

Short and Long Term Effects of Pomegranate (*Punica Granatum*) Extracts on Apoptosis in Rat Kidney Induced by Diethylnitrosamine and Phenobarbital

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Abstract: Pomegranate (*Punica granatum* L.) has strong anti-inflammatory, antioxidant, anti obesity, and anticancer effects. The effect of different pomegranate extracts, PE (peel extract), SOE (seed oil extract), and PJ (pomegranate juice) extract on levels of kidney caspase-3, DNAF (DNA fragmentation) and kidney function tests in rats treated and untreated with DEN (diethyl nitrosamine) and PB (Phenobarbital) during short (35 days) and long (154 days) period was studied. Injected of rats with DEN and PB caused an increased in the levels of DNAF, caspase-3 and kidney function tests, compared to the control in both period of study. Treatment of rats with PE, SOE, PJ pre, during, and post DEN and PB administration improved kidney function and decreased the levels of DNAF, and caspase-3 activities compared to the DEN group in both period of study, indicates that PE, SOE, PJ reduced and treatment apoptosis induced by DEN and PB. Treatment of healthy rats with PE, SOE, and PJ only for 35 days not increased kidney function or induced apoptosis for kidney tissues. Treatment with PJ alone in healthy kidney induced apoptosis which was higher than that induced by SOE and PE in case of long period study, this mean that fresh fruit or pomegranate juice safe for healthy in general at harvesting season only.

Key words: Kidney, apoptosis, diethylnitrosamine, phenobarbital, DNA fragmentation, pomegranate.

1. Introduction

Apoptosis is a general mechanism by which toxicants such as DEN can mediate a wide range of pathological organ responses, inflammation, and agenesis to tumour formation [1, 2]. DEN (Diethylnitrosamine) is a well known potent carcinogenic agent present in tobacco smoke, water, processed food, agricultural chemicals and pharmaceutical products [3, 4]. N-nitrosamines have been suggested to cause oxidative stress cell death due to involvement of free radicals which play an important role in the pathogenesis of kidney diseases [5]. PB (Phenobarbital), a derivative of barbituric acid, one of the classic AEDs (antiepileptic drugs), has recently become less popular due to its adverse effects, especially cognitive and behavioural side-effects [6]. Scientist reported that, PB is a nongenotoxic

hepatocarcinogen, and its administration at a high dose (500 ppm) after DEN injection promotes hepatocarcinogenesis in a rat model [7, 8].

Strategies to delay the onset of dialysis or to attenuate uremia often rely on dietary supplements [9]. Pomegranate is one of the oldest known drugs [10-12]. Pomegranate fruit presents strong anti-inflammatory, antioxidant, anticholesterol, antitumoral and pro-apoptotic activities [13-16].

Beneficial effects of pomegranate fruit due to the presence of ellagic acid, ellagitannins, puniceic acid and other fatty acids, flavonoids, anthocyanidins, anthocyanins, estrogenic flavonols, and flavones into pomegranate seeds, peel, and juice [14-17]. Still, the lack of pomegranate studies in short and long term with the growing amount of pomegranate consumption confirmed the need to investigate the effects of short and prolonged ingestion in health and disease establish the potential roles that may make pomegranate one of

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the most chosen foods of the future. The aim of this study included on: 1-study the effect of DEN and PB on normal kidney. 2- Study the effect of different pomegranate extract PE, SOE, and PJ on normal kidney for short and long term. 3- Study the chemoprevention of different pomegranate extract PE, SOE, PJ against of DEN and PB for short and long term. The studies focused on the changes levels of DNAF (DNA fragmentation), caspase-3 activity, and kidney function test.

2. Experimental Procedures

2.1 Chemicals

Caspase-3 assay kit was obtained from BioSource International, Inc. (Camarillo, CA, USA). An AxyPrep DNA gel extraction and purification kit was obtained from Montreal Biotechnologies Inc. (Dorval, PQ, Canada), DEN (Diethylnitrosamine) was purchased from Sigma- Aldrich (St. Louis, MO, USA).

2.2 Plant

Pomegranate plant from the family Lythraceae was purchased from the local market. The fruits were peeled mechanically. Then the seeds of the fruit containing the intact juice sacs were manually separated and filtered (PJ), and the filtrate was stored at 4°C until used [17]. The pomegranate peels were washed well in running water and dried in sun solar system. The dried clean peels were extracted with soaking in 70 % methanol (1: 6 w/v) for four hours. The methanolic extract was evaporated in rotary evaporator at 40 °C to obtain a sticky extract, PE (peel extract) (yield: 9 %) [18, 19]. The pomegranate hard white seeds were washed well in running water and dried in sun solar system. The dried clean seeds were mechanically crushed in crushing machine. Then crushed seeds were extracted with n-hexane (1: 5 w/v). The hexane extract containing seed oil was concentrated in rotary evaporator at 40 °C to obtain SOE (seed oil extract) (yield: 15 %).

2.3 Animals

160 adult male Sprague-Dawley rats weighing 100-110 g were obtained from faculty of medicine Alexandria University, Egypt. All rats were examined for health status and their room was designed to maintain the temperature at 25 °C, relative humidity at approximately 50% and 12 h light/dark photoperiod for 2 weeks prior to handling. The animals were then housed in stainless-steel cages, given standard diet and water throughout the study and observed daily for abnormal signs. After acclimatization, rats were divided into eight groups of twenty rats. All animal experiments were approved by the Ethics Committee of the Experimental Animal Care Society.

Control group (C): untreated rats.

(DEN) group: Rats were injected intraperitoneally, IP, with 200 mg of DEN/kg body mass (bm) as one dose [20]. At the beginning of the 2nd week, the animals were given drinking water containing 500 ppm of PB for 20 weeks [21, 22] (i.e. short term experimental period is 5 weeks = 35 days and long term experimental period is 22 weeks = 154 days).

(PE) group: Rats were treated orally (using oral gavages) with a daily dose 250 mg PE /kg bm [23] for 22 weeks. (i.e. short term experimental period is 5 weeks = 35 days and long term experimental period is 22 weeks = 154 days).

(PE-DEN) group: Rats were treated orally (using oral gavages) with a daily dose 250 mg PE /kg bm for 2 weeks. At the beginning of the 3rd week, rats were treated with DEN (as described previously) in addition to the PE treatment. At the beginning of the fourth week, rats were treated with PB with continuous intake of PE until the 22 week of the experiment (i.e., Rats were treated with PE pre, during, and post DEN and PB treatment) [20, 23] (i.e. short term experimental period is 5 weeks = 35 days and long term experimental period is 22 weeks = 154 days).

(SOE) group: Rats were treated orally (using oral gavages) with a daily dose 2 ml SOE /kg bm for 22 weeks [24]. (i.e. short term experimental period is 5

weeks = 35days and long term experimental period is 22 weeks = 154 days).

(SOE-DEN) group: Rats were treated orally (using oral gavages) with a daily dose 2 ml SOE /kg bm for 2 weeks. At the beginning of the 3rd week, rats were treated with DEN (as described previously) in addition to the SOE treatment. At the beginning of the fourth week, rats were treated with PB with continuous intake of SOE until the 22 week of the experiment (i.e., Rats were treated with SOE pre, during, and post DEN and PB treatment) [20, 24] (i.e. short term experimental period is 5 weeks=35days and long term experimental period is 22 weeks = 154 days).

(PJ) group: Rats were treated orally (using oral gavages) with a daily dose 1mL PJ/kg bm for 22 weeks.) [16] (i.e. short term experimental period is 5 weeks = 35days and long term experimental period is 22 weeks = 154 days).

(PJ-DEN) group: Rats were treated orally (using oral gavages) with a daily dose 1mL PJ/kg bm for 2 weeks. At the beginning of the 3rd week, rats were treated with DEN (as described previously) in addition to the PJ treatment. At the beginning of the fourth week, rats were treated with PB with continuous intake of PJ until the 22 week of the experiment (i.e., Rats were treated with PJ pre, during, and post DEN and PB treatment) [16, 20] (i.e. short term experimental period is 5 weeks = 35days and long term experimental period is 22 weeks = 154 days).

At the end of each period of studying (after 35 days or 150 days) of acclimatization, 80 animals were randomly taken from each group in each period to stop feeding for 12 h prior to killing. Rats were anaesthetized by diethylether and killed. Kidneys were removed immediately, and washed with cold saline solution (0.9% NaCl), weighed, divided into two parts and kept at -80°C until used for determination of DNAF (DNA fragmentation) and caspase-3 activity.

Unheparinized blood samples were collected, kept at room temperature for 15 min and then sera were separated by centrifugation at 3000 rpm at 2°C for 20

min. Sera were stored at -30°C until used for the determination of Kidney function test.

2.4 Biochemical Assay

2.4.1 Caspase-3 Assay (EC 3.4.22.56)

Caspase-3 activity was determined using a colorimetric kit according to the method of [25] kidney tissues were homogenized in four volumes of cold cell lysis buffer (50mM Tris-HCl buffer containing 0.2M NaCl and 1% Triton X-100, pH 6.8) using a Teflon glass homogenizer. The homogenates were centrifuged at 44,720 g for 3min at 4°C , and the supernatants were kept at -80°C . The supernatant ($50\ \mu\text{L} = 150\ \mu\text{g}$ protein) was put in a microplate reader, then $50\ \mu\text{L}$ (microliter) reaction buffer and $5\ \mu\text{L}$ (microliter) of 4 mM (millimolar) substrate were added, mixed well, and incubated at 37°C in the dark for 2 hours. The reaction rate was determined by measuring the absorbance of the produced yellow color at 405 nm (nanometer) against a blank using a microplate reader (Bio-Tek Instruments, Bad Friedrichshall, Germany). Fold increase in caspase-3 activity should be determined by direct comparison to the level of the control.

2.4.2 DNA Fragmentation

DNAF was determined in the kidney homogenate using agarose gel electrophoresis according to the method of [26]. Kidney tissues were homogenized in 1:5 weights per volume (1:5 w/v) 50 millimolar (mM) Tris-HCl buffer containing 20% sucrose and 50 millimolar (mM) EDTA, pH 7.6. DNA was isolated using a DNA purification kit. Then 15 microgram per lane of DNA ($15\ \mu\text{g}/\text{lane}$ DNA) was separated by electrophoresis on 1% agarose gel containing 25 microliter (μL) ethidium bromide at five volt per one centiliter ($5\ \text{V}/1\ \text{cm}$) for 2–3 hours, and visualized under UV light using a multiband transilluminator from Consort (Turnhout, Belgium).

2.4.3 Kidney Function Test

Creatinine, urea and uric acid concentrations were determined according to the methods of [27-29].

2.5 Statistical Analysis

All data are presented as means (X) ± S.D (standard deviation). Comparisons between the means of various treatment groups were analyzed using LSD (least significant difference) test. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using the statistical software SPSS v11.5 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1 Caspase-3 Activity

During the first period of study (35 days), caspase-3 levels in control (C) were 0.15 ± 0.13 lower than that in DEN group 0.72 ± 0.12 ; $p < 0.05$. The enzyme levels in PE, SOE and PJ groups were 0.20 ± 0.11 , 0.16 ± 0.08 and 0.16 ± 0.13 respectively compared to C; $p < 0.05$. The enzyme levels in PE-DEN, SOE-DEN, PJ-DEN groups were 0.25 ± 0.09 , 0.21 ± 0.11 and 0.25 ± 0.12 respectively compared to DEN; $p < 0.05$. (Table 1). After 154 days of study, caspase-3 levels in control (C) were 0.15 ± 0.13 lower than that in DEN group 0.87 ± 0.12 ; $p < 0.05$. The enzyme levels in PE, SOE and PJ groups were 0.30 ± 0.08 , 0.25 ± 0.13 and 0.36 ± 0.06 respectively compared to C; $p < 0.05$. The enzyme levels in PE-DEN, SOE-DEN, PJ-DEN groups were 0.45 ± 0.11 , 0.25 ± 0.09 and 0.46 ± 0.08 respectively compared to DEN; $p < 0.05$. (Table 1)

3.2 Kidney Function

During the first period of study (35 days), creatinine, urea and uric acid concentrations were increased significantly after administration of DEN and PB (1.004 ± 0.08 mg/dl, 46.657 ± 0.03 g/dl, 3.90 ± 0.4 mg/dl) respectively as compared to the C (0.625 ± 0.12 mg/dl, 44.157 ± 0.01 g/dl, 2.10 ± 0.01 mg/dl); $p < 0.05$. Creatinine, urea and uric acid concentrations were (0.543 ± 0.14 mg/dl, 40.161 ± 0.09 g/dl, 2.0 ± 0.22 mg/dl) in PE group, (0.544 ± 0.09 mg/dl, 44.154 ± 0.03 g/dl, 1.53 ± 0.21 mg/dl) in SOE group and (0.624 ± 0.01 mg/dl, 44.091 ± 0.13 g/dl, 2.1 ± 0.11 mg/dl) in PJ

group compared to C; $p < 0.05$. Creatinine concentrations in PE-DEN, SOE-DEN and PJ-DEN were 0.736 ± 0.07 mg/dl, 0.548 ± 0.11 mg/dl and 0.787 ± 0.01 mg/dl respectively compared to DEN; $p < 0.05$. Urea concentrations in PE-DEN, SOE-DEN and PJ-DEN were (43.900 ± 0.04 g/dl, 25.500 ± 0.09 g/dl, 44.092 ± 0.12 g/dl) respectively compared to DEN; $p < 0.05$. Uric acid concentrations in PE-DEN, SOE-DEN and PJ-DEN were (2.74 ± 0.09 mg/dl, 2.10 ± 0.11 mg/dl, 2.10 ± 0.21 mg/dl, respectively compared to DEN; $p < 0.05$ (Table 1).

After 154 days of study, creatinine, urea and uric acid concentrations were increased significantly after administration of DEN and PB (1.544 ± 0.13 mg/dl, 49.657 ± 0.07 g/dl, 4.90 ± 0.14 mg/dl) respectively as compared to the C (0.625 ± 0.01 mg/dl, 44.157 ± 0.08 g/dl, 2.10 ± 0.11 mg/dl); $p < 0.05$. Creatinine, urea and uric acid concentrations were (1.693 ± 0.12 mg/dl, 49.101 ± 0.09 g/dl, 2.5 ± 0.23 mg/dl) in PE group, (0.504 ± 0.06 mg/dl, 48.154 ± 0.09 g/dl, 1.55 ± 0.21 mg/dl) in SOE group and (1.694 ± 0.11 mg/dl, 48.171 ± 0.07 g/dl, 2.51 ± 0.16 mg/dl) in PJ group compared to C; $p < 0.05$. Creatinine concentrations in PE-DEN, SOE-DEN and PJ-DEN were 0.626 ± 0.05 mg/dl, 0.528 ± 0.11 mg/dl and 0.702 ± 0.12 mg/dl respectively compared to DEN; $p < 0.05$. Urea concentrations in PE-DEN, SOE-DEN and PJ-DEN were (42.990 ± 0.11 g/dl, 25.500 ± 0.08 g/dl, 43.071 ± 0.12 g/dl) respectively compared to DEN; $p < 0.05$. Uric acid concentrations in PE-DEN, SOE-DEN and PJ-DEN were (2.14 ± 0.19 mg/dl, 2.49 ± 0.21 mg/dl, 2.90 ± 0.15 mg/dl, respectively compared to DEN; $p < 0.05$ (Table 1). The changeable Percentage in the levels of biochemical parameters between 35 days and 154 days regarding to caspase-3, Creatinine, urea and uric acid has been shown in (Table 2).

3.3 DNAF (DNA Fragmentation) in Kidney Tissue

During the first period of study (35 days), the agarose gel electrophoresis showed very low DNAF (DNA laddering) in the kidney tissue of the Control. The

Table 1 Biochemical parameters of different studied groups.

Particulars(in	C	DEN	PE	PE-DEN	SOE	SOE-DEN	PJ	PJ-DENn
Short term study(35 days)								
Tissue								
Caspase -3 activity	0.15 ^a ± 0.13	0.72 ^b ± 0.12	0.20 ^c ± 0.11	0.25 ^d ± 0.09	0.16 ^a ± 0.08	0.21 ^e ± 0.11	0.16 ^a ± 0.13	0.25 ^d ± 0.12
Serum								
Creatinine (mg/dl)	0.625 ^a ± 0.12	1.004 ^b ± 0.08	0.543 ^c ± 0.14	0.736 ^d ± 0.07	0.544 ^c ± 0.09	0.548 ^c ± 0.11	0.624 ^a ± 0.01	0.787 ^e ± 0.01
Urea (g/dl)	44.157 ^a ± 0.01	46.657 ^b ± 0.03	40.161 ^c ± 0.09	43.900 ^a ± 0.04	44.154 ^a ± 0.03	25.500 ^d ± 0.09	44.091 ^d ± 0.13	44.092 ^d ± 0.12
Uric acid (mg/dl)	2.10 ^a ± 0.01	3.90 ^b ± 0.4	2.0 ^a ± 0.22	2.74 ^d ± 0.09	1.53 ^e ± 0.21	2.10 ^a ± 0.11	2.1 ^a ± 0.11	2.10 ^a ± 0.21
Long term study(154 days)								
Tissue								
Caspase -3 activity	0.15 ^a ± 0.12	0.87 ^b ± 0.12	0.30 ^c ± 0.08	0.45 ^d ± 0.11	0.25 ^e ± 0.13	0.25 ^e ± 0.09	0.36 ^f ± 0.06	0.46 ^d ± 0.08
Serum								
Creatinine (mg/dl)	0.625 ^a ± 0.01	1.544 ^b ± 0.13	1.693 ^c ± 0.12	0.626 ^a ± 0.05	0.504 ^d ± 0.06	0.528 ^e ± 0.11	1.694 ^c ± 0.11	0.702 ^f ± 0.12
Urea (g/dl)	44.157 ^a ± 0.08	49.657 ^b ± 0.07	49.101 ^c ± 0.09	42.990 ^d ± 0.11	48.154 ^e ± 0.09	25.500 ^f ± 0.08	48.171 ^e ± 0.07	43.071 ^d ± 0.12
Uric acid (mg/dl)	2.10 ^a ± 0.11	4.90 ^b ± 0.14	2.5 ^c ± 0.23	2.14 ^d ± 0.19	1.55 ^e ± 0.21	2.49 ^c ± 0.21	2.51 ^c ± 0.16	2.90 ^f ± 0.15

Group C - control rats; group DEN – rats treated with DEN and PB; PE (peel extract). PE-DEN (rats treated with PE pre, during, and post DEN and PB administration), SOE (seed oil extract), SOE-DEN (rats treated with SOE pre, during, and post DEN and PB administration), PJ (pomegranate juice), PJ-DEN (rats treated with PJ pre, during, and post DEN and PB administration).

Results are given as mean ± S.D. for ten rats. Values are expressed as mean ± S.D. for ten rats. Within each row, values with different letter are significantly different at p < 0.05.

Table 2 % of change between 35 days and 154 days regarding to Caspase -3 activity, Creatinine, Urea and Uric acid.

% of change between 35 & 154 days	C	DEN	PE	PE-DEN	SOE	SOE-DEN	PJ	PJ-DEN
Caspase -3 activity	0.0	↑20.8	↑50.0	↑80.0	↑56.3	↑19.0	↑125.0	↑84.0
Creatinine (mg/dl)	0.0	↑53.8	↑211.8	↓14.9	↓7.4	↓3.6	↑171.5	↓10.8
Urea (g/dl)	0.0	↑6.4	↑22.3	↓2.1	↑9.1	0.0	↑9.3	↓2.3
Uric acid (mg/dl)	0.0	↑25.6	↑25.0	↓21.9	↑1.3	↑18.6	↑19.5	↑38.1

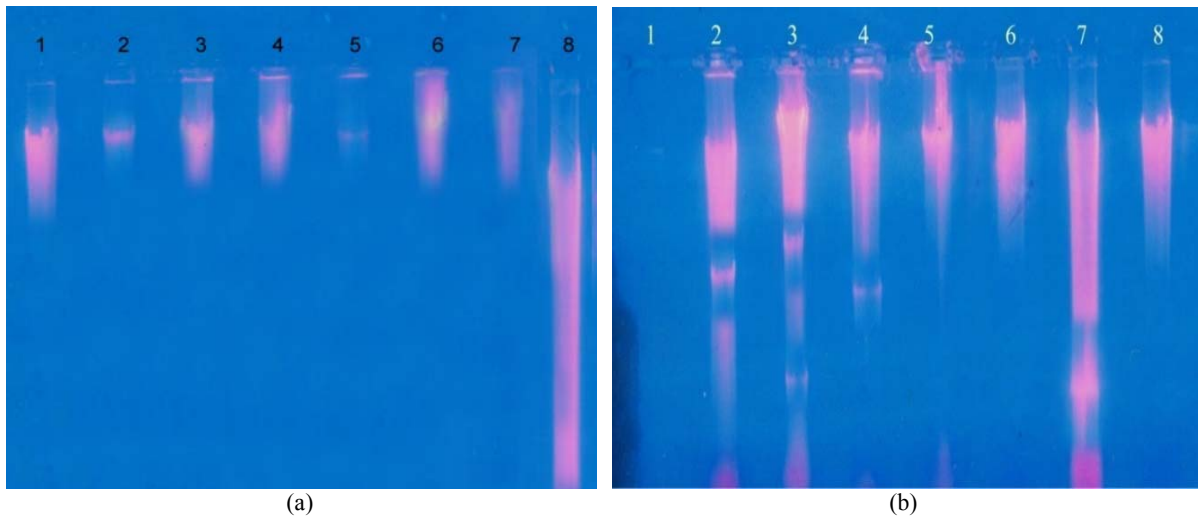


Fig. 1 DNA fragmentation in all studied groups in kidney tissue.

DNA intact band appears to be condensed near the application point with no DNA smearing suggesting no DNA fragmentation (DNAF). DEN administration and PB resulted in a massive DNAF compared to the C group. Treatment with PE, SOE, and PJ separately shows no DNA smearing suggesting no DNAF compared to control. Treatment with PE, SOE, PJ pre, during, and post DEN and PB administration decreased DNAF compared to the DEN group (Fig 1a). After 154 days of study, the agarose gel electrophoresis showed very low or undetectable DNA laddering (DNAF) in the kidney tissue of the Control. DEN administration and PB resulted in a massive DNAF compared to the C group. Treatment with PE, SOE, and PJ separately shows DNA smearing suggesting a massive DNAF compared to control. Treatment with PE, SOE, PJ pre, during, and post DEN and PB administration decreased DNAF compared to the DEN group (Fig. 1b).

DNA fragmentation separating in 1% agarose gel electrophoresis and visualized under UV. DNAF in C (control), DEN (diethyl nitrosamine- Phenobarbital), PE (peel extract). PE-DEN (rats treated with PE pre, during, and post DEN and PB administration), SOE (seed oil extract), SOE-DEN (rats treated with SOE pre, during, and post DEN and PB administration), PJ (pomegranate juice), PJ-DEN (rats treated with PJ pre, during, and post DEN and PB administration). Fig. 1a.

DNA fragmentation in all studied groups in short 35 days, control (1), PE (2), PE-DEN (3), SOE (4), SOE-DEN (5), PJ (6), PJ-DEN (7), DEN (8). Fig. 1b. DNA fragmentation in all studied groups in long 154 days, control (1), DEN (2).PE (3), PE-DEN (4), SOE (5), SOE-DEN (6), PJ (7), PJ-DEN (8).

4. Discussion

Scientist found that caspase-3 is a major mediator of both apoptotic and necrotic cell death. Increases in both caspase-1 and caspase-3 have been reported in ischemic injury to various organs, including liver, brain, heart and kidney [30]. Both pharmacological inhibitors and genetic trails have been used to inhibit caspases in vivo [31]. The present result has shown a significant elevation in caspase-3 activity and DNAF, creatinine, urea and uric acid levels after DEN and PB administration compared to the control group in both short (35 days) and long term (154 days) of study. This may be due to an enhanced generation and burst of ROS (reactive oxygen species) that accelerated peroxidation of native membrane lipids. Peroxidation of the mitochondrial membrane led to a loss of cell integrity, increase in membrane permeability, and alteration of Ca^{2+} homeostasis that contribute to cell death and alter kidney function tests [16, 32, 33]. In addition, some ROS interact with various tissue

compounds leading to dysfunction and injury to the kidney, liver and other organs (and [16, 32]. Elevation in caspase-3 activities and DNAF after DEN and PB administration indicates that DEN and PB caused apoptosis for kidney tissues, and apoptosis is an adaptive process of combating excessive damage. Elevated apoptosis within a diseased kidney should not always be viewed as harmful. The apoptotic process plays a role in the resolution of glomerular inflammation. Failure of these mechanisms might lead to disintegration of neutrophils within the inflamed glomerulus and the development of persistent inflammation leading to scarring [33, 34].

The injury of kidney probably due to the deleterious effect of DEN itself and/or its metabolites which includes ethylcarbonium ions, NO and ROS such as superoxide radicals O_2^- [35]. Ethylcarbonium ions bind to DNA forming adducts and generate superoxide radicals through lipid peroxidation of phospholipid membrane fatty acids [36]. DEN also induces iNOS gene expression and generates NO radicals which react nonenzymatically with O_2^- forming peroxynitrite ($ONOO^-$ a reactive nitrogen species) [35]. Furthermore, a continuous administration of PB simultaneous with DEN increases the release of ROS [21]. ROS can attack important biological molecules, such as lipids, proteins, DNA, enzymes, and RNA. Thus, ROS are involved in a number of degenerative diseases such as cancer, cirrhosis, diabetes, and Alzheimer's Disease [37].

Plants, vegetables, fruits and spices used in folk and traditional medicine have been accepted currently as one of the main sources of chemo preventive drug discovery and development [38]. In the present study, the animals treated with PE alone for 35 days showed a slight increase in caspase-3 with non change in DNAF levels and a slight decrease in creatinine, urea and uric acid levels as compared to the controls. These results agree with Settheetham et al, (1995) [39] who found that the administration of aqueous pomegranate PE

results in apoptotic DNA fragmentation and suppression of growth in two human Burkitt's lymphoma cell lines, Raji and P3HR-1. The slight increase in apoptosis in PE group than the control in this study may be due to the effect of tannin which is present in high concentration in PE and acts as proapoptotic. Otherwise, the treatment of rats with PE alone for 154 days has shown a significant elevation in caspase-3 activity and DNAF, creatinine, urea and uric acid levels compared to the control group, the treatment of rats with PE for a long period led to the accumulation of tannin in the kidney increases its effect and alter kidney function tests. Previous results reported that tannic acid causes DNA degradation through generation of reactive oxygen species. On the other hand, it exhibits antimutagenic and anticarcinogenic activities, and induces apoptosis in animal cells. It is known that most plant-derived polyphenolic antioxidants also act as pro-oxidants under certain conditions [40]. Other results reported that ellagic acid exhibited both antioxidant activity in V79-4 cells and apoptosis-inducing activity in human osteogenic sarcoma cells through the up-regulation of Bax and activation of caspase-3 [12, 41]. In vitro studies using different cancer cell lines demonstrated that pomegranate juice, seeds, and oil can inhibit cancer cell invasiveness and proliferation, cause cell cycle disruption, induce apoptosis, and inhibit tumor growth [42].

The present result has shown a significant decline in caspase-3 activity and DNAF, creatinine, urea and uric acid levels in PE-DEN compared to the DEN in studies, 35 days and 154 days, this indicated that PE have selective targeting effects to improve kidney function tests and injuries induced with DEN and PB. The renoprotective efficacy of pomegranate peels; attributed to antioxidant, anti-inflammatory and different signaling pathway mechanisms to their high polyphenolic contents. [43] Shaban et al, (2013), reported that pomegranate extracts (PE, SOE) reduced liver oxidative stress and apoptosis induced by DEN

for 23 weeks. Scientists demonstrate that pomegranate peel ethanol extract is a potent nephroprotective agent and suppresses Fe-NTA-induced renal oxidative damage in rats [44]. Ahmed and Zaki, (2009) [45] reported that animals administration of Pomegranate peel extract was significantly attenuated the toxicity and oxidative stress induced by chlorpyrifos-ethyl.

Administration of SOE alone showed that there were slight increases in caspase-3 activity with non change in DNAF levels as well as significant increase in caspase-3 activity, DNAF in short and long term of study respectively. Where in both short and long term studies, there were decreases in creatinine, urea and uric acid levels compared to the control group. These results may be due to effect of some steroid components of pomegranate seed oil and accumulated these competent inside the tissues, where Jin et al, (2006) [46] reported that steroid hormone could induce apoptosis of bone marrow-derived macrophages. The detailed mechanisms behind the apoptosis of macrophages induced by steroid hormone are not clear. Pomegranate seed oil decreased serum creatinine, urea, urine glucose and protein concentrations [47].

Otherwise treatment with SOE pre, during, and post DEN and PB administration showed a significant reduction in the levels of in caspase-3 activity and DNAF, creatinine, urea and uric acid levels after DEN and PB administration compared with the DEN group, this means that SOE contents inhibited the caspase-3 activity and DNA fragmentation in selective targeting way and this led to reduce apoptosis induced with DEN and improve kidney functions. It has been reported that SOE contents gamma-tocopherol, ursolic acid, 17-alpha-estradiol, estrone, estriol, testosterone, beta-sitosterol, coumesterol, campesterol, stigmasterol and puniceic acid acts as antioxidants and anti-apoptotic [43, 48, 49]. Where the anti-apoptotic selective targeting of pomegranate seed oil may be due to the effect of estrogenic components, through modulate intracellular Ca^{2+} , prevent inflammation, reduce caspase-3 activity as well as DNA fragmentation and subsequently, overall cell

death [48]. Otherwise Madsen-Bouterse et al, (2006) [50] reported that the mechanism of glucocorticoid regulation of spontaneous apoptosis and implicate steroid receptor activation and subsequent regulation of Bcl-2 family members. Pomegranate seed oil has a protective effect against hexachlorobutadiene and HgCl₂-induced nephrotoxicity in rats[47, 51].

Administration of PJ alone for 35 days in the present study showed that there was slight change in caspase-3 activity and DNAF, creatinine, urea and uric acid levels compared to the control group. While treatment with PJ alone for a long period 154 days showed increase in caspase-3 and DNAF, creatinine, urea and uric acid levels compared to the control group, this may be due to accumulation of pomegranate juice active compounds. It has been reported that whole fruit caused an elevation in creatinine level in vivo [52]. Pomegranate and its constituents have been safely consumed by humans for several millennia. Nevertheless, several animal studies and human clinical trials have investigated the toxicity of pomegranate over a long period (16).No adverse side effects have been noted in any of these studies, therefore considering safe to consume the fresh fruit or pomegranate juice in general at harvesting season only. Moreover, the health effect of pomegranate can vary due to geographical region, harvesting, and season, which can alter the fruit composition [53]. In addition, pomegranate juice administration in rats for 37 days has no toxic effect [17, 33, 54].

In both short and long term studies treatment with PJ pre, during, and post DEN and PB administration showed a reduction in the levels of in caspase-3 activity and DNAF, creatinine, urea and uric acid levels after DEN and PB administration compared with the DEN group, This means that, polyphenolic compounds in PJ play an important selective targeting role in quenching the free radicals resulted from the metabolism of DEN, thereby inhibiting lipid peroxidation and protecting membrane lipids from oxidative damage and in turn prevent apoptosis. Our

results agree with previous study which confirmed the protective role of pomegranate anthocyanins toward apoptosis [55, 56]. Where [57] Rezk, 2013 found that treatment pomegranate juice ameliorate rat renal damage induced by radiation, and this due to pomegranate polyphenolic components' (delphinidin-3,5-diglucoside, delphinidin-3-glucoside, cyanidin-3,5-diglucoside, cyanidin-3-glucoside, pelargonidin-3,5-diglucosid, pelargonidin-3-glucoside, ellagitannins gallotannins and condensed tannins (proanthocyanidins).

The antioxidant powers of PJ are related to their high polyphenolic contents and their variation [58]. The antioxidant effects of phenolic compounds of PJ are more potent than many other antioxidant compounds [58] (such as vitamins), and are able to scavenge ROS and reactive nitrogen species which resulted from DEN and PB metabolism [33, 59]. Punicalagin, one of the ellagitannins, is responsible for more than 50% of the antioxidant activity of the PJ [41]. In addition, ellagic acid decreases both the total hepatic CYP-450 and CYP2E1 that lead to alteration in the DEN and PB metabolism. Chlorogenic acid, caffeic acid, and some nonpolyphenolic compound in PJ such as serotonin are good inhibitors of the n-nitrosation reaction [33]. So polyphenols may be effective not only in protection against oxidative damage but also in inhibiting the formation of potent mutagenic and carcinogenic n-nitroso compound in vivo [11]. Ahmed et al. (2011) [60] found that PJ has significant effect on some oxidants/antioxidants enzymes of liver and kidney. This renoprotective effect of pomegranate extracts can be attributed to its high phenolic and steroid content and the mechanism of action may be through induction of various antioxidant enzymes and scavenging reactive oxygen species [61].

5. Conclusion

DEN and PB induced inflammation, apoptosis and toxicity for kidney tissues.

Treatments with pomegranate extracts (PE, SOE and

PJ) for short period (35 days) have no side effects on the normal kidney.

Treatments with pomegranate extracts (PE, SOE and PJ) for a long period (154 days) have side effects on the normal kidney.

The present study demonstrates that, administration of pomegranate extracts (PE, SOE, PJ) before, during and post DEN administration reduced apoptosis induced with DEN and PB in both period of study.

We concluded that the treatment with (SOE) is the best followed by (PJ), then (PE) against kidney injuries induced with (DEN).

The side effects of pomegranate extracts may be due to the long period of its administration, which led to accumulation of phenolic and tannin compounds and increase their effects.

The present study showed that pomegranate extract exerts a significant target protective effect against DEN and PB apoptosis in kidney by augmenting host antioxidant defense mechanisms. This extract is a promising agent for the prevention of chemical induced toxicity.

We recommend separating the compounds of pomegranate which have preventive and positive effects from the pomegranate extracts peel, seeds and juice. These compounds are considered economic sources. Using them in pure way in the treatment many types of disease such as cancers.

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