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# Effect of Pomegranate Hull Extract on Liver Neoplastic Changes in Rats: More than an Antioxidant

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**ABSTRACT**

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide. The current work was designed to elucidate the molecular mechanisms underlying the antitumorigenic effect of pomegranate hull extract (PHE) in livers of rats exposed to the hepatocarcinogen diethyl nitrosamine (DENA) with emphasis on oxidative stress, proliferation, and apoptosis. Male albino rats were divided into three groups: normal control, DENA group, and PHE group. PHE was given to rats orally 3 times weekly for 10 wk, 4 wk before and 6 wk after DENA (200 mg/kg, single i.p. dose). The results indicated a prophylactic effect of PHE against neoplastic changes in the liver, which was evidenced by the decrease of tumor size, liver index, and the anti-apoptotic protein Bcl-2; and the increase of glutathione. PHE group also showed decreased expression of liver cyclin D1 and $\beta$-catenin genes compared with DENA group. It is proved that PHE has antitumorigenic effect and could be a candidate for anticancer drugs.

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## Introduction

Hepatocellular carcinoma (HCC) is a leading primary malignancy of the liver. More than 700,000 newly diagnosed cases of HCC were reported in a survey conducted by the World Health Organization in 2008 (1). There was a significant increase in the incidence of HCC in the United States in the last two decades, which was significantly associated with chronic viral hepatitis, alcohol consumption, and nonalcoholic fatty liver disease (2).

The progression of HCC is a complex multistep process in which oxidative stress plays a key role (3). Excess reactive oxygen species (ROS) act to modulate gene expression, cell adhesion, cell metabolism, cell cycle, and cell death leading to oxidative DNA damage and an increase in chromosomal aberrations associated with cell transformation (4). ROS may also activate cellular signal pathways, such as those mediated by mitogen-activated protein kinase (MAPK), nuclear factor-$\kappa$B (NF-$\kappa$B), phosphatidylinositol 3-kinase (PI3K), p53, and $\beta$-catenin/Wnt leading to enhanced angiogenesis, proliferation, and apoptosis (5).

Pomegranate (*Punica granatum*), a large berry fruit, has been cultivated throughout the Middle East and the entire Mediterranean region (6). The pomegranate peels make up about 50% of the fruit, and they are rich in many compounds including phenolics, ellagitannins, proanthocyanidins, flavonoids, and complex polysaccharides (7). However, its biological properties are mainly associated with the presence of flavonoids and tannins. The peel is the part of the fruit with the highest polyphenolic contents, which give it the antioxidant property (8).

Pomegranate-derived phytoconstituents showed a significant chemopreventive effect against the dietary hepatocarcinogen diethyl nitrosamine (DENA)-induced liver tumorigenesis in rats by potent antioxidant mechanisms (9). Pomegranate extract also showed a promising role as anti-proliferative in MCF-7 and Hs578T breast cancer cells (10). In murine model, it was found that pomegranate extract acts to inhibit growth and angiogenesis of prostate tumors (7). Pomegranate phytochemicals also suppress the inflammatory cascade through modulation of NF-$_k$B signaling pathway, which participates in a number of interconnected signaling pathways implicated in malignancy, such as cell proliferation, cell survival, differentiation, apoptosis, invasion, angiogenesis, and metastasis (9).

PHE has multiple effects on other functions. Upon treatment with PHE, it was found that there was a significant increase in protein C and thrombin-antithrombin complex levels, and a decrease in platelet aggregation and fibrinogen concentration, in a dose-dependent manner (10).

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PHE was also found to affect other liver functions as metabolism through inhibition of CYP 3A4 activity. Carbamazepine (an antiepileptic drug) was found to be metabolized by CYP 3A4 to its active form, carbamazepine-10,11-epoxide. Therefore, co-administration of PHE and carbamazepine results in uncontrolled epileptic fits due to decreased carbamazepine-10,11-epoxide level in blood (11).

The current work was designed to elucidate the molecular mechanisms underlying the antitumorigenic effect of PHE in livers of rats exposed to DENA with emphasis on oxidative stress, proliferation, and apoptosis.

## Materials and methods

### Experimental design

The study was performed in accordance with the guidelines for the care and use of laboratory animals approved by Research Ethics Committee (Faculty of Pharmacy, Tanta University, Egypt). Male albino rats, weighing 100–120 g each, were utilized in the study. Rats were purchased from National Research Center (NRC), Dokki, Giza, Egypt. Rats were weighed and housed in aluminum cages for 2 wk under identical environmental conditions and allowed free access to standard pellet diet and water ad libitum. After acclimatization period, rats were weighed and randomly divided into three groups: Group 1 (normal control group; $n = 10$) given the vehicle for 10 wk. Group 2 (DENA group; $n = 10$) given distilled water orally for 10 wk and i.p. single dose of 200 mg/kg DENA (Sigma-Aldrich Inc, Japan) at the end of the fourth week (12). Group 3 (PHE group; $n = 10$) given PHE (Shaanxi Fuheng (FH) Biotechnology Co., Ltd, China) orally at a dose of 6 g/kg (4) three times weekly for 10 wk, 4 wk prior to and 6 wk after DENA i.p. injection. At the end of the experiment (10 wk), rats were weighed, anesthetized by ether, euthanized, and their livers were dissected. Fresh liver was washed twice with ice-cold saline, dried on clean paper towel, and weighed. Liver index was calculated as liver weight (g)/final body weight (g) $\times 100$. The liver was divided into five portions; one portion was preserved in 10% formalin for histopathological examination and the other portions were immediately frozen in liquid nitrogen and stored at−80°C.

### Quantitative RT-PCR analysis for cyclin D1 and β-catenin

Total RNA was extracted from liver tissue lysed by RNeasy Mini Kit (Qiagen Valencia, CA) and 1–5 μg total RNA was converted to cDNA by ThermoScript RNase H Reverse Transcriptase (TIAGEN OneStep RT-PCR Kit). About 1 μl of the cDNA was then used for quantitative PCR with SYBR Green I PCR Master Mix using the StepOnePlus Real-Time PCR System. The target gene $C_t$ values were normalized to the $C_t$ value of the housekeeping gene (Rat GAPDH) and expressed as relative copy number (RCN). Primers used in RT-PCR were prepared according to Kita et al. (13) and are presented in Table 1. The relative content of the gene amplification product was calculated using the $2^{-\Delta Ct}$ method. The real-time PCR instrument was adjusted according to the program: 95°C for 1 min (Predenaturation) and then 40 cycles (94°C for 15 s, 48°C for 15 s, and 72°C for 30 s)

### Determination of liver Bcl-2

Bcl-2 was measured in liver tissue by rat Bcl-2 ELISA kit (Sunred Biotechnology Company, Shanghai). The concentration of Bcl-2 was determined according to manufacturer procedure and expressed as μg/g tissue.

### Determination of reduced glutathione (GSH)

Liver GSH was estimated by colorimetric kit (Biodiagnostic, Cairo), which depends on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH to produce a dinitrophenyl thioether measured by spectrophotometry at 405 nm.

### Histopathological examination

Liver sections were prepared (3–5 μm thick) and stained with hematoxylin and eosin (H&E). The slides were examined blindly by a pathologist under light microscope. Images were viewed and recorded using Olympus microscope equipped with spot digital camera, using computer program MATLAB software in the Histochemistry and Cell Biology Department, Medical Research Institute, Alexandria University, Egypt.

**Table 1.** Primers for the studied genes.

| Gene | Reverse primer | Forward primer |
|---|---|---|
| Cyclin D1 | 5′-TAGTTGATTACTGGGGTACA-3′ | 5′-GGGAAGTTTTGTTCTCTTTG-3′ |
| β-Catenin | 5′-GAGCTTGCTTTCCTGATTGC-3′ | 5′-GCCAGTGGATTCCGTACTGT-3′ |
| GAPDH | 5′-TCCACCACCCTGTTGCTGTA-3′ | 5′-TGAACGGGAAGCTCACTGG-3′ |

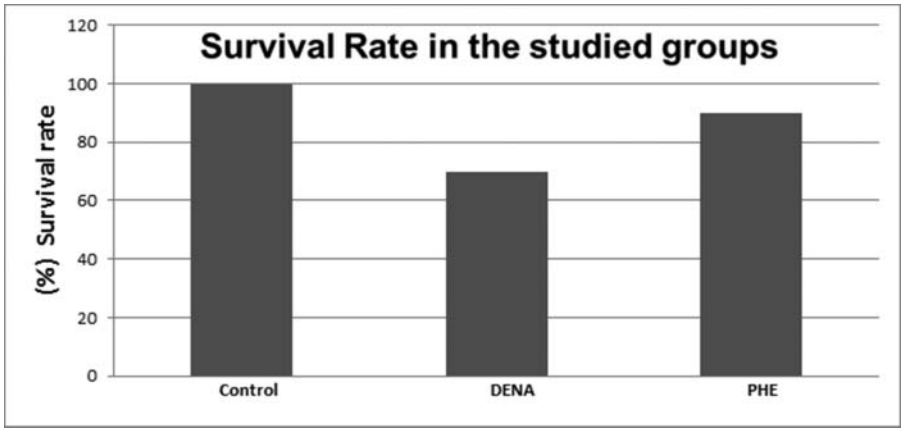


**Figure 1.** Survival rate in the studied groups. DENA, diethyl nitrosamine; PHE, pomegranate hull extract.

### *Statistical analysis*

Analysis of data was performed with statistical package for social science (SPSS) 20.0 (IBM Corporation, Armonk, NY, USA). All data are presented as mean ± SEM. Statistical comparison among groups was performed by one-way analysis of variance (ANOVA). Statistical significance was obtained at $P > 0.05$.

## Results

The survival rate was 70% in DENA group and 90% in PHE group compared to 100% in the normal control group (Figure 1). DENA group showed a significant increase in liver index (1.77-fold increase, $P > 0.05$) compared to normal control group, while treatment with PHE showed significant decrease ($P > 0.05$) in liver index when compared to DENA group (Figure 2). The liver index in PHE group returned to near its value in the normal control.

The sections from liver of normal control group showed normal cells with little infiltration of fat cells (Figure 3), while sections from DENA group showed that there is a large follicle of invasive tumor cells, newly formed blood vessels, surrounded by eosinophilic hepatocytes and apoptotic cells (Figure 4). Treatment with PHE reduced histopathological changes and showed area of recovering hepatocytes, minimum infiltrating lymphocytic cells, as well as newly formed hepatocytes (stem cells) (Figure 5). The size of hepatocellular foci was significantly decreased in PHE group (↓66.58%, $P > 0.05$) when compared to DENA group (Figure 6).

Single i.p injection of DENA significantly enhanced cyclin D1 gene expression (3.3-fold, $P > 0.05$) compared to normal control group while treatment with PHE reversed DENA action and decreased expression of

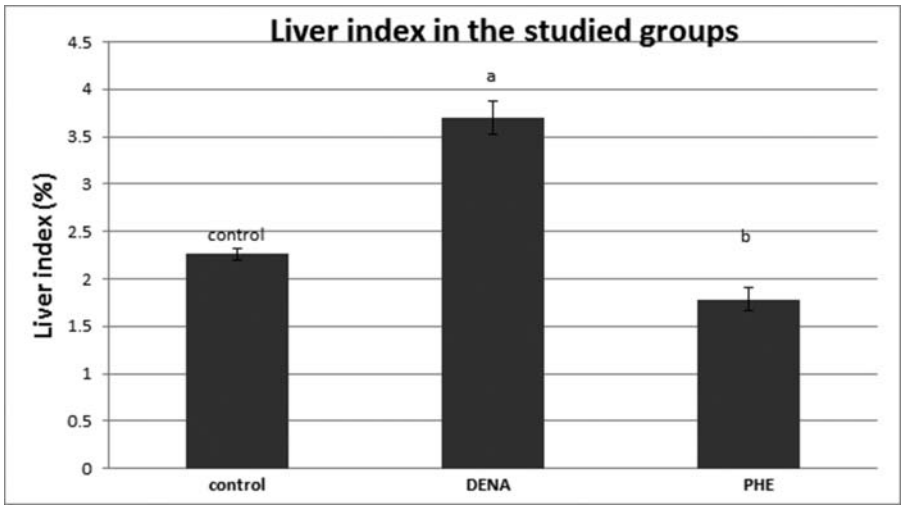


**Figure 2.** Liver index in the studied groups. Data are presented as mean ± SEM, $n = 8$. A: Significant versus control. B: Significant versus DENA. DENA, diethyl nitrosamine; PHE, pomegranate hull extract.

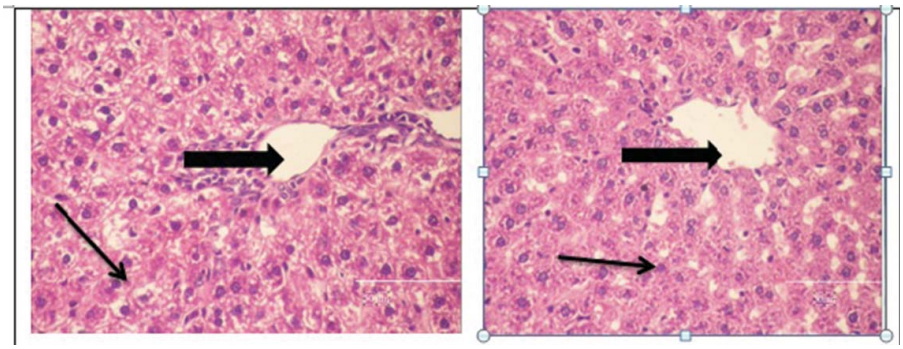

**Figure 3.** Sections of liver from normal control group.

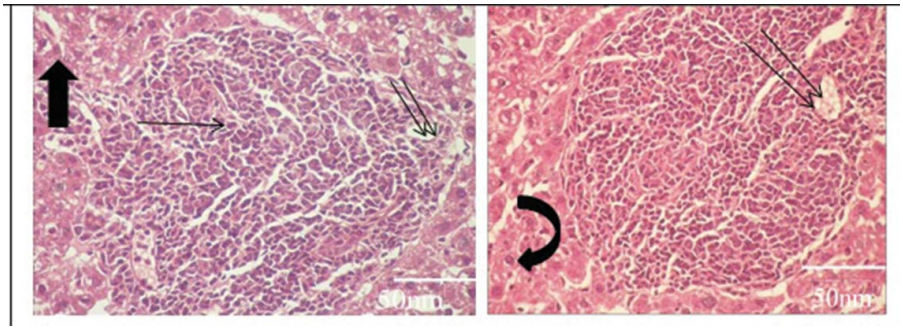

**Figure 4.** Sections of liver from DENA group.

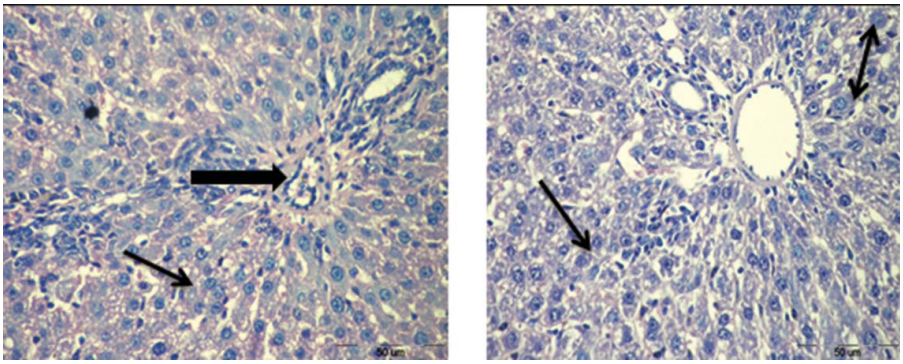

**Figure 5.** Sections of liver from PHE group.

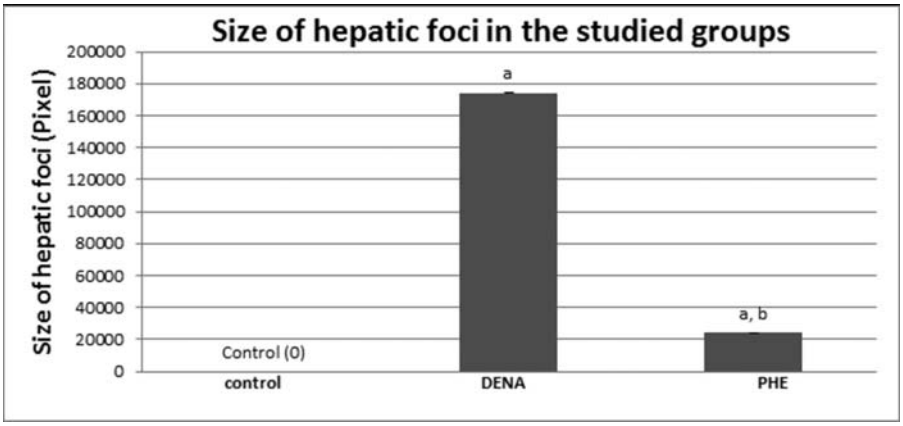


**Figure 6.** Size of hepatic foci in the studied groups. Data are presented as mean ± SEM, $n = 5$ for each group. A: Significant versus control. B: Significant versus DENA. DENA, diethyl nitrosamine; PHE, pomegranate hull extract.

**Table 2.** Effect of DENA and PHE on gene expression of cyclin D1 and $\beta$-catenin.

| Group | CyclinD1 (RCN) | $\beta$-Catenin (RCN) |
|---|---|---|
| Normal control | $0.28 \pm 0.164$ | $0.331 \pm 0.098$ |
| DENA | $1.72 \pm 0.444^{a}$ | $1.168 \pm 0.546^{a}$ |
| PHE | $0.02 \pm 0.01^{ab}$ | $0.026 \pm 0.004^{ab}$ |

Date are presented as mean $\pm$ SEM, $n = 6$ rats per group.
[a]Significant versus normal control.
[b]Significant versus DENA, RCN: relative copy number.

cyclin D1 gene significantly ($P > 0.05$) (Table 2). Treatment with PHE showed a significant decrease in $\beta$-catenin gene expression ($P > 0.05$) when compared to DENA group, while DENA group showed an increase in $\beta$-catenin gene expression (3.34-fold, $P > 0.05$) compared to normal control group (Table 2). The gene expression of cyclin D1 and $\beta$-catenin in PHE group was significantly lower than its value in the normal control group.

Figure (7) shows that Bcl-2 protein level was significantly increased in liver tissue of rats of DENA group (1.5-fold increase, $P > 0.05$) compared to normal control group, which was significantly reversed by PHE treatment ($P > 0.05$).

Liver GSH exhibited a significant decrease in DENA group (↓78.45%, $P > 0.05$) versus control group, but treatment with PHE increased GSH significantly when compared with DENA group (1.4-fold increase, $P > 0.05$) (Figure 8).

## Discussion

Because most chemotherapeutics have many adverse effects such as bone marrow suppression, oral mucositis, and alopecia, there is a new approach toward cancer prevention rather than treatment. The new trend toward complementary and alternative medicine is to use natural products (14).

This study aims to evaluate the antiproliferative, proapoptotic, and antioxidant effects of pomegranate in rats exposed to the hepatocarcinogen DENA carcinogen. In the present work, neoplastic changes in liver were induced in rats as evidenced by the histopathological examination. The liver tissues obtained from DENA group showed large follicle of invasive tumor cells, newly formed blood vessels, surrounded by eosinophilic hepatocytes and apoptotic cells. Moreover, the DENA group showed enlarged liver with greater liver index and the presence of hepatic foci indicating that DENA increase proliferation of hepatic cells. The present findings were in agreement with Heindryckx et al. (15), who reported that DENA-induced neoplastic changes were associated with a significant increase in liver size and liver index.

The histopathological changes induced by DENA, in the current work, were partially corrected by pomegranate treatment, where the liver sections showed area of recovering hepatocytes, minimum infiltrating lymphocytic cells, as well as newly formed hepatocytes (stem cells), together with a decrease in liver index and size of hepatic foci. According to (Figure 7), apoptosis is more common in PHE in correspondence with DENA group. Within the interstitial space, there are apoptotic cells with condensed cytoplasm, condensed and hyperchromatic chromatin, and fragmented nuclei (Figure 5). Therefore, basophilic appearance of figure of PHE group depends on its action as a proapoptotic on neoplastic changes and exposure of DNA fragments for binding to hematoxylin giving it a blue color (16). Also, it is evident from Figure 5 that new blood vessel is still present, which

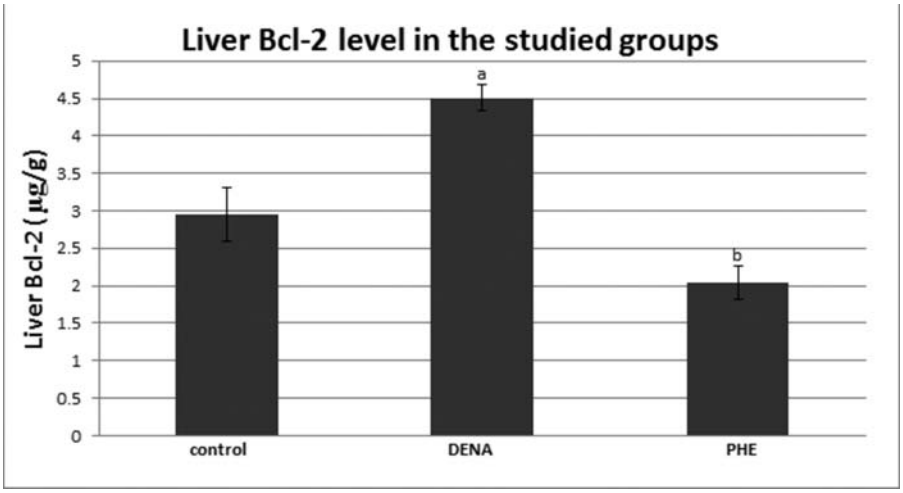


**Figure 7.** Liver Bcl-2 level in the studied groups. Data are presented as mean $\pm$ SEM, $n = 8$ rats per group. A: Significant versus control. B: Significant versus DENA. DENA, diethyl nitrosamine; PHE, pomegranate hull extract.

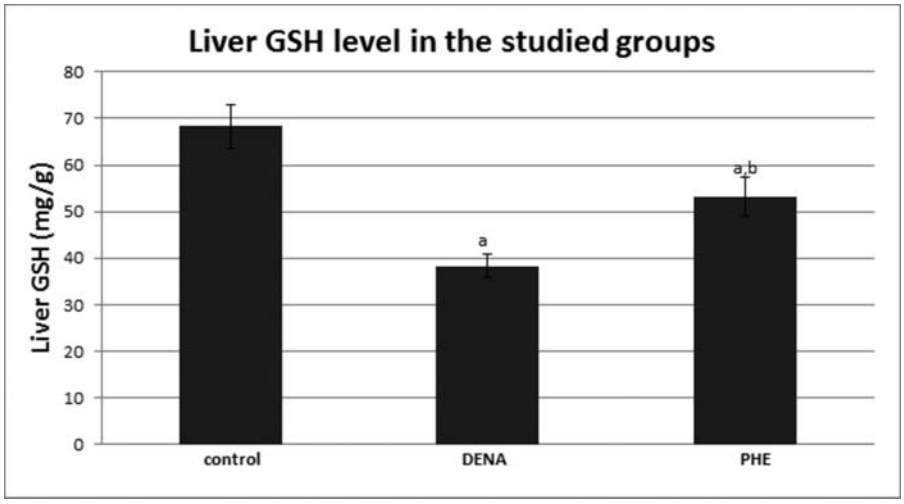


**Figure 8.** Liver GSH level in the studied groups. Data are presented as mean ± SEM, $n = 8$ rats per group. A: Significant versus control. B: Significant versus DENA. DENA, diethyl nitrosamine; PHE, pomegranate hull extract.



indicated that PHE did not affect angiogenesis. The authors concluded that PHE would be used as a prophylactic natural product against neoplastic changes and not as a treatment for metastatic cancer.

Our gene expression study supported the histopathological results and indicated that the tumorigenic effect of DENA was associated with a significant increase in gene expression of $\beta$-catenin and cyclin D1. $\beta$-catenin activation is crucial for liver development and regeneration (17). $\beta$-catenin acts both as a transcriptional co-regulator and an adaptor protein for intracellular adhesion and provides a mechanical linkage between intracellular junctions and cytoskeletal proteins (18). Stable $\beta$-catenin interacts with the lymphoid enhancer factor/T-cell factor (LEF/TCF) and is translocated into the nucleus as a complex of $\beta$-catenin/LEF/TCF to stimulate target gene transcription (19). Hyperphosphorylation of $\beta$-catenin by glycogen synthase kinase 3$\beta$ (GSK-3$\beta$) and casein kinase 1 (CK1) leads to its ubiquitination and proteasomal degradation (20). Decreased $\beta$-catenin expression in the cell membrane and its abundance in the nucleus or cytoplasm can lead to decreased intercellular adhesion and promote the invasion and metastasis of tumor cells (21). DENA increases the expression of $\beta$-catenin by stimulating release of ROS (4).

Our data showed that pomegranate treatment significantly decreased $\beta$-catenin gene expression. Downregulation of $\beta$-catenin gene by pomegranate could be explained by the fact that PHE contains ellagic acid, which suppresses carcinogenesis by preventing the constitutive activation of Wnt pathway through the upregulation of GSK-3$\beta$ resulting in degradation of $\beta$-catenin (22).

Cyclin D1, a cell cycle regulatory protein, is responsible for the transition from G1 to S phase (23). Dysregulated expression of cyclin D1 gene may contribute to cellular genomic instability and malignancy (24). Elevated expressions of cyclin D1 gene and protein disrupt G1/S regulatory point of the cell cycle, which leads to abnormal cell proliferation (25). Cyclin D1, together with its binding partners CDK4 and CDK6, promote cell cycle progression (6). Binding of $\beta$-catenin to TCF results in upregulation of cyclin D1 gene expression, which results in increasing cell proliferation, so inhibition of $\beta$-catenin gene amplification results in downregulation of cyclin D1 gene expression (26). As shown by our results, pomegranate treatment significantly decreased $\beta$-catenin gene expression and subsequently cyclin D1 gene expression. Another explanation for the effect of pomegranate on cyclin D1 gene expression is that PHE treatment also upregulates the level of the p21 and p27 (inhibitor of cyclin-CDK complexes) during G1 phase arrest and thereby initiates apoptosis (27).

The nuclear factor $\kappa$B (NF-$\kappa$B) is a transcription factor complex whose activation has often been linked to recurrence, poor survival, tumor progression, aggressiveness, and chemoresistance (27). DENA increases phosphorylation of p65 at Ser276, which is probably the key mechanism of NF-$\kappa$B activation (28). There is a strong positive correlation between NF-$\kappa$B and the expression of NF-$\kappa$B-regulated gene products including Bcl-2, cyclin D1, matrix metalloproteinase-9, and vascular endothelial growth factor (29). In the current work, PHE significantly decreased expression of cyclin D1 gene when compared with DENA group. PHE and its constituent ellagic acid have been demonstrated to inhibit

NF-$\kappa$B, which leads to downregulation of the expression of cyclin D1 and cyclin B1 (7). The observed protective effect of pomegranate, herein, could be attributed to the suppression of cyclin D1 and $\beta$-catenin genes in rat liver.

The present results indicated that DENA induced oxidative stress and decreased level of liver GSH. As explained by Ajiboye et al. (30), DENA treatment increases the level of ROS, such as hydrogen peroxide (H2O2), hydroxyl radicals (•OH), and superoxide anion radical (O2−). The high intracellular levels of ROS can lead to damaged mitochondria, DNA modification, and lipid peroxidation (31). Under oxidative stress the GSH is oxidized into GSSG, so GSH concentration is decreased subsequently. Furthermore, DENA metabolite can attack the nucleophilic sites of DNA, forming DNA adduct and thereby inducing carcinogenesis (23).

In our research, PHE antagonized the cancerous effect of DENA through increasing the amount of GSH, which may be due to the scavenging action of ellagic acid on both oxygen and hydroxyl radicals, and inhibition of lipid peroxidation formation. It has been shown that the two lactone groups of ellagic acid can act as a hydrogen bond donor and acceptor, which might be involved in the free radical scavenging action of ellagic acid (32).

Cancer also usually results from dysregulation of apoptotic pathway through increasing of anti-apoptotic protein Bcl-2 and decreasing of proapoptotic protein Bax resulting in apoptotic evasion and better survival of cancerous cells (33). Defects in programmed cell death mechanisms play important roles in tumor pathogenesis, allowing neoplastic cells to survive over intended lifespans, providing protection from oxidative stress and hypoxia as the tumor mass increase (34).

The proliferation/apoptosis imbalance was further demonstrated in the present work by the elevated level of the antiapoptotic protein Bcl-2 in livers of rats of DENA group. On the other hand, pomegranate significantly decreased Bcl-2 protein when compared with DENA group. Bcl-2 has been reported to be elevated in cancerous cells, and has an important role in development of cancer (35). DENA increases phosphorylation of p65 at Ser276, which is probably the key mechanism of NF-$\kappa$B activation (28) and thus increasing amount of Bcl-2 (29).

The Bcl-2 protein family acts as key regulators in the intrinsic or "mitochondrial" apoptosis pathway. Bcl-2 inhibits apoptosis by inhibiting the release of cytochrome c, apoptotic protease activating factor-2 (APAF-2) and apoptosis-inducing factor (AIF) from the mitochondria to the cytoplasm and by limiting the activation of caspase-3 through inhibition of its activator protein, APAF-1 (7).

Bcl-2 undergoes S-nitrosylation by nitric oxide (NO) in response to multiple apoptotic mediators, which results in inhibition of ubiquitin-proteasomal degradation of Bcl-2 (29). The pomegranate induced decrease of Bcl-2, which may be due to inhibition of NO production (36) and inhibition of S-nitrosylation of Bcl-2, and hence increased its ubiquitination, and promoted apoptotic cell death (37).

## Conclusion

In summary, PHE is proved to have antitumorigenic activity mediated by downregulation of $\beta$-catenin and cyclin D1 genes, and enhancement of antioxidant activity through increasing GSH level and proapoptotic properties by decreasing Bcl-2 level. Being a natural product, pomegranate could be a candidate for anticancer drugs with fewer side effects toward noncancerous tissues, which requires further investigations.

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