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Research article

Preventive effect of pomegranate juice against chemically induced bladder cancer: An experimental study

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ABSTRACT

Objectives: Pomegranate juice (PJ) is rich in important compounds with anti-cancer activities. This study aims to investigate the preventive effect of pomegranate juice (PJ) against bladder cancer (BC).

Methods: Eighty male Sprague Dawley rats were randomly classified into 4 equal groups: (1) Normal controls; (2) PJ group: supplied by PJ for 12 weeks; (3) Cancer-induced group: intake 0.05% v/v *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN) for 8 weeks; (4) Cancer-prevented group: BBN + PJ. After 12 weeks, all rats were sacrificed and their urinary bladder tissues were subjected to histopathological and immunohistochemical (p53) examinations, expression of interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), hypoxia-inducible factor 1 (HIF-1) and the tumor protein p53 (TP53) and analysis of oxidative stress markers.

Results: The development of BC was: 0/20 (0%) in normal, PJ and cancer-prevented groups and 20/20 (100%) in cancer-induced group. Significant neoplastic lesions were observed in cancer-induced group. Mild preneoplastic alterations were noticed in 25% (5/20) of cancer-prevented group. p53 immunostaining were significantly elevated in the cancer-induced group, which was decreased in the cancer-prevented group. The relative expressions of IL-6, TNF-α, HIF-1 and TP53 were significantly lower in the cancer-prevented group compared to the cancer-treated group. Correction in the oxidative stress markers were also observed in the cancer-prevented group.

Conclusion: PJ possesses a promising inhibitory effect on BC development, probably due to its anti-oxidant and anti-inflammatory properties.

1. Introduction

Bladder cancer (BC) is a worldwide problem that represents the fourth most common type of cancer in males and the eighth in females (Grasso, 2008). It is characterized by high mortality rates, especially, when becomes invasive or untreated (Malkowicz et al., 2007). Cigarettes smoking (Zeegers et al., 2000) and occupational exposure to certain aromatic compounds (Kogevinas et al., 2003) beside genetic factors (Wolff, 2007) represent the most important risk factors of BC. About 25% of patients with BC present with muscle invasive (Greene et al., 2002). The standard treatment includes chemotherapy, radiotherapy and surgery with an elevated rate of progression and recurrence (Dalbagni, 2010). Preventive strategies have become a concern to reduce the incidence and development of the disease, which has a positive impact on the economics of countries (Al-Zalabani et al., 2016; Kwan et al., 2018).

On the past few decades, efforts have been made to investigate the effects of natural products for prevention of many types of cancers with satisfying results (Chen and Chen, 2013; Wang et al., 2012). Cranberry juice concentrate (Prasain et al., 2008), Curcumin (Tian et al., 2008), broccoli sprout (Munday et al., 2008) and omega-3 (Parada et al., 2013) inhibit bladder cancer development in rat models. In a meta-analysis study, a reduction of BC risk was correlated with consumption of citrus fruits and cruciferous vegetables (Yao et al., 2014). In a prospective study, inverse relation was observed between risk of BC and intakes of

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lignan and flavonoids (Zamora-Ros et al., 2014). Green leafy vegetables and milk intake also reduces the incidence of BC (Di Maso et al., 2019).

Pomegranate (*Punica granatum L.*) is a sweet-tasting fruit cultivated by humans since ancient times. The edible part is rich in bioactive compounds such as trace elements, organic acids, polyphenols, flavonoids, alkaloids and sterols (Lansky and Newman, 2007). Most of these ingredients, particularly polyphenols and flavonoids, possess anti-cancer properties by cell cycle arrest, inhibition of cellular proliferation and activation of apoptosis (Hazafa et al., 2019). The chemical composition and the anti-oxidant properties vary according to part of the fruit and the cultivar (Orak et al., 2012). Generally, pomegranate has stronger anti-oxidant activity compared to β-carotene, vitamin E and ascorbic acid and green tea owing to its higher contents of polyphenols(Sharma et al., 2017). Pomegranate can affect pathways involved in cancer development such as cellular transformation and angiogenesis (Khan et al., 2008). It can modulate pro-apoptotic proteins, pro-inflammatory molecules and growth factors in different types of cancers (Sharma et al., 2017). Pomegranate juice (PJ) has displayed promise effect against prostate cancer in human clinical trials (Paller et al., 2013).

Few studies have been done regarding the protective effect of pomegranate against BC. All of them were *In vitro* studies (Chang et al., 2018; Lee et al., 2013; Zhou et al., 2015). Therefore, we decided in the present study to investigate the effectiveness of PJ in inhibiting BC induced by *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN) in rats. Histopathological, gene expression and biochemical studies were performed to understand the possible mechanism of BC inhibition by PJ.

2. Materials and methods

The present study was approved by the local Institutional Research Board under approval number: "RP.19.07.34" on 6 JUN 2019.

2.1. Chemicals

Unless otherwise mentioned, all the chemicals used in this study were of analytical grade obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA) or Merck (Darmstadt, Germany). Ultrapure deionized water was prepared using Milli-Q water purification system (Millipore, Billerica, MA, USA).

2.2. Instrumentation

Spectrophotometric measurements were recorded using Genway UV-VIS spectrophotometer equipped with Xe lamp (Model 7300, Cole-Parmer Ltd., Staffordshire, UK). StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA) was applied for quantitative real time polymerase chain reaction (qRT-PCR).

2.3. Preparation of pomegranate juice

Egyptian cultivar of pomegranate, namely wonderful100-1, was collected from local markets of Mansoura city, Egypt. The fruits were washed, dried and peeled. The edible part (seeds) of the fruit was ground in a blender and the obtained juice was separated by filtration. After centrifugation, the supernatant (PJ) was stored in dark bottles at -20 °C no longer than 3 days.

2.4. Determination of total phenolic and total flavonoid compounds in pomegranate juice

Total phenolic compounds were determined by a modified Folin–Ciocalteu method (Attard, 2013) and the results were expressed as mg gallic acid equivalent per litter (mg GAE L^{-1}). Total flavonoids were estimated by aluminium chloride method (Pękal and Pyrzynska, 2014) and were expressed as mg rutin equivalent/L (mg RE L^{-1}). Both phenolic and flavonoid compounds were determined spectrophotometrically at 420 and 630 nm, respectively.

2.5. Animals and experimental protocol

Eighty male Sprague Dawley rats (body weight 220–250 g, 4–5 months old) that bred in the animal research unit of our center were included in the study. They were housed in stainless steel cages (5 rats/cage) under standard environmental conditions (25 ± 1 °C, 45–75% relative humidity, 12 h dark/light cycle) and were provided by standard diet and drinking water. Experiments were performed as stated by the guide for the care and use of laboratory animals (Clark et al., 1997). The animals were divided in a random way into 4 equal groups (20 rats per group, Figure 1): (1) Control group, was given water without any treatment; (2) PJ group, each rat was supplied by 2.5 mL of PJ daily by oral gavage to provide a dose of about 20 mg gallic acid/kg body mass/day (Owumi et al., 2020); (3) Cancer-induced group, was treated with 0.05% BBN (v/v) in drinking water for 8 weeks (Lokeshwar and Soloway, 2001); (4) Cancer-prevented group, was received 0.05% BBN (for 8 weeks) and PJ. Every morning, the rats were followed up and their food and drinking water were replaced. The weight of each rat was recorded weekly during the period of the study. After 12 weeks, all rats were sacrificed and their urinary bladders were removed. The bladder of each rat was divided into three parts. One part was fixed in 10% formalin for pathological and immunohistochemical investigation. The second part was stored in RNA latter at -80 °C until gene expression analysis. The remaining part was rinsed with phosphate buffered saline, pH 7.4, to eliminate any blood residues and then accurately weighed and homogenized with ice-cold phosphate buffer (pH 7.4). The mixture was centrifuged for 15 min at 1500 rpm at 4 °C and the supernatant was collected in Eppendorf tube and was stored at -80 °C for analysis of Malondialdhyde (MDA), catalase (CAT), glutathione (GSH) and superoxide dismutase (SOD). The storage period didn't exceed 30 days.

2.6. Pathology and immunohistochemistry

Following automated dehydration through graded-alcohol series, cross tissue sections (4 μm) were embedded in paraffin, and stained with hematoxylin and eosin (H&E) for histopathological examination.

For immunohestochemical investigation, deparaffinized sections were incubated for 30 min with 0.3% methanolic solution of H_2O_2 and were heated in citrate buffer (pH 6.0) for 20 min in a microwave oven. Subsequently, an indirect immunoperoxidase technique was applied, using monoclonal antibodies p53 (monoclonal rabbit anti-human antibody, Abbexa Ltd., Cambride, UK, Cat. No #abx008610, dilution 1:100). Immunostaining was performed using Immuno-Pure Ultra-Sensitive ABC Peroxidase (Thermo Scientific Cat. No #32052) with (DAB) as chromogen. Breast carcinoma was used as positive control while stained sections without addition of a primary antibody were used as a negative control. The intensity of nuclear staining was graded on a semi-quantitative scale (none = 0, weak = 1, moderate = 2 and strong = 3), rating intensity in the dominant pattern within the tumor. In addition, the percentage of positively staining tumor cells was scored (negative, 1–9%, 10–49%, or >50%). The histopathology and immunohistochemistry investigations were performed in a blind manner.

2.7. Quantitative reverse transcription PCR reaction analysis of IL-6, TNF-α, HIF-1 and TP53

Total RNA was isolated and purified from urinary bladder tissues using Trizol extraction kits (Invitrogen Corporation, Grand Island, NY, USA) and its integrity was detected by agarose gel electrophoresis and ethidium bromide staining. Samples that exhibited two clear bands (18 S and 28 S) under ultraviolet light were utilized for real-time PCR. One μg of RNA was reverse transcribed using High-Capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA).

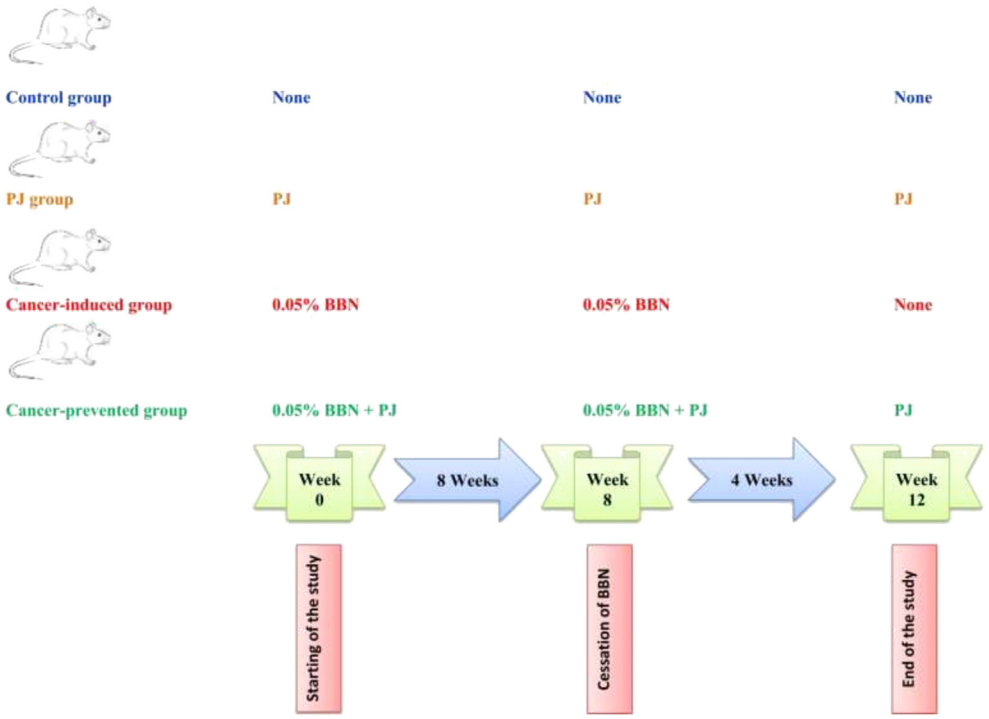

Figure 1. Scheme of the animal study design.

Quantitative RT-PCr was performed with SYPER Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) for IL-6, TNF-α, HIF-1 and TP53 as well as GAPDH as a housekeeping gene. The primer sequences for tested genes were: IL-6, forward: 5′-GAGACTTCCAGCCAGTTGCC-3′, reverse: 5′-TGAAGTCTCCTCTCCGGACTT-3'; TNF-α, forward: 5′-TACTGAACTTCGGGGTGATTGGTCC-3′, reverse: 5′-CAGCCTTGTCCCTTGAAGAGAACC-3'; HIF-1, forward: 5′-TGCTTGGTGCTGATTTGTGA-3′, reverse: 5′-GGTCAGATGATCAGAGTCCA-3'; TP53, forward: 5′-CCCCTGAAGACTGGATAACTGT-3′, reverse: 5′-TCTCCTGACTCAGAGGGAGC-3'; GAPDH (house-keeping gene), forward: 5′-TATCGGACGCCTGGTTAC-3′, reverse: 5′-CTGTGCCGTTGAACTTGC-3'. The cycling parameters of PCR analysis were adjusted based on the following program: 95 °C for 10 min (pre-denaturation), 40 cycles at 95 °C for 15 s (denaturation), 60 °C for 1 min (annealing) and 72 °C for 1 min (extension). Data analysis was carried out using ABI prism 7000 by equation $2^{-\Delta\Delta}$ct (Livak and Schmittgen, 2001).

2.8. Markers of oxidative stress

Oxidative parameters (MDA, CAT, GSH and SOD) in urinary bladder tissues were determined by spectrophotometric kits (Bio-diagnostic, Giza, Egypt) according to the manufacturer instructions. The concentrations were expressed per mass unit of the tissues.

2.9. Statistical analysis

All statistical calculations were carried out using SPSS-PC software version 25 (MAS Medical & Scientific Eq. Co, IL, USA). The continuous data were represented as mean ± standard deviation (SD) and were compared using paired sample ANOVA, Tukey's post hoc or Student's t-tests as relevant. On the other side, categorical data were expressed as frequency and percentage and compared by Chi-square. $p \leq 0.05$ is considered significant.

3. Results

3.1. Total phenolic, total flavonoid contents and anti-oxidant activities

The total phenolic and total flavonoid contents of the PJ were 1872.2 ± 70.7 mg GAE L^{-1} and 278.2 ± 8.7 mg RE L^{-1}, respectively.

3.2. Effect of pomegranate juice on the development of bladder cancer in rat models

3.2.1. General observation and macroscopic examination

All rats survived during duration of the experiment. No significant changes were found between the study groups concerning drinking, food consumption and behaviors (data not shown). The rats of control and PJ groups displayed a slightly body weight gain (38.6% and 40.0%, respectively) when compared with cancer-induced and cancer-prevented groups (30.8% and 36.6%, respectively), however, this difference was not statistically significant (Table 1). There was a significant increase in bladder/body weight ratio in the cancer-induced group and the cancer-prevented group at the end of the study ($p<0.001$) when compared to the normal controls. No abnormalities in the other organs (stomach, kidneys, liver, lungs and intestine) were observed after scarification. Urinary bladders of all members of the control and PJ groups had translucent appearance with no abnormal mass or any vascularization. All rats in cancer-induced group developed single (n = 13, 65%) or multiple (n = 7, 35%) bladder lesions. No lesions were observed in the cancer-prevented group, but 5 rats (25%) showed mild swollen bladder with appearance of congested blood vessels.

3.2.2. Histopathological investigations and p53 immunohistochemistry

All bladders from control and PJ groups had normal architecture without any signs of preneoplastic or neoplastic lesions (Table 2). On the other hand, all rats of cancer-induced group showed mucosal lesions

Table 1. Body weight and bladder/body weight ratio among the study groups.

	Normal group	PJ group	cancer-induced group	cancer-prevented group	*p1*	*p2*	*p3*	*p4*	*p5*
Initial body weight (g)	227.3 ± 3.6	229.5 ± 3.4	226.8 ± 4.0	228.0 ± 2.3	0.48	0.66	0.57	0.61	0.70
Final body weight (g)	315.1 ± 5.9	321.4 ± 6.4	296.6 ± 7.9	311.5 ± 8.4	0.37	0.29	0.40	0.66	0.42
Bladder/body weight ratio (%)	0.03 ± 0.01	0.03 ± 0.01	0.18 ± 0.06	0.06 ± 0.02	<0.001	0.80	<0.001	<0.01	<0.001

p1: Normal vs PJ vs cancer-induced vs cancer-prevented groups.
p2: Normal vs PJ.
p3: Normal vs cancer-induced.
p4: Normal vs cancer-prevented.
p5: cancer-induced vs cancer-prevented.

Table 2. Results of histopathological and immunohistochemistry examinations.

Variable	Normal group	PJ group	cancer-induced group	cancer-prevented group	*p1*	*p2*	*p3*	*p4*
	n (%)							
Neoplastic lesions								
Single	0 (0)	0 (0)	13 (65)	0 (0)	<0.001	<0.001	-	<0.001
Multiple	0 (0)	0 (0)	7 (35)	0 (0)	<0.001	<0.001	-	<0.001
Mucosal changes								
Degeneration of umbrella cells	0 (0)	0 (0)	10 (50)	0 (0)	<0.001	<0.001	-	<0.001
Mucosal thickness	0 (0)	0 (0)	20 (100)	5 (25)	<0.001	<0.001	<0.01	<0.001
Dysplasia								
Mild	0 (0)	0 (0)	10 (50)	5 (25)	<0.001	<0.001	<0.01	<0.01
Moderate	0 (0)	0 (0)	10 (50)	0 (0)	<0.001	<0.001	-	<0.001
Inflammation	0 (0)	0 (0)	20 (100)	5 (25)	<0.001	<0.001	<0.01	<0.001
Intramucosal hemorrhage	0 (0)	0 (0)	20 (100)	5 (25)	<0.001	<0.001	<0.01	<0.001
Lamina propria changes								
Tumor invasion	0 (0)	0 (0)	10 (50)	0 (0)	<0.001	<0.001	-	<0.001
Marked eosinophilic infiltrate	0 (0)	0 (0)	20 (100)	5 (25)	<0.001	<0.001	<0.01	<0.001
Congested capillaries	0 (0)	0 (0)	20 (100)	5 (25)	<0.001	<0.001	<0.01	<0.001
p53 immunostaining								
Negative (grade 0 intensity)	20 (100)	20 (100)	0 (0)	10 (50)	<0.001	<0.001	<0.001	<0.001
Positive								
Grade 1 intensity	0 (0)	0 (0)	0 (0)	10 (50)	<0.001	-	<0.001	<0.001
Grade 2 intensity	0 (0)	0 (0)	10 (50)	0 (0)	<0.001	<0.001	-	<0.001
Grade 3 intensity	0 (0)	0 (0)	10 (50)	0 (0)	<0.001	<0.001	-	<0.001

p1: Normal vs PJ vs cancer-induced vs cancer-prevented groups.
p2: Normal vs cancer-induced.
p3: Normal vs cancer-prevented.
p4: cancer-induced vs cancer-prevented.

including degeneration of umbrella cells (n = 10, 50%), mucosal thickness (n = 20, 100%), mild to moderate dysplasia (n = 20, 100%), inflammation and intramucosal hemorrhage (n = 20, 100%). Tumor invasion to lamina proporia with marked eosinophilic infiltrate (n = 20, 100%) was also noticed in the cancer-induced group. The histological examination of rats of the cancer-prevented group didn't exhibit neoplastic bladder lesions. Mild preneoplastic alterations were observed in 5 cases of this group (25%) including mucosal thickness and dysplasia, inflammation and capillary congestion (Table 2 & Figure 2). The bladders of control and PJ groups exhibited negative p53 immunostaining (grade 0 intensity). In cancer-induced group, tumor lesions presented high expression of p53 (grade 3 intensity). Mild expression (grade 1 intensity) of p53 was noticed in 50% (n = 10) of cancer-prevented group (Table 2 & Figure 3).

3.2.3. Real time PCR for inflammatory cytokines (IL-6 and TNF-α), hypoxia inducible factor (HIF-1) and antiapoptotic (TP53) genes

The relative quantifications (RQ) of IL-6, TNF-α, HIF and TP53 genes in urinary bladder tissues were presented in Table 3. As clearly shown, the expressions of IL-6, TNF-α, HIF-1 and TP53 genes in PJ group were statistically comparable to those of the controls ($p>0.05$). In cancer-induced group, the RQ values of IL-6, TNF-α, HIF and TP53 genes were statistically elevated compared to those in the normal group ($p<0.001$). Comparing with the controls, the expression of IL-6, TNF-α, HIF-1 and TP53 genes in the cancer-prevented group were also elevated and statistically significant. The genes were less expressed in the cancer-prevented group when compared with the cancer-induced group (almost half the values, $p<0.001$).

3.2.4. Markers of oxidative stress

As displayed in Table 4, there was a significant increase in level of MDA in bladder tissues of cancer-induced group when compared with that of the normal controls ($p<0.001$). Otherwise, significantly diminished levels of CAT, GSH and SOD were observed in cancer-induced group when compared with the normal group ($p<0.001$). No significant differences were noticed in tissue levels of MDA, CAT, GSH and SOD in PJ and the cancer-prevented groups when compared to those of the controls ($p>0.05$).

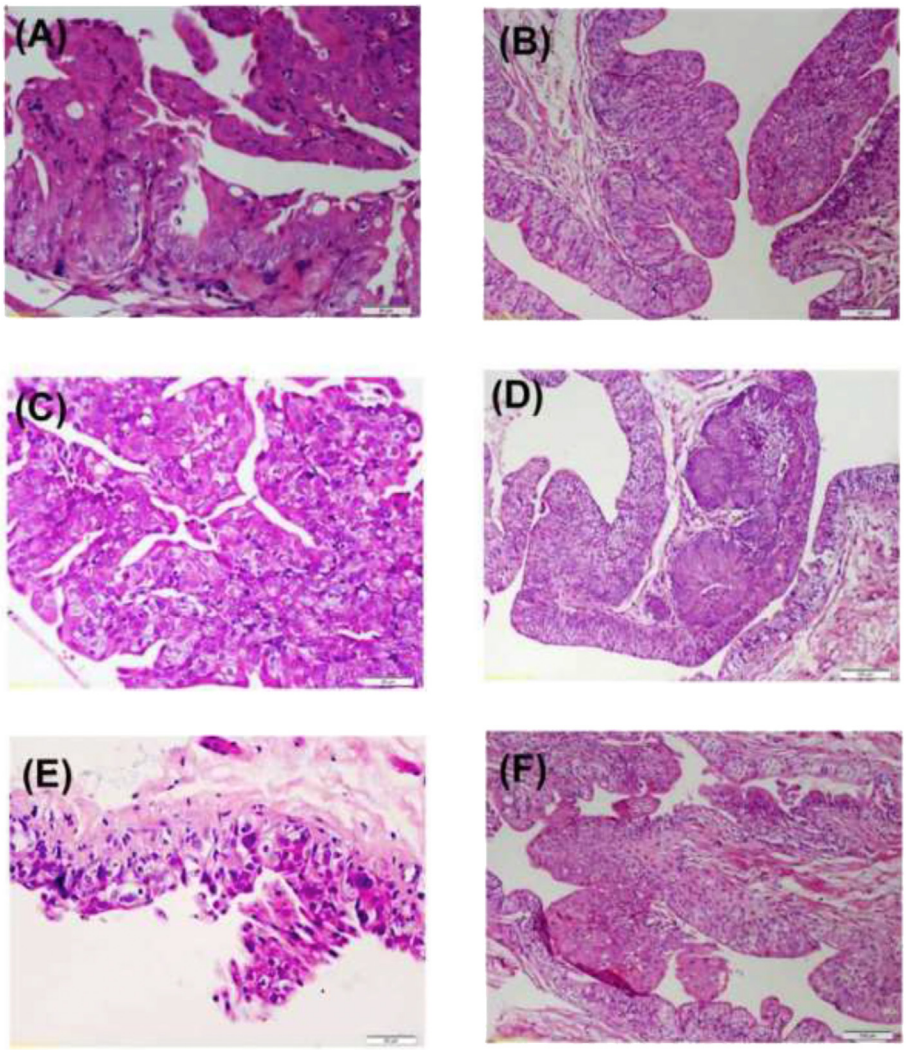

Figure 2. Microscopic examination using H & E. A: Papillary hyperplasia with mild dysplasia in the cancer-induced group. B: Papillary hyperplasia without any dysplasia in the cancer-prevented group. C: GI papillary transition cell carcinoma infiltrating lamina propria in cancer-induced group. D: Proliferative lesion formation (Brunn's nests) in the cancer-prevented group. E: Marked mucosal dysplasia along whole mucosal thickness and mild edema in lamina propria of cancer-induced group. F: Increased mucosal thickness with small focus of squamous metaplasia (intercellular desmosomes) in the cancer-prevented group.

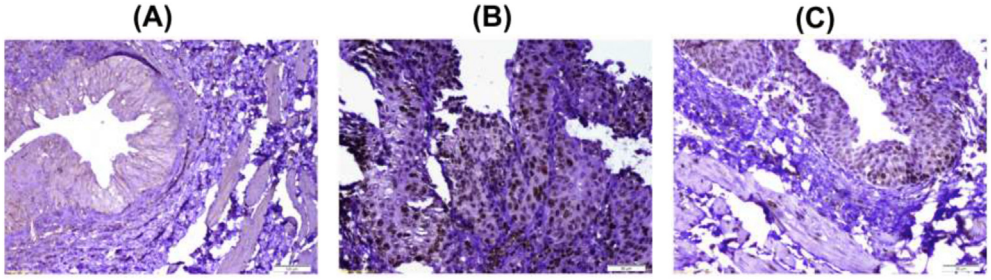

Figure 3. p53 immunostaining of urinary bladder samples. A: negative immunostaining in the normal group. B: Moderate to marked immunostaining intensity in >50% of cells of cancer-induced group. C: Mild immunostaining intensity in <9% of cells of cancer-prevented group

4. Discussion

Bladder cancer remains one of the most common malignancies threatening human health worldwide. More preventive strategies are then required to reduce morbidity especially in high risk people. Various phytochemicals have been investigated during the recent years as potential chemopreventive agents for BC (Koh et al., 2019). To the best of our knowledge, this is the first study exploring the effect of pomegranate or its derivative on preventing BC in experimental rat model. The major findings of the present study indicated that PJ prevents the induction of BC in rats exposed to BBN.

Pomegranate contains considerable amounts of important compounds including flavonoids and phenolic compounds which have antioxidant properties (Abid et al., 2017). In our study, the mean phenolic and flavonoid compounds were 1872.2 mg GAE L^{-1} and 278.2 mg RE L^{-1}, respectively.

Many models have been established for induction of BC in experimental animals (John and Said, 2017). We used BBN model in the present study as it resemble the human disease in histological characteristics (Grubbs et al., 2000). BBN can be given orally in drinking water and it is metabolized into an urothelium carcinogenic agent, namely *N*-butyl-*N*-(3-carboxypropyl)-nitrosamine, which has direct carcinogenic

Table 3. Results of gene expression.

Gene	Relative quantification of genes				*p1*	*p2*	*p3*	*p4*
	Normal group	PJ group	Cancer-induced group	Cancer-prevented group				
IL-6	1.26 ± 0.06	1.16 ± 0.12	5.03 ± 0.34	2.41 ± 0.35	<0.001	<0.001	<0.01	<0.001
TNF-α	1.21 ± 0.09	1.01 ± 0.06	8.10 ± 0.38	3.99 ± 0.38	<0.001	<0.001	<0.001	<0.001
HIF	1.02 ± 0.10	0.98 ± 0.07	3.08 ± 0.53	1.42 ± 0.22	<0.001	<0.001	<0.05	<0.001
TP53	1.35 ± 0.10	1.26 ± 0.06	10.35 ± 0.36	4.64 ± 0.34	<0.001	<0.001	<0.001	<0.001

p1: Normal vs PJ vs cancer-induced vs cancer-prevented groups.
p2: Normal vs cancer-induced.
p3: Normal vs cancer-prevented.
p4: cancer-induced vs cancer-prevented.

Table 4. Markers of oxidative stress.

Variable	Normal group	PJ group	Cancer-induced group	Cancer-prevented group	*p1*	*p2*	*p3*	*p4*
MDA (nmol g^{-1})	60.3 ± 6.5	61.4 ± 7.4	201.9 ± 28.5	66.2 ± 8.3	<0.001	<0.001	0.351	<0.001
CAT (U g^{-1})	19.4 ± 3.0	20.5 ± 3.8	11.9 ± 2.2	21.6 ± 4.0	<0.001	<0.001	0.243	<0.001
GSH (U g^{-1})	125.1 ± 13.3	128.5 ± 14.1	36.1 ± 5.0	111.8 ± 17.4	<0.001	<0.001	0.174	<0.001
SOD (U g^{-1})	13.1 ± 2.8	13.4 ± 3.3	2.6 ± 0.6	10.2 ± 2.0	<0.001	<0.001	0.109	<0.001

p1: Normal vs PJ vs cancer-induced vs cancer-prevented groups.
p2: Normal vs cancer-induced.
p3: Normal vs cancer-prevented.
p4: cancer-induced vs cancer-prevented.

effect on urinary bladder epithelium (Vasconcelos-Nóbrega et al., 2012). In the present study, all rats exposed to BBN developed BC with many pathological characteristics including hyperplasia, dysplasia and invasive neoplasms. These pathological features were markedly improved in rats supplied by PJ for 12 weeks starting from administration of BBN. The rats in cancer-prevented groups didn't show any signs of BC but mild pre-neoplastic lesions were observed in 25% of them. The ameliorations in the histopathological pattern of urinary bladder with the intake of PJ are in agreement with other experimental studies of skin (Afaq et al., 2010), breast (Bishayee et al., 2016), prostate (Adhami et al., 2012), colon (Sharma et al., 2017) and liver (Bhatia et al., 2013) cancers. In an *in vitro* study, ethanolic pomegranate extract reduced the proliferation of urinary bladder cell line carcinoma (T24 and J82) through apoptosis and cell cycle arrest (Lee et al., 2013).

To explore the mechanism(s) of chemopreventive effects of PJ against BBN induced BC, some investigations have been made including immunohistochemical staining of p53, gene expression of IL-6, TNF-α, HIF-1 and TP53 as well as indicators of oxidative stress.

The tumor suppressor p53 regulates cell cycle and apoptosis and therefore it possesses a significant role in malignancy. Inhibition of p53 pathway is a basic molecular defect in BC (Goebell et al., 2010). Our results which harmonizes with other studies (Oliveira et al., 2006) clarify impairment of p53 expression in BC as indicated by elevated immunostaining in cancer-induced group. This impact was clearly diminishing by treatment with PJ. Thus, PJ attenuates BC through induction of apoptosis. Pomegranate extract may induce BC cell apoptosis through mitochondrial damage, endothelium reticulum stress and death receptor signaling pathway (Lee et al., 2013).

Our findings revealed that BBN-induced BC in rats displayed significant increase in MDA activities in bladder tissues. In contrast, CAT, GSH and SOD were markedly reduced in the cancer-induced group when compared with those of the normal controls. Oral administration of PJ clearly restored the status of oxidative stress markers. These results suggest, at least in part, that the chemopreventive effect of PJ against BC is attributed to its anti-oxidant properties (Basiri, 2015). Due to these properties, PJ modulates many clinical diseases by enhancing the activity of antioxidant enzymes (Al-Gubory et al., 2016; Zarfeshany et al., 2014). It is surprising that the synergetic effect of pomegranate components is better than that of its individual components for prostate cancer cell line suppression (Lansky et al., 2005). Therefore, the use of PJ is preferable to the use of its components separately.

The gene expression results in our study supported the above mentioned findings. The administration of BBN was related with significant increase in expression of IL-6, TNF-α, HIF-1 and TP53 genes in bladder tissues among the cancer-induced group. IL-6 and TNF-α are pro-inflammatory cytokines that secreted by many cells and play a significant role in carcinogenesis (Han et al., 2016). Both stimulate the conversion of non-cancerous cells into cancerous cells (Landskron et al., 2014). IL-6 inactivates apoptosis and promotes proliferation of cancer cells by activation of Janus kinase (JAK)/signal transducers and activators of transcription (STATs) (Hodge et al., 2005). TNF-α involves in carcinogenesis by its significant role in necrosis, invasion and angiogenesis (Landskron et al., 2014). In our study, the expression of both IL-6 and TNF-α were corrected by PJ intake in the cancer-prevented group, although they remain higher than the normal controls. This highlights the anti-inflammatory role of PJ which is due to inhibitory effects on cyclooxygenase and lipoxygenase activities (Shukla et al., 2008).

In the present work, cancer-prevented group exhibited lower expression of HIF-1 than the cancer-induced group. Overexpression of HIF-1 gene can occur prior to histopathological proof of angiogenesis or cancer invasion (Unwith et al., 2015). Our results agree with a previous study which found that pomegranate extract hinder angiogenesis in prostate cancer by down-regulation of HIF-1 (Sartippour et al., 2008). These findings indicate that PJ can inhibit BBN induced BC through suppression of angiogenesis.

The tumor suppressor TP53 gene controls cell cycle and apoptosis and therefore it plays a critical role in carcinogenesis. Mutation of TP53 gene and subsequent production of dysfunctional p53 protein with prolonged half-life was detected in BBN administrated rats (El-Ashmawy et al., 2017). Intake of PJ in our study reduced expression of mutant TP53 gene among cancer-prevented group, therefore supporting the apoptotic and anticancer effects of PJ. The pro-apoptosis and anti-proliferative effects of pomegranate on BC cells might be due to regulation of p53/miR-34a axis (Zhou et al., 2015).

5. Conclusion

Our findings supply evidence for benefits in the preventive action of PJ against BC. Pomegranate juice contains valuable amounts of active components such as phenolic and flavonoid compounds and possesses strong anti-oxidant and anti-inflammatory activities. The present study elucidates that oral intake of PJ restore the status of oxidative stress and acts as free radical scavenger. The study also concluded that PJ corrects the expression of pro-inflammatory cytokines (IL-6 and TNF-1). It suppresses angiogenesis by down-regulation of HIF and supports apoptosis through reduction of the tumor suppressor gene p53. Further studies are recommended to investigate the effect of combined administration of PJ and anti-cancer drugs for treatment of BC.

Declarations

Author contribution statement

W. Mortada: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

A. Awadalla, S.M. Khater, N.M. Barakat and S.M. Husseiny: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

A.A. Shokeir: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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