Research Article

Updates on the Biochemical and Molecular Mechanisms of N-nitrosodiethylamine-induced Hepatocellular Carcinoma: Promising Therapeutic Role of *Punica granatum* Peel Extract

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ABSTRACT

This study was undertaken to elucidate the underlying biochemical and molecular mechanisms in favour of N-nitrosodiethylamine-induced hepatocellular carcinoma. Also, the aim of this work was extended to explore the efficacy of *Punica granatum* peel extract in retrogression of hepatocellular carcinoma (HCC) in rats. HCC group experienced significant elevation in the studied biochemical markers in serum paralleled by significant downregulation of ING-3 gene expression level and upregulation of Foxp-1 gene expression level in liver. Moreover, HCC group exhibited remarkable increase in β-catenin, survivin and Ki-67 expression in the liver as shown in the immunohistochemical analysis. Furthermore, histopathological investigation of liver tissue sections of rats in HCC group revealed typical anaplasia. In contrast, the treatment of HCC groups with *Punica granatum* peel extract resulted in significant depletion in the liver enzymes activity in association with significant reduction in the studied tumor markers and angiogenic markers. Upregulation of ING-3 and downregulation of Foxp-1 gene expression level in liver have been also detected due to treatment of HCC groups with *Punica granatum* peel extract. Furthermore, these groups displayed marked decrease in β-catenin, survivin and Ki-67 expression in the liver. Interestingly, treatment with *Punica granatum* peel extract elicited marked improvement in the histological feature of liver tissue of HCC groups. In conclusion, the present study indicated that the carcinogenic potency of N-nitrosodiethylamine targeted multiple systems on the cellular and molecular levels. Also, the present findings shed light on the promising anticancer activity of *Punica granatum* peel extract in recession of hepatocellular carcinoma induced chemically in the experimental model through its antiangiogenic, apoptotic and antiproliferative effciency.

Keywords: Hepatocellular carcinoma, *Punica granatum*, Tumor markers, Angiogenesis, Apoptosis, Proliferation.

INTRODUCTION

Hepatocellular carcinoma (HCC), like any other cancer, develops when there is a mutation to the cellular machinery that causes the cell to replicate at a higher rate and results in the cell growth avoiding apoptosis.[1] Among primary liver cancers, HCC represents the major histological subtype, accounting for 70% to 85% of the total liver cancer burden worldwide.[2] Globally, it is the fifth most common cancer and the second leading cause of cancer-related death.[3] In developing countries, incidence rates of HCC are two to three fold higher than in developed countries.[4]

In Egypt, HCC contributes to 14.8% of all cancer mortality with a higher incidence in males (17.3%) than in females (11.5%). It is the second most frequent cancer type in Egyptian males after bladder cancer and the eighth most frequent in Egyptian females.[5] Hospital-based studies have reported an overall increase in the relative frequency of all liver-related cancers in Egypt.[6] This rising incidence may be due to high prevalence of hepatitis C virus (HCV) and its complications and the fact that people born 20 years ago or earlier in Egypt has not been vaccinated against hepatitis B virus (HBV).[7,8]

Chronic inflammations of the liver and subsequent cirrhosis are highly correlated with hepatitis B and hepatitis C viral infections, alcohol abuse and metabolic liver diseases (diabetes and non alcoholic fatty liver disease) these are considered as the major risk factors for HCC. Additionally, obesity, environmental pollutants, consumption of food contaminated with the fungal toxins as aflatoxins that are produced by *Aspergillus flavus* in food grains and nitrosamine consumption are the strongest risk factors for HCC development.[9]

N-nitrosodiethylamine (NDEA) is an N-nitroso alkyl compound described as an effective hepatotoxin in the experimental animals, producing toxicity after repeated administration.[10] NDEA is found in a wide variety of foods such as cheese, soybeans, smoked, salted and dried fish, cured meat and alcoholic beverages.[11] NDEA is mainly metabolized in the liver by the action of cytochrome p450 enzymes and its reactive metabolites are primarily responsible for its hepatotoxic effects. NDEA is bioactivated to ethyldiazonium ion which alkylates DNA bases to form pro-mutagenic adducts such as O6-ethyldeoxyguanosine and O4 and O6-ethyldeoxythymidine and these bioactive molecules induce oxidative stress and cytotoxicity by damaging biomolecules such as DNA, lipids and proteins.[12]

Natural products are non-toxic natural extracts or compounds that compared with synthetic materials, generally produce less side effects.[13] Thus, they potentially provide an alternative or adjunct therapeutic

option for patients with cancer. According to the holy Quran, pomegranates grow in the gardens of paradise and the Quran has recited the *Punica granutum* twice as an example of god's good creations.[14] *Punica granutum,* popularly known as pomegranate, is belonging to Punicaceae family. The extract of *Punica granatum* has been reported to have various medicinal values; antioxidant, antibacterial, antidiabetic, cardioprotective and anticarcinogenic activity.[15-19] *Punica granatum* peel is a rich source of antioxidants, specially polyphenols, such as ellagic acid, querectin, kaempferol, luteolin and punicalagin.[20,21]

The current study was designed to elucidate the biochemical and molecular mechanisms of N-nitrosodiethylamine-induced hepatocellular carcinomain rats. Also, the present goal was extended to explore the potency of *Punica granatum* peel extract in retraction of hepatocellular carcinoma with special concern on its mechanism of action.

MATERIALS AND METHODS

Materials

Chemicals and Drug

N-nitrosodiethylamine (NDEA) (CAS no. 55-18-5) was purchased from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA). Doxorubicin was supplied from Pharmacia Italia S.P.A. Milan, Italy. All other chemicals used in the present study were of high analytical grade.

Plant Materials

Punica granatum fruits were purchased from local market, Cairo, Egypt. The plant was authenticated by Professor Ibrahim El-Garf, Professor of Plant Taxonomy, Department of Botany, Faculty of Science, Cairo University. Voucher Speciemens were kept in the museum of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Preparation of *Punica granatum* Peel Extract

Three kilograms of *Punica granatum* peel were separated from seeds of *Punica granatum* fruits. The peels were cut into small pieces and blended with 3000 ml of methanol 70% using electric blender. Then, the mixture was left for 72 hrs and filtered using filter paper. The filtrate was evaporated under reduced pressure at 45-50 ˚C using rotary evaporator to give a dry residue of crude aqueous methanolic extract (16.5% from fresh peel).[22]

Animals and Experimental Protocol

A total number of 60 adult female albino rats of Wister strain weighing 120-150 g were used in the present study. The animals were obtained from the Animal House Colony of the National Research Centre, Giza, Egypt. The animals were housed in polypropylene cages in an environmentally controlled clean air room with a temperature of 25±1˚C, an alternating 12h light/12h dark cycle, a relative humidity of 60 ± 5% and free access to tap water and a standard rodent chow (Wadi El Kabda Co., Cairo, Egypt). The animals were allowed to adapt to these conditions for 2 weeks before the beginning of the experimental protocol. The animal experimental protocol was approved by the Ethical Committee for Medical Research, National Research Centre, Egypt. After the acclimatization period, the animals were divided into 5 groups (12 rats/group) as follows:

1) Normal healthy rats received 1ml/rat normal saline orally and served as negative control group.

2) Hepatocellular carcinoma (HCC) group in which the rats were orally administered with NDEA (dissolved in 0.9% normal saline), in a dose of 20 mg/kg b.wt. five times a week during a period of 4 weeks and 10 mg/kg b.wt. for another 1 week (total: 5 weeks). Previously, NDEA was given as a single large dose;[23] however, in the current study, smaller doses were given daily throughout the experimental period to reduce the mortality rate. 20 mg/kg was administered at the beginning to initiate hepatocarcinogenesis, followed by 10 mg/kg for the promotion of liver cancer.[24]

3) Doxorubicin-treated group (HCC+Doxo) in which the rats were intraperitoneally (i.p.) treated with doxorubicin as reference drug in dose of 0.72 mg/rat which is equivalent to the human dose of 20 mg/m^2 according to Barnes and Paget[25] once weakly for 9 weeks.

4) Punica *granatum* (high dose)-treated group (HCC+*Punica granatum* H) in which the rats were orally administered with 2.25 g/kg.b.wt/day of *Punica granatum* peel extract five times a week during a period of 9 weeks and

5) *Punica* granatum (low dose)-treated group (HCC+*Punica granatum* L) in which the rats were orally administered with 1.125 g/kg.b.wt/day of *Punica granatum* peel extract five times a week during a period of 9 weeks.

Blood Sampling

After the completion of this round (14 weeks), all animals were fasted for 12 hours then the blood samples were collected from the retro-orbital venous plexus under diethyl ether anaesthesia. Blood samples were left to clot and the sera were separated by cooling centrifugation (4°C) at 1800 xg for 10 min. and stored immediately at -20°C in clean plastic Eppendorf pendng biochemical analysis.

Liver Tissue Sampling

After blood collection, the animals were sacrificed by cervical decapitation, dissected and the whole liver of each rat was rapidly excised and thoroughly washed with isotonic saline. The whole liver of each rat was divided into three portions; the first and second portions were fixed in formal saline (10%) for 24 h for

immunohistochemical investigation and histological examination respectively whereas, the third portion was snap-frozen directly in liquid nitrogen and stored at -80°C prior to RNA isolation for gene expression analysis.

Methods

Biochemical Analyses

Serum aspartate transaminase (AST) andalanine transaminase (ALT) activity was estimated using Quimica Clinica Aplicada S.A (Spain) kit according to the method described by Reitman and Frankel.[26] Serumalkaline phosphatase (ALP) activity was measured using Quimica Clinica Aplicada S.A.(Spain) kit according to the method described by Bowers and Mc Comb.[27] Serum alpha-fetoprotein (AFP) was determined by ELISA using kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer's instructions provided with AFP assay kit. Serum carcinoembryonic antigen (CEA) was quantified by ELISA using kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer's instructions provided with CEA assay kit. Serum glypican-3 (GPC-3) was detected by ELISA using kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer's instructions provided with GPC-3 assay kit. Serum vascular endothelial growth factor (VEGF) was determined by ELISA using kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer's instructions provided with VEGF assay kit. Serum hepatocyte growth factor (HGF) was assayed by ELISA using kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer's instructions provided with HGF assay kit. Serum epidermal growth factor (EGF) was measured by ELISA using kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer's instructions provided with EGF assay kit.

Molecular Genetic Analysis

Isolation of Total RNA

Total RNA was isolated from liver tissue of female rats by the standard TRIzol® Reagent extraction method (Invitrogen, USA). Briefly, tissue samples were homogenized in 1 ml of TRIzol® Reagent per 50 mg of the tissue. After wards, the homogenized sample was incubated for 15 minutes at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol® Reagent was added. Then the samples were shaked vigorously by vortex for 15 seconds and incubated at room temperature for 3 minutes. The samples were centrifuged at 12,000 x g for 15 minutes at 4 °C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a clean tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per 1 ml of TRIzol® Reagent was used for the initial homogenization. Afterwards, the samples were incubated at 15 to 30 °C for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4 °C.

The RNA was precipitated forming a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely and the RNA pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortex and centrifuged at 7,500 x g for 5 minutes at 4 °C.

The supernatant was removed and RNA pellet was air-dried for 10 minutes. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip.

Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, USA) to digest DNA residues and re-suspended in DEPC-treated water. Purity of total RNA was assessed by 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

Reverse Transcription (RT) Reaction

The complete Poly(A)$^+$ RNA isolated from female rat liver tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM consisted of 50 mM $MgCl_2$,5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50U M-MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000xg and transferred to the thermocycler. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time-polymerase chain reaction (sqRT-PCR).

Semi-Quantitative Real Time-Polymerase Chain Reaction (sqRT-PCR)

A Rotor-Gene Q - Qiagen (USA) was used to determine the rats cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM forward primer, 0.5 µL 0.2 µM reverse primer, 6.5 µL distilled water, and 5 µL of cDNA template. The reaction conditions were summarized in Table 1. Each experiment included a distilled water control. The semi quantitative values of RT-PCR (sqRT-PCR) of the studied genes were normalized on the expression value of ß-actin gene.

Primer sequences were as follows: ING3, F: 5′- agt ggc agg aag agc aaa aa-3′, R: 5′- tcc cac cat ctc tcc ata gg-3′ (GenBank NCBI Reference: NM_001034107.1), Foxp-1, F: 5'- CAG GCA GAT CCC CTA TGC AA-3', R: 5'-GGA CAG AGG GCC TTC AGC TT-3' [28] and ß-actin, F: 5′- CCC CAT CGA GCA CGG TAT TG-3′, R: 5′-ATG GCG GGG GTG TTG AAG GTC-3′.[29] At the end of each sqRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

Table 1: sqRT-PCR Reaction Program

	Temperature	Time
Step 1: (1 Cycle)		
a: Intial denaturation step	95.0 °C	03.00 min.
Step 2: (30 cycles)		
a: denaturation step	95.0°C	15 sec.
b: annealing step	55.0 °C	00.30 sec.
c: extension step	72.0 °C	00.30 sec.
Step 3: (71 cycles)		
a: melting curve analysis	60.0 °C	00.10 sec.

Then increased about 0.5 °C every 10 sec. up to 95 °C

Calculation of Gene Expression

The amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula:[30]

$$Ef = 10^{-1/slope}$$

$$Efficiency\ (\%) = (Ef - 1) \times 100$$

The relative quantification of the target gene to the reference gene was determined by using the ΔCT method if E for the target (ING3 and Foxp-1) and the reference primers (β-actin) are the same:

$$Ratio_{(reference/\ target\ gene)} = Ef^{C_T\ (reference)\ -\ C_T\ (target)}$$

Immunohistochemical Investigation

After fixation of liver specimens (first portion) in 10% formalin saline for 24 hours, washing was done in tap water then, ascending grade of ethyl alcohol (30%, 50%, 70%, 90% and absolute) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin (melting point 58-60°C) for 24 hours.

Sections were cut into 4µ thick by slidge microtome then, fixed on positive slides in a 65 °C oven for 1 hr. Slides were placed in a coplin jar filled with 200 mL of triology working solution (Cell Marque, CA-USA, cat.#920P-04) which combines the three pretreatment steps: deparaffinization, rehydration and antigen unmasking. Then, the jar is securely positioned in the autoclave which was adjusted so that temperature reached 120 °C and maintained stable for 15 min after which pressure is released. Thereafter, the coplin jar is removed to allow slides to cool for 30 min. Sections were then washed and immersed in Tris-buffer saline (TBS) to adjust the pH and these were repeated between each step of the immunohistochemical procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. Broad spectrum LAB-SA detection system (Invitrogen, USA, cat.#85-8943) was used to visualize any antigen-antibody reaction in the tissue.

Background staining was blocked by putting 3 drops of 10% goat non immuno serum blocker on each slide and incubating the slides in a humidity chamber for 10 min. Without washing, excess serum was drained and the working solution of the primary antibodies of β-catenin, survivin and Ki-67 mouse monoclonal (Thermo Scientific, USA, cat.#RM-2101-R7 for β-catenin, cat.#RB-9245-P1 for survvin and cat.#RB-9043-R1 for Ki-67) were prepared. Three drops of the working solution were applied. Then, the slides were incubated in the humidity chamber overnight at 4 °C. Hence forward, biotinylated secondary antibody from ultravision detection system anti-polyvalent HRP/DAB (Thermo Scientific, USA, cat.#TP-015-HD) was applied on each slide for 20 min followed by 20 min incubation with the streptavidin horse radish peroxidase (HRP) enzyme conjugate (Thermo Scientific, USA, cat.#TP-015-HD). Then, 3,3´-diaminobenzidine (DAB) chromogen (Thermo Scientific, USA, cat.#TP-015-HD) was prepared and 3 drops were applied on each slide for 2 min. DAB was rinsed, after which counterstaining with Mayer hematoxylin and cover slipping were performed as the final steps before slides were examined under the light microscope (Olympus Cx21 with attached digital camera).[31]

Image analysis was performed using the image J, 1.41a NIH, (USA) analyzer. β-catenin and survivin expressions were represented as area percentage of immunopositive cells, while, Ki-67 expression was represented as a count of immunopositive cells.

Histological Examination

After fixation of the second portion of liver tissue in formal saline (10%) for 24 hours, the tissue was then washed in running tap water, dehydrated in ascending grade of ethyl (30%, 50%, 70%, 90% and absolute alcohol). After that, specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 5µm thick by slidge microtome. The obtained tissue sections were mounted on clean glass slides. The slides were deparaffinized in xylol and then immersed in descending series of alcohol then, ordinary hematoxylin and eosin stain was used to stain the slides before examination under the light microscope (Olympus Cx21 with attached digital camera).[32]

Statistical Analysis

In the present study, the results were expressed as Mean + S.E of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) to compare significance between groups.[33] Difference was considered significant when P value was < 0.05.

RESULTS

Biochemical Analyses

The data depicted in Table 2 illustrated the effect of *Punica granatum* peel extract and doxorubicin treatment on the activity of liver enzymes (AST, ALT and ALP) in serum of rats bearing hepatocellular carcinoma (HCC). The present results demonstrated that there is significant increase (P<0.05) in serum AST, ALT and ALP activity in HCC group as compared to the negative control group. Insignificant change (P>0.05) in serum AST, ALT and ALP activity has been detected upon treatment of HCC with doxorubicin when compared with the untreated HCC bearing rats (HCC group). Meanwhile, the treatment of rats bearing HCC with either high (2.25g/kg.b.wt.) or low (1.125g/kg.b.wt.) dose of *Punica granatum* peel extract elicited significant reduction (P <0.05) in serum AST, ALT and ALP enzymes activity relative to the untreated HCC bearing rats (HCC group).

In comparison with doxorubicin-treated group, *Punica granatum* peel extract either high (2.25g/kg.b.wt.) or low (1.125g/kg.b.wt.) dose produced significant decrease (P< 0.05) in liver enzymes (AST, ALT and ALP) activity in serum of the treated rats (Table 2).

Table 2: Effect of treatment with *Punica granatum* peel extract and doxorubicin on serum AST, ALT and ALP activity in hepatocellular carcinoma rat model

Parameters Groups	AST (U/L)	ALT (U/L)	ALP (U/L)
Negative control	42.8 ± 0.6	26.7 ± 0.5	145.7 ± 4.3
HCC	80.3 ± 1.5[a]	50.1 ± 2.4[a]	284.1 ± 6.6[a]
HCC + Doxo	78.6 ± 2.2	47.5 ± 0.7	279.5 ± 13.3
HCC + *Punica granatum* H	65.2 ± 1.3[bc]	42.8 ± 0.4[bc]	235.3 ± 4.1[bc]
HCC +*Punica granatum* L	60.1 ± 1.4[bc]	38.3 ± 0.5[bc]	215.3 ± 2.4[bc]

HCC: hepatocellular carcinoma.

Doxo: Doxorubicin.

Punica granatum H: *Punica granatum* peel extract (high dose).

Punica granatum L: *Punica granatum* peel extract (low dose).

a: Significant change at P < 0.05 in comparison with the negative control group.

b: Significant change at P < 0.05 in comparison with HCC group.

c: Significant change at P < 0.05 in comparison with HCC + Doxo group.

The results in Table 3 represented the effect of *Punica granatum* peel extract and doxorubicin treatment on serum levels of AFP, CEA and GPC-3 in rats bearing hepatocellular carcinoma (HCC).

The current results revealed that there is significant increase (P<0.05) in serum AFP, CEA and GPC-3 levels in HCC group versus the negative control group. Insignificant decrease (P>0.05) in serum AFP level has been recorded in HCC bearing rats treated with doxorubicin as compared to untreated HCC bearing rats. Significant decrease (P<0.05) in serum CEA and GPC-3 levels has been detected in HCC bearing rats treated with doxorubicin relative to the untreated HCC bearing rats (Table 3). Similarly, the treatment of HCC bearing rats with high dose of *Punica granatum* peel extract (2.25g/kg.b.wt.) produced insignificant decrease (P > 0.05) in serum AFP level as compared to the untreated HCC bearing rats. Whereas, significant reduction (P < 0.05) in serum CEA and GPC-3 levels has been recorded in HCC bearing rats treated with high dose of *Punica granatum* peel extract relative to the untreated HCC bearing rats (Table 3). Treatment of rats bearing HCC with low dose *Punica granatum* peel extract (1.125g/kg.b.wt) showed significant reduction (P< 0.05) in serum AFP, CEA and GPC-3 levels vesus the untreated HCC bearing rats. Also, the data in Table 3 revealed that the treatment of rats bearing HCC with high dose of *Punica granatum* peel extract elicited insignificant reduction (P> 0.05) in serum AFP, CEA and GPC-3 levels, in comparison with doxorubicin-treated group. Treatment of HCC bearing rats with low dose of *Punica granatum* peel extract induced significant reduction (P<0.05) in serum AFP and GPC-3 levels versus doxorubicin-treated group. Meanwhile, treatment of rats bearing HCC with *Punica granatum* peel extract (low dose) produced insignificant reduction (P> 0.05) in serum CEA level in comparison with doxorubicin-treated group (Table 3).

Table 3: Effect of treatment with *Punica granatum* peel extract and doxorubicin on serum AFP, CEA and GPC-3 levels in hepatocellular carcinoma rat model

Parameters Groups	AFP (ng/mL)	CEA (ng/mL)	GPC-3 (pg/mL)
Negative control	19.1 ± 1.5	0.12 ± 0.01	9.4 ± 0.2
HCC	40.1 ± 2.9[a]	0.6 ± 0.06[a]	28.6 ± 0.6[a]
HCC + Doxo	36.9 ± 1.8	0.45 ± 0.04[b]	22.4 ± 0.3[b]
HCC + *Punica granatum* H	35.9 ± 2.4	0.44 ± 0.02[b]	21.8 ± 0.4[b]
HCC + *Punica granatum* L	30.3 ± 2.2[bc]	0.40 ± 0.03[b]	18.9 ± 0.3[bc]

HCC: hepatocellular carcinoma.

Doxo: Doxorubicin.

Punica granatum H: *Punica granatum* peel extract (high dose).

Punica granatum L: *Punica granatum* peel extract (low dose).

a: Significant change at P < 0.05 in comparison with the negative control group.

b: Significant change at P < 0.05 in comparison with HCC group.

c: Significant change at P < 0.05 in comparison with HCC + Doxo group.

The results presented in Table 4 revealed the effect of *Punica granatum* peel extract and doxorubicin treatment on serum levels of VEGF, HGF and EGF in rats bearing hepatocellular carcinoma (HCC). The present results demonstrated that there is significant increase (P<0.05) in serum levels of VEGF, HGF and EGF in HCC group as compared to the negative control group. Treatment of rats bearing HCC with doxorubicin resulted in significant decline (P < 0.05) in serum VEGF and HGF levels as

compared to the untreated HCC bearing rats. Insignificant depletion (P>0.05) in serum EGF level has been recorded in HCC bearing rats treated with doxorubicin with respect to the untreated HCC bearing rats (Table 4). Also, the data in Table 4 revealed that the treatment of HCC bearing rats with *Punica granatum* peel extract either high dose (2.25g/kg. b. wt.) or low dose (1.125g/kg. b. wt.) elicited significant reduction (P< 0.05) in serum VEGF and HGF levels relative to the untreated HCC bearing rats. Meanwhile, treatment of rats bearing HCC with high dose of *Punica granatum* peel extract produced insignificant suppression (P> 0.05) in serum EGF level as compared to the untreated HCC bearing rats. However, the treatment of HCC bearing rats with low dose of *Punica granatum* peel extract led to significant depletion (P< 0.05) in serum EGF level with respect to the untreated HCC bearing rats (Table 4). In comparison with doxorubicin-treated group, the present data showed that the treatment of rats bearing HCC with either high dose or low dose of *Punica granatum* peel extract produces insignificant depression (P> 0.05) in serum VEGF and EGF levels. Meanwhile, the treatment of rats bearing HCC with either high dose or low dose of *Punica granatum* peel extract resulted in significant reduction (P< 0.05) in serum HGF level relative to the doxorubicin-treated group (Table 4).

Table 4: Effect of treatment with *Punica granatum* peel extract and doxorubicin on serum VEGF, HGF and EGF levels in hepatocellular carcinoma rat model

Parameters Groups	VEGF (pg/mL)	HGF (ng/mL)	EGF (ng/L)
Negative control	6.0 ± 0.2	4.2 ± 0.05	1900 ± 53.5
HCC	18.0 ± 0.97[a]	28.8 ± 0.5[a]	2425 ± 123[a]
HCC + Doxo	14.4 ± 0.5[b]	16.9 ± 0.3[b]	2197 ± 87.7
HCC + *Punica granatum* H	14.1 ± 1.0[b]	16.0 ± 0.3[bc]	2168 ± 70.5
HCC + *Punica granatum* L	13.1 ± 0.98[b]	14.8 ± 0.2[bc]	2111 ± 102.5[b]

HCC: hepatocellular carcinoma.

Doxo: Doxorubicin.

Punica granatum H: *Punica granatum* peel extract (high dose).

Punica granatum L: *Punica granatum* peel extract (low dose).

a: Significant change at P < 0.05 in comparison with the negative control group.

b: Significant change at P < 0.05 in comparison with HCC group.

c: Significant change at P < 0.05 in comparison with HCC + Doxo group.

Molecular Genetic Analysis

Figure 1 illustrated the effect of treatment with *Punica granatum* peel extract and doxorubicin on the expression level of ING3 gene in the liver tissue of rats bearing hepatocellular carcinoma. The current results revealed that there is significant down regulation (P<0.05) in the expression level of ING3 gene in the liver tissue of rats bearing HCC when compared with the negative control group. Significant up regulation (P<0.05) in the expression level of ING3 gene has been detected in liver tissue of rats bearing HCC and treated with doxorubicin versus the untreated HCC bearing rats (Figure 1). Similarly, the treatment of rats bearing HCC with *Punica granatum* peel extract elicited significant upergulation (P< 0.05) in expression level of ING3 gene in the liver tissue as compared to the untreated HCC bearing rats. In comparison with doxorubicin-treated group, treatment of rats bearing HCC with high dose of *Punica granatum* peel extract resulted in insignificant change (P> 0.05) in the expression level of ING3 gene in the liver tissue, whereas, significant upregulation (P< 0.05) in the expression level of ING3gene in the liver tissue has been detected in HCC bearing rats treated with low dose of *Punica granatum* peel extract as compared to doxorubicin-treated group (Figure 1).

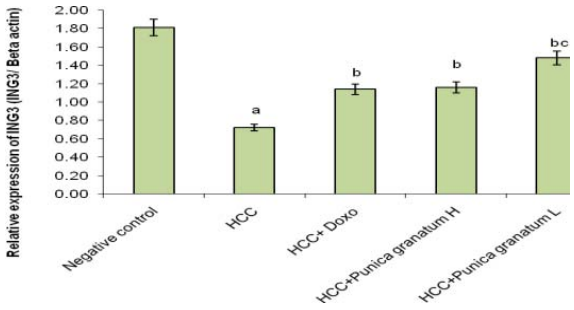

Figure 1: Effect of treatment with *Punica granatum* peel extract and doxorubicin on hepatic ING3 gene expression level in hepatocellular carcinoma rat model.

HCC: hepatocellular carcinoma.
Doxo: Doxorubicin.
Punica granatum H: *Punica granatum* peel extract (high dose).
Punica granatum L: *Punica granatum* peel extract (low dose).
a: Significant change at P < 0.05 in comparison with the negative control group.
b: Significant change at P < 0.05 in comparison with HCC group.
c: Significant change at P < 0.05 in comparison with HCC + Doxo group.

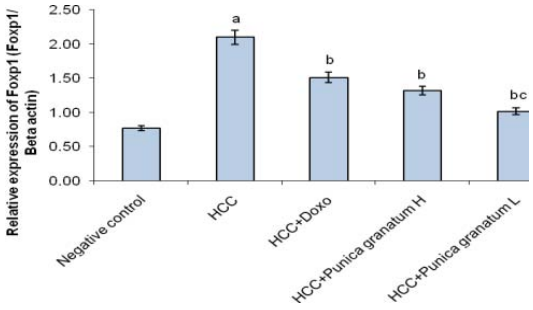

Figure 2: Effect of treatment with *Punica granatum* peel extract and doxorubicin on hepatic Foxp-1 gene expression level in hepatocellular carcinoma rat model.

HCC: hepatocellular carcinoma.
Doxo: Doxorubicin.
Punica granatum H: *Punica granatum* peel extract (high dose).
Punica granatum L: *Punica granatum* peel extract (low dose).
a: Significant change at P < 0.05 in comparison with the negative control group.
b: Significant change at P < 0.05 in comparison with HCC group.
c: Significant change at P < 0.05 in comparison with HCC + Doxo group.

Figure 2 represented the effect of treatment with *Punica granatum* peel extract and doxorubicin on the expression of Foxp-1 gene in the liver tissue of rats bearing hepatocellular carcinoma.

The present results showed that there is significant upregulation (P<0.05) in the expression level of Foxp-1 gene in the liver tissue of rats bearing HCC with respect to the negative control group.

Treatment of HCC bearing rats with doxorubicin elicited significant downregulation (P<0.05) in the gene expression level of Foxp-1 in the liver tissue versus the untreated of HCC bearing rats (Figure 2).

In the same manner, the treatment of rats bearing HCC with either high dose or low dose of *Punica granatum* peel extract induced significant downregulation (P< 0.05) in the expression level of Foxp-1 gene in the liver tissue in comparison with the untreated HCC bearing rats.

In comparison with doxorubicin-treated group, the treatment of rats bearing HCC with high dose of *Punica granatum* peel extract produced insignificant downregulation (P> 0.05) in the expression level of Foxp-1 gene in the liver tissue.

Whereas, significant downregulation (P< 0.05) in the expression level of Foxp-1 gene in the liver tissue was recorded in HCC bearing rats treated with low dose of *Punica granatum* peel extract as compared to doxorubicin-treated group (Figure 2).

Immunohistochemical Investigation

In the present study, the expression of β-catenin, survivin and Ki-67 in liver tissue of the different experimental groups has been investigated by immunohistochemical technique. Immunohistochemical staining of liver tissue section of rat in the negative control group using antibody against β-catenin showed area percentage of β-catenin immunopositive cells (3.50 %) (Figure 3).

While, immunohistochemical staining of liver tissue section of rat in HCC group using antibody against β-catenin showed sever increase in the area percentage of β-catenin immunopositive cells (30.9 %) (Figure 4). Immunohistochemical staining of liver tissue section of rat bearing HCC and treated with doxorubicin using antibody against β-catenin showed mild decrease in the area percentage of β-catenin immunopositive cells (23.3 %) (Figure 5).

Immunohistochemical staining of liver tissue section of rat bearing HCC and treated with high dose of *Punica granatum* peel extract using antibody against β-catenin showed moderate decrease in the area percentage of β-catenin immunopositive cells (16.4 %) (Figure 6).

Immunohistochemical staining of liver tissue section of rat bearing HCC and treated with low dose of *Punica granatum* peel extract using antibody against β-catenin showed marked decrease in the area percentage of β-catenin immunopositive cells (7.4 %) (Figure 7).

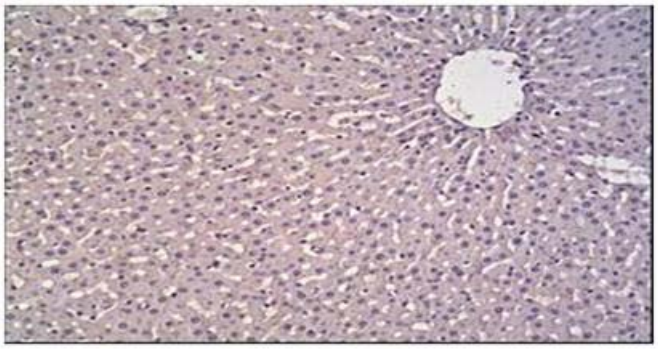

Figure 3: Photomicrograph for staining of liver tissue section of rat in the negative control group using antibody against β-catenin (80x).

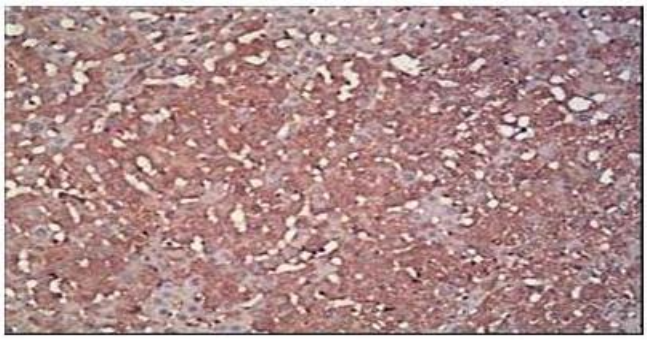

Figure 4: Photomicrograph for staining of liver tissue section of rat in HCC group using antibody against β-catenin (80x).

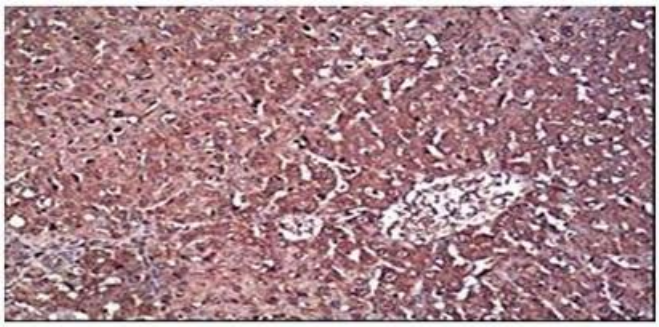

Figure 5: Photomicrograph for staining of liver tissue section of rat bearing HCC and treated with doxorubicin using antibody against β-catenin (80x).

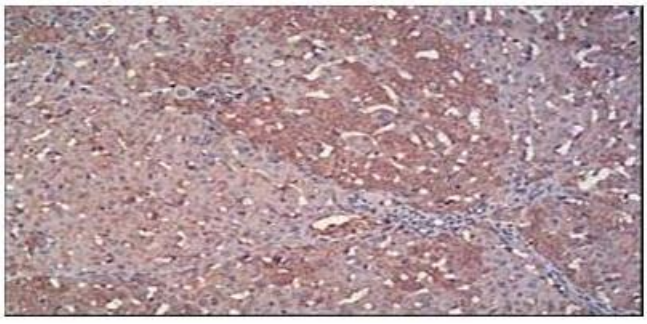

Figure 6: Photomicrograph for staining of liver tissue section of rat bearing HCC and treated with high dose of *Punica granatum* peel extract using antibody against β-catenin (80x).

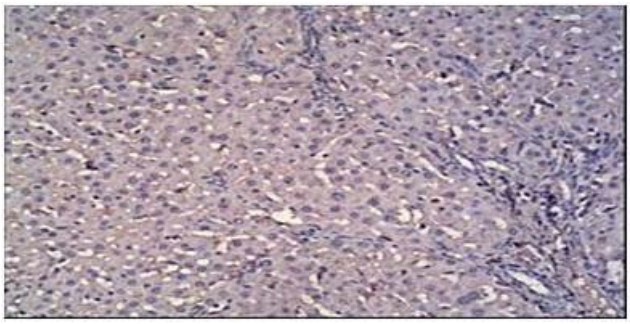

Figure 7: Photomicrograph for staining of liver tissue section of rat bearing HCC and treated with low dose of *Punica granatum* peel extract using antibody against β-catenin (80x).

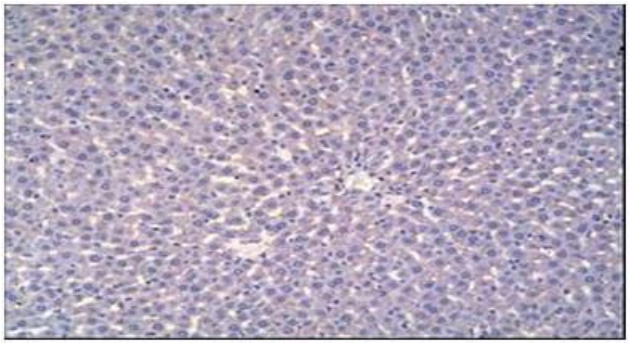

Figure 8: Photomicrograph for staining of liver tissue section of rat in the negative control group using antibody against survivin (80x).

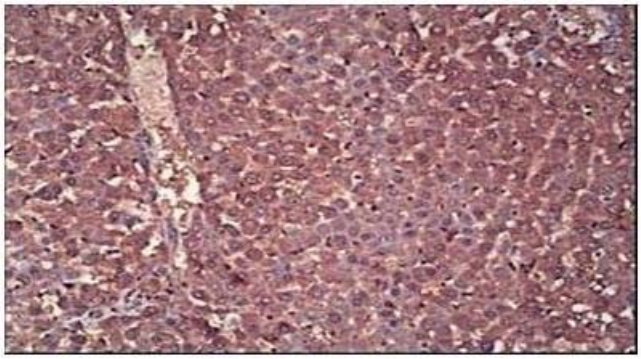

Figure 9: Photomicrograph for staining of liver tissue section of rat in HCC group using antibody against survivin (80x).

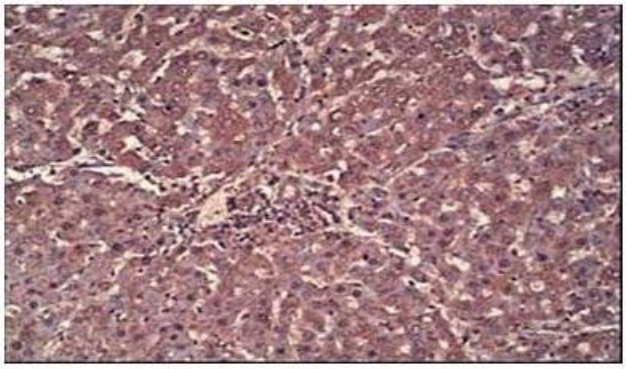

Figure 10: Photomicrograph for staining of liver tissue section of rat bearing HCC and treated with doxorubicin using antibody against survivin (80x).

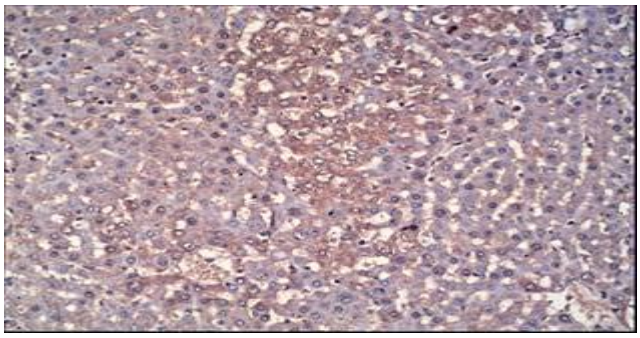

Figure 11: Photomicrograph for staining of liver tissue section of rat bearing HCC and treated with high dose of *Punica granatum* peel extract using antibody against survivin (80x).

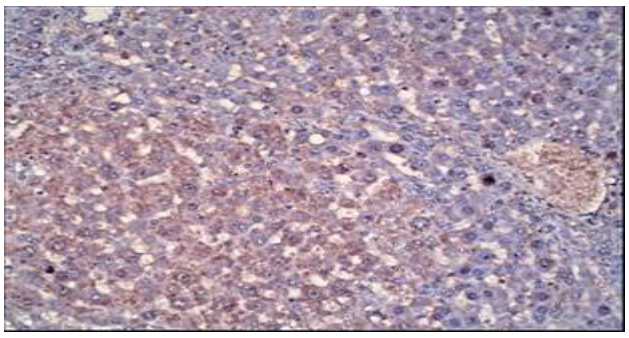

Figure 12: Photomicrograph for staining of liver tissue section of rat bearing HCC and treated with low dose of *Punica granatum* peel extract using antibody against survivin (80x).

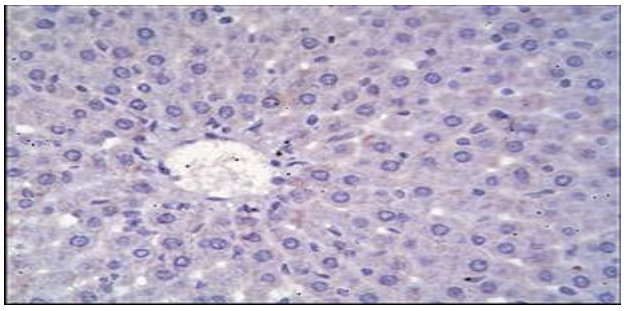

Figure 13: Photomicrograph for staining of liver tissue section of rat in the negative control group using antibody against Ki-67 (80x).

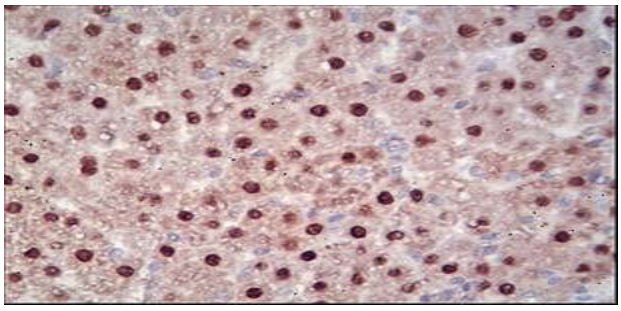

Figure 14: Photomicrograph for staining of liver tissue section of rat in HCC group using antibody against Ki-67 (80x).

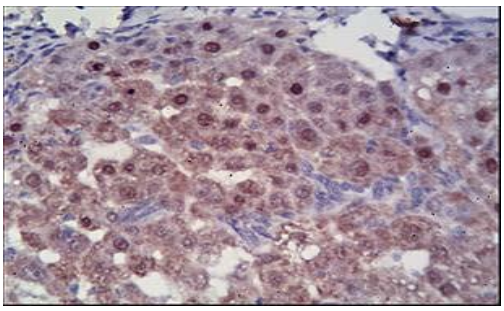

Figure 15: Photomicrograph for staining of liver tissue section of rat bearing HCC and treated with doxorubicin using antibody against Ki-67 (80x).

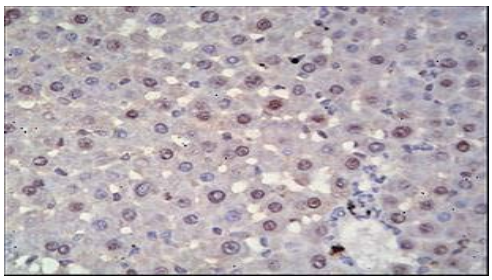

Figure 16: Photomicrograph for staining of liver tissue section of rat bearing HCC and treated with high dose of *Punica granatum* peel extract using antibody against Ki-67 (80x).

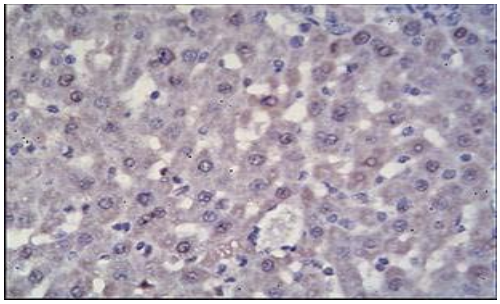

Figure 17: Photomicrograph for staining of liver tissue section of rat bearing HCC and treated with low dose of *Punica granatum* peel extract using antibody against Ki-67 (80x).

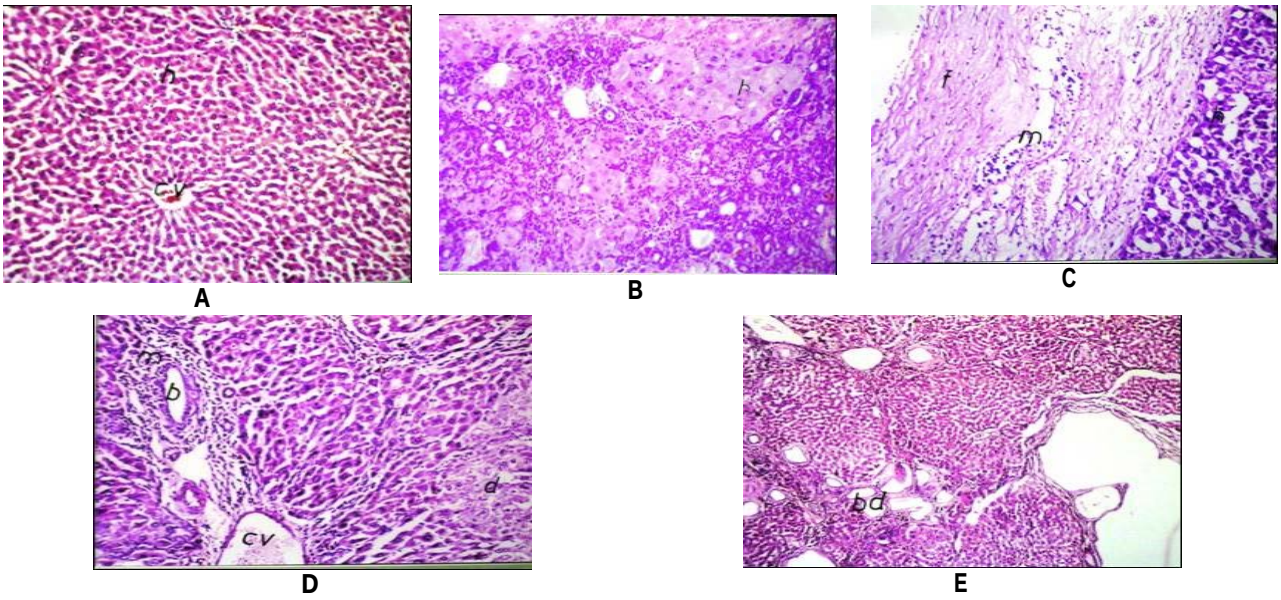

Figure 18: Photomicrograph of liver tissue section of rat in **(A):** The negative control group showing normal histological structure of the central vein (cv) and the surrounding hepatocytes (h) (H&E x 40), **(B):** HCC group showing anaplastic hepatocytes activities (h) charcacterized by polarity, pleomorphism and hyperchromachtic nuclei and the ratio between the nucleus and cytoplasm was 1:1 associated with acinar glandular structure formation (a) (H&E x 40), **(C):** HCC group and treated with doxorubicin showing thickening and fibrosis (f) with inflammatory cell infiltration (m) and dilated blood capillanes (v) in hepatic capsule. Also, many hepatocytes showed karomegaly and pyknotic nuclei (H&E x 40), **(D):** HCC group and treated with high dose of *Punica granatum* peel extract showing inflammatory cells infiltration in the portal area (m) with sever dilatation of in the portal vein (cv) and focal degenerated hepatocytes (d) (H&E x 40) and **(E):** HCC group and treated with low dose of *Punica granatum* peel extract showing proliferative cystic bile ducts (bd) (H&E x 16).

Immunohistochemical staining of liver tissue section of rat in the negative control group using antibody against survivin showed area percentage of survivin immunopositive cells (1.49 %) (Figure 8). Immunohistochemical staining of liver tissue section of rat in HCC group using antibody against survivin showed sever increase in the area percentage of survivin immunopositive cells (26.18 %) (Figure 9). Immunohistochemical staining of liver tissue section of rat bearing HCC and treated with doxorubicin using

antibody against survivin showed mild decrease in the area percentage of survivin immunopositive cells (20.51 %) (Figure 10). Immunohistochemical staining of liver tissue section of rat bearing HCC and treated with high dose of *Punica granatum* peel extract using antibody against survivin showed moderate decrease in the area percentage of survivin immunopositive cells (14.39 %) (Figure 11). Immunohistochemical staining of liver tissue section of rat bearing HCC and treated with low dose of *Punica granatum* peel extract using antibody against survivin showed moderate decrease in the area percentage of survivin immunopositive cells (6.76 %) (Figure 12).

Immunohistochemical staining of liver tissue section of rat in the negative control group using antibody against Ki-67 showed negative reaction (the number of immunopositive cells for Ki-67 expression was zero cell) (Figure 13). Immunohistochemical staining of liver tissue section of rat in HCC group using antibody against Ki-67 showed sever increase in the number of immunopositive cells for Ki-67 expression (65 cells) (Figure 14). Immunohistochemical staining of liver tissue section of rat bearing HCC and treated with doxorubicin using antibody against Ki-67 showed mild decrease in the number of immunopositive cells for Ki-67 expression (37 cells) (Figure 15). Immunohistochemical staining of liver tissue section of rat bearing HCC and treated with high dose of *Punica granatum* peel extract using antibody against Ki-67 showed mild decrease in the number of immunopositive cells for Ki-67 expression (28 cells) (Figure 16). Immunohistochemical staining of liver tissue section of rat bearing HCC and treated with low dose of *Punica granatum* peel extract using antibody against Ki-67 showed marked decrease in the number of immunopositive cells for Ki-67 expression (5 cells) (Figure 17).

Histological Examination

Histological examination of liver tissue section of rat in the negative control group stained with hematoxylin and eosin (H&E) showed normal histological structure of the central vein and the surrounding hepatocytes and no histopathological alteration was observed (Figure 18 A**)**. While, microscopic investigation of liver tissue section of rat in HCC group showed anaplastic hepatocytes activities indicated by polarity, pleomorphism and hyperchromachtic nuclei and the ratio between the nucleus and cytoplasm was 1:1 associated with acinar glandular structure formation (Figure 18 B**)**.

Photomicrograph of liver tissue section of rat bearing HCC and treated with doxorubicin showed thickening and fibrosis with inflammatory cell infiltration and dilated blood capillanes in hepatic capsule.

Also, many hepatocytes showed karomegaly and pyknotic nuclei (Figure 18 C**)**. Photomicrograph of liver tissue section of rat bearing HCC and treated with high dose of *Punica granatum* peel extract showed inflammatory cells infiltration in the portal area with sever dilatation of in the portal vein and focal degenerated hepatocytes (Figure 18 D). Photomicrograph of liver tissue section of rat bearing HCC and treated with low dose of *Punica granatum* peel extract showed proliferative cystic bile ducts (Figure 18 E**)**.

DISCUSSION

This study was constructed to elucidate the underlying biochemical and molecular mechanisms by which N-nitrosodiethylamine could induce hepatocellular carcinoma in rats. Also, the aim of the present study was extended to explore the possible therapeutic potential of *Punica granatum* peel extract against hepatocellular carcinoma with special concern on its mode of action. These goals were achieved through conducting liver function tests, relevant tumor and angiogenic as well as genetic markers, differentiated, antiapoptotic and proliferative indices.

A model close to human situation is the chemical induction of HCC with special carcinogens such as N-nitrosodiethylamine (NDEA). As it is widely accepted that metabolic activation of nitrosamines by cytochrome P450 enzymes to reactive electrophiles are required for their cytotoxic, mutagenic and carcinogenic activity. Because of its relatively simple metabolic pathway and potent carcinogenicity, NDEA has been used as an effective experimental model in the field of carcinogenesis and chemoprevention.[34]

The data of the present study showed that NDEA administration led to significant increase in liver enzymes activity AST, ALT and ALP in serum. These findings are in agreement with Willimsky[35] Pratt and Kaplan[36] reported that these enzymes are commonly elevated in patients with liver diseases and the elevated values of these enzymes in serum reflect the status of liver injury. NDEA is mainly metabolized in the liver by the action of cytochrome p450 enzymes and its reactive metabolites are primarily responsible for its hepatotoxic effects. NDEA is bioactivated to ethyldiazonium ion which alkylates DNA bases to form pro-mutagenic adducts such as O6-ethyldeoxyguanosine and O4 and O6-ethyldeoxythymidine.These bioactive molecules has the ability to induce oxidative stress and hepatotoxicity by damaging the biomolecules such as DNA, lipids and proteins.[12] The observed increment of liver enzymes in serum as a consequence of NDEA administration could be due to the production of free radicals during NDEA metabolism, which damage the hepatocellular membrane. As a result, these cytoplasmic enzymes are released into the systemic circulation.[37]

The current results revealed that the treatment of rats bearing HCC with *Punica granatum* peel extract produced significant decrease in serum AST, ALT and ALP enzymes activity. These findings are greatly supported by those of Rao and Dama[38] who reported that *Punica granatum* decreased the enzymatic activity of liver enzymes in

serum and alleviated the degree of liver damage. These effects might be related to that *Punica granatum* extract exhibits strong antioxidant activity, scavenges free radicals and ameliorates the damaging impact on the liver,[39] due to its high polyphenolic contents including ellagic acid and ellagitannins.[40] The inhibitory effect of *Punica granatum* on lipid peroxidation in rat liver microsomes is related to the ability of its phenolic and flavonoid compounds mainly ellagic acid[41,42] to inhibit hepatic oxidative enzymes (cytochrome P450 system).[16,39] Moreover, Abhijeet and Rupali[43] stated that *Punica granatum* possessed potential hepatoprotective activity which might be attributed to its flavonoids (anthocyanidins) content.

Punica granatum peel extract has been found to have a potent antioxidant activity due to its active compounds that are electron donors and can react with free radicals to convert them to more stable products and thus terminate radical chain reaction.[39,44] This indicates that *Punica granatum* peel extract could preserve the structural integrity of the hepatocellular membrane and liver cell architecture. Therefore, it could prevent the leakage of liver enzymes into serum and restore liver functions.[45]

The present data revealed that serum AFP level in rats bearing HCC displayed significant elevation. This finding comes in line with that of Sadiknah[46] and Sivaramakrishnan[47] who reported that rats injected with NDEA experienced significant increase in serum AFP level. It has been found a high AFP gene expression level in liver tissue of rats administered with NDEA.[48] Moreover, Zhou[49] demonstrated that the high serum concentration of AFP in HCC patients might be due to the tumor excretion of this protein. Furthermore, some clinical researches have indicated that the high percentage of AFP is closely related to poor differentiation and biologically malignant characteristics especially portal vein invasion of HCC.[50,51] The possible explanations for the reinitiation of AFP synthesis by neoplastic hepatocytes include either increased transcription of AFP gene or post-translational modification affecting AFP production. In rats which have been exposed to chemical carcinogens or those with HCC, AFP production is roughly proportional to the amount of transplantable mRNA present.[52] Steady state AFP transcription may begin prior to the development of histologically or symptomatic hepatocellular carcinoma.[53]

In view of the present data serum CEA level in rats bearing HCC showed significant increase. The increase in serum CEA levels upon NDEA administration was presumably associated with production rates of tumor, its location and stage, size, differentiation and vascularity.[54] Macnab[55] demonstrated that the tumor could cause a release of CEA from damaged liver cells adjacent to it. Experimental studies have shown that CEA is normally removed from circulating plasma by the liver.[56] Thus, the increased serum levels of CEA might therefore results from impaired hepatic uptake of CEA or CEA-like glycoproteins. It is well known that varying degrees of liver dysfunction occur in patients with HCC.

The current results revealed that NDEA administration led to significant increase in serum glypican-3 (GPC-3) level. Glypican-3 is a heparan sulfate proteoglycan that has an important role in cell growth and differentiation, and its function in tumorigenesis is tissue-dependent.[57] Moreover, the expression of GPC3, its receptor, and other growth factors coordinate signal transduction pathways that regulate cellular morphology and a variety of cellular behaviors, such as adhesion, proliferation, migration, survival, and differentiation. GPC-3 is differentially expressed during the invasive growth of liver cancer, suggesting that its expression might be involved in the initiation of liver cancer, and might be a critical step in liver cancer development.[58,59] Capurro[60] recorded overexpression of GPC-3 mRNA and protein in both serum and tissues of early-stage HCC. Also, Capurro[61] stated that GPC-3 could stimulate the growth of HCC cells by stimulating the Wnt pathway through facilitating the interaction between the Wnts and their signaling receptors. Chan[62] suggested that the upregulation of GPC-3 in some HCC tumors might be attributed to c-Myc gene amplification. As, it has been demonstrated that c-Myc transcriptionally regulates GPC3 expression by direct interaction with the GPC3 promoter. Together, these data indicate that the transcription factor c-Myc is a novel regulator of GPC3 gene and may accelerate the proliferation of liver cancer cells at least in part by upregulating the GPC3 pathway. Moreover, the exogenous overexpression of c-Myc increased the endogenous mRNA and protein levels of GPC3.[57] In accordance with our results, Suzuki[63] indicated that circulating levels of GPC-3 protein were significantly elevated in patient with HCC and the expression of mRNAs for GPC-3 was markedly elevated in the liver carcinoma tissues. Therefore, GPC-3 showed potential as a tumor biomarker for HCC that can be used molecularly targeted therapy.

Signal transducer and activators of transcription 3 (STAT3) is involved in oncogenesis through the upregulation of genes encoding apoptosis inhibitors [Bcl-xL, Mcl-1(Bcl-xL as well as Mcl-1 are a Bcl-2 family protein which can act as an apical molecule in apoptosis) and survivin], cell-cycle regulators (cyclin D1 and c-Myc (DNA binding protein concerning the regulation on metabolism of nucleic acid and the response to growth factor)), and inducers of angiogenesis (vascular endothelial growth factor). It has been found that STAT3 has an indirect role in GPC-3 expression *via* upregulation of c-myc and the poor control of the pathway mediated by STAT3 plays a role GPC-3 overexpression.[63] Therefore, STAT3 has recently drawn attention as a novel target for cancer therapy.[64,65]

In light of the present data, treatment of HCC bearing rats with *Punica granatum* peel extract significantly decreased

serum AFP, CEA and GPC-3 levels. The observed decline in serum levels of AFP as a consequence of treatment with *Punica granatum* peel extract might be related to the apoptotic effect exerted by ellagic acid, the active compound in *Punica granatum*. Ellagic acid can stimulate apoptosis and completely inhibit the proliferating capacity of the tumor by decreasing nuclear factor kappa B (NF-κB) activity, thereby activating the mitochondrial death pathway, which is associated with loss of mitochondrial membrane potential, cytochrome C release, and caspase-3 activation.[66]

The significant reduction in serum CEA levels in rats bearing HCC and treated with *Punica granatum* peel extract could be attributed to the modulating effect of ellagic acid on the oxidative stress. Ellagic acid is a naturally existing phenolic antioxidant found in *Punica granatum*.[67] It shows potent antioxidant effects by radical scavenging activity and the inhibition of lipid peroxidation.[68,69] Moreover, the anthocyanin, one of the active constituents of *Punica granatum* extract, is very reactive towards reactive oxygen species (ROS) because of its electron deficiency[70] and thus it can counteract oxidative stress. In addition, the antioxidant activity of *Punica granatum* might be related to its flavonoids constituents such as kaempferol and naringin.[71] The synergism between all of these antioxidant compounds in *Punica granatum* peel extract could counteract oxidative stress with consequence regression of serum CEA levels.

The suppressive effect of *Punica granatum* peel extract on serum GPC-3 level in HCC bearing rats, could be explained by more than one mechanism. First, the capability of ellagic acid to inhibit cancer cell growth, *via* reducing the expression of c-Myc[72] which is a direct regulator of GPC-3 gene.[57] Second, the ability of luteolin to induce cell cycle arrest at G0/G1 phase and apoptosis through increasing Bax/BclXL ratio and activating caspase-3 in hepatoma cell lines.[73] Luteolin has been found to induce apoptosis in hepatoma HLF cells by inactivating STAT3 protein, a negative regulator of Fas/CD95 signaling, through ubiquitin-dependent degradation and blocking of CDK5-dependent phosphorylation. This leads to the increased function of Fas/CD95 signaling, caspase-8 activation and ultimately apoptosis.[74] In fact, the antiapoptotic genes encoding c-Myc, Bcl-2, Bcl-xl, cyclin D1, and survivin are downstream targets of STAT3.[75] Therefore, the good control of STAT3 by luteolin present in *Punica granatum* peel extract resulted in the significant decline in GPC-3 serum level in the treated rats as shown in the present study.

The present results revealed that NDEA administration led to significant increase in serum VEGF, HGF and EGF levels. The increase in serum VEGF is greatly supported by the finding of El Mesallamy.[37] Moreover, Liu[76] reported that the expression of VEGF in NDEA administered rats was significantly upregulated. This finding might be attributed to the high angiogenic activity of NDEA-induced hepatocellular carcinoma in rats. Hiramoto[77] reported that NDEA decomposition is accompanied with concomitant release of NO in contact with reactive oxygen species. Ueno[78] suggested that ROS generated by the NDEA could activate the NF-κB-dependent pathways of neutrophil stimulation and enhance proinflammatory cytokine release and NO production. High levels of interleukin-1 (IL-1) or a combination of proinflammatory cytokines, including interleukin-1 (IL-1), tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, could induce iNOS expressionin hepatocytes under a variety of experimental conditions,[79,80] the excessive expression of iNOS leads to an accumulation of NO, which participates in cancer development.[81,82] As, the increased NO level in NDEA administered animals has been found to enhance angiogenesis by stimulating the synthesis of VEGF.[83,84] VEGF gene promotor contains many regulatory sequences including consensus binding sites for specificity protein-1(SP-1), activator protein-1(AP-1) and AP-transcription factors, as well as hypoxia-inducible factor-1(HIF-1).[85] The data using tumor cells, have revealed that NO stabilizes H IF-1 subunit leading to higher HIF-1 binding capacity and increased transcriptional activity of VEGF promotor.[86] Moreover, by upregulating hypoxia-inducible factor, hepatocyte growth factor (HGF) expression is increased in tumor and in the surrounding normal interstitial cells in association with increased expression of HGF receptor (c-met) in tumor and the endothelial cells. HGF c-met signaling induces upregulation of tumoral VEGF expression and endothelial VEGFR2 expression and downregulation of the endogenous inhibitors of angiogenesis.[87,88] Dual VEGF and c-met axis activity demonstrates the increased capillary formation *in vivo*, tubulogenesis *in vitro* and tumoral microvessel density.[88]

The significant increase in serum HGF level as a consequence of NDEA administration in rats observed in the present study is greatly supported by the finding of Burr.[89] HGF is the most potent growth factor for hepatocytes and has been reported to be elevated in a number of liver diseases, including acute and chronic hepatitis, liver cirrhosis, HCC, primary biliary cirrhosis, and hepatic failure,[90] as the expression of HGF is increased in response to liver injury.[91] Circulating pro HGF undergoes proteolytic conversion *via* extracellular proteases including HGF activator (HGFA).[91] Pro-HGFA is activated by thrombin in response to tissue injury and malignant transformation.[92,93] Stellate cells and myofibroblasts have been found to be stimulated to secrete HGF by tumor cell products and HGF has been found to stimulate tumor cell invasiveness in turn.[94] High c-met (HGF receptor) expression has been shown in invasive-type of HCC and has been associated with metastasis and reduced overall survival.[95,96] Moreover, the elevated HGF levels in HCC patients may reflect the impaired clearance of HGF due to severe liver damage or may be caused by increased HGF production to regenerate hepatocytes.[97]

It has been suggested that the elevated serum HGF is probably produced by infiltrating mesenchymal and cancer cells.[98] HGF is secreted primarily by mesenchymal

cells or by stellate and endothelial cells in the liver as an inactive precursor (pro HGF).[99] HGF transcription is upregulated by inflammatory modulators such as TNF-α, IL-1,transforming growth factor beta (TGF-beta) and VEGF in response to liver injury.[91,100] Based on inflammation and releasing of the proinflammatory cytokines stimulated by NDEA,[78] the nuclear transcription factor NF-κB controls a number of genes involved in inflammatory responses, cell cycle progression, inhibition of apoptosis and cell adhesion, hence it plays a critical role in promoting carcinogenesis and cancer progression.[101] Preiss and Sattar[102] reported that NF-κB activation of Kupffer cells, and macrophages within liver tissue increased the expression of HGF and consequently its protein level.

NDEA administration in the present work resulted in significant increase in serum EGF level. The principal angiogenic factors include VEGFs, PDGFs, TGF-alpha and -beta, basic fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), angiopoietins and interleukin-4 and -8.[103,104] It has been shown that some members of the NADPH oxidase (NOX1) family might play essential roles in regulating the autocrine growth and survival of liver tumor cells and this mechanism might be related to over activation of the angiogenic cascade mainly EGFR pathway. Early ROS production by NOX1 activates a Src/ERK pathway that promotes a positive feedback loop on NOX1 upregulation. In parallel, NOX1- induced ROS stimulates p38 and AKT activation which in turn induce TGF-β and EGFR expression with consequent activation of EGFR pathway to induce cell growth. This pathway might be common to tumoral liver cells, being inactive in normal cells.[105]

Treatment of HCC bearing rats with *Punica granatum* peel extract elicited significant reduction in serum levels of VEGF, HGF and EGF. The decreased serum level of VEGF as a consequence of *Punica granatum* treatment might be attributed to its polyphenolic constituent, ellagic acid. Wang[106] reported that ellagic acid exerts potent antiangiogenic activity *via* specifically and directly targeting vascular endothelial growth factor receptor-2 (VEGFR-2) and its signaling pathway. As ellagic acid could inhibit VEGF-induced phosphorylation of VEGFR-2 in endothelial cell as well as platelet-derived growth factor-induced phosphorylation of platelet-derived growth factor receptor (PDGFR) in smooth muscle cells, leading to the inhibition of downstream signaling triggered by these receptors.[107] Moreover, another active constituent of *Punica granatum* called anthocyanidins experienced anticarcinogenic effects through antiangiogenic, antimutagenic activities[108] and inhibition of cyclooxygenase-2 (COX-2) activity and nitric oxide production[109] leading to the reduction of VEGF level. This represents an indirect mechanism for regression of VEGF serum level in HCC bearing rats treated with *Punica granatum* peel extract. Additionally, recent research efforts have shown the efficacy of kaempferol which is a member of flavonols and abundantly found in *Punica granatum* extract[71] in impairing cancer angiogenesis both *in vitro* and *in vivo* through inhibiting VEGF secretion in human cancer cells. Kaempferol appears to inhibit VEGF expression and angiogenesis through an ERK-NF-κB-c-Myc-p21 pathway. Kaempferol administration has been shown to discourage ERK phosphorylation as well as NF-κB and c-Myc expression, the reduction of which, promotes p21 expression, which is a tumor suppressor protein known to antagonize VEGF secretion.[110] Furthermore, kaempferol exerts its effects on VEGF regulators asit lowers HIF levels, and it blocks AKT phosphorylation, impeding the signaling machinery that calls for increased VEGF production.[111]

The observed depletion in serum level of HGF upon treatment of HCC bearing rats with *Punica granatum* peel extract might be related to the chemopreventive effect exerted by its active phytochemicals. Bhatia[112] demonstrated that *Punica granatum* phytochemicals exerted chemoprevention against hepatocellular carcinoma through antiproliferative and proapoptotic mechanisms *via* modulating (NF-κB). Luteolin exhibited its antitumor activity by affecting numerous biochemical pathways critical for the regulation of cell survival, apoptosis, angiogenesis and metastasis, including PI3K/Akt, NF-κB, MAPKs, MMPs and E-cadherin.[113,114] Luteolin has been shown to inhibit metastasis of HepG2 cells by blocking hepatocyte growth factor (HGF) activity.[115] In particular, luteolin has been found to suppress phosphorylation of c-Met, the membrane receptor of HGF, and then inhibits activation of MAPK/ERKs and P13K-Akt pathways involved in HepG2 cell metastasis.[115]

The recorded decline in serum level of EGF due to treatment of HCC bearing rats with *Punica granatum* peel extract could be attributed to that both luteolin and quercetin have protein tyrosine kinase (PTK) inhibitory effect and they exert their antitumor effect by mediating epidermal growth factor receptor (EGFR) tyrosine kinase activity as they can inhibit autophosphorylation of EGFR.[116,117]

The results of the current study showed that the expression level of inhibitor of growth-3 (ING3) gene in liver tissues of NDEA administered rats was significantly downregulated. The tumor-suppressor ING3 has been shown to be involved in tumor transcriptional regulation, apoptosis and cell cycle arrest. Yang[118] demonstrated that the dysregulation of ING3 in HCC occurred at both transcription and translation levels. Downregulation of ING3 was found to be correlated with tumorigenesis and the progression of HCC.[119] It was demonstrated that ING3 was degraded by S-phase kinase-associated protein-2 (Skp2) mediated ubiquitin proteasome system.[120] In addition, there was a correlation between the downregulation of ING3 protein and tumor grades as it has been shown that the low expression of ING3 protein is correlated with more aggressive behavior of the tumor.[118] The deregulation of ING3 plays a continuous

role throughout the processing of cancer advancement and development.

Furthermore, the negative expression of ING3 is also associated with elevated AFP serum levels.[121] It has been reported that p53 negatively regulated AFP gene expression through alteration of chromatin structure at the core promoter.[122,123] Studies of Nagashima[124] and Luo[125] demonstrated that ING3 gene regulated p53. Thus, it is possible to postulate that ING3 could regulate the AFP gene through modifying p53 function. Mutations in p53 gene found in HCC have shown allelic loss at the 17p13.1 region of the chromosome.[126,127] ING3 has been shown to modulate p53-mediated transcription, cell cycle control, and apoptosis, possibly by modulating the NuA4 complex histone acetyltransferase activity. Thus, ING3 cooperates with p53 to regulate apoptosis death receptor/extrinsic apoptotic pathways.[124] In addition, ING3 has been found to upregulate Fas expression at both mRNA and protein levels.[128]

In the present study, NDEA administration resulted in overexpression of liver forkhead box transcription factor (Foxp-1) gene level in rats. Foxp-1, a member of F box family of ubiquitously expressed transcription factors, plays a critical role in cancer development.[129] Foxp-1 is unique because it can act as an oncogene and tumor suppressor depending upon the tissue type.[130] Significant upregulation of Foxp-1 protein in human primary HCC suggests its potential role in hepatic tumorigenesis.[131]

Foxp-1 has been identified as a potential target of miR-1 and the upregulation of miR-1 target Foxp-1 in hepatocellular carcinomas. This further explains the growth regulatory functions of miR-1 in the liver and probably in other tissues by predisposing these tissues to neoplastic transformation due to loss of miR-1. Recently, several studies have showed the deregulation of miR-1 in many types of tumor, such as hepatocellular,[132] prostate,[133,134] thyroid,[135] bladder[136] and renal[137] cancer. Numerous observations indicated an epigenetic deregulation of miRNAs expression in different tumors, including HCC. MiR-1 downregulation in human HCC is associated with promoter methylation.[131] Treatment of primary HCC cultures with the hypomethylating agent caused miR-1 reexpression, which in turn led to downregulation of miR-1 oncogenic targets, including FoxP1 which was overexpressed in HCCs, causing inhibition of cell cycle progression and cell survival.[131] As miR-1 has a specific targets including Foxp-1 that have roles in tumorigenesis, it is likely that miR-1's ability to reduce the expression of many tumor promoting genes could have a global influence on the suppression of tumor development.[131,138-140]

Treatment of HCC bearing rats with *Punica granatum* peel extract produced significant upregulation in the expression level of liver ING3 gene. This finding might be attributed to the apoptotic effect exerted by ellagic acid that activates expression of tumor suppressor genes p53 and p21, leading to cell cycle arrest at the G1/S phase and apoptosis.[141,142] Ellagic acid induces G0/G1-phase arrest of the cell cycle by decreasing expression of Cyclin-dependent kinase 2(CDK2)[142] S-phase arrest of the cell cycle by downregulation of the expression of cyclin A and B1[143] and whole cell cycle arrest by downregulation of Protein kinase C alpha (PKCα)[144] and cyclin D1.[72] Moreover, Horinaka[145] found that luteolin not only induced the mitochondrial pathway of apoptosis but also caspase 8/10 activation and death receptor (DR-5) expression in human malignant cells. These properties of ellagic acid and luteolin in *Punica granatum* peel extract are of great value in preserving gene expression level of liver ING3.

Treatment of HCC bearing rats with *Punica granatum* peel extract produced significant down regulation in the expression level of liver Foxp-1 gene. Luteolin, the substrate of catechol-Omethyltransferase (COMT),[146] has the ability to indirectly inhibit DNA methyltransferase-1 (DNMT-1) by elevating endogenous S-adenosyl homocysteine (SAH) concentration.[147] In fact, the indirect inhibitory effect of polyphenols with catechol (luteolin) or galloyl such as epigallocatechin gallate (EGCG) moiety on DNMTs activity has been demonstrated. These compounds could indirectly regulate DNMT activity by regulating the ratio of S-adenosyl methionine (SAM) and (SAH) during their metabolic methylation by COMT.[147,148] This means that these compounds are considered as hypomethylating agents that have the ability to cause reexpression of miR-1 with consequent downregulation of its targets genes including Foxp-1 gene.

The results of the current study showed that NDEA administration could up-regulate liver expression of β-catenin in rats. The molecular pathogenesis of HCC is multifactorial and is reliant upon dysregulation of multiple pathways including Wnt/β-catenin, mitogen activated protein kinase (MAPK), phosphatidylinositol-3kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), VEGF, PDGF, insulin-like growth factor (IGF), EGF, TGF-β and HGF.[149,150] One pathway of critical importance in HCC is the Wnt/β-catenin signaling. β-catenin is the central effector of the canonical Wnt signaling, which is a highly conserved pathway regulating critical cellular processes such as proliferation, differentiation, survival and self-renewal.[151,152] In the absence of Wnt, β-catenin is phosphorylated at amino-terminal serine and threonine residues and targeted for ubiquitination.[153] Upon binding of Wnt protein to its cell surface receptor Frizzled and co-receptor low density lipoprotein–related protein 5/6 (LRP5/6), a signal is transduced through disheveled that allows the inactivation of degradation complex comprised of glycogen synthase kinase 3b (GSK3b), adenomatous polyposis coli gene product (APC) and casein kinase Ia, which allows β-catenin to dissociate and translocate to the nucleus to bind to lymphoid enhancer-binding factor/T cell factor (LEF/TCF) family of proteins to transactivate target genes. The Wnt/β-catenin pathway has been implicated in a subset of HCC where activating mutations in the β-catenin gene (CTNNB1) have been

reported in HCC patients.[154,155] In HCC, accumulation of β-catenin was present in the early stage of HCC.[156,157] The previous studies investigated the correlation between β-catenin expression and the differentiation grades of HCC, and prognostic roles of β-catenin expression in HCC.[158,159] Suzuki[156] found β-catenin protein expression was located mainly in the cell membrane of highly differentiated HCC tissue with a low Ki-67 proliferation index. In contrast, cytoplasmic or nuclear β-catenin protein expression was observed in less differentiated HCC tissue with a high proliferation index. Thus it can be hypothesized that, to some extent, the change in localization of β-catenin expression reflects the differentiation of HCC tissues.[156]

In view of the present data, NDEA administration led to overexpression of liver survivin in rats. This result comes in line with Zhu[160] Survivin, a novel member of the Inhibitors of apoptosis proteins (IAP) family, inhibits the activation of caspase-3 and -7, which are downstream effectors of apoptosis, in cells exposed to apoptotic stimuli.[161] Also, expression of survivin may occur during early malignant transformation or following a disturbance in the balance between cell proliferation and death.[162] Previous studies have shown that survivin is expressed at a high level in 60%-100% of the most common human tumor types, including colon, pancreas, breast, lung, liver, brain, lymphoma, melanoma and prostate cancers.[163,164] Many cancer cell lines, including human hepatoma cells, also displayed resistance toward TRAIL-mediated apoptosis.[165,166] There is a growing body of evidence indicates that intracellular antiapoptotic pathways including NF-kB and Akt modulate the response to TRAIL in many cancer cells and contribute to resistance toward TRAIL.[167,168] NF-kB has been found to be directly activated by TRAIL[169-171] and it might upregulate antiapoptotic molecules, including cIAP1, and cIAP-2[172] and their members including survivin.

In light of the present data, the expression of Ki-67 in liver tissue of rats administered NDEA showed significant upregulation. This result is in agreement with Cui[173] who demonstrated that Ki-67 expressing rate in cancer tissue was higher than that in para-cancer tissue. Our results are in corformity with that of Pizem[174] who stated that PCNA and Ki-67 were useful for proliferative activity assessment of hepatocytes and their expressions were higher in HCC than in non-neoplastic liver. These authors demonstrated a statistically significant correlation between PCNA and Ki-67 proliferative indices in HCC as well as a positive correlation between tumor grade and Ki-67 index. Umemura[175] reported that high dose of NDEA in experimental animals induced cell proliferation associated with DNA damage, mutations and induction of HCC. On the other hand, study of Yeh[176] on human HCC with histological heterogeneity showed that differentiation of human HCC induced telomerase activation and Ki-67 expression.

Tumor cells consist of proliferating cells (S, G2, M, G1 stage), temporarily non-porliferating cells (G0) and non-proliferating cells (silent stage). Ki-67 can label proliferating cells in any stage except those in stage G0, while it is not expressed in cells in silent stage. Ki-67 is rapidly degraded or its antigenic determinant is disappeared after mitosis. So Ki-67 is considered as a kind of objective marker reflecting proliferating activity of cells.

Cyclooxygenase 2 (Cox-2) is expressed highly in cancer and it is found to be implicated in tumor progression, and its inhibition can reduce tumor growth and augment therapy.[177,178] Ogunwobi[179] reported that upregulation of Cox-2 could promote epithelial mesenchymal transition and carcinogenesis *in vitro*, and could take part in the development of HCC. Cui[180] found that Cox-2 deletion significantly inhibits Ki-67 expression in the HCC xenografts. This indicates that COX-2 overexpression promotes the G1-S transition of the cell cycle in HCC xenografts which means that Cox-2 gene overexpression enhances the expression of Ki-67. Cox-2 might be taking part in the pathogenesis of HCC. Over-expression of Cox-2 has been reported to be associated with hepatocarcinogenesis.

The present data revealed that the treatment with *Punica granatum* peel extract in HCC bearing rats resulted in significant downregulation in β-catenin expression in the liver tissue. Luteolin inhibits the translocation of β-catenin from the cytosol to nucleus in colon cancer *in vitro* and *in vivo*.[181,182] Inhibition of β-catenin is mediated by modulating the expression of phosphorylated glycogen synthase kinase 3β (p-GSK3β). Also, luteolin has been shown to inhibit the expression of cyclin D1, a downstream target of Wnt/β-catenin pathway.[181] Moreover, the expression of β-catenin was decreased significantly in HT29 and in HCT 116 cells treated with ellagic acid, however a marked increase in the expression of p-β-catenin levels was observed. The observed decrease in β-catenin levels and the increase in p-β-catenin expression suggests that ellagic acid effectively inactivated β-catenin and promoted its degradation and thereby modulating Wnt/ β-catenin signalling cascade.[183] Furthermore, it has been suggested that β-catenin/Tcf4 transcriptional activity was inhibited upon treatment with EGCG in HEK293 cells induced with β-catenin/Tcf4.[184] Wnt/β-catenin signaling was found to be inhibited by EGCG in breast cancer cells. Treatment with EGCG resulted in upregulation of high mobility group box (HMG-box containing protein-1), an important antagonist of Wnt signaling.[185,186] EGCG reduced both proliferation and invasiveness of breast cancer cells through induction of HMG-box containing protein 1 and the subsequent downregulation of Wnt/β-catenin signaling.

In light of the present data, treatment with *Punica granatum* peel extract in HCC bearing rats led to significant downregulation in survivin expression in the liver tissue. Li[187] reported that naringin which is a one of the flavonoid compounds present in *Punica granatum* extract[71] induced cell apoptosis and G1 cycle arrest by

modulation of p21 and survivin, through inactivating β-catenin signaling pathway. β-catenin pathway has been confirmed to be an essential regulator for the expression of survivin. As Zhu[188] reported that survivin expression is inhibited by down reguating β-catenin pathway. Moreover, Siegelin[189] demonstrated that some flavonoids such as kaempferol present in the *Punica granatum* extract exerted antitumor activity through inhibiting survivin. In line with this evidence, Jeong[190] stated that kaempferol could induce apoptosis through inhibition of survivin expression. More in details, it has been demonstrated that kaempferol mediated downregulation of phosphorylated Akt, thereby further reducing survivin protein level. The blockage of the serine/threonine kinase Akt activity by kaempferol is important for inhibition of survivin because active phosphorylated Akt enhances the stability of survivin.[189]

The observed downregulation in the expression of Ki-67 in liver tissue due to treatment of HCC bearing rats with *Punica granatum* peel extract might be attributed to the antiproliferative effect exerted by kaempferol. Ackland[191] reported that the reduction in cell proliferation is associated with the decreased expression of nuclear proliferation antigen Ki-67. Jeong[190] demonstrated that kaempferol caused a rapid inhibition of extracellular signal-regulated kinase (ERK) phosphorylation which upon activation contributes to the proliferative responses in cells and are considered to be an essential common element of mitogenic signaling.

Photomicrographs of liver tissue sections of rats in NDEA administered group (HCC group) showed anaplastic activity of hepatocytes characterized by polarity, pleomorphism and hyperchromachtic nuclei. In addition, the ratio between the nucleus and cytoplasm was 1:1 with the presence of acinar glandular structure formation.

Scherer and Emmelot[192] stated that in NDEA administered rats, carcinomas are preceded by the development of so-called altered foci and hyperplastic nodules. Also, Burr[89] reported that there was necroinflammation with perivenular inflammatory cells and macrophages in the liver tissue after NDEA administration. Gupta[193] found that NDEA administration caused vacuolization, loss of normal hepatocellular architecture and presence of pycnotic nuclei.

These findings might be attributed to that NDEA is primarily metabolized in the liver and the reactive generated metabolites are known to damage hepatocytes. NDEA is an N-nitroso alkyl compound described as an effective hepatotoxin and hepatotoxic in experimental animals, producing toxicity after repeated administration.[10]

Microscopic investigation of liver tissue section of rat bearing HCC and treated with high dose of *Punica granatum* peel extract showed focal regenerative reaction surrounding the focal necrosed hepatocytes. Also, the portal area showed inflammatory cells infiltration and seve dilatation in the portal vein.

Meanwhile hepatic parenchyma showed focal degeneration in the hepatocytes. These effects might be related to that *Punica granatum* extract exhibited the antitumor activity, with consequent amelioration of the carcinogenic impact of NDEA. *Punica granatum* has been extensively studied for its antitumor property.[194] This could be attributed to its high polyphenolic contents including ellagic acid and ellagitannins.[40]

Microscopic investigation of liver tissue section of rat bearing HCC and treated with low dose of *Punica granatum* peel extract showed proliferated cystic bile ducts, associated with regenerative reaction surrounding the nodular necrosis and degenerated hepatocytes. In addition, multiple newly formed bile ductules with fibrosis and inflammatory cells infiltration in between were observed.

These findings indicate the ability of this dose of *Punica granatum* peel extract to promote liver regeneration after counteracting the damaging impact of the carcinogenic agent.

The present results revealed that the treatment with doxorubicin (Dox) in HCC bearing rats caused marked increase in serum ALT, AST and ALP activity.

Dox has a metabolic activity that causes generation of free radicals and induction of oxidative stress leading to liver tissue injury.[195] Hence, Dox causes an imbalance between free oxygen radicals (ROS) and antioxidants enzymes resulting in liver damage,[196,197] as indicated by the increased serum indices of liver function including ALT, AST and ALP. The elevated serum levels of liver enzymes as indicators, for hepatocellular damage has been previously reported in Dox-induced hepatoxicity model.[198,199]

The current data revealed that the treatment with Dox in HCC bearing rats resulted in marked decrease in AFP, CEA and GPC-3 serum levels. This finding could be attributed to the powerful apoptotic property of Dox. Dox induces apoptosis *via* p53/Fas/Caspase-8 system.[200-202] This drug stimulates transcriptional activity of the proapoptotic molecule p53, which induces expression of the death ligand (Fas L) leading to activation of the Fas receptor. Activated Fas receptor binds, *via* its cytosolic domain, to adapter proteins such as the Fas-associated death domain (FADD) and the apoptosis initiator, caspase-8.[203] Activated caspase-8 cleaves Bid,[204,205] producing truncated Bid, which interacts with mitochondria to cause the release of cytochrome *c*. The latter activates caspase-9, transmitting apoptotic signals to the nucleus through downstream targets such as caspase-3 and endonucleases, ultimately causing DNA fragmentation and cell death.[203]

The present findings revealed that the treatment with Dox in HCC bearing rats elicited significant decrease in

serum VEGF. This result could be due to the antiangiogenic activity of Dox.

More in details, Lee[206] showed that Dox inhibited HIF-1 transcriptional activity by blocking the binding of hypoxia-induced HIF-1 to DNA. HIF-1a knockdown or inhibition increases the sensitivity of hypoxic tumor cells to Dox.[207,208] Because HIF-1 is a master regulator for many aspects in cancer biology, the inhibition of HIF-1a leads to the disruption of multiple important mechanisms for tumor cell survival, angiogenesis, and progression.

Although the main toxic effects of Dox on hepatocytes include: arrest cell cycle of hepatocytes,[209] oxidative stress and disruption of electron transport, generation of free radicals, and activation of NF-kB,[210] doxorubicin causes cancer cells death with direct toxic effects to living cells resulting in the release of doxorubicin-DNA complex into the intercellular space. This process prevents the release of the cytokines and growth factors including hepatocyte growth factor (HGF). This explains the reduction of serum HGF upon treatment of HCC bearing rats with Dox in the current work.

In light of the present data, the treatment with Dox in HCC bearing rats experienced insignificant change in serum EGF. Dox is known to produce ROS *via* one-electron reduction to the corresponding semiquinone free radicals that then react rapidly with oxygen to generate superoxide radical anions.[211] Thus, Dox treatment results in accumulation of ROS in HCT-116 and in various cell lines like H4IIE hepatoma cells.[212] Overproduction of ROS results in Akt phosphorylation[105] and by this way, Dox treatment could induce Akt phosphorylation with consequent activation of EGFR pathway.[105]

The present findings revealed that the treatment with Dox in HCC bearing rats caused remarkable upregulation in the expression level of ING-3 gene in the liver tissue. Deficiency of Fas expression is one of the mechanisms involved in the immune evasion by tumors. Several antitumor drugs, such as DOX causes apoptosis *via* Fas-mediated activation of caspase-8 *in vitro*.[213] However, the significance of Fas expression in *vivo* is still unclear. Dox has been found to be able to induce activation of caspase-8. The role of caspase-8 appeared to be restricted to apoptosis mediated by death receptors, such as CD95, TNF-R1, and the TRAIL receptors. But, it could be postulated that Dox induces apoptosis through molecular expression of ING-3 gene and in turn activation of caspase-8.[214-216]

The current results showed that the treatment with Dox in HCC bearing rats resulted in observable downregulation in Foxp-1 gene expression level in the liver tissue. Suggesting that DNA methyltransferase-1 (DNMT1) is one of the targets for Dox-induced apoptosis in cancer cells, it has been proposed that the expression levels of DNMT1 in tumor cells might be important criteria that should be taken into account to evaluate the selective cytotoxicity of the drug[217] and to determine the optimal dose regimen. Therefore, the interaction between DNMT1 and Dox might be represented on alternative mechanism for induction of apoptosis in cancer cell by Dox.[218]

The present data revealed that the treatment with Dox in HCC bearing rats induced marked downregulation in β-catenin, survivin and Ki-67 expression in liver the tissue. These findings might be attributed to the antiproliferative and apoptotic effects exerted by Dox.[219] There are two proposed mechanisms by which Dox could act in cancer cells, (i) intercalation into DNA and disruption of topoisomerase-II-mediated DNA repair and (ii) generation of free radicals and their damage to cellular membranes, DNA and proteins.[220]

Denard[221] reported that Dox inhibited proliferation through stimulating *de novo* synthesis of ceramide, which in turn activates CREB3L1, a transcription factor synthesized as a membrane bound precursor. Dox stimulated proteolytic cleavage of CREB3L1 by site-1 protease and site-2 protease, allowing the NH2 terminal domain to enter the nucleus. Within the nucleus, it could activate transcription of genes encoding inhibitors of the cell cycle including P[21]. Also, the apoptotic effect of Dox is linked to the redox activation of Dox by endothelial nitric oxide synthase (eNOS).[222] These mechanisms might be contributed in eliciting the observed downregulation of the proliferative and antiapoptotic markers as a consequence of Dox treatment in HCC bearing rats.

Histological investigation of liver tissue section of rats bearing HCC showed inflammatory cells forming granulomatous lesions and periportal fibrosis after Dox administration.

Dox has been shown to induce accumulation of inflammatory cells.[223] Moreover, it has been reported that Dox is able to cause accumulation of collagen fibers mainly in the portal area, manifested as an early sign of fibrosis.[224]

Furthermore, microscopic examination of liver tissue section of rats bearing HCC and treated with Dox revealed that many hepatocytes showed karyomegaly and pyknotic nuclei indicating apoptosis.

Apoptosis is a common feature of hepatotoxicity induced by many anti-carcinogenic drugs. It may precede necrosis or it may occur concurrently with necrosis.[225]

The influence of Dox on hepatocellular carcinoma comes from its interference with the synthesis of macromolecules, covalent DNA binding and DNA cross-linking, inhibition of topoisomerase II, arresting of tumor cell cycle progression in G2 phase, induction of apoptosis and generation of reactive oxygen radicals.[226]

In conclusion, the present findings shed light on the underlying biochemical and molecular mechanisms in favour of N-nitrosodiethylamine induce hepatocellular carcinoma.

Moreover, the present study provided new evidences about the potent anticancer activity of *Punica granatum* peel methanolic extract in regression of hepatocellular carcinoma in rats.

This effect could be ascribed to the antiangiogenic potential, apoptotic efficacy and antiproliferative capacity of the active phytochemicals in this extract.

These pathways are considered the most important targets in the therapeutic intervention against hepatocellular carcinoma.

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