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Effects of Some Pyrimidine Derivatives and Pomegranate Juice on Male Rat kidney Injuries Induced by Diethylnitrosamine and Carbon tetrachloride

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Abstract: The kidney possesses most of the common xenobiotic metabolizing enzymes, and is thus able to make an important contribution to the body's metabolism of drugs and foreign compounds. The effect of pyrimidine derivatives 6-amino-2-thiouracil (ATU), 2-thiouracil (TU) and 5-flurouracil (5FU), and pomegranate juice (PJ) on kidney nitric oxide (NO), malondialdehyde (MDA), DNA fragmentation (DNAF), caspase-3 levels and kidney function tests in rats treated with diethyl nitrosamine (DEN) and carbon tetra chloride CCl_4 was studied. The effect of PJ on rat not treated with DEN and CCl_4 was studied also. Administration of rats with DEN and CCl_4 caused an elevation in the levels of NO, MDA, DNAF, caspase-3 and kidney function tests, compared to the control. Treatment of rats with PJ pre, during, and post DEN and CCl_4 administration improved kidney function and decreased the levels of NO, MDA, DNAF, and caspase-3 activities better than that in DEN-5FU, DEN- ATU, DEN-TU groups compared to the DEN group, indicates that PJ reduced the oxidative stress and apoptosis induced by DEN and CCl_4 better than that in 5FU, ATU, TU. Administration of healthy rats with PJ only for 5 weeks not induced oxidative stress and apoptosis for kidney tissues. Treatment with 5FU after DEN and CCl_4 administration showed severe toxicity which was higher than that induced by DEN and CCl_4.

Keywords: Apoptosis, diethylnitrosamine, DNA fragmentation, thiouracil, fluorouracil, pomegranate juice.

1. Introduction

Kidney is a paired organ whose functions include removing waste products from the blood and regulating the amount of fluid in the body. Diseases of the kidney range from mild infection to life-threatening kidney failure. Normal function and development of the kidney has a demonstrable dependence on apoptosis [1]. Apoptosis, a morphological form of programmed cell death required for the control of cell populations, has been shown to have a role in the cell deletion associated with renal scarring [2, 3].

Diethylnitrosamine (DEN) has been found to be widely distributed in processed meats; tobacco smoke; whisky; smoked, salted, and dried fish; cheese; cured meat; and alcoholic beverages [4]. In foods, nitrosamines are produced from nitrites and secondary amines, which often occur in the form of proteins. Their formation can only

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occur under certain conditions, including strongly acidic conditions such as that of the human stomach. High temperatures, as in frying, can also enhance the formation of nitrosamines [5]. In general N-nitrosamines have been suggested to cause oxidative stress and cellular injury due to involvement of free radicals , such as nitric oxide radical, [6]. There has been recent interest in the concept that oxygen free radicals and nitric oxide (NO) play an important role in the pathogenesis of kidney diseases [7]. Carbon tetrachloride (CCl_4) is metabolized by cytochrome P-450 in endoplasmic reticulum and mitochondria with the formation of trichloromethyl peroxyl radicals a reactive oxidative free radical, which initiate lipid peroxidation [8].

The chemistry of pyrimidine and fused pyrimidine derivatives has been of increasing interest, since many of these compounds revealed several biological activities and useful applications as anticancer, antibacterial, antiviral, antifungal, hypoglycemic and diuretic agents [9]. Thiouracils and their nucleoside analogs are found in t-RNA among many prokaryotes [10]. The existence of uracil and thiouracil in many tautomeric forms has been revealed to be crucial to explain the mutation occurring during DNA duplication [11].

Chemoprevention is one of the strategies by which we can revert or delay the response of carcinogen. Dietary factors contribute about one third of potentially preventive cancer [12]. Pomegranate (Punica granatum L.) is one of the oldest edible fruits and has been used extensively in the folk medicine of many cultures. Pomegranate fruits are widely consumed fresh and in beverage forms as juice and wines [13]. Pomegranate fruit presents strong anti-inflammatory, antioxidant, antiobesity, and antitumoral properties, thus leading to an increased popularity as a functional food and nutraceutical source since ancient times [14, 15]. It can be divided into three parts: seeds, peel, and juice, all of which seem to have medicinal benefits. Several studies investigate its bioactive components as a means to associate them with a specific beneficial effect and develop future products and therapeutic applications. Many beneficial effects are related to the presence of ellagic acid, ellagitannins (including punicalagins), punicic acid and other fatty acids, flavonoids, anthocyanidins, anthocyanins, estrogenic flavonols, and flavones, which seem to be its most therapeutically beneficial components. However, the synergistic action of the pomegranate constituents appears to be superior when compared to individual constituents [5, 15, and 16].

The aim of this study included on: 1-study the effect of diethylnitrosamine (DEN) and carbon tetrachloride CCl_4 on normal kidney. 2- Study the effect of pomegranate juice (PJ)alone and the chemoprevention of PJ against of DEN and CCl_4. 3- treatment the effect of DEN and CCl_4 using pyrimidine derivatives 6-amino-2-thiouracil (ATU), 2-thiouracil (TU) and 5flurouracil (5FU) and (PJ) to know the best one with least adverse effects. The studies focused on the changes levels of DNA fragmentation (DNAF), caspase-3 activity, nitric oxide (NO), malondialdehyde (MDA) and kidney function test. The histopathological studies also were exanimate.

2. Experimental Procedures

I. Chemicals

A 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) Sulphanilamide, N-1-Napthyl ethylene diamine, Standard sodium nitrite, Diethylnitrosamine (DEN),

thiobarbituric acid (TBA), and tetramethoxypropan (TMP) were purchased from Sigma- Aldrich (St. Louis, MO, USA). Sodium dodecyle sulfate (SDS) were purchased from Fluka (St. Gallen, Switzerland). 5-fluoro-1H-pyrimidine-2, 4-dione (Fluorouracil (5-FU) was obtain from Ebewe Pharma, Ges.m.b.H.Nfg.KG, A-4866 Unterach, Austria. (6-amino-2-thiouracil (ATU) and 2-thiouracil (TU) were obtained from Aldrich and BDH companies. Pomegranate juice 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, and the filtrate was stored at 4C until used (20).

II. Animals

70 adult male Sprague-Dawely rats weighing 100-110 gram 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 ^{o}C, relative humidity at approximately 50% and 12 hours 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 seven groups of ten rats.

Control group(C): untreated rats.

(DEN) group: Rats were injected intraperitoneally, IP, with 200 milligram of DEN per kilogram body mass (200 mg of DEN/kg bm) as one dose, after one week they were injected subcutaneous (SC) with 3 milliliter of CCl_4 per kilogram per week for two weeks(3 ml of CCl_4 /kg/week for 2 weeks, [17]).

(DEN-5FU) group: Rats were injected with DEN and CCl_4 as mentioned in DEN group. Then rats were treated orally using gavages tube with 60 milligram of 5FU per kilogram body mass per day for six days(60 mg of 5FU/kg bm/day for 6 days) [17, 18].

(DEN –ATU) group: Rats were injected with DEN and CCl4 as mentioned before. Then they were treated orally with 60 milligram of ATU per kilogram body mass per day for six days (60 mg of ATU/kg bm/day for 6 days) [17, 18].

(DEN-TU) group: Rats were injected with DEN and CCl4 as mentioned before. Then they were treated orally with 60 milligram of TU per kilogram body mass per day for six days (60 mg of TU/kg bm/day for 6 days)[17, 18].

(PJ) group: rats were treated orally with a daily dose one milliliter of PJ per kilogram body mass for five weeks(1mL PJ/kg bm for 5 weeks) [5, 19, 20].

(PJ-DEN) group: rats were treated orally with a daily dose one milliliter of PJ per kilogrm body mass for one week. At the beginning of the second week, rats were treated with DEN (as described previously) in addition to the PJ treatment. Then rats were treated orally with PJ for six days (as described). [5, 17, 19, 20].

At the end of the experimental period, feeding was stopped 12 hours prior to killing. Rats were anaesthetized by diethylether and killed. Kidneys were removed immediately, and small portions were fixed in 10% formalin for histopathological examination. The remaining kidney tissues were washed with cold saline solution (0.9% NaCl), weighed, divided into four parts and kept at -80 ^{o}C until used for determination of DNA fragmentation (DNAF), caspase-3 activity, NO level, Lipid peroxidation. Unheparinized blood samples were collected, kept at room

temperature for 15 min and then sera were separated by centrifugation at 3000 revolutions per minute (rpm) at 2°C for 20 min. Sera were stored at -30 °C until used for the determination of kidney function test.

Ⅲ. Biochemical Assay

Caspase-3 assay (*EC 3.4.22.56*): Caspase-3 activity was determined using a colorimetric kit according to the method of [21]. kidney tissues were homogenized in four volumes of cold cell lysis buffer {50 millimolar (mM) Tris-HCl buffer containing 0.2 molar(M) NaCl and 1% Triton X-100, pH 6.8} using a Teflon glass homogenizer. The homogenates were centrifuged at 44.720 gram for 3min at 4 °C, and the supernatants were kept at – 80 °C. The supernatant 50 microliter equal 150 microgram of protein (50 µL = 150 µg protein) was put in a microplate reader, then 50 microliter (µL) reaction buffer and 5 microliter (µL) of 4 millimolar (mM) 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 nanometer (nm) 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.

DNA fragmentation: DNAF was determined in the kidney homogenate using agarose gel electrophoresis according to the method of [22]. kidney tissues were homogenized in 1:5 weight 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 µg/lane DNA) was separated by electrophoresis on 1% agarose gel containing 25 microliter (µL) ethidium bromide at five volt per one centiliter (5 V/1 cm) for 2–3 hours, and visualized under UV light using a multiband transilluminator from Consort (Turnhout, Belgium).

NO level: It was determined spectrophotometrically[23].

Malondialde-hyde levels:

Lipid peroxidation was determined calorimetrically by measuring the level of MDA, the end product of lipid peroxidation, according to the method of [24]. Fifty microliters of the crude homogenate or homogenizing buffer (blank) were incubated with 100 microliter (µL) of 8.1% of SDS, 750 microliter (µL) of 20% acetic acid containing HCl, pH 3.5, 750 microliter (µL) of 0.8% TBA, and 300 microliter (µL) of distilled water in boiling water bath for 45min. After cooling at room temperature, 500 microliter (µL) of distilled water and 2.5 milliliter (mL) of n-butanol/pyridine mixture (15:1 volume per volume, v/v) were added, mixed well, and centrifuged for 10 min at 1780 gram. The absorbance of the pink color was measured at 532 nanometer (nm) and the concentration of MDA was determined as nanomole per gram (nmol/g) kidney. Different concentrations of TMP (20–300 nanomole , nmol) were used as standard and treated in a similar way as the sample.

Kidney function test: Creatinine, urea and uric acid concentrations were determined according to the methods of [25, 26, 27] respectively.

Histopathological study: kidney tissues were fixed, processed and embedded in paraffin wax. Sections of 5 micromolar µm in thickness were cut and stained with hematoxylin and eosin.

Statistical analysis: All data are presented as means (X) ± standard deviation (S.D). Comparisons between the means of various treatment groups were analyzed using least significant difference (LSD) 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

CASPASE-3 ACTIVITY: The enzyme levels in control(C) were 0.21 ± 0.02 lower than that in DEN group 0.67 ± 0.02; $p< 0.05$. The enzyme levels in PJ group were 0.23 ± 0.01 compared to C; $p< 0.05$. The enzyme levels in DEN-5FU, DEN- ATU, DEN-TU and PJ-DEN were 0.44 ± 0.03, 0.41 ± 0.01, 0.29 ± 0.03 and 0.25 ± 0.03 respectively compared to DEN; $p< 0.05$. (Table.1)

DNA FRAGMENTATION (DNAF) IN KIDNEY TISSUE: The agarose gel electrophoresis showed very low or undetectable DNA laddering (DNAF) in the kidney tissue of the control. The DNA intact band appears to be condensed near the application point with no DNA smearing suggesting no DNAF. DEN administration and CCl_4 resulted in a massive DNAF compared to the C group. Treatment with PJ alone shows no DNA smearing suggesting no DNAF compared to control. Treatment with PJ pre, during, and post DEN and CCl_4

administration decreased DNAF compared to the DEN group. Treatment with 5FU and TU and ATU after administration of DEN and CCl4 decreased DNA fragmentation as compared with DEN group "Fig. 1,a and b".

NO LEVEL: NO levels in control(C) were 31.21 ±3.95 µm lower than that in DEN group 66.82 ±3.20µm; $p< 0.05$. NO levels in PJ group were 29.98 ±3.89 µm compared to C; $p< 0.05$. NO levels in DEN-5FU, DEN- ATU, DEN-TU and PJ-DEN were 35.53 ±3.76 µm, 36.43 ±3.71 µm, 34.49 ±3.66 µm and 32.01 ±3.75 µm respectively compared to DEN; $p< 0.05$. (Table.1)

MALONDIALDE-HYDE LEVELS: MDA levels in control(C) were 2.91 ± 1.10 nmol/g lower than that in DEN group 14.16 b ± 1.08 nmol/g; $p< 0.05$. MDA levels in PJ group were 3.01 ± 1.00 nmol/g compared to C; $p< 0.05$. MDA levels in DEN-5FU, DEN- ATU, DEN-TU and PJ-DEN were 23.12 ± 0.56 nmol/g, 7.39 ± 0.45 nmol/g, 10.14 ± 1.47 nmol/g and 5.11 ± 1.30 nmol/g respectively compared to DEN; $p< 0.05$. (Table.1)

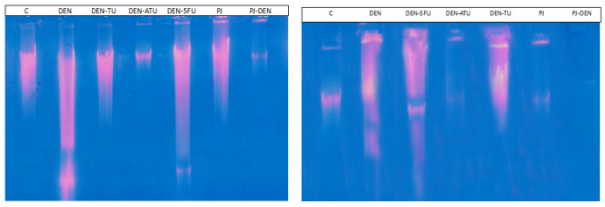
KIDNEY FUNCTION: Creatinine, urea and uric acid concentrations were increased significantly after administration of DEN and CCl4 (1.22 ± 0.12mg/dl, 39.85 ± 4.34g/dl, 3.90 ± 0.49mg/dl) respectively as compared to the C (0.89 ± 0.04mg/dl, 28.54 ± 3.82g/dl, 2.10 ± 0.11mg/dl); $p< 0.05$ (Table 1). Creatinine, urea and uric acid concentrations were (0.88 ± 0.03mg/dl, 29.35 ± 3.84g/dl, 2.10 ± 0.11mg/dl) in PJ group compared to C; $p< 0.05$(Table 1). Creatinine concentrations in DEN-5FU, DEN- ATU, DEN-TU and PJ-DEN were 1.04 ± 0.04mg/dl, 1.01 ± 0.06 mg/dl, 0.94 ± 0.07 mg/dl and 0.90 ± 0.05 mg/dl respectively compared to DEN; $p< 0.05$. (Table.1). Urea concentrations in DEN-5FU, DEN- ATU, DEN-TU and PJ-DEN were (62.32 ± 6.01g/dl, 39.39 ± 3.80g/dl, 37.99 ± 2.51g/dl, 31.24 ± 2.78g/dl) respectively compared to DEN; $p< 0.05$. (Table.1). Uric acid concentrations in DEN-5FU, DEN- ATU, DEN-TU and PJ-DEN were (2.0 ± 0.28mg/dl, 1.74 ± 0.07mg/dl, 1.53 ± 0.26mg/dl, 2.1 ± 0.11mg/dl) respectively compared to DEN; $p< 0.05$. (Table.1)

Table 1. Biochemical assay of different studied groups

Particulars	C	DEN	DEN-5FU	DEN-ATU	DEN-TU	PJ	PJ-DEN
Tissue							
Caspase–3 activities)	0.21 ^{a} ± 0.02	0.67 ^{b} ± 0.02	0.44 ^{c} ± 0.03	0.41 ^{d} ± 0.01	0.29^{e} ± 0.03	0.23 ^{a} ± 0.01	0.25 ^{a} ± 0.03
NO concentration (µm) in kidney tissues	31.21 ^{a} ±3.95	66.82 ^{b} ±3.20	35.53 ^{c} ±3.76	36.43 ^{c} ±3.71	34.49 ^{ac} ±3.66	29.98 ^{a} ±3.89	32.01 ^{a} ±3.75
Malondialdehyde (MDA) levels (nmol/g tissue	2.91^{a} ± 1.10	14.16 ^{b} ± 1.08	23.12 ^{c} ± 0.56	7.39 ^{d} ± 0.45	10.14 ^{e} ± 1.47	3.01^{a} ± 1.00	5.11^{a} ± 1.30
Serum							
Creatinine (mg/dl)	0.89 ^{a} ± 0.04	1.22 ^{b} ± 0.12	1.04 ^{c} ± 0.04	1.01 ^{bc} ± 0.06	0.94 ^{a} ± 0.07	0.88 ^{a} ± 0.03	0.90 ^{a} ± 0.05
Urea (g/dl)	28.54 ^{a} ± 3.82	39.85 ^{b} ± 4.34	62.32 ^{c} ± 6.01	39.39 ^{b} ± 3.80	37.99 ^{b} ± 2.51	29.35 ^{a} ± 3.84	31.24 ^{a} ± 2.78
Uric acid (mg/dl)	2.10 ^{a} ± 0.11	3.90 ^{b} ± 0.49	2.0 ^{ac} ± 0.28	1.74 ^{cd} ± 0.07	1.53 ^{d} ± 0.26	2.10 ^{a} ± 0.11	2.1 ^{a} ± 0.11

Legends

Group C - control rats; group DEN – rats treated with DEN and CCl4; group (DEN-5FU)–rats treated with 5FU post DEN and CCl4 administration. (DEN-ATU)–rats treated with ATU post DEN and CCl4 administration. (DEN-TU)–rats treated with TU post DEN and CCl4 administration. (PJ) rats treated with pomegranate juice alone. (PJ-DEN) rats treated with pomegranate juice before, during and post DEN and CCl4 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 $p<0.05$.



a b

DNA fragmentation separating in 1% agarose gel electrophoresis and visualized under UV. DNAF in C - control rats; group DEN – rats treated with DEN and CCl_4; group (DEN-5FU)–rats treated with 5FU post DEN and CCl_4 administration. (DEN-ATU)–rats treated with ATU post DEN and CCl_4 administration. (DEN-TU)–rats treated with TU post DEN and CCl_4 administration. (PJ) rats treated with pomegranate juice alone. (PJ-DEN) rats treated with pomegranate juice before, during and post DEN and CCl_4 administration.

Fig. 1. (a,b) DNA fragmentation (DNAF) in kidney tissue

Histopathologic Results

Histopathological examination of control showed normal rat kidney with no remarkable pathologic changes "Fig.2a". Treatment with DEN and CCl_4 showed unremarkable glomeruli. Proximal convolutes tubules showed mild severe cloudy swelling. Lumina of tubules were narrowed. Some were star shaped. Some cells lost their apices "Fig.2b". Treatment with PJ alone showed no pathological change "Fig.2c". Treatment with 5FU after DEN and CCl_4 administration showed unremarkable glomeruli. Proximal convolutes tubules showed severe cloudy swelling "Fig.2d". Treatment with ATU after DEN and CCl_4 administration showed unremarkable glomeruli. Proximal convolutes tubules showed moderate cloudy swelling "Fig.2e". Treatment with TU after DEN and CCl_4 administration showed unremarkable glomeruli. Proximal convolutes tubules showed mild cloudy swelling "Fig.2f,". Treatment with PJ before, during and after DEN and CCl_4 administration showed slightly tubular cell swelling "Fig.2g".

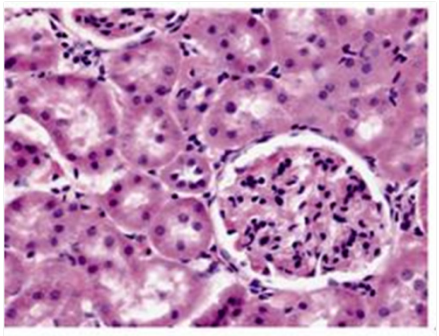


Fig.2a. Kidney histopathological examination of Control group. Higher magnification showed no pathological change. (H and E stain ×400)

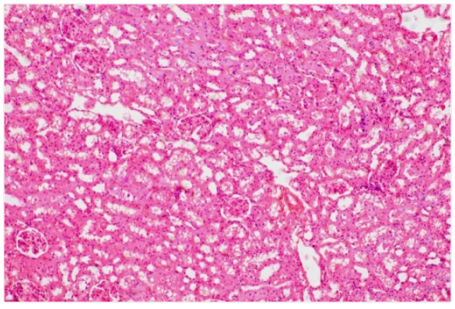


Fig.2b. Kidney histopathological examination of DEN group. Higher magnification showed unremarkable glomeruli. Proximal convolutes tubules showed mild sever cloudy swelling. Lumina of tubules were narrowed some were star shaped. Some cells lost their apices (H&E, x200).

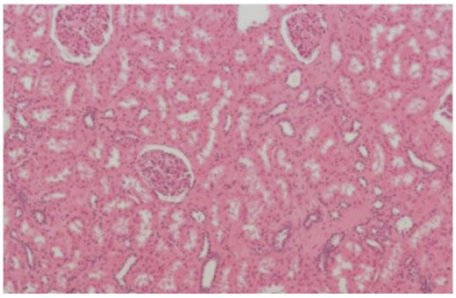


Fig.2c. Kidney histopathological examination of (PJ) group. Higher magnification showed no pathological change (H and E stain, ×200).

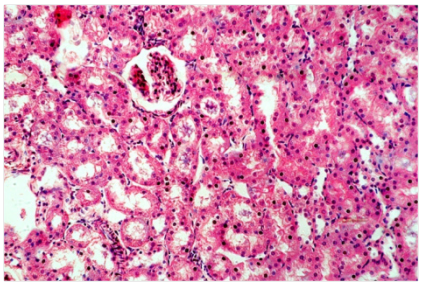


Fig.2d. Kidney histopathological examination of (DEN-5FU) group. Higher magnification showed unremarkable glomeruli. Proximal convolutes tubules showed sever cloudy swelling. Lumina of tubules were narrowed some were star shaped. Some cells lost their apices (H&E, x200).

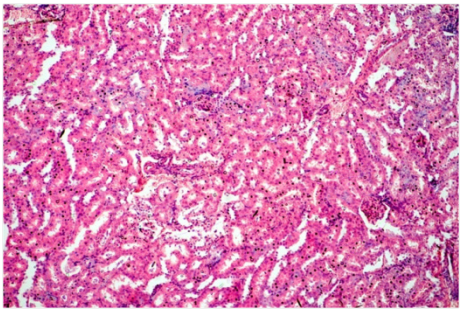


Fig.2e. Kidney histopathological examination of (DEN-ATU) group. Higher magnification showed unremarkable glomeruli. Proximal convolutes tubules showed moderate cloudy swelling. Lumina of tubules were narrowed some were star shaped. Some cells lost their apices.(H&E, x200).

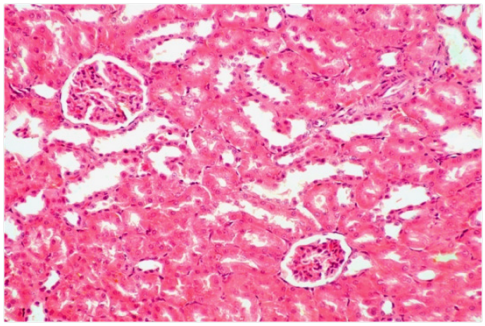


Fig.2f. Kidney histopathological examination of (DEN-TU) group. Higher magnification showed mild cloudy swelling (H&E, x200).

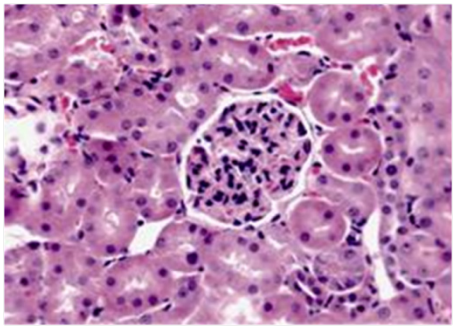


Fig.2g. Kidney histopathological examination of (PJ-DEN) group. Higher magnification showed slightly tubular cell swelling (H and E stain, ×400)

4. Discussion

Kidneys are pivotal in the elimination of numerous xenobiotics, including drugs and environmental chemicals, as well as endogenous metabolites. Kidneys have developed transport systems to prevent urinary loss of filtered nutrients, such as glucose, oligopeptides, and inorganic ions, as well as to facilitate the elimination of a variety of xenobiotics [28]. Reactive oxygen species (ROS) are generated during the detoxification of xenobiotics and drugs, and cause oxidative stress. Oxidative stress has been shown to be linked to kidney toxicity and diseases. Hence, it is associated with damage to a wide range of macromolecular species, including lipids, proteins, and nucleic acids. MDA is a major oxidation product of peroxidized polyunsaturated fatty acids, and increased MDA content is an important indicator of lipid peroxidation [5, 29]. The present study has shown a significant elevation in MDA and NO, creatinine, urea and uric acid levels after DEN and CCl_4 administration. In addition, DEN and CCl_4 administration led to elevation in caspase-3 activity and DNAF levels compared to the control group. Formation of lipid peroxides in the crude homogenates resulted in response to the administration of DEN and CCl_4. This may be due to an enhanced generation of superoxide radicals ($O2^{-}$) and Hydrogen peroxide radicals 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 due to alteration in the inner membrane potential [5, 29]. In addition, some reactive oxygen species (ROS) interact with various tissue compounds leading to dysfunction and injury to the kidney and other organs [5, 29]. Elevation in caspase- 3 activities and DNAF after DEN and CCl_4 administration indicates that DEN and CCl_4 caused apoptosis for kidney tissues, and apoptosis is an adaptive process of combating excessive damage. However, elevated apoptosis within a diseased kidney should not always be viewed as harmful. The apoptotic deletion of infiltrating neutrophils in glomerulonephritis limits neutrophil-mediated glomerular injury and may play 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 [3]. Our results illustrate that the endonuclease-dependent fragmentation shows mixed smearing and laddering DNA fragments, indicating that exposure to DEN generates ROS, which trigger DNA damage causing cell death by necrosis and apoptosis. Otherwise, the histopathologic results show that treatment with DEN and CCl_4 showed unremarkable glomeruli. Proximal convolutes tubules showed mild severe cloudy swelling. Lumina of tubules were narrowed. Some were star shaped. Some cells lost their apices.

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 [30]. Ethylcarbonium ions bind to DNA forming adducts and generate superoxide radicals through lipid peroxidation of phospholipid membrane fatty acids [31]. DEN also induces iNOS gene expression and generates NO radicals which react nonenzymatically with superoxide radicals forming peroxynitrite ($ONOO^-$ a reactive nitrogen species). $ONOO^-$ can induce protein oxidation since it reacts with susceptible amino acids, including arginine, cysteine, histidine, and lysine through a carbonylation process as well as it can react with aromatic amino acids forming 3-nitro aromatic amino acids [30].

Otherwise the inflammation of kidney in the present study may be related to the deleterious effect of CCl_4 itself and/or its metabolites. Trichloromethyl free radicals, a metabolite of CCl_4, combine with cellular lipids and proteins in the presence of oxygen and forming trichloromethyl peroxyl radicals, which attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical [32]. Thus, trichloromethyl peroxyl free radical leads to elicitation of lipid peroxidation and destruction of Ca^{2+} homeostasis, resulting in cell death [17]. The products of lipid peroxidation are considered mutagenic and carcinogenic as they cause damage to cellular macromolecules by generating ROS [17].

Treatment with 5FU after DEN and CCl_4 administration showed slightly decreases in the levels of caspase-3, DNAF, NO as well as serum creatinine, and uric acid levels but showed significant increases in the urea concentration and MDA level as compared to the DEN group, this result may be related to fluoride ion (F^-). This indicates that 5-FU and its metabolites induced lipid peroxidation [33]. Fluoride is concentrated to high levels within the kidney tubules [34]. "Kobayashi et al., (2004) [35] "illustrated that, the main mode of elimination of 5-FU is via renal glomerular filtration, but it is also eliminated via proximal tubule cells [36]. It has been reported that treatment with 5-FU results in the induction of proximal tubular cell necrosis. However, "Kuriyama et al., (1984) [37] "reported that, there is no report concerning the molecular mechanism of 5-FU-induced nephrotoxicity. "Hotta et al., (2005) [38] " found that, an evaluation of liver damage, renal damage, and glucose tolerance; serum alanine aminotransferase level, serum total bilirubin (T.Bil) level, and serum creatinine level during treatment with leucovorin (LV)/5-fluorouracil. 5-FU is metabolized in tissues to its active form, 5-fluoro-deoxyuridinemonophosphate, which inhibits thymidylate synthase. 5-FU is also catabolized primarily in the liver, as dihydrouracil, and the reduced compound is then cleaved to α-fluoro-β-alanine, ammonia, urea, and carbon dioxide which cause the hepatic and nephrotoxicity. The toxicity of 5-FU may be decreased if its catabolism is blocked by inhibiting dihydrouracil dehydrogenase [39]. Treatment with 5FU after DEN and CCl_4 administration in present study showed proximal convoluted tubules with severe cloudy swelling and unremarkable kidney glomeruli as compared to DEN group which showed mild to severe cloudy swelling.

The antithyroid and melanoma targeting properties of TU and ATU have been related to their inhibitory effects on distinct enzymatic activities, including thyroid iodide peroxidase, myeloperoxidase, eosinophil peroxidase, nNOS and tyrosinase [40]. Treatment with ATU and TU separately after DEN and CCl_4 administration showed decreases kidney caspase-3, DNAF, NO, MDA as well as creatinine, urea, and uric acid levels as compared to the DEN group. This means that ATU and TU decreased the kidney damage induced by DEN and CCl_4. These results agree with other studies who reported that, the antithyroid drugs which have a free SH group protect against acute nephrotoxicity in vivo [41]. Our histopathological results showed that treatment with ATU and TU separately after DEN and CCl_4 administration showed proximal convoluted tubules with moderate and mild cloudy swelling respectively and unremarkable kidney glomeruli as compared to DEN group which showed mild to severe cloudy swelling. The antioxidant effect may be due to the presence of organosulfur [42]. In case of ATU

treatment, MDA level was lower than that caused by TU treatment. This indicates that TU has a prooxidant activity than ATU probably due to the presence of basic amino group at C6 in ATU which decrease the oxidation process.

Administration of PJ alone in the present study showed that there was no change in kidney caspase-3, DNAF, NO, MDA as well as creatinine, urea, and uric acid levels compared to the control group. Treatment with PJ alone showed no histopathological changes as compared to C. 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 [5]. 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 [43]. In addition, pomegranate juice administration in rats for 37 days has no toxic effect [19, 20].

Treatment with PJ pre, during, and post DEN and CCl_4 administration showed a reduction in the levels of kidney caspase-3, DNAF, NO, MDA as well as creatinine, urea, and uric acid levels compared with the DEN group, This means that polyphenolic compounds in PJ play an important 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. “Lazze´ et al, 2003 [44]” confirmed the protective role of anthocyanins and their derivatives against lipid peroxidation, apoptosis, and DNA damage in rat smooth muscle and hepatoma cells induced by tertiary-butyl hydroperoxide. Polyphenols are important metabolic modulators by virtue of their ability to moderate and influence several cellular processes such as signaling, proliferation, apoptosis, redox balance, and so on [45]. The antioxidant potentials of PJ are attributed to their high polyphenolic contents and their variation [46]. The antioxidant effects of phenolic compounds of PJ are more potent than many other antioxidant compounds [46] (such as vitamins C and E), and are able to scavenge ROS and consequently reduced the MDA level [47]. Punicalagin, one of the ellagitannins, is responsible for more than 50% of the antioxidant activity of the PJ [48]. In addition, ellagic acid decreases both the total hepatic CYP-450 and CYP2E1 that lead to alteration in the DEN metabolism. Chlorogenic acid, caffeic acid, and some nonpolyphenolic compound in PJ such as serotonin are good inhibitors of the n-nitrosation reaction. 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 [49]. Hydroalcoholic extract of flowers of P. granatum has ameliorative potential in attenuating myoglobinuric renal failure and its renoprotective effects involve activation of PPAR-γ and nitric oxide-dependent signaling pathway [50]. The biochemical investigations of the present study were confirmed with the histopathologic results, since the treatment with PJ pre, during, and post DEN and CCl_4 administration reduced the toxicity of DEN. Therefore, DEN and CCl_4 showed unremarkable glomeruli. Proximal convolutes tubules showed mild sever cloudy swelling. Lumina of tubules were narrowed. Some were star shaped.

5. Conclusion

DEN and CCl_4 induced inflammation, apoptosis and toxicity for kidney tissues. The effects of TU and ATU on kidney function results are better than that of 5-FU but not reached to the control group.5-FU induced kidney damage which is higher than that induced by DEN and CCl_4. This due to the toxic effect of 5-FU and Fluoride ion

which resulted from its accumulation in the kidney. The toxic effects of ATU and TU are lower than that of 5-FU, this may be related to their metabolites which have no side effects beside they are not accumulate in kidney. Pomegranate juice administration in rats for 5 weeks has no toxic effect on kidney tissues. The present study showed that PJ extract exerts a significant protective effect against DEN and CCl_4 induced oxidative stress and apoptosis in kidney by augmenting host antioxidant defense mechanisms. This extract is a promising agent for the prevention of chemical induced toxicity through enhancing the antioxidative and drug metabolizing enzymes, as well as lowering the extent of lipid peroxidation. The treatment with PJ on kidney toxicity results are better than that of 5-FU, TU and ATU and results reached to the control group.

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