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RESEARCH ARTICLE

Anticancer and Antioxidant Activities of Polyphenolic Pomegranate peel Extracts Obtained by a Novel Hybrid Ultrasound-Microwave Method: *In vitro* and *in vivo* Studies in Albino Mice with HeLa, Colon, and HepG2 Cancerous Cell Lines

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ABSTRACT

Cancer is a deadly disease and ranks as the second leading cause of death globally, which predominantly arises as a result of continuous exposure of human beings to carcinogenic agents and environmental contaminants. Natural compounds can potentially treat up to 60% of cancer cases. Therefore, this study investigated the anticancer and antioxidant activities of pomegranate peel extracts, which were prepared using a novel hybrid ultrasound-microwave assisted extraction (HUME) method. The HUME demonstrated significant improvements in extraction yield and polyphenol contents compared to conventional methods. The anticancer activity of pomegranate peel extract (PPE) was evaluated against HeLa, colon, and HepG2 cell lines and *in vivo* colon cancer in albino mice. The PPE demonstrated potent antioxidant activity, with DPPH and ABTS radical scavenging capacities of 91% and 95%, respectively. This potent activity was attributed to its high levels of phenolic acids (181 mg GAE/g) and flavonoids (35 mg QE/g). The secondary metabolites of PPE were detected by MS/MS using negative and positive ionization modes; the most abundant detected compounds were 4-hydroxycoumarin, p-coumaric, and gallic acid. A 40 Albino mice were randomly assigned to four experimental groups: a control group, a pathogen-induced cancer group, a 5-fluorouracil-treated group, and a group administered pomegranate peel extract (PPE). Albino mice treated with the PPE showed significant ($P<0.05$) tumor volume reduction and tumor growth inhibition compared to the diseased group. Pomegranate peel extract (PPE) demonstrated *in vivo* anticancer activity against bacterial pathogen-induced colon cancer. This was evident by significantly ($P<0.05$) downregulated B-cell leukemia/lymphoma 2 (BCL2) and Hypoxia-inducible factor 1-alpha (HIF1-α) level in the serum by 50% and 30%, respectively, in the PPE-treated group compared to the infected control. Concomitantly, PPE treatment improved histopathological features in the colon tissue, where the glandular structures remained intact and normal histological structure. These findings suggest that pomegranate peel extracts obtained by HUME possess potent anticancer and antioxidant activities. Further research is warranted to elucidate the underlying mechanisms and explore their potential as promising therapeutic agents for cancer treatment.

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INTRODUCTION

Cancer is a significant public health issue and the leading cause of death globally (Ferlay *et al.*, 2020). It remains the second leading cause of morbidity and mortality after cardiovascular disease and microbial infections around the globe, and predominantly, all cancer cases have their roots in lifestyle and environment (Gaidai *et al.*, 2023; Guo *et al.*, 2024). Recognizing the high mortality and poor recovery rates linked to current chemotherapies, mainly because of their adverse effects (Anand *et al.*, 2023), there remains a critical need for innovative therapeutic approaches that can simultaneously improve treatment effectiveness and reduce toxicity (Sung *et al.*, 2021).

Conventional cancer treatments often fail to distinguish between malignant and healthy cells (Kaur *et al.,* 2023). Anticancer therapies should selectively target cancerous cells with minimal impact on surrounding healthy tissues (Liu *et al.,* 2024). 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 tumors (El-Saadony *et al.,* 2023; 2025). Around 35,000 phytochemicals from terrestrial and aquatic environments have been identified as potential complements to traditional cancer therapies (Lewandowska *et al.*, 2022).

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 (Mueed *et al.*, 2024; Alharbi *et al.*, 2024). 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 antiapoptotic proteins (Al-Quwaie *et al.,* 2023). The therapeutic value of natural compounds has gained increasing recognition, especially the bioactive components found in *Punica granatum* (Fakudze *et al*., 2022).

Pomegranate fruits *Punica granatum* L. is a family Lythraceae member grown in tropical and subtropical temperate zones on evergreen, deciduous small trees or shrubs (Das *et al.,* 2025). The pomegranate is an old plant cultivated in the Mediterranean, Middle East, and South Asia for several ages. Pomegranate peels, which constitute half the fruit weight, are often considered waste but are a rich source of phytochemicals (Fahmy and Farag, 2022). 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 (Teniente *et al.*, 2023). Furthermore, pomegranate peel extract (PPE) demonstrates diverse biological activities, encompassing anticancer, anti-inflammatory, neuroprotective, antiviral, and antibacterial effects (Xiang *et al*., 2022).

Numerous studies have explored the effects of Pomegranate peels, seeds, and pulp on ruminant and poultry health, focusing on parameters such as nutrient digestibility, ruminal fermentation, and growth performance (Abarghuei *et al.,* 2013; HamaKhan *et al.,* 2015; Addisu, 2016). For instance, research on Karadi lambs and dairy cows has demonstrated that incorporating pomegranate peels or extracts into diets can influence lipid profiles, blood biochemical parameters, and overall animal productivity (Jami *et al.*, 2012; Sadq *et al.,* 2015; Safari *et al.,* 2018). These effects are often attributed to the bioactive compounds present in pomegranate byproducts, including tannins and polyphenols, which can modulate digestive processes and metabolic outcomes (Addisu, 2016; Kotsampasi et al., 2017; Hatami *et al.,* 2018).

Inflammation develops chronic diseases initiated by prolonged oxidative stress, which occurs when an imbalance between harmful reactive oxygen species (ROS) and the body's defense mechanisms (Leyane *et al.,* 2022). This oxidative stress can then activate the NF-κB pathway, a central controller of inflammation (Saaoud *et al.,* 2024). This activation leads to increased expression of cyclooxygenase-2 (COX-2), the enzyme involved in the production of proinflammatory prostaglandins, and the induction of inducible nitric oxide synthase (iNOS), which generates nitric oxide (NO), a signaling molecule with complex roles in inflammation (Coutinho *et al.*, 2024).

Furthermore, inflammation can arise from invading pathogens, i.e., *Fusobacterium nucleatum*, *Bacteroides fragilis*, and certain species of *Escherichia coli* (Quaglio *et al.,* 2022). These invaders trigger an immune response characterized by the upregulation of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8) (Cao *et al.,* 2024). Simultaneously, the production of chemokines like CCL2 and CXCL8, which attract immune cells to the site of inflammation, is also increased. Conversely, the levels of anti-inflammatory cytokines, like interleukin-10 (IL-10), which help to dampen the inflammatory response, are often reduced (Song *et al.*, 2024).

Chronic inflammation (CI), a persistent and long-lasting inflammatory state, has been strongly implicated in the pathogenesis of a wide range of debilitating diseases (Wang *et al.,* 2021). These include atherosclerosis, the underlying cause of cardiovascular diseases (CVD); inflammatory bowel disease (IBD), a group of disorders affecting the digestive system; kidney disease; and diabetes Mellitus (Wu *et al.*, 2024). The global impact of CI is staggering, where recent studies suggested that it is a leading cause of death worldwide, with approximately half of all mortalities attributed to inflammation-related conditions and autoimmune diseases (Yin *et al.*, 2024).

Chronic inflammation can compromise the integrity of this crucial barrier, leading to increased permeability, a condition often referred to as "leaky gut" (Paray *et al.,* 2020). This breach allows macromolecules, including pathogens, exotoxins, and undigested fats, to cross from the intestinal lumen into the underlying tissues (Fasano, 2020). This increased permeability and the subsequent influx of foreign substances can contribute significantly to the development of colorectal cancer, particularly in individuals with pre-existing inflammatory bowel disease (Escalante *et al.*, 2024). Therefore, effective management of inflammation is paramount in preventing colorectal cancer, especially in the vulnerable IBD patient population.

While extensive research *in vitro* and *in vivo* has explored the potential therapeutic benefits of various compounds, including PPE, the specific *in vivo* cytotoxic effects of PPE on colon cancer and the precise molecular mechanisms by which it exerts its effects remain fully elucidated (Teniente *et al.,* 2023). Given the growing interest in utilizing industrial byproducts, such as pomegranate peel, this study employs advanced chromatographic techniques to investigate the chemical composition and bioactivity of its ethanolic extract. The study also evaluates the extract's antioxidant and cytotoxic effects on human cervical cancer cells (HeLa), colon cancer cells (Caco-2), and liver cancer cell (HepG2) lines. Then, the anticancer potential of pomegranate peel extract (PPE) on pathogen-induced colon cancer in albino mice will be tested by evaluating biochemical, metabolomics, and histological characteristics.

MATERIALS AND METHODS

Plant material and extraction process: Mature *Punica granatum* fruits were obtained from local markets, then ground to prepare pomegranate peel powder. The peels were manually separated, dried at 40°C, and finely ground using an electric blender (LM1A0, Moulinex, France). 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 24h. 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 72h (Khodadadi *et al.*, 2021).

Phytochemical analysis of bioactive compounds: The phenolic compounds in PPE were detected using High-Performance Liquid Chromatography with diode-array detection (HPLC-DAD). The separation process was conducted using an Inertsil ODS-3 guard column (150mm × 4.0mm, film thickness 4µm) 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, v/v)). 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 40min, it further changed from 10 % B to 0 % B. After injecting the sample, the phenolic compounds were identified using a diode array detector (DAD) set to a wavelength range of 200 - 600nm (Tokul-Ölmez *et al.*, 2020).

Characterization of PPE using Tandem mass spectrometry (MS/MS): The pomegranate peel extract was characterized qualitatively using a Shimadzu 8040 ultra-high sensitivity system as previously described (Belguidoum *et al.*, 2024). The MS/MS analysis was performed with electrospray ionization (ESI) under specific parameters.

Determination of antioxidant activity: The antioxidant activity of PPE was tested against DPPH (2,2- diphenyl-1-picrylhydrazyl, 24 µM) and ABTS (2,2'-azino- bis(3-ethylbenzothiazoline-6-sulfonic acid) radicals methods using JENEWAY spectrophotometer (UK) with Amari *et al.*, (2014); Altarawneh *et al.*, (2022). In the DPPH assay, a 50 µL sample was mixed with a 0.004 % DPPH solution and incubated in the dark for 30min, after which absorbance was measured at 517nm. A working solution was prepared for the ABTS assay by reacting 7mM ABTS with 2.45mM potassium persulfate and adjusting it with ethanol. A 20µL sample was then combined with 2 mL of this solution and incubated in the dark at room temperature for 6min. Absorbance was measured at 517 nm for DPPH and 734nm for ABTS. Trolox was used as a reference standard. The radical scavenging activity (S) was calculated using Eq 1.

$$\%\ Antioxidant\ activity = \frac{Abs\ control - Abs\ sample}{Abs\ control} x100$$

Where Abs sample is the absorbance of the solution with the sample, and Abs control is the absorbance of the solution in the absence of the sample.

Cytotoxic assay: The cytotoxicity of *P. granatum* peel extract was tested on human HeLa, Caco-2, and HepG2 cell lines (ATCC, USA). The cancerous cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Oxiod, UK) supplemented with 20ng/mL epidermal growth factor, 500ng/mL hydrocortisone, 0.01ng/mL insulin, 5% FBS and 1% Pen/Strep. The cells were maintained in standard conditions (humidified atmosphere with 5% CO_2 and 37 °C). The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was used to measure the cell viability by measuring the absorbance of the reaction mixture on 590nm after 24h of treatment with varying PPE extract concentrations (Zhang *et al.*, 2023).

Animal design model: Albino/c male mice were selected for this study. BALB/c mice were albino and had pink eyes and white hair. The "c" was added at F26 by Snell in 1932; it refers to coat color (Wang *et al.*, 2015). Forty BALB/c mice (6-8 weeks old) were bought and housed under 12h day/night cycles. All mice in the four groups had free access to water and a high-fat diet throughout the experimental period. These mice were maintained at 23°C with relative humidity (50%) and moisture (10%) (Ludgero-Correia *et al.*, 2012). The mice were separated into four groups (ten per group): group one was orally administered 0.5mL saline as a control. Induction of colon tumor using bacterial pathogens supported with protein and lipid-rich saturated fatty acid diet for groups 2, 3 and 4. Pathogenic bacteria, *Escherichia coli* and *Klebsiella* sp., were used at 10^6 CFU (colony-forming unit) in a single dose as the starting point of infection. Treatment was carried out for groups 3 & 4 with standard drug and PPE, respectively. Treatment of group 3 was performed via intraperitoneal administration of 5-fluorouracil (25 mg/kg) every 3 days for 2 weeks. Group 4 administered PPE (100 mg/kg).

Biochemical analysis of mice serum: At the end of the experiments, the animals were slaughtered, and blood samples were collected and centrifuged (3000 rpm for 10 min). Sera were isolated and then stored at -20°C until analysis. Using ELISA kits, the content of apoptosis key regulators, i.e., B cell lymphoma gene 2 (BCL2) and hypoxia-inducible factor 1 -α (HIF1-α) proteins (Biosource International Inc., California, USA) were investigated following the manufacturer's instructions (Gür *et al.*, 2011).

Histopathological examination: In parallel, animal colons were collected immediately from all mice after euthanasia using light ether. After being cleaned with a saline solution, the colon was first fixed in 10%; then, paraffin sections of 5 µm thickness were prepared and stained with hematoxylin and eosin (H & E). Paraffin slices of 5 µm thickness were prepared for microscopic examination and stained with H & E (Bastaki *et al.*, 2016).

Serum metabolomics analysis using GC-MS analysis: The serum metabolites of the studied groups were profiled using gas chromatography (Thermo Scientific Corp., USA) coupled with a thermal mass spectrometer detector. Metabolites separation were achieved under conditions described in previous work (Hassan *et al.*, 2020; Ammar *et al.*, 2021). The gas chromatography-mass spectrometry (GC-MS) data were cleaned, deconvoluted, and aligned using the MS-DIAL interface (Zhang *et al*., 2020).

Statistical analysis: All 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

Active compounds profile of PPE: The analysis of *P. granatum* peels revealed a significant concentration of phenolic compounds. Pyrocatechol was identified as the predominant compound, with a concentration of 19.5 mg/g (Table 1), followed by ellagic acid (5.2mg/g), epicatechin, and fisetin at 2.2mg/g and 1.9mg/g, respectively.

Table 1: Phenolic composition of PPE by HPLC-DAD (mg/g)

Phenolic compound	Retention time (min)	PPE (mg/g)
Fumaric acid	14.00	0.065±0.001
Protocatechic acid	24.5	0.01±0.001
4-oh-benzoic acid	30.9	0.09±0.01
Vanillic acid	34.5	0.32±0.02
Caffeic acid	35.3	0.02±0.01
Ellagic acid	50.11	5.2±0.09
p-Coumaric acid	40.77	0.09±0.002
Pyrocatechol	24.6	19.5±0.05
Theophylline	29.5	0.02±0.00
Vanillin	36.90	0.01±0.001
Hesperidin	47.31	0.02±0.05
Rutin	47.6	0.61±0.02
Fisetin	51.33	1.9±0.04
Quercetin	55.23	0.01±0.02
Curcumin	72.71	0.23±0.01
2,4-dihydroxybenz	41.21	0.01±0.001
Prophylgallate	46.92	0.02±0.002

n=3, data are presented mean±SE.

Characterization of PPE 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 phenolic compounds (vanillin, beta-carotene, folic acid, and maleic acid). Most of the compounds were detected in positive ionization modes.

Antioxidant potential of PPE: Table 3 shows the scavenging activity of PPE against DPPH and ABTS free radicals, where PPE (200µg/ml) scavenged 91% and 95% of free radicals, respectively. Furthermore, The IC50 of PPE was 120 and 85µg/ml against DPPH and ABTS free radicals because of the considerable content of phenolic and flavonoids (181 and 35 mg/g).

Anticancer activity of PPE: Fig. 1A-F shows the potent cytotoxic activity of PPE against three cancer cell lines (Hela, liver, and colon cancer cell lines). As the concentration of the extract is increased, there is a significant inhibition in cell viability, particularly at doses of 75 and 100µg/mL. The IC_{50} value against all cancer cells was 75µg/mL, which indicates the extract's effectiveness in reducing the cancer cell population by 50 %. PPE at 100µg/mL significantly ($P<0.05$) reduced the cancer cell viability by 23, 17, and 20% against Hela, liver, and colon cancer cell lines, respectively (Fig. 1G).

Table 2: The compounds identified in the PPE using LC/MS-MS results

Compound	Ionization mode (m/z)	Precursor ion	Product ion	PPE
Phenolic acids				
p-Coumaric acid	(MH)+	166.2	58.9	+
4-Hydroxy coumarin acid	(MH)-	161.1	117.6	+
Gallic acid	(MH)-	169.2	124.8	+
Folic acid	(MH)+	443.3	59.2, 25.6, 324.1	+
Maleic acid	(MH)+	118.3	85.9	+
Flavonoids				
Naringenin	(MH)+	271.22	192.1, 231.5	+
Myricetin	(MH)+	335.9	45.6, 71.6, 239.2	+
Quercetin	(MH)+	305.22	84.9	+
Rutin	(MH)+	610.55	72.8, 281.9	+
Vanillin	(MH)+	152.36	93.6	+
Beta-carotene	(MH)+	541.33	24.1, 201.33	+

Table 3: Antioxidant potential of PPE

Antioxidant poteintal	Value
Total phenolic (mg/g)	181±2.3
Total flavaniods (mg/g)	35±0.2
DPPH scavenging (%)	91±1.1
ABTS scavenging (%)	95±1.8
IC_{50} against DPPH (µg/ml)	120±5.2
IC_{50} against ABTS (µg/ml)	85±1.6

n=3, data are presented mean±SE

Effects of PPE on BCL2 and HIF1-α in mice sera with colon cancer: Induction of colon cancer by pathogenic bacteria caused significantly elevated levels of BCL2 and HIF1- α compared to the control group, as depicted in Fig. 2 (A & B). The PPE treatment significantly downregulated the levels of BCL2 and HIF1-α to nearly normal levels, as illustrated in Fig. 2 (A & B).

Histological studies on mice colon cancer tissues: Fig. 3 shows the histopathological analysis demonstrated the protective effects of PPE against bacterial infection-induced colon cancer in mice (H&E, 100x), whereas Fig. 3A showed the control group with normal colon tissue structure, including intact mucosa with glandular structures, submucosa, muscularis, and serosa. Fig. 3B, bacterial infection-induced colon cancer showed desquamation of the mucosal epithelium, significant inflammatory cell infiltration in the lamina propria of the mucosa and submucosa, and anaplasia of the glandular epithelium exhibiting malignant characteristics such as disorganization, hyperchromasia, polarity loss, and pleomorphism, along with surrounding inflammatory cell infiltration. *5*-fluorouracil-treated group after infection showed medium to mild lymphoid follicle hyperplasia in the submucosa, extending to the mucosa, accompanied by inflammatory cell infiltration (Fig. 3C), and PPE post-treated: the glandular structures remained intact and normal histological structure was maintained (Fig. 3D).

Oxidative stress markers in colon tissues: Analysis of colon tissue revealed that pathogen induction of colon cancer significantly ($P\leq0.05$) decreased glucose, calcium,

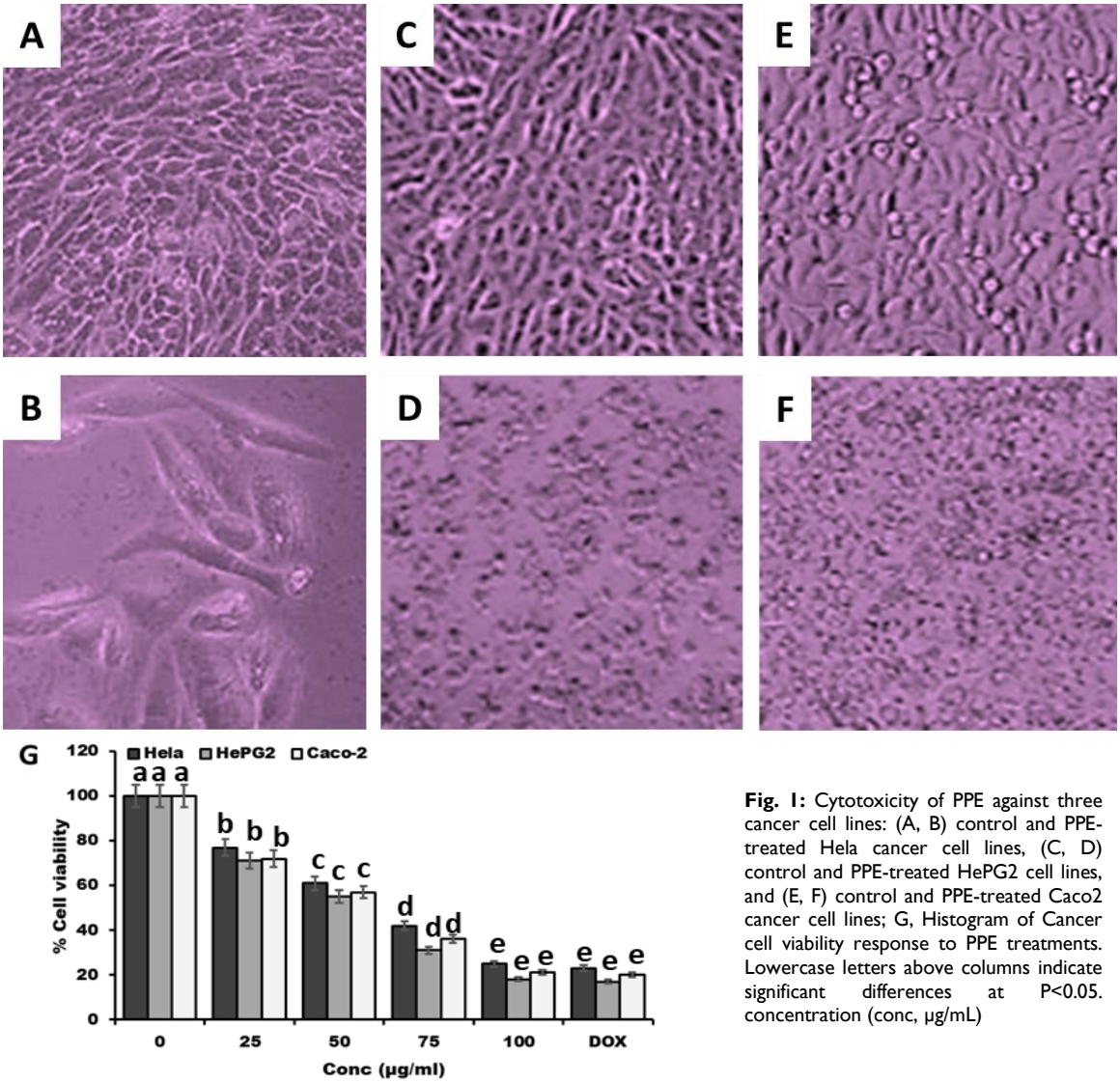

Fig. 1: Cytotoxicity of PPE against three cancer cell lines: (A, B) control and PPE-treated Hela cancer cell lines, (C, D) control and PPE-treated HePG2 cell lines, and (E, F) control and PPE-treated Caco2 cancer cell lines; G, Histogram of Cancer cell viability response to PPE treatments. Lowercase letters above columns indicate significant differences at P<0.05. concentration (conc, µg/mL)

and lactate dehydrogenase (LDH) levels compared to controls. PPE treatment significantly (P≤0.05) increased glucose levels in all treated groups compared to the pathogen group, restoring them to normal in the PPE-treated group. Calcium levels were significantly (P≤0.05) increased by the extract in all treated groups, achieving normal levels.

Furthermore, bacterial infection significantly (P≤0.05) reduced total antioxidant capacity (TAC) and the activity of catalase (CAT) and glutathione peroxidase (GPx) enzymes while significantly (P≤0.05) increasing lipid peroxidation (LPO) and total phenolic content (TPC) compared to controls. PPE treatment significantly (P≤0.05) increased TAC, CAT, and GPx while decreasing LPO and TPC in all treated groups compared to the infected group, with complete restoration to normal levels observed in the PPE-treated group.

GC-MS based metabolomics: In the present study, the GC-MS-based metabolomics approach has been applied to study the effect of PPE on the serum metabolic levels of BALB/c mice with pathogen-induced colon cancer, as shown in Fig. 4. The score plot of this model shows complete segregation between the untreated colon cancer group (G2) and mice treated with PPE (G4), as illustrated in (Fig. 4). The ratio between G2/G4 detected the fold change.

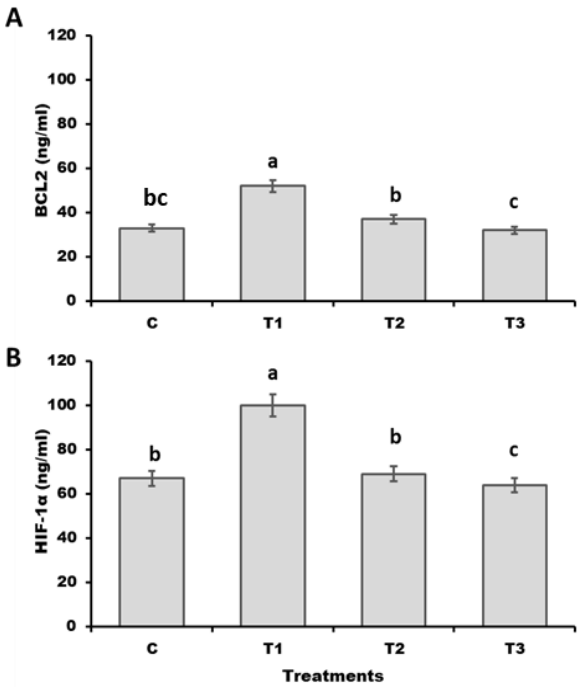

Fig. 2: Serum levels of BCL2 (A), and HIF1-α (B) in various studied groups. Data were expressed as mean ± SD (n=8 for each group). Different letters indicate significant differences at P<0.05.

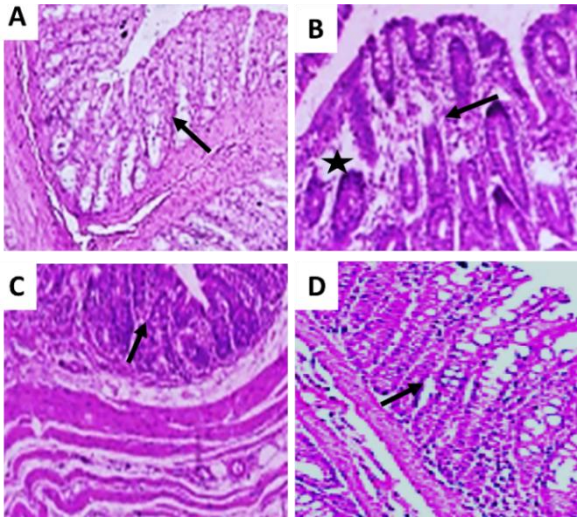

Fig. 3: Histopathological analysis demonstrated the protective effects of PPE against bacterial infection-induced colon cancer in rats (H&E, 100x). A) control colon tissues; (B) bacterial infection-induced colon cancer group; (C) 5-fluorouracil-treated group after infection; (D) PPE-treated group after infection.

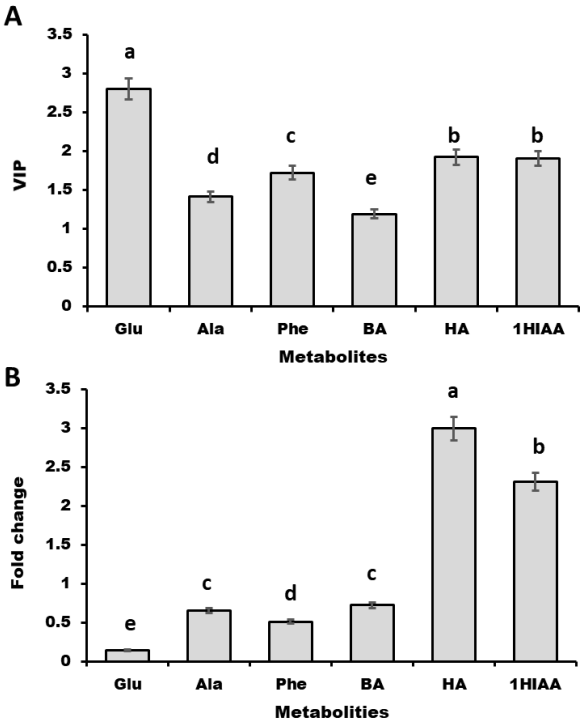

Fig. 4: Differential metabolite biomarkers as revealed for the multivariate OPLS-DA and univariate analysis of the studied groups *i.e.,* untreated mice with pathogen-induced colon cancer group (G2) vs mice treated with PPE group (G4). The VIP values of significant biomarkers (A) and fold change in biomarkers between untreated and PPE-treated groups (B).

Table 4: Impact of PPE on cellular components and oxidative stress markers in the colon tissues of albino mice with bacterial-induced colon cancer

Markers	C	T1	T2	T3
Glucose	3.77±0.1	1.2±0.2	2.69±0.2	3.71±0.6
LDH	1750±6.3	1045±11.2	1425±10.3	1552±9.8
Ca	0.19±0.02	0.2±0.01	0.18±0.03	0.17±0.02
TAC	1.5±0.1	0.5±0.02	0.91±0.02	1.66±0.1
CAT	44.9±0.98	11.9±0.5	43.69±1.2	44.45±1.6
POD	20.98±1.2	11.5±0.4	20.55±1.5	21.41±1.1
LPO	44.3±1.1	145.63±3.5	56.33±2.1	45.32±1.4
TPC	11.36±0.9	19.36±0.6	10.69±0.5	11.22±1.1

n=3, data are presented mean±SE. lactate dehydrogenase (LDH), total antioxidant capacity (TAC), catalase (CAT), peroxidase (POD), lipid peroxidation (LPO), and total phenolic content (TPC).

DISCUSSION

Colon cancer is the 3rd most common cancer and the 2nd leading cause of cancer-related deaths globally (Morgan *et al.*, 2023). Significant advances in the treatment of colon cancer were found either with resectable or metastatic tumors (Loree and Kopetz, 2017). Recent research demonstrated that specific pathogenic bacteria can significantly contribute to colon carcinogenesis through multiple mechanisms (Avril and DePaolo, 2021). Experimental studies utilizing rat models have been particularly valuable in elucidating these microbial influences, providing crucial insights into the complex relationship between gut microbiota and cancer development (Lindell *et al.*, 2022). These animal studies have revealed that pathogenic bacteria promote tumorigenesis through distinct and interrelated pathways, including the disruption of microbial homeostasis, induction of chronic inflammation, direct DNA damage, and modulation of host immune responses (Li *et al.*, 2022).

The process begins with microbial dysbiosis, where an imbalance in gut microbiota composition creates a pro-tumorigenic environment (Mignini *et al.*, 2023). Research in rat models has shown that antibiotic-mediated depletion of gut microbiota can reduce tumor incidence while deliberate colonization with pathogenic bacteria accelerates tumor formation (Paudel *et al.*, 2024). Particular attention focused on *Fusobacterium nucleatum*, which was consistently identified in human CRC patients and experimental rat models (Liu *et al.*, 2024). This pathogen promotes tumor progression by suppressing host immune responses, thereby creating favorable conditions for malignant growth (Wu *et al.*, 2022).

The inflammatory cascade represents another critical pathway, with enterotoxigenic *Bacteroides fragilis* (ETBF) as a prime example (Zhuang, 2022). ETBF secretes the *B. fragilis* toxin (BFT) that damages the intestinal epithelial cells, triggering chronic inflammation (Yang *et al.*, 2024). This persistent inflammation was stated to increase reactive oxygen species (ROS) production and proinflammatory cytokines such as IL-6 and TNF-α, promoting DNA damage and uncontrolled cellular proliferation (Antar *et al.*, 2023). Rat studies demonstrated that ETBF infection induces T helper 17 (Th17) immune responses strongly associated with CRC progression (Wu *et al*., 2022).

Perhaps more directly concerning is the genotoxic potential of certain bacterial species. Some pathogens produce toxins that damaged host DNA (Martin and Frisan, 2020). Notably, specific strains of *Escherichia coli* (particularly those carrying the pks genomic island) produce colibactin, a potent genotoxin that induces double-strand DNA breaks and chromosomal instability (Auvray *et al.*, 2021). Experimental evidence from rat models shows that exposure to colibactin-producing *E. coli* significantly increases the frequency of colonic adenocarcinomas (Wang and Fu, 2023). Similarly, *Salmonella Typhimurium* infection has been shown to promote CRC development in rats by activating the β-catenin signaling pathway, a key regulator of cell proliferation and tumorigenesis (Al-Qarraawi and Al-Awade, 2024). These findings highlight the diverse mechanisms pathogenic bacteria can initiate and promote colorectal carcinogenesis.

The immune system's role in this process is equally critical, as pathogenic bacteria developed sophisticated strategies to evade host defenses (Thakur *et al.*, 2019). Multiple studies have demonstrated that these microbes can suppress anti-tumor immunity by modulating regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (Haist *et al.*, 2021). *Fusobacterium nucleatum* inhibited natural killer (NK) cell activity, enabling tumor cells to evade immune surveillance (Pignatelli *et al.*, 2023). Rat models further revealed that bacterial infections increase immunosuppressive cytokines such as IL-10 and TGF-β production, creating a microenvironment that favors tumor growth and progression (Mir *et al.*, 2023).

The azoxymethane (AOM)-treated rat model has been particularly informative in studying these processes (Dzhalilova *et al.*, 2023). Research using this model has shown that ETBF colonization results in hyperproliferative crypts and tumor development at significantly higher rates than controls (Zhuang, 2022). Mechanistic studies have revealed that the BFT toxin activates NF-κB and STAT3 signaling pathways, which are known drivers of inflammation and tumor growth (Wu *et al.*, 2022). Similarly, rats infected with colibactin-producing *E. coli* exhibit increased aberrant crypt foci (ACF), recognized precursors to CRC (Wang *et al.*, 2021). The impact of *Fusobacterium nucleatum* has also been demonstrated, with infected rats developing larger and more invasive tumors than controls. This effect appears mediated through the bacterium's ability to enhance Wnt/β-catenin signaling, a pathway fundamental to CRC progression (Li *et al.*, 2021).

These findings have important therapeutic implications, suggesting multiple avenues for intervention. Microbiome-based strategies such as probiotics and fecal microbiota transplantation (FMT) promise to restore healthy microbial balance and potentially reduce CRC risk (Sahle *et al.*, 2024). Targeted approaches, including antibiotic therapies and specific anti-toxin agents (such as colibactin inhibitors), could help prevent tumor initiation in high-risk individuals. Additionally, immunomodulatory therapies designed to enhance antibacterial immune responses may help counteract the inflammation-driven aspects of carcinogenesis (Terveer, 2020).

Nevertheless, these treatments have many serious side effects. Therapeutic modalities using plant sources have been increasingly popular recently as safer options with fewer side effects than traditional anticancer drugs (Huang *et al.*, 2021). Pomegranates have been used in the treatment of several diseases. Moreover, pomegranate showed promising chemo-preventive activity against different types of cancer (Rauf *et al.*, 2025), such as breast, prostate, and lung cancers in cells, animal models, and humans (Sharma *et al.*, 2017).

Medicinal plants have historically been utilized for their diverse pharmacological properties to prevent and treat various human diseases. This therapeutic potential is mainly due to their rich array of primary and secondary metabolites, including flavonoids, phenolic compounds, alkaloids, and tannins (Selim *et al*., 2022; El-Saadony *et al.*, 2024a,b). Pomegranates, in particular, have demonstrated several medicinal benefits and are effective in managing diabetes, erectile dysfunction, obesity, reproductive disorders, and arthritis (Mueed *et al*., 2023; Rauf *et al.*, 2025).

In this context, extensive research conducted over the past two decades (Singh *et al.*, 2023) underscored the multifaceted pharmacological benefits of *P. granatum* L., commonly known as pomegranate. These benefits include anticancer, anti-inflammatory, antioxidant, and antimicrobial properties (Alsubhi *et al*., 2022). With the growing production of pomegranate products, peel extract (PPE) has emerged as a valuable byproduct (El Hosry *et al.*, 2023). 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 (Yassin *et al.*, 2021).

The investigation utilized HPLC-DAD and MS/MS techniques to analyze the pomegranate peel extract, revealing a wide range of secondary metabolites through phytochemical profiling (Saad *et al*., 2021; Ruan et al., 2022). The PPE contains a wide range of phenolic compounds, which vary depending on environmental factors, cultivar differences, and ripening stages (Kharchoufi *et al.*, 2018). *P. granatum* extract had a similar antioxidant capacity as Trolox. The lower IC50 values suggest that the pomegranate peel extract may exhibit substantial antioxidant effects at lower doses than Trolox, highlighting its impressive antioxidant potential (Sihag *et al.*, 2022). 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 (Benchagra *et al.*, 2021).

Research indicates that PPE is rich in gallic acid, ellagic acid, and punicalagin derivatives, critical components of its phenolic profile (Feng *et al.*, 2022). Pomegranate peel is exceptionally rich in phenolic acids, flavonoids, and ellagitannins, which contribute to its antioxidant activity and health benefits (Kharchoufi *et al.*, 2018). 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 (Derakhshan *et al.*, 2018).

Several *in vivo* studies have demonstrated that supplementation with pomegranate peel can enhance nutrient utilization and improve fermentation characteristics. For example, feeding pomegranate peel extract to dairy cows has been associated with improved nutrient digestion and altered ruminal fermentation patterns, potentially leading to better milk yield and composition (Abarghuei *et al.*, 2013; Shaani *et al.*, 2015; Kotsampasi *et al.*, 2017). Similarly, ensiled and dried pomegranate seeds have been evaluated for their nutritive value, with findings suggesting positive impacts on digestibility and animal performance (Taher-Maddah *et al.*, 2012; Eliyahu *et al.*, 2015; Karamnejad *et al.*, 2019). The presence of dietary tannins from pomegranate and other sources has also been reviewed for their role in modulating ruminal fermentation and production efficiency in cattle (Addisu, 2016; Bee *et al.*, 2016; Hatami *et al.*, 2018).

Beyond ruminants, pomegranate byproducts have been investigated in poultry and other livestock species. Research involving broilers and Japanese quail indicates that pomegranate peel powder can enhance antioxidant

status, improve growth performance, and positively affect gut health and carcass traits (Abbas *et al.,* 2017; Kamel *et al.,* 2021; Ahmadipour *et al.,* 2021). These benefits are linked to the antioxidant and antimicrobial properties of pomegranate constituents, which may help mitigate oxidative stress and support immune function (Ishfaq *et al.,* 2019; Aboelsoued *et al.,* 2019; Salles *et al.,* 2021). Additionally, studies have explored the effects of pomegranate peel on broilers challenged with *Escherichia coli*, demonstrating improvements in intestinal microbiota and growth performance (Xu *et al.,* 2024).

Further research has examined the combined effects of pomegranate peel with other feed additives, such as linseed oil or *Moringa oleifera*, showing synergistic benefits on growth, carcass traits, and muscle fatty acid composition in poultry (Elbaz, 2023; Maqsood *et al.,* 2024). In pigs, hydrolyzable tannins from pomegranate have been studied for their minimal impact on performance but notable effects on physiological traits (Bee *et al.,* 2016).

Collectively, the literature underscores the potential of pomegranate byproducts as sustainable, health-promoting feed additives across various animal production systems (Bist *et al.,* 2024; Younis *et al.,* 2025). Their multifaceted benefits—ranging from improved nutrient utilization and antioxidant capacity to antimicrobial effects—highlight their value in enhancing livestock productivity while reducing waste from the fruit processing industry.

Most chemo-preventive agents are antioxidant in nature. Fruits high in polyphenols are considered antioxidant and chemo-preventive (Imran *et al.,* 2023). Pomegranate has been shown to exert anticancer and antioxidant activity, which is generally attributed to its high content of polyphenols due to its effects of neutralizing free radicals (Turrini *et al.,* 2015). In this study, PPE showed high antioxidant capacity, which may be attributed to the excessive polyphenolic contents, represented by flavonoids and phenolic acids as free radical scavengers. The high antioxidant capacity may be attributed to excessive polyphenolic content, represented by flavonoids and phenolic acids (Sihag *et al.,* 2022; Rauf *et al.,* 2025).

One of the most distinguishing characteristics of cancer cells is their resistance to apoptosis. Oncogenes and tumor suppressor genes are well-established apoptosis regulators (Xia *et al.,* 2022). Regarding oncogenes, they can regulate apoptosis by producing antiapoptotic proteins, conferring cancer cells a survival benefit over normal cells (Wang *et al.,* 2023). So, these proteins are overexpressed in cancer cells and have a concurrent lower level in normal cells (Li *et al.,* 2012). Thus, disrupting the function of these antiapoptotic proteins is one of the approaches used to eradicate malignant cells with minimal effect in the surrounding normal cells (Radha and Raghavan, 2017).

Antiapoptotic protein BCL2 is encoded by the BCL2 (B cell lymphoma gene 2) gene family that produces either antiapoptotic proteins such as BCL2 or proapoptotic proteins such as Bcl-2 associated protein X (Bax) and Bcl-2 homologous antagonist/killer (Bak) (Poincloux *et al.,* 2009). Thus, the overexpression of BCL2 protein protects the malignant cells from apoptosis and triggers their propagation. BCL2 overexpression was first noted in B-cell follicular lymphoma (Miyaoka *et al.,* 2018). Subsequently, BCL2 overexpression was reported in several cancers, such as breast, lung, thyroid, nasopharyngeal, prostate, liver, ovarian, leukemia, neuroblastoma, and colorectal cancers (Credendino *et al.,* 2019; Zhou *et al.,* 2019; Jin *et al.,* 2021). The observed overexpression of BCL2 in many cancers confirms its vital role in cancer and makes it an ideal target for cancer therapy.

In the current study, we assessed the reducing effect of PPE on BCL2 serum levels in the bacterial pathogen-induced colon cancer mice group compared to the negative control group. In line with our findings, Larrosa *et al.* (2006) stated that ellagitannins (pomegranate Punicalagin) and its metabolite ellagic acid provoke apoptosis in human colon adenocarcinoma Caco-2 cells without affecting the normal colon cells, suggesting their anticancer effect of dietary ellagitannins in colon cancer which support our results. Recently and similar to our findings, Ganjouzadeh *et al.* (2022) reported the cytotoxicity activity of PPE on breast cancer cells via increasing Bax/Bcl-2 ratio and the intracellular ROS. Moreover, our results agreed with that of Cheshomi *et al.* (2022), who demonstrated a significant anticancer activity of ellagic acid of pomegranate extract on gastric cancer cell via reduction of BCL2 and stimulation of apoptosis in addition to inhibition of tumor growth in immunocompromised mice, supporting our study outcomes.

Interestingly, PPE showed an important anticancer activity against prostate cancer cells via reduction of BCL2 expression, inducing apoptosis, and impairs metastasis (Farooqi, 2021). Also, pomegranate extract showed an antiproliferative effect against oral cancer cells through the induction of mitochondrial dysfunction and apoptosis and the Bax/BCL2 ratio (Peng *et al.*, 2021), which agreed with our results. Most solid tumors, including colon cancer, are permanently or transiently exposed to hypoxia due to a deficient blood supply and aberrant vascularization (Matuszewska *et al.,* 2021). The hypoxia-inducible factors (HIFs) mediate the cellular response to hypoxia, thus encouraging modifications associated with cancer progression and metastasis (Lappano *et al.*, 2020). The transcription factor hypoxia-inducible factor 1 (HIF-1) is a member of the HIF family, consisting of an O2-regulated HIF-1α subunit assembled with a constitutively produced HIF-1b subunit (Albadari *et al.*, 2019).

Hypoxia controls HIF-1α activation via post-translational modifications. Oxygen leads to the post-translational hydroxylation of HIF-1α and promotes its degradation (Daly *et al.*, 2021). In contrast, the absence of oxygen stabilizes HIF-1α, allowing its binding to hypoxia-response elements in the nucleus, thus activating many HIF-target genes involved in cancer growth, metastasis, and anaerobic metabolism (Albanese *et al.,* 2020). Furthermore, HIF-1α disrupts DNA repair and, more importantly, suppresses apoptosis by altering the ratio between proapoptotic and antiapoptotic BCL-2 family members via triggering antiapoptotic proteins such as BCL2 and BCL-xl (Wang *et al.,* 2022). Thus, HIF-1α protein is overexpressed in several human cancers (Iovine *et al.*, 2016).

In the present study, we evaluated the serum level of HIF-1α in bacterial pathogen-induced colon cancer mice treated with the PPE group compared with the negative control group; consistent with our findings, the inhibition of HIF-1α and autophagy suppress colon cancer growth and proliferation (Albanese *et al.*, 2020). Similarly, suppressing

HIF-1α by L-carnosine dipeptide prevents colon cancer cells' resistance to 5- 5-fluorouracil, promoting its anticancer activity and stimulating apoptosis of colon cancer cells (Husari *et al.*, 2017).

In line with our findings, Husari *et al.*, (2017) reported pomegranate concentrate has chemotherapeutic activity against cigarette smoke-induced lung cancer in an animal model via inhibition of HIF-1α expression. In addition, treatment of lung cancer cells and tumor-bearing mice with ellagitanins significantly inhibited tumor growth via increased AMP-activated protein kinase and suppressed HIF-1α, suggesting that ellagitanins might be a promising anticancer agent (Duan *et al.*, 2020).

Using a based metabolomics approach to analyze the relationship between different metabolites and diseases is of great value (Galal *et al.*, 2022). Thus, it could support us with valuable knowledge about disease diagnosis, prognosis, and pathogenesis (Piras *et al.*, 2022). In the current study, numerous metabolites related to bacterial pathogen-induced colon cancer were observed via GC-MS-based metabolomics approach as 1H-indole-3-acetic acid, heptanoic acid, benzoic acid, alanine, phenylalanine, and glucose. This study showed a significant reduction of 1H-indole-3-acetic acid and heptanoic acid in mice treated with the PPE group compared with the untreated mice with the pathogen-induced colon cancer group.

In contrast, the PPE-treated mice group significantly increased benzoic acid, alanine, phenylalanine, and glucose. Indole derivatives are a type of serum metabolite, including indole-3-acetic acid, indole-3-propionic acid, serotonin, and other compounds. All these metabolites resulted from tryptophan metabolism (Konopelski and Mogilnicka, 2022). These protect the gastrointestinal tract from stress-induced illnesses such as cancers. These are produced mainly by the intestinal transformation of *Escherichia coli* (Xiao *et al.*, 2024). Recently and supporting our results, gut microbiota-derived tryptophan metabolites such as indole-3-acetic acid maintain gut and systemic homeostasis (Su *et al.*, 2022).

Conclusions: The novel hybrid ultrasound-microwave extraction method proved to be an efficient technique for obtaining polyphenolic-rich pomegranate peel extracts with significant anticancer and antioxidant properties. In vitro studies demonstrated potent cytotoxic effects against HeLa, colon, and HepG2 cancer cell lines, suggesting the potential of these extracts as natural chemotherapeutic agents. Additionally, *in vivo* studies in albino mice confirmed their antioxidant capacity, reducing oxidative stress markers while exhibiting minimal toxicity to normal cells. These findings highlight the therapeutic potential of pomegranate peel polyphenols as complementary agents in cancer treatment, offering a promising alternative to synthetic drugs with fewer side effects. Further research is warranted to elucidate the precise molecular mechanisms and optimize dosage formulations for potential clinical applications.

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