INDO AMERICAN JOURNAL OF PHARMACEUTICAL RESEARCH

PUNICA GRANATUM MITIGATES 7, 12-DIMETHYLBENZ[A]ANTHRACENE AND CCL_4-INDUCED OXIDATIVE STRESS AND HEPATIC PRECANCEROUS LESIONS IN WISTAR RATS

Osama M. Ahmed[1*], Mohamed B. Ashour[1], Hanaa I. Fahim[1], Sameh F. AbouZid[2], Ahmed R. G. [3], Mohamed A. Abdel Gaid[1]

[1]*Division of Physiology, Zoology Department, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt.*
[2]*Pharmacognosy Department, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt.*
[3]*Comparative Anatomy and Embryology Division, Zoology Department, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt.*

ARTICLE INFO

Article history
Received 09/07/2016
Available online
30/09//2016

Keywords
Pomegranate,
Oxidative Stress,
Liver Injury,
Pre-Cancerous Oval Cells,
Rats.

ABSTRACT

This study is designed to assess the preventive effects *Punica granatum* aril juice, aqueous extracts of seeds and husk and their mixture on the hepatic injury and hepatocarcinogenesis induced by 7,12-dimethylbenz(a)anthracene (DMBA) and carbon tetrachloride (CCl_4) administration in male Wistar rats. DMBA/CCl_4-administered rats were orally treated with *Punica granatum* aril juice at 10 ml/kg b.w./day, seeds and husk aqueous extracts at 400 mg/kg b.w./day and their mixture for 16 weeks to rats. The treatments with *Punica granatum* aril juice, seeds and husk aqueous extracts successfully attenuate the deleterious effects of DMBA/CCl_4 on serum ALT, AST, ALP and GGT activities as well as serum total bilirubin, albumin and globulin levels. The elevated oxidative stress and the deteriorated antioxidant defense system were markedly improved by treatments. The increased mRNA expressions of hepatic NF-κB, TNF-α and COX-2 in liver of DMBA/CCl_4-administered rats were significantly decreased by treatments. In contrast, the lowered p53 and Bcl-2 were significantly increased. The liver histological lesions, represented by inflammatory cell infiltration, necrosis of hepatocytes and emergence of pre-cancerous oval cells, in DMBA/CCl_4-administered rats, were amended by treatments with *Punica granatum* aril juice, seeds and husk extracts and their mixture. In conclusion, *Punica granatum* aril juice, aqueous extracts of seeds and husk and their mixture successfully mitigate DMBA and CCl_4-induced liver deleterious changes and precancerous lesions *via* their anti-oxidant, anti-inflammatory and anti-apoptotic actions.

Corresponding author
Osama M. Ahmed
Division of Physiology, Zoology Department,
Faculty of Science, Beni-Suef University, Egypt.
osamamoha@yahoo.com;
osama.ahmed@science.bsu.edu.eg
00201001084893

*Please cite this article in press as **Osama M. Ahmed** et al. Punica Granatum Mitigates 7, 12-Dimethylbenz [A] Anthracene and Ccl_4-Induced Oxidative Stress and Hepatic Precancerous Lesions in Wistar Rats. Indo American Journal of Pharmaceutical Research.2016:6(09).*

INTRODUCTION

Liver plays a major role in the detoxification and excretion of many endogenous and exogenous compounds [1]. Liver injury due to systemic drugs, food preservatives, agrochemicals and addiction to alcohol or impairments of its functions may lead to many complications in the health [2]. Hepatocellular carcinoma (HCC) is the fifth most common malignancy in men and the eighth in women worldwide and remains as one of the most lethal malignancies worldwide [3,4]. It accounts for as many as 600,000 deaths annually world-wide [5]. HCC is a complex disease associated with many risk factors and cofactors [6,7]. The etiology of HCC includes chronic infection with hepatitis B and C viruses, cirrhosis, and exposure to dietary and environmental hepatocarcinogens [8].

One of the environmental carcinogenic polycyclic aromatic hydrocarbons is 7,12-dimethylbenz[a]anthracene (DMBA) [9]. DMBA is one of the most chemical substances that actually cause tumors in one or more body organs [10-13]. It has been used to initiate animal model carcinogenesis in many organs as HCC [14], mammary carcinoma [15] and skin cancer [16]. Metabolism of DMBA by the oxidases enzymes often results in the formation of oxyradicals which bind to nucleophillic sites on cellular macromolecules thereby eliciting cancerous responses [17].

The carcinogenic actions of DMBA can be augmented by carbon tetrachloride (CCl_4) which was used in literatures as cytotoxic agent and used by others as cancer promoter [18,19]. CCl_4 biotransformation in the liver involves production of the highly lethal trichloromethyl free radical ($^{\bullet}CCl_3$) and peroxy trichloromethyl ($^{\bullet}OOCCl_3$) free radical through activation by drug metabolizing enzymes located in the endoplasmic reticulum [20,21]. The exacerbated oxidative stress is considered to be a direct cause of many pathological conditions such as liver damage and cancer [22-24] as well as inflammation and drug toxicity [25].Furthermore, it has been demonstrated that oxidative stress plays an important role in hepatocarcinogenesis [26].

Pomegranate, *Punica granatum* (*P. granatum*), belonging to family punicaceae is rich in antioxidant of polyphenolic class which includes tannins [27] and flavonoids [28]. *P. granatum* has been extensively used as a folk medicine by many cultures [29]. *P. granatum* juice (PJ) has been proposed as chemopreventive, chemotherapeutic, antiatherosclerotic and anti-inflammatory [30,31]. As well, *P. granatum* peel (husk) extract was utilized in Egyptian culture, as stated by Ismail *et al*. [32] in several common ailments such as inflammation, intestinal worms, diarrhea, cough and infertility. Major constituents in *P. granatum* pericarp (peel) are phenolic punicalagins, catechin, gallic acid, quercetin, rutin, flavonols, flavones, flavonones and anthocyanidins [33]. *P. granatum* fruit, juice and peel possess marked antioxidant, proapoptotic and antitumor efficacies which may be attributed to their polyphenolic constituents [34-37]. Various mediators of carcinogenesis are inhibited by the *P. granatum* active ingredients in *vitro*, for example, vascular endothelial growth factor [38], insulin-like growth factors [39] and cytokine stimulated NF-κB [40]. The most researches have also been directed towards using of total plant extracts mainly because of the synergistic effects of the mixture of plant metabolites and the multiple points of intervention in tumor chemoprevention. Tzulker *et al*. reported that the homogenates prepared from the whole fruit exhibited an approximately 20-fold higher antioxidant activity than the level found in the arils or seed sacs (fleshy or brightly colored cover of seed) juice [41], and the phenolic profile differs greatly from the two resources [42].

Therefore, this study was designed to assess the anticarcinogenic effects of *P. granatum* aril juice, seeds and husk extracts and their mixture on DMBA-initiated and CCl_4-promoted hepatocarcinogenesis.

MATERIALS AND METHODS

Experimental animal

Sixty male albino rats of Wistar strain weighing approximately 100-130 gram were used in this study. After two weeks of acclimatization period, the animals were subsequently divided to six groups ten in each group and housed in clean polypropylene cages and maintained in an air-conditioned animal house at temperature (20°C) with natural alternating light and dark cycles. The animals were supplemented with standard pellet diets and water *ad libitum*. All animal procedures are in accordance with rules and regulations of Experimental Animals Ethics Committee of Zoology Department, Faculty of Science, Beni-Suef University, Egypt. All efforts were done to decrease the suffering of animals.

Chemicals

Carcinogenic agent, 7,12-dimethylbenz(a)anthracene (DMBA) and mineral oil was purchased from Sigma Aldrich Company 3050 Spruce St. Saint-Louis, MO, United States of America. Carbone tetra-chloride (CCl_4) was purchased from Laboratory Chemical Trading Company for laboratory fine chemicals, Egypt. All other chemicals used in this experiment with high analytical grade.

Doses preparations and animals treatment

P. granatum fruits were purchased from the local market in Beni-Suef, Egypt. *P. granatum* fruits were washed with distilled water and manually peeled and fresh *P. granatum* crude juice (PJ) was prepared from the arils or seed sacs (fleshy or brightly colored cover of seed). Seeds and husk (rind; pericarp) were collected and dried in the shadow then powdered separately with a mechanical grinder. The obtained powders of seeds and husk were extracted in boiled water for fifteen minutes then filtered in clean bottles as infusion of *P. granatum* seeds extract (PSE) and *P. granatum* husk extract (PHE). *P. granatum* mixture of extracts and juice (PM) was prepared by adding equal volumes of juice, *P. granatum* seeds extract and *P. granatum* husk extract. DMBA solution prepared as 50 mg/kg body weight (b.w.) in mineral oil [43] was orally gavaged to five groups (each of ten rats) as carcinogenesis initiator. One group of them served as DMBA/CCl_4control and the other four groups were separately treated with PJ, PSE, PHE and PM preparations. The normal control group was administered mineral oil, saline and distilled water as vehicles of DMBA, $CCl_4$and *P. granatum* respectively.

After three weeks of DMBA intake, the five DMBA-administered groups were injected CCl_4 solution subcutaneously as carcinogenesis promoter at dose level of 3 ml/kg b.w./week [18] in the thoracic area for thirteen weeks. *P. granatum* juice (PJ) and mixture of aqueous extracts and juice (PM) were orally gavaged at dose level of 10 ml/kg b.w. according to Adukondalu *et al*. [44] and Patel *et al*. [45] daily for sixteen weeks after DMBA administration. As well, PSE and PHE were daily given at 400mg (infused 10 ml distilled water)/kg b.w. [46,47] for the same period.

Sampling

At the end of sixteenth week of DMBA administration and thirteenth week of CCl_4 injection, all groups were sacrificed. Blood samples were collected from jugular arteries and clear non-haemolysed serum were immediately separated and frozen at -30°C. Liver tissues were quickly removed then part of liver of each animal was fixed in neutral buffer formalin for histopathological studies. An additional part of liver was kept in sterilized tubes and frozen at -70°C till used for molecular studies. Another 0.5 gm of the liver of each rat was homogenized in 5 ml 0.9% sodium chloride solution. Homogenates were centrifuged at 3000 r.p.m. and separated supernatants were frozen till used for oxidative stress and antioxidants defense system markers measurement.

Biochemical analysis

Serum ALT and AST activities were measured using reagent kits purchased from Biosystems S.A (Spain) according to the methods of Gella *et al*. [48] and Young [49] respectively. Alkaline phosphatase (ALP) and total proteins were determined using reagent kits purchased from BIOMED Diagnostic (EGY-CHEM for lab technology), Egypt according to methods of Henry [50] and Vassault *et al*. [51] in the same order. Gamma-glutamyltransferase (γ-GT), total bilirubin and Albumin levels were measured using reagent kits purchased from Spectrum Company for biotechnology, Egypt according to the methods of Szasz *et al*. [52], Balistreri *et al*. [53] and Doumas *et al*. [54] respectively.

Oxidative stress and antioxidant defense parameters

GSH content was determined according to Beutler *et al*. [55] chemical method by using tissue homogenate supernatant instead of blood samples and readjusted the volumes. GST activity in liver homogenate was measured according to Mannervik and Gutenberg [56]. GPx activity was determined according to Matkovics *et al*. [57]. SOD was identified according to Marklund and Marklund [58] method. Total antioxidants capacity was measured using assay kit purchased from ABCAM Campany (El Emam Aly St., Heliopolis, Cairo, Egypt) and according to Csillag *et al*. [59]. Lipid peroxidation was determined according to the method of Preuss *et al*. [60]. Nitric oxide (NO) was measured according to Montgomery and Dymock [61] method using reagent kit purchased from BioDiagnostic Company 29 Tahreer St. Dokki, Giza, Egypt.

RNA isolation and gene's expression identification

Purification of RNA was carried up with Gene JET RNA Purification kits (Thermo Scientific Company, Thermo Fisher Scientific Inc. NYSE:TMO). Determination of RNA was carried up with UV spectrophotometer at 260nm and 280nm, then absorbance at 260nm was used for detection of total RNA quality and concentration. RNA concentration was calculated according to Sambrook and Russell formula [62]:

RNA (µg/µl) = absorbance at 260 nm × dilution × 40µg/ml /1000

For each tested sample, the ratio between the spectrophotometric readings at 260 nm and 280 nm (OD260/OD280) was used to provide an estimate of the purity of RNA, and the ratio in all samples ranged between 1.7 and 2.0.

Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was carried up with thermo cycler PCR (Pioneer, MYGenie 32 Thermal Block) using single step (Verso 1-Step RT-PCR ReddyMix kit) purchased from Thermo Scientific (Thermo Fisher Scientific Inc. NYSE:TMO). Primers of the tested genes of NF-κB, TNF-α, COX-2, IL-4, p53 and Bcl-2 are purchased from Biosearch Technologies (USA). The Sequence of the primers of Wister rats were represented in table 1.

Table 1: The sequences of forward and reverse primers of selected genes.

Gene	Forward primer	Reverse primer	References
β-actin (housekeeping gene)	5`TCACCCTGAAGTACCCCATGGAG3`	5`TTGGCCTTGGGGTTCAGGGGG3`	Shaker and Abdel-Halim [63]
NF-κB	5`TACCATGCTGTTTGGTTCA3`	5`TCAAGCTACCAATGACTTTC3`	EL-Swefy and Hassanen [64]
TNF-α	5`GCTGAGGTTGGACGGATAAA3`	5`AAAATCCTGCCCTGTCACAC3`	Sreeja *et al*. [65]
COX-2	5`GCTTTCTCCAACCTCTCCTACTACA3′	5`CATGGGAGTTGGGCAGTCA3`	Mohamed *et al*. [66]
IL-4	5`GGAACACCACGGAGAACG3`	5`GCACGGAGGTACATCACG3`	Zhou *et al*. [67]
p53	5`CAGCGTGATGATGGTAAGGA3`	5`GCGTTGCTCTGATGGTGA3`	Asiri [68]
Bcl-2	5`GGGATGCCTTTGTGGAACTA3`	5`CTCACTTGTGGCCCAGGTAT3`	Ashok and Sheeladevi [69]

Reddy mix (2x 1-step PCR) contains reaction buffer which has been optimized to allow both reverse transcription and PCR amplification to occurin the same reaction across a wide range of templates. RT enhancer was also included to remove contaminating DNA, eliminating the need for DNAase treatment. It degrades double stranded DNA during transcription of RNA and is inactivated after 2 minutes at 95°C while genes amplification started.

For 50 µl reaction volume, verso enzyme mix at 1 µl, 25 µl of 2x 1-step PCR reedy mix, 2.5 µl of RT enhancer, 1 µl of forward primer (10 µM) and 1 µl of reverse primer (10 µM) followed by 2 µl template RNA were mixed in sterilized PCR tubes. Then, the reaction mixture was completed with nuclease-free water. PCR tubes transferred to thermocycler PCR. The PCR program was adjusted at 50°Cfor 15 minutes, only one cycle for DNA synthesis, 95°C for 2 minutes for verso and RT-enhancer inactivation. After that 95°C for 20 seconds (denaturation) and 50-60°C (according to primer sequence) for 30 second annealing followed by extension at 72°C for one minute; this step repeated 30-35 cycles, then final extension at 72°C for 5 minutes only one cycle was adjusted.

The amplified genes were loaded and electrophoresed on agarose gel at 90 volts then gel transferred to gel documentation unit and photos were taken. Photos of gene bands were analyzed with GelDocu Advanced program from Raya for the Scientific Services, Giza, Egypt, and represented as numbers for statistical analysis.

Histopathological investigations

Animals were sacrificed under mild diethyl ether anesthesia and dissected. Liver was rapidly excised from each rat and then perfused in saline solution. Pieces from the liver were taken and fixed in 10% neutral buffer formalin for twenty four hours. Fixed organs were sent to Histopathology Department of National Cancer Institute (NIC), Cairo University, Egypt, for further processing, blocking in wax, sectioning and staining with haematoxylin and eosin (H&E) according to the method of Bancroft and Gamble [70].

Statistical analysis

Results were expressed as mean ± standard error (SE). PC-STAT was used for data analysis [71]. One-Way Analysis of Variance (ANOVA) followed by LSD at 5% and LSD at 1% to compare between the different groups. Percentage of changes were calculated by comparing DMBA/CCl_4-administered group with normal group and comparing DMBA/CCl_4-administered groups treated with various *P. granatum* infusions and juice with DMBA/CCl_4-administered control group. Values of $P>0.05$ were considered statistically non-significantly different, while values of $P<0.05$, $P<0.01$ and $P<0.001$ were significantly, highly significantly and very highly significantly different respectively.

RESULTS

The obtained data showed a highly significant increase in activities of ALT, AST, ALP and γGT in DMBA/CCl_4-administered rats recording percent changes of63.64%, 64.72%, 75.68% and 220.37% respectively as compared with normal group. These elevations were highly significantly decreased ($P<0.01$; LSD) as a result of treatment DMBA/CCl_4-administered rats with PJ, PSE, PHE and PM. One way ANOVA indicated that the effect between group serum enzymes related to liver function was very highly significant ($P<0.001$; F-probability) throughout the experiment (Table 2).

The total bilirubin and globulin concentrations were highly significantly increased ($P<0.01$; LSD) in DMBA/CCl_4-administered group as compared with normal rats; the recorded percentage changes were 95.89% and 68.07% respectively. In contrast, the concentrations of total and protein albumin levels were decreased ($P<0.01$; LSD); the recorded percentage changes were -20.07 and -63.50respectively. Accordingly, albumin/globulin ratio was vigorously depressed in DMBA/CCl_4-administered rats recording percentage change of -79.51%. The total bilirubin level was significantly ($P<0.05$; LSD) improved in DMBA/CCl_4-administered rats treated with husk extract but it was not significantly affected in juice, seeds extract and mixture treated groups. The lowered total protein level was increased significantly ($P<0.05$; LSD) as a result of treatment of DMBA/CCl_4-administered rats with *P. granatum* seeds extract and mixture. The depleted serum albumin concentration showed a highly significant increase ($P<0.01$; LSD) and returned near to normal values in DMBA/CCl_4-administered rats treated with *P. granatum*. Additionally, globulin level was highly significantly decreased ($P<0.01$; LSD) in juice and husk extract treated groups while it was non-significantly decreased ($P>0.05$; LSD) in seeds extract and mixture treated groups. Albumin/globulin ratio was highly significantly elevated in treated groups ($P<0.01$; LSD).One way-ANOVA depicted that the effect between groups on serum total protein level was significant ($P<0.05$; F-probability) while the effect between groups on total bilirubin, albumin and globulin levels as well as A/G ratio was very highly significant ($P<0.001$; LSD) throughout the experiment (Table 3).

Liver GSH level and total antioxidant capacity (TAC) were highly significantly declined ($P<0.01$; LSD) in DMBA/CCl_4-administered group recording percentage changes of -57.21 and -45.44 respectively. Conversely, GSH level was highly significantly elevated ($P<0.01$; LSD) in DMBA/CCl_4-administered rats treated with the tested agents except for those treated with the mixture. Moreover, TAC was highly significantly increased ($P<0.01$; LSD) in DMBA/CCl_4-administered rats treated with *P. granatum* juice and mixture while it was non-significantly increased ($P>0.05$; LSD) in DMBA/CCl_4-administered rats treated with seeds and husk extracts. On the contrary, liver lipid peroxidation and NO level were highly significantly increased ($P<0.01$; LSD) in DMBA/CCl_4-administered rats recording percentage changes of 89.54 and 62.42% respectively as compared with normal control group. On the other hand, lipid peroxidation in liver of DMBA/CCl_4-administered rats treated with PJ, PSE, PHE and PM was highly significantly decreased ($P<0.01$; LSD) recording percentage changes of -47.99, -42.56, -54.51 and -50.06% respectively as compared with DMBA/CCl_4 control rats. What's more, nitric oxide concentration was highly significantly decreased ($P<0.01$; LSD) in *P. granatum* treated groups recording percentage changes of -54.07, -58.13 and -48.17% as a result of treatment with PJ, PHE and PM respectively

while it was only significantly (P<0.05; LSD) decreased in PSE treated group with percentage changes -30.72% when compared with DMBA/CCl_4-administered control (Table 4).

Experiment outcomes also revealed a highly significant decrease (P<0.01; LSD) in the activity of antioxidant enzymes GPx, GST and SOD in liver tissue of DMBA/CCl_4-administered rats; the recorded percentage changes were -21.26, -70.60 and -32.38% respectively. Treatment of DMBA/CCl_4-administered rats with PJ produced ahighly significant increase of antioxidant parameters (P<0.01; LSD); the recorded percentage changes were 37.01, 105.15 and 46.51% for GPx, GST and SOD in that order as compared with corresponding DMBA/CCl_4-administered control group. Furthermore, the daily treatment of DMBA/CCl_4-administered rats with PSE resulted in a highly significant increase (P<0.01; LSD) in GST and SOD activities recording percentage changes of 175.09 and 32.02% respectively. Additionally, PHE induced a highly significant increase in the activities of antioxidant enzymes with P<0.01 and percentage changes of 36.50, 174.55 and 33.08% for GPx, GST and SOD respectively. As well, PM treatment caused a highly significant increase in liver antioxidant enzymes (P<0.01; LSD) that reached percentage changes of 43.17, 144.04 and 55.99% for GPx, GST and SOD respectively as compared with DMBA/CCl_4-administered control (Table 5).

Table 2: Effects of *P. granatum* aril juice, seeds extract, husk extract and their mixture on serum enzymes ALT, AST, ALP and γGT activities in DMBA/CCl_4-administered rats.

Parameters / Treatment	ALT (U/L)	% change	AST (U/L)	% change	ALP (U/L)	% change	γ-GT (U/L)	% change
Distilled water	48.88 ± 0.70^{d}	-	63.04± 2.42^{b}	-	446.80 ± 7.72^{c}	-	5.30 ± 0.64^{b}	-
DMBA/CCl_4	79.99 ± 3.10^{a}	63.64	103.84 ±3.96^{a}	64.72	784.97 ± 49.12^{a}	75.68	16.98 ± 1.36^{a}	220.37
DMBA/CCl_4 and PJ	68.88 ± 2.53^{b}	-13.88	49.120±5.85^{bc}	-52.69	368.85 ± 39.26^{c}	-53.01	4.63 ± 0.73^{b}	-72.73
DMBA/$CCl_4$and PSE	59.99 ± 3.21^{c}	-25.00	40.080 ±4.72^{c}	-61.40	561.83 ± 39.10^{b}	-28.42	5.79 ± 0.52^{b}	-65.90
DMBA/$CCl_4$and PHE	58.88 ± 3.06^{c}	-26.39	52.280±4.34^{bc}	-49.65	207.37 ± 19.26^{d}	-73.58	5.01 ± 0.24^{b}	-70.49
DMBA/$CCl_4$and PM	61.10 ± 0.70^{c}	-23.61	64.200± 8.91^{b}	-38.17	247.76 ± 29.8^{d}	-68.43	4.28 ± 0.51^{b}	-74.79
F-probability	P<0.001		P<0.001		P<0.001		P<0.001	
LSD at 5% level	7.149		15.660		97.244		2.169	
LSD at 1% level	9.628		21.092		130.961		2.921	

- Data are expressed as mean ±mean standard error. Number of detected samples in each group is six.
- Means, which share the same superscript symbol (s) are not significantly different.
- Percentage changes were calculated by comparing DMBA/CCl_4-administered control group with normal group (dist. water) and DMBA/CCl_4-administered treated groups with DMBA/CCl_4-administered control group.

Table 3: Effects of P. granatum aril juice, seeds extract, husk extract and their mixture on serum total bilirubin (Total bil.), total protein, albumin (A), globulin and (G) albumin/globulin (A/G) ratio in DMBA/CCL4-administered rats.

Parameters / Treatment	Total bil. (mg/dl)	% change	Total protein (g/dl)	% change	A (g/dl)	% change	G (g/dl)	% change	(A/G) ratio	% change
Distilled water	0.73±0.19^{c}	-	5.03±0.08^{ab}	-	3.37±0.12^{a}	-	1.66 ± 0.10^{bc}	-	2.05±0.12^{b}	-
DMBA/CCl_4	1.43±0.06^{a}	95.89	4.02 ±0.36^{c}	- 20.07	1.23 ±0.18^{b}	- 63.50	2.79 ± 0.18^{a}	68.07	0.42±0.23^{c}	-79.51
DMBA/CCl_4 and PJ	1.21±0.11^{ab}	-15.38	4.61±0.18^{abc}	10.19	2.72 ± 0.35^{a}	121.13	1.89 ± 0.35^{b}	-32.25	1.93±0.59^{b}	359.52
DMBA/CCl_4 and PSE	1.26±0.10^{ab}	-11.88	5.1 ±0.19^{ab}	27.11	2.89 ± 0.10^{a}	134.95	2.22 ± 0.29^{ab}	-20.43	1.45±0.23^{b}	245.23
DMBA/CCl_4 and PHE	1.07±0.07^{b}	-25.17	4.24±0.57^{bc}	5.47	3.13 ± 0.38^{a}	154.47	1.11 ± 0.19^{c}	-60.21	3.04±0.28^{a}	623.80
DMBA/CCl_4 and PM	1.36±0.05^{ab}	-4.89	5.46±0.20^{a}	35.82	3.18 ± 0.15^{a}	158.53	2.27 ± 0.20^{ab}	-18.63	1.53 ±0.23^{b}	264.28
F-probability	P<0.001		P<0.05		P<0.001		P<0.001		P<0.001	
LSD at 5% level	0.310		0.887		0.696		0.671		0.8770	
LSD at 1% level	0.418		1.194		0.937		0.904		1.181	

- Data are expressed as mean ±mean standard error. Number of detected samples in each group is six.
- Means, which share the same superscript symbol (s) are not significantly different.
- Percentage changes were calculated by comparing DMBA/CCl_4-administered control group with normal group (dist. water) and DMBA/CCl_4-administered treated groups with DMBA/CCl_4-administered control group.

Table 4: Effects of *P. granatum* aril juice, seeds extract, husk extract and their mixture on liver GSH, TAC, MDA and NO content in DMBA/CCl_4-administered rats.

Parameters / Treatment	GSH (nmole/100 mg tissue)	% change	TAC nmole/mg tissue	% change	MDA (nmole/100mg tissue/hr)	% change	NO nmole/g tissue	% change
Distilled water	86.48 ± 5.19^{a}	-	12.50 ± 1.19^{a}	-	34.33 ± 2.84^{bc}	-	76.36 ± 9.46^{b}	-
DMBA/CCl_4	37.00 ± 2.65^{d}	-57.21	6.82 ± 0.80^{d}	-45.44	65.07 ± 2.52^{a}	89.54	126.20±20.43^{a}	62.42
DMBA/CCl_4 and PJ	84.12 ± 2.01^{a}	127.35	10.01 ± 0.79^{bc}	46.77	33.84 ± 3.16^{bc}	-47.99	57.96 ± 5.17^{b}	-54.07
DMBA/$CCl_4$and PSE	95.37 ± 3.54^{a}	157.75	7.33 ± 0.32^{d}	7.47	37.37 ± 1.98^{b}	-42.56	87.43 ± 12.55^{b}	-30.72
DMBA/$CCl_4$and PHE	66.93 ± 4.69^{b}	80.89	8.83 ± 0.34^{cd}	29.47	29.60 ± 1.10^{c}	-54.51	52.83 ± 11.25^{b}	-58.13
DMBA/$CCl_4$and PM	49.73 ± 4.33^{c}	34.40	11.78 ± 1.00^{ab}	72.72	32.49 ± 3.22^{bc}	-50.06	65.40 ± 12.77^{b}	-48.17
F-probability	P < 0.001		P < 0.001		P < 0.001		P < 0.01	
LSD at 5% level	11.281		2.341		7.460		36.943	
LSD at 1% level	15.193		3.152		10.046		49.752	

-Data are expressed as mean ±mean standard error. Number of detected samples in each group is six.
-Means, which share the same superscript symbol(s) are not significantly different.
-Percentage changes were calculated by comparing DMBA/CCl_4-administered control group with normal group (dist.water) and DMBA/CCl_4-administered treated groups with DMBA/CCl_4-administered control group.

Table 5: Effects of *P. granatum* aril juice, seeds extract, husk extract and their mixture on liver GPx, GST and SOD activities in DMBA/CCl_4-administered rats.

Parameters / Treatment	GPx (mU/100 mg tissue)	% change	GST (U/100 mg tissue)	% Change	SOD (U/g tissue)	% Change
Distilled water	52.39 ± 0.43^{ab}	-	180.21 ± 6.10^{a}	-	15.27 ± 0.92^{ab}	-
DMBA/CCl_4	41.25 ± 4.39^{c}	-21.26	52.97 ± 2.98^{d}	-70.60	10.33± 049^{c}	- 32.38
DMBA/CCl_4 and PJ	56.52 ± 1.99^{a}	37.01	108.67 ± 8.18^{c}	105.15	15.13 ± 0.27^{ab}	46.51
DMBA/$CCl_4$and PSE	46.68 ± 2.65^{bc}	13.16	145.72 ± 11.42^{b}	175.09	13.63 ± 0.69^{b}	32.02
DMBA/$CCl_4$and PHE	56.31 ± 3.05^{a}	36.50	145.43 ± 10.68^{b}	174.55	13.74 ± 0.10^{b}	33.08
DMBA/$CCl_4$and PM	59.06 ± 3.43^{a}	43.17	129.27 ± 17.05^{bc}	144.04	16.11 ± 0.58^{a}	55.99
F-probability	P < 0.001		P < 0.001		P < 0.001	
LSD at 5% level	8.471		30.023		2.039	
LSD at 1% level	11.408		40.432		2.746	

-Data are expressed as mean ±mean standard error. Number of detected samples in each group is six.
-Means, which share the same superscript symbol(s) are not significantly different.
-Percentage changes were calculated by comparing DMBA/CCl_4-administered control group with normal group (dist. water) and DMBA/CCl_4-administered treated groups with DMBA/CCl_4-administered control group.

Regarding mRNA genes expression, the results revealed that hepatic NF-κB, TNF-α and COX-2 expressions were significantly increased while IL-4expression was significantly decreased relative to a housekeeping gene β-actin expression (Figures 1-4) in the DMBA/CCl_4-administered group as compared with normal group. The treatments of DMBA/CCl_4-administered rats with PJ, PSE, PHE and PM produced a significant decrease of the elevated NF-κB, TNF-α and COX-2 expressions while they did not significantly affect IL-4 expression (Figures 1-4).The mRNA expressions of hepatic p53 and Bcl-2 were significantly depleted the DMBA/CCl_4-administered group as compared with normal group (Figures 5 and 6).The lowered mRNA expression p53 and Bcl-2 due to DMBA/CCl_4-administeration were significantly increased as a result of treatment of DMBA/CCl_4-administered group treated with PJ, PSE, PHE and PM (Figures 5 and 6).

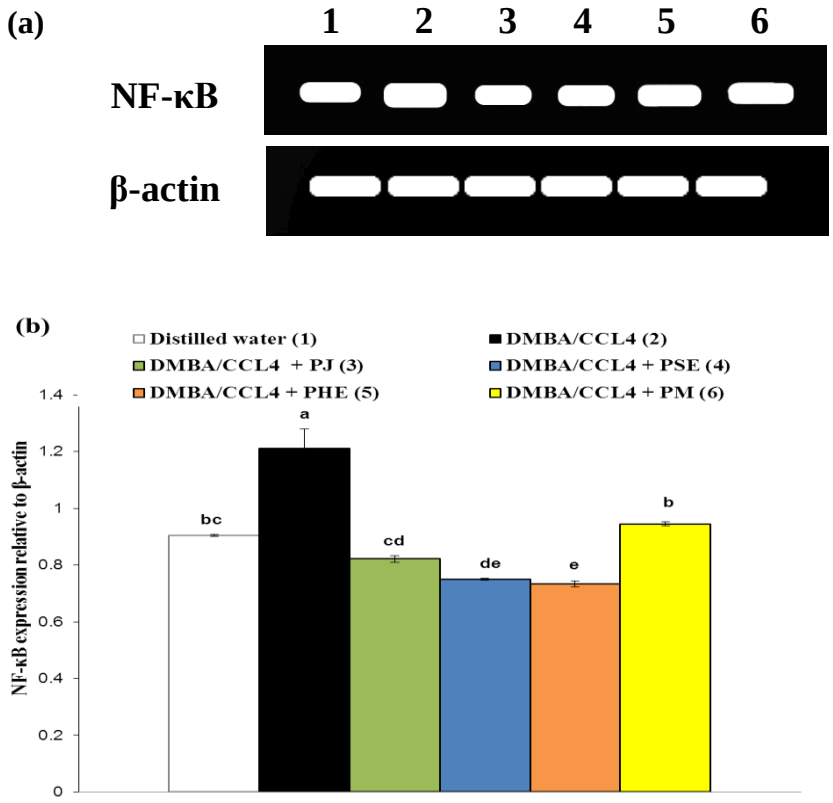


Figure 1: Effects of PJ, PSE, PHE and PM on liver mRNA expression of NF-κB relative to β-actin in DMBA/CCl_4-administered rats. (a) Gel photograph depicting representative PCR products. (b) It corresponded to the densitometric analysis of PCR products. Means, which share the same superscript symbol(s), are not signficantly different. Number of detected samples in each group is three.

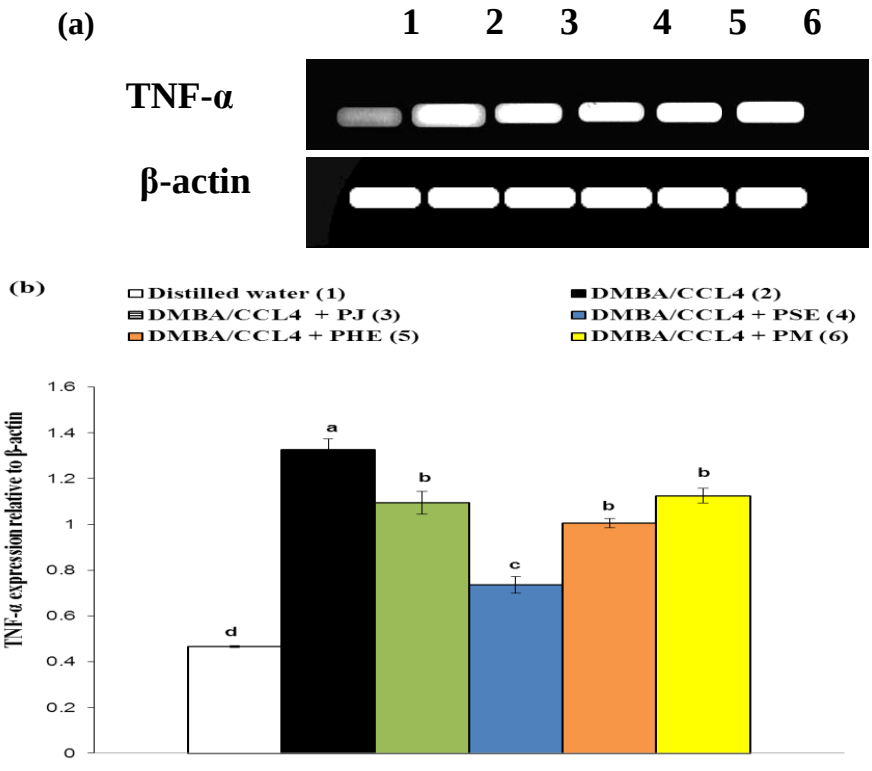


Figure 2: Effects of PJ, PSE, PHE and PM on liver mRNA expression of TNF-γ relative to β-actin in DMBA/CCl_4-administered rats. **(a)** Gel photograph depicting representative PCR products. **(b)** It corresponded to the densitometric analysis of PCR products. Means, which share the same superscript symbol(s), are not signficantly different. Number of detected samples in each group is three.

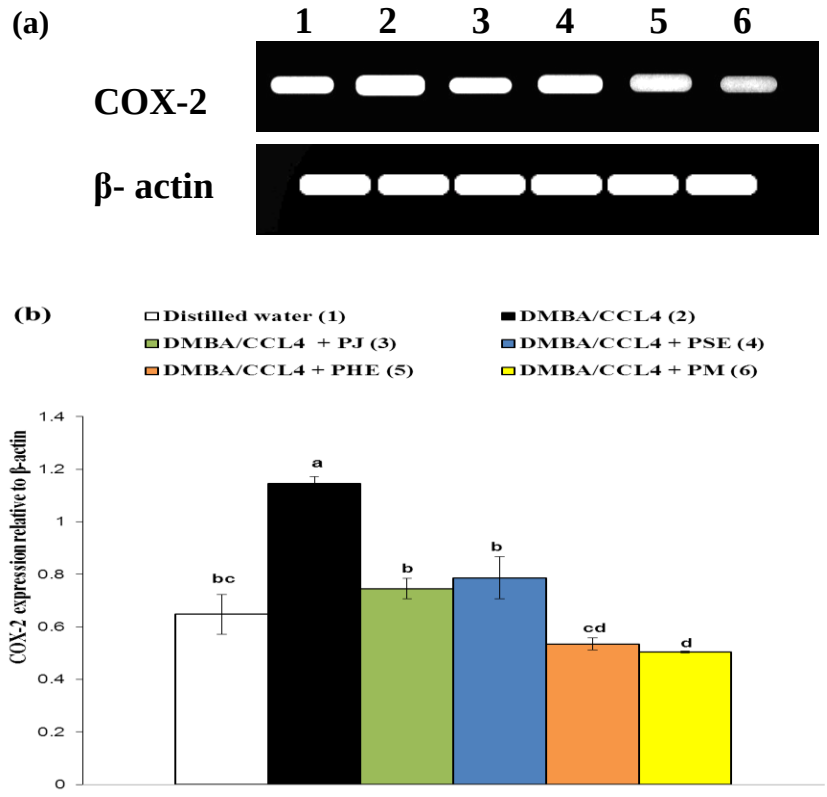


Figure 3: Effects of PJ, PSE, PHE and PM on liver mRNA expression of COX-2 relative to β-actin in DMBA/CCl_4-administered rats. **(a)** Gel photograph depicting representative PCR products. **(b)** It corresponded to the densitometric analysis of PCR products. Means, which share the same superscript symbol(s), are not signficantly different. Number of detected samples in each group is three.

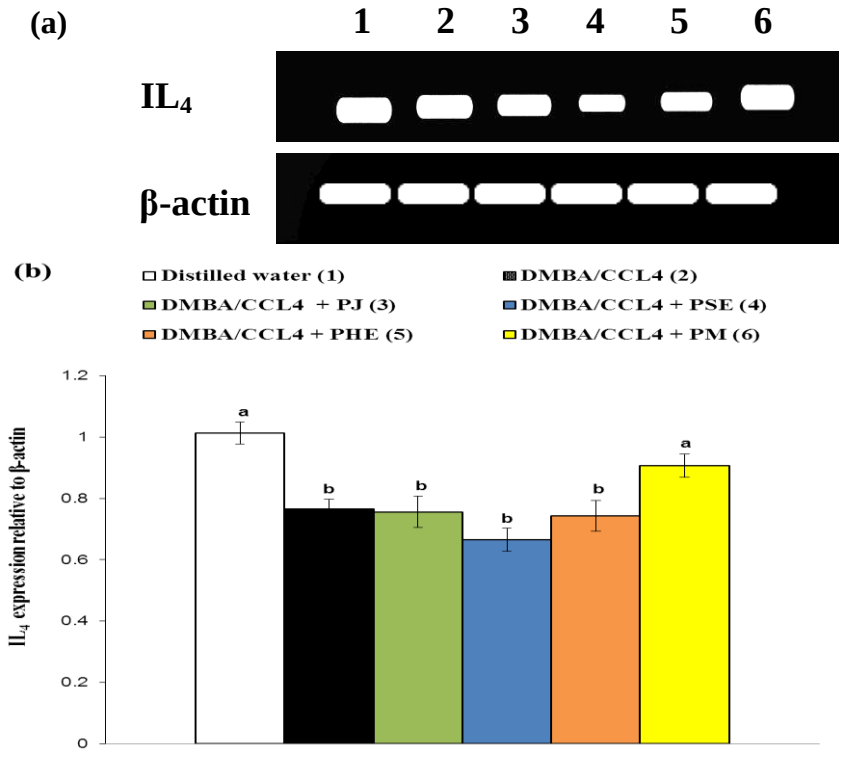


Figure 4: Effects of PJ, PSE, PHE and PM on liver mRNA expression of IL_4 relative to β-actin in DMBA/CCl_4-administered rats. (a) Gel photograph depicting representative PCR products. (b) It corresponded to the densitometric analysis of PCR products. Means, which share the same superscript symbol(s), are not signficantly different. Number of detected samples in each group is three.

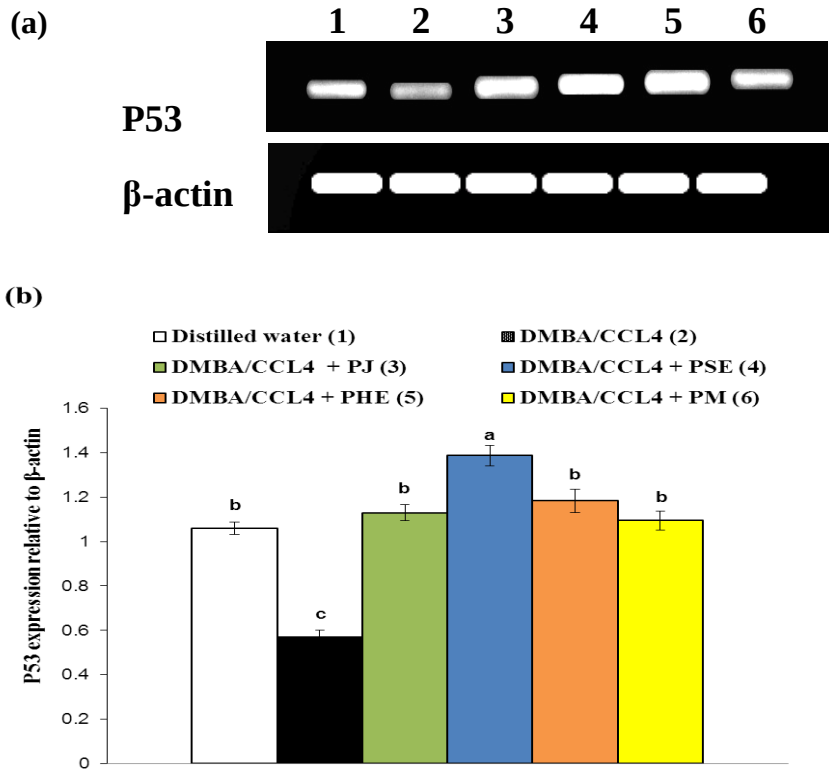


Figure 5: Effects of PJ, PSE, PHE and PM on liver mRNA expression of P53 relative to β-actin in DMBA/CCl_4-administered rats. (a) Gel photograph depicting representative PCR products. (b) It corresponded to the densitometric analysis of PCR products. Means, which share the same superscript symbol(s), are not signficantly different. Number of detected samples in each group is three.

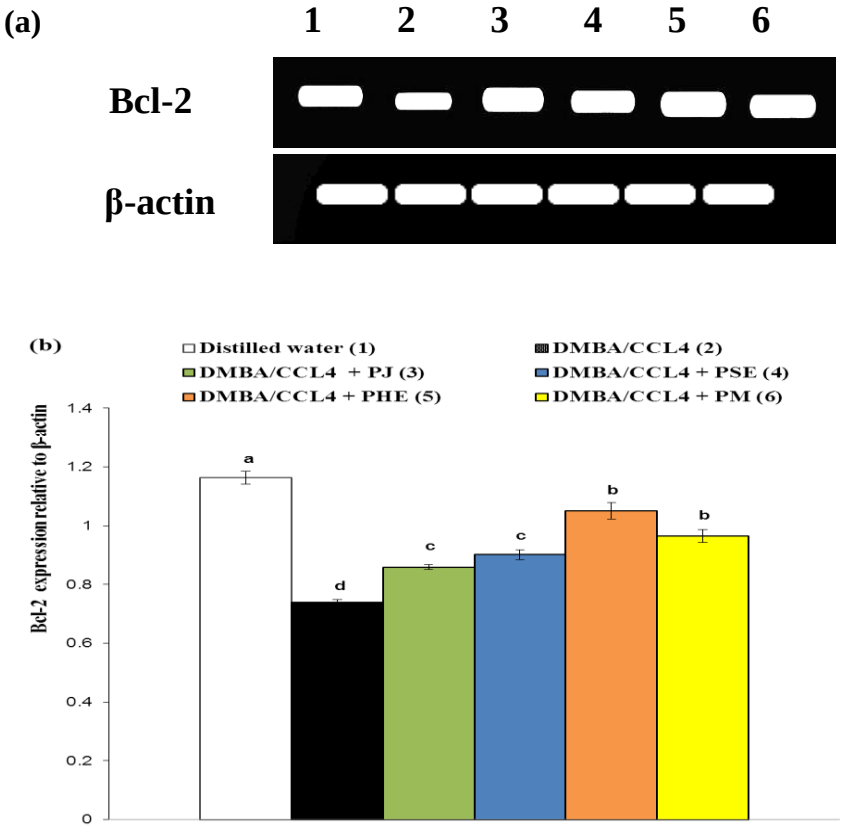


Figure 6: Effects of PJ, PSE, PHE and PM on liver mRNA expression of Bcl-2 relative to β-actin in DMBA/CCl_4-administered rats. (a) Gel photograph depicting representative PCR products. (b) It corresponded to the densitometric analysis of PCR products. Means, which share the same superscript symbol(s), are not signficantly different. Number of detected samples in each group is three.

Liver tissue histological examination of DMBA/CCl_4-administered rats revealed massive destructive alteration which varied from vacuolization, fatty changes of hepatocytes in hepatic lobules and hyperchromacia, karyomegally of their nuclei (Figures 8A and8B) to multifocal hepatic necrosis associated with inflammatory cells infiltration in addition to dilated hyperemic central vein. Precancerous lesions also appeared as oval cells. Treatment of DMBA/CCl_4-administered rats with *P. granatum* aril juice produced slight alterations represented by mild hydropic degeneration of hepatocytes in the peripheral zone (Figure 9). As well, DMBA/CCl_4-administered rats treated with *P. granatum* seeds extract exhibited slight vacuolation and hydropic degeneration of hepatocytes with congestion of hepatic sinusoids as illustrated in figure 10. In addition, husk aqueous extract treatment produced mild hydropic degeneration of hepatocytes and Kupffer cells activation (Figure 11). The mixture of aril juice and aqueous extracts of seeds and husk revealed that no histopathological changes appeared in liver tissues as demonstrated in figure 12. Thus, the mixture of aril juice and aqueous extracts seems to be the most potent in the amendment of liver lesions produced by DMBA and CCl_4.

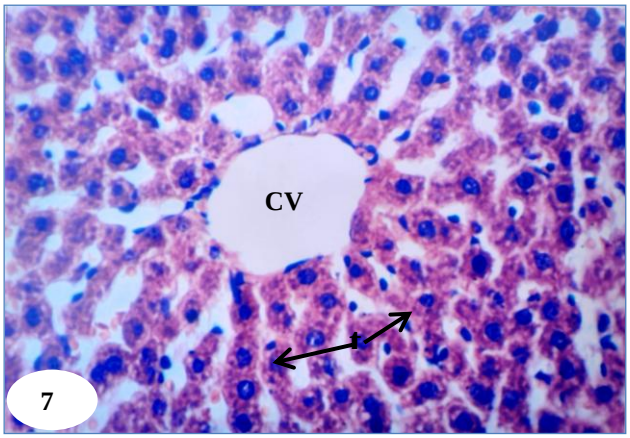
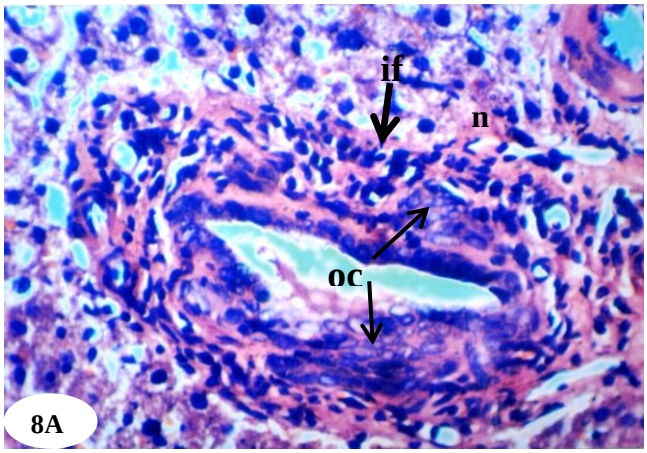
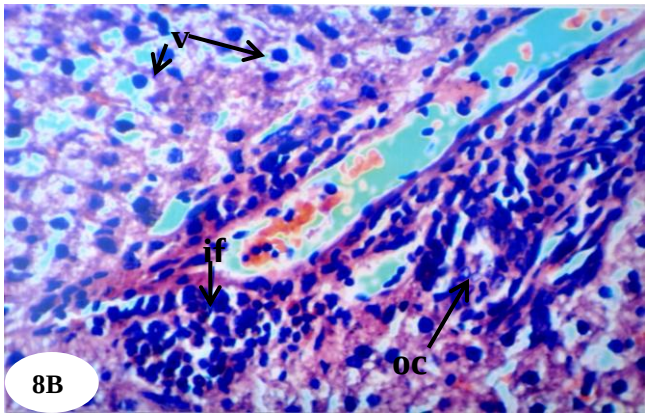


Figure 7: Photomicrograph of liver section of normal rat showing the normal histological structure of the central vein (cv) and hepatocytes arranged in trabeculae (t). (H&E; X 400)





Figures 8: Photomicrographs 8A and 8B of liver sections of DMBA/CCl_4-administered rats showing inflammatory cells infiltration (if), necrosis of hepatic cells (nc) and oval cells (oc) of proliferated fibrous connective tissue. (H&E; X 400)

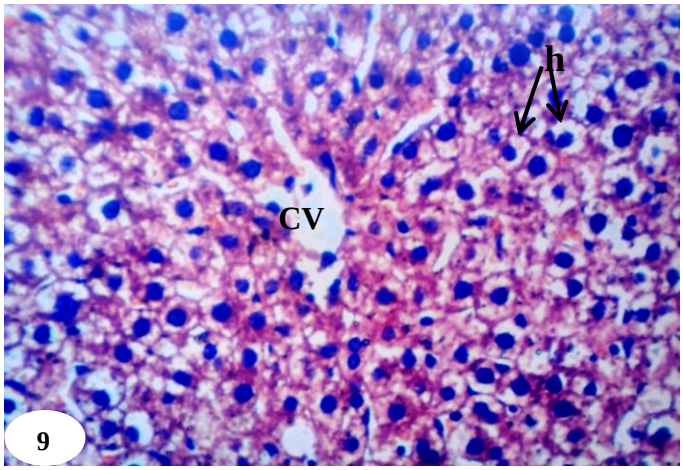


Figure 9: Photomicrograph of liver section of DMBA/CCl4-administered rat treated with *P. granatum* aril juice showing only hydropic degeneration of hepatocytes (hd). (H&E; X 400).

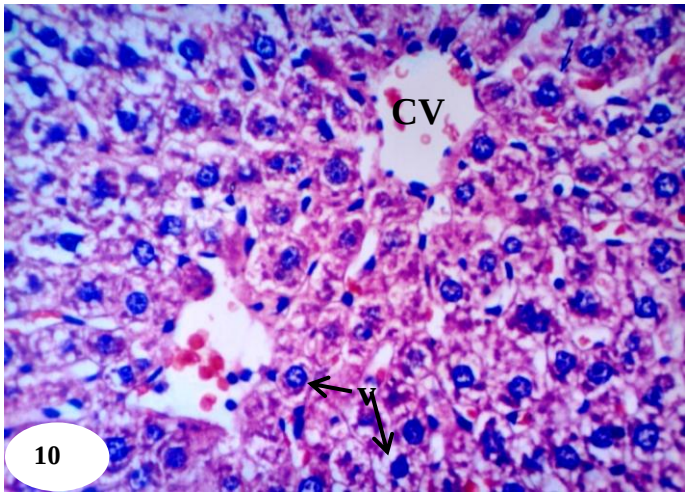


Figure 10: Photomicrograph of liver section of DMBA/CCl4-administered rat treated with *P. granatum* seeds extract showing slight vacuolation of hepatocytes (v). (H&E; X 400)

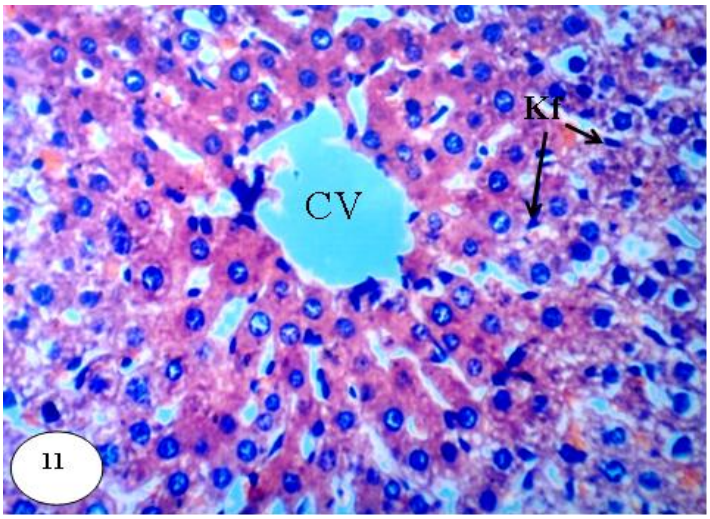


Figure 11: Photomicrograph of liver section of DMBA/CCl4-administered rat treated with *P. granatum* husk extract showing Kupffer cells activation (Kf). (H&E; X400)

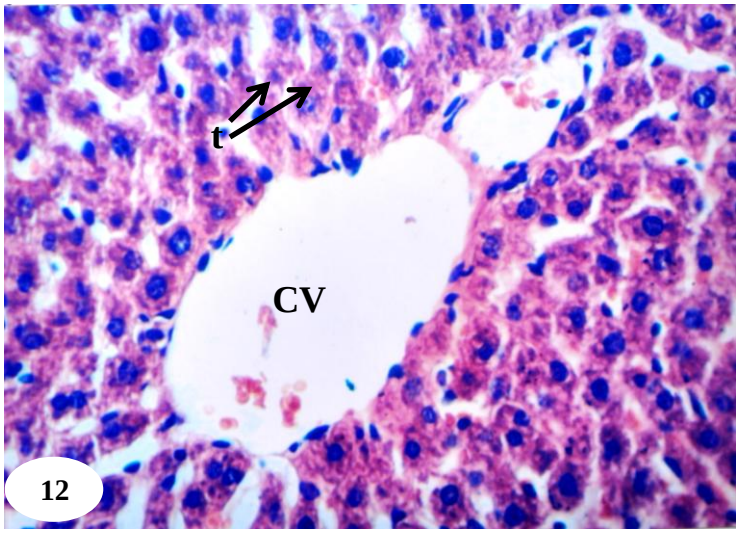


Figure 12: Photomicrograph of liver section of DMBA/CCl_4-dministered rat treated with *P. granatum* mixture of juice and extracts showing no histopathological changes and normal liver tissue. (H&E; X400)

DISCUSSION

Experimental animal model models in cancer research are developed to imitate human carcinogenesis. Suitable animal models are essential to promote our understanding of the molecular, cellular and pathophysiological mechanisms of hepatocaringenesis and for the development of new cancer preventive and therapeutic strategies. In this study, rat model of hepatocarcingensis induced by DMBA as initiator [14] and CCl_4 as promotor of the process [18] is used to assess the preventive effects of pomegranate against DMBA/CCl_4-induced hepatotoxicity and carcinogenicity.

The present study revealed an increase in the activities of serum enzymes ALT, AST, ALP and GGT in DMBA/CCl_4-administered rats reflecting the harmful and destructive effects of DMBA and CCl_4 on liver tissue. It is worth mentioning from previous publications that these enzymes are normally located in serum and they are elevated due to loss of structural integrity and damage of hepatic tissue and leakage to blood stream [72]. So, they are used as diagnostic measures of liver damage [73,74]. These results are in accordance with El Kholy *et al*. [75] and Dakrory *et al*. [76] who used DMBA to induce hepatotoxicity and liver disorders models. These outcomes confirmed previous studies of Ozdemir *et al*. [77] that used 50 mg/kg b.w. of DMBA as single doses in albino rats. The present results are also in accordance with Cheng *et al*. [78] who stated that CCl_4 causes acute hepatocyte injuries, altered membrane integrity and as a result, enzymes leak outand elevate in blood. In addition, Khan *et al*. [79] found that CCl_4administration at dose of 3 ml/kg b.w. twice per week for four weeks induced hepatotoxicity in Sprague Dawley rats.

In the current study, the serum total bilirubin level and globulin levels were significantly increased while the serum total protein and albumin levels as well as A/G were significantly decreased in DMBA/CCl_4-administered rats. The increase in serum total bilirubin may be owing to blockage of bile ductules as a result of inflammation and fibrosis in the portal triads and/or due to regurgitation of conjugated bilirubin from the necrotic hepatocytes to sinusoids [80]. As albumin is quantitatively the most important protein in plasma synthesized by the liver and is a useful indicator of hepatic function. Thus, the decrease in serum albumin as well as total protein levels reflects the impairment in liver function due to DMBA and CCl_4 administration.

The elevated serum ALT, AST, ALP and GGT activities in DMBA/CCl_4-administered rats, in the current study were significantly decreased as a result of treatment with *P. granatum* juice, seeds extract, husk extract and their mixture. Serum total bilirubin and globulin levels were also decreased while albumin and total protein levels were increased as a result of these treatments. These previous changes may reflect the amendment of liver functions and integrity. The improvement in liver functions parameters in *P. granatum* treated groups may provide an evidence of preventive effects *P. granatum* aril juice, seeds extract, husk extract and their mixture against DMBA and CCl_4. The preventive effects can be influenced by some mechanisms as preventing metabolic activation of carcinogen, increasing the detoxification of the carcinogens and blocking the interaction of carcinogen with cellular macromolecules [81]. The *P. granatum* aril juice and seeds and husk extracts may have powerful antioxidants compounds that scavenged and inhibited the metabolic activated carcinogens or assisted the excretion of carcinogenic metabolites of DMBA. These results are in accordance with Yehia *et al*. [82] and Osman *et al*. [83] using *P. granatum* juice and *P. granatum* husk extract respectively. The crude juice of *P. granatum* arils appeared to have more potential as a health supplement rich in natural antioxidants, and improve protein synthesis.

These results are also in concurrent with those obtained by Luangpirom *et al*. [84] using ethanol-intoxicated mice. Moreover, the hepatoprotective activity of *P. granatum* aqueous extract has been evaluated by Khalil [85], who reported that the acute elevation of AST, ALT, lactate dehydrogenase and liver damage by acetaminophen were reduced in *P. granatum* mixture pretreated group. Furthermore, Leelavinothan and Ramasamy [86] revealed that the treatment with ellagic acid (EA), a principal constituent in *P. granatum* reduced the activities of the elevated liver enzymes. The study concluded that administrations of EA at 50 mg/kg b.w.

significantly decreased the activities of hepatic marker enzymes in serum compared with other doses of EA and this can be attributed to the antioxidant properties of EA.

On the other hand, DMBA induces the production of reactive oxygen species (ROS) that resulted in lipid peroxidation, depletion of cell antioxidant defense systems and DNA damage [87]. The antioxidant defense system, represented in this study by hepatic GSH content and GPx, GST and SOD activities as well as TAC in DMBA/CCl_4-administered animals were exhausted due to increased free radical production. These results are in concordance with the study of Arulkumaran *et al*. [88] and Koul *et al*. [89] on male Balb/c mice while they are in discordance with Amin [90] who found slight elevation in GSH level after DMBA administration to female Wister rats.

The increase in lipid peroxidation (expressed by MDA production) and nitric oxide as a result of DMBA administration is reported in many other studies [75,91]. Furthermore, Talas *et al*. [92] concluded that DMBA injection induces an increase of NO levels in the rat liver. The peroxidation of unsaturated fatty acids in biological membranes leads to the decrease of membrane fluidity and the disruption of membrane integrity and function [93]. As well, CCl_4 consecutive doses administration to rats augmented ROS production and increases lipid peroxidation in hepatic cells and induces liver damage and necrosis [21]. The increased incidence of oxidative stress and lipid peroxidation are implicated in carcinogenic processes [94] and free radicals are involved both in the initiation as well as promotion stage of tumourigenesis [90]. These events and increased free radicals, MDA and NO exhibited histopathological perturbations as vacuolization, multifocal hepatic necrosis associated with inflammatory cells infiltration and precancerous lesions marked by oval cells proliferation in liver of DMBA and CCl_4 administered group.

On the other hands, results of the present study showed a decrease in MDA and nitric oxide of DMBA/CCl_4-administered animals treated with *P. granatum* aril juice and seeds and husk extracts as well as their mixture as compared with DMBA/CCl_4-administered control. The reduction of lipid peroxidation in biological systems could be attributed to the free radical scavenging by antioxidant defense system [95]. Antioxidant properties of *P. granatum* aril juice and seeds and husk extracts may be mediated by enhancement of antioxidant enzyme activities and by their intrinsic free radical scavenging properties [96]. *P. granatum* extracts contains many antioxidant compounds such as ascorbic acid, vitamin E, polyphenols, tannins, pro-anthocyanidins and flavonoids [97,98]. These results confirm previous studies that used different parts and concentrations of *P. granatum*. Study of Atilgan *et al*. [99] concluded that administration of *P. granatum* juice significantly reduced the lipid peroxide levels in the serum of rats. Basu *et al*. [100] findings revealed a significant lowering of lipid peroxidation with a 4-week *P. granatum* polyphenol supplementation (POMx) (2 capsules/day) in diabetic patients. The present data also revealed an increase in the antioxidant parameters in DMBA/CCl_4-administered rats treated with *P. granatum* aril juice, seeds extract, husk extract and their mixture. These results are in accordance with many previous publications which proved the antioxidant activities of *P. granatum* fruit, fruit juice, peel extracts and seed extracts [35,101,102]. Furthermore, Al-Olayan *et al*. [103] declared that the supplementation of rats with *P. granatum* juice pre- and concurrent with CCl_4 injection caused a significant increase in GSH content not only when compared with CCl_4 group but also with the control group. Increased antioxidant and decreased oxidative stress markers of DMBA/CCl_4-administered groups treated with *P. granatum* aril juice and seeds and husk aqueous extracts were associated with the amelioration of deleterious histopatholigical perturbations in liver tissue. Slight alteration appeared in liver tissues of treated groups and no alterations appeared in mixture group which authenticate the synergistic effect of the total fruit extract.

In trial to assess the effects of the *P. granatum* seeds and husk extracts and aril juice as well as their mixture on the inflammatory status of DMBA/CCl_4-administered rats, the expression of hepatic COX-2, proinflammatory mediators, NF-κB and TNF-α, as well as anti-inflammatory cytokine, IL-4, were investigated.

P. granatum seeds and husk extracts and aril juice markedly suppressed mRNA expression of NF-κB in DMBA/CCl_4-administered rats to some extent which are in agreement with results of Afaq *et al*. [104], Ahmed *et al*. [105] and Schubert *et al*. [106] on normal human cells, including epidermal keratinocytes, chondrocytes, and vascular endothelial cells respectively. The decrease in mRNA expression of NF-κB in treated groups than that in DMBA/CCl_4-administered group may lead to decline of pro-inflammatory and other mediators .The over expression of TNF-α in the liver cells of DMBA/CCl_4 group can activate NF-κB as indicated by West *et al*. [107]. Consequently, the activation of NF-κB leads to the transcription of hundreds of genes which have kB binding sites; the most of these genes are concerned with the regulation of inflammation, immune responses and cell survival [108]. On the other hand, COX-2 expression decreased in *P. granatum* juice and fruit extracts treated groups reflecting the therapeutic effects of *P. granatum*. This result is in accordance with Jaganathan *et al*. [109] who mentioned that *P. granatum* aril juice inhibits NF-κB activation and expression of COX-2 in HT-29 cells. In contrast, COX-2 increased in DMBA/CCl_4-administered group and there are a correlation between enhanced COX-2 expressions and increase in cell proliferation as shown by Meei *et al*. [110]. In contrast to hepatic COX-2 and TNF-α, the expression of IL-4 was significantly decreased in DMBA/CCl_4-administered rats but it was not significantly altered as a result of treatment of DMBA/CCl_4-administered rats with PJ, PSE, PHE and PM. As stated by Bogdan *et al*. [111], IL-4 is a biological mediator that plays anti-inflammatory roles so it inhibits the production and release of pro-inflammatory mediators [111]. So down-regulation of IL-4 in DMBA/CCl_4-administered rats may lead to the increased production and release of pro-inflammatory mediators, such as TNF-α.

In order to assess the effect on apoptosis in the present study, the mRNA expression of hepatic p53 and Bcl-2 were detected by RT-PCR technique.

The p53 and Bcl-2 expression, in the current study, was increased in DMBA/CCl_4-administered groups treated with *P. granatum* and revealed the chemoprevention power of *P. granatum* aril juice and seeds and husk aqueous extracts as well as their mixture. The

transcriptional promoter p53 can regulate the transcription and expression of a variety of target genes required for cell cycle arrest and apoptosis, including Bcl-2 [112]. The elevation in mRNA expression of tumor suppressor p53 which normally prevents cell proliferation is stimulating apoptosis while the decline of p53 expression in DMBA/CCl_4-administered group can promote tumorigenesis. Bcl-2 which encodes a 26-kDa protein and blocks programmed cell death without affecting cellular proliferation [113] increased in some treated groups and prevented apoptosis and tissue destructive effects due to DMBA and CCl_4 administration

CONCLUSION

P. granatum aril juice and aqueous seeds and husks extracts as well as their mixture have a preventive effects against hepatotoxicity and hepatocarcinogenesis induced by DMBA and CCl_4 thereby *P. granatum* formula may hardly participate in cancer prevention and treatment in the next years.

ACKNOWLEDGEMENTS

The authors acknowledged Prof. Dr. Mahmoud Badawy El-Begawy, Professor of Histopathology, Faculty of Veterinary, Beni-Suef University, Egypt and Prof. Dr. Kawkab Abd El Aziz Ahmed, Professor of Histopathology, Faculty of Veterinary, Cairo University, for her great help in the examination of liver sections and description of histopathological changes.

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. Yeasmin T, Akhter QS, Siddika ST, Karim F. Effect of *Terminalia chebula* (haritaki) on serum aspartate aminotransferase, alanine aminotransferase in paracetemol induced liver damage in Wister albino rats. J Banglad Soc Physiol. 2015; 10(1):1-5.
2. Sapakal VD, Ghadge RV, Adnaik RS, Naikwade NS, Magdum CS. Comparative hepatoprotective activity of Liv-52 and livomyn against carbon tetrachloride induced hepatic injury in rats. Inter J Green Pharm. 2008; 2:79–82.
3. Bosch FX, Ribes J, Díaz M, Cléries R. Primaryliver cancer: Worldwide incidence and trends. Gastroenterology. 2004 ;127:S5–S16.
4. Bhatti AH, Dar FD, Waheed A, Shafique K, Sultan F, Shah NH. Hepatocellular carcinoma in Pakistan: National trends and global perspective. Gastroenterol Res Pract. 2016; Article ID 5942306, 10 pages
5. Ishikawa T. Strategy for improving survival and reducing recurrence of HCV-related hepatocellular carcinoma. Worl J Gastroenterol. 2013;19(37):6127–30.
6. Di Bisceglie AM. Epidemiology and clinical presentation of hepatocellular carcinoma. J Vasc Interv Radiol. 2002; 13:169–71.
7. Gomaa AI, Khan SA, Toledano MB,Waked I, Taylor-Robinson SD. Hepatocellular carcinoma: Epidemiology, risk factors and pathogenesis. Worl J Gastroenterol. 2008; 14(27):4300–8.
8. Mastron JK, Siveen KS, Sethi G, Bishayee A. Silymarin and hepatocellular carcinoma: a systematic, comprehensive, and critical review. Antican Drug. 2015; 26(5):475–86.
9. Rengarajan T, Rajendran P, Nandakumar N, Lokeshkumar B, Rajendran P, Nishigaki I. Exposure to polycyclic aromatic hydrocarbons with special focus on cancer. Asian Pac J Trop Biomed. 2015; 5(3):182–9.
10. Sugiyama T, Osaka M, Koami K, Maeda S, Ueda N. 7,12-DMBA-induced rat leukemia: a review with insights into future research. Leuk Res. 2002; 26(12):1053-68.
11. Abel EL, Angel JM, Kiguchi K, DiGiovanni J. Multi-stage chemical carcinogenesis in mouse skin: Fundamentals and applications. Nat Protoc. 2009; 4(9):1350-62.
12. Abdel-Rahman S, Haggag A, Elmaghraby A. Activation of PTEN tumor suppressor gene expression by Eruca sativa seeds extract against rat mammary gland carcinogenesis induced by DMBA. Australian Journal of Basic and Applied Sciences. 2015; 9(23):431-6.
13. Gurushankar K, Nazeer SS, Jayasree RS, Krishnakumar N. Evaluation of antitumor activity of hesperetin-loaded nanoparticles against DMBA-induced oral carcinogenesis based on tissue autofluorescence spectroscopy and multivariate analysis. J Fluoresc. 2015; 25:931-9
14. Monga J, Chauhan CS, Sharma M. Chemopreventive efficacy of (+)-catechin-rich aqueous extract of *Acacia catechu* Willd. heartwood against 7,12-dimethylbenz[a]anthracene-induced hepatocarcinoma in Balb/c mice. J Environ Pathol Toxicol Oncol. 2012; 31(4):313-23.
15. Khan HBH, Vani S, Palanivelu S, Panchanadham S. Erythrocyte protoporphyrin fluorescence as a biomarker to monitor the anticancer effect of *Semecarpus Anacardium* in DMBA induced mammary carcinoma rat model. J Fluor. 2015; 25(4):907-15.
16. Ebenezar J, Aruna PR, Ganesan S. Native fluorescence spectroscopic characterization of DMBA induced carcinogenesis in mice skin for the early detection of tissue transformation. Analyst. 2015; 140(12):4170-81.
17. Giri U, Sharma SD, Abdulla M, Athar M. Evidence that in situ generated reactive oxygen species act as a potent stage I tumor promoter in mouse skin. Biochem Biophys Res Commun. 1995; 209(2):698-705.
18. Hussain T, Siddiqui HH, Fareed S,Vijayakumar M, Rao CV. Evaluation of chemopreventive effect of *Fumaria indica* against N-nitrosodiethylamine and CCl4-induced hepatocellular carcinoma in Wistar rats. Asian Pacif J Trop Med. 2012; 5:623-9.
19. Moustafa D, Gamal-Eldeen AM, Saleh S, El-Daly SM. The Pharmacological effect of gum arabic on liver hyperplasia in the presence or absence of laser beam. Int J Inno Res Dev. 2014; 3(7):269-73.
20. Slater TF, Sawyer BC. The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative reactions in rat liver fractions *in vitro*. Inhibitory effects of free radical scavengers and other agents. Biochem J. 1971; 123:823-8.

21. Weber LW, Boll M, Stampf A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. Crit Rev Toxicol. 2003; 33:105-36.
22. Ahmed FF, Cowan DL, Sun AY. Detection of free radical formation in various tissues after acute carbontetrachloride administration in gerbil. Life Sci. 1987; 41:2469-75.
23. Kurata M, Suzuku M, Agar NS. Antioxidant systems and erythrocyte life span in mammals. Comp Biochem Physiol B. 1993; 106(3):477-87.
24. Pellegriti G, Frasca F, Regalbuto C, Squatrito S, Vigneri R. Worldwide increasing incidence of thyroid cancer: update on epidemiology and risk factors. J Cancer Epidemiol. 2013; 2013:965212.
25. Johnkennedy N, Adamma E. The protective role of *Gongronema latifolium* in acetaminophen induced hepatic toxicity in Wistar rats. Asia. Pacif J Trop Biomed. 2011; 2011:S151-S154.
26. Miyanishi K, Hoki T, Tanaka S, Kato J. Prevention of hepatocellular carcinoma: Focusing on antioxidant therapy. Worl J Hepatol. 2015; 7(3):593-9.
27. De Nigris F, Balestrieri ML, Williams-IgnarroS,D'Armiento FP, Fiorito C, Ignaro LJ, Napoli C. The influence of pomegranate fruit extract in comparison to regular pomegranate juice and seed oil on nitric oxide and arterial function in obese Zucker rats. Nitric oxide. 2007; 17(1):50-54.
28. Sudheesh S, Presannakumar G, VijayaKumar S, Vijayalakshmi NR. Hypolipidemic effect of flavonoids from *Solanum melongena*. Plant Foods Human Nutri. 1997; 51(4):321-30.
29. Langley P. Why a pomegranate? BMJ 2000; 321:1153-4.
30. Malik A, Afaq F, Sarfaraz S, Adhami VM, Syed DN, Mukhtar H. Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. Proc Natl Acad Sci USA. 2005; 102(41):14813-8.
31. Rozenberg O, Howell A, Aviram M. Pomegranate juice sugar fraction reduces macrophage oxidative state, whereas white grape juice sugar fraction increases it. Atheroscler. 2013; 188(1):68-76.
32. Ismail T, Sestili P, Akhtar S. Pomegranate peel and fruit extracts: A review of potential anti-inflammatory and anti-infective effects. J Ethnopharmacol. 2005; 143(2):397-405.
33. Jurenka JS. Therapeutic applications of pomegranate (*Punica granatum L.*): A Review. Altern Med Rev. 2008; 13(2):128-44.
34. Afaq F, Saleem M, Krueger CG, Reed JD, Mukhtar H. Anthocyanin- and hydrolyzable tannin-rich *Punica granatum* fruit extract modulates MAPK and NF-kappaB pathways and inhibits skin tumorigenesis in CD-1 mice. Inter J Canc. 2005; 113:423-33.
35. Kaur G, Jabbar Z, Athar M, Alam MS. Pomegranate (*Punica granatum*) flower extract possesses potent antioxidant activity and abrogates Fe-NTA induced hepatotoxicity in mice. Food Chem Toxicol. 2006; 44(7): 984-93.
36. Adhami VM, Khan N, Mukhtar H. Cancer chemoprevention by *Punica granatum*: laboratory and clinical evidence. Nutr Canc. 2009; 61(6): 811–5.
37. Zahin M, Aqil F, Ahmad I. Broad spectrum antimutagenic activity of antioxidant active fraction of *Punica granatum L*. peel extracts. Mutat Res. 2010; 703(2):99-107.
38. Toi M, Bando H, Ramachandran C, Melnick SJ, Imai A, Fife RS, Carr RE, Oikawa T, Lansky EP. Preliminary studies on the anti-angiogenic potential of pomegranate fractions *in vitro* and *in vivo*. Angiogen. 2003; 6(2):121-8.
39. Koyama S, Cobb LJ, Mehta HH, Seeram NP, Heber D, Pantuck AJ, Cohen, p. Pomegranate extract induces apoptosis in human prostate cancer cells by modulation of the IGF-IGFBP axis. Growth Horm IGF Res. 2010; 20(1):55-62.
40. Rettig MB, Heber D, An J, Seeram NP, Rao JY, Liu H, Klatte T, Belldegrun, A, Moro A, Henning SM, Mo D, Aronson WJ, Pantuck A. Pomegranate extract inhibits androgen-independent prostate cancer growth through a nuclear factor-kB-dependent mechanism. Mol Canc Ther. 2008; 7(9):2662-71.
41. Tzulker R, Glazer I, Bar-Ilan I, Holland D, Aviram M, Amir R. Antioxidant activity, polyphenol content and related compounds in different fruit juices and homogenates prepared from 29 different pomegranate accessions. J Agric Food Chem. 2007; 55(23):9559-70.
42. Gil MI, Tomas-Barberan FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agric Food Chem 2000; 48(10): 4581-9.
43. Singhal R, Shankar K, Badger TM, Ronis MJ. Estrogenic status modulates aryl hydrocarbon receptor-mediated hepatic gene expression and carcinogenicity. Carcinogenesis. 2008; 29:227-36.
44. Adukondalu D, KumarYS, VishnuYV, KumarRS, Rao YM. Effect of *Punica granatum* juice pre-treatment on the transport of carbamazepine across rat intestine. DARU J Pharmac Sci. 2010; 18(4):254-9.
45. Patel C, Dadhaniya P, Hingorani L, Soni MG. Safety assessment of pomegranate fruit extract: acute and subchronic toxicity studies. Food Chem Toxicol. 2008; 46(8):2728-35.
46. Hemmati AA, Rezaie AR, Darabpour P. Preventive effects of pomegranate seed extract on bleomycin-induced pulmonary fibrosis in Rat. Jundishapur J Nat Pharm Prod. 2013; 8(2):76-80.
47. Akter S, Sarker A, Hossen MS. Antidiarrhoeal activity of rind of *Punica granatum*. Inter Curr Pharm J. 2013; 2(5):101-4.
48. Gella FJ, Olivella T, Cruz pastor M, Arenas J, Moreno R, Durban R,Gomez JA. A simple procedure for routine determination of aspartate aminotransferase and alanine aminotransferase with pyridoxal phosphate. Clin Chem Acta. 1985; 153:241-47.
49. Young DS. Effects of Drugs on Clinical Laboratory Tests. 5th ed. American Association for Clinical Chemistry Press. Washington, DC; 2000.
50. Henry RJ. Clinical Chemistry, Principles and Technics. Harper and Row Publishers. New York; 1964.
51. Vassault A, Grafmeyer D, Naudin CI, Dumont G, Bailly M, Henny J, Gerhardt MF, Georges P. Protocole de validation de techniques. Ann Biol Clin. 1986; 44:686–745.

52. Szasz G, Persijn JP, Coll E. Kinetic Method for quantitative determination of gammaglutamyl transpeptidase. Z Klin Chem Klin Biochem. 1974; 12:228.
53. Balistreri WF, Shaw LM. Liver function. In: Tietz, N.W., ed. Fundamentals of clinical chemistry. 3rd ed. Philadelphia: WB Saunders; 1987. pp 729-761.
54. Doumas BT, Watson WA, Biggs HG. Albumin standard and the measurement of serum albumin with bromocresol green. Clin Chem Acta 1971; 31:87–96.
55. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med. 1963; 61(5):882–8.
56. Mannervik B, Gutenberg C. Glutathione transferase (Human placenta). Meth Enzymol. 1981; 77:231–5.
57. Matkovics B, Sasvari M, Kotorman M, Varga IS, Hai DQ, Varga C. Further prove on oxidative stress in alloxan diabetic rat tissues. Acta Physiol Hung. 1997/1998; 85:183–92.
58. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem. 1974; 47(3):469-74.
59. Csillag A,Kumar BV,Szabo K, Szilasi M, Papp Z, Szilasi ME, Pazmandi K.,BoldoghI, Rajnavolgyi E, Bacsi A, Laszlo JF. Exposure to inhomogeneous static magnetic failed beneficially affects allergic inflammation in a murine model. J R Soc Interface. 2014; 11(95):1-11.
60. Preuss HG, Jarrell ST, Scheckenbach R, Lieberman S, Anderson RA. Comparative effect of chromium vanadium and *Gymnema sylvestre* on sugar-induced blood pressure elevation in SHR. J Am Coll Nutr. 1998); 17(2):116-3.
61. Montgomery HAC, Dymock, JF. The determination of nitrite in water. Analyst 1961; 86:414–6.
62. Sambrook J, Russell DW. Molecular Cloning Laboratory Manual. 5thed. Beijing: Chemical Industry Press; 2008.
63. [Shaker OG, Abdel-Halim M. Connexin 26 in psoriatic skin before and after two conventional therapeutic modalities: methotrexate and PUVA Eur J Dermatol. 2012; 22(2):218-24.
64. [El-Swefy S, Hassanen SI. Improvement of hepatic fibrosis by leukotriene inhibition in cholestatic rats. Ann Hepatol. 2009; 8(1):41–9.
65. Sreeja S, R, Priyadarshini E, Bhavani K, Anuradha CV. Substitution of soy protein for casein prevents oxidative modification and inflammatory response induced in rats fed high fructose diet. ISRN Inflammation. 2014; Article ID 641096, 8 pages
66. Mohamed HE, El-Swefy SE, Hasan RA, Hasan AA. Neuroprotective effect of resveratrol in diabetic cerebral ischemic-reperfused rats through regulation of inflammatory and apoptotic events. Diabetol Metabol Syndrom. 2014; 6 (88):1-14.
67. Zhou B, Luo G, Wang C, Niu R, Wang J. Effects of fluoride on expression of cytokines in the hippocampus of adult rats. Fluoride 2014; 47(3):191-8.
68. Asiri YA. Probucol attenuates cyclophosphamide-induced oxidative apoptosis, p53 and Bax signal expression in rat cardiac tissues. Oxid Med Cell Longev. 2010; 3(5):308-16.
69. [Ashok I, Sheeladevi R. Biochemical responses and mitochondrial mediated activation of apoptosis.On long-term effect of Aspartame in rat brain. Redox Biol. 2010; (2):820-31.
70. Bancroft JD, Gamble M Theory and practice of histological techniques. 5th edition. London, Churchill Livingstone; 2002.
71. Roa M, Blane K, Zonneberg M. One Way Analysis, version 1A(C). PC-STAT, University of Georgia, Athens, USA; 1985.
72. Kelava T, Ćavar I, Vukojević K, Saraga-Babić M, Čulo F. The effect of glucagon and cyclic adenosine monophosphate on acute liver damage induced by acetaminophen. Histol Histopathol. 2013; 28(2):245-55.
73. Sanjiv C. The liver book: A comprehensive guide to diagnosis, treatment and recovery. Atria Jimcafe Company; 2002.
74. Kumar R, Kaur R, Singh AP, Arora S. Diminution of hepatic response to 7, 12- dimethylbenz(a)anthracene by ethyl acetate fraction of *acacia catechu* willd. Through modulation of xenobiotic and anti-oxidative enzymes in rats. PloS one 2014; 9(2):1-10.
75. El Kholy W, Serag H, Zakaria A, El Metwaly A. The Potency of Some Natural Products on Dimethyl Benz(a)anthracene (DMBA) Induced Hepatotoxicity in Rats. Egyptian J Hos Med. 2013; 53:1036-48.
76. Dakrory AI, Fahmy SR, Soliman AM, Mohamed AS, Amer SAM. Protective and Curative Effects of the Sea Cucumber *Holothuria atra* Extract against DMBA-Induced Hepatorenal Diseases in Rats. BioMed Research International. 2015; Article ID 563652, 11 pages
77. Ozdemir I, Selamoglu Z, Ates B, Gok Y, Yilmaz I. Modulation of DMBA-induced biochemical changes by organoselenium compounds in blood of rats. Indian J Biochem Biophys. 2007; 44(4):257-9.
78. Cheng HL, Hu YY, Wang RP, LiuC,Liu P, Zhu DY. Protective actions of salvianolic acid A on hepatocyte injured by peroxidation in vitro. Worl J Gastroenterol. 2000; 6(3):402-04.
79. Khan RA, Khan MR, Sahreen S. CCl_4-induced hepatotoxicity: protective effect of rutin on p53, CYP2E1 and the antioxidative status in rat. BMC Comp Alter Med. 2012; 12:178-90.
80. Ahmed OM. Histopathological and biochemical evaluation of liver and kidney lesions in streptozotocin diabetic rats treated with glimepiride and various plant extracts. JUA Biol. 2001; 16A:585-625.
81. Chandra Mohan KV, Kumaraguruparan R, Prathiba D, Nagini S. Modulation of xenobiotics metabolizing enzymes and redox status during chemoprevention of hamster buccal carcinogenesis by bovine lactoferrin. Nutr. 2006; 22:940-6.
82. Yehia HM, Al-Olayan EM, Elkhadragy MF. Hepatoprotective Role of the pomegranate (*Punica Granatum*) Juice on Carbon Tetrachloride-Induced Oxidative Stress in Rats. Life Sci J. 2013; 10(4):1534-44.
83. Osman M, Ahmed M, Mahfouz S, Elaby S. Biochemical studies on the Hepatoprotective effects of pomegranate and Guava ethanol extracts. New York Sci. 2011; J4:27-41.
84. Luangpirom A, Junaimuang T, Kourchampa W, Somsapt P, Sritragool O. Protective effect of pomegranate (*Punica granatum Linn.*) juice against hepatotoxicity and testicular toxicity induced by ethanol in mice. ABAH Bioflux 2013; 5(1):87-93.

85. Khalil EAM. A hepatoprotective effect of an aqueous extract of pomegranate (*Punica granatum L.*) rind against acetaminophen treated rats. Egypt J Hosp Med. 2004; 16:112-8.
86. Leelavinothan P, Ramasamy S. Effect of ellagic acid on cyclosporine A-induced oxidative damage in the liver of rats. Fundam Clin Pharmacol. 2008; 22(4):395-401.
87. Bharali R, Tabassum J, Azad MRH. Chemomodulatory effect of *Moringa oleifera, Lam*. on hepatic carcinogen metabolizing enzymes, antioxidant parameters and skin papillomagenesis in mice. Asian Pac J Canc Prev. 2003; 4:31-139.
88. Arulkumaran S, Ramprasathm VR, Shanthi P, Sachdanandam P. Alteration of DMBA-induced oxidative stress by additive action of a modified indigenous preparation- Kalpaamruthaa. Chem Biol Interact. 2007; 167(2):99-106.
89. Koul A, Mohan V, Bharati S. *Azadirachta indica* mitigates DMBA-induced hepatotoxicity: a biochemical and radiometric study. Indian J Biochem Biophys. 2014; 51(1):37-45.
90. Amin A. (2008).Chemopreventive effects of Chlorella on the antioxidant system in 7,12-dimethylbenz(a)anthracene-induced oxidative stress in liver. Intern J pharmcol. 4(3):169-176.
91. Arroyo-Acevedo J, Chávez-asmat RJ,Anampa-Guzmán A,Donaires R, Ráez-Gonzáles J. Protective Effect of *Piper aduncum* Capsule on DMBA-induced Breast Cancer in Rats. Breast Cancer (Auckl) 2015; 9:41-8.
92. Talas ZS, Bayraktar N, Ozdemir I, Gok Y, Yilmaz I. The effects of synthetic organoselenium compounds on nitric oxide in DMBA-induced rat liver. J Environ Biol. 2009; 30(4): 591-593.
93. Sahreen S, Khan MR, Khan RA, Shah NA. Effect of *Carissa opaca* leaves extract on lipid peroxidation, antioxidant activity and reproductive hormones in male rats. Lipid Heal Dis. 2013; 12(90): 1-10.
94. Khanzode SS, Muddeshwar SD, Khanzode SD, Dakhale GN. Antioxidant enzymes and lipid peroxidation in different stages of breast cancer. Free Radic Res. 2004; 38:81-85.
95. Yuan LP, Chen FH, Ling L, Bo H, Chen ZW, Li F, Zhong MM, Xia LJ. Protective effects of total flavonoids of *Bidens bipinnata L.* against carbon tetrachloride-induced liver fibrosis in rats. J Pharm Pharmacol. 2008; 60(10):1393-402.
96. Ahmed MAE, El Morsy EM, Ahmed AAE. *Punica granatum* extract protects against cerebral ischemia/reperfusion injury and preserves brain DNA integrity in rats. Life Sci. 2014; 110(2):61–69.
97. Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S. Evaluation of antioxidant properties of pomegranate peel extract in comparison with *Punica granatum* pulp extract. Food Chem. 2006; 96(2):254-60.
98. Rahimi HR, Arastoo M, Ostad SN. A Comprehensive review of pomegranate (*Punica granatum*) properties in toxicological, pharmacological, cellular and molecular biology researches. Iran J Pharm Res. 2012; 11:385-400.
99. Atilgan D, Parlaktas B, Uluocak N, Gencten Y, Erdemir F, Ozyurt H, Erkorkmaz U, Aslan H. Pomegranate (*Punica granatum*) juice reduces oxidative injury and improves sperm concentration in a rat model of testicular torsion-detorsion. Exp Therapeut Med. 2014; 8(2):478-482.
100. Basu A,Newman, ED, Bryant AL, Lyons TJ, and Betts NM. Pomegranate Polyphenols Lower Lipid Peroxidation in Adults with Type 2 Diabetes but Have No Effects in Healthy Volunteers: A Pilot Study. J Nutr Metab. 2013:1-7.
101. Stowe CB. The effects of pomegranate juice consumption on blood pressure and cardiovascular health. Com Ther Clin Pract. 2011; 17:113–5.
102. Fawole OA, Makunga NP, Opara UL. Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. BMC Comp Alter Med. 2012; 12(200):1-11.
103. Al-Olayan EM, El-Khadragy MF, Metwally DM, Abdel MoneimAE. Protective effects of pomegranate (*Punica granatum*) juice on testes against carbon tetrachloride intoxication in rats. BMC Complement Altern Med. 2014; 14(164):1-9.
104. Afaq F, Malik A,Syed D, Maes D,Matsui MS, Mukhtar H. *Punica granatum*fruit extract modulates UV-B-mediated phosphorylation of mitogen-activated protein kinases and activation of nuclear factor kappa B in normal human epidermal keratinocytes paragraph sign. Photochem Photobiol. 2005; 81(1):38-45.
105. Ahmed S, Wang N, Hafeez BB, Cheruvu VK, Haqqi TM. *Punica granatum L.* extract inhibits IL-1beta-induced expression of matrix metalloproteinases by inhibiting the activation of MAP kinases and NF-kappaB in human chondrocytes in vitro. J Nutr. 2005; 135(9): 2096-102.
106. Schubert SY, Neeman I, Resnick N. A novel mechanism for the inhibition of NF-kappaB activation in vascular endothelial cells by natural antioxidants. FASEB J. 2002; 16:1931-3.
107. West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. Annu Rev Cell Dev Biol. 2006; 22:409-37.
108. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene. 1999; 18:6853-66.
109. Jaganathan SK, Vellayappan MV, Narasimhan G, Supriyanto E. Role of pomegranate and citrus fruit juices in colon cancer Prevention. Worl J Gastroenterol. 2014; 20(16): 4618-25.
110. Meei LS, Kuo FC, Yen JS, Wan WL, Shoei YLS, Shing HL. Activation of phosphoinositide 3-kinase in response to inflammation and nitric oxide leads to the up-regulation of cyclooxygenase-2 expression and subsequent cell proliferation in mesangial cells. Cellul Sign. 2005; 17(8):975-84.
111. Bogdan C, Paik J, Vodovotz Y, Nathan C. Contrasting mechanism for suppression of macrophage cytokine release by transforming growth factor- and interleukin-10. J Biol Chem. 1992; 267:23301-08.
112. Burmakin M, Shi Y, Hedstrom E, Kogner P, Selivanova G. Dual targeting of wild type and mutant p53 by small molecule RITA results in the inhibition of N-Myc and key survival oncogenes and kills neuroblastoma cells *in vivo* and *in vitro*. Clin Canc Res. 2013; 19: 5092–103.
113. Reed JC. Bcl-2 and the regulation of programmed cell death. J Cell Biol. 1994; 124:1–6.

5487847845**160715**