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Original Research Article

Chemical composition, cytotoxic potential and antioxidant properties of *Punica granatum* peel extract

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Abstract

Purpose: *To evaluate the antioxidant activity of pomegranate peel extract and its effects on the survival of colorectal cancer cells (Caco-2) and kidney cells (HEK293).*
Methods: *An ethanol extract was prepared from the outer layer of pomegranate peels. The chemical composition of the extract was examined using high-performance liquid chromatography with diode-array detection (HPLC-DAD) and liquid chromatography with tandem mass spectrometry (LC-MS-MS). Antioxidant activity was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assays. Cytotoxicity was tested against Caco-2 colorectal cancer cells and HEK293 normal kidney cells using MTT assay.*
Results: *The pomegranate peel extract was rich in phenolic compounds and demonstrated significant antioxidant activity, with IC_{50} values comparable to Trolox in DPPH and ABTS assays. The extract exhibited selective and concentration-dependent cytotoxicity against Caco-2 cells, with an EC_{50} of 121.05 ± 13.12 µg/mL, while showing minimal toxicity towards HEK293 cells.*
Conclusion: *Pomegranate peel extract's high phenolic content and potent antioxidant properties highlight its potential as a therapeutic agent against colorectal cancer. The findings suggest that this extract could serve as a viable, natural alternative with reduced toxicity for colorectal cancer treatment.*

Keywords: *Pomegranate peel extract, Phenolic compounds, HPLC-DAD, LC-MS/MS, Antioxidant activity, Cytotoxicity*

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INTRODUCTION

Colorectal cancer remains a major global public health concern. Despite advancements in chemotherapy, there is an ongoing need for innovative therapeutic approaches that enhance efficacy while minimizing toxicity, given the high mortality and low recovery rates associated with

current treatments, mainly due to the adverse side effects of existing therapies [1].

Conventional cancer treatments often fail to distinguish between malignant and healthy cells. Anticancer therapies should selectively target cancerous cells with minimal impact on surrounding healthy tissues. This challenge has accelerated the search for new anticancer drugs, particularly those derived from plants, which tend to have fewer side effects when used to treat tumours. Around 35,000 phytochemicals from terrestrial and aquatic environments have been identified as potential complements to traditional cancer therapies [2].
Various plant-derived metabolites, including phenols, terpenoids, saponins, and alkaloids, have demonstrated chemoprotective effects against different types of cancer cells, with the capacity to trigger either cell cycle arrest or apoptosis. These bioactive compounds work through multiple mechanisms, such as promoting apoptosis and reducing DNA damage induced by oxidative stress by disrupting cellular checkpoints or decreasing levels of anti-apoptotic proteins. The therapeutic value of natural compounds has gained increasing recognition, especially the bioactive components found in *Punica granatum* [3].

The pomegranate peel possesses a higher concentration of antioxidants than the juice, making it a valuable source of bioactive compounds. It contains proteins, polysaccharides, minerals, and phenolic substances [4]. Furthermore, pomegranate peel extract (PPE) demonstrates diverse biological activities, encompassing anticancer, anti-inflammatory, neuroprotective, antiviral, and antibacterial effects [5].

Given the growing interest in utilizing industrial by-products such as pomegranate peel, this study uses advanced chromatographic techniques to examine its ethanolic extract's chemical composition and bioactivity. The study also evaluates the extract's antioxidant and cytotoxic effects on human colorectal cancer (Caco-2) and embryonic kidney (HEK293) cell lines.

EXPERIMENTAL

Plant material and extraction process

In October 2022, Mature *P. granatum* fruits were obtained from the market in Mostaganem City, located in West Algeria, to prepare pomegranate peel powder. The peels were manually separated, dried at 40 °C, and finely ground using an electric blender. The resulting fine powder was stored at -20 °C in a freezer for future use.

The extraction process involved mixing 10 g of the powder with 70 % ethanol and agitating it on an orbital shaker for 24 h. The mixture was then filtered under vacuum and concentrated using a rotary evaporator. Subsequently, it was frozen and lyophilized using a lyophilizer at -80 °C under pressure for 72 h [6].

Phytochemical analysis of bioactive compounds

This was achieved by using High-performance liquid chromatography with diode-array detection (HPLC-DAD). Separation process was conducted using an Inertsil ODS-3 guard column (150 mm × 4.0 mm, film thickness 4 µm) at a temperature of 35 °C. The extracts were dissolved to create stock solutions (8 mg/mL) in a methanol and water mixture (80/20 volume ratio). The samples were pre-filtered using an Agilent 0.45 µm PTFE filter. The mobile phase consisted of a solution containing 0.5 % acetic acid in aqueous (A) and methanolic (B) solvents. The gradient elution method varied the solvent composition as follows: 0 to 20 % B (0 - 0.01 min), 20 to 60 % B (0.01 - 0.02 min), 60 to 80 % B (0.02 - 15.2 min), and 100 % B (15.2 - 30 min). Between 30 and 35 min, the composition shifted from 100 % B to 10 % B, and from 35 to 40 min, it further changed from 10 % B to 0 % B. After injecting the sample, compound identification was conducted using a diode array detector (DAD) set to a wavelength range of 200 - 600 nm [7].

Characterization by liquid chromatography with tandem mass spectrometry (LC-MS-MS)

The *P. granatum* peel extract was analyzed qualitatively using a Shimadzu 8040 ultra-high sensitivity LC system as previously described [8]. The MS/MS analysis was performed with electrospray ionization (ESI) under specific parameters.

Determination of antioxidant activity

Antioxidant activity was assessed using spectrophotometric methods with DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radicals [9,10]. In the DPPH assay, a 50 µL sample was mixed with a 0.004 % DPPH solution and incubated in the dark for 30 min, after which absorbance was measured at 517 nm. A working solution was prepared for the ABTS assay by

reacting 7 mM ABTS with 2.45 mM potassium persulfate and adjusting with ethanol. A 20 µL sample was then combined with 2 mL of this solution and incubated in the dark at room temperature for 6 min. Absorbance was measured at 517 nm for DPPH and 734 nm for ABTS, using Trolox as a reference standard. The radical scavenging activity (S) was calculated using Eq 1.

$$S\ (\%) = ((A_{control}-A_{sample})/A_{control})100 \ldots\ldots\ldots (1)$$

where A_{sample} is the absorbance of the solution with sample and $A_{control}$ is the absorbance of the solution in the absence of sample.

Cytotoxic assay

The cytotoxicity of *P. granatum* peel extract was tested on human colon cancer (Caco-2) and healthy embryonic kidney (HEK293) cells. Both cell types were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum and penicillin/streptomycin. The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was used to measure cell viability at 590 nm after 24 h of treatment with varying extract concentrations [11].

Statistical analysis

Experiments were done in triplicate and analyzed with a *T*-test and one-way ANOVA (Dunnett) using GraphPad Prism (Version 9) and Microsoft Excel spreadsheet. *P*-value ≤ 0.05 was considered significant.

RESULTS

Phytochemical report of bioactive compounds

The analysis of *P. granatum* revealed a significant concentration of phenolic compounds. Pyrocatechol was identified as the predominant compound, with a concentration of 16.16 ± 0.05 mg/g, as supported by the data in Table 1 and Figure 1. The pomegranate peel exhibited a diverse range of phenolics, with ellagic acid being prominent at 3.97 ± 0.09 mg/g and epicatechin and fisetin present at 1.35 ± 0.09 mg/g and 1.30 ± 0.04 mg/g, respectively.

Phytochemical characterization Using LC-MS/MS

Table 2 provides detailed information on the compounds. Three phenolic acids were identified: 4-hydroxycoumarin, p-coumaric, and gallic acids. Additionally, the analysis detected several phytoconstituents, such as flavonoids (naringenin, myricetin, quercetin, and rutin) and other significant compounds (vanillin, beta-carotene, folic acid, and maleic acid).

Antioxidant activity

Table 3 presents the results of the PPE's antioxidant activity evaluation. The IC_{50} values for PPE were 0.980 ± 0.01 mg/mL for DPPH assay and 0.332 ± 0.04 mg/mL for ABTS assay. In comparison, Trolox had IC_{50} values of 0.727 ± 0.02 mg/mL for DPPH and 0.127 ± 0.03 mg/mL for ABTS.

Table 1: Phenolic composition of *P. granatum* extract by HPLC-DAD (mg/g)

Phenolic compound	Retention time (min)	*P. granatum* (mg/g)
Fumaric acid	14.014	-
Protocatechic acid	24.625	-
Pyrocatechol	24.658	16.16±0.05
Theophylline	29.449	-
4-oh-benzoic acid	30.867	0.08±0.02
Vanillic acid	34.758	0.29±0.03
Epicatechin	35.278	1.35±0.09
Caffeic acid	35.28	-
Vanillin	36.915	Trace
p-Coumaric acid	40.874	Trace
2,4-dihydroxybenz	41.086	-
Prophylgallate	46.984	-
Hesperidin	47.381	-
Rutin	47.527	0.48±0.02
Ellagic acid	50.005	3.97±0.09
Fisetin	51.243	1.30±0.04
Quercetin	55.19	-
Curcumin	72.622	Trace

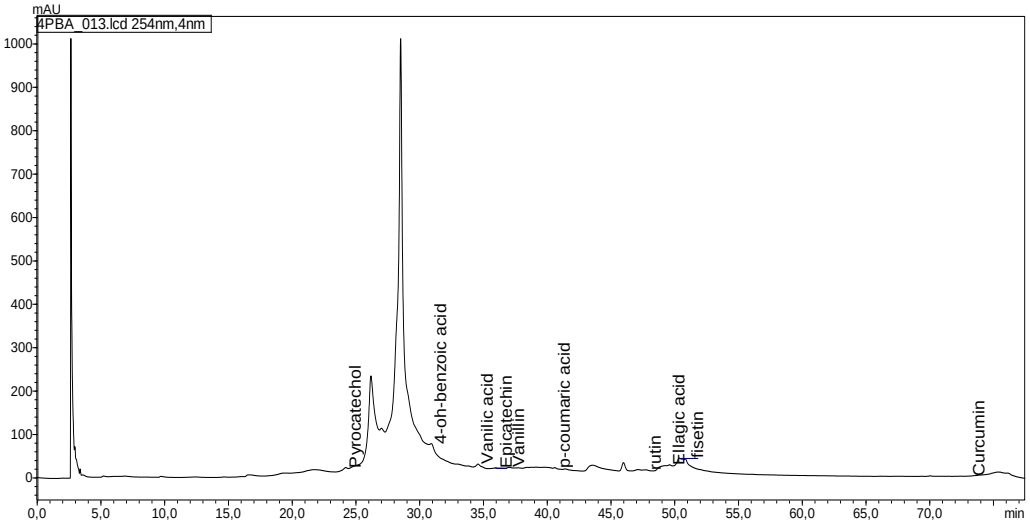

Figure 1: HPLC–DAD chromatogram of *P. granatum* extract at 254 nm

Table 2: The compounds identified in the *P. granatum* peel extract based on the LC/MS-MS results

Compound	Ionization mode (m/z)	Precursor ion	Product ion	*P. granatum*
Naringenin	$(MH)^+$	273.10	191.10 232.20	ND
Myricetin	$(MH)^+$	336.25	46.15 72.15 238.40	D
Quercetin	$(MH)^+$	303.10	85.05	D
Rutin	$(MH)^+$	611.20	73.20 282.20	D
Vanillin	$(MH)^+$	153.10	93.20	D
Beta-carotene	$(MH)^+$	537.20	199.25 23.10	D
p-Coumaric acid	$(MH)^+$	165.10	59.10	D
4-Hydroxy coumarin acid	$(MH)^-$	160.80	117.10	D
Gallic acid	$(MH)^-$	168.80	125.10	ND
Folic acid	$(MH)^+$	442.90	59.10 426.45 323.45	
Maleic acid	$(MH)^+$	117.10	85.20	D

***Note*:** D: detected, ND: not detected

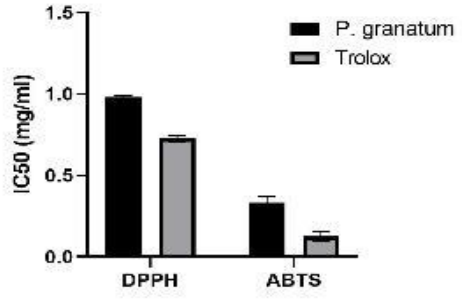

Figure 1: Antioxidant activities of the ethanol extract of pomegranate

Cytotoxic activity

The extract obtained from the rind of the pomegranate fruit has a toxic effect on Caco-2 colon cancer cells that varies depending on the dose. As the concentration of the extract is increased, there is a significant decline in cell viability, particularly at doses of 125 and 250 µg/mL. The EC_{50} value, 121.05 ± 13.12 µg/mL, signifies the extract's effectiveness in lowering cancer cell population by 50 %. On the other hand, extract demonstrates low toxicity levels towards HEK293 embryonic kidney cells, resulting in a consistently high survival rate at various dosages. Significant reduction in viability is only detected at the highest concentrations, indicating that the extract is less toxic to healthy cells at lower dosages. The results emphasize the extract's unique capacity to eradicate cancer cells while sparing healthy cells from harm, indicating its promise as a targeted therapeutic intervention.

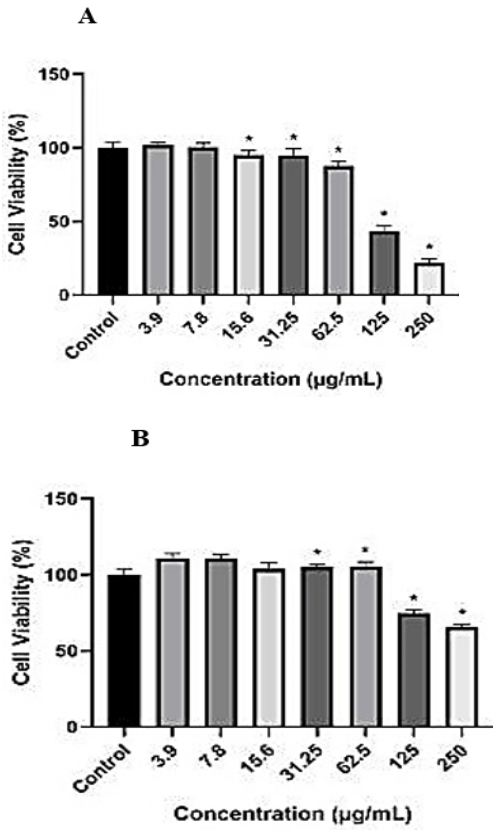

Figure 2: Cytotoxic effects of pomegranate peel extract on (A) Caco-2 and (B) HEK293 cell lines after 24 h of treatment

DISCUSSION

Cancer remains a pressing global health issue, recognized as one of the most critical medical challenges of our time. Although several cancer therapies have been approved for clinical use [12], conventional treatments often result in considerable side effects and can lead to drug resistance. Consequently, there is a pressing need for novel anticancer agents that offer high efficacy and low toxicity.

Medicinal plants have historically been utilized for their diverse pharmacological properties to prevent and treat various human ailments. This therapeutic potential is mainly due to their rich array of primary and secondary metabolites, including flavonoids, phenolic compounds, alkaloids, and tannins. Pomegranates, in particular, have demonstrated several medicinal benefits and are effective in managing diabetes, erectile dysfunction, obesity, reproductive disorders, and arthritis.

In this context, extensive research conducted over the past two decades [13] has underscored the multifaceted pharmacological benefits of *P. granatum* L., commonly known as pomegranate. These benefits include anticancer, anti-inflammatory, antioxidant, and antimicrobial properties. With the growing production of pomegranate products, peel extract (PPE) has emerged as a valuable by-product. PPE is notably rich in primary bioactive compounds, often in higher concentrations than those found in the fruit's edible parts, and is abundant in antioxidants and phytochemicals.

The investigation utilized HPLC-DAD and LC-MS/MS techniques to analyze the pomegranate peel extract, revealing a wide range of secondary metabolites through phytochemical profiling. The PPE contains a wide range of phenolic compounds, which vary depending on environmental factors, cultivar differences, and ripening stages [14]. *P. granatum* extract had a similar antioxidant capacity as Trolox. The lower IC_{50} values suggest that the pomegranate peel extract may exhibit substantial antioxidant effects at lower doses than Trolox, highlighting its impressive antioxidant potential. Furthermore, a significant correlation was observed between polyphenol concentration and antioxidant efficacy, reinforcing that polyphenols neutralize free radicals and protect essential biomolecules from oxidative damage.

Research indicates that PPE is rich in gallic acid, ellagic acid, and punicalagin derivatives, critical components of its phenolic profile. Pomegranate peel is exceptionally high in phenolic acids, flavonoids, and ellagitannins, contributing to its antioxidant activity and health benefits [14]. The pomegranate peel demonstrates greater antioxidant activity and a higher concentration of phenolic compounds than other parts of the fruit. The accumulation of phenolic compounds in the peel likely reflects its role as a protective barrier for the fruit [15].

Further investigations determined the effect of pomegranate peel extract on Caco-2 colon cancer cells and HEK293 embryonic kidney cells. Results showed that while healthy kidney cells were largely unaffected, the extract exhibited dose-dependent cytotoxicity against colon cancer cells. The pomegranate peel extract has an EC_{50} value of 121.05 ± 13.12 µg/mL for colon cancer cells, suggesting its potential in cancer research. Its effectiveness against HeLa, HCT116, and MCF7 cell lines is attributed to its flavonoids (hesperidin, rutin, catechin, kaempferol) and phenolic compounds (ferulic acid, pyrogallol, ellagic acid), which have known anticancer properties [16].

Previous studies reported that ellagic acid from pomegranates triggers apoptosis in Caco-2 colon

cancer cells by activating the intrinsic apoptotic pathway without adversely affecting healthy colonic cells [17]. Further studies have indicated that pomegranate peel extract inhibits cell growth, reduces cell viability, and prevents invasion in HeLa cells, demonstrating its dose-dependent anticancer effects. The antioxidant and anticancer properties of pomegranate pericarp and seeds are attributed to the synergistic effects of polyphenols [18].

CONCLUSION

Ethanolic extract derived from pomegranate peel has significant bioactive characteristics, such as antioxidant capabilities and specific toxicity toward colon cancer cells. The extract has promise for pharmacological applications, namely in cancer therapy. Determination of the molecular mechanisms and clinical efficacy of pomegranate peel extract (PPE) is essential to ascertain its therapeutic potential and facilitate its incorporation into cancer treatment protocols.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was conducted by the authors listed in this article, and all responsibilities for claims related to the content of this article will be borne by the authors. Sena Bakhti contributed to the methodology, formal analysis, and original draft writing. Ahmed Bekada and Mohammed Bouzouina provided supervision, with Bouzouina also overseeing revisions and editing. Mohamad Khairi Zainol contributed resources, investigation, and revisions. Mohammed Aness Bekada participated in revisions, while Cansel Cakir, Mehmet Ozturk, Amine Hafis Abdelsalam, and Sevki Arslan contributed to the methodology, revision, and review. All authors read and approved the manuscript for publication.

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