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RESEARCH

Green Extraction Enhances Antioxidant and Cytotoxic Properties of Pomegranate By-Products Against Human Cancer Cell Lines

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Abstract

The aim of this study was to determine the anticancer effects of extract obtained from pomegranate by-products by green extraction (ultrafiltration and enzymatic extraction) (GE) on prostate (LNCaP), ovarian (A2780), breast (MCF-7), and colon (Caco-2) cancer cell lines compared to the traditional extraction (TE) and pomegranate juice (PJ). The GE sample demonstrated the highest total or individual phenolic content, and antioxidant activity. The major phenolics in all samples were determined to be ellagic acid, punicalagin A and punicalagin B. The 50% and 100% doses of the PJ exhibited a significant reduction in the viability of all cancer cells except LNCaP cells (only 100%) ($p<0.05$). The TE sample showed a significant decrease in the viability of all cancer cells except the MCF-7 cell line (at all doses) at doses other than 1 µM ($p<0.05$). The GE sample caused a significant decrease in all cancer cells at all doses (1, 5, 25, 50, 100 and 150 µM) ($p<0.05$). Consequently, the GE sample applied with the green method was effective at all doses in reducing the viability of cancer cells.

Keywords Cancer · Antiproliferative · Pomegranate by-product · Extract · Green extraction

Introduction

Cancer remains the second leading cause of mortality worldwide [1]. Among women, breast cancer is the second most common cause of cancer-related death [1, 2], and epithelial ovarian cancer exhibits the highest mortality rate among all gynecological cancers [1]. In men, prostate cancer is the second leading cause of cancer-related mortality. Colorectal cancer is the third most common cause of cancer-related mortality among men and the fourth among women [1]. Major risk factors of cancer include tobacco use, excessive alcohol consumption, obesity, physical inactivity, infectious agents, prolonged exposure to ultraviolet radiation (sunlight), and insufficient intake of fruit and vegetables [3]. Another critical contributor is oxidative stress, which results from an imbalance between antioxidants and pro-oxidants [4].

Chemotherapeutic agents, though widely employed in cancer treatment, are often associated with cancer progression and/or recurrence [2]. Therefore, there is an increasing interest in the development of non-toxic, cost-effective, accessible and efficacious alternatives to conventional therapies. Recent studies have demonstrated a strong inverse association between the consumption of fruit- and vegetable-rich diet and cancer incidence. This is largely attributed to their potent anti-cancer properties, high concentrations of phytochemicals (polyphenolic and flavonoid compounds), and minimal side effects [4, 5].

Pomegranate (*Punica granatum*) has been utilized in traditional medicine throughout history. During the processing of pomegranates, a substantial proportion (40–50%) of by-products are generated. These by-products are rich in bioactive components (hydrolysable tannins, phenolic acids, and flavonoids) [6] and possess antioxidative, antibacterial, antifungal, anti-inflammatory, and antiproliferative properties [7]. Numerous studies have reported the potent chemotherapeutic potential of pomegranate, which operates by modulating multiple molecular pathways and influencing gene expression [4, 8]. Pomegranate peel and juice have been

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shown to decrease the proliferation, migration, and colony formation of human prostate cancer cell lines DU145 and PC-32, via the regulation of mTOR/S6K signaling pathway, apoptosis and morphological changes, DNA fragmentation, and PARP cleavage by increasing the proliferation of COX-2 and MMP2/MMP9 [9].

Some antioxidant-rich phenolic compounds present in pomegranate by-products are either bound within the plant matrix or exist in large molecular structure, which limits their bioavailability and results in suboptimal antioxidant capacity [10]. Therefore, the application of novel extraction techniques is recommended, as conventional solvent extraction methods are often inadequate for efficiently isolating bioactive compounds [11]. Green and novel extraction methods offer several advantages, including reduced solvent consumption, time and energy efficiency, and environmentally sustainable processing. Enzyme-assisted extraction is a particularly promising biological approach for the production and recovery of phenolic compounds with enhanced stability and antioxidant activity. This process involves the enzymatic hydrolysis of cell wall components, facilitating the release of phenolic compounds [12]. Ultrafiltration, a membrane-based separation technique, is commonly used to isolate target components based on molecular size. It is considered a favorable method due to its energy efficiency, high selectivity, and the absence of thermal processing [13]. For example, in our previous study, phenolic compounds were extracted from the pomegranate juice (PJ) by-products using a combined green extraction approach involving enzymatic liquefaction and ultrafiltration [14].

To the best of our knowledge, the use of pomegranate by-product extracts enriched in phenolic compounds through enzymatic extraction combined with ultrafiltration has not been previously investigated for their anticancer effects on cancer cell lines under in vitro conditions. The application of ultrafiltration following enzymatic extraction may yield a phenolic-rich extract with enhanced biological activity and elevated phenolic content. The aim of this study was to evaluate and compare potential anticancer effects of PJ and phenolic-enriched extracts obtained from pomegranate by-products using enzymatic extraction and ultrafiltration. These effects were assessed on prostate (LNCaP), ovarian (A2780), breast (MCF-7), and colon (Caco-2) cancer cell lines.

Materials and Methods

Materials

The pomegranates were procured from a local market in Konya, Türkiye. The enzymes Pectinex Ultra SP-L (3300 PGNU/g, containing pectinase, hemicellulase and beta-glucanase) and Viscozyme-L (300 AGU/mL, containing beta-glucanase, pectinase, hemicellulase and xylanase) were obtained from Novozymes (Bagsvaerd, Denmark), while tannase (≥150 U/g) was purchased from Sigma-Aldrich (Germany). The ultrafiltration membrane used in this study was a Sartocon Slice flat sheet membrane with a 50 kDa molecular weight cut-off (MWCO) made of polyethersulfone, obtained from Sartorius (Goettingen, Germany). All chemicals used were of analytical reagent grade and purchased from Merck (Germany).

Cell culture materials, MCF-7 human breast cancer cell line, A2780 human ovarian cancer cell line, Caco-2 human colon cancer cell line, LNCaP human prostate cancer cell line, Minimum Essantiel Medium (MEM), fetal bovine serum (FBS, 10%), glutamine (1%) and penicillin (100 U/mL) were purchased from Biochrome (Berlin, Germany).

Preparation of Pomegranate Juice (PJ) and By-Product Extracts

PJ

Pomegranates cut in half were manually squeezed using a juicer. Following the squeezing the PJ, the residual by-products (peels, pulps, and seeds) were dried in a tray dryer (TK-LAB, Eksis Makine, Isparta, Türkiye) at 50 °C for 10 h until the moisture content was reduced to ≤3%. The dried material was then ground using a steel mill (Alveo, Istanbul, Türkiye) to obtain a fine powder. This powder was subjected to two different extraction approaches: a traditional method and a green extraction method involving enzymatic liquefaction followed by ultrafiltration.

Traditional Extraction (TE)

Powdered pomegranate by-products were extracted following the method described by Mikucka et al. [15], with slight modifications. The powdered material was incubated with aqueous ethanol (80%) at a solid-to-solvent ratio of 1:10 (w/v) in a water bath (Daihan WisebathWSB-30, Gangwon, South Korea) at 50 °C for 6 h with continuous agitation at 100 rpm. After incubation, the mixture was centrifuged (Awel Industries, Centrifuge MF 20, France) at 10,000 rpm for 5 min, followed by filtration through 150 µm filter paper. The solvent in resulting supernatant was evaporated with a rotary evaporator (Heidolph Hei-Vap Core, Schwabach, Germany) at 50 °C, and subsequently stored at −20 °C until further analysis.

Green Extraction (GE)

Based on the findings of Aydın et al. [16], the extract exhibiting the highest total phenolic content and antioxidant activity was selected for evaluation against various cancer cell lines in the present study. Powdered pomegranate by-products were rehydrated in a buffer solution (pH 5) at a solid-to-liquid ratio of 1:6 (w/v) for 1 h. Then, the mixture was supplemented with Viscozyme L (2%, v/w), Pectinex (1%, v/w), and tannase (0.2%, v/w), calculated based on the initial powder weight. The enzymatic reaction was carried out in a water bath at 50 °C for 2 h with continuous agitation at 100 rpm. The reaction was then terminated by heating the mixture at 90 °C for 3 min. Following enzymatic hydrolysis, aqueous ethanol (80%) was added at a ratio of 1:10 (w/v) relative to the initial powder weight, and the mixture was incubated again at 50 °C for 2 h at 100 rpm. After incubation, the samples were centrifuged, and the resulting supernatant was passed through a 50 kDa ultrafiltration membrane. The retentate (> 50 kDa) was collected and stored under the same conditions as the extract obtained by the traditional extraction method.

Total Phenolic Content

To determine the total phenolic content, 0.5 mL of the sample was mixed with 2.5 mL of 0.2 M Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate solution [17]. The mixture was incubation in a water bath at 50 °C for 5 min. After cooling, absorbance was measured at 760 nm using a spectrophotometer (Libra S60, Biochrom Ltd., Cambridge, England). Total phenolic content was expressed as mg gallic acid equivalent (GAE) *per* mL (mg GAE/mL), based on a calibration curve generated using gallic acid solutions in the range of 20–100 mg/L, with the equation (Fig. S1):

$$Concentration = (Absorbance - 0.0201)/0.0114$$

DPPH Radical Scavenging Antioxidant Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging antioxidant activity of the samples was determined according to the method described by Fernández-León et al. [18]. A freshly prepared 60 µM DPPH solution in methanol (950 µL) was mixed with 50 µL of the extract. The mixture was vortexed and incubated in the dark at room temperature for 30 min. Absorbance was then measured at 516 nm using a spectrophotometer. Antioxidant activity was expressed as mg of Trolox equivalent antioxidant activity (TEAA) per mL (mg TEAA/mL), based on a calibration curve prepared with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard solutions in the range of 20–100 mg/L, using the equation Fig. S2:

$$Concentration = (Absorbance + 0.0004)/(-0.0022)$$

Identification of Phenolics by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS)

Phenolic compounds in the PJ and extract samples were identified and quantified using an ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) system (Ultimate 3000 UPLC & TSQ Fortis, Thermo Fisher Scientific Inc., Massachusetts, USA), following the method of Yenil et al. [19] with modifications. A C18 column (Hypersil Gold RP, 1.9 µm, 50 × 2.1 mm) was used for chromatographic separation. Mobile phase A (water: acetonitrile in a ratio of 95:5 containing 0.1% formic acid and 4 mM ammonium formate) and mobil phase B (acetonitrile: water in a ratio of 95:5, with the addition of 0.1% formic acid and 4 mM ammonium formate) solutions were used for separation.

For sample preparation, 10 mg of sample was dissolved in 20 mL of a methanol-acetonitrile solution (50:50, v/v) containing 0.1% (v/v) formic acid. The mixture was vortexed for 5 min and centrifuged at 4000 rpm for 15 min at 4 °C. The supernatant was filtered through a 0.2 µm PTFE membrane filter and subsequently diluted tenfold with mobile phase A prior to injection into the UHPLC-MS/MS system.

Cells Culture

The study was carried out in the laboratories of Department of Physiology, Faculty of Medicine, Inonu University. The cancer cell lines used included LNCaP (prostate), A2780 (ovarian), MCF-7 (breast) and Caco-2 (colon). LNCaP and A2780 cells were grown in 25 cm^2 culture flasks in RPMI-1640 medium containing FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 5 mL MEM Non-Essential Amino Acids Solution (NEAAS). MCF-7 cells were fed with medium prepared by adding 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 5 mL MEM (NEAAS) and 1 mL insulin into Dulbecco's Modified Eagle Medium (DMEM) High Glucose. Caco-2 cells were also fed with DMEM F-12 medium supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 5 mL MEM (NEAAS) and 1 mL insulin.

All cells were maintained in a humidified 5% CO_2 incubator (Esco CO_2 incubator) at 37 °C, and the culture medium was refreshed twice weekly. Once the cells reached confluence, they were detached using a trypsin-ethylenediamine tetraacetic acid (EDTA) solution. Cell viability was assessed by trypan blue (0.4%) exclusion and counted using an inverted microscope [20]. Experimental procedures were initiated when cell viability exceeded 90%. Confluent cells were removed from the flasks using trypsin-EDTA solution, counted and transferred to 96-well plates at a density of 1.5×10^4 cells per well. The plates were incubated for 24 h to allow cell adherence prior to treatment [20, 21].

MTT (3-(4,5-Dimethyl-2-thiazolyl)−2,5-diphenyl-2 H-tetrazolium bromide) Assay Method

Anti-cancer agents of PJ (1, 25, 50 and 100%) or pomegranate by-product extracts (1, 5, 25, 50, 100 and 150 µM) were prepared by dissolving in distilled water at various concentrations. For the control group, the cell's native medium was used (for example, MCF-7 cells were fed with medium prepared by adding 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 5 mL MEM (NEAAS) and 1 mL insulin into Dulbecco's Modified Eagle Medium (DMEM) High Glucose). These solutions (100 µL per well) were added to the culture wells following the initial 24-h incubation of the cancer cell lines (LNCaP, A2780, MCF-7, and Caco-2). The treated cells were then incubated for an additional 24 h in a humidified CO_2 incubator. To assess the cytotoxic effects of the extracts on cell viability, the MTT assay was employed. This colorimetric assay is based on the reduction of the yellow tetrazolium salt MTT to an insoluble blue-violet formazan product by mitochondrial succinate dehydrogenase in metabolically active cells [22, 23]. The intensity of the resulting color correlates with the number of viable cells, allowing for quantitative analysis of cytotoxicity.

Statistical Analysis

All samples were produced in duplicate. Total phenolic content and antioxidant activity measurements were conducted in parallel, and the results were expressed as mean values ± standard deviations. Statistical analysis was performed using one-way analysis of variance (ANOVA) with JMP software (version 5.0.1a; SAS Institute Inc., USA). Significant differences between group means were determined using Tukey's post hoc test, with a significance level set at $p \leq 0.05$.

Cytotoxicity data were analyzed using IBM SPSS Statistics for Windows, version 24.0 (SPSS Inc., Chicago, IL). Group comparisons were performed using the Kruskal-Wallis H Test. When statistically significant differences were identified, pairwise comparisons were conducted using the Mann-Whitney U test with Bonferroni correction. A p-value of <0.05 was considered statistically significant. Furthermore, MTT assay results for all cell lines were illustrated using GraphPad Prism, version 8.0.1 (GraphPad Software Inc., La Jolla, CA, USA).

Results and Discussion

Total Phenolic Content

Table 1 The total phenolic content and antioxidant activity of pomegranate juice and by-product extracts[1]

Samples	Total phenolic content	DPPH (radical scavenging activity)
PJ[2]	0.45 ± 0.00^c mg GAE/mL	1.92 ± 0.11^c mg TEAA/mL
TE[3]	21.29 ± 0.25^b mg GAE/g	79.93 ± 1.80^b mg TEAA/g
GE[4]	81.30 ± 0.42^a mg GAE/g	375.70 ± 6.93^a mg TEAA/g

[1] A statistical difference exists between the means presented with different letters in the same column ($p \leq 0.05$). [2] *PJ* Pomegranate juice, [3] *TE* Extract obtained from solvent extraction, [4] *GE* Extract obtained from green extraction

As shown in Table 1, the highest total phenolic content (TPC) value was observed in the GE sample, whereas the lowest was recorded in the PJ sample. The extracts obtained from pomegranate by-products exhibited significantly higher TPC values compared to PJ. Application of the green extraction method to the pomegranate by-products resulted in a 3.8-fold increase in TPC compared to the TE. In a study comparing the TPC of fresh PJ and ethanol extracts from pomegranate peel and seed, the peel extract showed the highest TPC, while PJ had the lowest [24]. Shin and Lee [25] reported that utilizing enzymatic extraction using Viscozyme L facilitated the breakdown of cell wall polysaccharides in immature citrus, thereby enhancing the release of phenolic compounds. Similarly, Aydin et al. [14] demonstrated that combining Pectinex and Viscozyme L enzymes with ultrafiltration significantly improved the extraction efficiency of phenolic compounds from pomegranate by-products. Other studies have also shown that sequential water-based extraction and ultrafiltration of pomegranate peels [26], as well as combined enzymatic extraction and ultrafiltration on distilled rose petals [27], led to increased TPC.

Phenolic Compound Profile

Table 2 presents the phenolics profiles (12 compounds) of pomegranate by-products extracted using TE and GE techniques and PJ, as determined by UHPLC-MS/MS. The total amount of phenolics in both the TE and GE samples was higher than that observed in the PJ sample. The predominant phenolic compounds identified in the TE and GE samples were punicalagin B, punicalagin A and ellagic acid, in descending order. In contrast, ellagic acid content of PJ was higher than punicalagin B and punicalagin A. The green extraction method notably enhanced the recovery of key phenolic compounds, with ellagic acid, punicalagin B, and punicalagin A showing increases of approximately 5.6-, 4.9-, and 4.8-fold, respectively, compared to the traditional method.

Eroglu Ozkan et al. [2] reported that punicalagin was the most abundant phenolic compound in pomegranate extracts (Ekşilik Nar and Izmir 1513). Similarly, several other studies have identified punicalagin as the predominant phenolic compound in pomegranate peel, juice and seed [28]. Enzymes such as cellulase, hemicellulase, β-glucosidase,

Table 2 Individual phenolic composition (mg/kg) of pomegranate by-product extracts obtained from different extraction methods by determining UHPLC-MS/MS

Invidual phenolic compounds	PJ[1]	TE[2]	GE[3]
ferulic acid	81.7	243.8	1272.8
gallic acid	62.6	977.8	4555.6
ellagic acid	364.8	14381.0	80348.4
fumaric acid	11.3	4446.5	21478.3
punicalagin A	142.9	29689.0	143145.5
punicalagin B	165.8	45385.6	222061.9
punicalin	40.3	2481.6	13288.1
granatin	13.4	2184.7	11951.8
pellargonidin	29.1	2656.8	15154.3
cyanidin	21.2	1336.7	6035.2
cyanidin-3-O-glucoside	n.d.	4487.3	23576.5
delphinidin-3-O-glucoside	n.d.	5904.7	31364.4
Total	933.1	114175.5	574242.8

[1] *PJ* Pomegranate Juice, [2] *TE* Extract obtained by traditional extraction, [3] *GE* Extract obtained by green extraction. *n.d* not detected

xylanase, β-glucanase and pectinase play a critical role in degrading polysaccharides, thereby facilitating the release of various phenolic compounds [29]. Additionally, tannase enhances the hydrolysis of complex polyphenols into low-molecular-weight phenolics, increasing both their bioavailability and biological activity [30]. Consequently, the GE sample exhibited a higher content of phenolics in comparison to the TE sample, likely due to the enzymatic breakdown of bound phenolics and structural polyphenols. These findings are consistent with prior research, which demonstrated that pomegranate peel contains high levels of punicalagin and that green extraction methods result in enhanced phenolic yield.

Antioxidant Activity

The antioxidant activity of the PJ and the extracts, as assessed by DPPH radical scavenging activity, is presented in Table 1. Among the samples, the GE extract exhibited the highest antioxidant activity. Notably, the antioxidant activity of the GE extract was approximately 4.7-times greater than that of the TE extract. These findings are consistent with the phenolic content results, indicating a strong correlation between total phenolic concentration and antioxidant activity across the samples.

Kupnik et al. [24] reported that ethanol extracts of pomegranate peel exhibited significantly higher DPPH radical scavenging activity than fresh PJ, which demonstrated the lowest activity. Similar findings were also noted by Shahkoomahally et al. [31]. Consistent with the present results, studies by Aydin et al. [14] and Dushkova et al. [27] showed that the combination of enzymatic extraction and ultrafiltration significantly enhanced antioxidant activity in pomegranate by-products and rose petals, respectively. Shin and Lee [25] likewise observed that enzymatic treatment with Viscozyme L increased the antioxidant capacity of immature citrus extracts by converting flavonoid glycosides to aglycones—forms with higher bioactivity. According to Andishmand et al. [32], the high antioxidant activity of such extracts is closely linked to their phenolic composition. Supporting this, Wang et al. [33] found a strong correlation between punicalagin and ellagic acid levels and antioxidant activity in pomegranate peels. Therefore, the high antioxidant activity observed in the GE extract in this study can be attributed to its elevated contents of ellagic acid, punicalagin A, and punicalagin B, aligning well with existing literature.

Pomegranate Phenolics on Cell Viability

Figures 1, 2, 3 and 4 (Tables S1-S2) illustrate the effects of PJ, TE and GE samples on the viability of prostate (LNCaP), ovarian (A2780), breast (MCF-7) and colon (Caco-2) cancer cells after 24 h of exposure to different doses. In the Caco-2 cancer cell line, PJ significantly reduced cell viability at 50 and 100% concentrations compared to the control (Fig. 1a, Table S1). The TE extract led to a significant decrease at all concentrations except 1 µM (Fig. 1b, Table S2), while the GE extract reduced viability at all tested concentrations (Fig. 1c, Table S2; $p<0.05$). Similar trends were observed in A2780 cells: PJ was effective at 50 and 100% (Fig. 2a, Table S1), whereas TE induced significant reductions at all concentrations except the lowest dose (Fig. 2b, Table S2). GE exhibited significant cytotoxic effects at all concentrations in A2780 cells (Fig. 2c, Table S2; $p<0.05$).

In the MCF-7 cell line, significant reductions ($p<0.05$) in viability were observed at 50 and 100% dose of PJ (Fig. 3a, Table S1), all doses of TE (Fig. 3b, Table S2) and GE (Fig. 3c, Table S2) samples. In LNCaP cells, viability was significantly reduced at 100% PJ (Fig. 4a, Table S1), 25–150 µM TE (Fig. 4b, Table S2), and across all GE concentrations (Fig. 4c, Table S2; $p<0.05$). The presence of ellagic acid, punicalagin B and punicalagin A in all extracts—particularly in higher concentrations in the GE sample—suggests that these compounds play a pivotal role in reducing cancer cell viability. Therefore, among all samples, GE demonstrated the strongest and most consistent cytotoxic effect across all cell lines and doses tested.

Punicalagin and ellagic acid, which are predominantly found in pomegranate peel, have been extensively studied for their anticancer potential due to mechanisms such as anti-estrogenic, anti-angiogenetic, anti-inflammatory, anti-proliferative, and anti-metastatic activities [9, 34]. Chaves et al. [9] reported that both PJ and peel extracts inhibited the proliferation of prostate cancer cells, with the peel extract demonstrating a more pronounced effect. Similarly,

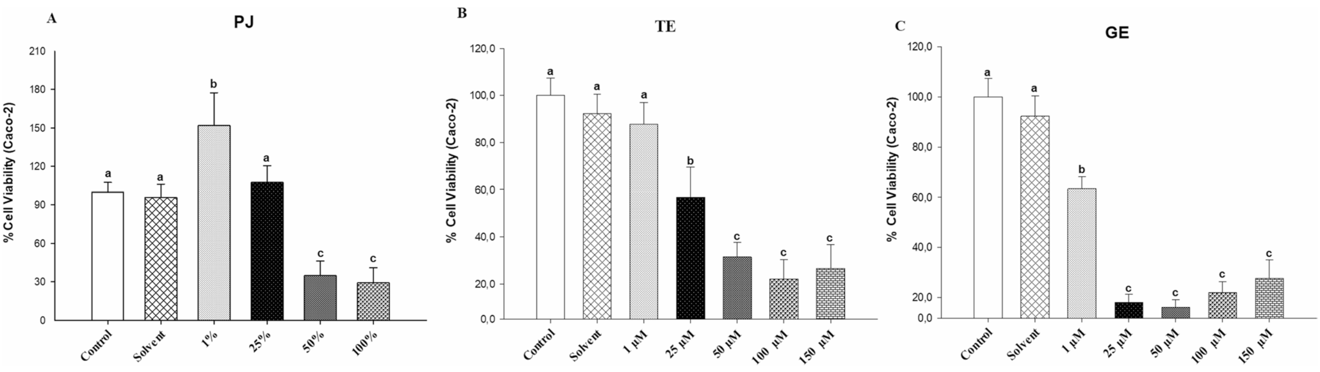

Fig. 1 Dose-dependent cell viability results of PJ (**A**), TE (**B**) and GE (**C**) on Caco-2 cells. Each data point is the average of 10 viability measurements. Different letters indicate statistical difference between groups; a, b, c, d ($p<0.05$). (PJ: Pomegranate Juice; TE: Traditional Extraction; GE: Green Extraction)

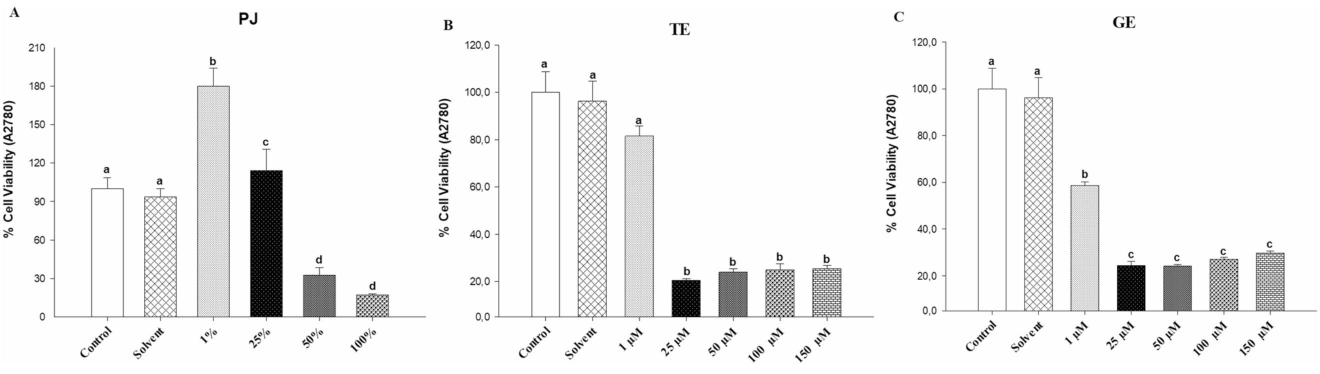

Fig. 2 Dose-dependent cell viability results of PJ (**A**), TE (**B**) and GE (**C**) on A2780 cells. Each data point is the average of 10 viability measurements. Different letters indicate statistical difference between groups; a, b, c, d ($p<0.05$). (PJ: Pomegranate Juice; TE: Traditional Extraction; GE: Green Extraction)

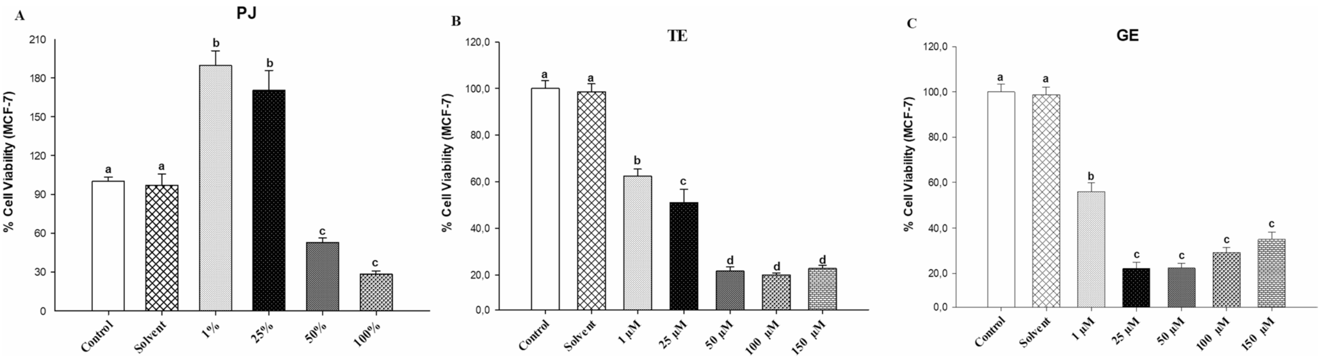

Fig. 3 Dose-dependent cell viability results of PJ (**A**), TE (**B**) and GE (**C**) on MCF-7 cells. Each data point is the average of 10 viability measurements. Different letters indicate statistical difference between groups; a, b, c, d ($p<0.05$). (PJ: Pomegranate Juice; TE: Traditional Extraction; GE: Green Extraction)

Gonzalez-Castillo et al. [35] observed a decrease in cell viability in NIH-3T3 and HeLa cancer cells following exposure to punicalin and ellagic acid isolated from pomegranate peel extract decreased. The analysis demonstrated that there was no statistically significant difference in cell death between the NIH-3T3 and Hela cells at a concentration of 500 mg/L. Eroglu Ozkan et al. [2] demonstrated that a PJ extract (Izmir 1513 cultivar), containing bioactive compounds such as cyanidin, cyanidin-3-O-glucoside, and punicalagin, significantly inhibited the growth of the MCF-7 breast cancer cell line after 24 h. In another study, Kolesarova et al. [8] assessed the effects of pomegranate peel extract on the viability of ovarian cancer cells (OVCAR-3) and found a significant reduction in the viable cancer cells across all

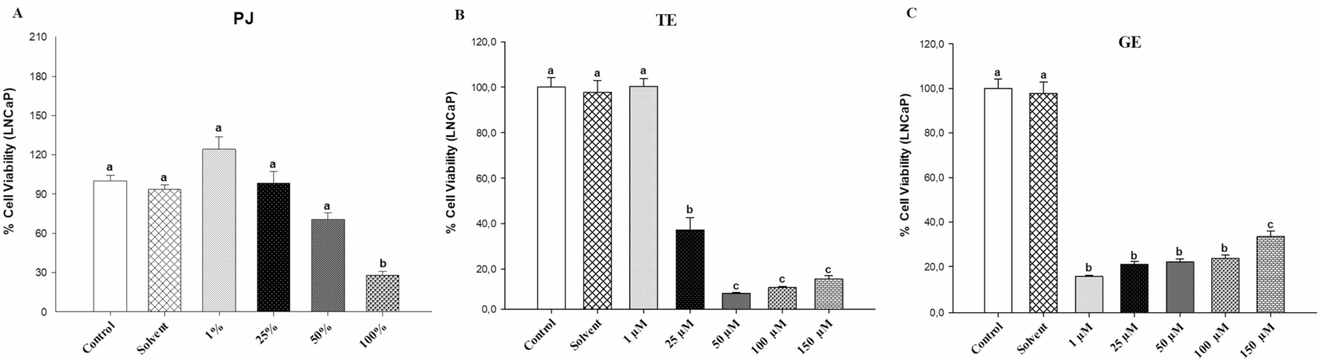

Fig. 4 Dose-dependent cell viability results of PJ (**A**), TE (**B**) and GE (**C**) on LNCaP cells. Each data point is the average of 10 viability measurements. Different letters indicate statistical difference between groups; a, b, c, d ($p<0.05$). (PJ: Pomegranate Juice; TE: Traditional Extraction; GE: Green Extraction)

treated groups compared to control. The authors highlighted pomegranate peel as a rich source of punicalagins A and B, as well as rutin, with potent antioxidant and chemopreventive properties against ovarian cancer.

A study has revealed that punicalagin induces mitochondrial-mediated apoptosis and suppresses the viability of cervical cancer (ME-180) cells in a dose-dependent manner. It has been demonstrated that the punicalagin has altered several cell-signalling pathways involved in apoptosis and cellular proliferation. Notably, the compound exerted antagonistic effects on NF-κB signaling pathways, thereby impeding cancer cell progression [36]. The anticancer potential of pomegranate against breast cancer has also been well-documented. Reported mechanisms include anti-estrogenic and anti-aromatase effects, modulation of the transforming growth factor-beta (TGF-β)/Smads pathway activity, suppression of pro-inflammatory cytokines and chemokines, reduction in vascular endothelial growth factor (VEGF) concentrations, downregulation of genes involved in DNA damage and expression of estrogen-responsive genes, and disruption of estrogen resistance [34]. In a related study, Shirode et al. [37] found that a standardized extract of pomegranate fruit peels, comprising 95% glycone ellagitannins and free ellagic acid, significantly inhibited the proliferation of MCF-7 cells in a concentration- and time-dependent manner. This inhibition was characterized by the induction of cell cycle arrest in the G2/M phase, culminating in the subsequent induction of apoptosis.

Kilit and Aydemir [38] demonstrated that punicalagin derived from pomegranate peel exhibited a statistically significant cytotoxic effect on various human cancer cell lines, including human prostate (22RV-1), pancreatic (PANC-1), brain (U87), and non-small cell lung cancer (A549) cells, across all tested concentration (25, 50, 100 and 200 µg/mL) following a 24-hour exposure. Similarly, Sudha et al. [39] reported that pomegranate fruit extract, administrated at concentrations of 5, 10, and 20 mg/mL, suppressed the progression of human pancreatic (Suit-2) and colon (colo205) cancer cells using a chick chorioallantoic membrane model. Furthermore, González-González et al. [5] demonstrated that hydrolysable and condensed polyphenol extracts obtained from the pomegranate peel via ultrasound-microwave assisted extraction significantly reduced the viability of HeLa and HepG2 cells in a dose-dependent manner (25 mg/L). The findings indicated a strong correlation between increased extract concentration and exposure time with elevated levels of cancer cell death. Overall, the results align with previous literature, supporting the anticancer potential of pomegranate-derived polyphenols.

Conclusions

High antioxidant activity and anticancer properties of pomegranate by-products are well established. However, previous studies have primarily investigated these effects using extracts obtained via conventional solvent-based methods. In the present study, for the first time, a bioactive-rich extract was developed through a green extraction process combining enzymatic treatment and ultrafiltration. This green extract exhibited significantly enhanced phenolic content and antioxidant activity, and it effectively reduced the viability of LNCaP, A2780, MCF-7, and Caco-2 cancer cells in a dose-dependent manner.

The GE sample demonstrated a superior cytotoxic effect compared to both the traditionally extracted sample (TE) and fresh pomegranate juice (PJ), correlating with its higher concentrations of ellagic acid, punicalagin A, and punicalagin B. These findings not only validate the therapeutic potential of pomegranate by-products but also establish that optimizing extraction methods can substantially enhance their bioactivity.

Overall, this study highlights the importance of sustainable extraction techniques for the valorization of food waste and supports the use of green-extracted pomegranate by-products as promising candidates for functional food ingredients or complementary therapeutic agents in cancer-related applications.

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Data Availability Data will be made available on request.

Declarations

Competing interests The authors declare no competing interests.

Ethical Approval This article does not contain any studies with human participants or animals.

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