Applied Food Research 5 (2025) 101296

Contents lists available at ScienceDirect

Applied Food Research

journal homepage: www.elsevier.com/locate/afres

Extraction optimization of *Citrus medica* L. whole fruit: phytochemical profile, pectin recovery and antioxidant activity in Caco-2 cells

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ARTICLE INFO

Keywords:
Citrus medica L.
Full Factorial Design
Antioxidant Activity
Untargeted-Targeted Analysis
Pectin

ABSTRACT

This study aims to increase the antioxidant yield from fresh whole fruits of *Citrus medica* cv. Diamante Liscia applying the Full Factorial Design (FFD), which allowed to obtain an optimized extract (OE). The optimal extraction conditions obtained by FFD were 60 min, 85.30 % EtOH/H_2O and 47.22 °C, reporting the following data: 3.79±0.09 mgGAE/g, 4.45±0.03 mgTE/g and 10.62±0.26 mgTE/g for Total Polyphenol Content (TPC), 2,2-diphenyl-1-picryl hydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP), respectively. To provide a comprehensive metabolic profile of samples prepared as part of the experimental design, LC—HRMS-based untargeted analysis was performed using a combination of MS-DIAL, MS-CLEANR, and MS-FINDER software suites. Pearson correlation established the relationship between antioxidant activity evaluated by TPC, DPPH, and FRAP and peaks area of specific metabolites. Of these, rutin, hesperidin, and diosmin were confirmed and quantified as OE's characteristic component. Furthermore, after recovering bioactive compounds of interest, pectin with a high yield of 14.78 ± 1.26 % was recovered from the exhausted material.

The OE showed antioxidant properties on intestinal Caco-2 cells by stimulating antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, catalase, and NAD(P)H dehydrogenase. This study reports for the first time the antioxidant properties of *C. medica* whole fruit extract highlighting its potential as therapeutic agent in preventing oxidative stress-related diseases, such as inflammatory bowel disease.

1. Introduction

Inflammatory Bowel Disease (IBD) is a chronic condition characterized by inflammation of the mucosal layer in the gastrointestinal tract that often leads to complications such as an increased risk of colorectal cancer (Sato, Tsujinaka, Miura, Kitamura, Suzuki & Shibata, 2023). Although the etiology is not fully understood, it is widely accepted that the disease results from an interplay of genetic predisposition, environmental factors, immune system hyperactivity, and an alteration of intestinal microbial composition (Haag & Siegmund, 2014; Strober & Fuss, 2011). In the context of IBD, chronic inflammation is both a cause and consequence of oxidative stress. In fact, the inflammatory cells that infiltrate the intestinal mucosa release large quantities of Reactive Oxygen Species (ROS), which if not counterbalanced by endogenous defences, generate an imbalance condition known as oxidative stress, causing damage to cells and tissues (Rezaie, Parker & Abdollahi, 2007). Some studies have reported that patients with inflammatory bowel disease show reduced levels of key regulators of antioxidant defenses such as glutathione and superoxide dismutase but also alterations of lipid peroxidation products such as malondialdehyde (MDA) (Kruidenier & Verspaget, 2002). These findings suggest that enhancing antioxidant defenses could be a potential therapeutic strategy for managing IBD. *Citrus medica* Linn. cv. Diamante Liscia belonging to the Rutaceae family, commonly known as citron or cedar, was the first citrus introduced to the Mediterranean area (Benedetto, Carlucci, Faraone, Lela, Ponticelli, Russo et al., 2023; Luro, Venturini, Costantino, Paolini, Ollitrault & Costa, 2012). Although *C. medica* is widely used in traditional Ayurvedic medicine for various therapeutic purposes (Haridas,

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https://doi.org/10.1016/j.afres.2025.101296
Received 18 May 2025; Received in revised form 23 July 2025; Accepted 26 August 2025
Available online 27 August 2025
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Sasidhar, Nath, Abhithaj, Sabu & Rammanohar, 2021), currently it is underutilized in nutraceutical field. Like most fruits of the genus Citrus, it is rich in bioactive compounds such as terpenes, polyphenols, coumarins and carotenoids considered multi-target agents against different disorders especially for their free radical scavenging and anti-inflammatory properties (Mateus, Teixeira, Barros, Almeida, Silva & Sanches-Silva, 2024). These activities are ascribable to the presence of bioactive compounds like flavonoids (hesperetin, hesperidin, rutin, diosmin), phenolic acids (chlorogenic acid, ferulic acid, coumaric acid) known to possess hydroxyl groups that can neutralize free radicals (Bocco, Cuvelier, Richard & Berset, 1998). In recent years growing demand for natural products and extracts is leading to the development of appropriate extraction methods or alternative extraction methods in terms of reduction of organic solvents, raw materials, time and costs. In the pharmaceutical industry the choice of extraction methods is a crucial step to obtain enriched extracts in bioactive compounds of interest. By using emerging techniques as well as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), enzyme-assisted extraction (EAE), ultrasound assisted extraction (UAE), microwave assisted extraction (MAE) researchers can reduce the time and energy required for the extraction of desired compounds from natural sources (Herrero, 2024). Although these techniques are highly innovative, they require increased costs for industrial scale-up. On the other hand, an approach to optimize solvent and energy consumption using conventional extraction methods as well as maceration or digestion could be a viable alternative to ensure a safe and high-quality extract. To achieve sustainability, the study focuses on using a non-toxic solvent and an easy process scalable at industrial level. Therefore, the aim was to investigate the influence of extraction parameters (temperature, time, percentage of solvent EtOH/H_2O) to obtain an extract with high antioxidant activity from whole fresh fruits of *C. medica*. To perform this outcome, the Full Factorial Design (FFD) was applied as experimental model (Ponticelli, Carlucci, Mecca, Todaro, Milella & Russo, 2024; Veličković, Stamenković, Todorović & Veljković, 2013). More in details, in this study, 27 different extracts were carried out and by fitting the obtained experimental data into a multi-parametric polynomial equation, the model provided ideal predictive values for obtaining an optimized extract with high total phenolic content and antioxidant activity. Not less important, from the exhausted material after the extraction of bioactive compounds, the pectin was recovered to promote zero waste theory which could represent an added value for the industry. For the first time, an optimized extract was obtained from whole fresh fruits of *C. medica* cultivar Diamante Liscia using an eco-friendly approach. The latter approach allows for shorther extraction times, the use of green solvents like water and ethanol and the re-use of waste materials, such as the exhausted material for pectin extraction.

The optimized extract selected for its antioxidant activity was analyzed for its phytochemical profile using untargeted-targeted approach. Additionally, its *in vitro* cell-free effects and the molecular pathways involved in oxidative stress that characterize IBD were confirmed in intestinal Caco-2 cells.

2. Materials and methods

2.1. Chemicals

The reagents 2,4,6-tripyridyl-s-triazine (TPTZ), iron (III) chloride ($FeCl_3 \bullet 6H_2O$), Folin-Ciocalteu reagent, carbonate sodium (Na_2CO_3), 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, absolute ethanol, sodium phosphate monobasic (NaH_2PO_4), analytical standards including rutin, hesperedin, diosmin were purchased from Sigma-Aldrich S.p.a. (Milano, Italy). All solvents used in the experiment for sample preparation and LC—HRMS analysis were purchased from Merck (Germany). Methanol and 2-propanol were obtained as gradient grade LC; acetic acid, ammonium formate, and ammonium acetate were of purity ≥ 99.9 %, and formic acid of purity ≥ 98 %. Distilled water was prepared using the purifying system Milli Q RG – Millipore. Reagents for cell culture were obtained by Euroclone (Euroclone, Italy). Thiazolyl Blue Tetrazolium Bromide (MTT), HOECHST 33258 Stain Solution, Bradford Reagent, *N*-acetyl-l-cystein (NAC) were purchased by Sigma Aldrich (Sigma, St. Louis, MO, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was supplied by Invitrogen (Invitrogen, Carlsbad, CA, USA).

2.2. Plant material and extraction procedure

Whole ripe fruits of *C. medica* were harvested in Calabria region (Italy) in October 2021. To ensure uniformity of the starting material and prevent the potential deterioration of the samples during the experiment, the samples were cut and homogenized into 1–2 cm pieces by using a blade mill, then aliquoted and stored at −20 °C until extraction. For the extract preparation, 25 g of homogenized whole fruits were subjected to dynamic maceration (a process which involves agitation of the mixture of plant material and solvent at 25 °C) and digestion (a process which involves agitation of the mixture of plant material and solvent at 55 °C and 75 °C) as extraction procedures. After the extraction, the solutions were filtered (mesh 10–50 μm), and then the solvent was removed with a rotary evaporator at 37 °C. Extractive yield was calculated and dried extracts were stored at −20 °C until their use. Exhausted whole fruit after the extraction of compounds of interest was dried in oven at 40 °C. Dried material was pulverized and then the pectin was extracted in hot water. Precisely, 5 g of exhausted material of *C. medica* powder were combined with a liquid/solid ratio of 40:1 *v/w*. The extraction process was carried out at 90 °C for 180 min in accordance with optimum extraction conditions obtained applying Response Surface Methodology by Pasandide et al. (Pasandide, Khodaiyan, Mousavi & Hosseini, 2017a). Once the extraction was complete, the mixture was centrifuged at 4500 × *g* for 15 min. Next, the mixture was mixed with an equal volume of 98 % ethanol and stored at 4 °C for 12 h. Afterward, the supernatant was separated through centrifugation (4500 × *g*, 20 min) and dried using rotary evaporator at 37 °C until a constant weight was achieved. In both extraction methods, the yields were calculated according to the following formula (Eq. (1)):

$$\% = \frac{dried\ extracts\ (g)}{starting\ material\ (g)} \cdot 100 \quad (1)$$

2.3. Fourier transform infrared (FT-IR) spectroscopy

Fourier transform infrared (FT-IR) spectroscopy of pectin was determined by Jasco FT-IR 460 Plus interferometer. The target sample was prepared by mixing and pressing KBr and the starting powders. Three replicate spectra were collected for each sample. The spectra were further processed using the Essential FTIR software (version 1.50.282).

2.4. Degree of esterification (DE)

The DE of pectin was determined by the titrimetric method with minor variation. 3 mL of ethanol was added to 0.2 g of dehydrated pectin and dissolved in 20 mL of distilled water (Revision, 2008). The samples were stirred until the pectin dissolved completely. Two drops of phenolphthalein reagent were added to the samples and titrated with 0.1 N sodium hydroxide (V1). Then, 10 mL of 0.1 N sodium hydroxide was added to samples and was stirred for 15 min for hydrolysis. After that, 10 mL of 0.1 N hydrochloric acid was added to samples and stirred until the disappearance of pink color. Finally, the samples were titrated with 0.1 N sodium hydroxide until the appearance of pink color (V2). The DE was calculated from the following equation (Eq. (2)):

$$DE(\%) = \frac{V2}{V1 + V2} \cdot 100 \quad (2)$$

2.5. LC—HRMS analysis

Chromatographic separation was performed on the ACQUITY BEH octadecyl C_{18} column (150 × 2.1 mm; 1.7 μm) from Waters using the Agilent Technologies 1290 Infinity II LC System; the injection volume was set to 3.0 μl and the column temperature to 60 °C. The mobile phase system consisted of (A) H_2O:MeOH (95:5) and (B) IPA:MeOH:H_2O (65:30:5) both containing formic acid (0.1 %) and ammonium formate (5 mM). The gradient was as follows: 0–2 min, flow 0.30 mL/min, 100–98 % A; 2–10 min, 0.30 mL/min, 98–0 % A; 10–17 min 0.30 mL/min 0 % A. The column was finally equilibrated for 2.5 min under the initial conditions. The detection of compounds was carried out on the Agilent 6560 Ion Mobility Q-TOF, operated in positive and negative ionization polarity. The following MS conditions were used: drying gas flow rate 12 l/min; drying gas temperature 180 °C; sheath gas flow rate 11 l/min; sheath gas temperature 350 °C; nozzle voltage 250 V, − 250 V; capillary voltage 3000 V, − 3000 V; nebulizer 40 psig. The parameters of the Auto MS/MS measuring mode were as follows: mass range 50–1200 m/*z* (MS) and 50–1200 m/*z* (MS/ MS); acquisition rate 3 spectra/s (MS) and 5 spectra/s (MS/MS); collision energy 20 eV; 5 precursor ions/cycle.

2.6. Sample preparation and LC–HRMS data processing

All extracts obtained under different extraction conditions from the whole fresh fruits of *C. medica*, including the optimized sample (OE), were dissolved in 70 % MeOH at 100 mg/mL and sonicated for 10 min. After sonication in ultrasonic bath (Sonorex Super 510H, Bandelin electronics, Germany), the extracts were centrifuged at 6000 × *g* for 10 min at 25 °C and then the supernatants were collected and used for the untargeted analysis. The following workflow was employed: MS data through the use of MS-DIAL (version 4.9221218) for spectral deconvolution and data alignment, MS-CleanR for feature filtration, and MS-Finder v.3.30 for peak annotation (Fraisier-Vannier, Chervin, Cabanac, Puech, Fournier, Durand et al., 2020). The raw MS data of both ionization modes were converted in .abf format using Abf converter (https://www.reifycs.com/AbfConverter/). The raw data in positive and negative mode were separately uploaded on MS-DIAL (downloaded from http://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/).

Process, threshold of peak intensity was selected as 1000 amplitude and linear weighted moving average method was preferred for smoothing and peak width was adjusted as 5. In deconvolution process, sigma window value was selected as 0.5 while retention time and mass tolerance were selected 2 min and 15 ppm for gap filling process. In peak alignment process, retention time tolerance and MS1 tolerance were set to 0.05 min and 0.04 Da. After alignment, feature detection and normalization by TIC using MS-DIAL, all detected features in positive and negative were cleaned with MS-CleanR by selecting all filters with a minimum blank ratio set to 0.8, a maximum relative standard deviation (RSD) set to 40, and a relative mass defect (RMD) ranging from 50 to 3000. The maximum mass difference for feature relationships detection was set to 0.005 Da, and the maximum RT difference was set to 0.025 min. Two peaks were kept in each cluster: the most intense and the most connected within the graph (adducts, neutral loss, dimers). Correlational analysis was performed on the untargeted screening data to identify potential bioactive compounds, and the correlation coefficients were calculated based on the spectrophotometric antioxidant assays (TPC, DDPH, FRAP) values versus peaks area. The features were annotated by MS FINDER: the MS1 and MS2 tolerances were set to 5 and 15 ppm, respectively, formula prediction and structure elucidation were exclusively processed with C, H, O, N, and P atoms. For the quantitative analysis a mix of analytical standards at 1 μg/mL in 70 % MeOH was prepared. A specific volume of the mix standard solution was then pipetted into a clean vial obtaining different concentrations of mix standard (10–25–50–100–250–500–1000 ng/mL). After adding the standard, the solvent was carefully evaporated by N_2. Once the solvent was evaporated, 500 μL of OE at different concentrations (100–10–1–0.2–0.1–0.01 mg/mL) was added in each vial containing the standard mix. The working range was determined by the linearity of the signal by subtracting the area of the compound present in the OE from that of the OE spiked with the standard mix. The mean of peak area value of six sample repetitions in succession, were used to calculate relative standard deviation (RSD) repeatability.

2.7. Total phenolic content (TPC)

The TPC was evaluated throughout the Folin-Ciocalteau reagent method. In detail, 75 μL of the extract were mixed with 500 μL of Folin-Ciocalteau reagent, 500 μL Na_2CO_3 solution (10 % *w/v*) and water until the final volume of 1.5 mL. The mixture was incubated for 1 hour into the dark, and its absorbance was read at 725 nm. The TPC of the sample was expressed in milligrams of gallic acid equivalents (GAE) per gram of dried extract ± standard deviation (SD) of the triplicate.

2.8. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH assay is an antioxidant method based on reducing a violet methanol solution containing the free radical DPPH by antioxidant molecules. The reduction of the free radical DPPH into the non-radical DPPH—H is measured spectrophotometrically by evaluating the colour variation from violet to yellow at 515 nm. 50 μL of different dilutions of Trolox or extract were added to 200 μL of methanol solution of DPPH (100 μM) in a 96 well plate. The absorbance was measured at 515 nm after 30 min of incubation in the dark at room temperature. Decreasing the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. Results were expressed as mg of Trolox Equivalent (mgTE)/g of dried extract. Each reaction was performed in triplicate (Carlucci, Ponticelli, Russo, Labanca, Costantino, Esposito et al., 2024).

2.9. Ferric reducing antioxidant power (FRAP) assay

FRAP assay measures the potential antioxidant activity of sample thanks to the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by the antioxidant molecules present in the sample. More in detail, 25 μL of extract/standard were added to 225 μL of FRAP reagent. FRAP reagent was composed by acetate buffer 300 mM pH 3.6, $FeCl_3 \bullet 6H_2O$ 20 mM in distilled water and TPTZ 10 mM in HCl 40 mM in a proportion of 10:1:1. The mixture was incubated at 37 °C for 40 min in the dark. The absorbance of the solution was measured at 593 nm. The increased absorbance of the reaction mixture indicates an increase in the reduction capacity. Each reaction was performed in triplicate. Trolox was used as standard, and the results were expressed as mg of Trolox equivalent per gram of dried extract (mgTE/g) (Ponticelli, Carlucci, Mecca, Todaro, Milella & Russo, 2024).

2.10. Experimental design

A 3^3 full factorial design of experiments was used to understand the effects of three different independent variables: time (X_1), solvent percentage (X_2), temperature (X_3), on the antioxidant activity of *C. medica*'s whole fresh fruits extract. To achieve this purpose, three levels of time ($X_1 = 1, 3, 6$ h), solvent ($X_2 = 15, 55, 95$ % EtOH/H_2O) and temperature ($X_3 = 25, 50, 75$ °C), were selected, resulting in a total of 27 sequential experiments. The natural and coded values of independent variables for each factor are defined in **Table S1**. The significance of the selected independent variables, considered alone and in combination, on the dependent variables (total phenolic content determined with TPC assay and antioxidant activity evaluated with DPPH and FRAP assays) of *C. medica*'s whole fresh fruits extract was evaluated by the analysis of variance (ANOVA). Experimental data were modeled considering the following second-order polynomial equation (Eq. (3)):

$$Y_n = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_2X_3 + b_1X_1^2 + b_2X_2^2 + b_3X_3^2 \quad (3)$$

Where Y_n indicates the responses (dependent variables), X_i indicates the independent variables (time, solvent and temperature), while β_0, β_i, β_{ii}, β_{ij} (i, j, k = 1, 2, 3), are the constant, linear, quadratic, and cross-product coefficients, respectively (Veličković, Stamenković, Todorović & Veljković, 2013).

2.11. Caco-2 cells

Human colon epithelial cells, Caco-2 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS, streptomycin (100 μg/mL), penicillin (100 units/mL) and 2 mM glutamine. Cells were incubated in a humidified atmosphere with 5 % CO_2 at 37 °C and sub-cultured at 80–90 % confluence. The extract was dissolved in DMSO and diluted to the tested concentrations with fresh medium. Dimethyl sulfoxide (DMSO)-treated cells (0.4 % *v/v*) were considered as control (CTRL) for all the experiments. For experimental studies Caco-2 cells were plated and used when fully differentiated.

2.12. Cell viability assay

Cell viability was analyzed following the enzymatic reduction of MTT to formazan, catalyzed by mitochondrial succinate(Lela, Ponticelli, Carlucci, Stevens, Faraone, Tzvetkov et al., 2024). Briefly, 1.5 × 10^4 cells/well were seeded in a 96-well plate and treated with different concentrations of OE (10–400 μg/mL). After 24 and 48 h, 100 μL of MTT solution (0.75 mg/mL) was added to each well. After 2 h, the incubation buffer was removed and the blue MTT–formazan product was extracted with DMSO and the absorbance was measured at 540 nm (SPECTROstarNano BMG Labtech, Ortenberg, Germany). To generate a condition of oxidative stress in Caco-2 cells and to determine exposure time affecting cell viability, *tert*-butylhydroperoxide (TBH) a known and strong pro-oxidant agent was added at different concentrations (0.25, 0.50, 1, 5, 7 mM), to the cells and cell viability was measured by MTT assay. Moreover, the protective effect of the extract was also evaluated against cytotoxic-induced effect of TBH at 1 mM for 3 h. For this assay, cells were seeded and pre-treated with OE extract (100 and 200 μg/mL) or NAC (10 mM) for 24 h, and then stressed with TBH (1 mM) for 3 h. Cell viability was measured by the MTT assay as reported above.

2.13. Observation of morphological changes

The fluorescent dye Hoechst 33258 was used to detect morphological changes like DNA condensation and nuclear fragmentation in Caco-2 cells. After 24 h pre-treatment with OE or NAC and 3 h TBH treatment, cells were washed with PBS and fixed in 4 % formalin for 20 min. Fixed cells were then stained with 10 μg/mL Hoechst 33258 at room temperature for 10 min in the dark to stain nuclei. Cells were observed and photographed under a fluorescence microscope (FLoid Cell Imaging Station, LIFE Technologies, Nikon 80i, Thermo Fisher Scientific, Waltham, MA, USA) (Rinaldi, Miglionico, Nigro, D'Orsi, Chiummiento, Funicello et al., 2021).

2.14. Evaluation of intracellular ROS

The intracellular accumulation of ROS in Caco-2 cells under chemical inducing conditions was evaluated by flow cytometry by measuring the oxidation of the nonfluorescent probe DCFH-DA to its fluorescent reduced form 2′,7′-dichlorofluorescein (DCF). Cells were seeded in a 24-well plate (1 × 10^5 cells/well) and treated with different concentration of OE extract (100, 200 μg/mL), and NAC 10 mM, for 24 h followed by the addition of TBH (1 mM for 3 hrs). After treatment cells were stained with DCFH-DA (10 μM, 30 min at 37 °C), and fluorescence was measured by BD FACSCanto II (BD Pharmingen, San Jose, CA, US) (λex 485 nm and λem 515–540 nm) (Lela, Ponticelli, Caddeo, Vassallo, Ostuni, Sinisgalli et al., 2022).

2.15. Lipid peroxidation

To estimate the lipid peroxidation, thiobarbituric acid reactive substance (TBARS) assay was performed (Morresi, Cianfruglia, Sartini, Cecati, Fumarola, Emanuelli et al., 2019). Malondialdehyde (MDA) is an aldehyde which forms a pink complex when reacts with thiobarbituric acid (TBA). Caco-2 cells were seeded into 12 well-plates (2 × 10^5 cells/well) and treated with NAC (10 mM) or 100 and 200 μg/mL of *C. medica* optimized extract for 24 h in serum free medium. After treatment, 1 mM of TBH was added for 3 h to induce oxidative stress. Cell culture medium was mixed with trichloroacetic acid (TCA, 10 %) and thiobarbituric acid (0.67 %) and incubated at 95 °C for 15′. After cooling at room temperature, samples were centrifuged at 1500 rpm for 10 min and the absorbance was measured at 532 nm. The MDA equivalents were calculated and expressed as nmol equivalent of MDA.

2.16. Determination of catalase activity

For measuring catalase activity, Caco-2 cell lysate (10 μL) was added to 480 μL of PBS (50 mM, pH: 7.0) and 100 μL H_2O_2 (40 mM) to initiate the reaction. The absorbance was spectrophotometrically determined at 240 nm. The activity of catalase was calculated from the equation Specific activity (units/mg of protein/min) = $A_{240\ nm}$ (1 min) * 1000/ 43.6 * mg of protein (Alam, Bristi & Rafiquzzaman, 2013).

2.17. Western blot analysis

Caco-2 cells were seeded in a 12-well plate (2 × 10^5 cells/well) and treated with 100 and 200 μg/mL of OE and NAC (10 mM) for 24 h followed by the addition of TBH (1 mM, for 3 h). Cell lysates were prepared using RIPA buffer (0.2 % SDS, 50 mM Tris–HCl pH 8, 1 % sodium deoxycholate, 150 mM NaCl, 1 % Igepal) supplemented with a phosphatase and protease inhibitors cocktail (Lela, Carlucci, Kioussi, Choi, Stevens, Milella et al., 2024). Protein content was determined by Bradford assay. Equal amounts of proteins were subjected to 10 % SDS-PAGE gels (*w/v*) and transferred to nitrocellulose membranes. As for primary antibodies, anti-*β*-ACTIN (1:5000), anti-SOD2 (1:1000), were purchased from Thermo Fisher Scientific, Milan, Italy; while anti-GPx-1/2 (1:400), anti-NQO1 (1:100) and anti-CAT (1:500) antibodies from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. The primary antibodies were captured with suitable peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive bands were visualized using iBright 1500 Imaging system. Densitometric analysis was performed by using Image J software and results were expressed as percentage of the value in comparison to the control sample (100 %).

2.18. Quantitative rt-pcr

Caco-2 cells were seeded into 12-well plates (2 × 105 cells/well) and treated with 100 and 200 μg/mL of OE and 10 mM NAC for 24 h followed by the addition of TBH (1 mM, for 3 h). After treatment, RNA was isolated by using a specific kit (Qiagen, Hilden, Germany) and stored at −80 °C until further analysis. The concentration and purity of the RNA was measured using SPECTROstar^{Nano} BMG Labtech, Ortenberg, Germany. All PCR primers were designed toward available *Homo sapiens* sequences and are reported in our previous work (Lela, Russo, De Biasio, Gorgoglione, Ostuni, Ponticelli et al., 2023). The cDNA amplification was performed by real time PCR using iTAQTM Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) by the StepOnePlusTM Real-Time PCR System (Applied Biosystems, USA). Samples were tested in triplicates and the gene expression was normalized to levels of *β*-actin. The comparative threshold cycle method (ΔΔCt) was used to quantify

Table 1
Independent and dependent variables with the respective code levels, results of total phenolic content (TPC) and antioxidant activity (DPPH, FRAP).

Run	Yield %	Independent variables			Dependent variables		
		X_1 Time (h)	X_2 Solvent (%EtOH/H_2O)	X_3 Temperature (°C)	TPC[1] mgGAE/g DW	DPPH[2] mgTE/g DW	FRAP[3] mgTE/g DW
1	4.28 ± 0.13	3 (0)	55 (0)	25 (−1)	2.57 ± 0.05^{i}	3.93 ± 0.01^{b,c,d,}	7.64 ± 0.07^{d,e,f}
2	5.36 ± 0.17	3 (0)	55 (0)	50 (0)	2.96 ± 0.06^{d,e,f,g,h,i}	3.37 ± 0.34 ^{h,i,j}	9.31 ± 0.30^{e,f}
3	5.80 ± 0.81	3 (0)	55 (0)	75 (+1)	2.36 ± 0.12^{i}	1.68 ± 0.28^{j,k}	7.55 ± 0.07^{d,e,f}
4	5.27 ± 0.36	3 (0)	15 (−1)	25 (−1)	2.77 ± 0.03^{f,g,h,i}	2.56 ± 0.16^{f,g,h}	6.62 ± 0.10^{e,f,g}
5	7.35 ± 0.14	3 (0)	15 (−1)	50 (0)	2.89 ± 0.09^{d,e,f,g,h,i}	3.26 ± 0.36^{d,e,f}	8.74 ± 0.45^{b,c,d,e}
6	6.21 ± 0.53	3 (0)	15 (−1)	75 (+1)	2.80 ± 0.30^{e,f,g,h,i}	2.03 ± 0.34 ^{h,i,j,k}	6.52 ± 0.09^{e,f,g}
7	3.98 ± 0.08	3 (0)	95 (+1)	25 (−1)	2.72 ± 0.20 ^{g,h,i}	3.48 ± 0.02^{c,d,e}	8.36 ± 1.63^{b,c,d,e,f}
8	4.45 ± 0.15	3 (0)	95 (+1)	50 (0)	3.07 ± 0.30^{b,c,d,e,f,g,h}	3.21 ± 0.07^{d,e,f}	8.28 ± 1.40^{c,d,e,f}
9	5.86 ± 0.03	3 (0)	95 (+1)	75 (+1)	2.78 ± 0.07^{e,f,g,h,i}	2.34 ± 0.21 ^{h,i,j}	8.16 ± 0.20^{c,d,e,f}
10	2.37 ± 0.24	1 (−1)	55 (0)	25 (−1)	3.42 ± 0.07^{a,b,c,d}	4.49 ± 0.13^{b}	7.33 ± 0.80^{e,f}
11	3.50 ± 0.13	1 (−1)	55 (0)	50 (0)	3.35 ± 0.13^{a,b,c,d,e,f}	4.21 ± 0.37^{a}	12.07 ± 0.01^{a}
12	3.50 ± 0.12	1 (−1)	55 (0)	75 (+1)	3.03 ± 0.39^{c,d,e,f,g,h}	2.11 ± 1.04 ^{h,i,j}	7.67 ± 1.42^{d,e,f}
13	4.22 ± 0.10	1 (−1)	15 (−1)	25 (−1)	2.77 ± 0.11^{i}	3.72 ± 0.08^{c,d,e}	6.00 ± 0.08^{f,g}
14	4.97 ± 0.15	1 (−1)	15 (−1)	50 (0)	3.05 ± 0.10^{c,d,e,f,g,h}	3.42 ± 0.06^{c,d,e}	10.84 ± 0.74^{a,b}
15	4.68 ± 0.59	1 (−1)	15 (−1)	75 (+1)	3.09 ± 0.06^{b,c,d,e,f,g,h}	2.12 ± 0.02 ^{h,i,j}	7.06 ± 0.25^{e,f}
16	2.91 ± 0.11	1 (−1)	95 (+1)	25 (−1)	3.68 ± 0.08^{a,b}	3.44 ± 0.17^{c,d,e}	7.53 ± 0.63^{d,e,f}
17	4.46 ± 0.02	1 (−1)	95 (+1)	50 (0)	3.86 ± 0.26^{a,b,c,d,e}	4.30 ± 0.16^{b,c,}	9.91 ± 1.60^{a,b,c,d}
18	4.21 ± 0.32	1 (−1)	95 (+1)	75 (+1)	3.58 ± 0.36^{a,b,c}	3.10 ± 0.18^{e,f,g}	8.75 ± 0.29^{b,c,d,e}
19	5.36 ± 0.24	6 (+1)	55 (0)	25 (−1)	2.86 ± 0.12^{d,e,f,g,h,i}	3.65 ± 0.02^{b}	10.86 ± 0.15^{a,b}
20	4.27 ± 1.53	6 (+1)	55 (0)	50 (0)	3.31 ± 0.20^{a,b,c,d,e,f,g,h}	3.57 ± 0.19^{c,d,e}	10.55 ± 0.30^{a,b,c}
21	5.85 ± 0.55	6 (+1)	55 (0)	75 (+1)	2.93 ± 0.07^{d,e,f,g,h,i}	1.73 ± 0.01^{i,j,k}	6.25 ± 1.79^{e,f,g}
22	6.62 ± 0.59	6 (+1)	15 (−1)	25 (−1)	2.78 ± 0.01^{e,f,g,h,i}	2.52 ± 0.48^{f,g,h}	7.90 ± 0.82^{d,e,f}
23	6.31 ± 0.13	6 (+1)	15 (−1)	50 (0)	2.67 ± 0.04 ^{h,i,}	2.03 ± 0.19 ^{h,i,j,k}	6.38 ± 0.10^{h}
24	5.59 ± 0.24	6 (+1)	15 (−1)	75 (+1)	2.72 ± 0.25 ^{g,h,i}	1.29 ± 0.05^{k}	4.44 ± 0.28 ^{g,h}
25	4.30 ± 0.44	6 (+1)	95 (+1)	25 (−1)	3.31 ± 0.07^{a,b,c,d,e,f,g}	3.53 ± 0.09^{c,d,e}	8.93 ± 0.25^{e,f,g}
26	6.08 ± 0.37	6 (+1)	95 (+1)	50 (0)	3.04 ± 0.29^{c,d,e,f,g,h}	2.62 ± 0.20^{f,g,h}	10.65 ± 1.41^{a,b,c}
27	5.31 ± 0.34	6 (+1)	95 (+1)	75 (+1)	3.43 ± 0.29^{a}	2.42 ± 0.20 ^{g,h,i}	8.39 ± 0.24^{b,c,d,e,f}

[1] TPC: total phenolic content expressed as milligrams of Gallic acid equivalents per grams of dry extract.
[2] DPPH: 2,2-diphenyl-1-picrylhydrazyl expressed as milligrams of Trolox equivalents per gram of dry extract;.
[3] FRAP: ferric reducing antioxidant power expressed as milligrams of Trolox equivalents per grams of dry extract; Results are expressed as mean ± standard deviation of mg/g dry weight (DW). In each column, significant differences ($p < 0.05$) between all samples of the same column are highlighted with different superscript letters (a–k).

the relative amounts of product transcripts.

2.19. Statistical analysis

Data were expressed as mean ± standard deviation (Mean ± SD). Statistical analysis was performed using GraphPad Prism 8 Software. Inc. (San Diego. CA. USA) and p values ≤ 0.05 were considered statistically significant.

3. Results and discussion

3.1. Model adequacy

With the increasing global prevalence of inflammatory bowel disease (IBD), there is a growing need for novel natural products rich in bioactive compounds, particularly antioxidants, that offer promising therapeutic potential in mitigating the inflammation and oxidative stress associated with this condition. To meet this demand with the possibility of industrial scale-up using minimal expensive equipment, it is essential to optimize the parameters that can affect the extraction of bioactive compounds such as temperature, extraction time, and solvent. Among these, the selection of an appropriate solvent is crucial for the extraction of compounds of interest, both in terms of extraction yield and industrial costs. For these reasons, different concentrations of ethanol (15, 55, 95 %), a safe solvent with a medium polarity range, have been selected. Several studies (Asfaw, Tadesse, Tessema, Woldemariam, Chinchkar, Singh et al., 2024; Mondal, Saha, Sarkar, Hossen, Hossain, Khalipha et al., 2021; Pasandide, Khodaiyan, Mousavi & Hosseini, 2017b; Suri, Singh & Nema, 2021) employed different extraction methods, extraction time, temperature and starting material (e.g. peel or waste). Therefore, taking these investigations as a starting point, this study aimed to optimize the extraction process from whole fresh *C. medica* fruits for the first time by selecting 1, 3 and 6 h and 25, 50 and 75 °C. In particular, the effect of the chosen variables on the antioxidant activity of *C. medica*'s fresh fruit was evaluated using the 3^3 full factorial experimental matrix, with the addition of 3 replications of the central point. The experimental matrix, containing uncoded levels along with the mean and standard error of total phenolic content and antioxidant activity, is listed in Table 1. Results from ANOVA are reported in **Table S2**. For statistical data analysis, no data transformation (e.g., Natural Log, Square roots, Base 10 Log, etc.) was employed. As can be observed in **Table S2,** in all cases, the model's statistically significant p-values provide evidence that the proposed model accurately estimates the tested responses. This is also confirmed when the Fit statistics are considered (**Table S3**), as the regression coefficient (R^2) values for the TPC, DPPH, and FRAP assays are 0.75, 0.81, and 0.77, respectively. Also noteworthy is the evaluated Adequate Precision, which indicates the signal-to-noise ratio. Normally, a ratio greater than 4 is preferred (Ponticelli, Carlucci, Mecca, Todaro, Milella & Russo, 2024). In the present study, ratios of 8.88, 10.82, and 11.37 were obtained for TPC, DPPH, and FRAP assays, respectively. This is indicative of an adequate signal, suggesting that the built model can be used to optimize the design. Finally, to validate the normal distribution of the model residuals, normal residual plots were also considered. As shown in **Figure S1**, the residuals are randomly distributed around the central line, indicating a good fit. Based on the obtained results, the optimization analysis was performed on the three dependent variables obtained from TPC, DPPH, and FRAP.

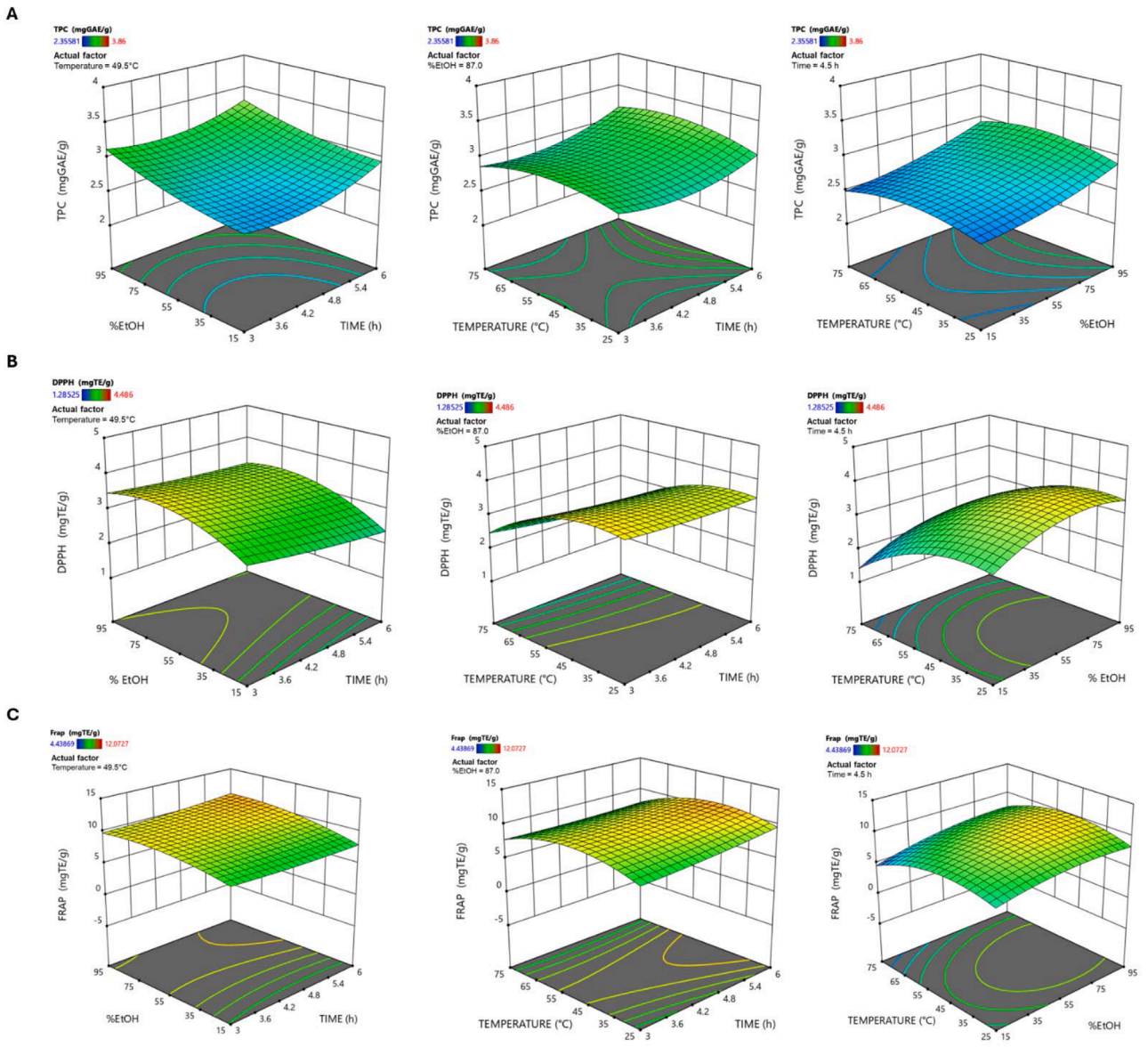

Fig. 1. 3D Surface Plots illustrating the relationships between the independent variables (time, solvent, and temperature) and depentent variables: TPC (A), DPPH (B), and FRAP (C).

3.2. Effect of extraction conditions on total phenolic content and antioxidant activity

Another fundamental point for industrial process is the extraction yield, above all to ensure the maximum amount of valuable compounds from the raw materials. As reported in Table 1, the extraction yields range from 2.37 ± 0.24 % (run 10) to 7.35 ± 0.14 % (run 5). *C. medica* fruit is a rich source of bioactive compounds such as phenolic acids and polyphenols, therefore a quali-quantitative spectrophotometric assay was performed in order to investigate the Total Phenolic Content (TPC). Among all the extracts obtained using different extraction parameters, TPC varied between 2.36 ± 0.12 and 3.86 ± 0.26 mgGAE/g DW. Our data analysis showed that the lowest total phenolic content was obtained using $X_1 = 3$ h, $X_2 = 55$ % EtOH, and $X_3 = 75$ °C as extractive parameters. On the other hand, the higher concentration was gained by applying $X_1 = 1$ h, $X_2 = 95$ % EtOH, and $X_3 = 50$ °C as extractive conditions. ANOVA results highlighted that extraction solvent (X_2, p-value = 0.0027) showed a higher significant effect on TPC, followed by the extraction time (X_1, p-value = 0.0301) (Fig. 1A). In fact, the ethanol percentage ranging from 70−100 % was found to be the most effective extraction solvent for *Citrus medica* L. fruit (Fratianni, Cozzolino, De Feo, Coppola, Ombra & Nazzaro, 2019; Francesco Menichini, Tundis, Loizzo, Bonesi, D'Angelo, Lombardi et al., 2016). The predicted equation (Eq. (4)) for total phenols extracted from *C. medica* whole fresh fruit is:

$$\begin{aligned} Y_{TPC} = {} & 2.78253 + 0.115407\ X_1 + 0.204675\ X_2 + 0.00468557\ X_3 \\ & - 0.0178507 X_1 X_2 + 0.015178\ X_1 X_3 + 0.0169666\ X_2 X_3 \\ & + 0.0430563\ X_1 X_2 X_3 + 0.156655\ X_1^2 + 0.0751057\ X_2^2 \\ & - 0.15244\ X_3^2 \end{aligned} \quad (4)$$

Where Y_{TPC} indicates the total phenolic content expressed in mgGAE/g dried weight while X_1, X_2, and X_3 represent the three variables. Based on this equation, the multiple response optimization was predicted: $X_1 = 1$ h, $X_2 = 95$ % EtOH, and $X_3 = 45.70$ °C, which should give a predicted concentration of 3.69 mgGAE/g (95 % PI 3.125 – 4.265).

Antioxidant activity was evaluated using both DPPH and FRAP tests, which are well established assays in this field. In our extracts, the lowest radical scavenging activity value evaluated by the DPPH assay (1.29 ± 0.05 mgTE/g) was obtained using $X_1 = 6$ h, $X_2 = 15$ % EtOH, and $X_3 =$

75 °C as extractive parameters, while the higher value (4.49 ± 0.13 mgTE/g) was gained by applying $X_1 = 1$ h, $X_2 = 55$ % EtOH, and $X_3 = 25$ °C as extractive conditions. Based on the FFD, the predicted model equation (Eq. (5)) for the DPPH scavenging activity is reported as follow:

$$Y_{DPPH} = 3.36141 - 0.189458\ X_1 + 0.356325\ X_2 - 0.648918\ X_3 + 0.0642474\ X_1X_2 - 0.00592094\ X_1X_3 + 0.0079452\ X_2X_3 - 0.0710205\ X_1X_2X_3 + 0.0465737\ X_1^2 - 0.438029\ X_2^2 - 0.49789\ X_3^2 \quad (5)$$

Where Y_{DPPH} indicates the radical scavenging activity expressed in mgTrolox/g of dried weight while X_1, X_2, and X_3 represent the three variables. Considering this quadratic polynomial equation, the response optimization to maximize the radical scavenging activity was performed, obtaining the following extraction parameters: $X_1 = 1$ h, $X_2 = 65.10$ % EtOH, and $X_3 = 34.59$ °C with a predicted response of 4.26 mgTE/g (95 % PI 3.214 – 5.314). Among all tested extraction parameters, temperature (X_3, p-value < 0.0001) showed the most significant effect on the extracts' radical scavenging activity, followed by the extraction solvent (X_2, p-value = 0.0083) (Fig. 1B). The significance of their quadratic terms also confirms this effect (X_3^2, p-value = 0.012; X_2^2, p-value = 0.0187).

Regarding FRAP test, the highest value (12.07 ± 0.01 mgTE/g DW) was obtained using the following conditions: $X_1 = 1$ h, $X_2 = 55$ % EtOH, and $X_3 = 50$ °C, while the lowest value (4.44 ± 0.28 mgTE/g DW) was obtained using $X_1 = 6$ h, $X_2 = 15$ % EtOH, and $X_3 = 75$ °C as extractive conditions. The relationship between the FRAP test and the extraction parameters is shown as follows (Eq. (6)):

$$Y_n = 9.86272 + 0.16177\ X_1 + 1.08664\ X_2 - 0.785406\ X_3 + 0.362944\ X_1X_2 - 0.573359\ X_1X_3 + 0.419645\ X_2X_3 + 0.220539\ X_1X_2X_3 + 0.191855\ X_1^2 - 0.863888\ X_2^2 - 2.06991\ X_3^2 \quad (6)$$

Where Y_{FRAP} indicates the Ferric Reducing Antioxidant Power expressed as mgTE/g DW while X_1, X_2, and X_3 represent the three variables. Considering this equation, the response optimization for FRAP was estimated: $X_1 = 6$ h, $X_2 = 87.72$ %, and $X_3 = 43.18$ °C with a predicted value of 10.99 mgTE/g DW (95 % PI 8.497 – 13.497). As reported in Fig. 1C, extraction solvent (X_2, p-value = 0.0008) plays a crucial role in the response of FRAP, followed by extraction temperature (X_3, p-value = 0.0082). Also, in this case, the significance of their quadratic terms confirms their influence (X_2^2, p-value = 0.0377; X_3^2, p-value < 0.0001). Noteworthy is the significance of the negative synergistic effect of time and temperature ($X_1 \times_3$, p-value = 0.0038), which suggests that using long times in conjunction with high temperatures may reduce the effectiveness of the process.

Overall, the highest values in terms of TPC, DPPH and FRAP were obtained using an extraction time of 1 hour. This is a more attractive option for industrial-scale up than an extraction time of 6 h. Furthermore, our findings are in line with those reported in several other studies confirming that EtOH/H_2O mixture enhance the extraction of antioxidant compounds, whereas high temperatures have a negative effect on antioxidant activity (Barrales, Silveira, Barbosa, Ruviaro, Paulino, Pastore et al., 2018; Huang, Xue, Niu, Jia & Wang, 2009; Phucharoenrak, Muangnoi & Trachootham, 2022). In Fig. 1, the 3D Surface Plots allow visualization of how changes in extraction time, ethanol percentage, and temperature affect the responses

3.3. Multiple response optimization

The ultimate goal of the optimization process in our study, is to identify the precise combination of extraction parameters that will produce an extract with the highest total phenolic content and antioxidant activity. The multiple response optimization based on the results from each FFD analysis showed that maintaining an extraction time of 1 hour, an ethanol concentration of 85.30 %, and a temperature of 47.22 °C would achieve the maximum levels of all desired outcomes with an extraction yield of 3.56 ± 0.09 % *w/w*. Using these extraction parameters, the mathematical model has predicted the following responses for TPC (3.600 mg/GAE/g DW, 95 % PI = 3.046; 4.155), DPPH (4.051 mg/TE/g DW, 95 % PI = 2.995; 5.108) and FRAP (10.117 mg/TE/g DW, 95 % PI = 7.642; 12.592) respectively. To validate the optimized extraction parameters, an additional extraction was performed using the same solvent-to-drug ratio and the following extraction variables: $X_1 = 1$ h, $X_2 = 85.30$ % EtOH, and $X_3 = 47.22$ °C. In **Table S4,** are reported the predicted values from the FFD analysis and the confirmed experimental data. The model predicted values were confirmed, obtaining the following results: 3.79 ± 0.09 mg GAE/g DW, 4.79 ± 0.04 TE/g DW,

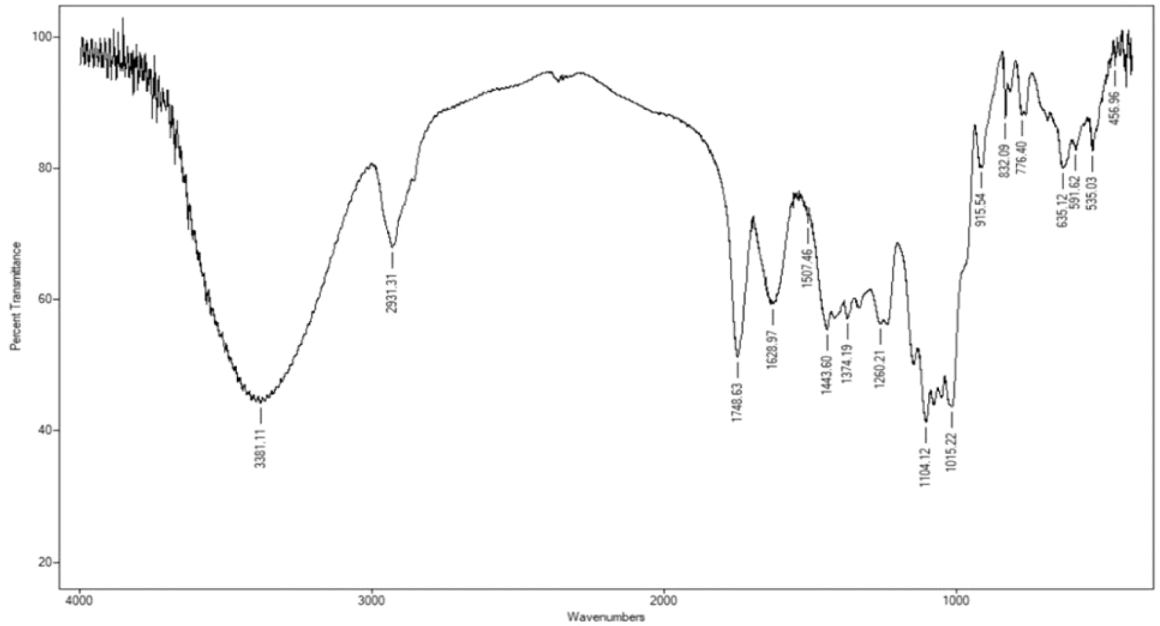

Fig. 2. FTIR spectra of pectin obtained from the exhausted whole fruit of *C. medica*.

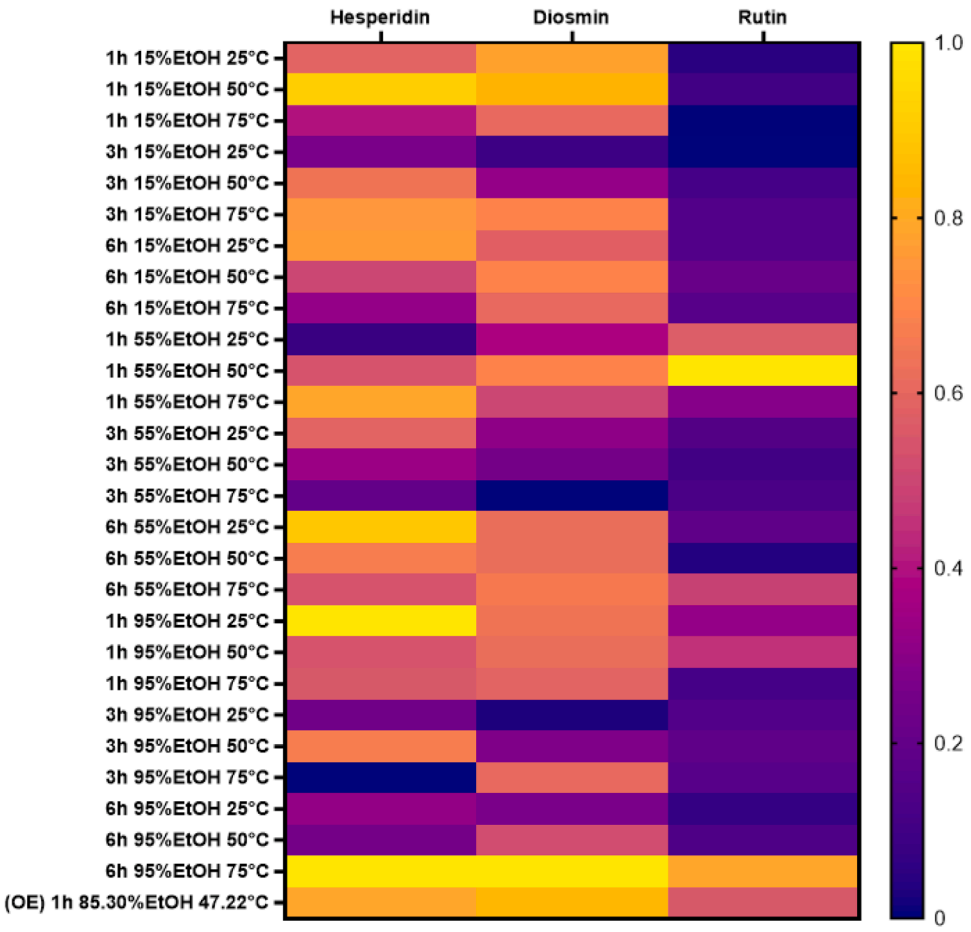

Fig. 3. Heatmap visualization of Rutin, Hesperidin, and Diosmin content in 27 extracts derived from the RSM experimental design plus the optimized sample (OE) obtained using 1 h, 85.30 % EtOH, 47.22 °C. Data represents normalized peak areas (0–1 scale) obtained from MS-DIAL software, reflecting relative abundance of each compound within the extracts.

10.06 ± 0.34 mg TE/g DW for TPC, DPPH, and FRAP, respectively.

Despite the lack of specific studies on *C. medica* ethanol whole fruit extract, OE showed stronger antioxidant activity than range values of 0.38−1.48 μM Trolox/g for DPPH and FRAP reported by Zhao et al., employing ultra-sonicator with 50 % methanol for 30 min and different starting material (Zhao, Duan, Guo, Dou, Dong, Zhou et al., 2015). In addition, OE exhibited higher total phenolic content in comparison with the aforementioned work (2.74 ± 1.12 mg GAE/g DW) and with a previous study conducted by Hasan et al. (Hasan, Roy, Alam, Hoque & Zzaman, 2022), reporting a range of 5.12 to 7.48 mg GAE/100 g for *C. medica* peel extracted with methanol for 2–3 days at room temperature.

Conversely, the total phenolic content of OE (3.79 ± 0.09 mg GAE/g DW) appears to be lower than the values reported in previous studies using single sections of the fruit (e.g. pulp, peel), given that, in this study, the whole fruit was used. In fact, Menichini et al. reported values range from 109.4 ± 2.9 mg/100 g FW (mature endocarp) to 262.6 ± 3.7 mg/100 g FW (immature endocarp) (Federica Menichini, Loizzo, Bonesi, Conforti, De Luca, Statti et al., 2011) suggesting that there may also be a significant decrease in phenolic compounds during ripening. This hypothesis was also confirmed by Mateaus et al. and Wu et al. (Mateus, Teixeira, Barros, Almeida, Silva & Sanches-Silva, 2024; Wu, Li, Yang, Zhan & Tu, 2013). Unfortunately, our results are not directly comparable due to the different starting material, reference standard, and to the fact that they performed an exhaustive maceration with 70 % aqueous alcohol 96 *h* × 5 times. This discrepancies highlights that the OE's antioxidant activity is attributed to the compounds present in all sections of the fruit, including the mesocarp, which represents 70 % of the fruit composition.

3.4. Pectin recovery

In this study, we not only optimized the extraction of bioactive compounds with promising antioxidant activity from whole fresh fruits, but also aimed to increase the value of the exhausted extraction material. In fact, in the context of optimizing industrial processes for the recovery of bioactive substances, it is equally important to aim for zero waste production. Although there are a lot of studies on the recovery of pectin from the genus Citrus (Kanmani, Dhivya, Aravind & Kumaresan, 2014; Kurita, Fujiwara & Yamazaki, 2008; Lin, Liu, Wang, Dai, Wang & Zhang, 2024), as far as is known, there are no studies on the pectin recovery from the exhausted material after the extraction of compounds of interest with potential biological activity. Several studies reported only extraction methods to obtain pectin, especially from the peel or waste of citrus fruits (Chandrasekar, Carullo, Saitta, Krishnamachari, Bellesia, Nespoli et al., 2024; Du, Zhang, Waterhouse, Zhou, Xu, Wang et al., 2024). It is now known that the different extraction parameters affect the chemical, physical, and technological characteristics of pectin. The calculated degree of esterification 76.25 ± 5.43 % indicated that the pectin extracted could be classified as high methoxyl pectin (HMP). In Fig. 2 is reported the FTIR spectrum of the pectin extracted in water at 90 °C for 180 min, whereas the typical functional groups of the pectin are showed in **Table S5.** The wide peak between 3200 and 3600 cm^{-1} is related to OH groups. The peak at around 2931.31 cm^{-1} is attributed to C—H of CH, CH_2 and CH_3 groups (Wang, Chen & Lü, 2014). Also, the peak at about 1748.63 cm^{-1} is due to the vibration of the $C = O$ of the

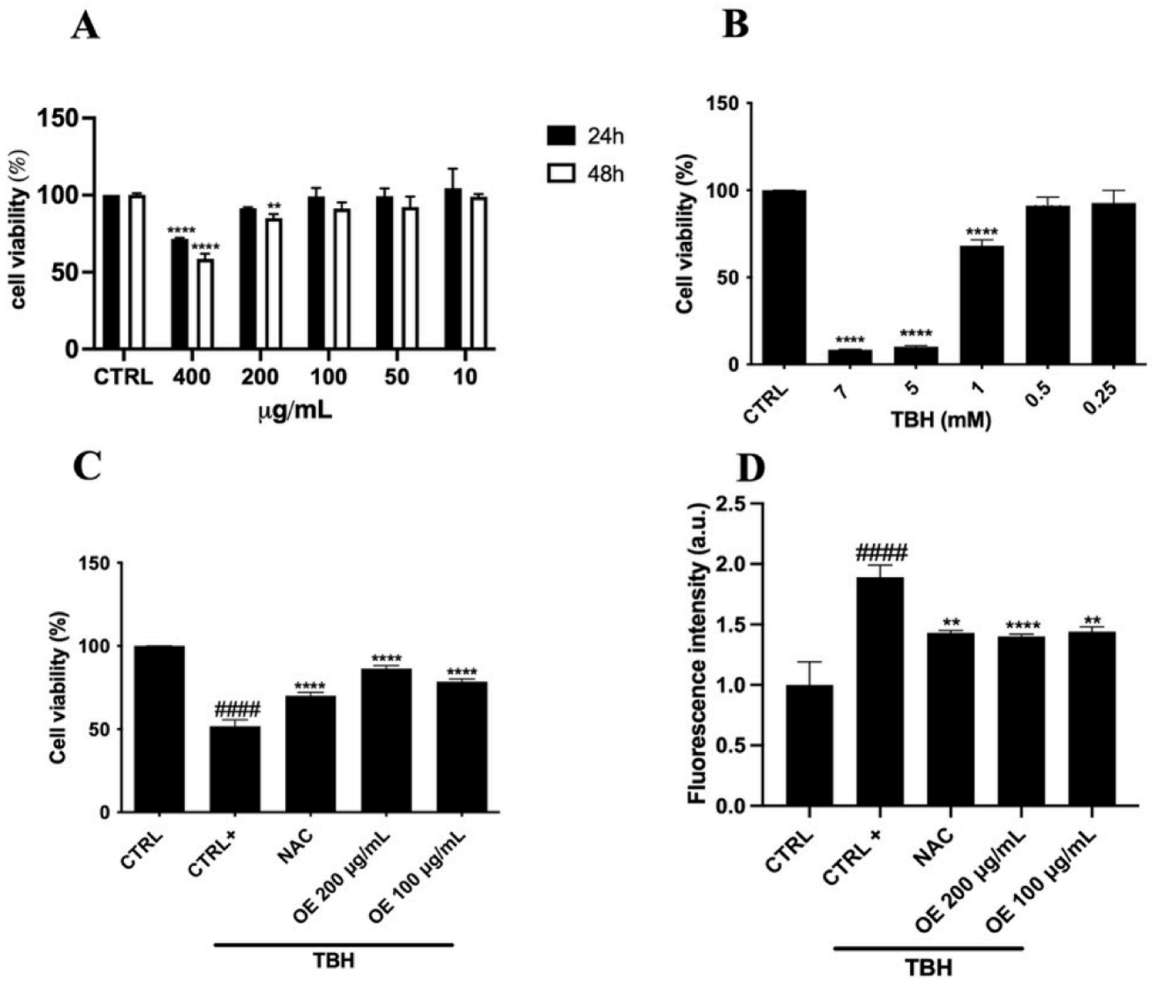

Fig. 4. (A) Effect of *C. medica* optimized extract (OE) on cell viability evaluated by MTT assay in Caco-2 cells. (B) Cytotoxicity induced by *tert*-butyl hydroperoxide (TBH) after 3 h incubation period in Caco-2 cells. (C) Protective effect of *C. medica* optimized extract (OE) or *N*-acetylcysteine (NAC, positive control) in cell viability against stress induced by 1 mM TBH in Caco-2 cells. Caco-2 cells were treated with OE or NAC for 24 h, then stressed by TBH for 3 h. Data were normalized as % control (CTRL). (D) Effect of OE extract or NAC on intracellular ROS generation in Caco-2 cells under TBH-stressed conditions. Data are expressed as the mean ± standard deviation (SD) of three independent experiments ($n = 3$) and analyzed by one-way ANOVA followed by Tukey's post-hoc test. #### $p < 0.0001$ vs. untreated cells (CTRL), **** $p < 0.0001$, ** $p < 0.01$ vs. TBH stressed cells (CTRL+).

methyl ester group ($COOCH_3$). Carboxylate groups have two peaks: one peak is related to asymmetrical stretching vibration at 1628.97 cm^{-1}, and another one is due to weaker symmetric vibrating at 1443.60 cm^{-1}. The two strong absorptions at 1007.30 and 1110.67 cm^{-1} are attributed to glycosidic linkage between sugar units (Gnanasambandam & Proctor, 2000). According to Pasandide et al., increasing the extraction time is crucial for the extraction of the polysaccharide trapped in plant cells. In this case, optimized extraction conditions were used, obtaining a yield of 14.78 ± 1.26 %; slightly lower than the yield of 22.34 % obtained by Pasandide et al. (Pasandide, Khodaiyan, Mousavi & Hosseini, 2017a). This difference can certainly be attributed to the ripeness of the fruits, but also to the fact that, in this case, the whole citrus fruit was first subjected to digestion for 1 hour with 85.30 % ethanol at 47.22 °C to extract bioactive compounds.

3.5. LC—HRMS untargeted-targeted analysis

To better understand the differences between the obtained extracts and their different correlation with the selected antioxidant assays, a comprehensive metabolic profiling was performed on 28 samples (27 prepared as part of the FFD experimental design plus the optimized extract). The untargeted screening was performed using a combination of MS-DIAL, MS-CLEANR, and MS-FINDER software suites. Once the final annotated peak list was obtained, Pearson correlation was used to establish the relationships between antioxidant activity (assessed by TPC, DPPH, FRAP) and the peaks area of specific metabolites in each extract. Thus, inspecting the **Table S6**, 39 putative structures with positive correlation were reported as potential antioxidants. These compounds can be collectively categorized into flavonoids, coumarins, phenols, terpenoids, fatty acids and amino acids. Of these, MS-FINDER software allowed the putative identification of 23 compounds at level 3 based on the comparison of the predicted or *in silico* generated spectra to the experimental data. Imperatorin/isoimperatorin was identified at confidence level 2 by comparison of the observed mass spectrum with a reference spectrum from MassBank or the literature (Duan, Guo, Liu, Liu & Li, 2014; Zhao et al., 2015). As shown in the heatmap (Fig. 3), increasing the ethanol concentration while reducing both the extraction time and temperature significantly improves the diosmin extraction yield. As reported in **Table S7**, rutin (4.27 ± 1.35 mg/100 g DW), hesperidin (29.83 ± 8.82 mg/100 g DW), diosmin (50.83 ± 6.64 mg/100 g DW) were confirmed and quantified in the OE using authentic standards, demonstrating that the untargeted analysis by MS-FINDER software predicted the identification of these compounds with high score level. These latter compunds are well-known bioactive metabolites, naturally found in citrus fruits and are associated with numerous health benefits. For instance, diosmin is well known for its effectiveness in supporting

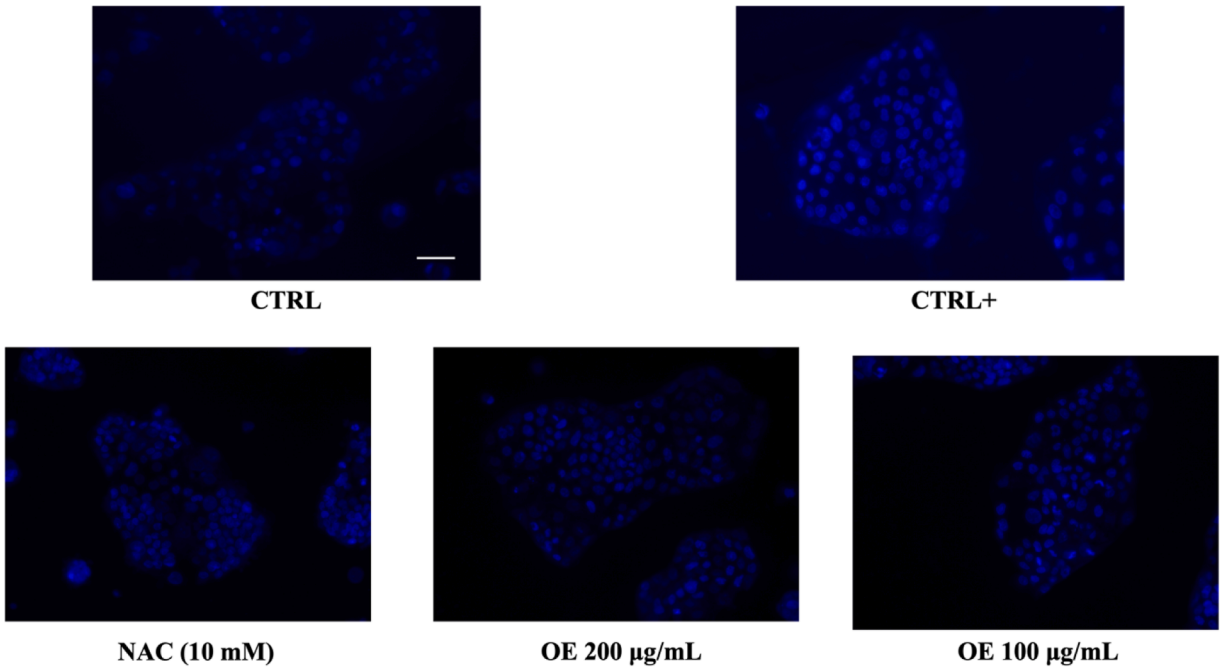

Fig. 5. Nuclear staining of Caco-2 cells by Hoechst 33258. Caco-2 cells were pre-treated with *Citrus medica* optimized extract (OE) or NAC (*N*-acetylcysteine, positive control) for 24 h and then stressed with *tert*-butyl hydroperoxide (TBH) for 3 h. The dose-dependent changes in nuclear morphology were observed under fluorescence microscope. Scale bars: 100 µm.

the treatment of chronic venous insufficiency (CVI) as well as in preventing oxidative stress restoring the activity of cellular antioxidant enzymes (Wójciak, Feldo, Borowski, Kubrak, Płachno & Sowa, 2022). Similar to diosmin, the antioxidant activity of hesperidin is ascribable to the presence of hydroxyl groups which characterize both structures. In addition, imperatorin/isoimperatorin (furocoumarin), was also identified in pomelo cold-pressed essential oil and other cultivars of *C. medica* showing good radical scavenging capacity (Li, Cheng, Zhang, Li, Han & Liang, 2021; Ma, Chen, Chen, Wu, Zhu, He et al., 2023).

3.6. Effect of OE on cell viability, morphology and intracellular ROS

To further support the antioxidant potential of *C. medica* optimized extract evaluated by spectrophotometric assays, intestinal Caco-2 cell line was used. Caco-2 cells, represent an extensively used model to study intestinal absorption, metabolism, inflammation and oxidative stress in the context of gastrointestinal health (Iftikhar, Iftikhar, Zhang, Gong & Wang, 2020; Morresi, Vasarri, Bellachioma, Ferretti, Degl′ Innocenti & Bacchetti, 2022). The safety of the extract was first determined using the MTT test. Treatment with OE reduced cell viability by 30 % and approximately 50 % at 400 µg/mL after 24 and 48 h, respectively (Fig. 4A). Based on this result, further experiments were conducted over 24 h using doses of 200 and 100 µg/mL. Oxidative stress plays a significant role in the pathogenesis and progression of inflammatory bowel diseases (IBD). Excessive production of ROS in the inflamed intestinal mucosa leads to cellular damage, including lipid peroxidation, DNA damage, and protein oxidation (Bourgonje, Feelisch, Faber, Pasch, Dijkstra & van Goor, 2020). Additionally, oxidative stress markers were found to be elevated in the intestinal tissues of IBD patients (Muro, Zhang, Li, Zhao, Jin, Mao et al., 2024). To induce oxidative stress, Caco-2 cells were incubated with the strong prooxidant TBH. Cells were incubated with different concentrations of TBH for 3 h, and cell viability was measured by the MTT assay. As reported in Fig. 4B there was a significant dose-dependent decrease in cell viability indicating a cytotoxic effect. A 30 % inhibition of cell viability was observed at 1 mM, and this concentration was used in subsequent experiments. Indeed, the protective effect of OE extract or NAC on cell death induced by TBH (1 mM) was measured after 3 h of incubation. As expected, TBH (CTRL+) reduced cell viability compared to untreated cells (CTRL, Fig. 4C). Treatment of Caco-2 cells with OE (100 and 200 µg/mL) or NAC (10 mM) prevented TBH-induced cell death. Notably, OE provided a significant increase in cell survival (30 % and 25 % at 200 and 100 µg/mL, respectively, compared to CTRL+). TBH is a strong source of ROS therefore intracellular levels were assessed in Caco-2 cells by considering the oxidation of the nonfluorescent probe DCFH-DA to the fluorescent form 2′,7′-dichlorofluorescein (DCF). TBH (CTRL+) induced an approximately 2-fold increase in fluorescence intensity in Caco-2 cells, meaning an increase in intracellular ROS, compared to untreated cells (CTRL, Fig. 4D). However, pretreatment with OE extract dose-dependently reduced ROS levels by 30 % at 200 µg/mL, even more than the antioxidant molecule NAC, used as a positive control. This finding is consistent with the work of Hong et al., who reported the protective effect of the ethanolic extract of *C. medica* var. sarcodactylis by reducing intracellular ROS concentration in human epidermal keratinocytes (HaCaT) stimulated by H_2O_2 (Mengsa, Kun, Pei & Jun 2022). *C. medica*'s ability to counteract or prevent oxidative stress may be largely due to its phenolic content, as phenolic compounds are known for their ability to donate hydrogen atoms to neutralize free radicals. In fact, the presence of flavonoids, and other polyphenolic compounds could contribute to its ability to scavenge free radicals, thereby protecting cells from oxidative damage. Among them, hesperidin is one of the most powerful ROS scavenger. This activity is associated with the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for the elimination of free radicals, i.e. the catechol in ring B (Wilmsen, Spada & Salvador, 2005). Furthermore, in human hepatocytes, hesperidin prevented TBH-induced cell damage by upregulating heme oxygenase-1 (HO-1), a cytoprotective enzyme, resulting in decreased intracellular ROS production. Diosmin also showed protective potential in endothelial cells subjected to H_2O_2 stress by restoring the activity of cellular antioxidant enzymes (Purba, Mayangsari, Setyaningsih, Chansuwan & Sirinupong, 2024). Oxidative stress is a critical factor influencing cell survival; the use of the lipophilic and cell permeable dye Hoechst 33258 allowed to assess cell viability and apoptosis in response to oxidative stress. Hoechst dye selectively

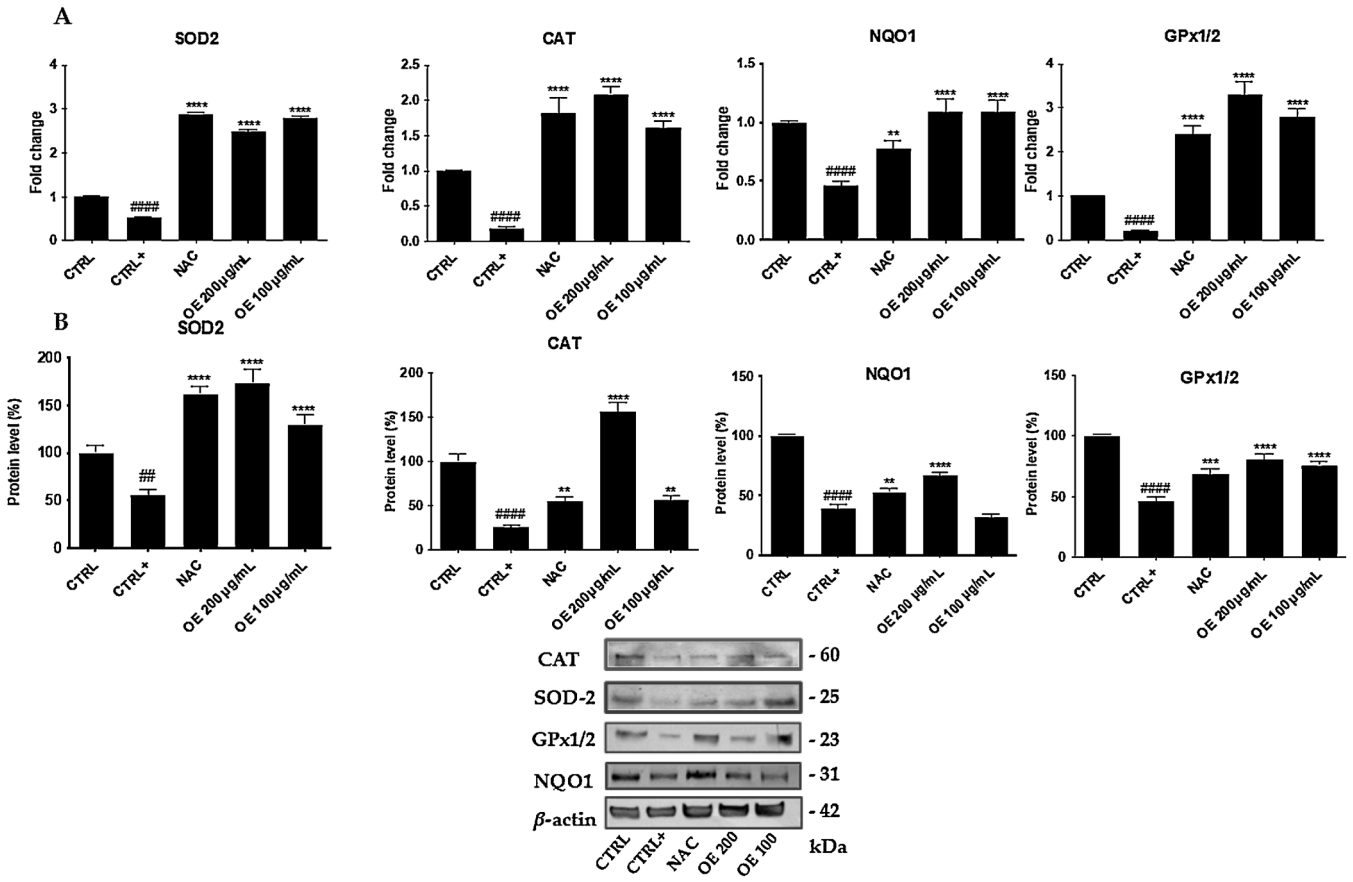

Fig. 6. Effect of optimized extract (OE) of *Citrus medica* on (A) gene and (B) protein expression. OE significantly induces the expression of the antioxidant enzymes superoxide dismutase (SOD2), catalase (CAT), glutathione peroxidase (GPx) and NAD(P)H dehydrogenase (quinone 1) (NQO1) in Caco-2 cells treated for 24 h with the extract followed by 3-hour stress with TBH. NAC: *N*-acetylcysteine, positive control. Data are normalized to the housekeeping gene β-actin and are shown as treated/control fold change ± standard deviation (SD), from three independent experiments ($n = 3$). Cell lysate was subjected to western blot analysis and protein content was normalized against β-actin. Densitometric analysis of the immunoreactive bands are expressed as the mean ± SD of three independent experiments ($n = 3$) and analyzed by one-way ANOVA followed by Tukey's post-hoc test #### $p < 0.0001$, ## $p < 0.01$ vs. control cells (CTRL); **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ compared to TBH-treated control cells (CTRL+).

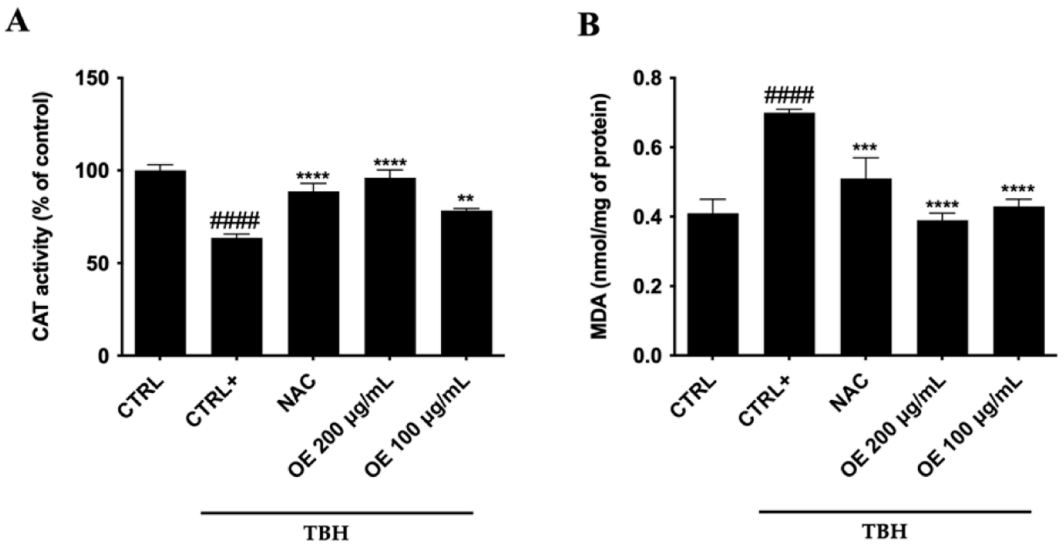

Fig. 7. Effect of *C. medica* optimized extract (OE) on A) antioxidant enzyme catalase (CAT) activity and B) inhibition of lipid peroxidation in Caco-2 cells. Cells were pretreated with OE and NAC (*N*-acetylcysteine used as positive control) followed by the induction of oxidative stress with TBH for 3 h. Data are expressed as the mean ± standard deviation (SD) of three independent experiments ($n = 3$) and analyzed by one-way ANOVA followed by Tukey's post-hoc test. #### $p < 0.0001$ vs. untreated cells (CTRL), **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ vs. TBH stressed cells (CTRL+).

binds to DNA, allowing identification of live and dead cells based on nuclear morphology and membrane integrity. As shown in Fig. 5, untreated cells (CTRL) presented a regularly shaped nuclear morphology and homogeneous staining. In contrast, Hoechst 33258 staining showed that nuclear DNA condensation and nuclear fragmentation occurred after treatment with TBH, 1 mM (CTRL+). TBH-treated cells, in fact, undergo apoptosis phenomena appearing with bright blue dots representing condensed chromatin. The addition of the extract, however, reduced the accumulation of the fluorescent dye, increased Caco-2 cell viability and reported protective effects to the cells whose nuclei were less bright in comparison to TBH-treated cells.

3.7. Effect of OE on the expression of antioxidant enzymes and lipid peroxidation

As far as it is known, there are no studies on the cultivar Diamante Liscia of *C. medica*, therefore, the exact mechanism through which the extract exerts its antioxidant effects remains to be fully elucidated. To detect the change in gene and protein expression induced by treatment with OE in a stressed model of Caco-2 cells, qRT-PCR and western blot were used, respectively. TBH treatment reduced the expression of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and NAD(P)H dehydrogenase (quinone 1) (NQO-1). However, in cells pre-treated for 24 h with OE, both gene (Fig. 6A) and protein (Fig. 6B) levels of antioxidant enzymes were increased, demonstrating that the extract exerts a protective effect from oxidative stress by acting on these proteins. More specifically, treatment with OE restored basal levels or even increased them in a manner comparable to the effect of NAC. Furthermore, OE also affected catalase activity. As reported in Fig. 7A, CAT enzymatic activity was significantly reduced by 40 % in TBH-stressed cells (CTRL+), compared to untreated cells (CTRL). Caco-2 cells pre-treatment with OE or NAC statistically increased the enzymatic activity. Specifically, OE exerted a dose-dependent effect restoring basal level at 200 μg/mL. Moreover, the present study explored the effect of the optimized extract on malondialdehyde (MDA) levels in Caco-2 cells, a known byproduct of polyunsaturated fatty acid peroxidation and indicator of oxidative stress (Del Rio, Stewart & Pellegrini, 2005). As expected, TBH caused oxidative damage in Caco-2 cells by increasing MDA levels almost twofold compared to untreated cells (CTRL, Fig. 7B). Pre-incubation with two concentrations of OE extract or NAC for 24 h significantly reduced cellular MDA levels compared to TBH-treated cells (CTRL+) by restoring basal MDA levels. This result confirms the protective effect of the OE extract against oxidative stress. The elevated MDA levels induced by TBH can led to cellular dysfunction and health diseases. Treatment with the extract resulted in a significant reduction in MDA levels suggesting that *C. medica* has a protective effect against lipid peroxidation, thereby reducing oxidative damage. Other citrus species have reported similar effects. Ademosun et al. demonstrated that *C. limon, C. aurantium* and *C. maxima* peel extracts reduced lipid peroxidation in heart tissue homogenate (Ademosun, Adebayo & Oboh, 2019). Overall, these results described the potential mechanisms through which *C. medica* extract could protect Caco-2 cells from oxidative stress.

4. Conclusion

This study underscores the importance of optimizing extraction processes for bioactive compounds from *C. medica*, specifically focusing on enhancing the yield of antioxidants from fresh whole fruits. Thus, Full Factorial Design allowed obtaining the optimal condition for the simultaneous maximum extraction in terms of phenols evaluated by TPC, and antioxidant activity measured by DPPH and FRAP tests. The optimal experimental conditions include time of 1 h, ethanol concentration of 85.30 % and 47.22 °C as temperature. Our findings indicate that the FFD represented a valid model in this study to select the best extraction conditions. Particularly, extraction time plays a critical role; prolonged extraction can lead to degradation of phenolic compounds, suggesting that 1 h may be more beneficial both for preserving antioxidant activity and reducing extraction time during the industrial working process. Our approach not only maximizes the extraction of bioactive compounds, but also adds value to the exhausted material including the recovery of pectin. This study reported for the first time the combined FFD approach with untargeted analysis to find out the key bioactive compounds potentially linked with the antioxidant activity evaluated by well-established in vitro assays as well as TPC, DPPH and FRAP. Additionally, MS-DIAL, MS-CLEANR, and MS-FINDER software suites predicted with high score level the presence of antioxidant compounds such as diosmin, hesperetin, rutin in the optimized extract (OE). Moreover, OE demonstrated antioxidant properties in Caco-2 cell line, highlighting its potential as a therapeutic agent in preventing or mitigating oxidative stress-related diseases, such as inflammatory bowel diseases. The use of natural antioxidants from food sources, such as *C. medica*, offers a promising alternative to synthetic antioxidants, which have been associated with various side effects.

Ethical statement - studies in humans and animals

It is hereby declared that no studies involving human participants or animals were conducted for this research.

Funding

This research was funded by Project PHENYLOVE, funded under PNRR mission 4 component 2 “ON Foods (Food and Nutrition Research and Innovation Network Sustainability, Safety and Security - Working ON Foods)” code PE000003, CUP H93C22000630001”

CRediT authorship contribution statement

Vittorio Carlucci: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tereza Jaegerova:** Writing – review & editing, Software, Methodology, Investigation, Data curation. **Ludovica Lela:** Writing – review & editing, Software, Methodology, Investigation, Data curation. **Immacolata Faraone:** Writing – review & editing. **Luigi Milella:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis. **Maria Ponticelli:** Writing – review & editing, Software, Methodology. **Jana Hajslova:** Writing – review & editing, Supervision, Resources, Formal analysis.

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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2025.101296.

Data availability

Data will be made available on request.

References

Ademosun, A. O., Adebayo, A. A., & Oboh, G. (2019). Modulatory effect of some citrus (Citrus limon, Citrus reticulata, Citrus maxima) peels on monoamine oxidase, phosphodiesterase-5 and angiotensin-1 converting enzyme activities in rat heart homogenate. *Journal of Complementary Integrative Medicine, 16*(1), Article 20180067. https://doi.org/10.1515/jcim-2018-0067

Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal, 21*(2), 143–152. https://doi.org/10.1016/j.jsps.2012.05.002

Asfaw, T. B., Tadesse, M. G., Tessema, F. B., Woldemariam, H. W., Chinchkar, A. V., Singh, A., Upadhyay, A., & Mehari, B. (2024). Ultrasonic-assisted extraction and UHPLC determination of ascorbic acid, polyphenols, and half-maximum effective concentration in Citrus medica and Ziziphus spina-christi fruits using multivariate experimental design. *Food Chemistry: X, 22*, Article 101310. https://doi.org/10.12980/APJTB.4.2014APJTB-2014-0273

Barrales, F. M., Silveira, P., Barbosa, P.d. P. M., Ruviaro, A. R., Paulino, B. N., Pastore, G. M., Macedo, G. A., & Martinez, J. (2018). Recovery of phenolic compounds from citrus by-products using pressurized liquids—An application to orange peel. *Journal Of Food Bioproducts Processing, 112*, 9–21. https://doi.org/10.1016/j.fbp.2018.08.006

Benedetto, N., Carlucci, V., Faraone, I., Lela, L., Ponticelli, M., Russo, D., Mangieri, C., Tzvetkov, N. T., & Milella, L. (2023). An insight into citrus medica linn.: A systematic review on phytochemical profile and biological activities. *Plants, 12*(12), 2267. https://doi.org/10.3390/plants12122267

Bocco, A., Cuvelier, M.-E., Richard, H., & Berset, C. (1998). Antioxidant activity and phenolic composition of citrus peel and seed extracts. *Journal Of Agricultural Food Chemistry, 46*(6), 2123–2129. https://doi.org/10.1021/jf9709562

Bourgonje, A. R., Feelisch, M., Faber, K. N., Pasch, A., Dijkstra, G., & van Goor, H. (2020). Oxidative stress and redox-modulating therapeutics in inflammatory bowel disease. *Trends In Molecular Medicine, 26*(11), 1034–1046. https://doi.org/10.1016/j.molmed.2020.06.006

Carlucci, V., Ponticelli, M., Russo, D., Labanca, F., Costantino, V., Esposito, G., & Milella, L. (2024). Nutraceutical valorization of exhausted olive pomace from Olea europaea L. Using advanced extraction techniques. *Plants, 13*(16), 2310. https://doi.org/10.3390/plants13162310

Chandrasekar, C. M., Carullo, D., Saitta, F., Krishnamachari, H., Bellesia, T., Nespoli, L., Caneva, E., Baschieri, C., Signorelli, M., & Barbiroli, A. G. (2024). Valorization of citrus peel industrial wastes for facile extraction of extractives, pectin, and cellulose nanocrystals through ultrasonication: An in-depth investigation. *Carbohydrate Polymers, 344*, Article 122539. https://doi.org/10.1016/j.carbpol.2024.122539

Del Rio, D., Stewart, A. J., & Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism Cardiovascular Diseases, 15*(4), 316–328. https://doi.org/10.1016/j.numecd.2005.05.003

Du, Y., Zhang, S., Waterhouse, G. I., Zhou, T., Xu, F., Wang, R., Sun-Waterhouse, D., & Wu, P. (2024). High-intensity pulsed electric field-assisted acidic extraction of pectin from citrus peel: Physicochemical characteristics and emulsifying properties. *Food Hydrocolloids, 146*, Article 109291. https://doi.org/10.1016/j.foodhyd.2023.109291

Duan, L., Guo, L., Liu, K., Liu, E.-H., & Li, P. (2014). Characterization and classification of seven Citrus herbs by liquid chromatography–quadrupole time-of-flight mass spectrometry and genetic algorithm optimized support vector machines. *Journal of Chromatography A, 1339*, 118–127. https://doi.org/10.1016/j.chroma.2014.02.091

Fraisier-Vannier, O., Chervin, J., Cabanac, G., Puech, V., Fournier, S., Durand, V., Amiel, A., André, O., Benamar, O. A., & Dumas, B. (2020). MS-CleanR: A feature-filtering workflow for untargeted LC–MS based metabolomics. *Analytical Chemistry, 92*(14), 9971–9981. https://doi.org/10.1021/acs.analchem.0c01594

Fratianni, F., Cozzolino, A., De Feo, V., Coppola, R., Ombra, M. N., & Nazzaro, F. (2019). Polyphenols, antioxidant, antibacterial, and biofilm inhibitory activities of peel and pulp of Citrus medica L., Citrus bergamia, and citrus medica cv. Salò cultivated in southern Italy. *Journal Of Molecules, 24*(24), 4577. https://doi.org/10.3390/molecules24244577

Gnanasambandam, R., & Proctor, A. (2000). Determination of pectin degree of esterification by diffuse reflectance Fourier transform infrared spectroscopy. *Food Chemistry, 68*(3), 327–332. https://doi.org/10.1016/S0308-8146(99)00191-0

Haag, L.-M., & Siegmund, B. (2014). Exploring & exploiting our 'other self'–Does the microbiota hold the key to the future therapy in Crohn's? *Best Practice Research Clinical Gastroenterology, 28*(3), 399–409. https://doi.org/10.1016/j.bpg.2014.04.001

Haridas, M., Sasidhar, V., Nath, P., Abhithaj, J., Sabu, A., & Rammanohar, P. (2021). Compounds of Citrus medica and Zingiber officinale for COVID-19 inhibition: In silico evidence for cues from Ayurveda. *Future journal of pharmaceutical sciences, 7*, 1–9. https://doi.org/10.1186/s43094-020-00171-6

Hasan, M. M., Roy, P., Alam, M., Hoque, M. M., & Zzaman, W. (2022). Antimicrobial activity of peels and physicochemical properties of juice prepared from indigenous citrus fruits of Sylhet region, Bangladesh. *Journal Of Heliyon, 8*(7). https://doi.org/10.1016/j.heliyon.2022.e09948

Herrero, M. (2024). Towards green extraction of bioactive natural compounds. *Analytical Bioanalytical Chemistry, 416*(9), 2039–2047. https://doi.org/10.1007/s00216-023-04969-0

Huang, W., Xue, A., Niu, H., Jia, Z., & Wang, J. (2009). Optimised ultrasonic-assisted extraction of flavonoids from Folium eucommiae and evaluation of antioxidant activity in multi-test systems in vitro. *Journal Of Food chemistry, 114*(3), 1147–1154. https://doi.org/10.1016/j.foodchem.2008.10.079

Iftikhar, M., Iftikhar, A., Zhang, H., Gong, L., & Wang, J. (2020). Transport, metabolism and remedial potential of functional food extracts (FFEs) in Caco-2 cells monolayer: A review. *Food Research International, 136*, Article 109240. https://doi.org/10.1016/j.foodres.2020.109240

Kanmani, P., Dhivya, E., Aravind, J., & Kumaresan, K. (2014). Extraction and analysis of pectin from citrus peels: Augmenting the yield from citrus limon using statistical experimental design. *Iranica Journal of Energy Environment, 5*(3), Article e64538. https://doi.org/10.5829/idosi.ijee.2014.05.03.10

Kruidenier, L.a., & Verspaget, H. (2002). oxidative stress as a pathogenic factor in inflammatory bowel disease—Radicals or ridiculous? *Alimentary Pharmacology Therapeutics, 16*(12), 1997–2015. https://doi.org/10.1046/j.1365-2036.2002.01378.x

Kurita, O., Fujiwara, T., & Yamazaki, E. (2008). Characterization of the pectin extracted from citrus peel in the presence of citric acid. *Carbohydrate Polymers, 74*(3), 725–730. https://doi.org/10.1016/j.carbpol.2008.04.033

Lela, L., Carlucci, V., Kioussi, C., Choi, J., Stevens, J. F., Milella, L., & Russo, D. (2024a). Humulus lupulus L.: Evaluation of phytochemical profile and activation of bitter taste receptors to regulate appetite and satiety in intestinal secretin tumor cell line (STC-1 Cells). *J Molecular Nutrition Food Research, 68*(21), Article 2400559. https://doi.org/10.1002/mnfr.202400559

Lela, L., Ponticelli, M., Caddeo, C., Vassallo, A., Ostuni, A., Sinisgalli, C., Faraone, I., Santoro, V., De Tommasi, N., & Milella, L. (2022). Nanotechnological exploitation of the antioxidant potential of humulus lupulus L. extract. *Food Chemistry, 393*, Article 133401. https://doi.org/10.1016/j.foodchem.2022.133401

Lela, L., Ponticelli, M., Carlucci, V., Stevens, J. F., Faraone, I., Tzvetkov, N. T., & Milella, L. (2024b). Insight into the interaction of humulus lupulus L. Specialized metabolites and gastrointestinal bitter taste receptors: In vitro study in STC-1 cells and molecular docking. *J Journal of Natural Products, 87*(8), 2021–2033. https://pubs.acs.org/doi/10.1021/acs.jnatprod.4c00532.

Lela, L., Russo, D., De Biasio, F., Gorgoglione, D., Ostuni, A., Ponticelli, M., & Milella, L. (2023). Solanum aethiopicum L. From the Basilicata region prevents lipid absorption, fat accumulation, oxidative stress, and inflammation in OA-treated HepG2 and caco-2 cell lines. *Plants, 12*(15), 2859. https://doi.org/10.3390/plants12152859

Li, G., Cheng, Y., Zhang, T., Li, Y., Han, L., & Liang, G. (2021). Characterization of oxygenated heterocyclic compounds and in vitro antioxidant activity of pomelo essential oil. *Drug Design, Development Therapy*, 937–947. https://doi.org/10.2147/DDDT.S299678

Lin, X., Liu, Y., Wang, R., Dai, J., Wang, L., & Zhang, J. (2024). Extraction of pectins from renewable grapefruit (Citrus paradisi) peels using deep eutectic solvents and analysis of their structural and physicochemical properties. *International Journal of Biological Macromolecules, 254*, Article 127785. https://doi.org/10.1016/j.ijbiomac.2023.127785

Luro, F., Venturini, N., Costantino, G., Paolini, J., Ollitrault, P., & Costa, J. (2012). Genetic and chemical diversity of citron (Citrus medica L.) based on nuclear and cytoplasmic markers and leaf essential oil composition. *Phytochemistry, 77*, 186–196. https://doi.org/10.1016/j.phytochem.2011.12.013

Ma, Q.-G., Chen, J., Chen, L.-H., Wu, G., Zhu, M.-N., He, N.-X., Wang, Q.-Y., Sang, Z.-P., Zhu, C.-Q., & Wu, Y.-Z. (2023). Citrus medica var. Sarcodactylus (Siebold ex Hoola van Nooten) Swingle: An insight into its botany, traditional uses, phytochemistry, and pharmacological properties. *Phytochemistry Reviews, 22*(5), 1247–1279. https://doi.org/10.1007/s11101-023-09865-0

Mateus, A. R. S., Teixeira, J. D., Barros, S. C., Almeida, C., Silva, S., & Sanches-Silva, A. (2024). Unlocking the potential of citrus medica L.: Antioxidant capacity and phenolic profile across peel, pulp, and seeds. *Molecules (Basel, Switzerland), 29*(15), 3533. https://doi.org/10.3390/molecules29153533

Mengsa, H., Kun, X., Pei, L., & Jun, L. (2022). Five Rutaceae family ethanol extracts alleviate H2O2 and LPS-induced inflammation via NF-κb and JAK-STAT3 pathway in HaCaT cells. *Chinese Journal of Natural Medicines, 20*(12), 937–947. https://doi.org/10.1016/S1875-5364(22)60217-6

Menichini, F., Loizzo, M. R., Bonesi, M., Conforti, F., De Luca, D., Statti, G. A., de Cindio, B., Menichini, F., & Tundis, R. (2011). Phytochemical profile, antioxidant, anti-inflammatory and hypoglycemic potential of hydroalcoholic extracts from Citrus medica L. cv Diamante flowers, leaves and fruits at two maturity stages. *Food Chemical Toxicology, 49*(7), 1549–1555. https://doi.org/10.1016/j.fct.2011.03.048

Menichini, F., Tundis, R., Loizzo, M. R., Bonesi, M., D'Angelo, D., Lombardi, P., & Mastellone, V. (2016). Citrus medica L. cv Diamante (Rutaceae) peel extract improves glycaemic status of Zucker diabetic fatty (ZDF) rats and protects against oxidative stress. *J Journal of Enzyme Inhibition Medicinal Chemistry, 31*(6), 1270–1276. https://doi.org/10.3109/14756366.2015.1115400

Mondal, M., Saha, S., Sarkar, C., Hossen, M. S., Hossain, M. S., Khalipha, A. B. R., Islam, M. F., Wahed, T. B., Islam, M. T., & Rauf, A. (2021). Role of citrus medica L. Fruits extract in combatting the hematological and hepatic toxic effects of carbofuran. *J Chemical Research in Toxicology, 34*(8), 1890–1902. https://doi.org/10.1021/acs.chemrestox.1c00166

Morresi, C., Cianfruglia, L., Sartini, D., Cecati, M., Fumarola, S., Emanuelli, M., Armeni, T., Ferretti, G., & Bacchetti, T. (2019). Effect of high glucose-induced oxidative stress on paraoxonase 2 expression and activity in caco-2 cells. *Journal Of Cells, 8*(12), 1616. https://doi.org/10.3390/cells8121616

Morresi, C., Vasarri, M., Bellachioma, L., Ferretti, G., Degl' Innocenti, D., & Bacchetti, T. (2022). Glucose uptake and oxidative stress in caco-2 cells: Health benefits from Posidonia oceanica (L.) Delile. *Marine Drugs, 20*(7), 457. https://doi.org/10.3390/md20070457

Muro, P., Zhang, L., Li, S., Zhao, Z., Jin, T., Mao, F., & Mao, Z. (2024). The emerging role of oxidative stress in inflammatory bowel disease. *Frontiers in Endocrinology, 15*, Article 1390351. https://doi.org/10.3389/fendo.2024.1390351

Pasandide, B., Khodaiyan, F., Mousavi, Z. E., & Hosseini, S. S. (2017a). Optimization of aqueous pectin extraction from Citrus medica peel. *Carbohydrate Polymers, 178*, 27–33. https://doi.org/10.1016/j.carbpol.2017.08.098

Pasandide, B., Khodaiyan, F., Mousavi, Z. E., & Hosseini, S. S. (2017b). Optimization of aqueous pectin extraction from Citrus medica peel. *J Carbohydrate Polymers, 178*, 27–33. https://doi.org/10.1016/j.carbpol.2017.08.098

Phucharoenrak, P., Muangnoi, C., & Trachootham, D. (2022). A green extraction method to achieve the highest yield of limonin and hesperidin from lime peel powder (Citrus

aurantifolia). *Journal Of Molecules, 27*(3), 820. https://doi.org/10.3390/molecules27030820

Ponticelli, M., Carlucci, V., Mecca, M., Todaro, L., Milella, L., & Russo, D. (2024). Extraction optimization of Quercus cerris L. Wood chips: A comparative study between full factorial design (FFD) and artificial neural network (ANN). *Antioxidants, 13*(9), 1115. https://doi.org/10.3390/antiox13091115

Purba, C. C., Mayangsari, Y., Setyaningsih, W., Chansuwan, W., & Sirinupong, N. (2024). Bioactive compounds of Citrus hystrix peel ethanolic extract and their antioxidant potential under hydrogen peroxide-induced oxidative stress in Caco-2 cells. *Future Foods : A Dedicated Journal For Sustainability In Food Science, 9*, Article 100350. https://doi.org/10.1016/j.fufo.2024.100350

Revision, C. (2008). United States Pharmacopeia, the National Formulary. *United States Pharmacopeial Convention. Incorporated*.

Rezaie, A., Parker, R. D., & Abdollahi, M. (2007). Oxidative stress and pathogenesis of inflammatory bowel disease: An epiphenomenon or the cause? *Digestive Diseases Sciences, 52*, 2015–2021. https://doi.org/10.1007/s10620-006-9622-2

Rinaldi, R., Miglionico, R., Nigro, I., D'Orsi, R., Chiummiento, L., Funicello, M., Lupattelli, P., Laurenzana, I., Sgambato, A., & Monné, M. (2021). Two novel precursors of the hiv-1 protease inhibitor darunavir target the upr/proteasome system in human hepatocellular carcinoma cell line hepg2. *Cells, 10*(11), 3052. https://doi.org/10.3390/cells10113052

Sato, Y., Tsujinaka, S., Miura, T., Kitamura, Y., Suzuki, H., & Shibata, C. (2023). Inflammatory bowel disease and colorectal cancer: Epidemiology, etiology, surveillance, and management. *Cancers, 15*(16), 4154. https://doi.org/10.3390/cancers15164154

Strober, W., & Fuss, I. J. (2011). Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology, 140*(6), 1756–1767. https://doi.org/10.1053/j.gastro.2011.02.016. e1751.

Suri, S., Singh, A., & Nema, P. K. (2021). Recent advances in valorization of citrus fruits processing waste: A way forward towards environmental sustainability. *Journal Of Food Science Biotechnology*, 1–26. https://doi.org/10.1007/s10068-021-00984-y

Veličković, A. V., Stamenković, O. S., Todorović, Z. B., & Veljković, V. B. (2013). Application of the full factorial design to optimization of base-catalyzed sunflower oil ethanolysis. *Journal Of Fuel, 104*, 433–442. https://doi.org/10.1016/j.fuel.2012.08.015

Wang, X., Chen, Q., & Lü, X. (2014). Pectin extracted from apple pomace and citrus peel by subcritical water. *Food Hydrocolloids, 38*, 129–137. https://doi.org/10.1016/j.foodhyd.2013.12.003

Wilmsen, P. K., Spada, D. S., & Salvador, M. (2005). Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. *Journal Of Agricultural Food Chemistry, 53*(12), 4757–4761. https://doi.org/10.1021/jf0502000

Wójciak, M., Feldo, M., Borowski, G., Kubrak, T., Płachno, B. J., & Sowa, I. (2022). Antioxidant potential of diosmin and diosmetin against oxidative stress in endothelial cells. *Molecules, 27*(23), 8232. https://doi.org/10.3390/molecules27238232

Wu, Z., Li, H., Yang, Y., Zhan, Y., & Tu, D. (2013). Variation in the components and antioxidant activity of Citrus medica L. var. Sarcodactylis essential oils at different stages of maturity. *Journal of Industrial Crops Products, 46*, 311–316. https://doi.org/10.1016/j.indcrop.2013.02.015

Zhao, P., Duan, L., Guo, L., Dou, L.-L., Dong, X., Zhou, P., Li, P., & Liu, E.-H. (2015). Chemical and biological comparison of the fruit extracts of Citrus wilsonii Tanaka and Citrus medica L. *Food Chemistry, 173*, 54–60. https://doi.org/10.1016/j.foodchem.2014.10.010