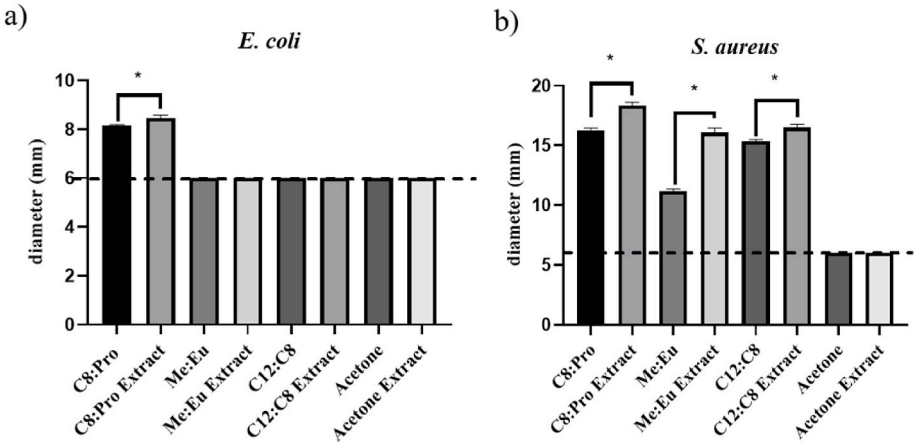


**Fig. 2.** Measurements of inhibition halo diameter of all samples against a) Gram-negative (*E. coli*), b) Gram-positive (*S. aureus*) bacteria. The dashed line indicates the diameter of the disks. Data indicated as mean ± SD. *$p < 0.05$, as the statistical significance compared between OP Extract and pure HDES. OP (Orange peel), HDES (Hydrophobic deep eutectic solvents), C8:Pro (Octanoic acid: L-Proline), Me: Eu (Menthol: Eucalypthol), C12:C8 (Dodecanoic acid: Octanoic acid).

activity of the studied HDES is most likely due to the H-bonding or electrostatic attraction of the DES components with the polysaccharide or peptide chains of the peptidoglycans. The antibacterial potential of HDES has already been reported by other authors (Rodrigues et al., 2020; Silva et al., 2021). Terpenes, such as menthol, have been characterized as antibacterial, antifungal, and antiviral (Silva et al., 2021). Terpenes and fatty acids are known to have antibacterial effects on both Gram-positive and Gram-negative bacteria. For this reason, they are already used in the cosmetics sector as natural preservatives and additives. This inhibition can also be explained by pH changes, the value of which decreases with the elongation of the organic acid carbon chain (Silva et al., 2021). It would be desirable to prevent extract-solvent separation to acquire synergistic bioactivity, according to the behavior demonstrated by HDES and the carotenoid-rich extracts obtained.

### 3.6. *In vitro* bioaccessibility

An *in vitro* static digestion assay was performed to investigate the bioaccessibility of OP-HDES extracts through the determination of not saponified carotenoids (β-carotene, β-cryptoxanthin, zeaxanthin, lutein)

and antioxidant capacity (by ORAC method). This study focused on native carotenoids extracted from orange peel using acetone and HDES. Table 2 shows the initial content of carotenoids and ORAC, and the amounts after the *in vitro* static digestion. Based on these contents, the bioaccessibility of carotenoids and antioxidant capacity was calculated and shown in Fig. 3. As can be seen, carotenoids in the HDES extracts have similar or statistically significant ($p < 0.05$) higher bioaccessibility than acetone-extracted carotenoids. Zeaxanthin has a bioaccessibility of 11.0% in the acetone extract, which was statistically significant higher ($p < 0.05$) when compared to the HDES extracts. Lutein also demonstrated a statistically significant increased bioaccessibility ($p < 0.05$) in acetone (4.2%) compared to the other HDES. Then, the bioaccessibility of β-carotene was significantly ($p < 0.05$) improved in Me:Eu (7.6%) extract. The bioaccessibility of β-cryptoxanthin was improved just in the C8:Pro (10.1%). Other authors have reported that the bioaccessibility of bioactive compounds can be enhanced by DES. In the case of Neuls, Espino, Fernandez, & Silva, 2022, who reported that 43% of the anthocyanins from olive pomace remained available with lactic acid: glucose. Although the literature has shown that more hydrophobic carotenoids, such as carotenes, are generally less bioaccessible than those that are more polar, such as free xanthophylls (Murador et al., 2021) and this could explain the results of the present study, where zeaxanthin and β-cryptoxanthin showed higher bioaccessibility. It is well known that the food matrix affects the bioaccessibility of carotenoids and one of the main factors that can affect the absorption of carotenoids in the human gut is the higher amounts of fibers that can be present in oranges (Priyadarshani, 2017). To avoid this effect, in the present research was evaluated carotenoids extracted using DES, not in a food matrix, therefore the impact of the fiber content should not be considered.

Fig. 3 also shows the bioaccessibility (%) of antioxidant capacity by ORAC method, where there was no significant difference ($p < 0.05$) between the acetone (24.3%) and the C8:Pro (24%) extracts. The bioaccessibility of antioxidant activity using HDES has not been reported previously. However, the results presented are consistent with other studies using DES for the extraction of other bioactive compounds, where DES was shown to improve the stability and bioaccessibility of polyphenols during *in vitro* static digestion and, consequently, the antioxidant capacity (Popović et al., 2022; Xia et al., 2023; Zannou et al., 2022). The interaction between HDES and carotenoids is likely to be responsible for their improved bioaccessibility compared to acetone extract. According to Kivelä et al. (2022) heating conditions may cause certain hydrogen bonds to break and the structure of HDES to expand, increasing the percentage of available binding sites. The internal structure may reform, and the viscosity may increase to incorporate carotenoids into the complex at a relatively low digestion temperature (37 °C). This allows the carotenoid to be fixed to protect it from oxidative destruction and the gastrointestinal environment. Strong hydrogen bonding interactions are typically formed between active substances and deep eutectic solvents (Brahmi-Chendouh et al., 2022).

To the best of our knowledge, our work is the first report to investigate the *in vitro* bioaccessibility of carotenoids extracted by HDES. The information presented here validates and clarifies the potential of HDES to enhance the bioactivity and bioaccessibility of by-product extracts.

## 4. Conclusion

By-products provide an enormous potential for innovative food bioadditives as they are considered to be an important source of high-value-added compounds. HDES represent an attractive option to replace organic solvents in the extraction of biomolecules due to their non-toxicity. In the present work, the potential use of HDES for OP revalorization was investigated, and the results support that HDES are potential solvents for obtaining bioactive compounds, such as carotenoids, from various plant sources or food wastes. Furthermore, the observed antiproliferative, antioxidant, and antibacterial activities of HDES-OP extracts, and the fact that OP already contains active compounds, highlight the great potential for industrial application of the extracts obtained by HDES. In contrast to the majority of the studies on the bioaccessibility of carotenoids, the present study analyzed orange peel extracts obtained using HDES. Therefore, HDES are not only greener and more efficient extraction solvents, but also capable of increasing the bioaccessibility of natural active ingredients. Our results support the potential use of OP for health and nutrition applications. Further research before their use in food products, such as *in vivo* studies, is essential to ensure that these extracts can be used in the food and pharmaceutical industries.

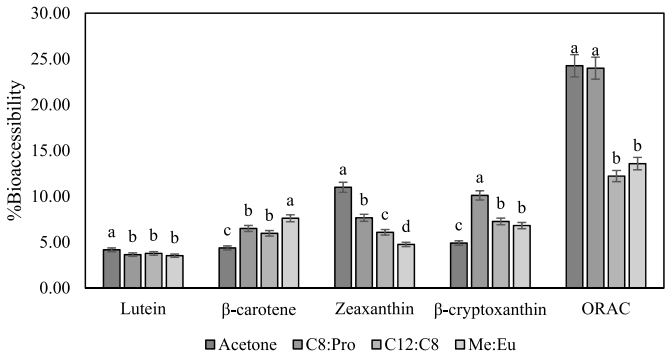


**Fig. 3.** Bioaccessibility (%) of carotenoids and antioxidant activity (ORAC) for each extract. Results are expressed as mean ± standard deviation. Different lowercase letters (a–d) indicate statistically significant differences between the extracts ($p < 0.05$).

## CRediT authorship contribution statement

**Adriana Viñas-Ospino:** Writing – original draft, Investigation, Formal analysis. **Isabel Sá-Nogueira:** Supervision, Methodology. **Ana Rita Duarte:** Supervision, Investigation. **Daniel López-Malo:** Writing – review & editing, Methodology, Data curation. **María José Esteve:** Supervision, Project administration, Funding acquisition. **Ana Frígola:** Validation, Conceptualization. **Jesús Blesa:** Writing – review & editing, Supervision, Investigation. **Alexandre Paiva:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.104684.

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