SYNERGIC EFFECT OF ENZYME ASSISTED EXTRACTION AND NATURAL DEEP EUTECTIC SOLVENTS FOR BIOACTIVES RECOVERY IN ORANGE PEEL WASTE

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Running title: Bioactives recovery in orange peel by enzymes and NADES

Highlights:

- extraction of bioactive compounds is more influenced by the type of solvent than by the enzymatic treatment
- the pretreatment with 3% pectinase followed by extraction with LP:MA proved to be the most promising experimental condition
- supplementation of the3 % pectinase LP:MA extract with intestinal cells resulted in the counteraction of the cytotoxic effects of the solvent at sublethal concentrations

Abstract

Citrus wastes are a source of valuable compounds that have potential application in food industries. Despite the several methods employed for bioactive recovery, most of them are detrimental for both human health and the environment. Recently, the use of "green" extraction methods with natural deep eutectic solvents (NaDES) represent a promising ecofriendly approach. Moreover, the employment of enzymes could facilitate the breakdown of food matrix-bioactive interactions and enhance biomolecules extraction. The present study aimed to investigate the potential synergistic application of combined enzyme assisted extraction (EAE) and NaDES formulation on polyphenols and proteins/peptides recovery from orange peel, in comparison with conventional extraction methods. Hesperidin appeared to be the most abundant polyphenol, and its extraction seemed higher with malic acid containing NaDES, regardless the EAE used. In terms of antioxidant capacity, the ABTS assay had the highest capacity with both aqueous and ethanolic extraction, while the DPPH method revealed the highest values with L-proline:malic acid (LP:MA) pre-treated with cellulase, 3% pectinase and 0.75% protease. Moreover, the level of protein hydrolysis and peptide content was generally higher in malic acid:glucose containing NaDES. The extract obtained from the most promising condition in term of bioactives extracted and antioxidant activities, identified as 3% pectinase followed by LP:MA, show a cytoprotective effect at 10 μL/mL against the vehicle LP:MA by using in vitro an intestinal cell model. In conclusion, EAE and NaDES represent a combined effective and sustainable method of recovering bioactives from citrus waste, as opposed to the conventional extraction methods.

1. INTRODUCTION

Approximately 90 million tons of food waste are generated per year within the European Union, constituting a substantial challenge and a matter of concern for the sustainability of the food chain [1]. In this line, a substantial amount of waste and by-products are generated during the process of citrus fruit production. Several citrus fruits are cultivated worldwide, and their annual production has grown considerably and rapidly due to rising consumer demand. Approximately 50% of global fruit production is allocated for juice manufacturing, a process that generates 25 million tons of waste on an annual basis to produce citrus juices. The fruit that undergoes the most processing is orange (*Citrus sinensis*), generating approximately 16 million tons of waste per year [2].

Citrus processing waste represents a significant reservoir of nutritionally valuable compounds, including peptides and polyphenols, which have recently been linked to several health effects in humans [3]. These characteristics render citrus waste a promising source of ingredients for novel and/or added-value products in the medical, cosmetic, and food/feed sectors. Given the ubiquity of citrus peels, it is imperative to explore strategies that would facilitate the augmentation of their utilisation [4].

Conventional extraction with solvents is the most widely applied technique from an industrial perspective for the extraction of bioactive compounds from vegetable matrices. The safety of the process, in terms of avoiding potentially hazardous chemicals and environmentally friendly waste product disposal, is an essential aspect that should be considered in estimating both production cost and environmental impact. Since traditional processes involve the use of organic solvents, which have a negative environmental impact, there is a great interest in exploring new and sustainable methods for the processing of by-products to recover specific molecules.

In recent years, there has been a notable advancement in the field of green extraction techniques, with several novel methods being employed to optimise the extraction of bioactive compounds from food waste. Among these emerging techniques, natural deep eutectic solvents (NaDES) have emerged as a particularly promising candidate [5]. NaDES are comprised of two or more natural components (in a given molar ratio), which interact through specific hydrogen bonds, thereby forming a eutectic mixture [6]. The positive aspects related to NaDES use include their biodegradability, recyclability, extremely low vapor pressure, low costs, low toxicity of most or all their components, and the possibility to be included in food products [7]. In this context, a phenolic extract from orange peels obtained with NaDES has recently been added to orange juices with a view to extending their shelf-life and improving sensorial properties [8].

In defining the extraction conditions of bioactive compounds from food waste, it is fundamental to acknowledge that most of bioactive compounds are bound or entrapped within complex matrices. In this frame, polyphenols have been observed to covalently bind to other food constituents, including carbohydrates and proteins [9, 10]. Consequently, their extraction by organic solvents may be difficult due to the formation of insoluble covalent complexes with cell-wall polymers through ester and glycosidic bonds. In this context, enzymes could facilitate the breakdown of these interactions and enhance the extraction yield of biomolecules. The nature of cell wall structure suggests that enzyme preparations for cell wall hydrolysis must contain a mixture of cellulases, pectinases, and proteases. Even though enzyme-assisted extraction (EAE) has been demonstrated to be a successful combination with other traditional extraction methods [11], to the best of our knowledge, only a limited number of studies combined the effect of EAE and NaDES for polyphenols recovery and other bioactives [12-14].

The present study aimed to investigate the potential synergistic application of the combined EAE and NaDES on polyphenol and small protein fragments (peptides) recovery from orange peel in comparison with the single treatment and conventional extraction methods. Citrus peel was treated with various concentrations of pectinase, cellulase, and protease, either separately or in appropriate combinations. The phenolic composition and content were determined by chromatographic techniques, while the antioxidant capacities, peptides content, and protein fragmentation were spectrophotometrically assessed. The protein profile was determined by electrophoretic techniques. The data obtained were then utilised to ascertain the most promising extraction condition in terms of bioactive compound extraction and antioxidant capacities by a full factorial design statistical approach, to evaluate its potential impact on intestinal cell viability.

2. MATERIALS AND METHODS

2.1 Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Lonza (Basel, Switzerland). Pectinase from *Aspergillus* (89216), cellulase from *Aspergillus niger* (C1184), protease from *Aspergillus saitoi* (P2143), and other solvents were of the highest analytical grade from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2 NaDES formulation

COSMOtherm software was employed to model the solubilization of hesperidin in various NaDES. In summary, the discrete Fourier transform was employed to optimize the shape and density of

molecules during the first phase. Each molecule was optimized for this objective using the COSMO-BP-TZVP software programs. The COSMO computations were subsequently conducted utilizing the BP_TZVP_C30_19.CTD parametrization with BIOVIA COSMOtherm version 20.0.0, 2024. COSMOtherm software was used as a tool to predict the activity coefficient of hesperidin in 75 % of NaDES, as well as to simulate the solubility of hesperidin in the different NaDES. According to the best results, six different types of NaDES combinations were selected using the COSMOtherm software according to hesperidin solubility ($\gamma_{solutes}$) [15] and prepared: i. 1:1:1 (mol/mol/mol) choline chloride:L-proline:malic acid (ChLPMA); ii. 1:1 (mol/mol) L-proline:malic acid (LPMA); iii. 1:1 (mol/mol) betaine:malic acid (BeMA); iv. 5:1 (mol/mol) lactic acid:glucose (LAGlu); v. 1:1 (mol/mol) malic acid:glucose (MAGlu); vi. 1:3 (mol/mol) choline chloride:lactic acid (ChLA). The reagents were mixed in the indicated molar ratio and subjected to rigorous stirring at 80ºC in a water bath until a transparent liquid was achieved. To mitigate viscosity and enable the migration of bioactive compounds from the matrix into the solvent, the addition of 25% distilled water was implemented, as previously described [16].

2.3 Sample preparation and bioactive extraction

The orange peels were obtained from *Citrus sinensis* Navel cultivar, which were provided by a local agricultural cooperative in Carlet, Spain. Following a thorough cleansing with distilled water, the peels were manually separated from the pulp and subsequently crushed using a kitchen blender. Five ml of phosphate-buffered saline (PBS) (24.6 mM NaH_2PO_4, 75.4 mM NaH_2PO_4, pH 4.5) alone or containing 0.75% or 3% cellulase, protease, pectinase, or all enzymes was added to 1 g of orange peel. The mixture was left at 37°C for 2 hours under constant stirring. At the end of the incubation period, the enzymes were heat-inactivated at 95°C for 5 minutes. The extraction of the bioactive compounds was then carried out at 50°C for 30 minutes by stirring, with the addition of 5 mL of NaDES. Phosphate-buffered saline (PBS), or 50% (v/v) ethanol (Et-OH) were used as comparison solvents. Following the completion of extraction, the samples were subjected to centrifugation at 800g for 10 minutes at room temperature. The clear upper layer was then removed, and the samples were frozen at -20°C until further analysis.

2.4 Total antioxidant activity

The total antioxidant capacity (TAC) was evaluated both with ABTS and DPPH assays [17]. These assays utilised the capacity of the antioxidant molecules to reduce the radical cation of 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+\cdot}$) and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$). Ten and 8 µl of each sample were added to 990 µl of 80 µM $ABTS^{+}$ and 192 µl of 100 µM $DPPH^{\cdot}$,

respectively. The quenching of the absorption at 734 nm for 1 min and at 517 nm for 30 min for $ABTS^{\cdot+}$ and $DPPH^{\cdot}$, respectively, was monitored. The resulting data were then compared to the concentration-response curve of a standard solution of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and expressed as mM.

2.5 Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) assay was performed following the reduction of ferric ion-ligand complex to the intensely blue-coloured ferrous one in an acidic medium by antioxidants present in the sample as previously reported by Jo et al. with slight modifications [18]. Eight µl of the sample was added to 192 µL of a 1.64 mM Fe(III)/0.82 mM tripyridyltriazine complex, and the quenching of the light at 595 nm over a period of 30 minutes was monitored. The resulting data were then compared to the concentration-response curve of a standard solution of trolox and expressed as mM.

2.6 High Performance Liquid Chromatography

The chromatographic separation of bioactive compounds extracted with NaDES and conventional solvents was performed on solid-phase extraction (SPE) according to Anticona et al. [19]. Chromatographic analyses were performed using a column of C18 (250 × 4.6 mm, 5 µm particle size; Luna PFP2, Phenomenex, Torrance, USA) and an HPLC system (Agilent Technologies 1120 Compact LC) with an ultraviolet light detector, as described by Anticona et al. [19]. Mobile phase A consists of 5% formic acid in MilliQ Water (v/v), and phase B consists of 40% A phase in acetonitrile (v/v). Mobile phases were used to create the following elution gradient: 0 – 10 min, 100% A; 10 – 20 min, 85% A; 20 – 50 min, 82% A; 50 – 75 min, 0% A; 75 – 80 min, 100% A. The flow rate remained constant (0.8 mL/min) with an injection volume of 20 µL. Chromatograms were acquired at a wavelength of 280 nm. All samples were filtered with nylon filters (0.22 µm) before injection. The quantification was conducted in accordance with the external standard method, utilising a calibration curve within the range of 0–50 µg/ml. This process was executed under the same conditions as the samples. The quantification parameters are provided in Supplementary Table 1.

2.7 Total protein fragments

The total protein fragments on orange peel extract were determined spectrophotometrically by the o-phthalaldehyde (OPA) assay [20]. This assay is based on the reaction of OPA and 2-mercaptoethanol with primary amine released during the lysis of a protein substrate. This results in the formation of a

colorimetric adduct with a peak of absorption at 335 nm. Total protein fragments were expressed as mM glutamic acid equivalent.

2.8 Peptides concentration

The peptide concentration on orange peel extract was spectrophotometrically determined using the Pierce Quantitative Colorimetric Peptide Assay kit (Pierce Chemical Co, Dallas, TX, USA), following the manufacturer's instructions. The method is based on copper reduction by the accessible amide backbone of peptides under alkaline conditions followed by the chelator coupling with the reduced copper to form a bright red complex with a peak of absorption at 480 nm. Peptide content was expressed μg/ml, respectively.

2.9 Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis

Precipitation of proteins in orange peel extracts was performed according to Méchin et al. [21] using trichloroacetic acid as precipitating agent. The resulting washed protein pellet was then dried, resuspended in an equal volume of non-reducing buffer (0.125 M Tris-HCl, pH 6.8, 50% glycerol, 1.7% SDS, 0.01% bromophenol blue), and heated in boiling water for 5 minutes. Sodium dodecylsulfate-polyacrylamide gel electrophoresis was carried out using home-cast 12% polyacrylamide gels [22]. Gels were stained with Coomassie Blue R250 and a grayscale image of the polyacrylamide gels was obtained through a benchtop image scanner.

2.10 Selection of the most promising extract for cell culture experiments

The results of the full factorial design (Design Expert, v.10.0.03, Stat-Ease Inc., Minneapolis, MN, USA) were modelled considering simultaneously the main and interaction effects of three factors: enzyme (no enzymes, cellulase, protease, pectinase or all enzymes); enzyme concentration (0.75% or 3%); solvents (ChLPMA, LPMA, BeMA, LAGlu, MAGlu, ChLA, EtOh, and PBS). The optimization was performed for the combination of factor levels simultaneously satisfy the maximisation criteria placed on each of the responses and factors. Being several responses and factors, all goals were combined into one desirability function [23], with the objective of maximizing all the responses.

2.11 Caco-2 cell culture and supplementation

Caco-2 cells were cultivated in 75 cm^2 sterile flasks at 37°C, 95% air, 5% CO_2 in high-glucose Dulbecco's Modified Eagle's Medium (DMEM), with the following supplements: foetal heat-inactivated foetal bovine serum (10%), non-essential amino acids (1% v/v), 100 U/mL penicillin and 100 μg/mL streptomycin. On a weekly basis, cells were seeded at a density of 3.3 x 10^4 cell/mL in a

new flask, with the medium being refreshed every 48 h. For the experimental phase of the study, Caco-2 cells were seeded in 24-well plates at a density of 1×10^5 cells/well and cultured for a period of 21 days [24]. Following this, the cells appeared fully differentiated and polarised, resembling the morphological and functional characteristics of mature enterocytes. The cells were then supplied with 1 mL of serum-free DMEM for a period of 4 hours, containing varying concentrations of a 3% Pec-LPMA extract (2, 5, 10, 20, and 30 µl). To circumvent any potential interference from the vehicle, a subset of cells was also supplied with an equivalent amount of LPMA (designated as the control cells).

2.12 Cell cytotoxicity

The viability of the cells was determined by converting the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt to its formazan product, which was then detected at 560 nm using a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland) [25]. The cell viability was expressed as a percentage of the viability of the control cells, which were assigned a value of 100%.

2.13 Statistical analysis

All data are means ± standard deviation (SD) of two independent extractions. Statistical analysis was applied by the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test ($p < 0.005$ was considered as significant).

3. RESULTS AND DISCUSSION

3.1 Combined effect of EAE and NaDES on total antioxidants recovery

Antioxidants can be defined as substances capable of preventing or counteracting the action of reactive oxygen species thus prevent cellular oxidative damage [26]. Among the *in vitro* antioxidant assay methodologies, the electron transfer-based assays, encompassing ABTS and FRAP, along with the electron/hydrogen atom transfer (mixed) assays, exemplified by the DPPH assay, have emerged as the prevailing practices [27]. The TAC, as determined by the ABTS and DPPH assay, and FRAP in orange peel extracts for the ten most promising conditions is presented in descending order in Figure 1, while the full range of conditions is reported in Supplementary Table 2.

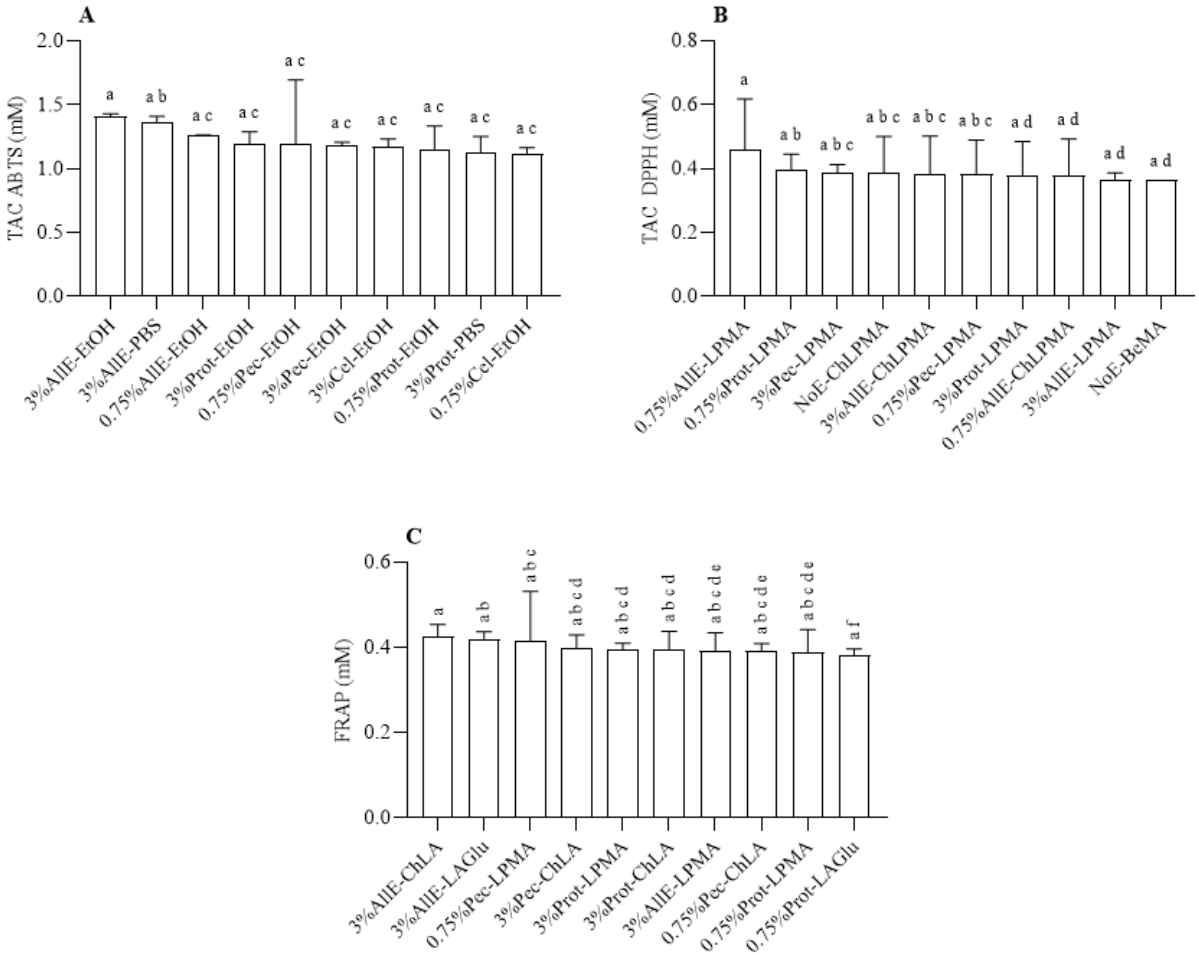

Figure 1. Total antioxidant capacity by ABTS (A) and DPPH (B) assay, and ferric reducing antioxidant power (C) in orange peel extracts for the ten most promising conditions represented in descending order. Data are expressed as mM and are means ± standard deviation of two extraction and chromatographic analysis. Statistical analysis was by one-way ANOVA (all $p<0.05$) with Tukey multiple comparison as post hoc test (different uppercase letters indicate at least a $p<0.05$ significant variation).

With respect to the antioxidant capacity, the main findings indicate that the extraction of bioactive compounds is more influenced by the type of solvent rather than the enzymatic treatment. Indeed, irrespective of the EAE, antioxidants extracted with Et-OH and PBS were observed to show the highest antioxidant capacity, as evaluated by the ABTS assay. Conversely, the DPPH method revealed the highest antioxidant activity in samples extracted with LPMA. In contrast, the capacity of the antioxidants presents in the samples to reduce the ferric ions does not appear to be predominantly associated with a particular solvent or EAE.

As different polyphenols can exhibit varying antioxidant capacities depending on the methods employed [28], the phenol composition and content in orange peel extracts for the ten most promising conditions is presented in descending order in Figure 2, while the full range of conditions is reported in Supplementary Table 3.

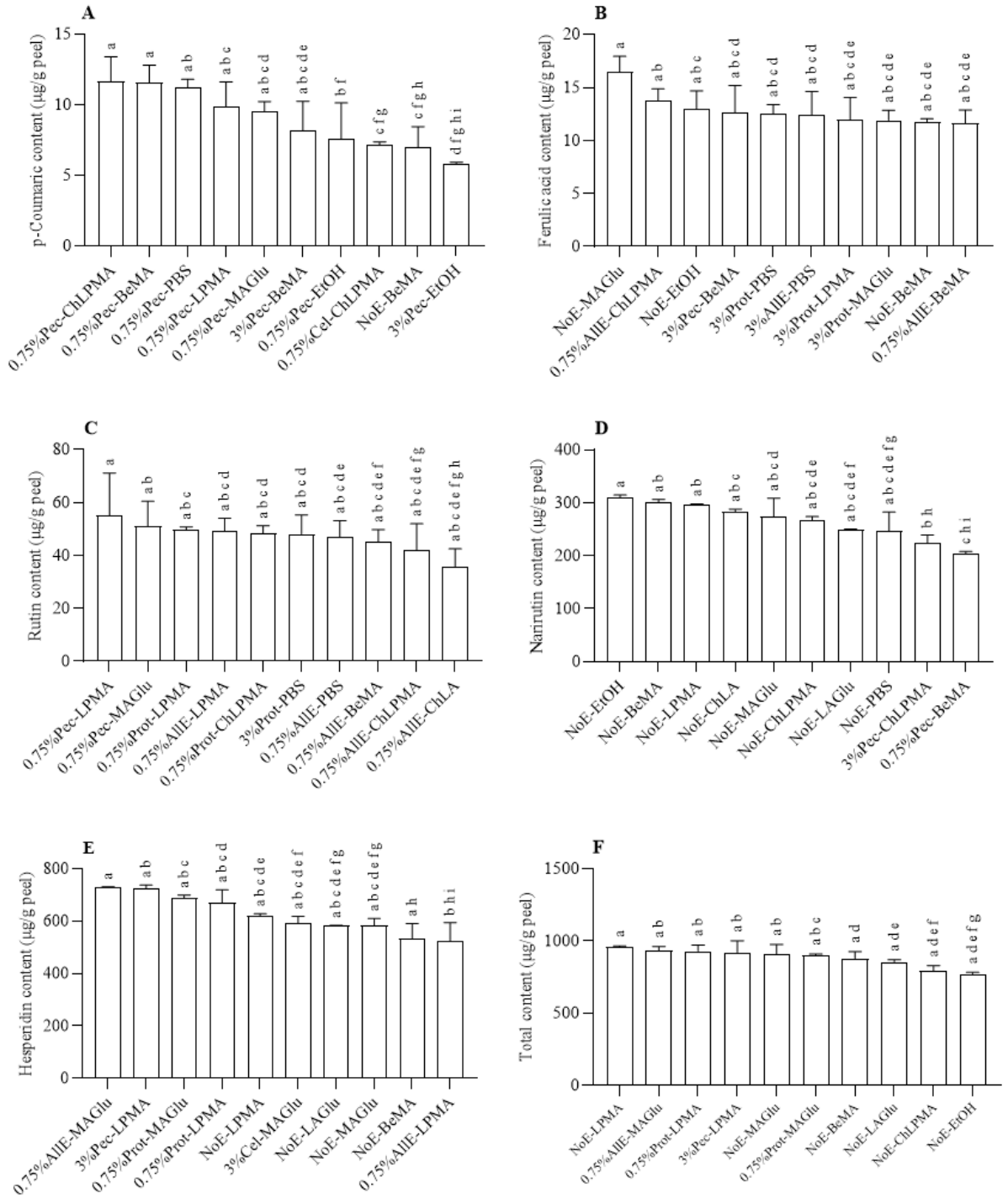

Figure 2. Polyphenol content in orange peel extracts. p-Coumaric (A), ferulic acid (B), rutin (C), narirutin (D), hesperidin (E), and total phenol (F) content in orange peel extracts for the ten most promising conditions represented in descending order. Data are expressed as µg/g orange peel and are means ± standard deviation of two extraction and chromatographic analysis. Statistical analysis was by one-way ANOVA (all $p<0.05$) with Tukey multiple comparison as post hoc test (different uppercase letters indicate at least a $p<0.05$ significant variation).

In this study, 5 main free phenolic compounds were identified and quantified in orange peel extracts. As outlined in Supplementary Table 3, the identified orange peel phenolics belong to three distinct classes: phenolic acids (*p*-coumaric and ferulic acid), flavonol (rutin), and flavanones (narirutin and hesperidin). Among these, rutin, narirutin, and hesperidin are the glycosylated forms of quercetin, naringenin, and hesperetin, respectively. Concurrently, *p*-coumaric acid was found to be less abundant than ferulic acid, followed by rutin, narirutin, and hesperidin in increasing order. The efficacy of extraction appears to be contingent upon the type of phenolic compound, with the nature of the enzymatic treatment or the type of solvent employed exerting a more significant influence. Concerning the p-coumaric acid content, it was determined that orange peels subjected to treatment with the enzyme pectinase exhibited elevated levels of the substance, irrespective of the type of solvent employed. On the contrary, the narirutin content was higher in orange peel extracts obtained without any enzymatic treatment, regardless of the type of solvent used. In contrast, the extraction efficiency of hesperidin and total phenolic compounds was found to be higher when NaDES solvents with malic acid were employed, regardless of the EAE used. In agreement, it has been reported that DESs extracted more polyphenols with higher molecular weights and more diverse phenolic compounds than methanol from mangosteen peel. The high extraction efficiency may be due to the large solvent accessible surface area, long lifetime of H-bonds between NaDES and extract, and low intermolecular interaction energy [29].

Pearson correlation analyses were conducted to predict the relationship between the antioxidant activity and the polyphenol species content. The results obtained from the DPPH assay demonstrated a weak (r = 0.2-0.39) to moderate (r = 0.4-0.59) significant positive correlation with ferulic acid (r = 0.39; $R^2 = 0.15$), rutin (r = 0.42; $R^2 = 0.18$), narirutin (r = 0.35; $R^2 = 0.12$), hesperidin (r = 0.42; $R^2 = 0.18$), and total phenol content (r = 0.48; $R^2 = 0.23$). Furthermore, an almost negligible yet positive correlation was identified between rutin content and FRAP (r = 0.26; $R^2 = 0.07$). This observation aligns with the findings from previous studies that have established a strong correlation between polyphenol content and DPPH assay outcomes [30]. Conversely, an absence of correlation has been documented between FRAP and ABTS values and polyphenol content [31].

The feebler or absent correlation between free phenolic content and FRAP and TAC by ABTS assay is probably because the antioxidant activity results from a different type of polar extractable bioactive component with antioxidant activity, such as citric acid, essential oil and ascorbic acids, frequently present in citrus peel [32-35]. Moreover, even though orange peel has been shown to be a significant source of carotenoids [36], it is believed that their contribution to the total antioxidant capacity is negligible. This assertion is primarily founded upon the observation that carotenoids, being apolar molecules, are inherently incompatible with the polarity exhibited by the solvents employed in the

study. Secondly, carotenoids may demonstrate a negligible relationship with antioxidant assays [37, 38], implying minimal contribution of carotenoids towards the observed antioxidant activities. To facilitate a more profound comprehension of the function of individual molecules, the utilisation of nuclear magnetic resonance (NMR) spectroscopy may emerge as a pivotal instrument in subsequent research endeavours. ^{1}H NMR-based metabolomics has been demonstrated to be a particularly effective and efficient approach to assess the entirety of discernible components present within food products [39, 40].

As reported here, and as evidenced by the research that has been carried out on the subject [41], the impact of diverse solvents on the extraction of antioxidants and bioactive compounds from plant samples is amply described. In recent years, polyphenols were successfully extracted from cocoa by-products using NaDES for subsequent use in food fortification. This method was found to be a viable option since the extraction solvent was not removed, and it was estimated to be sensory acceptable and safe [42]. Concurrently, hydroalcoholic solvents exhibited a high level of antioxidant extraction efficiency. Kong et al. evidenced as the water extracts of *Barringtonia racemosa* leaves had the highest polyphenols yield and antioxidant activities, followed, in descending order, by the ethanol > ethyl acetate > hexane extracts [43]. Similarly, Kaczorová et al. demonstrated that *Achillea* species extracts obtained using polar solvents (water and ethanol) exhibited a higher level of antioxidant activity when evaluated using the DPPH assay, in comparison to those extracted using non-polar solvents such as chloroform and ether [44]. Zengin et al. recently demonstrated that NaDES 1:2 Ch:urea and 5:1 LP:xylitol possessed a superior capacity for the extraction of polyphenols from *Cytinus hypocistis* in comparison to water and ethanol. However, it should be noted that alcoholic and aqueous extracts exhibited an augmented antioxidant capacity [45]. Furthermore, Gomez-Urios demonstrated the efficacy of LA:Glu and LP:MA, but not MA:Glu and ChFru, in extracting phenolic compounds from orange peels when compared to a hydroalcoholic extraction [15]. Given the high NaDES number, in a recent study, Milošević et al. evaluated the ability to extract antioxidant compounds from strawberry tree fruit (*Arbutus unedo*) using 20 different NaDES. The results obtained from this study indicate that the extraction capacity of phenolic compounds varies significantly depending on the type of NaDES employed. Furthermore, the higher recovery of phenolic compounds does not necessarily equate to a higher antioxidant capacity [46].

Overall, it is evident that polarity plays a crucial role in this process, with higher extraction rates being achieved with polar solvents with hydrogen bonding capacity [47]. The degree of polarity can be expressed in terms of the molar transition energy (E_{NR}), with higher polarity solvents having lower E_{NR} values [48]. The knowledge of the polarity of a NaDES can be of significant use for the extraction of target compounds, with the organic acid-based and amino-acid-based NaDES generally being the

most suitable for this purpose [49]. According to Gomez-Urios et al. [50] and Karadendrou et al. [51], the E_{NR} values for ChLPMA, LPMA, BeMA, LAGlu, MAGlu, ChLA, ethanol and water were 49.18, 48.32, 48.71, 47.89, 46.56, 47.97, 52.17, and 48.20 Kcal/mol, respectively. Consequently, it may be posited that the comparable levels of polyphenol recovery and antioxidant activity may be attributable, at least in part, to the comparable E_{NR} values observed among the utilized solvents. In addition to the role of the solvent and bioactive compounds profile in determining antioxidant capacity, the method employed is also a contributing factor to the observed differences. Despite their similar objectives, the significant discrepancies in the absolute antioxidant capacity values measured by the DPPH and ABTS assays are attributable to intrinsic factors. The DPPH assay is chiefly concerned with the measurement of hydrogen-donating antioxidants at a wavelength of 517 nm, whilst the ABTS assay is designed to measure hydrogen- and electron-donating antioxidants at a wavelength of 734 nm. The DPPH assay employs the use of organic solvents, such as methanol or ethanol, which influence antioxidant solubility and reactivity in a manner divergent from the aqueous environment typically utilised in the ABTS assay. Furthermore, the kinetics of the reactions also play a role. Phenolic compounds with multiple hydroxyl groups exhibit higher reactivity with the ABTS radical cation than with the DPPH radical, resulting in higher values in the ABTS assay [28, 47]. Also, the compositional profile of bioactive compounds, regardless of the content, has been demonstrated to exert a considerable impact on antioxidant activity. Indeed, it has been reported that glycosylated polyphenols exhibit a reduced ability as hydrogen donors and are less effective as antioxidants in comparison to their free aglycone forms due to substitution of the hydroxyl group [52].

Regarding the role of EAE on bioactives recovery, Li et al. [53] demonstrated that treatment with varying concentrations of Celluzyme MX - ranging from 0.5% to 5% - in conjunction with various types of commercial enzymes (Celluzyme CL and Kleepase AFP) at a concentration of 1.5%, did not result in a significant alteration to the recovery of phenolic compounds in orange peels. A potential rationale for the enzymes' inefficiency could be attributed to the distinct nature of polyphenols present within citrus peels. While polyphenols can be found in food matrices in both the free, easily extractable form and the matrix-bound form, various studies have demonstrated that in citrus, they are predominantly found in the free form [54-57]. This could render the use of enzymes to aid in extraction not predominantly helpful.

Concurrently, a multitude of experimental conditions, including time, temperature, enzyme type, and concentration, may exert a substantial influence on the extraction of phenolic and antioxidant compounds. Indeed, Nishad et al. demonstrated using a response surface methodology that the optimum conditions for the extraction of phenolic substances involved the use of Viscozyme at an

enzyme concentration of 0.84%, for a duration of 4.87 hours, and with a solvent-solid ratio of 30.94 ml/g [58].

3.2 Combined effect of EAE and NaDES on protein recovery

Notwithstanding the dearth of protein in citrus peel [59], that they may serve as a source of bioactive peptides [60], there is a scarcity of research on protein in citrus residues.

The total protein fragments and peptides content in orange peel extracts for the ten most promising conditions are presented in Figure 3, while in Supplementary Table 4 the full range of conditions is shown. Information on the nature of proteins in the various extracts are provided by SDS-PAGE tracing reported in Supplementary Figure 1.

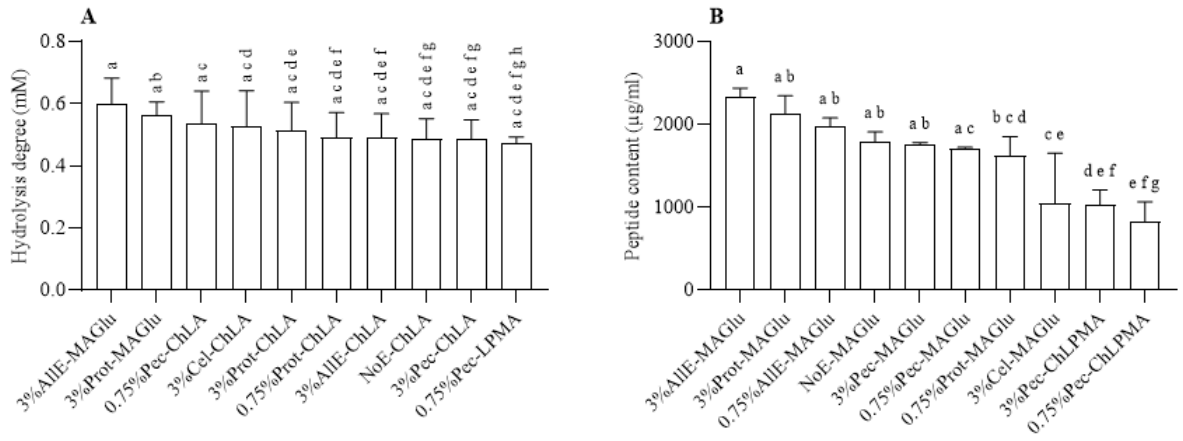

Figure 3. Total protein fragments (A) and peptide content (B) in orange peel extracts for the ten most promising conditions represented in descending order. Data are expressed as mM and µg/ml and are means ± standard deviation of two extraction and spectrophotometric analysis. Statistical analysis was by one-way ANOVA (all $p<0.05$) with Tukey multiple comparison as post hoc test (different uppercase letters indicate at least a $p<0.05$ significant variation

It is evident that the peptide content, as well as the extent of protein hydrolysis, appears to be affected by the type of solvent than by the EAE. It has been demonstrated that, irrespective of the EAE, the level of protein hydrolysis and peptide content was generally higher when MAGlu is utilised as the extraction solvent, reasonably by a higher efficient solubilization.

Guzmán-Lorite demonstrated that NaDES with more extreme pH values (both basic and acidic) exhibited a greater capacity for protein extraction in pomegranate peel [61]. Similarly, Karimi et al. demonstrated that all NaDES tested exhibited a lower extraction efficiency than alkaline extraction at a pH of 12, yet higher than at a pH of 9 [62]. The extreme pH levels could potentially promote the solubilisation of proteins, which are expected to exhibit predominant positive charges at low pH values and negative charges at high pH values [63]. In the present study, the lowest pH value was observed in MAGlu (1 vs. 2.06, 2.01, 2, 1.03, 1.03, 6.71, and 4.5 for ChLPMA, LPMA, BeMA,

LAGlu, ChLA, 50% Et-OH, and PBS, respectively) [50]. This finding, in conjunction with the lowest E_{NR} value, may have led, at least in part, to a slightly greater extraction of polar protein fragments with MAGlu. A comparable effect was identified in the evaluation of the protein profile through SDS-PAGE, which shows the presence of soluble proteins with a M_r between 25 and 30 kDa, primarily in orange peel extracts obtained through NaDES. As recently highlighted by Sánchez-Elvira et al., comparable results were also obtained on lemon peels [64]. Using capillary gel electrophoresis, Vergara-Barberán et al. show the presence of three abundant proteins with molecular weights of 24, 27, and 36 kDa in orange peel extract. The protein at 24 kDa was identified as a germin-like protein, which is known to be the major allergen in orange fruits, while the protein at 27 kDa has been attributed to superoxide dismutase [65].

Even for protein recovery, the application of EAE is dependent on the operational conditions, including food matrix, selection of the enzyme, substrate, and enzyme ratio, enzyme-specific temperature and pH, and extraction time [66]. For instance, as Liaset et al. [67] demonstrated, alcalase and pepsin, compared to neutrase, were the enzymes that yielded the highest protein recovery in fish by-product. Furthermore, the research conducted by Braspaiboon *et al*. demonstrated using alcalase that, under optimal conditions, the maximum protein recovery was achieved at a solids-to-liquids ratio of 7.25%, an enzyme substrate ratio of 0.9%, and a treatment duration of 4.88 hours [68].

Pearson correlation analyses were also conducted to predict the relationship between the peptide content and the protein fragments and antioxidant capacities. The results obtained from the peptides content evidenced a strong (r = 0.6-0.79) significant positive correlation with the protein fragments (r = 0.62; R^2 = 0.38) but a moderate (r = 0.4-0.59) negative correlation with the total antioxidant capacities evaluated by the ABTS assay (r = -0.41; R^2 = 0.17).

Despite the paucity of studies assessing the presence of bioactive peptides in orange peel, several studies have emphasised their presence in various food by-products of plant origin. These by-products have been shown to possess several biological effects including antihypertensive and anti-diabetic activities [69]. In this study, a weak but significant correlation was demonstrated between the content of peptides and antioxidant activity, as measured exclusively by the ABTS assay and indicated above. It can be hypothesised that the observed negative correlation is attributable, at least in part, to the predominance of the phenolic fraction over the peptide fraction, which possesses antioxidant activity.

3.3 Selection of the most promising extract to be supplemented in cell culture

To incorporate a response within the optimisation criteria, a model employing the analysis of variance (ANOVA) method was constructed for each response variable. In certain instances, the data underwent mathematical transformations to meet the assumptions of normality and reduce skewness.

Non-significant model terms were then removed through a stepwise simplification process, ensuring compliance with the model hierarchy and preserving overall statistical integrity. The performance of the model was evaluated by means of several statistical parameters, including the coefficient of determination (R^2), the adjusted R^2, the predicted R^2, the adequate precision, and the lack-of-fit (LoF) test p-values. The model specifications are documented in Table 1.

Response	**PC**		**TAC-ABTS (log)**		**TAC-DPPH**		**FRAP (log)**		**PF (square root)**		**Peptides content**	
Source	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value
Model	4.89	0.000	385.38	0.000	49.22	0.000	67.88	0.000	11.44	0.000	28.91	0.000
A -Enzyme	3.01	0.038	535.54	0.000	59.20	0.000	90.52	0.000	6.44	0.001	36.66	0.000
B- Conc	5.37	0.024	35.02	0.000	25.95	0.000	0.31	0.583	6.61	0.013	10.82	0.000
C - NaDES	5.63	0.000					93.32	0.000	16.76	0.000		
AB							8.39	0.000	5.63	0.002		
R^2	0.51		0.99		0.90		0.95		0.77		0.85	
adj R^2	0.40		0.98		0.88		0.94		0.70		0.82	
pred R^2	0.26		0.98		0.86		0.92		0.60		0.77	
Ad Precision	9.27		57.90		26.76		31.81		14.11		21.87	

Table 1. Ad Precision: adequate precision**;** adj R^2: adjusted R^2; ABTS: 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl; EtOH: ethanol; FRAP: ferric reducing antioxidant power; PF: protein fragments; pred R^2: predicted R^2; PC: polyphenol content; TAC: total antioxidant capacity.

All the models, except for FRAP, demonstrate a substantial impact of enzyme and enzyme concentration, resulting in a significant effect ($p<0.05$) of the two main factors. Enzyme concentration factor does not exhibit a significant effect ($p>0.05$) on FRAP but was considered as model term due to the significant interaction of enzyme x enzyme concentration. NaDES determined a significant effect only for PC, FRAP and PF. Furthermore, FRAP and PF were significantly ($p<0.001$) affected by the interaction between enzyme and enzyme concentration. The coefficient of determination ranged from 0.51 for polyphenols to 0.99 for TAC-ABTS, with reasonable agreement with adjusted R^2 and R^2 in prediction. The signal-to-noise ratio, expressed as adequate precision, consistently exceeded 4, confirming the fitness of the model in the design space.

Successive optimisation of the process was conducted via the desirability function methodology [23], with the objective of maximising all the responses, assigning the highest weight (+++++ instead of +++) to polyphenols and peptide concentration. The statistical analysis yielded results indicating that the optimal condition was obtained by pectinase at 3.00% combined with LP:MA, obtaining a desirability of 0.440. This was selected for cell culture experiments.

3.4 Effect of LP:MA with 3% pectinase extract and solvent supplementation on cell viability

Figure 4 reported cell viability of Caco-2 cells after a 4-hour period of supplementation with LP:MA with 3% pectinase extract and the solvent alone at varying concentrations.

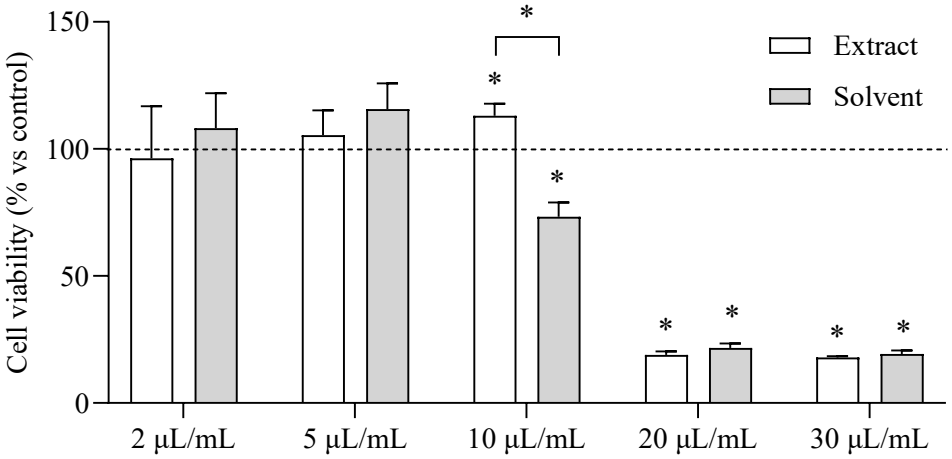

Figure 4. Cell viability evaluated by MTT assay in Caco-2 cells supplemented 3% pectinase LP:MA orange peel extract and solvent alone at different concentration. Data are expressed as percentage versus control unsupplemented cells (assigned to 100%, dashed line) are means ± standard deviation of two independent experiments each performed in duplicate. Statistical analysis was by unpaired t-test comparing supplemented cells versus unsupplemented ones and, within the same concentration, extract-supplemented cells with solvent supplemented ones (* $p < 0.05$).

No decrease on cell viability was detected at both 2 µL/mL and 5 µL/mL of LP:MA with 3% pectinase orange peel extract or solvent alone. At 10 µL/mL concentration, 3% pectinase LP:MA orange peel extract and the solvent alone resulted in an increase of 13% and a decrease of 26.7% in cell viability, respectively. Conversely, at concentrations of 20 µL/mL and 30 µL/mL, both the extract and the solvent exhibited a cell viability of approximately 20%. Moreover, only at 10 µL/mL concentration, cell viability appeared higher in extract supplemented cell in comparison with the solvent supplemented ones.

In a recent study, Popović et al. [70] examined the cytotoxic profile of twelve choline chloride-based NaDES. The researchers observed that the level of cell survival depended mostly on the concentration of the NaDES in the cell medium, as well as on the chemical constitution of the investigated systems. The study found that an acidic hydrogen bond donors showed the highest cytotoxic effects in all the cell lines investigated.

Therefore, it is plausible that the decrease in cell viability observed at the highest concentration examined can be ascribed to the acidification of the culture medium following the incorporation of the solvent. It is imperative that future experimentations incorporate this potentiality, with the objective being the neutralisation of NaDES' naturally acidic characteristics. It is worth to note that at 10 µL/mL concentration, the presence of the extract is such that the detrimental effect of the solvent is overwhelmed. While extracts containing elevated concentrations of bioactives can be regarded as high-value products, a comprehensive understanding of their cellular effects is fundamental to evaluate their potential as a value-added functional ingredient [71]. It has been demonstrated that polyphenols and bioactive peptides do not universally increase cell viability in intestinal cells. While

they have shown beneficial effects, such as protecting the gut barrier [72] and acting as prebiotics [73], some studies indicate that high concentrations of certain polyphenols and peptides can decrease cell viability [74], triggering apoptosis [75] and cell death [76], particularly in cancer cells. The effect is concentration-dependent, with lower doses often being beneficial and higher doses potentially resulting in cell death.

4. CONCLUSION

The combined use of EAE and NaDES is shown to represent an effective, sustainable, and synergistic method in comparison to traditional solvent extractions for the recovery of bioactive compounds from food waste. In consideration of the antioxidant capacity, peptide content and extent of protein hydrolysis, the primary findings suggest that the extraction of bioactive compounds is more influenced by the type of solvent than by the enzymatic treatment. Conversely, the efficacy of extraction of polyphenols appears to be contingent upon the type of phenolic compound. Overall, the pretreatment with pectinase followed by extraction with LPMA proved to be the most promising experimental condition, and it was found that supplementation of the extract with intestinal cells resulted in the counteraction of the cytotoxic effects of the solvent at sublethal concentrations. Globally, the findings presented here will be helpful to the commercial value of the byproducts and increase the recovery of biologically active molecules to which numerous beneficial effects can be used in the formulation of functional foods to improve the health level and safety of the population.

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