Food Bioscience 60 (2024) 104466

Contents lists available at ScienceDirect

Food Bioscience

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

Lycopene enriched extra virgin olive oil: Biological activities and assessment of security profile on cells

Lorenza Marinaccio [a], Gokhan Zengin [b], Onur Bender [c], Rumeysa Dogan [c], Arzu Atalay [c], Domiziana Masci [d], Federica Flamminii [e], Azzurra Stefanucci [a,*], Adriano Mollica [a]

[a] Department of Pharmacy, "G. D'Annunzio" University of Chieti-Pescara, 66100, Chieti, Italy
[b] Department of Biology, Science Faculty, Selcuk University, Konya, 42130, Turkey
[c] Biotechnology Institute, Ankara University, Ankara, Turkey
[d] Department of Basic Biotechnological Sciences, Intensivological and Perioperative Clinics, Catholic University of Sacred Heart, Largo Francesco Vito 1, Rome, 00168, Italy
[e] Department of Medical, Oral and Biotechnological Sciences, "G. D'Annunzio" University of Chieti-Pescara, 65100, Chieti, Italy

ARTICLE INFO

Keywords:
Food enrichment
Carotenoids
Ultrasound-assisted extraction
Antioxidants
Inhibition effect

ABSTRACT

The production of tomato products is massive in Italy resulting in a huge amount of tomato waste as seeds, peels and stems. The aim of our work is to re-utilize these matrices through the extraction of carotenoids, especially lycopene to obtain enriched extra virgin olive oil (EVOO). The extraction has been obtained by ultrasound-assisted extraction (UAE) technique using directly extra virgin olive oil as extraction solvent, due to its lipophilic nature. The quantification of lycopene has been done through HPLC-DAD, resulting in a good concentration of lycopene per gram of oil (0.9 mg/g oil). Then the lycopene enriched EVOO was evaluated *in vitro* to determine its antioxidant and enzyme inhibitory activity in comparison with the EVOO itself. These data were associated to the cytotoxicity assays and *in vivo* histological bioassays, aiming to highlight its safe and healthy profile on human normal cells.

1. Introduction

Carotenoids are lipophilic pigments usually found in fruits but also produced by microorganisms and plants, vegetables and fish. About 600 carotenoids have been identified, but only a small number was found in human tissues, lycopene is among them (Imran et al., 2020). Tomato represents a rich source of carotenoids, mostly lycopene, which account for 80–90% of the overall carotenoids, and in minor part β-carotene (2–3%) (Dumas et al., 2003; Strati & Oreopoulou, 2011). Lycopene ($C_{40}H_{56}$) is a tetraterpene composed of 8 isoprene units, characterized by 13 C–C double bonds of which 11 are linearly conjugated (Kong et al., 2010; Papaioannou & Karabelas, 2012). Numerous researchers investigated the role of lycopene for preventing health disorders (Caseiro et al., 2020; Imran et al., 2020). Lycopene of natural origin is commercially available, but it's expensive and easily purchasable only at small lots, thus the development of effective and economic extraction and purification procedures are of great interest (Marinaccio et al., 2024; Papaioannou & Karabelas, 2012). The extraction of carotenoids from tomato wastes starting to gain interest due to the abundance of this kind of by-products and the beneficial effects of the compounds contained in them (Strati & Oreopoulou, 2011).

Conventional and unconventional methods have been applied to recover pigments from these by-products. Sample extraction using solvents with decreasing polarity allows to retrieve different compounds with the lack of specialized apparatus and the simplicity of execution (Marinaccio et al., 2024). However some drawbacks of the conventional methods (*e.g.* Soxhlet, maceration, distillation etc.) are the large amounts of solvent required, long time processes and the high consumption of energy, the generation of hazardous volatile organic compounds, low process efficiency, use of separate evaporators, and reagent residues after evaporation and harmful effects on the environment. The ultrasound-assisted extraction (UAE) has been widely studied and applied to obtain extracts enriched in pigments like carotenoids. The UAE can be performed on fresh by-products or dried samples, as described by Linares and Rojas (2022) and Chemat-Djenni et al. (2010). Briefly, the mechanisms allow the decrease of the particle size, promoting the interaction between the matrix and the extraction solvent and ameliorating the pigments release and solubilisation (Linares &

* Corresponding author.
E-mail address: a.stefanucci@unich.it (A. Stefanucci).

https://doi.org/10.1016/j.fbio.2024.104466
Received 15 April 2024; Received in revised form 29 May 2024; Accepted 30 May 2024
Available online 31 May 2024

Rojas, 2022). A large number of papers reported the extraction of lycopene from tomato or tomato wastes using UAE as extraction technique (Eh & Teoh, 2012; Kumcuoglu et al., 2014; Lianfu & Zelong, 2008; Strati & Oreopoulou, 2011).

Also, Bendini et al. (2015) have explored the possibility to obtain lycopene enriched olive oil by co-milling proves of olives and tomato by-products. The choice of the extraction solvent depends on the chemical structure of the carotenoids. Xanthophylls are soluble in hydrophilic solvents, while carotenes are more soluble in non-polar solvents. The main problem is the toxicity of these kind of solvents (Strati & Oreopoulou, 2011). Today the involvement of hydrocarburic compounds derived from petroleum distillation such as *n*-hexane, which is the most common solvent in carotenoid extraction, are strictly regulated by European directives on registration, evaluation, authorization and restriction of chemicals (REACH) (Kultys & Kurek, 2022).

Green extraction methods are taking the stage in light of their fast extraction procedure, reduced thermal effects on the bioactive compounds without production of hazardous volatile residues, low water consumption and waste-water development (Martins et al., 2023). This method is based on environmentally friendly, safe and non-toxic solvents by renewable biomass sources (*e.g.* starch, wood, and vegetable oils). Vegetable oil could be a good alternative to common solvents for the extraction of beneficial compounds in terms of environmental and human safety (Fadda et al., 2022). Some applications have been extensively documented by Tiwari et al. (2019), which describe the extraction of carotenoids by UAE from carrot pomace, in linseed oil. Civan and Kumcuoglu (2019) optimized a method to extract carotenoids and capsaicin from red Jalapeño peppers using olive oil. In a similar fashion, Chutia and Mahanta (2021) described the extraction of carotenoids using olive oil from passion fruit peel. Also Bhimjiyani et al. (2021) optimized an UAE method of carotenoids from sea buckthorn pomace with linseed oil. Considering that the quote of lipids in the diet is crucial for the uptake of carotenoids (van het Hof, 1999), the development of a method in which the vegetable oil works as extraction medium and vehicle at the same time, guarantees the concomitant intake of lipids and carotenoids. Vegetable oils are ideal vehicle for carotenoid fortification due to their lipophilic nature, regularity of consumption by the target population, production in strategic regions, sensory alteration of the food when compared to the non-fortified matrix, adequate stability and bioavailability as relates to consumption (Dary & Mora, 2002). Taking into account all these aspects, this work reports an easy and eco-friendly procedure for lycopene's extraction using tomato peels waste as plant matrix by combining the previously developed ultrasound assisted extraction (UAE) (Kumcuoglu et al., 2014) with the use of EVOO as green solvent. With our procedure, it is possible to obtain a lycopene enriched extra virgin olive oil in one simple passage; furthermore we assessed its safety as super food and its suitability as dietary supplement, determining its antioxidant activity, enzyme inhibition effects, and cytotoxicity through *in vitro* assays. Such novel procedure aimed to reach the Recommended Daily Intake for lycopene (25.2 mg in a daily diet for an adult) within the enriched EVOO (https://ods.od.nih.gov/HealthInformation/nutrientrecommendations.aspx).

2. Materials and methods

2.1. Reagents and instruments

Acetonitrile, methanol and tetrahydrofuran were all for HPLC (≥99.9%) and used as such for analytical purposes. HPLC system: Agilent HPLC-DAD 1100, C18 column (Agilent Eclipse Plus, C18 3.5 μm, 4.6 × 100 mm, USUXR16700, USA), T column: 30 °C, Injection volume: 100 μL, Mobile phase: MeOH/ACN = 9:1 + 0.125% TEA, Time run:15 min, Flow rate: 1.2 mL/min. Lycopene analytical standard ≥85.0% (HPLC) was purchased by Sigma-Aldrich (Milano), shipped in dry ice and stored in an amber glass at −65 °C before its use. UAE was performed with a Lab-scale ultrasound sonicator (Cole-Parmer, Illinois, USA) with frequency, power and amplitude of 20 kHz, 400 W and 70 %, respectively; centrifugation with an Eppendorf centrifuge 5702.

2.2. Sample preparation and extraction procedure

Tomato skins waste furnished by S.A.L.P.A. (Società Abruzzese Lavorazione Prodotti Agricoli) were conserved at −20 °C. After lyophilisation for 48h, the skins were grounded in a blender until the obtainment of a powder. Approximately 1 g of lyophilized and grounded powder was put in a flask along with 20 mL of extra virgin olive oil. An ultrasound-assisted extraction was performed for 20 min at 36 °C. After centrifugation (2750×*g* (4400 rpm); 20 min, 25 ± 2 °C). The sample was filtered as routinely done before HPLC analysis, then a red-coloured oil was obtained. It was conserved in a brown glass bottle in the fridge before its use. For antioxidant and enzyme inhibitory assays, the sample solutions were carefully formulated in ethanol at a concentration of 2 mg/mL. All chemicals were freshly prepared, with special attention to keeping them in an ice bath for the upcoming use.

2.3. HPLC-DAD analysis

A mixture of THF/ACN/MeOH = 1.5/3/5.5 was used to solubilize the analytical standard of lycopene and each sample in a ratio of 1:1. Mobile phase: methanol/acetonitrile 9:1 + 0.125% TEA isocratic mode. Time run: 15 min; Column temperature 30 °C; wavelength 475 nm; flow rate: 1.2 mL/min; Injection volume: 100 μ L; Calibration curve 0.5–500 mg/L Y = 28.80x-75.65 R^2 = 1.00.

2.4. Antioxidant assays

Spectrophotometric methods were used for the assessment of the total content of phenols and flavonoids described in the paper (Slinkard & Singleton, 1977) The total antioxidant capacity of the extracts was evaluated using the previously reported assays (Grochowski et al., 2017). Results from the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, Cupric reducing antioxidant capacity (CUPRAC), and ferric reducing antioxidant power (FRAP) tests were reported as milligrams of Trolox equivalents (TE) per gram of extract. Moreover, the antioxidant potential of the extracts was determined by the phosphomolybdenum assay (PBD), denoted in terms of millimoles of Trolox equivalents (TE) per gram of extract, while metal chelating activity (MCA) was quantified as milligrams of disodium edetate equivalents (EDTAE) per gram of extract (Grochowski et al., 2017).

2.5. Enzyme inhibition activity

The cholinesterase inhibitory activity (AChE and BChE) of the extracts was obtained as equivalents of galantamine per gram of the plant sample using Elmann's method. The tyrosinase inhibitory activity of the extracts was evaluated as equivalents of kojic acid per gram of the plant sample using dopachrome method. The α-amylase inhibitory activity of the extracts was obtained as equivalents of acarbose per gram of the plant sample using the iodine/potassium iodide method. The α-glucosidase inhibitory activity of the extracts was measured as equivalents of acarbose per gram of the plant sample using the chromogenic PNPG method. All protocols in detail were described by Grochowski et al. (Grochowski et al., 2017).

2.6. Cell culture

The PNT1A cell line, which is a normal human prostatic epithelial cell line that lacks androgen receptor (AR) and prostate-specific antigen (PSA) expression, was used in this study. The PNT1A cells (catalog no: 95012614) were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Wiltshire, England) and cultured in RPMI

1640 Medium (catalog no: A1049101) (Gibco, Waltham, MA, USA) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin (Stefanucci et al., 2024). The cells were cultured in polystyrene cell culture flasks and incubated in a humidified standard incubator with 5% CO_2 at 37 °C. When the cells reached 80% density, they were subcultured using trypsin-EDTA 0.25% (Gibco, Waltham, MA, USA). Prior to each main experiment, cell counting was performed using a hemocytometer, and cell viability was assessed using the Trypan Blue (Gibco, Waltham, MA, USA) exclusion method (Bender & Atalay, 2018).

2.7. iCelligence cell proliferation assay

The effect of EVOO and lycopene-enriched EVOO (EVOO + Lycopene) on cell proliferation was evaluated using the iCELLigence cell analysis system (Agilent Technologies (formerly ACEA Biosciences, San Diego, CA, USA), which can monitor changes in cellular behavior, including proliferation, migration, and invasion, in real-time and without any labeling (Bender, Celik, et al., 2023; Mahomoodally et al., 2018). Prior to the experiment, the iCELLigence instrument was placed in a 37 °C incubator with 5% CO_2. The experiment was started by measuring the background with 200 μL of the growth medium, and then PNT1A normal prostate cells were seeded in E-Plate L8 wells at a density of 2.0 x 10^4 and incubated in a laminar flow cabinet for 30 min. Subsequently, the E-plates were placed in the iCELLigence instrument and incubated for 24 h to allow for cell growth (Bender, Gunduz, et al., 2018). The cells were treated after 24 h, with various concentrations of EVOO or EVOO + Lycopene (62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL) in duplicate. Docetaxel (Taxotere®) and growth medium were used as positive and negative controls, respectively. The iCELLigence system was set to measure the cell index every 15 min for 72 h. The data were analyzed using the RTCA Data Analysis Software 1.0 (Agilent Technologies, formerly ACEA Biosciences).

2.8. Cellular morphology assays

2.8.1. Inverted microscopy

To evaluate the morphological effects of serial concentrations of EVOO or EVOO + Lycopene on PNT1A cells, Leica DM IL LED inverted microscope equipped with a DFC-290 camera (Leica, Wetzlar, Germany) was used (Alafnan et al., 2023; Bender, Shoman, et al., 2023). Briefly, the PNT1A cells were seeded into 6-well cell culture plates with 2.0 × 10^5 cells per well in 1 mL of growth medium. After 24 h of incubation, cells were treated with increasing concentrations of EVOO or EVOO + Lycopene (62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL) for 72 h. As negative control, growth medium was used. At the end of the incubation period, the medium was discarded, the wells were carefully washed with PBS. Then, cells were observed for morphological changes assessment under an inverted microscope. All experiments were conducted in duplicates.

2.8.2. Crystal violet staining

Crystal violet staining was performed to assess and visualize the effects of EVOO or EVOO + Lycopene on cellular morphology (Bender, Llorent-Martínez, et al., 2018). PNT1A cells were seeded at a density of 2.0 × 10^5 cells/well in 6-well cell culture plates and incubated overnight at 37 °C and 5% CO_2. The following day, the cells were treated with varying concentrations of EVOO or EVOO + Lycopene (ranging from 125 μg/mL to 1000 μg/mL) for 72 h. Then, media was discarded, and the cells washed twice with ice-cold PBS. The cells were fixed with 100% methanol for 10 min at room temperature. After the methanol was aspirated, the cells were stained with 0.5% crystal violet solution for 15 min at room temperature (25 ± 2 °C). Stained cells were washed with distilled water, and allowed to air dry. Subsequently, the cells were photographed under an inverted microscope, Leica DM IL LED, equipped with a DFC-290 camera (Leica, Wetzlar, Germany).

2.9. Statistical analysis

Each experiment was repeated three times to ensure accuracy and reliability. The findings are depicted as mean values accompanied by standard deviation (SD). To assess variances between these samples, the student t-test ($\alpha = 0.05$) was employed. This statistical analysis was conducted using the GraphPad v. 9.0 program.

3. Results and discussion

3.1. HPLC-DAD quantification of lycopene UAE extract

The potential beneficial effects of lycopene on human health and its expensive cost made its extraction from food waste an interesting topic. The extraction of this carotenoid requires the use of a lipophilic solvent given its chemical nature. To overcome the environmental and safety drawbacks of the common organic solvents, an edible oil was used for the extraction. Both the extraction technique (UAE) and the solvent used render the process entirely green, furthermore EVOO represents both the vehicle and final enriched product minimizing the sample's treatments. The result is the production of an oil enriched of the lipophilic compounds contained in the tomato peels waste, in particular of lycopene which represents the main fat-soluble carotenoid in this matrix. After the extraction process, the EVOO changes its colour from yellow to red due to the presence of this red pigment. The lycopene content of the EVOO was assessed by HPLC-DAD. Carotenoids are characterized by their chemical liability, which imply a meticulous manipulation. The analytical methods generally used for the determination of carotenoids are not suitable for the quantification of lycopene because of its lipophilic nature and low solubility in the solvents, *e.g.* methanol (Barba et al., 2006; Bicanic et al., 1995). In our work the determination of lycopene content, was performed using the method developed by Barba et al. (2006) with slight modifications. The column was set at 30 °C to avoid the separation of the pigments (Barba et al., 2006). The correct balance of the mobile phase has a pivotal role; indeed an excess of methanol can cause the lycopene precipitation while the excess of non-polar solvent could induce deformation of the chromatographic peaks linked to the polarity difference (Barba et al., 2006). The use of TEA prevents or reduces the degradation of carotenoids on-column (Barba et al., 2006; Hart & Scott, 1995; Rodriguez-Amaya, 2003). The lycopene content in the enriched EVOO oil is 0.9 ± 0.2 mg/g expressed as milligrams of lycopene per gram of oil. The result is the mean of three different experiments ± the standard deviation. Considering that the daily intake is 25.2 mg and about 37 g for lycopene and EVOO respectively in a diet for an adult, this quantity perfectly fits the recommendation of EFSA, the European Food Safety Authority (https://www.efsa.europa.eu/it).

3.2. Antioxidant assays

After determining the lycopene content, the antioxidant activity of the fortified oil was evaluated and compared with the baseline antioxidant activity of the EVOO. The results are shown in Table 1. First, we determined the total bioactive compounds in the EVOO and the fortified oils. Total phenolic content in the fortified oil (30.95 mg GAE/g) was higher than that of the EVOO (24.72 mg GAE/g). However, the total flavonoid content in the EVOO (0.25 mg RE/g) was 3.5 times higher than that of enriched oil (0.07 mg RE/g). This fact can be explained by the formation of a complex between lycopene and flavonoids. This fact was also supported by some researchers (Han et al., 2012). Lycopene enrichment increased total phenolic content in certain oil samples (Montesano et al., 2006), consistent with our findings. However, in the Folin-Ciocalteu assay, interactions between lycopene and the Folin reagent can occur, which can lead to false positive results. At this point, the *in vitro* antioxidant assays may be more useful to understand the effect of lycopene fortification. The antioxidant activity of the EVOO and

Table 1
Total phenolic, flavonoid content and antioxidant properties of the tested samples.

Samples	TPC (mg GAE/g	TFC (mg RE/g)	DPPH (mg TE/g)	ABTS (mg TE/g	CUPRAC (mg TE/g)	FRAP (mg TE/g)	MCA (mg EDTAE/g)	PBD (mmol TE/g)
EVOO	24.72 ± 1.12[b]	0.25 ± 0.03[a]	0.76 ± 0.05[b]	11.66 ± 0.38[a]	42.59 ± 1.06[b]	24.40 ± 1.13[b]	3.31 ± 0.41[b]	0.96 ± 0.03[a]
EVOO with enriched lycopene	30.95 ± 0.50[a]	0.07 ± 0.01[b]	7.88 ± 0.74[a]	10.60 ± 0.36[b]	49.74 ± 1.07[a]	27.38 ± 1.66[a]	3.81 ± 0.24[a]	0.97 ± 0.08[a]

Values are reported as mean ± SD of three parallel measurements. TPC: Total phenolic content; TFC: Total flavonoid content; MCA: Metal chelating assay; PBD: Phosphomolybdenum. GAE: Gallic acid equivalent; RE: Rutin equivalent; TE: Trolox equivalent; EDTAE: EDTA equivalent.

the fortified oil were investigated using various methods, including radical quenching (DPPH and ABTS), reducing power (CUPRAC and FRAP), phosphomolybdenum, and metal chelating. DPPH and ABTS are the most common tests for evaluating antioxidant properties and reflect the hydrogen donation ability of antioxidants (Bibi Sadeer et al., 2020). In the DPPH assay, enrichment with lycopene increased the radical scavenging ability compared to EVOO. DPPH and ABTS are the most common tests for evaluating antioxidant properties and reflect the hydrogen donation ability of antioxidants. In the DPPH assay, enrichment with lycopene increased the radical scavenging ability compared to EVOO. The contradictory results can be explained by the different nature of these radicals. Both hydrophilic and lipophilic antioxidant systems can be tested with the ABTS method, while the DPPH method utilizes a radical dissolved in organic media and is suitable for hydrophobic systems. The assays based on the reduction of metal ions reflect the electron donating ability of the antioxidant and a high reducing ability indicates a high antioxidant potential. To this end, CUPRAC and FRAP assays were performed. In both assays, the fortified oil exhibited higher reducing abilities compared to EVOO. Müller et al. (2011) reported that lycopene was the most active carotenoid in the FRAP assay compared to β-carotene and zeaxanthin and this ability can explained by the presence of conjugated double bonds in its structure. In addition, several studies have shown that the enrichment with lycopene was positively influenced the reducing power of the tested samples (Naz et al., 2013). Similar to CUPRAC and FRAP assays, the phosphomolybdenum assay involves the reduction of Mo(VI) to Mo(V) by antioxidants under acidic conditions. Because not only phenolic but also nonphenolic antioxidants can play a role in the assay, it is considered a total antioxidant test. In contrast to FRAP and CUPRAC assays, the EVOO and enriched oils showed very similar performance in the phosphomolybdenum assay. This fact can be explained by the breakdown of lycopene at higher temperatures (>90 °C). Metal chelation ability may be closely related to controlling the production of hydroxyl radicals in the Fenton reaction. As can be seen from Table 1, the metal chelating ability of the lycopene fortified EVOO (3.81 mg EDTAE/g) was higher than EVOO (3.31 mg EDTAE/g). From this point on, the lycopene can positively influence the observed ability to form metal chelates. At first glance, some structural features of lycopene suggest the possible mechanism of metal chelation. Like other carotenoids, lycopene is a highly unsaturated molecule and can isomerize. Lycopene may chelate metal ions through multiple mechanisms, both through the pi electrons in the polyene chain and through the lone pairs of electrons on the carbonyl or hydroxyl groups. Overall, lycopene fortification has a positive impact on antioxidant properties and may therefore be useful for the development of health-promoting applications, including functional foods.

3.3. Enzyme inhibitor activity

The theory of enzyme inhibition is linked to tackling global health problems such as diabetes, obesity, cancer or Alzheimer's disease. According to the theory, by inhibiting key enzymes, the pathological symptoms of these diseases can be reduced. For example, by inhibiting amylase and glucosidase, blood sugar levels can be controlled after a high-carbohydrate diet (Kashtoh & Baek, 2023). To this end, we tested the inhibitory properties of EVOO and lycopene-enriched oils against cholinesterase, tyrosinase, amylase and glucosidase. The results are shown in Table 2. The AChE inhibition of EVOO was higher than that of lycopene-enriched EVOO. However, their BChE inhibition was very close. In other enzymes, namely inhibition of tyrosinase, amylase and glucosidase, the ability of lycopene-enriched EVOO was higher than that of EVOO. From this point on, lycopene enrichment can be considered a valuable ingredient for further applications. Consistent with our results, Ronanki et al. (2018) evaluated the inhibitory effect of tomato-fortified rice on amylase and glucosidase and reported higher efficacy in tomato-fortified than non-fortified samples. In addition, Kim (2011) provided information on the inhibitory mechanism of lycopene on cytokine expression in pancreatic acinar cells. Similarly, Morais et al. (2018) reported a correlation between lycopene and tyrosinase inhibitory effects of *Dalbergia ecastaphyllum*. Báo et al. (2023) found a significant tyrosinase-inhibiting effect of the nano form of lycopene. In another study by Shahraki and Daneshmand (2023), the anti-tyrosinase effect of lycopene-loaded solid lipid nanoparticles was investigated, and the particles could alleviate melanogenesis in melanoma cells. In summary, with the exception of cholinesterase inhibition, lycopene enrichment positively influenced the observed inhibitory properties. In this sense, reinforcement can be useful for designing functional products.

3.4. Cytotoxicity assay

Finally, the cytotoxicity of the enriched EVOO and the morphological changes on PNT1A normal prostate Ecell line were investigated. Assessing the effects of EVOO and EVOO + Lycopene on cell viability in a normal cell line is crucial for understanding their safety and therapeutic potential. Therefore, the iCELLigence real-time cell analysis system was used to evaluate the effect of EVOO and EVOO + Lycopene on cell proliferation and determine their cytotoxicity in the PNT1A normal prostate cell line. For this assay, the PNT1A cells were seeded into iCELLigence E-plates, which have gold-plated sensor electrodes at the bottom of each well. Through impedance readings, these specialized plates allow for real-time monitoring of cell viability (Bird & Kirstein, 2009; Ke et al., 2011; Kho et al., 2015; Lazarova et al., 2015). Twenty-four hours after cell seeding, the cells were treated with EVOO or EVOO + Lycopene at various concentrations (62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL). As a positive control group, cells were treated with docetaxel. The cells were monitored, and measurements were taken every 15 min for a total of 96 h, including a 72-h period following the EVOO or EVOO + Lycopene treatment. The normalized cell index graph was given in Fig. 1A and B. Furthermore, the cell viability percentages for each dosage at certain time points were calculated as mentioned in previous study (Bender, Celik, et al., 2023), and presented in Table 3 for EVOO and Table 4 for EVOO + Lycopene.

As illustrated in Fig. 1, the PNT1A non-cancerous cell line shows a similar cell growth dynamic when treated with EVOO or EVOO + Lycopene in a time-dependent manner. Notably, EVOO or EVOO + Lycopene treatments did not significantly affect cell viability compared to the control group. However, the lowest cell viability was observed at the highest concentrations of EVOO (1000 μg/mL) after 48 and 72 h of

Table 2
Enzyme inhibitory effects of the tested samples.

Samples	AChE inhibition (mg GALAE/g)	BChE (mg GALAE/g)	Tyrosinase (mg KAE/g)	Amylase (mmol ACAE/g)	Glucosidase (mmol ACAE/g)
EVOO	2.22 ± 0.01[a]	4.42 ± 0.30[a]	10.17 ± 1.56[b]	1.17 ± 0.03[b]	1.43 ± 0.12[b]
EVOO with enriched lycopene	1.93 ± 0.09[b]	4.41 ± 0.23[a]	12.36 ± 1.56[a]	1.42 ± 0.01[a]	1.59 ± 0.08[a]

Values are reported as mean ± SD of three parallel measurements. GALAE: Galanthamine equivalent; KAE: Kojic acid equivalent; ACAE: Acarbose equivalent.

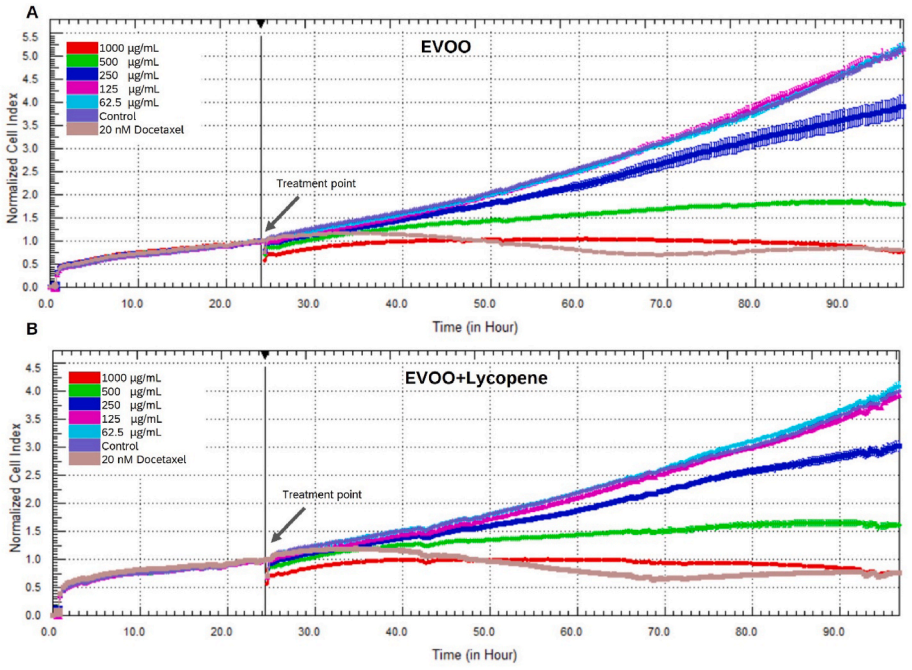

Fig. 1. RTCA assay for the determination of cytotoxicity profile of EVOO (A) and EVOO enriched with lycopene (B).

Table 3
PNT1A cell viability rates (%) at 24-, 48-, and 72- hours treatments with *EVOO* or Docetaxel (Min %Control).

Time point	Cell Viability (% ± SEM)						
	Control	*EVOO* (µg/mL) Docetaxel					
		1000	500	250	125	62.5	20 nM
24 h	99.27 ± 0.00	51.54 ± 0.01	72.23 ± 0.00	87.66 ± 0.01	92.07 ± 0.00	95.01 ± 0.00	51.33 ± 0.00
48 h	99.22 ± 0.00	30.34 ± 0.01	52.27 ± 0.01	84.53 ± 0.04	92.07 ± 0.00	95.01 ± 0.00	21.72 ± 0.00
72 h	98.90 ± 0.01	14.75 ± 0.00	34.97 ± 0.01	75.83 ± 0.05	92.07 ± 0.00	95.01 ± 0.00	15.67 ± 0.00

Table 4
PNT1A cell viability rates (%) at 24-, 48-, and 72- hours treatments with *EVOO + Lycopene* or Docetaxel (Min %Control).

Time point	Cell Viability (% ± SEM)						
	Control	*EVOO + Lycopene* (µg/mL) Docetaxel					
		1000	500	250	125	62.5	20 nM
24 h	97.80 ± 0.00	54.67 ± 0.01	75.04 ± 0.01	87.58 ± 0.01	93.02 ± 0.01	97.38 ± 0.03	55.32 ± 0.00
48 h	94.46 ± 0.00	32.07 ± 0.00	53.35 ± 0.02	81.06 ± 0.01	91.87 ± 0.00	94.96 ± 0.01	23.03 ± 0.00
72 h	94.18 ± 0.01	18.10 ± 0.00	38.54 ± 0.01	71.59 ± 0.02	91.31 ± 0.01	94.71 ± 0.01	17.34 ± 0.00

incubation, with viability rates of 30.34% ± 0.01 and 14.75% ± 0.00, respectively. Similarly, for EVOO + Lycopene, the lowest cell viability was observed at the highest concentrations (1000 µg/mL) after 48 h and 72 h, with viability rates of 32.07% ± 0.00 and 18.10% ± 0.00, respectively. These findings suggest that longer exposure to high concentrations of EVOO or EVOO + Lycopene can slightly decrease PNT1A cell viability compared to the control. Furthermore, based on these results, lycopene does not exhibit cytotoxic effects beyond those observed with EVOO treatment on PNT1A cells. Also, the results shown in Fig. 1, Table 3, and Table 4 indicate that treatments with EVOO or EVOO + Lycopene at a concentration of 1000 µg/mL had a similar viability profile to that of 20 nM docetaxel over all time periods. At 24 h, the cell viability for EVOO treatments ranged from 51.54% ± 0.01–99.27% ± 0.00 (Table 3) and EVOO + Lycopene treatments ranged from 54.67% ± 0.01 (Table 4) while the viability for Docetaxel was 51.33% ± 0.00 and 55.32% ± 0.00, respectively. Based on the 24- to 72-h exposure time, it was observed that doses ranging from 1000 µg/mL to 250 µg/mL of both EVOO and EVOO + Lycopene led to a slight decrease in cell viability compared to the control group. However, even at these concentrations, the treated cells still exhibited relatively high cell viability rates at 24 h. Particularly, concentrations of EVOO or EVOOL at 250 µg/mL and below demonstrates higher cell viability throughout all time periods. Based on these findings, it can be concluded that EVOO + Lycopene is not toxic to the PNT1A cell line at concentrations below 500 µg/mL across all exposure times. Especially, concentrations of EVOO or EVOO + Lycopene at 250 µg/mL and below demonstrate higher cell viability throughout all time periods. These results suggest that EVOOL at lower concentrations has a favourable effect on cell viability and does not induce cytotoxicity in the PNT1A cell line. Taken together, all these results indicated that Lycopene does not exert any cytotoxic effects

above the effects of EVOO on the cells and is not harmful to their viability. Overall, these results highlight that EVOO or EVOO + Lycopene did not induce any significant toxicity or harm to the PNT1A cells, as indicated by their high cell viability rates. The concentrations of 500 μg/mL, 250 μg/mL, 125 μg/mL, and 62.5 μg/mL deserve particular attention in further investigations and potential applications.

3.5. Morphological evaluations of EVOO and EVOO + lycopene on body cells

We further validated our findings by performing additional assays that enabled us to assess the morphological changes on PNT1A cells following treatment with EVOO or EVOO + Lycopene. These assays provide valuable insights into the characteristics and overall appearance of the cells, allowing us to gain a comprehensive understanding of the effects of EVOO or EVOO + Lycopene treatments on cellular morphology.

For first assay, the PNT1A cells were treated with various concentrations of EVOO or EVOO + Lycopene (62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL) for 72 h and photographed under inverted microscope to visualize any morphological changes (Fig. 2). Furthermore, we performed crystal violet staining, which is a colorimetric method that dyes cellular nuclei and can be used for various purposes. In this assay, PNT1A cells were seeded and incubated for 24 h. Subsequently, cells were treated with different concentrations of EVOO or EVOO + Lycopene (ranging from 125 μg/mL to 1000 μg/mL). Cells were photographed after 72 h of treatments (Fig. 3).

The control group, untreated PNT1A cells, displayed a polygonal shape and strong adhesion to the bottom of the plate, reflecting their epithelial nature (Figs. 2 and 3). Moreover, these untreated cells exhibited elongated cytoplasmic regions and maintained membrane integrity, resulting in a larger cell volume (Figs. 2 and 3). In Fig. 2, cells treated with a concentration of 1000 μg/mL of EVOO or EVOO + Lycopene showed noticeably reduced viability compared to the control. At a magnification of 400× (Fig. 3), relatively fewer cells were observed at 1000 μg/mL concentration for both treatments, and the polygonal membrane structures of the cells became more rounded. At a concentration of 500 μg/mL of EVOO or EVOO + Lycopene, cell viability was slightly decreased (Fig. 2); however, the cell membranes maintained a more structurally intact appearance compared to the 1000 μg/mL concentration (Fig. 3). Notably, in Fig. 2 concentrations of EVOO or EVOO + Lycopene within the range of 250 μg/mL and 62.5 μg/mL demonstrated higher cell viability. Correspondingly, in Fig. 3 the cells exhibited a polygonal shape similar to the control group and displayed a higher cell volume when treated with EVOO or EVOO + Lycopene concentrations at 250 μg/mL and below.

Consequently, these morphological observations are consistent with the results obtained from the iCELLigence assay. There were no significant differences observed between the treatment of EVOO or EVOO + Lycopene on the cellular morphology. Also, our findings indicate that EVOO or EVOO + Lycopene treatments do not significantly alter the cellular morphology of PNT1A cells, except at concentrations higher than 500 ug/mL. At these higher concentrations, cellular viability decreases, and noticeable changes in cellular morphology become apparent. At concentrations of 250 μg/mL and below, both EVOO and EVOO + Lycopene treatments resulted in higher cell viability and minimal changes in the cellular morphology of PNT1A cells. The integrity of the cell structure and cellular properties were maintained at doses of 250 μg/mL and below.

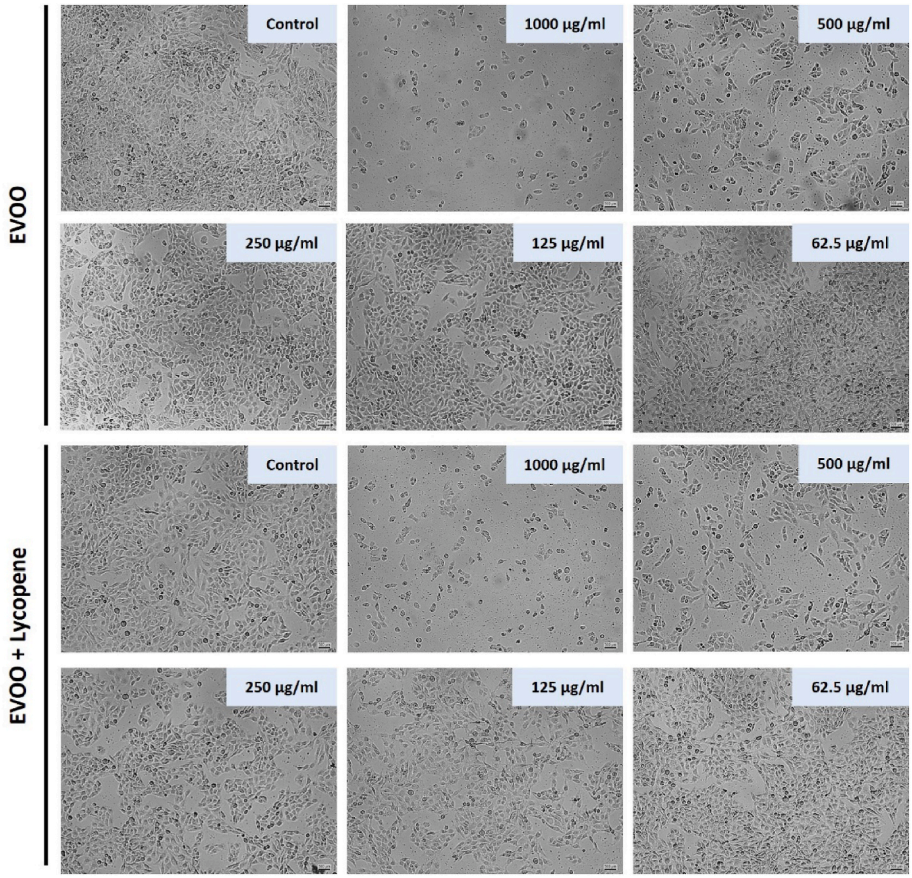

Fig. 2. Morphological evaluations of EVOO and EVOO + Lycopene on PNT1A cells.

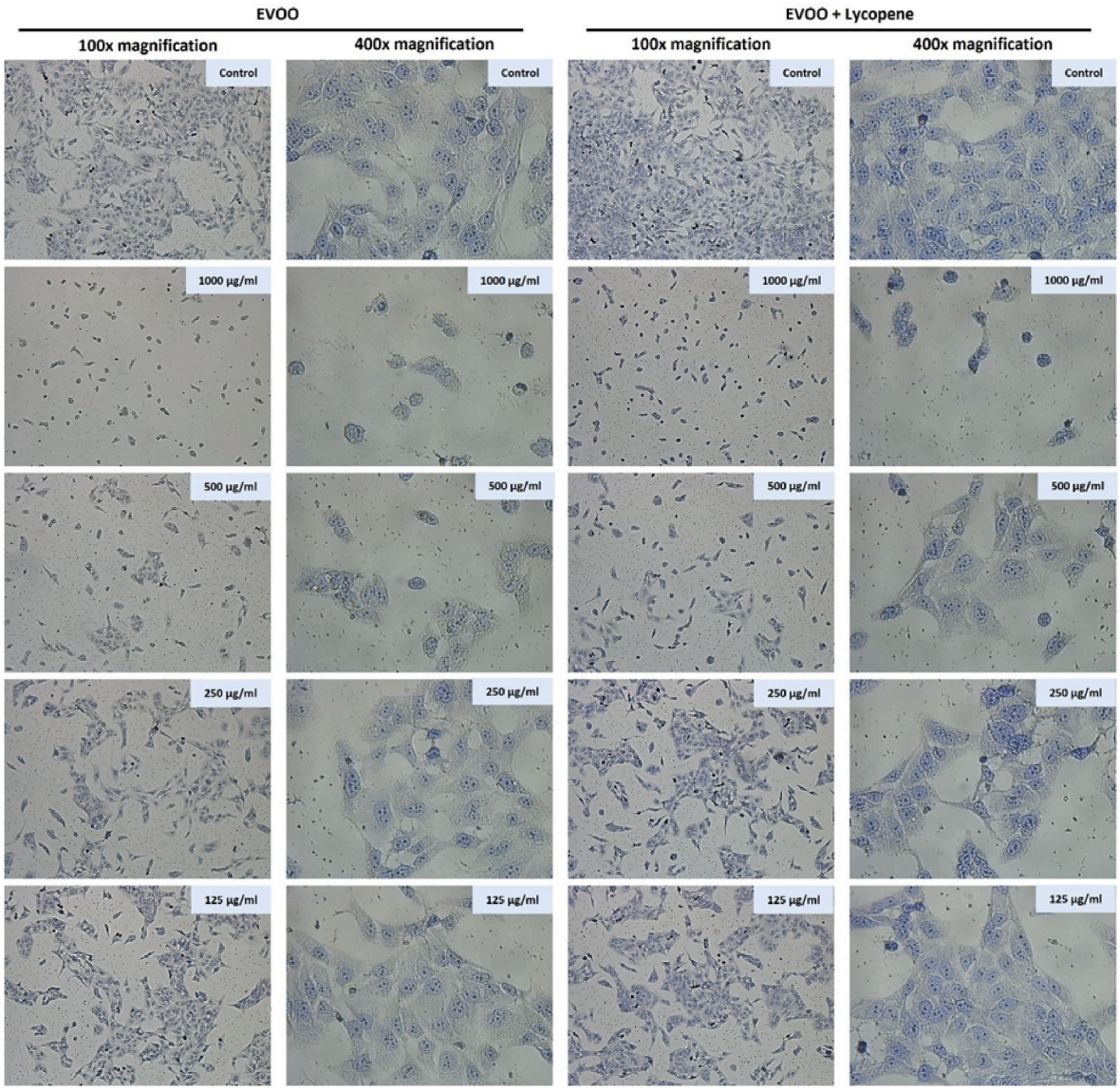

Fig. 3. Crystal violet staining on PNT1A cells treated with EVOO and EVOO + Lycopene.

4. Conclusions

The consumption of food rich in carotenoids is of primary importance in human diet due to the well-known beneficial properties on health. Lycopene is a carotenoid mostly found in red and orange fruits, such as tomato, which daily intake for an adult is 25.2 mg as recommended by EFSA. Since the presence of these fruits is not always guaranteed in a daily diet, it would be possible to assume lycopene using an EVOO as extraction solvent/vehicle. Despite other research groups have already produced lycopene enriched olive oil by other procedures (Bendini et al., 2015; Kumcuoglu et al., 2014), we used a green procedure in mild condition to achieve the production of a safe enriched extra virgin olive oil. The bioactive compound has been extracted using a green UAE procedure from tomato peels as industrial waste and then it was quantified by HPLC-DAD. The safety profile and beneficial *in vitro* activities of this product have been evaluated and compared with the pure EVOO, confirming its cell protecting effect on PNT1A cells and strong antioxidant activity *in vitro*. These results suggest the potential use of lycopene-enriched EVOO as a novel food, dietary supplement and nutraceutic in human diet. In conclusion, this work opens concrete perspectives on the production of enriched foods starting from food wastes, promoting sustainable economy through new-food production chains.

Fundings

This work has been partially funded *by the European Union -NextGenerationEU under the Italian Ministry of University and Research (MUR) National Innovation Ecosystem* grant *ECS00000041 -VITALITY -CUP* ***[D73C22000840006]***.

CRediT authorship contribution statement

Lorenza Marinaccio: Validation, Methodology, Investigation. **Gokhan Zengin:** Visualization, Formal analysis, Data curation. **Onur Bender:** Visualization, Methodology, Data curation. **Rumeysa Dogan:** Visualization, Methodology, Data curation. **Arzu Atalay:** Visualization, Methodology, Data curation. **Domiziana Masci:** Formal analysis, Data curation. **Federica Flamminii:** Formal analysis, Data curation. **Azzurra Stefanucci:** Writing – review & editing, Writing – original draft, Project administration, Conceptualization. **Adriano Mollica:** Supervision, Resources.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgements

A.S. and A.M. acknowledge the Next Generation EU, PON Ricerca ed Innovazione 2014–2020 for L.M. PhD. Program.

References

Alafnan, A., Dogan, R., Bender, O., Celik, I., Mollica, A., Malik, J. A., Rengasamy, K. R. R., Break, M. K. B., Khojali, W. M. A., & Alharby, T. N. (2023). Beta Elemene induces cytotoxic effects in FLT3 ITD-mutated acute myeloid leukemia by modulating apoptosis. *European Review for Medical and Pharmacological Sciences, 27*, 3270–3287.

Báo, S. N., Machado, M., Da Silva, A. L., Melo, A., Cunha, S., Sousa, S. S., Malheiro, A. R., Fernandes, R., Leite, J. R. S., & Vasconcelos, A. G. (2023). Potential biological properties of lycopene in a self-emulsifying drug delivery system. *Molecules, 28*, 1219.

Barba, A. O., Hurtado, M. C., Mata, M. S., Ruiz, V. F., & De Tejada, M. L. S. (2006). Application of a UV–vis detection-HPLC method for a rapid determination of lycopene and β-carotene in vegetables. *Food Chemistry, 95*, 328–336.

Bender, O., & Atalay, A. (2018). Evaluation of anti-proliferative and cytotoxic effects of chlorogenic acid on breast cancer cell lines by real-time, label-free and high-throughput screening. *Marmara Pharmaceutical Journal, 22*.

Bender, O., Celik, I., Dogan, R., Atalay, A., Shoman, M. E., Ali, T. F. S., Beshr, E. A. M., Mohamed, M., Alaaeldin, E., Shawky, A. M., Awad, E. M., Ahmed, A. F., Younes, K. M., Ansari, M., & Anwar, S. (2023). Vanillin-based indolin-2-one derivative bearing a pyridyl moiety as a promising anti-breast cancer agent via anti-estrogenic activity. *ACS Omega, 8*, 6968–6981.

Bender, O., Gunduz, M., Cigdem, S., Hatipoglu, O. F., Acar, M., Kaya, M., Grenman, R., Gunduz, E., & Ugur, K. S. (2018). Functional analysis of ESM 1 by si RNA knockdown in primary and metastatic head and neck cancer cells. *Journal of Oral Pathology & Medicine, 47*, 40–47.

Bender, O., Llorent-Martínez, E. J., Zengin, G., Mollica, A., Ceylan, R., Molina-Garcia, L., Fernández-de Córdova, M. L., & Atalay, A. (2018). Integration of *in vitro* and *in silico* perspectives to explain chemical characterization, biological potential and anticancer effects of *Hypericum salsugineum*: A pharmacologically active source for functional drug formulations. *PLoS One, 13*, Article e0197815.

Bender, O., Shoman, M. E., Ali, T. F. S., Dogan, R., Celik, I., Mollica, A., Hamed, M. I. A., Aly, O. M., Alamri, A., Alanazi, J., Ahemad, N., Gan, S. H., Malik, J. A., Anwar, S., Atalay, A., & Beshr, E. A. M. (2023). Discovery of oxindole-based FLT3 inhibitors as a promising therapeutic lead for acute myeloid leukemia carrying the oncogenic ITD mutation. *Archiv der Pharmazie, 356*, Article e2200407.

Bendini, A., Di Lecce, G., Valli, E., Barbieri, S., Tesini, F., & Gallina Toschi, T. (2015). Olive oil enriched in lycopene from tomato by-product through a co-milling process. *International Journal of Food Sciences & Nutrition, 66*(4), 371–377.

Bhimjiyani, V. H., Borugadda, V. B., Naik, S., & Dalai, A. K. (2021). Enrichment of flaxseed (Linum usitatissimum) oil with carotenoids of sea buckthorn pomace via ultrasound-assisted extraction technique: Enrichment of flaxseed oil with sea buckthorn. *Current Research in Food Science, 4*, 478–488.

Bibi Sadeer, N., Montesano, D., Albrizio, S., Zengin, G., & Mahomoodally, M. F. (2020). The versatility of antioxidant assays in food science and safety—Chemistry, applications, strengths, and limitations. *Antioxidants, 9*, 709.

Bicanic, D., Franko, M., Jalink, H., Dukić, R., Bozóki, Z., & Linssen, J. (1995). Study of saturated triglycerides in oil based on the cw transverse CO_2 laser excited photothermal deflection signals. *Infrared Physics & Technology, 36*, 617–622.

Bird, C., & Kirstein, S. (2009). *Real-time, label-free monitoring of cellular invasion and migration with the xCELLigence system*. New York: Nature Publishing Group US.

Caseiro, M., Ascenso, A., Costa, A., Creagh-Flynn, J., Johnson, M., & Simões, S. (2020). Lycopene in human health. *LWT-Food Science & Technology, 127*, Article 109323.

Chemat-Djenni, Z., Ferhat, M. A., Tomao, V., & Chemat, F. (2010). Carotenoid extraction from tomato using a green solvent resulting from orange processing waste. *Journal of Essential Oil Bearing Plants, 13*, 139–147.

Chutia, H., & Mahanta, C. L. (2021). Green ultrasound and microwave extraction of carotenoids from passion fruit peel using vegetable oils as a solvent: Optimization, comparison, kinetics, and thermodynamic studies. *Innovative Food Science & Emerging Technologies, 67*, Article 102547.

Civan, M., & Kumcuoglu, S. (2019). Green ultrasound-assisted extraction of carotenoid and capsaicinoid from the pulp of hot pepper paste based on the bio-refinery concept. *LWT-Food Science & Technology, 113*, Article 108320.

Dary, O., & Mora, J. O. (2002). Food fortification to reduce vitamin A deficiency: International Vitamin A Consultative Group recommendations. *The Journal of Nutrition, 132*, 2927S–2933S.

Dumas, Y., Dadomo, M., Di Lucca, G., & Grolier, P. (2003). Effects of environmental factors and agricultural techniques on antioxidantcontent of tomatoes. *Journal of the Science of Food and Agriculture, 83*, 369–382.

Eh, A. L.-S., & Teoh, S.-G. (2012). Novel modified ultrasonication technique for the extraction of lycopene from tomatoes. *Ultrasonics Sonochemistry, 19*, 151–159.

Fadda, A., Sanna, D., Sakar, E. H., Gharby, S., Mulas, M., Medda, S., Yesilcubuk, N. S., Karaca, A. C., Gozukirmizi, C. K., & Lucarini, M. (2022). Innovative and sustainable technologies to enhance the oxidative stability of vegetable oils. *Sustainability, 14*, 849.

Grochowski, D. M., Uysal, S., Aktumsek, A., Granica, S., Zengin, G., Ceylan, R., Locatelli, M., & Tomczyk, M. (2017). *In vitro* enzyme inhibitory properties, antioxidant activities, and phytochemical profile of Potentilla thuringiaca. *Phytochemistry Letters, 20*, 365–372.

Han, R.-M., Zhang, J.-P., & Skibsted, L. H. (2012). Reaction dynamics of flavonoids and carotenoids as antioxidants. *Molecules, 17*, 2140–2160.

Hart, D. J., & Scott, K. J. (1995). Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. *Food Chemistry, 54*, 101–111.

Imran, M., Ghorat, F., Ul-Haq, I., Ur-Rehman, H., Aslam, F., Heydari, M., Shariati, M. A., Okuskhanova, E., Yessimbekov, Z., & Thiruvengadam, M. (2020). Lycopene as a natural antioxidant used to prevent human health disorders. *Antioxidants, 9*, 706.

Kashtoh, H., & Baek, K.-H. (2023). New insights into the Latest advancement in α-amylase inhibitors of plant origin with anti-diabetic effects. *Plants, 12*, 2944.

Ke, N., Wang, X., Xu, X., & Abassi, Y. A. (2011). The xCELLigence system for real-time and label-free monitoring of cell viability. *Mammalian Cell Viability: Methods and Protocols*, 33–43.

Kho, D., MacDonald, C., Johnson, R., Unsworth, C. P., O'Carroll, S. J., Du Mez, E., Angel, C. E., & Graham, E. S. (2015). Application of xCELLigence RTCA biosensor technology for revealing the profile and window of drug responsiveness in real time. *Biosensors, 5*, 199–222.

Kim, H. (2011). Inhibitory mechanism of lycopene on cytokine expression in experimental pancreatitis. *Annals of the New York Academy of Sciences, 1229*, 99–102.

Kong, K.-W., Khoo, H.-E., Prasad, K. N., Ismail, A., Tan, C.-P., & Rajab, N. F. (2010). Revealing the power of the natural red pigment lycopene. *Molecules, 15*, 959–987.

Kultys, E., & Kurek, M. A. (2022). Green extraction of carotenoids from fruit and vegetable byproducts: A review. *Molecules, 27*, 518.

Kumcuoglu, S., Yilmaz, T., & Tavman, S. (2014). Ultrasound assisted extraction of lycopene from tomato processing wastes. *Journal of Food Science and Technology, 51*, 4102–4107.

Lazarova, I., Zengin, G., Bender, O., Zheleva-Dimitrova, D., Uysal, S., Ceylan, R., Gevrenova, R., Aktumsek, A., Acar, M., & Gunduz, M. (2015). A comparative study of Bulgarian and Turkish *Asphodeline lutea* root extracts: HPLC–UV profiles, enzyme inhibitory potentials and anti-proliferative activities against MCF-7 and MCF-10a cell lines. *Journal of Functional Foods, 15*, 254–263.

Lianfu, Z., & Zelong, L. (2008). Optimization and comparison of ultrasound/microwave assisted extraction (UMAE) and ultrasonic assisted extraction (UAE) of lycopene from tomatoes. *Ultrasonics Sonochemistry, 15*, 731–737.

Linares, G., & Rojas, M. L. (2022). Ultrasound-assisted extraction of natural pigments from food processing by-products: A review. *Frontiers in Nutrition, 9*, Article 891462.

Mahomoodally, M. F., Atalay, A., Picot, M. C. N., Bender, O., Celebi, E., Mollica, A., & Zengin, G. (2018). Chemical, biological and molecular modelling analyses to probe into the pharmacological potential of Antidesma madagascariense lam.: A multifunctional agent for developing novel therapeutic formulations. *Journal of Pharmaceutical and Biomedical Analysis, 161*, 425–435.

Marinaccio, L., Zengin, G., Bender, O., Cichelli, A., Novellino, E., Stefanucci, A., & Mollica, A. (2024). Ultrasound assisted lycopene extraction from tomato skin waste by volatile natural deep eutectic solvent. *Food Chemistry Advances*, Article 100656.

Martins, R., Barbosa, A., Advinha, B., Sales, H., Pontes, R., & Nunes, J. (2023). Green extraction techniques of bioactive compounds: A state-of-the-art review. *Processes, 11*, 2255.

Montesano, D., Cossignani, L., D'Arco, G., Simonetti, M. S., & Damiani, P. (2006). Pure lycopene from tomato preserves extra virgin olive oil from natural oxidative events during storage. *Journal of the American Oil Chemists' Society, 83*, 933–941.

Morais, D. V.d., Costa, M. A. P.d. C., Santa Bárbara, M. F., Silva, F.d. L., Moreira, M. M., Delerue-Mato, C., Guimaraes Dias, L. A., Estevinho, M. L. M., & Carvalho, C. A. L. D. (2018). Antioxidant, photoprotective and inhibitory activity of tyrosinase in extracts of Dalbergia ecastaphyllum. *PLoS One, 13*, Article e0207510.

Müller, L., Fröhlich, K., & Böhm, V. (2011). Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. *Food Chemistry, 129*, 139–148.

Naz, A., Butt, M. S., Pasha, I., & Nawaz, H. (2013). Antioxidant indices of watermelon juice and lycopene extract. *Pakistan Journal of Nutrition, 12*, 255.

Papaioannou, E. H., & Karabelas, A. J. (2012). Lycopene recovery from tomato peel under mild conditions assisted by enzymatic pre-treatment and non-ionic surfactants. *Acta Biochimica Polonica, 59*.

Rodriguez-Amaya, D. B. (2003). Food carotenoids: Analysis, composition and alterations during storage and processing of foods. *Forum of nutrition, 56*, 35–37.

Ronanki, S., Bethapudi, A., Maheswari, K. U., Boga, N., & Suneetha, W. J. (2018). *In vitro* studies on alpha amylase and alpha glucosidase inhibitory activities of tomato powder incorporated rice. *International Journal of Current Research, 8*, 42739–42742.

Shahraki, O., & Daneshmand, S. (2023). Lycopene-loaded solid lipid nanoparticles: Preparation, characterization, ROS-scavenging, and *in vitro* anti-melanogenesis evaluations. *Letters in Drug Design and Discovery, 20*, 1768–1774.

Slinkard, K., & Singleton, V. L. (1977). Total phenol analysis: Automation and comparison with manual methods. *American Journal of Enology and Viticulture, 28*, 49–55.

Stefanucci, A., Marinaccio, L., Llorent-Martínez, E. J., Zengin, G., Bender, O., Dogan, R., Atalay, A., Adegbite, O., Ojo, F. O., & Onaolapo, A. Y. (2024). Assessment of the in-vitro toxicity and in-vivo therapeutic capabilities of Juglans regia on human prostate cancer and prostatic hyperplasia in rats. *Food Bioscience, 57*, Article 103539.

Strati, I. F., & Oreopoulou, V. (2011). Effect of extraction parameters on the carotenoid recovery from tomato waste. *International Journal of Food Science and Technology, 46*, 23–29.

Tiwari, S., Upadhyay, N., Singh, A. K., Meena, G. S., & Arora, S. (2019). Organic solvent-free extraction of carotenoids from carrot bio-waste and its physico-chemical properties. *Journal of Food Science and Technology, 56*, 4678–4687.

van het Hof, K. H. (1999). *Dietary factors that affect carotenoid bioavailability* (pp. 1–194). Wageningen University and Research.