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Antibacterial and antioxidant effects of punicalagin extracts from *Punica granatum* peels

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ABSTRACT

The growing recognition of the health-promoting potential of compounds derived from natural sources, particularly those exhibiting antimicrobial and antioxidant activities, has resulted in a surge of research publications dedicated to exploring the functional attributes of these target compounds for applications in functional foods and preventive healthcare. Pomegranate has been identified as a source of beneficial health effects, attributed to its rich content of bioactive components, including polyphenols, tannins, and anthocyanins. This study investigates the effects of punicalagin on the viability and proliferation of A549 cells, a human alveolar basal epithelial adenocarcinoma cell line. The cells were treated with varying concentrations of punicalagin (10, 25, 50, 100, and 200 μg mL^{-1}), with assessments conducted after a 72-hour incubation period. Cell viability was evaluated using the MTT assay, which measures metabolic activity, and results indicated a dose-dependent response to punicalagin treatment. Specifically, lower concentrations (10 μg mL^{-1}) exhibited minimal cytotoxicity (viability: 86.04 ± 3.09%), while higher concentrations (200 μg mL^{-1}) significantly reduced cell viability to 51.17± 0.94%, indicating cytotoxic effects at elevated doses. Additionally, the study highlighted that even at non-cytotoxic concentrations, treated cells displayed reduced proliferative potential compared to control groups, suggesting that punicalagin may impair cell growth. Punicalagin treatment also demonstrated a similar trend, were higher concentrations negatively impacted cell proliferation. The findings underscore the importance of concentration in determining the cytotoxic and proliferative effects of these compounds on A549 cells, providing insights into their potential therapeutic applications and mechanisms of action. Overall, this research contributes

to the understanding of how natural compounds like punicalagin can influence cancer cell behaviour, paving the way for future studies aimed at developing effective cancer treatments.

Keywords: Anti-bacterial, Antioxidant, Punicalagin, MTT assay.
Article type: Research Article.

INTRODUCTION

Antimicrobial resistance (AMR) represents a critical global public health concern, culminating in an increase in hospital admissions and mortality rates, with forecasts indicating an alarming 10 million fatalities each year by the year 2050 as a consequence of AMR. The inappropriate utilization of antimicrobial pharmacological agents has led to the emergence of resistant microbial strains, thereby complicating therapeutic interventions and escalating healthcare expenditures. There exists a rising interest in exploring plant-derived remedies as feasible alternatives to synthetic antimicrobial agents, as natural products frequently encompass bioactive constituents endowed with antimicrobial efficacy (Salam *et al*. 2023). Pomegranate (*Punica granatum*) is a fruit extensively consumed and recognized for its myriad health benefits, having been utilized in traditional medicinal practices for centuries. The pomegranate is systematically categorized within the Punicaceae family. It is delineated as a deciduous shrub exhibiting distinct characteristics (Kahramanoglu & Usanmaz 2016). The agricultural production of pomegranate primarily transpires in geographical locales such as Afghanistan, China, Iran, India, Pakistan, and the United States. Pomegranate is integral to the maintenance of human health. An array of scholarly investigations has delineated the presence of antioxidants and bioactive phenolic constituents in pomegranate, which are pivotal for the enhancement of human health (Qahir *et al*. 2021). The advantageous compounds that confer positive effects on human well-being are located within both the fruit and its juice, with a particularly elevated concentration found in the peel of the fruit. Consequently, the pomegranate has attained recognition as a medicinal plant. The peels of the fruit are abundant in bioactive constituents and possess considerable economic potential within the food processing sector. Researchers are increasingly directing their focus toward the investigation of beneficial phytochemicals present in fruit peels, aspiring to harness these compounds for various cosmetic and pharmaceutical applications to advance human welfare (Mo *et al*. 2022). Among the multitude of fruits, the pomegranate peel is exceptionally rich in phytochemicals. The peels of pomegranates have attracted substantial interest from researchers, emphasizing their significance in the fields of medicine and nutrition. The compounds encapsulated within the peels are classified into numerous categories, inclusive of phenolic acids, flavonoids, and tannins, among others (Balwinder Singh *et al*. 2017). Pomegranate encompasses a diverse array of phytoactive constituents, such as flavonoids and tannins, which contribute to its antioxidant, anti-inflammatory, and antimicrobial properties. Pomegranate peels, which are frequently regarded as waste, are particularly abundant in these bioactive compounds and have demonstrated notable antimicrobial efficacy against various pathogenic microorganisms (Sreekumar *et al*. 2014; Pirzadeh *et al*. 2020). The extraction methodology employed to procure pomegranate peel extracts (PPEs) can profoundly affect their antimicrobial potency, thereby necessitating the exploration of varied extraction strategies (Qahir *et al*. 2021). Microwave-assisted extraction (MAE) is underscored as an innovative technique that can augment the yield and quality of bioactive constituents derived from pomegranate peels. By repurposing food waste, such as pomegranate peels, this research advocates for environmental sustainability while simultaneously addressing the pressing issue of AMR (Sreekumar *et al*. 2014). Punicalagin extracts derived from pomegranate peels have exhibited broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacterial strains. Empirical studies have demonstrated that these extracts are efficacious against pathogens including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Salmonella typhi* (Abutayeh *et al*. 2024). The extracts have manifested significant inhibitory effects against *Staphylococcus aureus*, encompassing both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA; Mendes *et al*. 2023). The extracts are effective against *E. coli*, *P. aeruginosa*, and *S. typhi*, with notable inhibition zones observed in well-diffusion assays (Shleghm *et al.* 2024; Koosehlar *et al*. 2024). Finding novel active principles is necessary since multidrug-resistant bacteria are a significant issue in clinical practice. Pomegranate has been shown to have antibacterial capabilities against a variety of bacteria, including both Gram-positive and Gram-negative ones. Because of the presence of secondary metabolites, specifically phenolic, flavonoid, anthocyanin, and hydrolyzable tannin, there has been demonstrated antibacterial action (Shleghm *et al.* 2024). It is recommended that researchers develop novel natural remedies to

enhance the quality of life and lessen clinical symptoms in patients with lung infections, particularly in developed countries. The current study evaluated the antioxidant and antibacterial properties of punicalagin on *Streptococcus pneumoniae*.

MATERIALS AND METHODS

Punicalagin extraction

Punica granatum fruits were procured from a local market and subsequently peeled, and the peels were dried in a shaded environment. Punicalagin extract was obtained through the maceration technique. The dried orange peels were immersed in a 10% potassium hydroxide (KOH) solution for a duration of one night, after which the mixture was subjected to filtration. Following the Soxhlet extraction and maceration processes, the resulting filtrate was adjusted to a pH of 3-4 using a 6% acetic acid solution. The concentrated liquid was then stored in a refrigerator at temperatures between 4 and 6 °C overnight, forming a solid crystalline substance. This mixture was filtered once more, and the crude punicalagin was isolated as an amorphous powder using a Buchner funnel (Sharifnia *et al.* 2023).

Cell culture

The cell line A549, which represents human alveolar basal epithelial adenocarcinoma, has been propagated at 37 °C with 5% CO_2 in the ambient environment. A DMED fresh medium supplemented with 10% FBS and 1% penicillin/streptomycin made up the culture media. After the cells achieved 85–95% confluence, the media was aspirated, and 0.25% (w/v) trypsin-EDTA was used to separate the cell monolayer. Using a hemocytometer, the detached cells were counted after being stained with trypan blue and suspended in a full-growth medium (Safdarpour *et al*. 2022).

Cell viability

After treating A549 cells with Punicalagin (10, 25, 50, 100, and 200 µg mL^{-1}, repeated three times), the cells' vitality was assessed using a fluorescence microscope and staining with the viability/cytotoxicity kit. Using a 3-(4, 5-dimethylthiazol-2-yl) - 2, 5-diphenyltetrazolium bromide (MTT) test, the metabolic activity of the treated cells was determined. The cells were collected and treated with the MTT solution (5 mg mL^{-1}) for 4 hours after they had aggregated after being punicalagin-treated. After solubilizing the MTT crystals with dimethyl sulfoxide (Sigma), the absorbance at 570 nm was measured with a microplate reader. Due to the 72-hour duration of the therapy, cell cultures treated with punicalagin were used for the MTT test to evaluate proliferation. The following formula was used for quantification (Mohammadi *et al*. 2024):

Percentage of cell viability (%) = Optical Density $_{570}$ Treatment/Optical Density $_{570}$ Control ×100%

Microorganism

The efficacy of Punicalagin antibacterial characteristics was evaluated concerning *Streptococcus pneumoniae* (ATCC 49619).

The inoculum preparation

The inoculum solutions were prepared from five colonies of 24-hour cultures, and the strains were subsequently injected into the Mac Conkey agar medium before the experiment. For twenty seconds, the colonies were shaken while suspended in 5-mL sterile saline (0.85% NaCl). The density was measured using 5×10^8 CFU mL^{-1}, or turbidity equal to 0.5 McFarland Standard.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Determination

Using the IC_{50} calculation of the MTT technique, the MIC values for plant extracts retrieved at multiple concentrations using the broth microdilution procedure were determined. The microbe inoculum was generated and the turbidity was adjusted to 0.5 McFarland in Mueller-Hinton Broth (Himedia, India). After diluting the wells, a final turbidity of around 1×10^8 CFU mL^{-1} was achieved. DMSO was used as the solvent to generate a plant extraction concentration of 200 mg mL^{-1}. Based on earlier research, serial doubling concentrations of the oils were generated in 96-well microtiter plates, ranging from 10.9 to 0.34 mg mL^{-1}. Each plate was added with positive and negative controls, and it was then incubated for 24 hours at 37 °C. The IC_{50} estimate provided the

foundation for these conclusions (Yehia & Altwaim 2023). Diffusion testing of agar gel was also performed using Mueller-Hinton agar plates. A quantity of organism equal to around 3×10^6 colony-forming units mL^{-1} was added for each replication. The width of the inhibitory zone associated with the disks soaked in 1, 5, and 10 mg mL^{-1} of essential oil and incubated at 37 °C for 16–18 hours was also prepared for room temperature settling and sterile control of the Petri dishes following a 24-hour incubation period at 37 °C. The inhibitory zone around the disc was measured and quantified in mL. It was found that the diameters of 8 to 12 mm, larger than 12 mm, and less than 8 mm were categorized as vulnerable, moderately susceptible, and resistant, respectively (Sultana *et al*. 2010; Mohammadi *et al*. 2024).

The activity of antioxidants reduces the power and DPPH

The Yen and Duh technique was used to determine the Fe^{3+} reduction power of plant peel extract with certain modifications. In short, the produced extract was mixed with 3-mL phosphate buffer (0.2 Molar) and 3-mL potassium ferricyanide (1%). The mixture was incubated for 30 minutes at 50 °C. Following the incubation period, 5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 5000 rpm for 7 minutes. An aliquot of 0.5 mL of 0.1% ferric chloride was combined with 1 mL of the upper layer. The absorbance of the resulting solution was measured at 700 nm. A stronger reducing power is indicated by a greater amount of absorption. Regression analysis findings were interpolated to find the EC_{50} value or the effective concentration at which the absorbance was 0.5. Quercetin and ascorbic acid were used as positive controls (Yen & Duh 1993). The DPPH radical scavenging test was calculated using the procedure that had been previously outlined. The scavenging rate on DPPH radicals was computed using the following formula: Scavenging rate (%) = 100 × (A blank – A sample)/A blank, where A sample is the tested compound's absorbance and A blank is the absorbance of the control reaction (which contains all reagents except the tested compound). Regression analysis interpolation yielded the extract concentration required to scavenge 50% of DPPH free radicals, or IC_{50} (µg mL^{-1}), which was used to represent the antiradical activity. Three copies of the determination were made, and the outcomes were reported as mean values ± standard deviation (SD). As positive controls, ascorbic acid, quercetin, and gallic acid were employed (Selles *et al*. 2020).

Statistical analysis

Using the Graph Pad Prism 5.04 software, all statistical analyses of biochemical estimates were performed using the One-Way ANOVA analysis and Dunnett's post hoc test and t-tests. In every study, a difference was considered significant if the *p*-value was less than 0.05. Significant differences are indicated by the asterisks: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

RESULTS

Cell Proliferation

Fig. 1 shows the A549 cell's viability rate (%) after 72 h incubated with the different concentrations of punicalagin (10, 25, 50, 100, and 200 µg mL^{-1}). Statistically, there was a significant difference between the control and treatments viability in the five examined concentrations after inoculation ($p < 0.0001$). Punicalagin treatment for 3 days manifested low cytotoxicity at 10 µg mL^{-1} (viability: 86.04 ± 3.09%), 25 µg mL^{-1} (viability: 70.16 ± 0.52 %), 50 µg mL^{-1} (viability: 62.06 ± 1.83 %), and 100 µg mL^{-1} (viability: 53.58 ± 1.36 %). However, as the concentration of punicalagin increased up to 200 µg mL^{-1}, the viability of treated cells gradually decreased, indicating the cytotoxic effect of punicalagin in higher concentrations. Essentially, the group that received 200 µg mL^{-1} punicalagin showed a markedly reduced relative viability (51.17± 0.94%), indicating that the cells under investigation were somewhat sensitive to cytotoxic effect of punicalagin at higher concentrations. The punicalagin-treated cells multiplied over time. Even in the lowest concentration group that did not display cytotoxicity, on the third day of the culture, they did, however, show inferior proliferative potential in comparison to the controls. According to the results, cell viability was dose-dependent and punicalagin treatment at high concentrations may have somewhat reduced A549 cells' capacity to proliferate.

Antioxidant activity

In this study, the antioxidant properties of extracted punicalagin were evaluated through the DPPH radical scavenging assay and the assessment of reducing power. The examination of antioxidant activity was based on the capacity of antioxidants to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The EC_{50} value for the reducing efficacy of punicalagin is presented in Table 1. The DPPH assay, which measures the ability of antioxidants to

scavenge the stable DPPH radical, was employed to evaluate the radical scavenging potential of the samples. As indicated in Table 1, the reference compounds quercetin and ascorbic acid exhibited EC_{50} values of 27.2 ± 0.5 µg mL^{-1} and 19.98 ± 0.12 µg mL^{-1}, respectively, while punicalagin demonstrated an EC_{50} value of 22.56 ± 0.12 µg mL^{-1}. In comparison to the reference substances, punicalagin exhibited an IC_{50} value of 98.82 ± 0.25 µg mL^{-1}.

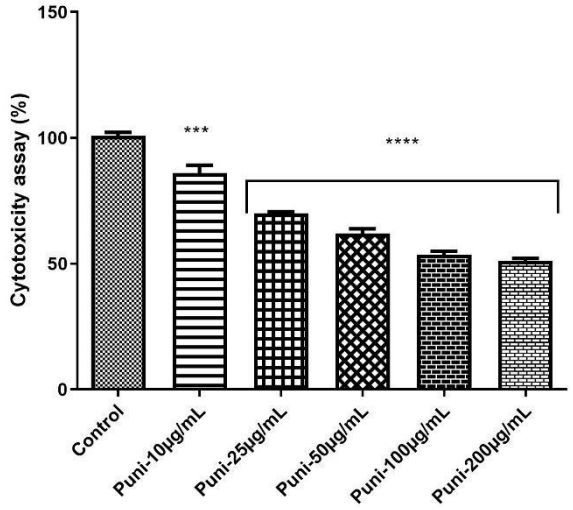

Fig. 1. The A549 cells were treated with a range of concentrations of Punicalagin (10, 25, 50, 100, and 200 µg mL^{-1}) for 72 h in comparison to the control group, and cell viability percent were measured.

Table 1. Punicalagin antioxidant activity in reducing power.

	Reducing power (EC_{50}; µg mL^{-1})	**DPPH (EC_{50}; µg mL^{-1})**
Quercetin	27.2 ± 0.5	13.18 ± 0.25
Gallic acid	-	6.9 ± 0.12
Ascorbic acid	19.98 ± 0.12	6.20 ± 0.12
Punicalagin	22.56 ± 0.12	98.82 ± 0.25

MIC and MBC assay

MIC, as an appropriate antimicrobial activity assessment, demonstrated that punicalagin had antimicrobial activities against *Streptococcus pneumoniae* in selected doses. The largest zones of inhibition were observed at the concentration of 20 µg mL^{-1} for the experienced pathogen. Based on the observed zone of inhibition, the plant extracts' antibacterial activity ability depended on the concentrations of punicalagin used. At 5 µg mL^{-1} *S. pneumoniae* had no inhibition zone, whereas 10 µg mL^{-1} (6.4 mm), 20 µg mL^{-1} (17.9 mm), and 50 µg mL^{-1} (21.5mm) showed zone of inhibition. Also, the lowest MIC value of 5 mg mL^{-1} (Table 2) in the presence of punicalagin was observed against *S. pneumoniae*. The MIC value of punicalagin against the bacterium was 5 µg mL^{-1}. The mean value zone of inhibition assessments revealed that punicalagin antibacterial activity depended on the plant extract concentrations. Generally, zones of inhibition mean the antibacterial activity of the *S. pneumoniae* which depends on its concentrations.

Table 2. Zone of inhibition against *Streptococcus pneumoniae* by punicalagin.

Pathogens	**Zone of inhibition (mm)**				*Gram*
	Conc. of punicalagin (µg mL^{-1})				
	5	**10**	**20**	**50**	
S. pneumoniae	-	6.4	17.9	21.5	Positive
	Zone of inhibition (mm)				
Penicillin (10 µg/disc)	18.2				Positive

DISCUSSION

Punica granatum, commonly known as pomegranate, has been widely studied for its bioactive compounds, particularly punicalagin, which are rich in its peel. These compounds have shown significant potential in exhibiting antibacterial and antioxidant properties, making them valuable for various applications in medicine, food preservation, and health promotion. This response synthesizes the findings from the provided papers to explore the antibacterial and antioxidant effects of punicalagin extracts from pomegranate peels (Mendes *et al.*

2023; Yehia & Altwaim 2023). Punicalagin, a polyphenol found in pomegranate peel extracts (PPEs), is primarily credited for its antimicrobial activity. The study highlights that PPEs exhibit significant antibacterial effects against various bacteria, including antibiotic-resistant strains. Additionally, pomegranate peels are rich in bioactive compounds with antioxidant properties, contributing to their overall health benefits. The qualitative screening of local pomegranate peels confirmed the presence of phenolic compounds, including punicalagin, which enhance both antimicrobial and antioxidant activities (Abutayeh *et al*. 2024; Shleghm *et al.* 2024). The antibacterial activity of punicalagin extracts is attributed to their ability to disrupt bacterial cell membranes and interfere with essential biochemical processes. For instance, punicalagins can modulate bacterial membrane permeability, leading to cell death (Shleghm *et al.* 2024). The extracts have been shown to deactivate extended-spectrum beta-lactamase (ESBL) enzymes, which are responsible for antibiotic resistance in certain bacteria, such as ESBL-producing *E. coli*. Punicalagin extracts have been found to enhance the efficacy of conventional antibiotics when used in combination. For example, microwaved aqueous extracts of pomegranate peels, when combined with gentamicin, showed improved antibacterial activity against gentamicin-resistant *P. aeruginosa*. This highlights the potential of punicalagin extracts as adjuvants in combination therapies to combat antibiotic resistance (Abutayeh *et al*. 2024). The antibacterial activity of punicalagin extracts has been consistently demonstrated in laboratory settings, with minimum inhibitory concentrations (MICs) ranging from 12.5 to 25 µg μL^{-1} for various bacterial strains (Abutayeh *et al*. 2024; Almasehali *et al*. 2024). Based on the observed zone of inhibition, the plant extracts antibacterial activity depended on the concentrations of punicalagin used. At 5 µg mL^{-1}, *S. pneumoniae* had no inhibition zone, whereas 10 µg mL^{-1} (6.4 mm), 20 µg mL^{-1} (17.9 mm), and 50 µg mL^{-1} (21.5mm) displayed zone of inhibition. Also, the lowest MIC value of 5 mg mL^{-1} in the presence of punicalagin was observed against *S. pneumoniae*. The MIC value of punicalagin against the bacterium was 5 µg mL^{-1}. In animal models, pomegranate peel extracts have been shown to reduce bacterial loads and improve survival rates. For example, in a rat model of *E. coli* infection, treatment with pomegranate peel extract significantly reduced *E. coli* levels in faeces and improved survival rates (Shleghm *et al*. 2024). Punicalagin extracts are rich in phenolic compounds, flavonoids, and tannins, which are responsible for their strong antioxidant activity. These compounds scavenge free radicals and reduce oxidative stress, as demonstrated in various assays such as DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate)] radical scavenging assays. The ethanolic extract of pomegranate peels exhibited significant antioxidant activity, with an IC_{50} value of 46.05 ppm, comparable to ascorbic acid at lower concentrations. The extracts showed strong radical scavenging activity, highlighting their potential to mitigate oxidative stress-related diseases (Alghamdi *et al*. 2024; Roziafanto *et al*. 2024). As indicated in the present study, the reference compounds quercetin and ascorbic acid exhibited EC_{50} values of 27.2 ± 0.5 µg mL^{-1} and 19.98 ± 0.12 µg mL^{-1}, respectively, while punicalagin demonstrated an EC_{50} value of 22.56 ± 0.12 µg mL^{-1}. In comparison with some other studies, punicalagin exhibited an IC_{50} value of 98.82 ± 0.25 µg mL^{-1}. The antioxidant activity of punicalagin extracts is closely linked to their phytochemical composition. Punicalagins are the primary ellagitannins responsible for the antioxidant activity of pomegranate peels. Flavonoids and phenols contribute to the free radical scavenging properties of the extracts (Mendes *et al*. 2023; Roziafanto *et al*. 2024). The antioxidant activity of punicalagin extracts can be enhanced by optimizing extraction conditions such as temperature, solvent ratio, and extraction duration. For instance, maceration at 27 °C for 30 minutes with a simplicia/solvent ratio of 1:3 yielded extracts with high phenolic and flavonoid content, resulting in superior antioxidant activity (Roziafanto *et al*. 2024). Punicalagin, a major bioactive compound in pomegranate peels, plays a central role in both antibacterial and antioxidant activities. Its ability to disrupt bacterial membranes and scavenge free radicals makes it a promising natural compound for therapeutic and food applications (Mendes *et al*. 2023). Punicalagin has been shown to inhibit bacterial growth by modulating membrane permeability and interfering with bacterial enzymes. Punicalagin's strong antioxidant activity helps protect against oxidative stress, reducing the risk of chronic diseases such as cancer, cardiovascular disorders, and neurodegenerative diseases (Alghamdi *et al*. 2024). Punicalagin extracts have shown potential in treating bacterial infections, particularly those caused by multidrug-resistant strains. Their ability to enhance the efficacy of conventional antibiotics makes them valuable in combination therapies (Abutayeh *et al*. 2024). The antimicrobial and antioxidant properties of punicalagin extracts make them suitable for use as natural food additives and preservatives. They can prevent food spoilage and extend shelf life by inhibiting the growth of foodborne pathogens (Abu-Niaaj *et al*. 2024).

CONCLUSION

In conclusion, the study emphasizes the dual nature of punicalagin as both a potential therapeutic agent and a compound that can exhibit cytotoxic effects at high concentrations. The antioxidant activity of punicalagin extracts contributes to overall health by reducing oxidative stress and inflammation. This makes them valuable in the prevention and management of chronic diseases. Punicalagin extracts from pomegranate peels exhibit significant antibacterial and antioxidant effects, driven by their rich phytochemical composition. These extracts have shown promise in addressing antibiotic resistance, reducing bacterial loads, and mitigating oxidative stress. Their potential applications span therapeutic, food, and health promotion sectors, highlighting the importance of further research to fully harness their benefits.

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