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Comparative analysis of chemical composition and biological activities of *Punica granatum* L. Extracts: UPLC-MS/MS profiling, antioxidant capacity, enzyme inhibition, and molecular docking

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ABSTRACT

Pomegranate (*Punica granatum* L.) has been valued for its medicinal and nutritional benefits across traditional healing systems. This study investigated the phytochemical profile, antioxidant capacity, and enzyme inhibitory potential of mature pomegranate leaves. Methanol and ethanol extracts were prepared and analyzed using UPLC-MS/MS, multiple antioxidant assays (Fe^{3+} reduction, CUPRAC, FRAP, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$), and targeted enzyme inhibition tests. The methanol extract exhibited the highest phenolic and flavonoid content (409.44 μg GAE/mg and 64.52 μg QE/mg), with ellagic acid, gallic acid, syringic acid, and luteolin identified as major compounds. Both extracts showed concentration-dependent antioxidant activity, with the methanol extract demonstrating a significantly stronger Fe^{3+} reducing capacity ($p < 0.05$) and superior $ABTS^{\bullet+}$ scavenging activity compared to standard antioxidants ($p < 0.05$). For carbonic anhydrase I and II, the extracts showed notable inhibition (IC_{50} = 0.0221, 0.0344 μg/mL), although not reaching the efficacy of acetazolamide (IC_{50} = 0.0061, 0.0072 μg/mL). Both extracts exhibited potent acetylcholinesterase inhibitory activity (IC_{50} = 0.0017 μg/mL), closely approximating the efficacy of tacrine (IC_{50} = 0.0010 μg/mL). The ethanol extract also showed remarkable α-glucosidase inhibition (IC_{50} = 0.0052 μg/mL), being 2.8-fold more potent than the acarbose (IC_{50} = 0.0147 μg/mL)—a noteworthy result, as natural compounds rarely exceed pharmaceuticals in such assays. Molecular docking confirmed ellagic acid's interaction with the target enzymes. These findings underscore pomegranate leaves as a valuable natural source of bioactive compounds, with promising applications in the management of diabetes, neurodegenerative disorders, and glaucoma —attributable to their rich phenolic content, strong antioxidant capacity, and notableenzyme inhibitory activities.

1. Introduction

Punica granatum L. (pomegranate) is a perennial medicinal plant of Mediterranean origin belonging to the Lythraceae family and is widely cultivated throughout the world. This species has been extensively used by many civilizations throughout history for the treatment of various ailments such as infections and inflammatory diseases, as well as atherosclerosis, diabetes, hypertension, hyperlipidemia, and cancer (Ge et al., 2021; Machado et al., 2023). The diverse biological properties identified in the fruit, peel, juice, flowers, roots, and leaves of *P. granatum* demonstrate the potential of this species in therapeutic and food applications (Saparbekova et al., 2023). Leaves, as the main part responsible for maintaining the vital functions of the plant, are a reservoir of various bioactive components, including phenolic compounds, fatty acids, and essential oils (Machado et al., 2023). These leaves are traditionally used in the treatment of throat infections, thrush, urinary tract infections, fever, flu, and pneumonia (Battineni et al., 2017). This widespread use in traditional medicine has also inspired

Abbreviations: $ABTS^{+}$, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AChE, Acetylcholinesterase; BHA, Butylated hydroxyanisole; BHT, Butylated hydroxytoluene; CA, Carbonic anhydrase; $DMPD^{\bullet+}$, *N*, *N*-dimethyl-*p*-phenylenediamine radicals; $DPPH^{\bullet}$, 1,1-diphenyl-2-picrylhydrazyl radicals; PGM, *Punica granatum* leaf methanol extract; PGE, *Punica granatum* leaf ethanol extract; GAE, Gallic acid equivalents; QE, Quercetin equivalent; α-Gly, α-glycosidase.

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modern scientific research.

Research has established numerous health benefits of pomegranate leaf extracts across various therapeutic areas. Studies indicate these extracts can maintain weight equilibrium and reduce cholesterol levels (Wang et al., 2018), offer protection against diabetic kidney complications, and provide anti-inflammatory, enzyme-inhibiting, and cell-toxicity effects (Bekir et al., 2013). Multiple investigations have confirmed their efficacy against bacterial infections (Trabelsi et al., 2020) and regulating blood glucose levels (Pottathil et al., 2020). Scientists have also recognized their tumor-suppressing capabilities (Wong et al., 2021), along with dermatological applications such as the prevention of scalp disorders and the potential to promote hair growth (Bhinge et al., 2021). The diverse medicinal properties stem from a rich array of bioactive substances, particularly phenolic compounds, flavonoid structures, and tannic constituents (Ge et al., 2021; Trabelsi et al., 2020; Wang et al., 2018; Zhang et al., 2010). While composition varies depending on growth location, extraction methodology, and harvest timing, researchers consistently identify several key compounds, including ellagic and gallic acids, punicalagin, and catechin variants. Notably, ellagic acid and related molecules provide cell protection by preventing oxidative damage, stabilizing lipid, and promoting cell survival mechanisms (Yu et al., 2024). *P. granatum* leaves contain a wide range of bioactive compounds, which have attracted significant attention due to their potential therapeutic benefits. However, the extraction of these compounds presents severalchallenges. Factors such assolvent selection, extraction duration, and variability in raw materials can significantly affect the yield and stability of bioactive compounds. The solvent used may not extract all bioactive components equally, and prolonged extraction times may lead to the degradation of sensitive compounds when exposed to light, heat, or oxygen. Additionally, variations in plant growth conditions, harvest time, and geographical origin contribute to inconsistency in the bioactive content, making standardization of the extraction process difficult. These challenges represent significant barriers to reproducible results and optimizing of *P. granatum* leaf extracts for pharmacological applications (Du et al., 2025; Marcelino et al., 2023; Pirzadeh et al., 2021).

Recent research has shown a strong correlation between the phenolic composition of various medicinal plants and their antioxidant and enzyme-inhibitory activities (Huyut et al., 2017; Saravanakumar et al., 2019; Şenol et al., 2023; Taslimi & Gulçin, 2018). Similarly, Llorent-Martínez et al., 2017 emphasized that the superior biological activities of plant extracts are directly related to their high phenolic content. The enzymes we focused on in our study, carbonic anhydrase (CAs) I-II, acetylcholinesterase (AChE), and α-glycosidase (α-Gly), have significant clinical value. CA-inhibiting agents, which play roles in various physiological processes such as intraocular pressure regulation, bone resorption, and acid-base balance, are widely used in the treatment of glaucoma (Ghorai et al., 2020). AChE inhibitors are used to alleviate symptoms of Alzheimer's disease (Jha et al., 2018). Consistent with the findings of Ojo et al., molecular docking analyses demonstrated that ellagic acid exhibited strong binding affinities toward key target proteins, highlighting its potential as a promising bioactive compound (Ojo et al., 2021). α-Gly is an intestinal enzyme involved in carbohydrate digestion, and its inhibition is considered a valuable therapeutic strategy for reducing postprandial hyperglycemia in diabetes management (Yu et al., 2024).

Despite their significant therapeutic and bioactive potential, *P. granatum* leaves have not been sufficiently researched and evaluated in the development of food and pharmaceutical products (Machado et al., 2023). Additionally, while the season-independent availability of pomegranate leaves provides an important advantage, studies on standardization and technological development related to the species' ethnopharmacopoeia remain limited. A critical gap in the current literature is the absence of comprehensive studies that concurrently assess the phytochemical profile, antioxidant capacity through multiple mechanisms, and enzyme inhibitory potential of pomegranate leaf extracts against diverse therapeutic targets. Furthermore, there is insufficient comparative data on how different extraction solvents like methanol and ethanol affect the resulting biological activities of pomegranate leaf extracts, even when detailed phytochemical profiling is available for one extract type.

In the present study, methanol and ethanol were selected as extraction solvents based on their established efficiency in extracting a wide range of bioactive compounds from plant materials (Patra et al., 2022). The choice of appropriate solvents plays a critical role in the efficient extraction of polyphenols. Methanol, being more polar, efficiently extracts lower molecular weight polyphenols, anthocyanins, terpenoids, saponins, and flavones, while ethanol effectively extracts tannins, polyphenols, flavanols, and alkaloids Additionally, ethanol offers advantages for potential pharmaceutical and food applications due to its safety for human consumption (Dai & Mumper, 2010). Using both solvents provides a more comprehensive evaluation of extractable bioactive compounds from pomegranate leaves, as the polarity of targeted compounds is a critical factor in extraction efficiency (Altemimi et al., 2017; Azmir et al., 2013).

This study aims to determine the comprehensive phytochemical profile of *P. granatum* leaf methanol (PGM) and ethanol (PGE) extracts, evaluate their antioxidant capacities through various mechanisms, and investigate their inhibitory potential against enzymes associated with diabetes, neurodegenerative diseases, and glaucoma. In this context, the reducing powers of the extracts (through Fe^{3+} reduction, CUPRAC, and FRAP analyses), radical scavenging capacities (with $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and $DMPD^{\bullet+}$ tests), and inhibitory effects on selected target enzymes (CA I-II, AChE, and α-Gly) were evaluated. Additionally, the phytochemical composition of the leaf extracts was characterized in detail by UPLC-MS/MS analysis, and the interaction mechanisms of the main bioactive components with target enzymes were aimed to be elucidated through molecular docking studies.

2. Materials and methods

2.1. Chemicals and reagents

Acetylcholinesterase from electric eel (*Electrophorus electricus*) with a purity of ≥100 units/mg protein (Type VI-S), acetylcholine iodide, α-Gly from *Saccharomyces cerevisiae* with a purity of ≥10 units/mg protein, and *p*-nitrophenyl-D-glucopyranoside were acquired from Sigma-Aldrich (Steinheim, Germany). CA I and CA II were purified from human erythrocytes isolated and supplied by a certified blood bank. The purification was performed using Sepharose-4B-L-tyrosine affinity chromatography according to established protocols (Burmaoglu et al., 2022). (1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), *N*,*N*-dimethyl-*p*-phenylenediamine ($DMPD^{\bullet+}$), and 2,9-dimethyl-1,10-phenanthroline (Neocuproine)) and reference standards (butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Trolox, α-tocopherol) were purchased from Sigma-Aldrich. Standard phenolic compounds for UPLC-MS/MS analysis were obtained from Sigma-Aldrich. Additional reagents and solvents were sourced from either Sigma-Aldrich or Merck (Darmstadt, Germany).

2.2. Plant material and extraction procedure

P. granatum mature leaves were harvested from Şanlıurfa Province (Bozova District), Türkiye, in September 2023. The collected leaves were dried at room temperature in a shaded, dry area. After drying, the leaves were ground to a fine powder using liquid nitrogen and a porcelain mortar. For extraction, 50 g of the powdered leaf material was combined with 250 mL of solvent (methanol or ethanol). The extraction process was conducted in a shaking water bath at 50 °C over a 72-h period. The mixture underwent filtration every 24 h, with fresh solvent added following each filtration step (Aggul et al., 2022; Demir

et al., 2016). The filtered extracts were combined and concentrated using a rotary evaporator (SCILOGEX RE100-Pro, USA) at 50 °C. The resulting dry extract was transferred to an amber glass container and maintained at 4 °C until further analysis.

2.3. Phytochemical compound characterization using UPLC-MS/MS

Phenolic compound profiling of *P. granatum* leaf extract was performed using an Agilent 1290 Infinity UPLC system paired with an Agilent 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The mass spectrometer was equipped with an electrospray ionization (ESI) source and operated in negative multiple reaction monitoring (MRM) mode according to previously described methods (Tohma et al., 2019). Separation was achieved on a Zorbax SB-C18 column (4.6 × 100 mm, 3.5 µm) at a column temperature of 30 °C. The mobile phase consisted of solution A (0.1 % formic acid in water) and solution B (0.1 % formic acid in acetonitrile). The gradient program was set as follows: initial 5 % B maintained until 4 min; increased to 20 % B by 7 min; increased to 90 % B by 14 min; held at 90 % B until 15 min; decreased to 5 % B at 15.1 min; and maintained at 5 % B until 17 min for column re-equilibration. For each analysis, 5 µL of sample was injected at a flow rate of 0.4 mL/min with a total analysis time of 17 min. The mass spectrometer parameters were established as follows: nitrogen gas temperature of 350 °C with 12 L/min flow rate; sheath gas temperature of 250 °C with 5 L/min flow rate; and nebulizer pressure of 55 psi. A full mass scan was conducted in the range of 50–1300 *m/z*. The Agilent MassHunter Workstation software was utilized for data collection and processing. MRM transitions and detailed UPLC-MS/MS validation parameters for all targeted phenolic compounds are indicated in Supplementary File S1.

2.4. Determination of total phenolic content

The total phenolic content of *P. granatum* leaf extracts was quantified using a modified version of the method described by Singleton and Rossi (1965), with adjustments as outlined by Gülçin et al. (2005). Results were expressed as gallic acid equivalents (GAE) (Köksal et al., 2017). The assay procedure involved combining 0.5 mL of the extracts with 1.0 mL of Folin-Ciocalteu reagent according to the protocol detailed by Çakmakçı et al. (2015). The solution was then supplemented with 0.5 mL of 1 % sodium carbonate solution and thoroughly mixed. Following a 2-h incubation period at room temperature under dark conditions, absorbance measurements were taken at 760 nm, using water as the blank reference. The phenolic content was calculated and reported as mg GAE per gram of extract.

2.5. Determination of total flavonoid content

Total flavonoid content was measured using the aluminum chloride ($AlCl_3$) colorimetric method as described by Gülçin et al. (2019). The assay was performed by combining 0.5 mL of the extracts (PGM and PGE) solution with 1.5 mL of 95 % methanol, followed by the addition of 1.5 mL of 10 % $AlCl_3$ solution, 0.5 mL of 1.0 M potassium acetate solution, and 2.3 mL of distilled deionized water. The mixture was incubated in the dark at 25 °C for 40 min, after which absorbance was recorded at 415 nm, with distilled water serving as the blank reference. Results were calculated and presented as mg quercetin equivalent (QE) per gram of extract, according to the method outlined by Kalin et al. (2015).

2.6. Antioxidant assays

Multiple complementary antioxidant assays were employed to ensure comprehensive evaluation, as no single method can fully reflect the overall antioxidant potential. The selected methods represent different reaction mechanisms: (1) electron transfer-based assays (Fe^{3+} reduction, CUPRAC, and FRAP) that measure the reducing capacity of antioxidants, and (2) radical scavenging assays (DPPH, ABTS, and DMPD) that evaluate the ability to neutralize different types of free radicals. These assays were chosen based on sample compatibility, analytical sensitivity, and widespread use in literature (Gülçin, 2012; Munteanu & Apetrei, 2021). To prevent radical stability issues, we maintained controlled incubation conditions (temperature and light).

2.6.1. Fe^{3+} reducing assay

The ferric ion (Fe^{3+}) reducing the power of *P. granatum* leaf extracts was assessed using a modified method based on protocols established by Oyaizu (1986) and subsequently modified by Gülcin (2020). This assay measures the capacity of extracts to reduce $Fe^{3+}(CN^{-})_6$, resulting in the formation of Perl's Prussian blue following the addition of excess Fe^{3+} ions. For the procedure, varying concentrations (10–30 µg/mL) of *P. granatum* leaf extracts (0.75 mL) were mixed with 1.25 mL of phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of potassium ferricyanide (1 % $K_3Fe(CN)_6$). After incubation at 50 °C for 30 min, the reaction mixture was supplemented with 1.25 mL of 10 % trichloroacetic acid (TCA). Finally, 0.5 mL of 0.1 % $FeCl_3$ was added, and the absorbance of the resulting solution was determined spectrophotometrically at 700 nm.

2.6.2. Cupric ions (Cu^{2+}) reducing capacity – CUPRAC assay

The cupric ions (Cu^{2+}) reducing capacity of *P. granatum* leaf extracts was evaluated using the CUPRAC assay, alongside reference antioxidants (BHA, BHT, α-tocopherol, and Trolox). This method utilizes neocuproine as the chromogenic oxidizing agent for the detection of antioxidant activity (Ak & Gülçin, 2008; Apak et al., 2006). The assay protocol involved combining 1.0 M acetate buffer, 10 mM $CuCl_2$ solution, and 7.5 mM neocuproine solution with extract samples at concentrations ranging from 10 to 30 µg/mL. After thorough homogenization of the reaction mixture, distilled water was added to bring the final volume to 2 mL. The solution was then maintained at room temperature for an incubation period of 30 min. Measurements were conducted against deionized water, which served as the blank reference. The antioxidant capacity was determined based on the increase in absorbance (450 nm) values observed for the samples.

2.6.3. Fe^{3+}-TPTZ reducing ability – FRAP assay

The Fe^{3+}-reducing antioxidant power (FRAP) assay was employed to evaluate the ability of *P. granatum* leaf extracts to reduce the TPTZ-Fe^{3+} complex under acidic conditions. Standard antioxidants, including BHA, BHT, α-tocopherol, and Trolox, were used as positive controls for comparison. The assay was conducted by combining 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution, 20 mM $FeCl_3$ solution, and 0.3 M phosphate buffer (pH 3.6) with the extracts at concentrations between 10 and 30 µg/mL. The reaction mixture was subjected to incubation at 37 °C for a period of 30 min. Subsequently, absorbance measurements were recorded at 593 nm, with buffer solution serving as the blank reference. As noted by Göçer and Gülçin (2011), the absorbance readings demonstrated a concentration-dependent increase, indicating higher reducing capacity with increasing extract concentration.

2.6.4. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity

The DPPH• scavenging method was utilized to assess the free radical neutralizing capacity of *P. granatum* leaf extracts, alongside reference antioxidants BHA, BHT, α-tocopherol, and Trolox. This assay evaluates the ability of compounds to donate hydrogen atoms to the stable DPPH radicals, resulting in their reduction. For experimental implementation, extracts at various concentrations (10 to 30 µg/mL) were combined with 0.1 mM DPPH• solution. The resulting mixtures were maintained at room temperature under dark conditions for 30 min. Following incubation, spectrophotometric measurements were taken at 517 nm, with ethanol employed as the blank reference. As described by Gaurav et al. (2023), the scavenging efficiency is inversely related to absorbance

values, where lower absorbance readings indicate greater DPPH radical neutralization by the test samples.

2.6.5. N,N′-dimethyl-p-phenylenediamine dihydrochloride ($DMPD^{\cdot+}$) deradicalization activity

The ability of *P. granatum* leaf extracts to inhibit $DMPD^{\cdot+}$ cation radical formation was evaluated using the $DMPD^{\cdot+}$ scavenging assay. For the preparation of $DMPD^{\cdot+}$ solution, 105 mg of $DMPD^{\cdot+}$ was dissolved in 5 mL of solution, and subsequently, 1 mL of this preparation was combined with 100 mL of acetate buffer (0.1 M, pH 5.3). The mixture was then subjected to 10 min of agitation under dark conditions. The $DMPD^{\cdot+}$ scavenging capacity assessment involved combining a mixture of 0.1 M phosphate buffer (pH 5.3), 0.05 M $FeCl_3$, and $DMPD^{\cdot+}$ solution with the extracts (PGM and PGE) and reference antioxidants (BHA, BHT, α-tocopherol, and Trolox) at concentrations between 10 and 30 μg/mL. Following a 1-h incubation period, absorbance was measured spectrophotometrically at 505 nm, with buffer solution used as the blank reference. The observed absorbance values were used to calculate the $DMPD^{\cdot+}$ scavenging efficiency of the samples (Fogliano et al., 1999; Karagecili et al., 2023).

2.6.6. Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) radical ($ABTS^{\cdot+}$) scavenging activity

The capacity of *P. granatum* leaf extracts to neutralize $ABTS^{\cdot+}$ radicals was evaluated according to the protocol established by Re et al. (1999), alongside reference antioxidants BHA, BHT, α-tocopherol, and Trolox. The $ABTS^{\cdot+}$ was generated through the reaction between 7.0 mM $ABTS^{\cdot+}$ solution and 2.45 mM potassium persulfate ($K_2S_2O_8$). Before testing, the absorbance of the $ABTS^{\cdot+}$ solution was standardized to 0.750 ± 0.025 at 734 nm by dilution with 0.1 M buffer (pH 7.4). Extract samples at concentrations ranging from 10 to 30 μg/mL were then introduced to this adjusted $ABTS^{\cdot+}$ solution. The reaction mixtures were maintained for 30 min, after which absorbance measurements were recorded at 734 nm. Ethanol was employed as the blank reference for measurements. The efficacy of $ABTS^{\cdot+}$ radical scavenging was determined by monitoring the reduction in absorbance values, with greater decreases indicating enhanced radical neutralization capacity (Gaurav Anwar et al., 2023; Re et al., 1999).

2.7. Enzyme inhibitory capacity assays

The inhibition of CA I and II isoenzymes was evaluated using enzymes purified from human erythrocytes *via* Sepharose-4B-L-Tyrosine sulphonamide affinity chromatography. The purification process involved precipitating erythrocytes by centrifugation, separating serum, and adjusting pH to 8.7 with solid Tris. The prepared sample was loaded onto an affinity column equilibrated with Tris-Na_2SO_4/HCl (22 mM/25 mM, pH 8.7). CA I and II isoenzymes were eluted using sodium acetate/$NaClO_4$ solution with pH adjusted to 5.6 at 25 °C. Protein concentration was determined using the Bradford method, and enzyme purity was verified by SDS-PAGE analysis. The esterase activity of CA I and II isoenzymes, as well as inhibition by extracts, was monitored through absorbance measurements at 348 nm (Huyut et al., 2017; Şenol et al., 2023; Taslimi & Gulçin, 2018).

The AChE inhibition analysis employed Ellman et al. (1961) utilizing acetylthiocholine iodide as the primary substrate. The analytical principle relies on quantifying the yellow chromophore produced through the DTNB (5,5′-dithiobis (2-nitrobenzoic) acid) reaction. The experimental procedure involved preparing reaction mixtures containing *P. granatum* leaf extracts (50–200 μL), Tris-HCl buffer solution (100 μL, 1.0 M, pH 8.0), and AChE enzyme preparation. Following thermal equilibration at 20 °C for 10 min, the reaction was initiated by introducing substrate and DTNB solutions (50 μL each). Enzyme inhibition was quantified through spectrophotometric measurements at 412 nm (Karageçili et al., 2023).

For α-Gly inhibition analysis, the method described by Tao et al. (2013) was employed, using *p*-nitrophenyl-D-glycopyranoside (p-NPG) as the substrate. The reaction mixture was prepared by combining 40 μL of extract with 200 μL of phosphate buffer-containing enzyme (0.15 EU/mL, pH 7.4). After pre-incubation, 50 μL of p-NPG solution (5 mM in phosphate buffer, pH 7.4) was added. Following incubation at 30 °C, the enzymatic reaction was monitored spectrophotometrically at 405 nm to determine inhibition rates (Özaslan et al., 2022).

2.8. Molecular docking studies

Molecular docking analysis was performed to investigate interactions between ellagic acid (the major compound) and target enzymes (CA I, CA II, AChE, and α-Gly) using the Schrödinger Molecular Modeling Suite Maestro 13.5 program (https://doi.org/10.1002/slct.202303054). The workflow comprised: (i) Ligand preparation: Chemical structures of ellagic acid and reference inhibitors (acetazolamide, tacrine, and acarbose) were retrieved as SMILES codes from PubChem and optimized using Maestro's LigPrep software (Schrödinger Release 2023–1: LigPrep, LLC, New York, NY 2023). (ii) Protein preparation: Crystal structures of target enzymes were obtained from the RCSB Protein Data Bank (PDB IDs: 4WR7 for CA I, 5AML for CA II, 4TVK for AChE, and 3L4Y for α-Gly) and processed using Protein Preparation Wizard in Schrödinger software (v.13.5.128) to optimize structures. (iii) Grid generation: Active site regions were defined using the receptor grid generation tool in Maestro 13.5. (iv) Docking simulations: Binding affinities and potential interactions between ligands and enzymes were calculated using the ligand docking module of Schrödinger Molecular Modeling Suite.

The re-docking approach is used to validate the molecular docking methodology. In this process, co-crystal ligands are separated from target proteins for further processing and re-docked to their respective proteins to validate successful docking. After performing the re-docking procedure, the best conformations of target compounds are superimposed with co-crystal ligands. In the validation process, poses with RMSD values below 2 Å are generally accepted as a criterion for determining correct binding structure prediction (Guney et al., 2023).

2.9. Statistical analysis and data evaluation

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation. In antioxidant activity tests, differences between PGM, PGE, and standard antioxidants were evaluated using One-Way ANOVA followed by Tukey's post-hoc test (SPSS 25, IBM). Values of $p < 0.05$ were considered significant. In radical scavenging tests (DPPH, ABTS, and DMPD) and enzyme inhibition studies, IC_{50} values were calculated using regression analysis in Excel. The concentration-response relationship was evaluated by the regression coefficient (r^2).

3. Results

3.1. Phytochemical analysis

3.1.1. Total phenolic and flavonoid contents

Quantitative analysis revealed that PGM contained substantially higher levels of bioactive compounds compared to PGE. Specifically, PGM contained 409.44 μg GAE/mg of total phenolic compounds and 64.52 μg QE/mg of flavonoids. In comparison, PGE contained 265.00 μg GAE/mg of phenolic compounds and 50.86 μg QE/mg of flavonoids.

3.1.2. UPLC-MS/MS profile of phytochemical compounds

Detailed phytochemical profiling of PGE using UPLC-MS/MS identified and quantified 10 phytochemical compounds out of 35 standard compounds analyzed, as presented in Table 1 and Fig. 1. Ellagic acid was identified as the major compound (34,695.8928 ng/mL), in agreement with previous findings on pomegranate leaves, as discussed in detail in

Table 1
Phytochemical profiling and quantification of phytochemical compounds in *Punica granatum* L. leaf extract using UPLC-MS/MS.

No	Analytes	RT	PGE (ng/mL)
1	Quinic Acid	2.359	14.3158
2	Fumaric Acid	3.971	387.9811
3	Gallic Acid	5.490	3408.1290
4	Pyrogallol	6.609	n.d.
5	Keracyanin Chloride	10.436	n.d.
6	Cyanidin-3-o-glucoside	10.452	n.d.
7	Chlorogenic Acid	10.860	n.d.
8	Catechin	11.167	n.d.
9	Peonidin-3-o-glucoside	11.220	n.d.
10	4-OH-Benzoic Acid	11.290	n.d.
11	Epicatechin	11.298	n.d.
12	Epigallocatechin Gallate	11.408	n.d.
13	Caffeic Acid	11.534	n.d.
14	Vitexin	11.699	n.d.
15	Vanillic Acid	11.840	n.d.
16	Hesperidin	12.030	n.d.
17	Naringin	12.031	n.d.
18	Ellagic Acid	12.036	34,695.8928
19	Syringic Acid	12.185	495.6746
20	p-Coumaric Acid	12.318	375.4048
21	Taxifolin	12.397	n.d.
22	Ferulic Acid	12.447	32.0906
23	Rosmarinic Acid	12.497	n.d.
24	Sinapic Acid	12.518	n.d.
25	Vanillin	12.638	n.d.
26	Myricetin	12.759	n.d.
27	Resveratrol	13.185	n.d.
28	Luteolin	13.356	325.7642
29	Quercetin	13.470	75.5758
30	Apigenin	13.966	11.1540
31	Naringenin	13.994	n.d.
32	Isorhamnetin	14.122	n.d.
33	Chrysin	15.489	n.d.
34	Galangin	15.546	n.d.
35	Curcumin	15.613	n.d.

R.T.: Retention time, PGE: *Punica granatum* L. leaf ethanol extract, n.d.: not detected.

Section 4. Other phenolic compounds detected in significant quantities included gallic acid (3408.1290 ng/mL), syringic acid (495.6746 ng/mL), fumaric acid (387.9811 ng/mL), and p-coumaric acid (375.4048 ng/mL). Flavonoids, including luteolin (325.7642 ng/mL), quercetin (75.5758 ng/mL), and apigenin (11.1540 ng/mL), were also identified. Additionally, quinic acid (14.3158 ng/mL) and ferulic acid (32.0906 ng/mL) were detected in minor amounts.

3.2. Antioxidant activity

3.2.1. Fe^{3+} reduction, CUPRAC, and FRAP reducing abilities results

The reducing abilities of PGM and PGE were evaluated using three complementary assays (Fe^{3+} reducing, CUPRAC, and FRAP) and compared with standard antioxidants (BHA, BHT, Trolox, and α-Tocopherol) across concentrations ranging from 10 to 30 μg/mL as presented in Fig. 2, with the results at 30 μg/mL specifically detailed in Table 2. Both extracts showed concentration-dependent reducing ability in all assays, with activity increasing proportionally with concentration from 10 to 30 μg/mL. The comparative analysis between extracts and standards was most pronounced at the highest tested concentration (30 μg/mL).

In the Fe^{3+} reducing assay, the methanol extract (30 μg/mL) exhibited significantly stronger reducing capacity (2.64 ± 0.06) compared to the ethanol extract (1.74 ± 0.08, $p < 0.05$). The methanol extract demonstrated comparable activity to the standard antioxidant BHA (2.78 ± 0.04, $p > 0.05$), while showing higher reducing ability than Trolox (1.71 ± 0.12, $p < 0.05$) and α-Tocopherol (1.16 ± 0.01, $p < 0.05$). However, BHT displayed the strongest Fe^{3+} reducing capacity (3.26 ± 0.06) among all tested samples, significantly higher than both extracts ($p < 0.05$). The high correlation coefficients ($r^2 > 0.9400$) confirmed the reliability of these measurements.

The CUPRAC assay revealed that both methanol (2.54 ± 0.04) and ethanol (2.49 ± 0.02) extracts exhibited comparable Cu^{2+} ion reducing capacities ($p > 0.05$). Moreover, both extracts significantly outperformed the standard antioxidants BHA (1.80 ± 0.02, $p < 0.05$), Trolox (0.97 ± 0.04, $p < 0.05$), and α-tocopherol (1.44 ± 0.02, $p < 0.05$). Only BHT (3.11 ± 0.09) showed significantly higher activity than both extracts ($p < 0.05$). The correlation coefficients ($r^2 > 0.9200$) validated the accuracy of these results.

For the FRAP assay, which measures Fe^{3+} reducing antioxidant power, the methanol extract (1.86 ± 0.02) showed slightly higher activity than the ethanol extract (1.80 ± 0.01), though this difference was not statistically significant ($p > 0.05$). Both extracts exhibited comparable reducing ability to Trolox (1.87 ± 0.05, $p > 0.05$) and α-Tocopherol (1.68 ± 0.06, $p > 0.05$), while BHA (1.99 ± 0.01) and particularly BHT (2.75 ± 0.02) demonstrated significantly stronger Fe^{3+} reducing capacity ($p < 0.05$ for both compared to the extracts). The high correlation coefficients ($r^2 > 0.9520$) confirmed the reliability of the FRAP assay data.

3.2.2. $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and $DMPD^{\bullet+}$ scavenging results

The multi-radical scavenging capacity of PGM and PGE was

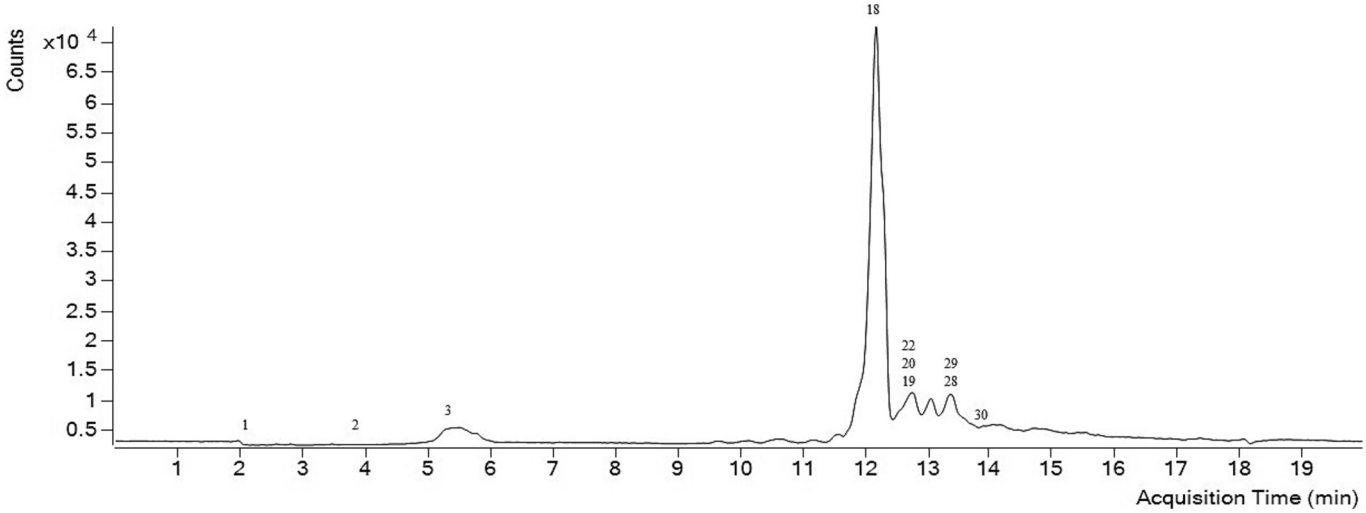


Fig. 1. UPLC-MS/MS multiple reaction monitoring chromatograms of phenolic compounds from *Punica granatum* leaf ethanol extract.

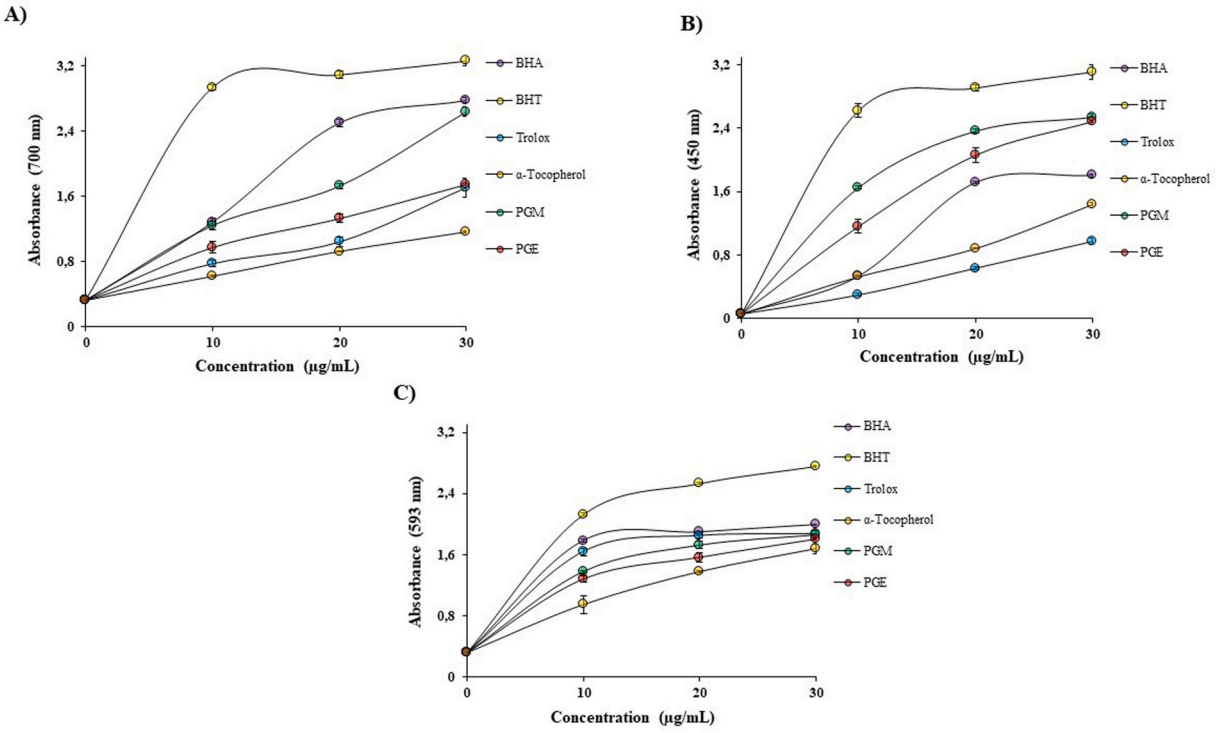


Fig. 2. The reducing capabilities of *Punica granatum* leaf ethanol, methanol extract and standards: Fe^{+3} reduction **(A)**, Cu^{+2} reduction **(B)**, Fe^{+3}-TPTZ reduction **(C).** Values represent mean ± SD (n = 3). BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene. PGE: *Punica granatum* L. leaf ethanol extract, PGM: *Punica granatum* leaf methanol extract.

Table 2
The reducing abilities *Punica granatum* L. leaf extracts (30 µg /mL).

Assay	Unit	Extracts		BHA	BHT	Trolox	AT
		Methanol	Ethanol				
Fe^{3+} reducing	λ_{700}	2.64 ± 0.06[b]	1.74 ± 0.08 [a]	2.78 ± 0.04[b]	3.26 ± 0.06[a,b]	1.71 ± 0.12[a]	1.16 ± 0.01[a,b]
	r^2	0.9868	0.9942	0.9815	0.9474	0.9832	0.9993
CUPRAC	λ_{450}	2.54 ± 0.04	2.49 ± 0.02	1.80 ± 0.02[a,b]	3.11 ± 0.09[a,b]	0.97 ± 0.04[a,b]	1.44 ± 0.02[a,b]
	r^2	0.9984	0.9990	0.9292	0.9604	0.9990	0.9952
FRAP	λ_{593}	1.86 ± 0.02	1.80 ± 0.01	1.99 ± 0.01[a,b]	2.75 ± 0.02[a,b]	1.87 ± 0.05	1.68 ± 0.06[a,b]
	r^2	0.9912	0.9842	0.9522	0.9798	0.9739	0.9998

AT: α-Tocopherol, BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene.
Values are mean ± SD (n = 3). One-way ANOVA with Tukey's test ($p < 0.05$). [a] $p < 0.05$ *vs.* PGM, [b] $p < 0.05$ *vs.* PGE.
AT: α-Tocopherol, BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene.

evaluated using three different radical systems ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, and $DMPD^{\bullet+}$) and compared with standard antioxidants. Fig. 3 illustrates the concentration-dependent radical scavenging effects across different concentrations, demonstrating clear dose-response relationships for both extracts, while Table 3 summarizes the IC_{50} values derived from these inhibition curves.

In the $DPPH^{\bullet}$ assay, both extracts demonstrated potent activity with IC_{50} values of 6.9315 µg/mL (methanol) and 7.3739 µg/mL (ethanol). The methanol extract showed 1.2-fold and 1.7-fold stronger $DPPH^{\bullet}$ scavenging ability than BHA (IC_{50}: 8.3512 µg/mL) and BHT (IC_{50}: 11.9508 µg/mL), respectively, while the ethanol extract was 1.1-fold and 1.6-fold more potent. Statistical analysis confirmed significant differences between the extracts and BHT ($p < 0.05$), with the methanol extract also showing significantly better activity than BHA ($p < 0.05$). The methanol extract's activity was comparable to Trolox (IC_{50}: 7.0015 µg/mL), while the ethanol extract's activity matched α-Tocopherol (IC_{50}: 7.3739 µg/mL). The high correlation coefficients ($r^2 > 0.9350$) confirmed the reliability of these findings.

In the $ABTS^{\bullet+}$ assay, both extracts exhibited exceptional radical scavenging activity, with the ethanol extract (IC_{50}: 2.8063 µg/mL) being 1.1-fold more effective than the methanol extract (IC_{50}: 3.0535 µg/mL). Both extracts substantially surpassed the standard antioxidants, with the methanol extract being 2.1-fold, 1.7-fold, and 8.7-fold more potent than BHA (IC_{50} = 6.3591 µg/mL), Trolox (IC_{50}: 5.1727 µg/mL), and α-Tocopherol (IC_{50}: 26.6595 µg/mL), respectively. The ethanol extract showed even greater potency, being 2.3-fold, 1.8-fold, and 9.5-fold more effective than BHA, Trolox, and α-Tocopherol, respectively. The activity of BHT (IC_{50}: 2.9496 µg/mL) was comparable to that of the extracts (1.03-fold difference with methanol extract and 1.05-fold difference with ethanol extract). Statistical analysis of absorbance measurements confirmed that both extracts performed significantly better than Trolox and DMPD ($p < 0.05$). The strong correlation coefficients ($r^2 > 0.93$) validated the accuracy of these results.

In the $DMPD^{\bullet+}$ assay, both extracts showed moderate activity, with the methanol extract (IC_{50}: 49.5105 µg/mL) performing slightly better than the ethanol extract (IC_{50}: 53.3190 µg/mL). However, both extracts exhibited significantly lower activity than standard antioxidants ($p < 0.05$), with the methanol extract being 2.0-fold and 3.8-fold less potent than BHA (IC_{50}: 24.7553 µg/mL) and Trolox (IC_{50}: 13.0783 µg/mL), respectively. Similarly, the ethanol extract was 2.2-fold and 4.1-fold less

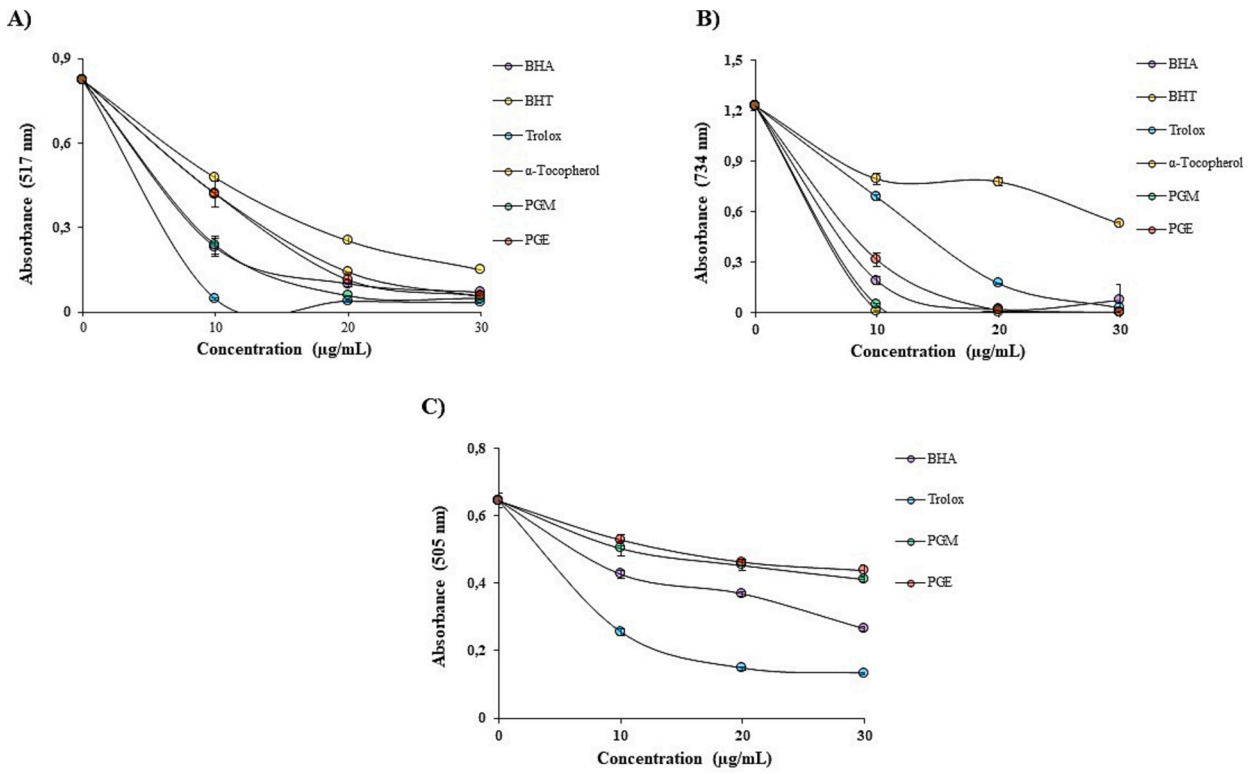


Fig. 3. Comparison of radical scavenging activities (IC_{50}, µg/mL) of *Punica granatum* leaf ethanol, methanol extract and reference antioxidants against **(A)** $DPPH^{\bullet}$, **(B)** $ABTS^{\bullet+}$, and **(C)** $DMPD^{\bullet+}$ radicals.
Values represent mean ± SD (n = 3). BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene. PGE: *Punica granatum* L. leaf ethanol extract, PGM: *Punica granatum* leaf methanol extract.

Table 3
Multi-radical scavenging activity assessment of *Punica granatum* L. leaf extracts.

Assay	Unit	Extract		BHA	BHT	Trolox	AT
		Methanol	Ethanol				
$DPPH^{\bullet}$	IC_{50}, µg/mL	6.9315	7.3739	8.3512	11.9508	7.0015	7.3739
	r^2	0.9930	0.9971	0.9817	0.9992	0.9359	0.9994
$ABTS^{\bullet+}$	IC_{50}, µg/mL	3.0535	2.8063	6.3591	2.9496	5.1727	26.6595
	r^2	0.9419	0.9948	0.9786	0.9354	0.9936	0.9158
$DMPD^{\bullet+}$	IC_{50}, µg/mL	49.5105	53.3190	24.7553	–	13.0783	–
	r^2	0.9892	0.9998	0.9724	–	0.9896	–

AT: α-Tocopherol, BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene.

effective. BHT and α-Tocopherol activities were not determined in this assay. The high correlation coefficients ($r^2 > 0.9720$) confirmed the reliability of these measurements.

3.3. Enzyme inhibition results

The enzyme inhibition properties of PGM and PGE were evaluated against four physiologically important enzymes: CAs I and II, AChE, and α-Gly. Fig. 4 illustrates the concentration-dependent inhibition profiles, while Table 4 presents the calculated IC_{50} values compared with reference inhibitors.

For CA I isoenzyme inhibition, both extracts showed activity with IC_{50} values of 0.0221 µg/mL and 0.0234 µg/mL for PGM and PGE, respectively. Reference inhibitor Acetazolamide exhibited an IC_{50} value of 0.0061 µg/mL, with PGM and PGE showing approximately 3.6-fold and 3.8-fold higher IC_{50} values.

In the CA II isoenzyme inhibition assay, PGE showed an IC_{50} value of 0.0240 µg/mL compared to PGM (IC_{50}: 0.0344 µg/mL), representing a 1.4-fold difference. Both extracts showed higher IC_{50} values than Acetazolamide (IC_{50}: 0.0072 µg/mL), with PGM being 4.8-fold and PGE 3.3-fold higher.

Both extracts displayed AChE inhibition with identical IC_{50} values (0.0017 µg/mL), which was 1.7-fold higher than the reference inhibitor tacrine (IC_{50}: 0.0010 µg/mL).

In the α-Gly inhibition assay, both extracts demonstrated inhibitory activity with IC_{50} values of 0.0073 µg/mL (PGM) and 0.0052 µg/mL (PGE). Compared to the clinical reference inhibitor acarbose (IC_{50}: 0.0147 µg/mL), PGM and PGE showed 2.0-fold and 2.8-fold lower IC_{50}, respectively.

3.4. Molecular docking analysis of ellagic acid interactions with target enzymes

Table 5 presents the computational analysis of binding interactions between ellagic acid (the major bioactive compound) and four target enzymes (CA I, CA II, AChE, and α-Gly), while Fig. 5 illustrates these interactions in both 2D and 3D formats. For CA I (PDB: 4WR7), ellagic acid exhibited a reasonable binding affinity with a docking score of

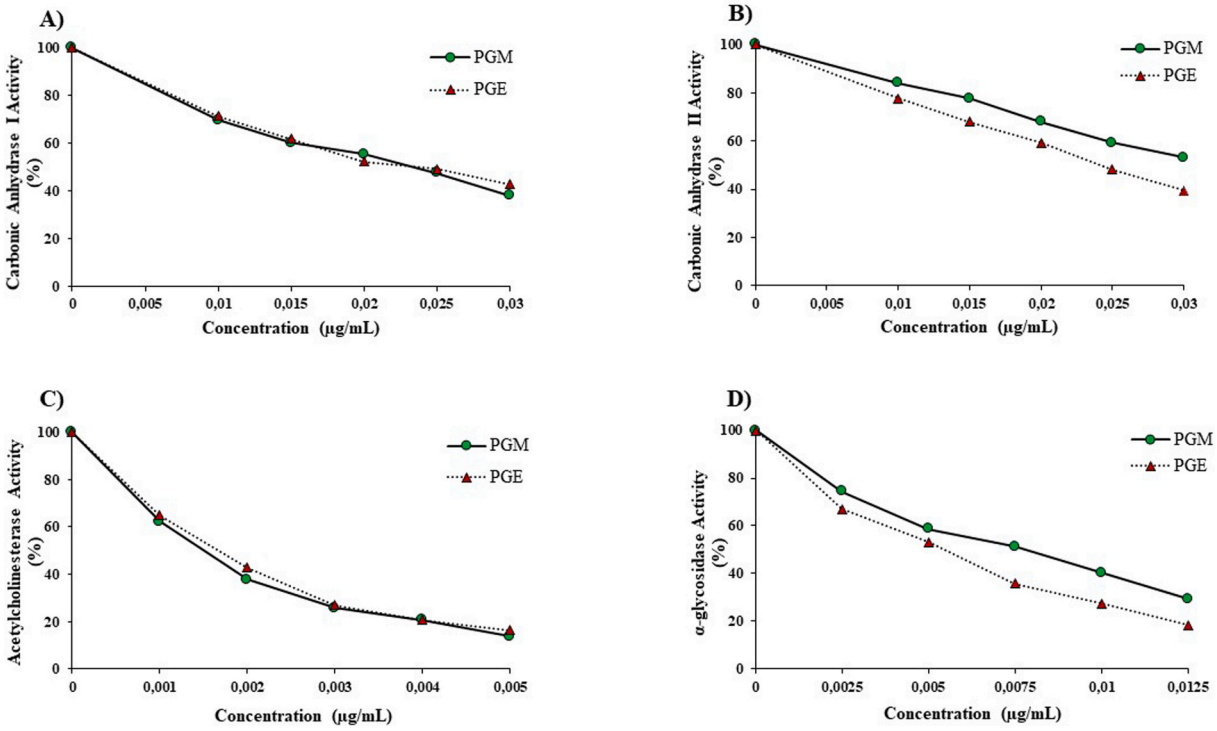


Fig. 4. Enzyme inhibitory activities (IC_{50}, µg/mL) of *Punica granatum* leaf ethanol, methanol extract and reference inhibitors against **(A)** CA I, **(B)** CA II, **(C)** AChE, and **(D)** α-Gly.
Values represent mean ± SD (n = 3). CA: carbonic anhydrase, AChE: acetylcholinesterase, α-Gly: α-glycosidase, PGE: *Punica granatum* L. leaf ethanol extract, PGM: *Punica granatum* leaf methanol extract.

Table 4
Enzyme inhibition activities of *Punica granatum* L. leaf extracts.

Enzyme	Extract IC_{50} (µg/mL)		Reference Inhibitor	References IC_{50} (µg/mL)
	Methanol	Ethanol		
CA I	0.0221 (r^2:0.9906)	0.0234 (r^2:0.9901)	Acetazolamide	0.0061 (r^2:0.9983)
CA II	0.0344 (r^2:0.9873)	0.0240 (r^2:0.9892)	Acetazolamide	0.0072 (r^2:0.9850)
AChE	0.0017 (r^2:0.9912)	0.0017 (r^2:0.9947)	Tacrine	0.0010 (r^2:0.9856)
α-Gly	0.0073 (r^2:0.9895)	0.0052 (r^2:0.9946)	Acarbose	0.0147 (r^2:0.9922)

Reference inhibitors: Acetazolamide for carbonic anhydrases (CAs) I and II, tacrine for acetylcholinesterase (AChE), and acarbose for α-glycosidase (α-Gly)

−5.2 kcal/mol, though this was less favorable than acetazolamide (−6.0 kcal/mol). For CA II (PDB: 5AML), ellagic acid demonstrated a stronger binding affinity (−5.6 Kcal/mol) compared to acetazolamide (−4.2 Kcal/mol). For AChE (PDB: 4TVK), ellagic acid showed an impressive binding affinity (−11.5 Kcal/mol), approaching that of tacrine (−13.8 Kcal/mol). In the case of α-Gly (PDB: 3L4Y), ellagic acid showed moderate binding affinity (−6.226 Kcal/mol) compared to acarbose (−14.705 Kcal/mol).

3.5. Docking validation studies

The re-docking method was applied in this study to verify the accuracy of the docking process. As shown in Fig. 6, the RMSD (root mean square deviation) values for ligands 3TV, 51 J, TJH, and NR4 were calculated as 1.0595, 1.8722, 1.7187, and 1.6261 Å, respectively.

Table 5
Molecular docking interactions of the major phenolic compound of *Punica granatum* L. (Ellagic acid) with CAs I and II, AChE, α-Gly.

Ligand – Protein complex	PDB id	Affinity (Kcal/mol)	Interactions at the active pocket	
			Type of interaction	Residue information
Ellagic acid – CA I	4WR7	−5.2	H Bond	GLN 92, HIE 67, ASN 69
Ellagic acid – CA II	5AML	−5.6	π-π stacking H Bond	PHE 13 (5.14 Å) ASN 67 (1.96 Å)
Ellagic Acid – AChE	4TVK	−11.5	π-π stacking H Bond	THR 121(5.17 Å) PHE 330 (4.40, 5.15, 5.02 Å), TYR 121(5.17 Å), TYR 334 (4.98, 5.46 Å) PHE 288 (2.05 Å)
Ellagic Acid – α-Gly	3L4Y	−6.2	H Bond	ASP 203 (1.79 Å) ARG 526 (2.07 Å)

*CA: Carbonic anhydrase, AChE: Acetylcholinesterase, α-Gly: α-glycosidase.

4. Discussion

Our analysis demonstrates significant levels of beneficial compounds in pomegranate leaf extracts, with methanol-based extraction yielding 409.44 µg GAE/mg phenolics and 64.52 µg QE/mg flavonoids, while ethanol-based methods produced 265.00 µg GAE/mg phenolics and 50.86 µg QE/mg flavonoids. Such findings align with existing research documenting the substantial bioactive properties of these plant materials (Machado et al., 2023; Saparbekova et al., 2023; Trabelsi et al., 2020; Yu et al., 2024; Zhang et al., 2010). When examining relative concentrations, pomegranate foliage often surpasses both conventional medicinal herbs and standard fruit plants in phenolic and flavonoid abundance. Turkish investigators identified methanol-extracted

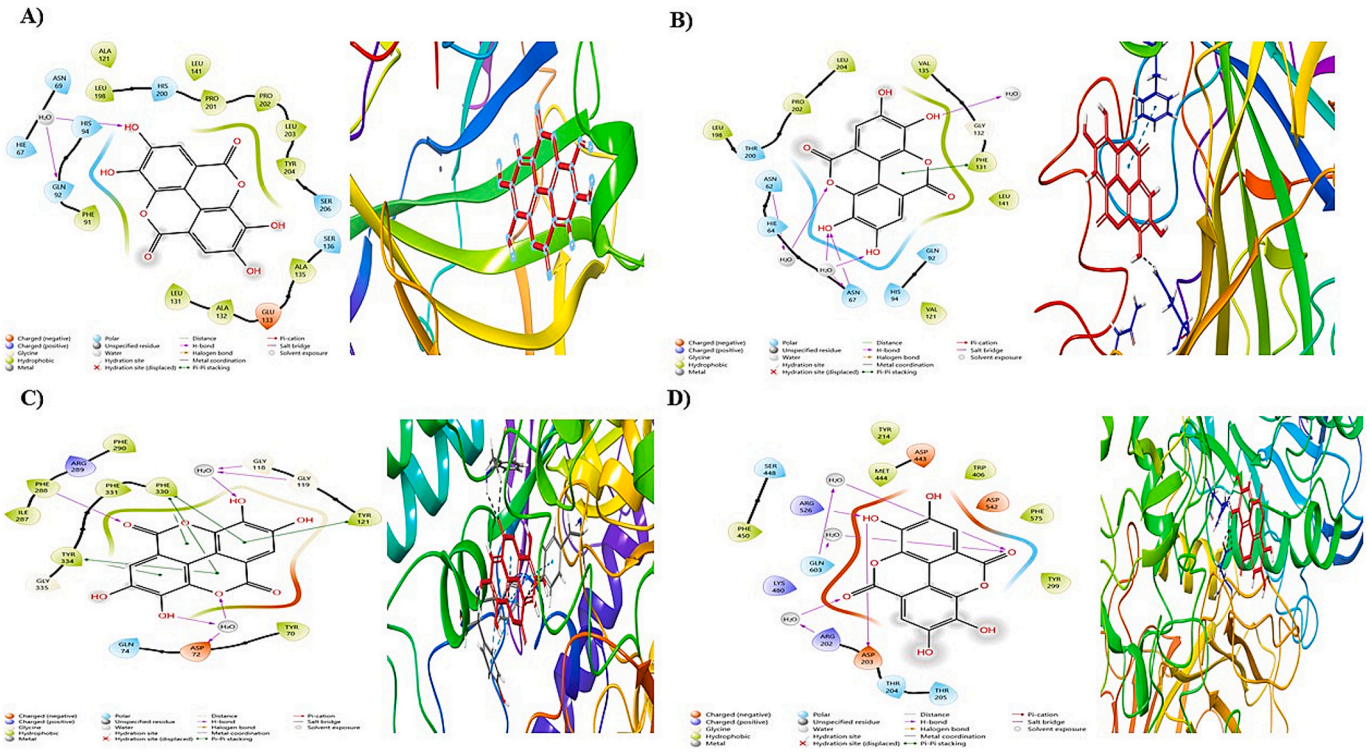


Fig. 5. Molecular docking analysis showing 2D and 3D interactions of ellagic acid with target enzymes: **(A)** CA I, **(B)** CA II, **(C)** AChE, and **(D)** α-Gly.
CA: carbonic anhydrase; AChE: acetylcholinesterase; α-Gly: α-glycosidase.

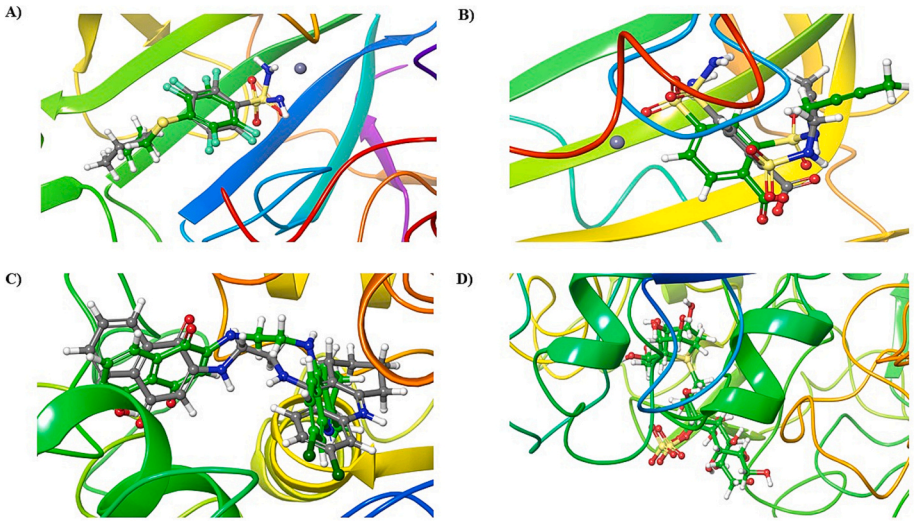


Fig. 6. *Re*-docking validations of enzyme-ligand complexes: (A) CA I-3TV, (B) CA II-51 J, (C) AChE-TJH, (D) α-Gly-NR4, Co-crystallized ligands are shown in gray, while re-docked ligands are represented as ball and stick models in green. The zinc ion (Zn^{2+}) is depicted as a gray sphere.
CA: carbonic anhydrase; AChE: acetylcholinesterase; α-Gly: α-glycosidase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pomegranate leaves as containing the highest phenolic levels among various fruit tree foliage samples (Uysal et al., 2016). Similarly, Thai researchers found ethanol-derived pomegranate leaf material contained superior concentrations of both compound types (3.13 mg GAE/g phenolics; 237 μg QE/g flavonoids) when comparing ten plant species with known health benefits (Kaewnarin et al., 2014). Examinations of Tunisian Gabsi varieties revealed leaf phenolic acid measurements (721.9 mg FAE/kg) substantially exceeding those from peel and flower components (164.5 and 173.3 mg FAE/kg) (Fellah et al., 2018; Fellah et al., 2020) Indian samples showed regional variations, with Maharashtra specimens yielding 126.667 mg RE/g flavonoids (Mestry & Juvekar, 2017) and Gujarat material containing 102.02 mg QE/g (Ankita et al., 2015). Our measured flavonoid values (64.52 and 50.86 μg QE/mg) appear comparatively lower, though such differences likely result from cultivar selection, growing location, harvest timing, and processing methodology rather than analytical discrepancies.

As a result of UPLC-MS/MS analysis, 10 phytochemical compounds were identified in PGE, among which ellagic acid stood out as the major bioactive compound. The dominance of ellagic acid is particularly noteworthy given its well-characterized antioxidant, anti-inflammatory,

and enzyme inhibitory properties (Mohammadinejad et al., 2022). The detected value (34,695.8928 ng/mL) is significantly higher than the ellagic acid content reported in pomegranate peels grown in Tunisia (35.89 mg/100 g) (Elfalleh et al., 2011). The presence of ellagic acid in such high concentration is consistent with the literature, as it is among the most frequently identified phenolic acids in pomegranate leaves along with gallic acid (Pottathil et al., 2020). Similarly, in our study, gallic acid (3408.1290 ng/mL) was detected as the compound with the second-highest concentration in the extract after ellagic acid. It has been reported that punicalagin and gallagic acid concentrations in leaf extracts of the Wonderful variety in Israel were higher than ellagic acid and gallic acid (Orgil et al., 2014). This difference may be related to the fact that ellagic acid and gallic acid are precursors for ellagitannins such as punicalagin (Ono et al., 2012) and reflect the influence of the pomegranate variety used growing conditions, and extraction method. Additionally, other phenolic compounds such as syringic acid (495.6746 ng/mL), fumaric acid (387.9811 ng/mL), and p-coumaric acid (375.4048 ng/mL) were detected in notable amounts in the extract. A study from Turkey demonstrated that aqueous extracts of pomegranate leaves contained significantly higher concentrations of gallic acid (6.92 mg/g *vs.* 0.73 mg/g) and chlorogenic acid (22.25 mg/g *vs.* 3.32 mg/g) compared to methanol extracts (Uysal et al., 2016). The choice of extraction solvent is a critical factor in the extraction of phenolic compounds, as some phenolic acids are specific to certain solvents: p-hydroxybenzoic acid and benzoic acid were found only in methanolic *P. granatum* leaf extract, while ferulic acid was found only in water extracts (Uysal et al., 2016). Flavonoids such as luteolin (325.7642 ng/mL), quercetin (75.5758 ng/mL), and apigenin (11.1540 ng/mL) were also detected at lower concentrations. These findings are consistent with the main flavonoids identified in pomegranate leaves in the literature; apigenin, luteolin, quercetin, rutin, catechin, and kaempferol and their derivatives have been commonly detected in various pomegranate leaf extracts (Ankita et al., 2015; Farag et al., 2014; Mestry & Juvekar, 2017; Pottathil et al., 2020). In the literature, it has been reported that pomegranate peel contains tannins ranging from 193 to 420 mg/g and flavonoids ranging from 84 to 134 mg/g in dry matter (Pottathil et al., 2020). The high phenolic content we detected in leaf extracts in our study confirms that not only the fruit of pomegranate but also its leaves are a valuable source of bioactive compounds. These findings are also consistent with the content detected by de Lima et al. (2021) in their phytochemical analysis of the crude extract and different fractions (hexanoic, ethyl acetate, and aqueous fractions) of *P. granatum* leaves.

In the Fe^{3+} reduction test, it was observed that PGM (2.64 ± 0.06) exhibited a significantly stronger reduction capacity compared to PGE (1.74 ± 0.08, $p < 0.05$). PGM demonstrated a reduction capability comparable to the standard antioxidant BHA ($p > 0.05$) and significantly higher than Trolox ($p < 0.05$) and α-Tocopherol ($p < 0.05$). The effect of solvent choice on the antioxidant potential of extracts was most evident in this test. This finding is consistent with the study by Kaneria et al. (2012), the researchers showed that different extraction techniques and solvents significantly affected the antioxidant capacity of pomegranate leaves. They particularly noted that sequential extraction with acetone was especially effective in extracting antioxidants from *P. granatum* leaves obtained from India, showing higher $DPPH^{\bullet}$ and stronger FRAP compared to pomegranate stem. The CUPRAC method measures the capacity of extracts to reduce Cu^{2+} ions, and in this test, PGM (2.54 ± 0.04) and PGE (2.49 ± 0.02) were observed to exhibit similar activity ($p > 0.05$). Both extracts demonstrated significantly superior Cu^{2+} reduction capabilities compared to standard antioxidants BHA, Trolox, and α-Tocopherol ($p < 0.05$). In the FRAP analysis, PGM (1.86 ± 0.02) showed a slight superiority compared to PGE (1.80 ± 0.01, $p > 0.05$), and both extracts exhibited Fe^{3+} reduction power comparable to Trolox and α-Tocopherol ($p > 0.05$). The fact that the FRAP test measures antioxidant capacity, particularly in acidic environments indicates that the extracts maintain their efficacy under different pH conditions. When the results of the three reduction tests are evaluated holistically, it becomes apparent that the capacity of *P. granatum* leaf extracts to reduce metal ions is a crucial component of their antioxidant mechanism.

Our antioxidant evaluation revealed exceptional free radical neutralization capabilities in both pomegranate leaf extracts. Against $DPPH^{\bullet}$ radicals, PGM demonstrated superior performance (IC_{50}: 6.93 μg/mL) compared to PGE (IC_{50}: 7.37 μg/mL), both significantly surpassing reference antioxidants BHA and BHT ($p < 0.05$). This extraction solvent-dependent variation aligns with Zhang et al. (2010) observations regarding the relationship between phenolic/flavonoid concentrations and antioxidant potential. Even more remarkable were the results from $ABTS^{\bullet+}$ radical scavenging assessments, where both extracts achieved impressive neutralization rates (IC_{50}: 2.81–3.05 μg/mL), significantly exceeding the effectiveness of BHA, Trolox, and α-Tocopherol ($p < 0.05$). These findings align with research by Yu et al. (2021), who documented Portuguese pomegranate leaf preparations outperform established medicinal herbs, such as sage and rosemary, in terms of antioxidant capability. Multiple comparative analyses support this pattern of exceptional antioxidant activity. Kolar et al. (2021) and Rummun et al. (2013) independently confirmed that pomegranate's non-edible tissues, particularly the leaves, exhibit stronger antioxidant properties than the traditionally used peel and seeds. de Lima et al. (2021) further established a direct connection between the chemical constituents identified in foliar tissue and the electron-donating mechanisms observed during both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ testing protocols. When challenged with $DMPD^{\bullet+}$ radicals, however, the extracts showed less pronounced scavenging abilities (IC_{50}: 49.51–53.32 μg/mL), performing significantly worse than BHA and Trolox ($p < 0.05$), suggesting that their protective mechanisms may function selectively depending on the specific reactive species encountered. Despite the methanol extract containing higher levels of phenolic compounds (409.44 *vs.* 265.00 μg GAE/mg) and flavonoids (64.52 *vs.* 50.86 μg QE/mg), the ethanol extract demonstrated comparable or superior activity in several assays, particularly ABTS. This suggests that antioxidant capacity depends not only on the quantity of phenolics but also on their specific structures, potential synergistic interactions, and possibly non-phenolic antioxidants not detected by standard assays (Gülcin, 2020).

The integration of molecular docking with enzyme inhibition data provided insights into potential inhibitory mechanisms, though docking scores alone cannot predict actual biological activity. For CA I, the predicted binding affinity of ellagic acid (−5.2 Kcal/mol) was lower compared to acetazolamide (−6.0 Kcal/mol), which aligns with our findings where PGM and PGE (IC_{50}: 0.0221 and 0.0234 μg/mL, respectively) were approximately 3.6–3.8 fold less potent than acetazolamide (IC_{50}: 0.0061 μg/mL).

Interestingly for CA II, the docking score of ellagic acid (−5.6 kcal/mol) was stronger than acetazolamide (−4.2 Kcal/mol), yet experimentally, PGM and PGE (IC_{50}: 0.0344 and 0.0240 μg/mL, respectively) were still 3.3–4.8 fold less potent than acetazolamide (IC_{50}: 0.0072 μg/mL). This discrepancy highlights the limitations of single-compound docking simulations, which cannot fully capture the complex interactions that occur in multi-component extracts, including potential allosteric effects, binding site competition, or cooperative interactions between different compounds.

For AChE, the docking results suggested a high binding affinity of ellagic acid (−11.5 Kcal/mol) compared to tacrine (−13.8 Kcal/mol), which correlates well with our experimental findings where the extracts (IC_{50}: 0.0017 μg/mL) were only 1.7-fold less potent than tacrine (IC_{50}: 0.0010 μg/mL). This value is consistent with the strong AChE inhibition activity of the ethanol extract of pomegranate leaves reported by Bekir et al. (2013). These findings highlight the extraordinary potential of pomegranate leaf as an AChE inhibitor, particularly for disorders characterized by cholinergic deficiency, such as Alzheimer's disease. In research conducted by Jha et al. (2018), they found that ellagic acid was safe in the neuroblastoma (SH-SY5Y) cell line and exhibited strong antioxidant activity in the $DPPH^{\bullet}$ test. Additionally, ellagic acid was

reported to effectively inhibit the AChE enzyme obtained from the electric eel (*Electrophorus electricus*). More importantly, it was reported that administration of ellagic acid (30 days, 50 mg/kg, oral) to rats in a streptozotocin-induced Alzheimer's disease model provided significant decreases in oxidative stress, inflammatory markers (GFAP, and CRP), AChE activity, and amyloid-β plaque formation, increased levels of synaptophysin, a neural connection marker, and protected neural structures. This suggests that pomegranate leaf extracts may exhibit protective effects in neurodegenerative diseases not only through enzyme inhibition but also by reducing neuroinflammation and oxidative stress.

The most intriguing discrepancy between docking predictions and experimental results was observed in α-Gly inhibition. Despite ellagic acid showing a substantially lower docking score (−6.2 Kcal/mol) compared to acarbose (−14.7 Kcal/mol), the extracts (PGM and PGE with IC_{50}: 0.0073 and 0.0052 μg/mL, respectively) exhibited approximately 2- and 2.8-times stronger inhibition than acarbose (IC_{50}: 0.0147 μg/mL). The superior inhibitory activity likely results from synergistic effects among multiple compounds in the extracts, as gallic acid, luteolin, and quercetin also possess α-Gly inhibitory properties (Proença et al., 2017). Additionally, compounds may bind to allosteric sites or through multiple binding modes not captured in our single-compound docking simulation, or the extracts may contain unidentified potent inhibitors (Jhong et al., 2015; Rasouli et al., 2017).

The ability of pomegranate leaf extract to inhibit the α-Gly *in vitro* suggests a potential mechanism for regulating postprandial blood glucose levels, as this enzyme plays an important role in polysaccharide digestion (Yu et al., 2024). In a study conducted by Patel et al. (2014), the ethyl acetate fraction of pomegranate leaf extract showed multiple effects in experimental models, including enzyme inhibition, increased glycogenesis, improved glucose uptake by muscles, and reduced intestinal glucose absorption independent of pancreatic insulin release. These findings, while promising, require validation in clinical settings. Similarly, the strong enzyme inhibitory activity by silver nanoparticles synthesized with aqueous pomegranate leaf extract, as reported by Saratale et al. (2018), represents an interesting approach that warrants further investigation. From a theoretical perspective, our observed 2.0–2.8-fold greater *in vitro* potency compared to acarbose could be advantageous, considering that acarbose therapy (50–100 mg thrice daily) often causes gastrointestinal side effects in up to 30 % of patients (Derosa & Maffioli, 2012). While our findings suggest potential therapeutic applications, further studies on bioavailability, safety, and *in vivo* efficacy would be necessary before clinical consideration.

Evaluating the safety profile of pomegranate leaf extracts used in our study is of critical importance for their potential therapeutic applications. Pomegranate leaves have long been used in traditional medicine and are generally considered safe. *In vitro* studies conducted by Elbatanony et al. (2019) have shown that pomegranate leaf extracts are safe in normal and cancer cells at certain concentrations; however, Bekir et al. (2013) indicated that cytotoxic effects may vary depending on the extraction solvent used. *In vivo*, acute toxicity tests conducted by Das and Barman (2012) revealed a non-toxic profile at certain doses in animal models. A systematic review conducted by Zare et al. (2024), including 66 clinical studies, indicated that pomegranate and its extracts are generally safe, with gastrointestinal problems and allergic reactions occurring rarely. However, the insufficiency of clinical studies specifically focusing on the therapeutic and toxicological properties of pomegranate leaves indicates that more comprehensive research is needed in this area. Additionally, the potential risks of residues from organic solvents used in extraction processes should be considered, and appropriate purification processes should be applied for human use (Yu et al., 2024).

Although this study reveals the rich phytochemical profile and various biological activities of pomegranate leaf extracts, several limitations should be acknowledged. Our research is limited to *in vitro* conditions, and it is important to emphasize that these results may not directly translate to *in vivo* efficacy or clinical outcomes due to complex physiological factors, including absorption, metabolism, and excretion. Extraction challenges, including solvent selection effects, compound degradation during processing, and raw material variability due to growth conditions, impact the standardization of results. The absence of cytotoxicity and safety assessments is another significant limitation, as is the potential presence of solvent residues that could affect the extracts' safety profile. Additionally, the poor bioavailability of phenolic compounds remains a challenge for therapeutic applications. Compounds detected in our analysis may not represent all bioactive components, and our molecular docking focused only on ellagic acid, overlooking potential contributions from other compounds. Being limited to samples from a single geographical region further restricts the generalizability of our findings. Future research should address these limitations through *in vivo* validation, safety assessments, bioavailability enhancement strategies, and more comprehensive mechanistic studies to fully realize the therapeutic potential of this valuable plant resource.

The antioxidant and enzyme inhibitory effects observed in pomegranate leaf extract suggest its potential use as a dietary supplement in the management of oxidative stress-related diseases. Furthermore, the inhibition of α-glycosidase and acetylcholinesterase enzymes indicates that the bioactive compounds in the leaf may serve as lead candidates for drug development. These findings support the pharmacological potential of pomegranate leaf and provide a scientific basis for translational studies.

5. Conclusion

This study has comprehensively compared for the first time the inhibition activity of *P. granatum* leaves on CA I, CA II, AChE, and α-Gly, revealing superior efficacy to acarbose, especially in α-Gly inhibition. The high ellagic acid content and rich bioactive component profile determined by UPLC-MS/MS analysis explain the molecular basis of these activities, while the presence of other metabolites like gallic acid likely contributes to the synergistic effects observed. While these findings support the potential pharmaceutical applications of *P. granatum* leaves, particularly for diabetes management, our *in vitro* results require *in vivo* validation, and concerns regarding solvent residues and bioavailability need to be addressed. Clinical trials are needed to confirm efficacy and safety before therapeutic use. With these considerations, pomegranate leaves represent a promising resource for natural therapeutic development that warrants further investigation.

CRediT authorship contribution statement

Lale Duysak: Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Adem Ertürk:** Formal analysis, Data curation. **Merve Becit-Kızılkaya:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Betul Aksoy:** Formal analysis. **İlhami Gülçin:** Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization.

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Ethical statement

Ethics approval was not required for this research. This study does not involve any human or animal testing.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.117139.

Data availability

Data will be made available on request.

References

Aggul, A. G., Demir, G. M., & Gulaboglu, M. (2022). Ethanol extract of myrtle (*Myrtus communis* L.) berries as a remedy for streptozotocin-induced oxidative stress in rats. *Applied Biochemistry and Biotechnology, 194*(4), 1645–1658.

Ak, T., & Gülçin, I. (2008). Antioxidant and radical scavenging properties of curcumin. *Chemico-Biological Interactions, 174*(1), 27–37.

Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D. G., & Lightfoot, D. A. (2017). Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. *Plants, 6*(4), 42.

Ankita, P., Deepti, B., & Nilam, M. (2015). Flavonoid rich fraction of *Punica granatum* improves early diabetic nephropathy by ameliorating proteinuria and disturbed glucose homeostasis in experimental animals. *Pharmaceutical Biology, 53*(1), 61–71.

Apak, R., Güçlü, K., Özyürek, M., Esin Karademir, S., & Erçağ, E. (2006). The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. *International Journal of Food Sciences and Nutrition, 57*(5–6), 292–304.

Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., … Omar, A. K. M. (2013). Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering, 117*(4), 426–436.

Battineni, J. K., Boggula, N., & Bxakshi, V. (2017). Phytochemical screening and evaluation of anti-emetic activity of *Punica granatum* leaves. *European Journal of Pharmaceutical and Medical Research, 2017*(4), 4.

Bekir, J., Mars, M., Souchard, J. P., & Bouajila, J. (2013). Assessment of antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of pomegranate (*Punica granatum*) leaves. *Food and Chemical Toxicology, 55*, 470–475.

Bhinge, S. D., Bhutkar, M. A., Randive, D. S., Wadkar, G. H., Todkar, S. S., Savali, A. S., & Chittapurkar, H. R. (2021). Screening of hair growth promoting activity of *Punica granatum* L.(pomegranate) leaves extracts and its potential to exhibit antidandruff and anti-lice effect. *Heliyon, 7*(4).

Burmaoglu, S., Kazancioglu, E. A., Kazancioglu, M. Z., Sağlamtaş, R., Yalcin, G., Gulcin, I., & Algul, O. (2022). Synthesis, molecular docking and some metabolic enzyme inhibition properties of biphenyl-substituted chalcone derivatives. *Journal of Molecular Structure, 1254*, Article 132358.

Çakmakçı, S., Topdaş, E. F., Kalın, P., Han, H., Şekerci, P., Köse, L. P., & Gülçin, İ. (2015). Antioxidant capacity and functionality of oleaster (*Elaeagnus angustifolia* L.) flour and crust in a new kind of fruity ice cream. *International Journal of Food Science and Technology, 50*(2), 472–481.

Dai, J., & Mumper, R. J. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules, 15*(10), 7313–7352.

Das, S., & Barman, S. (2012). Antidiabetic and antihyperlipidemic effects of ethanolic extract of leaves of Punica granatum in alloxan-induced non–insulin-dependent diabetes mellitus albino rats. *Indian Journal Of Pharmacology, 44*(2), 219–224.

Demir, G. M., Gulaboglu, M., Aggul, A. G., Baygutalp, N. K., Canayakin, D., Halici, Z., & Suleyman, H. (2016). Antioxidant and antidiabetic activity of aqueous extract of *Myrtus communis* L. berries on streptozotocin-induced diabetic rats. *IOSR Journal of Pharmacy and Biological Sciences, 11*, 11–16.

Derosa, G., & Maffioli, P. (2012). α-Glucosidase inhibitors and their use in clinical practice. *Archives of Medical Science, 8*(5), 899–906.

Du, J., Wang, H., Zhong, L., Wei, S., Min, X., Deng, H., … Huang, Y. (2025). Bioactivity and biomedical applications of pomegranate peel extract: A comprehensive review. *Frontiers in Pharmacology, 16*, 1569141.

Elbatanony, M. M., El-Feky, A. M., Hemdan, B. A., & El-Liethy, M. A. (2019). Assessment of the antimicrobial activity of the lipoidal and pigment extracts of *Punica granatum* L. leaves. *Acta Ecologica Sinica, 39*(1), 89–94.

Elfalleh, W., Tlili, N., Nasri, N., Yahia, Y., Hannachi, H., Chaira, N., Ying, M., & Ferchichi, A. (2011). Antioxidant capacities of phenolic compounds and tocopherols from Tunisian pomegranate (*Punica granatum*) fruits. *Journal of Food Science, 76*(5), C707–C713.

Ellman, G. L., Courtney, K. D., Andres, V., Jr., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology, 7*(2), 88–95.

Farag, R. S., Abdel-Latif, M. S., Emam, S., & Tawfeek, L. S. (2014). Phytochemical screening and polyphenol constituents of pomegranate peels and leave juices. *Agriculture Soil Science, 1*(6), 86–93.

Fellah, B., Bannour, M., Rocchetti, G., Lucini, L., & Ferchichi, A. (2018). Phenolic profiling and antioxidant capacity in flowers, leaves and peels of Tunisian cultivars of *Punica granatum* L. *Journal of Food Science and Technology, 55*, 3606–3615.

Fellah, B., Rocchetti, G., Senizza, B., Giuberti, G., Bannour, M., Ferchichi, A., & Lucini, L. (2020). Untargeted metabolomics reveals changes in phenolic profile following *in vitro* large intestine fermentation of non-edible parts of *Punica granatum* L. *Food Research International, 128*, Article 108807.

Fogliano, V., Verde, V., Randazzo, G., & Ritieni, A. (1999). Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. *Journal of Agricultural and Food Chemistry, 47*(3), 1035–1040.

Gaurav Anwar, N., Zahiruddin, S., & Ahmad, S. (2023). TLC-bioautography-MS-based identification of antioxidant, α-amylase and α-glucosidase inhibitory compounds in a polyherbal formulation "Sugreen-120". *Pharmacognosy Magazine, 19*(2), 254–268.

Gaurav Sharma, I., Khan, M. U., Zahiruddin, S., Basist, P., & Ahmad, S. (2023). Multi-mechanistic and therapeutic exploration of nephroprotective effect of traditional ayurvedic polyherbal formulation using in silico, *in vitro* and in vivo approaches. *Biomedicines, 11*(1), 168.

Ge, S., Duo, L., Wang, J., Yang, J., Li, Z., & Tu, Y. (2021). A unique understanding of traditional medicine of pomegranate, *Punica granatum* L. and its current research status. *Journal of Ethnopharmacology, 271*, Article 113877.

Ghorai, S., Pulya, S., Ghosh, K., Panda, P., Ghosh, B., & Gayen, S. (2020). Structure-activity relationship of human carbonic anhydrase-II inhibitors: Detailed insight for future development as anti-glaucoma agents. *Bioorganic Chemistry, 95*, Article 103557.

Göçer, H., & Gülçin, İ. (2011). Caffeic acid phenethyl ester (CAPE): Correlation of structure and antioxidant properties. *International Journal of Food Sciences and Nutrition, 62*(8), 821–825.

Gülçin, I. (2012). Antioxidant activity of food constituents: An overview. *Archives of Toxicology, 86*(3), 345–391.

Gülcin, I. (2020). Antioxidants and antioxidant methods: An updated overview. *Archives of Toxicology, 94*(3), 651–715.

Gülçin, İ., Beydemir, Ş., Şat, G., & Küfrevioğlu, Ö. (2005). Evaluation of antioxidant activity of cornelian cherry (*Cornus mas* L.). *Acta Alimentaria, 34*(2), 193–202.

Gülçin, İ., Tel, A. Z., Gören, A. C., Taslimi, P., & Alwasel, S. H. (2019). Sage (*Salvia pilifera*): Determination of its polyphenol contents, anticholinergic, antidiabetic and antioxidant activities. *Journal of Food Measurement and Characterization, 13*, 2062–2074.

Guney, M., Aggul, A. G., Erturk, A., & Gulcin, I. (2023). *In vitro* and *in silico* studies of 3-hydroxyflavone-based sulfonate esters: Potent acetylcholinesterase, carbonic anhydrase and α-glycosidase inhibitors. *ChemistrySelect, 8*(40), Article e202303054.

Huyut, Z., Beydemir, Ş., & Gülçin, İ. (2017). Inhibition properties of some flavonoids on carbonic anhydrase I and II isoenzymes purified from human erythrocytes. *Journal of Biochemical and Molecular Toxicology, 31*(9), Article e21930.

Jha, A. B., Panchal, S. S., & Shah, A. (2018). Ellagic acid: Insights into its neuroprotective and cognitive enhancement effects in sporadic Alzheimer's disease. *Pharmacology Biochemistry and Behavior, 175*, 33–46.

Jhong, C. H., Riyaphan, J., Lin, S. H., Chia, Y. C., & Weng, C. F. (2015). Screening alpha-glucosidase and alpha-amylase inhibitors from natural compounds by molecular docking in silico. *Biofactors, 41*(4), 242–251.

Kaewnarin, K., Niamsup, H., Shank, L., & Rakariyatham, N. (2014). Antioxidant and antiglycation activities of some edible and medicinal plants. *Chiang Mai Journal of Science, 41*(1), 105–116.

Kalin, P., Gülçin, İ., & Gören, A. C. (2015). Antioxidant activity and polyphenol content of cranberries (*Vaccinium macrocarpon*). *Records of Natural Products, 9*(4), 496.

Kaneria, M. J., Bapodara, M. B., & Chanda, S. V. (2012). Effect of extraction techniques and solvents on antioxidant activity of pomegranate (*Punica granatum* L.) leaf and stem. *Food Analytical Methods, 5*, 396–404.

Karageçili, H., Yilmaz, M. A., Alwasel, S. H., Arık, M., & Gülçin, İ. (2023). Comprehensively revealing the profile of *Pistacia vera* L. cv. Siirt turpentine-antioxidant, antidiabetic, anti-Alzheimer, and antiglaucoma effects. *Records of Natural Products, 17*(5), 918–937.

Karagecili, H., Yılmaz, M. A., Ertürk, A., Kiziltas, H., Güven, L., Alwasel, S. H., & Gulcin, İ. (2023). Comprehensive metabolite profiling of Berdav propolis using LC-MS/MS: Determination of antioxidant, anticholinergic, antiglaucoma, and antidiabetic effects. *Molecules, 28*(4), 1739.

Köksal, E., Bursal, E., Gülçin, İ., Korkmaz, M., Çağlayan, C., Gören, A. C., & Alwasel, S. H. (2017). Antioxidant activity and polyphenol content of Turkish thyme (*Thymus vulgaris*) monitored by liquid chromatography and tandem mass spectrometry. *International Journal of Food Properties, 20*(3), 514–525.

Kolar, F. R., Lingasur, S. M., Kumathalli, T. M., & Gurikar, S. A. (2021). Comparative analysis of phytochemical constituents and antioxidant activity of two pomegranate (*Punica granatum* L.) cultivars. *Israel Journal of Plant Sciences, 69*(1–2), 100–109.

de Lima, L. B., da Silva, W. A. V., Dos Santos, E. C. F., Machado, J. C. B., Procópio, T. F., de Moura, M. C., … Soares, L. A. L. (2021). Evaluation of antioxidant, antibacterial and enhancement of antibiotic action by *Punica granatum* leaves crude extract and enriched fraction against multidrug-resistant bacteria. *Chemistry & Biodiversity, 18*(12), Article e2100538.

Llorent-Martínez, E. J., Ortega-Barrales, P., Zengin, G., Mocan, A., Simirgiotis, M., Ceylan, R., … Aktumsek, A. (2017). Evaluation of antioxidant potential, enzyme inhibition activity and phenolic profile of *Lathyrus cicera* and *Lathyrus digitatus*: Potential sources of bioactive compounds for the food industry. *Food and Chemical Toxicology, 107*, 609–619.

Machado, J. C. B., Ferreira, M. R. A., & Soares, L. A. L. (2023). *Punica granatum* leaves as a source of active compounds: A review of biological activities, bioactive compounds, food, and technological application. *Food Bioscience, 51*, Article 102220.

Marcelino, S., Mandim, F., Taofiq, O., Pires, T. C., Finimundy, T. C., Prieto, M. A., & Barros, L. (2023). Valorization of *Punica granatum* L. leaves extracts as a source of bioactive molecules. *Pharmaceuticals, 16*(3), 342.

Mestry, S. N., & Juvekar, A. R. (2017). Aldose reductase inhibitory potential and anti-cataract activity of *Punica granatum* Linn. Leaves against glucose-induced

cataractogenesis in goat eye lens. *Oriental Pharmacy and Experimental Medicine, 17*, 277–284.
Mohammadinejad, A., Mohajeri, T., Aleyaghoob, G., Heidarian, F., & Kazemi Oskuee, R. (2022). Ellagic acid as a potent anticancer drug: A comprehensive review on *in vitro*, in vivo, in silico, and drug delivery studies. *Biotechnology and Applied Biochemistry, 69*(6), 2323–2356.
Munteanu, I. G., & Apetrei, C. (2021). Analytical methods used in determining antioxidant activity: A review. *International Journal of Molecular Sciences, 22*(7), 3380, 2021.
Ojo, O. A., Ojo, A. B., Okolie, C., Nwakama, M. A. C., Iyobhebhe, M., Evbuomwan, I. O., … Batiha, G. E. S. (2021). Deciphering the interactions of bioactive compounds in selected traditional medicinal plants against Alzheimer's diseases via pharmacophore modeling, auto-QSAR, and molecular docking approaches. *Molecules, 26*(7), 1996.
Ono, N. N., Bandaranayake, P. C., & Tian, L. (2012). Establishment of pomegranate (*Punica granatum*) hairy root cultures for genetic interrogation of the hydrolyzable tannin biosynthetic pathway. *Planta, 236*, 931–941.
Orgil, O., Schwartz, E., Baruch, L., Matityahu, I., Mahajna, J., & Amir, R. (2014). The antioxidative and anti-proliferative potential of non-edible organs of the pomegranate fruit and tree. *LWT- Food Science and Technology, 58*(2), 571–577.
Oyaizu, M. (1986). Studies on products of browning reaction antioxidative activities of products of browning reaction prepared from glucosamine. *The Japanese Journal of Nutrition and Dietetics, 44*(6), 307–315.
Özaslan, M. S., Sağlamtaş, R., Demir, Y., Genç, Y., Saraçoğlu, İ., & Gülçin, İ. (2022). Isolation of some phenolic compounds from *Plantago subulata* L. and determination of their antidiabetic, anticholinesterase, antiepileptic and antioxidant activity. *Chemistry & Biodiversity, 19*(8), Article e202200280.
Patel, A. N., Bandawane, D. D., & Mhetre, N. K. (2014). Pomegranate (*Punica granatum* Linn.) leaves attenuate disturbed glucose homeostasis and hyperglycemia mediated hyperlipidemia and oxidative stress in streptozotocin induced diabetic rats. *European Journal of Integrative Medicine, 6*(3), 307–321.
Patra, A., Abdullah, S., & Pradhan, R. C. (2022). Review on the extraction of bioactive compounds and characterization of fruit industry by-products. *Bioresources and Bioprocessing, 9*(1), 14.
Pirzadeh, M., Caporaso, N., Rauf, A., Shariati, M. A., Yessimbekov, Z., Khan, M. U., … Mubarak, M. S. (2021). Pomegranate as a source of bioactive constituents: A review on their characterization, properties and applications. *Critical Reviews in Food Science and Nutrition, 61*(6), 982–999.
Pottathil, S., Nain, P., Morsy, M. A., Kaur, J., Al-Dhubiab, B. E., Jaiswal, S., & Nair, A. B. (2020). Mechanisms of antidiabetic activity of methanolic extract of *Punica granatum* leaves in nicotinamide/streptozotocin-induced type 2 diabetes in rats. *Plants, 9*(11), 1609.
Proença, C., Freitas, M., Ribeiro, D., Oliveira, E. F. T., Sousa, J. L. C., Tomé, S. M., … Fernandes, E. (2017). α-Glucosidase inhibition by flavonoids: An *in vitro* and in silico structure-activity relationship study. *Journal of Enzyme Inhibition and Medicinal Chemistry, 32*(1), 1216–1228.
Rasouli, H., Hosseini-Ghazvini, S. M., Adibi, H., & Khodarahmi, R. (2017). Differential α-amylase/α-glucosidase inhibitory activities of plant-derived phenolic compounds: A virtual screening perspective for the treatment of obesity and diabetes. *Food & Function, 8*(5), 1942–1954.
Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*(9–10), 1231–1237.
Rummun, N., Somanah, J., Ramsaha, S., Bahorun, T., & Neergheen-Bhujun, V. S. (2013). Bioactivity of nonedible parts of *Punica granatum* L.: A potential source of functional ingredients. *International Journal of Food Science, 2013*(1), Article 602312.
Saparbekova, A., Kantureyeva, G., Kudasova, D., Konarbayeva, Z., & Latif, A. (2023). Potential of phenolic compounds from pomegranate (*Punica granatum* L.) by-product with significant antioxidant and therapeutic effects: A narrative review. *Saudi Journal of Biological Sciences, 30*(2), Article 103553.
Saratale, R. G., Shin, H. S., Kumar, G., Benelli, G., Kim, D. S., & Saratale, G. D. (2018). Exploiting antidiabetic activity of silver nanoparticles synthesized using *Punica granatum* leaves and anticancer potential against human liver cancer cells (HepG2). *Artificial Cells, Nanomedicine, and Biotechnology, 46*(1), 211–222.
Saravanakumar, K., Sarikurkcu, C., Sarikurkcu, R. T., & Wang, M.-H. (2019). A comparative study on the phenolic composition, antioxidant and enzyme inhibition activities of two endemic Onosma species. *Industrial Crops and Products, 142*, Article 111878.
Şenol, H., Çelik Turgut, G., Şen, A., Sağlamtaş, R., Tuncay, S., Gülçin, İ., & Topçu, G. (2023). Synthesis of nitrogen-containing oleanolic acid derivatives as carbonic anhydrase and acetylcholinesterase inhibitors. *Medicinal Chemistry Research, 32*(4), 694–704.
Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*(3), 144–158.
Tao, Y., Zhang, Y., Cheng, Y., & Wang, Y. (2013). Rapid screening and identification of α-glucosidase inhibitors from mulberry leaves using enzyme-immobilized magnetic beads coupled with HPLC/MS and NMR. *Biomedical Chromatography, 27*(2), 148–155.
Taslimi, P., & Gulçin, İ. (2018). Antioxidant and anticholinergic properties of olivetol. *Journal of Food Biochemistry, 42*(3), Article e12516.
Tohma, H., Altay, A., Köksal, E., Gören, A. C., & Gülçin, İ. (2019). Measurement of anticancer, antidiabetic and anticholinergic properties of sumac (*Rhus coriaria*): Analysis of its phenolic compounds by LC–MS/MS. *Journal of Food Measurement and Characterization, 13*, 1607–1619.
Trabelsi, A., El Kaibi, M. A., Abbassi, A., Horchani, A., Chekir-Ghedira, L., & Ghedira, K. (2020). Phytochemical study and antibacterial and antibiotic modulation activity of *Punica granatum* (pomegranate) leaves. *Scientifica, 2020*(1), 8271203.
Uysal, S., Zengin, G., Aktumsek, A., & Karatas, S. (2016). Chemical and biological approaches on nine fruit tree leaves collected from the Mediterranean region of Turkey. *Journal of Functional Foods, 22*, 518–532.
Wang, D., Özen, C., Abu-Reidah, I. M., Chigurupati, S., Patra, J. K., Horbanczuk, J. O., … Atanasov, A. G. (2018). Vasculoprotective effects of pomegranate (*Punica granatum* L.). *Frontiers in Pharmacology, 9*, 544.
Wong, T. L., Strandberg, K. R., Croley, C. R., Fraser, S. E., Venkata, K. C. N., Fimognari, C., … Bishayee, A. (2021). Pomegranate bioactive constituents target multiple oncogenic and oncosuppressive signaling for cancer prevention and intervention. *Seminars in Cancer Biology, 73*, 265–293.
Yu, M., Gouvinhas, I., Chen, J., Zhu, Y., Deng, J., Xiang, Z., Oliveira, P., Xia, C., & Barros, A. (2024). Unlocking the therapeutic treasure of pomegranate leaf: A comprehensive review on phytochemical compounds, health benefits, and future prospects. *Food Chemistry: X, 23*, Article 101587.
Yu, M., Gouvinhas, I., Rocha, J., & Barros, A. I. (2021). Phytochemical and antioxidant analysis of medicinal and food plants towards bioactive food and pharmaceutical resources. *Scientific Reports, 11*(1), Article 10041.
Zare, H., Ardekani, E. A., Tavakoli, A., Bradley, R., Tavakoli, F., & Pasalar, M. (2024). Reporting of adverse effects of pomegranate in clinical studies: A systematic review. *Journal of Complementary and Integrative Medicine, 21*(2), 154–166.
Zhang, L., Gao, Y., Zhang, Y., Liu, J., & Yu, J. (2010). Changes in bioactive compounds and antioxidant activities in pomegranate leaves. *Scientia Horticulturae, 123*(4), 543–546.