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Promising anticancer activity of pomegranate peels extract (PPE) against bacterial pathogens-induced colon cancer in mice model

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Abstract

Colorectal cancer is one of the most invaded lethal types of malignancy worldwide. It's an important issue to find prophylactic and curative agents against that life-threatening ailment, preferably from cheap, natural, commonly available nutraceuticals. Herein, total pomegranate peel extract was tested for its significance as an anticancer agent against bacterial pathogens-induced colon cancer in mice model. The phytochemical investigation was carried out via quantitative estimation of phenolic and flavonoid contents along with metabolomic analysis. In this study, Bagg Albino/c (BALB/c) mice were classified into 4 groups including negative control group, untreated mice with pathogen-induced colon cancer group, mice treated with 5-flurouracil and mice treated pomegranate peels extract (PPE). B cell lymphoma gene 2 (BCL2) and hypoxia-inducible factor 1 -α (HIF1-α) proteins were measured using enzyme-linked immunosorbent assay (ELISA) kits in all groups. Histopathological changes occurred in the colon of 4 groups were evaluated. The bioactive extract was found to possess a potent antioxidant capacity (357.6 ± 0.5 and 408.4 ± 0.55 mg Trolox equivalents/g) for 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) and 2, 2`-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) assays, respectively, owing to its high amount of phenolic acids (174.0±0.65) mg gallic acid equivalent/g and flavonoids (23.2 ±0.3 mg catechin equivalent/g). Furthermore, metabolomics investigation by liquid chromatography/electrospray ionization - tandem mass spectrometry (LC-ESI-MS/MS) analysis revealed the identification of 73 metabolites from varying chemical classes, most abundantly gallotannin and ellagitannin derivatives, flavonoids and their glycosides, pentacyclic triterpenes, coumarins, in addition to many phenolic and fatty acids. In vivo experiment, the metabolomics investigation by gas chromatography - mass spectrometry (GC-MS) revealed a decrease in 1H-indole-3-acetic acid and heptanedioic acid in mice treated PPE compared with untreated mice with pathogen-induced colon cancer group. Moreover, an increase in benzoic acid, alanine, phenylalanine, and glucose were observed in mice treated PPE compared with untreated mice. Finally, reduction in of BCL2 and HIF1-α serum levels in treated groups compared to untreated group. PPE showed anticancer activity against bacterial pathogen-induced colon cancer in vivo via reduction of serum levels of BCL2 and HIF1-α as apoptosis controlling factors leading to histopathological improvement of colon of PPE treated group.

Keywords: pomegranate; anticancer activity; colorectal cancer

1. Introduction

It is recognized that cancers are still the most dangerous diseases worldwide. Colon cancer is one of the four major cancer-causing determinants in many countries, among prostate, breast and lung tumors [1]. Despite the presence of notable advances in surgical techniques and chemotherapy, colorectal

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cancer survival stays dismal [2]. Thus there is a necessity for a new and safe medication for treatment of colon cancer.

Pomegranate fruits, Punica granatum L. family Lythraceae, are grown in the tropical to subtropical temperate zones, on evergreen, deciduous small trees or shrubs. The pomegranate is an old plant that had been cultivated in the Mediterranean regions, Middle East, and South Asia for several ages. Pomegranate peels constitute half of the whole fruit weight and are considered garbage, but are yet a very rich source of phytochemicals. Many studies had investigated the chemical composition and the bioactivities of pomegranate peels. They were found to be a richer source of biologically active metabolites even higher than other fruit parts [3]. They contained polyphenolic compounds, predominantly the hydrolysable tannins even more than aril and fruit juice. The red color of peels is due to the presence of anthocyanidins, in higher quantities than the flesh and edible part of the fruit [4]. They also contained other flavonoids, polysaccharides, and microelements. It was reported that peels had a good healing effect on colon cancer [5]. They also possessed powerful antioxidant activity, antimicrobial, anti-mutagenic, and anticancer activities [3].

The current study aimed to test the anticancer activity of pomegranate peels extract (PPE) on pathogen-induced colon cancer in Bagg Albino/c (BALB/c) male mice via evaluation of biochemical, metabolomics, and histological characteristics.

2. Experimental:

2.1. Plant material

Pomegranate fruits (Punica granatum L.; family Lythraceae) were collected from the local market at Cairo, Egypt. They were manually peeled and separated. Peels were left to be dried in shade, cut into small pieces, ground by an electric grinder into a fine powder, and kept in a cool, dry place.

2.2. Extraction procedure

The crude extract was prepared by maceration of 100 g. powdered peels of P. granatum in 70% ethanol. It was then kept at room temperature and shaken every 2 h for one day. Thereafter, it was filtered by using Whatman filter paper and evaporated by a vacuum pump Rotary evaporator (Heidolph, Germany) at 40 °C, which yielded crude pomegranate peel extract (PPE), weighed 26.5 g.

2.3. Phytochemical investigation and antioxidant capacity of PPE

2.3.1. Quantitative estimation for polyphenolic contents:

Spectrophotometric assays were followed to quantitatively determine the polyphenolic composition of PPE. each experiment were carried out against the reagent blank, using gallic acid as standard according to the Folin-Ciocalteu method for determining total phenolic content [6], and catechin as standard for flavonoid content [7]. A calibration curve was made in each case using the relevant standard and results were expressed as mg of gallic acid equivalent (mg GAE) for total phenols, and mg of catechin equivalent (CE) for flavonoids, per gram sample. After a repeated appraisal, the findings were shown as mean ± standard deviation.

2.3.2. Radical scavenging activity assays:

Free radical scavenging capacity of PPE using Trolox as standard was determined on both 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) and 2, 2`-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) radical scavenging assays [8]. The results were expressed as mg Trolox equivalents (TE)/g sample, as mean ± standard deviation, upon triplicate testing

2.4. Metabolomic analysis of PPE by LC-ESI-MS/MS:

The analysis of PPE was performed using liquid chromatography/electrospray ionization - tandem mass spectrometry (LC-ESI-MS/MS) with an Exion LC AC system for separation and AB SCIEX Triple Quad 5500+ MS/MS system (SCIEX; Darmstadt, Germany), equipped with electrospray ionization (ESI). The separation was performed on an Ascentis® C18 column (4.6×150 mm, 3 µm, Sigma-Aldrich; St Louis, MO). Two eluents were comprised

as the mobile phases A: 0.1% formic acid and B: acetonitrile. The mobile phase gradient was programmed as follows: 10 % B at 0-2 min, 10-90% B from 2-30 min, 90% B from 30-36 min, 10% at 36.1, 10% from 36.1-40 min with flow rate 0.7 mL/min and injection volume 10 µL. For MS/MS analysis, negative ionization mode was applied with a scan from m/z 100 to 1000 for MS1 with the following parameters: curtain gas: 25 psi; IonSpray voltage: -4500 V; source temperature: 500 °C; ion source gas 1 & 2 were 45 psi and from m/z 50 to 1000 for MS2 with a declustering potential: -80 V; collision energy: -35 V; collision energy spread: 15 V. Processing of compounds was performed using MS-DIAL software (version 4.70) and Fiehn HILIC library.

2.5. Animal design model

Bagg Albino/c (BALB/c) male mice were selected for this study. BALB/c mice are albino and have pink eyes and white hair. The "c" was added at F26 by Snell at 1932. Forty BALB/c mice (6-8 weeks old) were bought from the National Research Centre (NRC), Giza, Egypt and housed in the high groups under 12-hr day/night cycles. All mice had free access to water and the high-fat diet throughout the experimental period in all four groups. These mice were maintained at 23 ± 2 °C with 50 % ± 10 % of moisture. The NRC's Ethics Committee approved this animal study, which was conducted in conformity with international guidelines for the care and welfare of laboratory animals [Approval no: 1143042021]. The mice were separated into the following four groups (eight mice per group): group one only orally administered 0.5 mL saline as controls. Induction of colon tumor using bacterial pathogens supported with protein and lipid rich with saturated fatty acid diet carried out for groups 2, 3 & 4. The pathogenic bacteria included *Escherichia coli* and *Klebsiella sp.* were used at 10^6 CFU (colony-forming unit) single doses as starting point of infection part. 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 (20 mg/kg) every 3 days for 2 weeks. Group 4 administered PPE (200 mg/kg).

Thus, the BALB/c male mice were randomly designed into four experimental groups as the following:

Group 1 (negative control group): includes normal mice and administered 0.5 mL saline orally.

Group 2 (untreated colon cancer group): includes the untreated bacterial pathogen-induced colon cancer mice group.

Group 3 (5-fluorouracil treated group): includes bacterial pathogen-induced colon cancer mice treated with 5-fluorouracil as the standard drug group.

Group 4 (pomegranate peel extract PPE treated group): includes bacterial pathogen-induced colon cancer mice treated with PPE group.

2.6. Biochemical analysis of mice serum

At the end of the experiments, the animals were slaughtered and blood samples were withdrawn 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 regulator i.e., B cell lymphoma gene 2 (BCL2) and hypoxia-inducible factor 1 -α (HIF1-α) proteins (Biosource International Inc., California, USA) were investigated in accordance with the manufacturer's instructions.

2.7. Histopathological examination

In parallel, animal colons was collected immediately from all mice after euthanasia using light ether. After being cleaned with a saline solution, colon was first fixed in 10% then, 5 µm thickness paraffin sections were prepared, stained with hematoxylin and eosin (H & E). For the microscopic examination, 5 µm thick paraffin slices were prepared, stained with H & E.

2.8. Serum metabolomics analysis using GC-MS analysis

The serum metabolites of the studied groups were profiled using a gas chromatography instrument (Thermo Scientific Corp., USA), coupled with a thermo mass spectrometer detector. Metabolites separation was achieved under conditions previously described in our previous work [9, 10]. The gas chromatography - mass spectrometry (GC-MS) data were cleaned, deconvoluted and aligned using the MS-DIAL interface. QC pooled samples were used to reduce the systematic error caused by instrumental fluctuations through removing features with high relative standard deviation (RSD). Normalization,

Log transformation and Pareto scaling were applied to the filtered features prior to chemometric analysis. Principal component analysis (PCA), projection of latent structure discriminate analysis (PLS-DA) in addition to pairwise comparison by orthogonal projection to latent structure discriminate analysis (OPLS-DA) were applied using "ropls" package 27 under R 3.3.2 environment for better understanding of the subtle common point and discrepancies of such complicated data. Univariate analysis was also implemented by analyzing the differences between the normalized quantities of metabolites using t-test adjusted by calculating the false discovery rate (q values) and metabolites fold changes. The selected potential metabolites were searched and identified by comparing their retention indices (RI) and fragmentation patterns by those available in Golm, Fiehn BinBase and RIKEN databases [11].

2.9. Statistical analysis

The mean ± standard deviation was used to express the findings from the biochemical study. One-way analysis of variance (ANOVA) and the Tukey comparison test were used to analyse the data using the GraphPad Prism program (version 8.00), at $p < 0.05$ to indicate significant difference.

3. Results

3.1. Quantitative Estimation for Polyphenolic Contents:

Quantitative assessment of the total phenolic and flavonoid amounts by spectrophotometric methods of PPE demonstrated the richness of the extract of such polyhydroxylated compounds, where it recorded 174.0±0.65 mg GAE/g and 23.2 ±0.3 mg CE/g for phenolics and flavonoids, respectively.

3.2. Estimating the Antioxidant Capacities:

Measuring the antioxidant capacity of PPE as free radical scavenger showed potent actions as the results were 357.6 ± 0.5 and 408.4 ± 0.55 mg TE/g for DPPH and ABTS assays, respectively. The high antioxidant capacity may be attributed to the excessive amount of polyphenolic contents, represented by flavonoids and phenolic acids.

3.3. Metabolomics examination by LC-ESI-MS/MS:

It has previously been reported that P. granatum leaves, fruits, and peels are abundant sources of phenolic compounds, specifically the hydrolysable tannins; gallotannins, ellagitannins as well as gallic acid derivatives, besides flavonols and their glycosides. For that great diversity in chemical content, LC-ESI-MS/MS analysis has been used to investigate the structural identification of some phytochemicals in PPE. Probable identification of compounds was carried out after visualizing the chromatogram and processing the abundant peaks using PeakView® software 1.2. The possible confirmation of the abundant compounds was based on their molecular ions and comparing their MS-MS fragmentation pattern with those reported in different literature and the respectful databases; PubChem, MoNA database and the global natural product social molecular networking (GNPS) libraries. Tentatively identified compounds, in negative ionization mode, were listed in Table 1.

3.4. Characterization of the tentatively identified compounds:

LC-ESI-MS/MS analysis of PPE had revealed the probable identification of 73 compounds from different chemical classes, phenolic, carboxylic and fatty acids, gallic acid, ellagic acid and their derivatives and their corresponding glycosides, besides widespread number of derivatives, isomers and glycosides of flavonoids. The identification was done among the conjugates showing the same molecular ions [M-H]- based on their characteristic fragmentation peaks Table (1).

A group of pentacyclic triterpenoidal compounds (aglycones) from the lupine, oleanane, and ursane types, were detected here for the first time, as far we know. They were all characterized by giving [M-H] - peak as the main predominant base peak, and being fragmented distinctive losses of water molecule [M-H-18]-, methyl group [M-H-30]- or both, in addition to the loss of COO- at (44 Da) and HCOOH at (46 Da).

Three spectra were showing the same mol. ion at [M-H]- 455.20, two of them displayed similar

fragmentation patterns, at Rt. 20.92 and 21.12 min, with distinct fragments at m/z 407 from [M-H-H2O-CH3]- and were identified as either oleanolic or ursolic acid. As they are 2 isomers with the lone difference is in the position of one methyl group; CH3 is located at C-19, in ursolic instead of at C-20 in oleanolic, it is hard to be differentiated just by mass fragmentation. The third chromatogram showed more detailed fragmentation pattern; at m/z 411 and 409 from the elimination of COO- at (44 Da) and HCOOH at (46 Da), respectively, m/z 425 [M-H-2CH3]-. In addition to the presence of distinct peaks at 255 and 227 which are characteristic to betulinic acid, as was identified by [25], in their HPLC-ESI MS-MS analysis on standard triterpene compounds.

Distinguishing compounds to P. granatum are the isocoumarins brevifolin and methyl brevifolincarboxylate. Compounds (64, 65 and 66), Rt. 3.85, 4.37, 5.63 had parent ions at [M-H]- at 247.28 and fragmentation peaks at m/z 229 [M-H-18]- indicating the loss of H2O molecule, 219.05 [M-H-28 (CO-)]-, 203.04 [M-H- 44 (COO-]-, 191.05 [M-H-CO, -CO (-28-28)]- and 175.04 [M-H- 44 - 28 (COO- - CO)]-. After reviewing the literature, this compound was tentatively identified as brevifolin and its isomers [13] [23]. It was previously isolated from Punica granatum leaves extract and identified by NMR data (Nawwar et al, 1994). Compounds 62 and 63, two identical fragmentation patterns were found at Rt. 3.17, 3.77 min. showing the same molecular ion at [M −H]− at m / z 304.94, and at MS2, peaks at m/z 272.99, for (M-H-OCH3- (-32) and m/z 245, 217.05 (100), 189.1 due to consecutive loss of (CO-) were detected. this was identical to methyl brevifolincarboxylate [13].

3.5. Effects of PPE on BCL2 and HIF1-α in mice sera with colon cancer

Induction of colon cancer by pathogenic bacteria caused significant elevated levels of BCL2 and HIF1-α compared to control group as depicted in Figure 1 (A & B). In contrast, PPE treatment reduced significantly the levels of BCL2 and HIF1-α to nearly normal conditions as illustrated in Figure 1 (A & B).

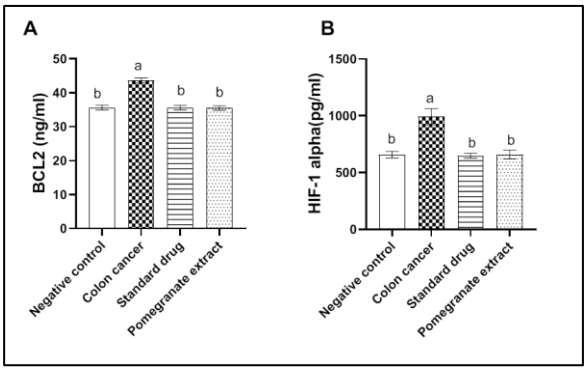

Figure 1: Serum levels of BCL2 (A), and HIF1-α (B) in various studied groups. Data were expressed as mean ± SD (n = 8 for each group). Different letters indicate significant difference at $p< 0.05$.

3.6. Histopathological Examination

Colons from negative control mice were histologically examined, revealing normal tissue features without inflammatory cell infiltration as observed in (Figure 2A). In contrast, colon cancer mice demonstrated dysplasia, anaplasia and hyperchromasia in the epithelial cells lining the glandular structure and inflammatory cell infiltration in colon tissues (Figure 2B). While the colon tissue section of the standard drug group showed some damaged necrotic cells and loss of acinar patterns of the colon glands as shown in (Figure 2C). Finally, the PPE group showed considerable improvement with a reduction of crypt dysplasia and a few inflammatory cell infiltration as illustrated in (Figure 2D).

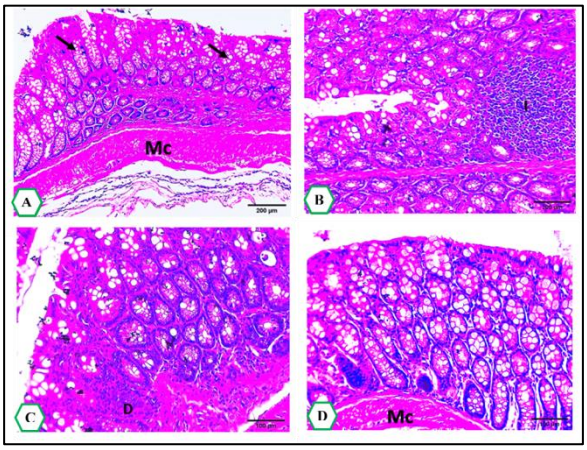

Figure 2: Representative microscopic examination of an H&E-stained mice colon tissue. Histological examination of A: negative control group, B: untreated mice with pathogen-induced colon cancer group, C: mice treated with 5-flurouracil group as a

standard drug, D: mice treated with pomegranate peel extract (PPE) group.

3.7. GC-MS based metabolomics

In the present study, 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 Table 2. The score plot of this model shows complete segregation between untreated colon cancer group and mice treated with PPE as illustrated in (Figure 3).

4. Discussion

Colon cancer is the 3rd most common cancer and the 2nd leading cause of cancer-related deaths globally [27]. Significant advances in the treatment of colon cancer were found either with resectable or metastatic tumors. Nevertheless, these treatments have many serious side effects. Therapeutic modalities using plant sources have been increasingly popular recently as safer options with lesser side effects compared with traditional anticancer drugs [28]. Pomegranate has been used in the treatment of several diseases and ailments. Moreover, pomegranate showed a promising chemopreventive activity against different types of cancer, such as, breast, prostate, and lung cancers in cells, animal models and humans [29].

In the present study, the metabolic profiling of pomegranate peel extract revealed the prescence of 73 phytoconstituents including polyphenolics, such as flavonoids, gallotannin and ellagitannins derivatives (gallic acid, ellagic acid) in addition to other components, such as minerals, glycosides, alkaloids, and fatty acids. The tentative identification of compounds performed by comparison of their low resolution electrospray ionization mass spectroscopy (LRESIMS) pseudomolecular ion peaks, retention times, and ms2 daughter ions. Further confirmation performed via GNPs clustering. Given the fact that cytotoxic potential of flavonoid and polyphenolic compounds mainly ellagitannins reported in several publications which encourage the observed activity of PPE [30].

Due to the scarcity of studies about the anticancer effect of PPE in pathogen induced-colon cancer, in this study, we attempt to evaluate the antiproliferative function of PPE against bacterial pathogen-induced colon cancer. To the best of our knowledge, we are the first group to investigate this issue.

Most chemopreventive agents are antioxidant in nature. Fruits that are high in polyphenols are reported to have antioxidant and chemopreventive. Pomegranate has been shown to exert anticancer and antioxidant activity, which is generally attributed to its high content of polyphenols due to their effects of neutralizing free radicals [31]. In this study, PPE showed high antioxidant capacity may be attributed to the excessive amount of polyphenolic contents, represented by flavonoids and phenolic acids as free radical scavenger. The high antioxidant capacity may be attributed to the excessive amount of polyphenolic contents, represented by flavonoids and phenolic acids.

One of the most distinguishing characteristics of cancer cells is their resistance to apoptosis. Currently, oncogenes and tumor suppressor genes are well-established regulators of apoptosis [32-34]. Regarding oncogenes, they can regulate apoptosis via the production of antiapoptotic proteins conferring cancer cells a survival benefit over normal cells [35, 36]. So, these proteins are overexpressed in cancer cells and with a concurrent lower level in normal cells [37]. 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 [38].

Antiapoptotic protein BCL2 is encoded by *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) [39]. Thus, overexpression of BCL2 protein protects the malignant cells from apoptosis and triggers their propagation. BCL2 overexpression was first noted in B-cell follicular lymphoma [40, 41]. Subsequently, BCL2 overexpression was reported in several cancers such as breast, lung, thyroid, nasopharyngeal, prostrate, liver, ovarian, leukemia, neuroblastoma and colorectal cancers [42-51]. 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 effect of PPE on BCL2 serum levels in bacterial pathogen-induced colon cancer mice group compared to the negative control group, untreated bacterial pathogen-induced colon cancer mice group, bacterial pathogen-induced colon cancer mice group treated with 5-fluorouracil as standard drug group. Interestingly, the BCL2

serum level in PPE treated group reduced significantly compared to the untreated colon cancer group. Moreover, the reduction in BCL2 serum level in PPE treated group was comparable to that of 5-fluorouracil treated and negative control groups. Thus, our results suggested a potential anticancer role of PPE in treatment of bacterial pathogen induced-colon cancer via significant reduction of antiapoptotic BCL2 level.

In line with our findings, Larrosa et al. 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 [52].

Recently and similar to our findings,[53] reported the cytotoxicity activity of PPE on breast cancer cells via increasing Bax/Bcl-2 ratio and the intracellular ROS. Moreover, our results were agreed with that of [54] who demonstrated that 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 which support our study outcomes.

Interestingly, PPE showed an important anticancer activity against prostate cancer cells via reduction of BCL2 expression, inducing apoptosis, and impairs metastasis [55-57]. Also, pomegranate Extract showed antiproliferative effect against oral cancer cells through induction of mitochondrial dysfunction and apoptosis and Bax/BCL2 ratio [58] which agreed with our results.

A hypoxic microenvironment is a trait shared by tumor cells. Most solid tumors, including colon cancer, are permanently or transiently exposed to hypoxia due to a deficient blood supply and aberrant vascularisation. The hypoxia-inducible factors (HIFs) mediate the cellular response to hypoxia, thus encouraging modifications associated with cancer progression and metastasis [59].

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 [60].

Hypoxia controls HIF-1α activation via post-translational modifications. The presence of oxygen leads to the post-translational hydroxylation of HIF-1α and promotes its degradation. 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 [61]. 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 [62-64]. Thus, HIF-1α protein is overexpressed in several human cancers [65].

In the present study, we evaluated the serum level of HIF-1α in bacterial pathogen-induced colon cancer mice treated with PPE group compared with the negative control group, untreated bacterial pathogen-induced colon cancer mice group, and bacterial pathogen-induced colon cancer mice treated with 5-fluorouracil group. Excitingly, a PPE treated group showed a significant decrease in HIF-1α serum level when compared to the untreated colon cancer group. In addition, no statistically significant difference was observed between PPE treated group and either the negative control group or 5-fluorouracil treated group. Therefore, these findings proposed a potential antiproliferative effect of PPE in treatment of bacterial pathogen induced-colon cancer through reduction of the HIF-1α level in blood.

Consistently with our findings, Zakaria et al. suggested that inhibition of HIF-1α and autophagy supress colon cancer growth and proliferation [60]. Similarly, suppression of HIF-1α by L-carnosine dipeptide prevents colon cancer cells resistance to 5-fluorouracil promoting its anticancer activity and stimulates colon cancer cells apoptosis [65].

In line with our findings, Husari et al. reported pomegranate concentrate has chemotherapeutic activity against cigarette smoke induced lung cancer in an animal model via inhibition of HIF-1α expression [66]. 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 [67].

Using GC-MS based metabolomics approach to analyze the relationship between different metabolites and diseases is of great value. Thus, it could support us with valuable knowledge about disease diagnosis, prognosis, and pathogenesis [68]. 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, heptanedioic acid, benzoic acid, alanine, phenylalanine, and glucose.

This study showed a significant reduction of 1H-indole-3-acetic acid and heptanedioic acid in mice treated with PPE group compared with the untreated mice with pathogen-induced colon cancer group. In contrast, benzoic acid, alanine, phenylalanine, and glucose were increased significantly in PPE treated mice group compared with the untreated mice with pathogen-induced colon cancer group.

Indole derivatives are a type of serum metabolite including indole, indole-3-acetic acid, indole-3-

propionic acid, serotonin, and other compounds. All these metabolites resulted from tryptophan metabolism. These are vital in protecting the gastrointestinal tract from stress-induced illnesses such as cancers. These are mostly produced by the intestinal transformation of Escherichia coli [69].

In this study, the high serum level of 1H-indole-3-acetic acid observed in the untreated pathogen-induced colon cancer mice group occurs due to the effect Escherichia coli on tryptophan metabolism producing a high level of 1H-indole-3-acetic acid metabolite. In contrast, the reduced serum level of 1H-indole-3-acetic acid noted in mice treated with PPE group indicates the great effect of PPE on intestinal microbiota influencing the synthesis of indole derivatives. So, PPE regulates gut microbiota-derived tryptophan metabolites which may help its anticancer activity against bacterial pathogen induced colon cancer. Recently and supporting to our results, su et al, reported that gut microbiota-derived tryptophan metabolites such indole-3-acetic acid maintain gut and systemic homeostasis [70].

5. Conclusion

In conclusion, PPE showed a promising anticancer activity against bacterial pathogen-induced colon cancer in BALB/c male mice. This anticancer activity occurs via its high antioxidant activity, reduction of serum levels of important antiapoptotic proteins; BCL2 and HIF1-α, significant histopathological improvement of colon of PPE treated mice and via controlling gut microbiota-derived tryptophan metabolites.

Table 1

The tentatively identified compounds from PPE via HPLC/ESI MS-MS analysis:

The chemical class	**Rt.**	**Mol. Ion $(M-H)^-$ (*m/z*)**	**ESI MS-MS fragments (*m/z*)**	**Tentative Identification**	**Reference**
Organic acids					
1.	1.00	204.97	169.04, 158.91, **143**, 124.99, **110.98 (100)**	Citric acid methyl ester	[12]
2.	1.02	132.99	**115.00 (100), 100.8**	malic acid	[13]
3.	1.15	179.04	**163.95, 151.00, 135.00 (100), 112.95, 111**	caffeic acid	---
4.	1.19	191.2	**173**, 155, **129**, **111** (100)	Citric acid	[12]
5.	1.45	167.9	167.8, 153, 123, 108	Vanillic acid; (4-Hydroxy-3-methoxy benzoic acid)	--
6.	1.57	153.6	153 (100), 135, 117	Protocatechuic acid (PCA) = 3,4-dihydroxybenzoic acid	--
7.	1.87	181.4	163, 131, 113, 101, 89, 71, 59	Dulcitol (D-Galactitol)	MONA
8.	1.92	194.7	195.01, 177.01, 159, 129, 111, 75	Gluconic acid	[14]
9.	2.19	163.5	145.05, 128, 119, 117, 69	Coumaric acid= Hydroxycinnamic acid isomer	[15]

10.	2.40	335.07	317.07, 3.2.98, 290.79, 275.0, 273.11, 190.9, 173.03, **171.13 (100)**, 169, 125.1, 111.03	*O*-caffeoyl shikimic acid	[16]
11.	2.56	163.5	145, 135, 119, 117, 79	Coumaric acid isomer	[15]
12.	2.63	261.05	**125.05 (100)**, 217.1, 199, **187.12**, 127.14	9-(2,3-dihydroxypropoxy) -9-oxononanoic acid	GNPS library, MONA
13.	7.93	329.15	329.21, 311.23, 293.15, 283.25, 246.96, 229.12, 211.15 (100), 193.2, 183.21, 171.18, 139.12	Trihydroxy-octadecenoic acid isomer	[12]
14.	8.50	329.14	329.22, 312.95, 311.24, 298.87, 283.23, 270.96, 269.93, 258.1, 243, 229.09, 211.18, 201.19, 171.15 (100), 155.19, 153.11, 139.13	Trihydroxy-octadecenoic acid isomer	[12]
15.	9.06	311.13	311.17, 293.16, 281, 265.06, 253.10, 249.25 (100), 225.15, 185.24, 171.25	dihydroxy-octadecadienoic acid isomer	[12]
16.	9.24	293.09	**236.17**, **231.27**, 221.16 (100), 220.2, 205.22, 192.99, 177.17, 148.14	Hydroxy-octadecatrienoic acid isomer	[12]
17.	9.6	209.06	209.15, 191.05, 173, 155.01, 137.07, 118.98 179.07, 175.07, 165.04, 163.04,153.02, 137.07, 121, 111.18	Glucaric acid (saccharic acid)	--
18.	10.83	313.15	313.19, 295.13, 298.02, 284.97, 277.13, 267.1, 255.03, 253.1, 237.15, 223.12, 201.13 (100), 171.14, 165.12, 153.1, 127.1, 125.12	dihydroxy octadecenoic acid	[12]
19.	12.69	295.15	295.14, 280.07, 277.2, 267.18, 233.23, 183.12 (100),171.09, 155.18, 139.18	Hydroxy-octadecadienoic acid	[12]

20.	13.13	293.13	**113.07 (100)**, 291.00, 275.17, 249.22, 247.17, 221.20, 217.02, 195.2, 175.09, 179.15, 177.14, 165.21, 149.15, 147.15, 141.12, 139.12	Hydroxy-octadecatrienoic acid isomer	[12]
21.	14.43	295.14	295.19 (100), 277.21, 251.29, 183.22, 181.2, 155.18, 139.18, 137.19	Hydroxy-octadecadienoic acid isomer	
22.	14.53	271.15	**271.19, 253.18, 225.24 (100), 197.33, 227.64, 223.32, 209.42, 183.19**	hydroxy palmitic acid (hydroxy-hexadecanoic acid)	--
Hydrolysable tannins					
23.	1.34	480.89	481, **301**. (100), 462.91, 436.86, 420.97, 275.04	hexahydroxy diphenyl-glucose (HHDP-glucose)	[13]
24.	1.40	462.91	462.87, 300.99, 274.92, 271.97, 271.08, **257.04**, 191, **185.04**, 173, 169.1, **111**	Ellagic acid-*O*-hexoside	[13]
25.	1.46	446.9	446.93, 300 (100), 301, **283.92**, **257.13**, **229.05**, 185,173.22, 156.1	Ellagic acid-*O*-rhamnoside	[17]
26.	1.49	432.90	432.89, 300.03 (100), 300.97, **272.98**, **225.14**, 125.01, 111.08	Ellagic acid-*O*-pentoside	[17]
27.	2.25	169.6	151, 135 (100), 79.0, 69.9	Gallic acid isomer (trihydroxy benzoic acid)	--
28.	3.03	169.5	**125.00 (100)**, **151.91**, **134.98**, 97.2, 79.9, 69.02	Gallic acid	--
29.	3.16	632.85	633 (100) 589.06, 464, 463.01, 301.12, 275.03, 244.94, 169.13, 125.28, 110.96	*O*-Galloyl-hexahydroxy diphenol-Glucopyranose (Galloyl-HDDP-glucose) [Strictinin or Corilagin]	[13]

30.	4.82	490.90	490.91, 475.93, 460.83, 328.02 (100), 362.88, 313.06, 297.86, 268.18, 254.95	Di-O-methyl ellagic acid-O-hexoside	[18]
31.	5.02	328.94	**329.02, 313.99, 298.91 (100), 283.2, 285.95, 270.98, 227.13, 215.07**	Di-O-methyl ellagic acid	[18]
32.	5.07	490.90	490.94, 475.95, 458.82, 328.09 (100), 329, 313.17, 298.07, 4285, 0, 270.09, 239.01	Di-O-Methyl ellagic Acid-O-hexoside	[18]
33.	5.27	328.94	**314.03, 298.89 (100), 283.33, 271.06, 243,12**	Di-O-methyl ellagic acid	[18]
34.	7.58	328.93	329.22, 314.01, 313.06, 299 (100), 283.27, 271.06, 229.08, 179.02	Di-O-methyl ellagic acid	[18]
35.	7.66	608.97	608.99, 354.96, 343.12, 325.1, **301.12** (100), 286.1, **283.12**, **257.12**, 125.11	ellagic acid-*O*-rhamno-hexoside	---
36.	7.68	301.01	300.9, **283.96, 257.03**, 245.04, 229.02, 201.04, **185.13**,	Ellagic acid	[19]
37.	7.75	644.97	645, 609.01, 301.09 (100), **283.14**, 258.12, **257.18**, 158, 151.01, 125.06	ellagic acid-*O*-rhamno-hexoside derivative	
38.	7.88	331.17	331.23 (100), 313.28, 169.08, 125.12, 211.18	Galloyl-O-hexoside (Glucogallin)	---
39.	7.97	608.98	609.02 (100), **447**, 301.11 (80), 283, 257.1, 227.1, 124.94	ellagic acid-*O*-rhamno-hexoside	--
40.	8.19	300.93	301.02 (100), 284.03, **257.08**, **185**, 173	Ellagic acid isomer	---
Flavonoids and their glycosides					
41.	2.00	448.93	449.01, 269.03, **287.03**, 283.02, 311.01, **259.05** 151.11, **125.06**, **153.07** **177.14, 179.14** **character. To the aglycone**	Dihydrokaempferol O-hexoside	[20]

42.	3.27	464.93	464.95, 446.87, 436.97, 303.04, 284.99, 274.98, 259.06, 217.07, 169.02, 151, 125 (100)	Dihydroquercetin-O-hexoside (Taxifolin-O-glucoside)	--
43.	5.3	608.93	608.96, 463.19, 446.92, **301.11, 285**, **255.08, 207.07, 179.03, 151.06, 121.08**	quercetin-*O*-hexoside-*O*-rhamnoside	[20]
44.	5.41	462.9176 63574219	462.91, **301**, 300.01 (100), **283.17**, **271.04**, **255.14**, 229.1, **179.03**, **151.09**	quercetin-O-hexoside	[20]
45.	5.60	432.95	432.92, 300 (100), 301.01, **270.99**, 271.89, **255.07**, 220.92, 211.06, **179.05**, 165.1, **151.04, 107.03**	Quercetin-O-pentoside	---
46.	6.11	592.95	**285.01, 284.04, 257.09, 255.12, 227.16, , 151.09, 107,**	kaempferol-*O*-rutinoside	--
47.	6.22	446.95	**284.95, 284.03 (100),** 257.05, **255.13**, **227.16**, **179.14** , 151, 107	kaempferol-O-hexoside	[21]
48.	6.49	446.92	**285.01 (100) 271, 254.94, 175.15,**	kaempferol-*O*-hexoside	[21]
49.	7.17	578.96	578.98, **416.97, 271.01 (100)**, 229.2, 193.06, 151.06, 119.11, 107.03	Naringenin-*O*-rhamno-glucoside	---
50.	7.41	578.99	579.02 (100), 543.22, +**417**, **271.01**, 269.06, 235.07, 193.05, **181.07**, **151.01**, 119.02, 107.03	Naringenin-*O*-rhamno-hexoside	---
51.	7.49	446.93	**284.96 (100)**, **257.27**, **241.33**, **175.25, 107.13**	luteolin-*O*-hexoside	[21]
52.	7.52	430.95	431, **268.03** (100), 269.04, **311.15**, 255.22, **240.99**, 187.16, 185.11, 125.06	Apigenin -*O*-hexoside	MONA

53.	8.55	342.94	343.16, 327.98, 313.04 (100), 297.91, 285.01, 270.07, 257, 217.12, 186.23, 177.03	Tri-*O*-methylquercetin	---
54.	9.17	592.99	593.03, 309.07, **285.03** (100), 283.44, 270.12, 269.14, **175.22**, 151, **132.92**	luteolin-*O*-rutinoside	---
55.	9.37	268.98	269.03 (90), 251.08, 241, 227.1, 225.1, **151.05**, 149.03, 136.94, **121.02**, **117.03 (100), 107.01**	Apigenin	
56.	10.39	342.94	343.01, 328, 313 (100), 297.9, **284.97**, **269.97**, 257.04, 217.04, **179.02**	Tri-*O*-methylquercetin	--
57.	11.35	315.17	315.17, **299.92**, 297.13, **286.92**, **269.19 (100)**, 251.23, 253.06, 183.11, 171.16, 155.14	Rhamnetin or isorhamnetin (methyl quercetin isomer)	----
58.	11.38	446.95	417.16, 347.33, 331.1, **301.09**, 300.1, **285.05**, 269.09, **179.08**, 159, 153	quercetin-O-rhamnoside	[17]
59.	11.77	315.16	**315.26 (100), 313.32, 297.21, 285.09,** 279.3, **269.35, 225.24, 171.25, 155.23**	methyl quercetin isomer	----
60.	18.69	301.1	**285**.09, **255.21**, **217.12** (100), 203.05, 187.11, 135.05, 119.05	Quercetin	--
61.	19.92	301.12	301.07 (100), 284.04, 285.16, 255.31, 217.23, 185.16	Quercetin isomer	--
Coumarins					
62.	3.17	304.94	272.96; 245.03; **217.04** (100); 201.13; 189.1; 173.11; 161.1; 145.09; 133.11	Methyl brevifolincarboxylate isomer	[22]
63.	3.77	304.95	272.96, 245.02; 217.0084; 173.0185; 161.0211; 145.0259;	Methyl brevifolincarboxylate isomer	[22]

			133.0261; 117.0297; 105.0318		
64.	3.85	247.01	**247.03** (100), 229.02, 219.08, 229.02, 203.06, **191.06**, 173.12, 145.09, 119.14	Brevifolin isomer	[23]
65.	4.37	246.96	**247.03** (100), 219.08, 229.06, 203.06, **191.06**, 173.12, 145.09, 119.14	Brevifolin isomer (Geranium)	[22]
66.	5.63	246.96	**247.03** (100), 219.08, 229.06, 203.06, **191.06**, 173.12, 145.09, 119.14	Brevifolin isomer	[23]
Pentacyclic triterpenoid					
67.	9.89	499.14	499.14 (100), 481.08, 469.19, 451.18, 453.05, 433, 407.26, 393.29, 353.31, 153.1, 111.05	Serjanic acid oleanane type	GNPS library
68.	11.40	487.20	487.2 (100), 409.28, 411.39, 423, 391.26, 393, 379, 375	asciatic acid ursane type	[24], GNPS library
69.	13.23	469.17	469.2 (100), 451.17 433.01, 425.18, 423.26 , 407.13, 355.13	Glycyrrhetic acid (Enoxolone) Glycyrrhetinic acid Uralenic acid	*(HMDB) & GNPS library
70.	19.45	455.11	455.18 (100), 437.4, 425.19, 411.18, 409.24, 407.16, 391.12, 363.21, 365.49, 353.28, 255.23, 153.09	betulinic acid	[25]
71.	20.92	455.20	**455.18 (100), 407.35, 377**	oleanolic acid / ursolic acid	[26]
72.	21.12	455.19	455.16 (100), 457.04, 456.38, - 18-30= 407.46	oleanolic acid / ursolic acid	[26]
Other compounds					
73.	1.74	121.0	121.05 (100), 108.	Hydroxybenzaldehyde	--

The bold numbers refer to the characteristic fragmentation peaks. (100) denotes for the most abundant peak (Base Peak B.P.)

Table 2

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 significant biomarkers are ordered according to their VIP values.

Name	VIP	Metabolites fold change (G2/G4)	P value	Q value
Glucose	2.748077	0.134456576	0.002633	0.026675
Alanine	1.3524	0.638105976	0.001057	0.01489
Phenyl alanine	1.673982	0.472160575	0.004085	0.034948
Benzoic acid	1.18031	0.701922089	0.004324	0.036073
Heptanedioic acid	1.846901	2.939275472	0.007937	0.049125
1H-Indole-3-acetic acid	1.820392	2.222838335	0.000212	0.006566

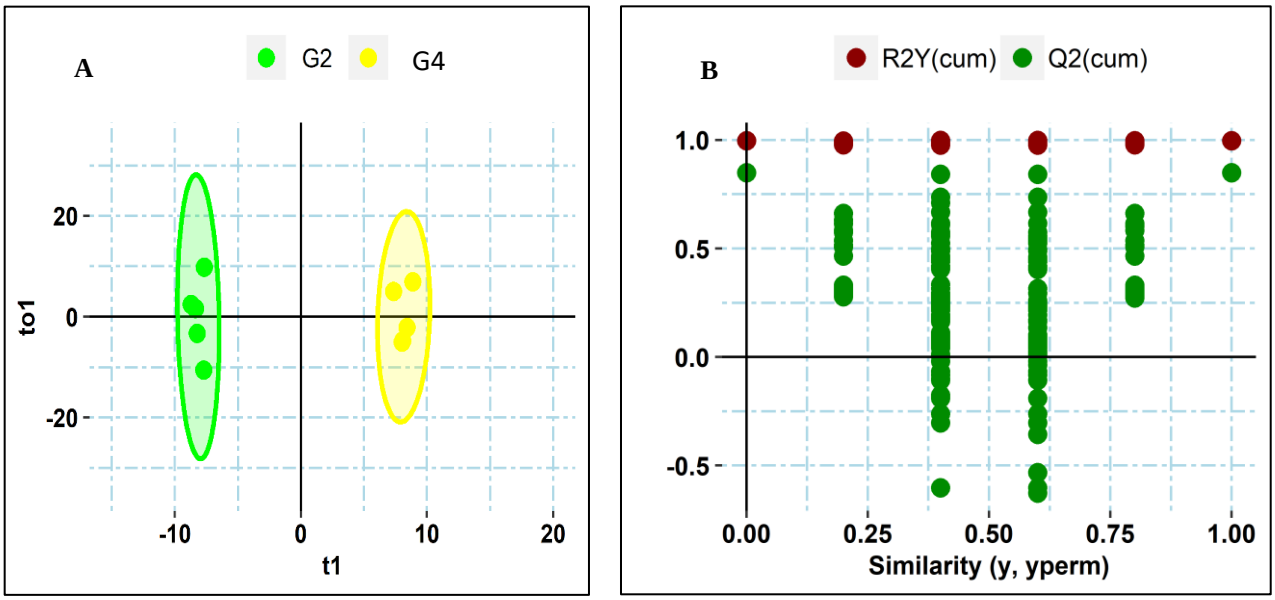

Figure 3: Multivariate modeling of the GC-MS metabolomics dataset. (A) OPLS-DA score plot shows complete separation along the predictive component (t1). (B) PLS-DA permutation plot shows a significant model with pR2Y and pQ2 of (0.01) (permutation number: 1000). G2, untreated mice with pathogen-induced colon cancer group & G4, mice treated with PPE group.

6. Conflicts of interest

There are no conflicts to declare.

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