Received: 25 July 2018 | Revised: 14 November 2018 | Accepted: 9 January 2019

DOI: 10.1002/jbt.22305

WILEY

Involvement of PI3K/Akt pathway in the protective effect of hesperidin against a chemically induced liver cancer in rats

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Abstract

Hesperidin is a flavanone glycoside that is found in the *Citrus* species and showed antioxidant, hepatoprotective as well as anticancer activity. This study investigated the effect of hesperidin on the PI3K/Akt pathway as a possible mechanism for its protective effect against diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC). Adult Wistar rats were divided into Control group (received drug vehicle); DEN group (received 100 mg/L of DEN solution for 8 weeks), and hesperidin + DEN group (received 200 mg/kg body weight of hesperidin/day orally for 16 weeks + DEN solution as DEN group). Our findings showed that the administration of hesperidin significantly decreased the elevation in liver function enzymes, serum AFP level, and oxidative stress markers. Moreover, hesperidin administration suppressed DEN-induced upregulation of PI3K, Akt, CDK-2 protein expression, and preserved the integrity of the liver tissues from HCC formation. In conclusion, the hepatoprotective activity of hesperidin is mediated via its antioxidation and downregulation of the PI3K/Akt pathway.

KEYWORDS
animal models, antioxidant, cancer prevention, cell signaling, flavonoids

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer in adults and it is counted as the third among cancer-related mortality worldwide.[1,2] HCC is usually developed after chronic hepatitis and liver cirrhosis. It arises commonly in chronic hepatitis B and C viral infections, alcohol abuse, nonalcoholic fatty liver disease, and nonalcoholic steatohepatitis.[3,4] Furthermore, other liver diseases such as hemochromatosis, Wilson's disease, and α-1 antitrypsin deficiency are associated with HCC development.[5] Among predisposing factors to HCC development is the exposure to the genotoxic hepatocarcinogen; nitrosamines.[6] Nitrates and nitrites, which are usually added as food preservatives and coloring agents to processed meats, are precursors of nitrosamines. They combine with the amine content of the food either on cooking or in acidic pH of the stomach to form nitrosamines.[7,8] Moreover, occupational exposure to nitrosamines can occur in the rubber and herbicides factories as well as research laboratories.[6] Diethylnitrosamine (DEN) is one of the most common nitrosamines existing in food.[7] The carcinogenicity of DEN is attributed to its direct DNA alkylating effect and production of reactive oxygen species (ROS) after its bioactivation by CYP450 enzymes in the liver.[9,10] In addition, prolonged DEN exposure promotes continuous cycles of hepatocytes necrotic death-inflammation-regeneration and eventually fibrosis and cirrhosis. Importantly, the sustained ROS production had been reported to activate cell-survival signaling cascades such as the PI3K/Akt pathway.[11–13] Therefore, DEN-induced HCC imitates the same sequential progression of human HCC with poor prognosis. Additionally, DEN-induced HCC animal models are established for understanding the molecular pathways involved in hepatocarcinogenesis.[14]

Phytochemicals, such as flavonoids of herbs and fruits showed chemopreventive effects against multiple human cancers.[15] Hesperidin is a flavanone glycoside that is found in the *Citrus* species.[16,17] It has been reported with antioxidant, anti-inflammatory, and antifibrotic activities. Interestingly, hesperidin exhibited antioxidant hepatoprotective activity against CCl4-induced hepatocytes damage and lipopolysaccharide-induced hepatotoxicity in a dose-dependent manner.[18,19] Moreover, it protected against the inflammatory and oxidative hepatocyte injury mediated by antineoplastic drugs.[20,21] Many studies

reported that hesperidin possesses an anticancer effect.[22] It exhibited a chemopreventive effect against DEN-induced renal carcinogenesis by induction of apoptotic proteins and decreasing the biomarkers of both inflammation and cell proliferation.[23]

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway regulates various cellular responses and it has been targeted by human cancer therapy. The PI3Ks are a family of lipid kinases that the phosphorylates the 3′-OH group of the inositol ring of phosphatidylinositols on the plasma membrane.[24,25] These enzymes are activated by either upstream tyrosine kinase receptors or G protein-coupled receptors and they mediate multiple cellular processes such as inflammation, metabolism, and cancer progression. The serine/threonine kinase (Akt) is the main downstream effector of PI3K signaling. Interestingly, the PI3K/Akt signaling pathway is highly mutated and activated in various cancers since it mediates cancer cell proliferation, survival, migration, and angiogenesis.[25–27] Importantly, it has been reported that PI3K/Akt cascade is involved in HCC progression, vascular invasion, and metastasis, and it is correlated with poor prognosis and survival in HCC patients.[28] Therefore, in this study, we investigated the ability of hesperidin to protect against DEN-induced HCC in rats and its effect on PI3K/Akt signaling cascade.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult male Wistar rats (160–180 g) were obtained from Nahda University Animal House at Beni-Suef, Egypt. Rats were kept under standard environmental conditions with supplied food and water ad libitum. All experimental procedures performed in the study were approved by the Institutional Animal Care and Use Committee at Beni-Suef University.

2.2 | Experimental design

Thirty male Wistar rats were divided into three groups (10 rats each) as follows:

Control group: Rats were given corn oil by oral gavage daily (from week 1 to week 16).

DEN group: Diethylnitrosamine solution (DEN; Sigma-Aldrich, St. Louis, MI) was given in drinking water (100 mg/L) for 8 weeks, as the same dose that was used previously[29–33] to induce HCC in rats. From week 9 onward, rats were given only DEN-free water.

Hesperidin + DEN group: Hesperidin (H5254; Sigma-Aldrich) was dissolved in corn oil and given by oral gavage to rats at a dose of 200 kg body weight (b.wt.) daily from weeks 1 to 16, as the same dose used previously by Omar et al[20] while DEN solution was given in the drinking water (100 mg/L) to rats for only the first 8 weeks.

After week 16, rats were fasted for 12 hours overnight then blood samples were collected from retro-orbital plexus, left to coagulate for 30 minutes at room temperature and finally centrifuged for sera preparation. After that, rats were killed by cervical decapitation under anesthesia and livers were dissected and washed in saline. Thirty milligrams of the liver were rapidly removed and preserved in protease inhibitor buffer at −80°C for Western blot analysis. Twenty percent of tissue homogenates were prepared, whereas the liver sections were homogenized in ice-cold phosphate-buffered saline and centrifuged by a cooling centrifuge at 3000 rpm for 20 minutes. The supernatants were collected and stored until analysis.

2.3 | Biochemical measurements

The serum activities of alanine transaminase (ALT) and aspartate transaminase (AST) were measured kinetically by ALT and AST assay kits, respectively (Spinreact, Girona, Spain). Alkaline phosphatase (ALP) activity was measured by ALP kit (Gesellschaft Fur Biochemica Und Diagnostica MbH, Wiesbaden, Germany). Serum albumin, total and direct bilirubin concentrations were determined using kits provided by Diamond Diagnostics Co (MA) according to the manufacturer's procedures. The serum α-fetoprotein (AFP) level was measured by Rat Alpha-Fetoprotein ELISA Kit (MyBiosource, San Diego, CA) according to the kit's protocol. Concentrations of malondialdehyde (MDA) and nitric oxide (NO) in addition to the activities of catalase (CAT), glutathione peroxidase (GPx), were measured by kits provided by Biodiagnostic Co (Giza, Egypt) according to the manufacturer's procedures.

2.4 | Histopathological examination of liver tissues

Liver samples from the right lobe were dissected and fixed in 10% neutral-buffered formalin. After that, graded ethanol solutions were used to dehydrate the fixed tissues, which were cleared in xylene and embedded in paraffin wax. Using hematoxylin and eosin (HE) stain, 5-μm thick sections were examined under a light microscope at ×100 magnification.

2.5 | Western blot analysis

Twenty micrograms of extracted proteins from each sample were loaded into the wells of 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After gel electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes. Following this, the membranes were blocked and incubated separately overnight at 4°C with the following primary antibodies: PI3K antibody (PA5–32550; Thermo Fisher Scientific, Waltham, MA), phosphorylated Akt antibody (PA5–38251; phosphorylation site at serine 124; Thermo Fisher Scientific, Waltham, MA), and CDK2 antibody (PA5–79024; Thermo Fisher Scientific, Waltham, MA). The blots were washed thoroughly and horseradish peroxidase (HRP)-secondary antibody was added to the blots and incubated for 1 hour at room temperature. The blots were visualized by the chemiluminescent substrate (cat no. 170-5060; Clarity Western ECL substrate; Bio-Rad, Hercules, CA) and the bands were quantified using Bio-Rad image analysis software.

TABLE 1 The liver function tests in control, DEN, and hesperidin + DEN groups

Parameter	Control group	DEN group	Hesperidin + DEN group
ALT, IU/L	46 ± 2	97 ± 8*	67 ± 6#
AST, IU/L	131 ± 9	246 ± 19*	209 ± 20
ALP, U/L	208 ± 22	795 ± 36*	539 ± 34###
Albumin, g/dL	4 ± 0.1	4 ± 0.0	4 ± 0.1
Total bilirubin, mg/dL	0. 3 ± 0.1	0.8 ± 0.1*	0.78 ± 0.1
Direct bilirubin, mg/dL	0.11 ± 0.0	0.141 ± 0.0	0.141 ± 0.0
Indirect bilirubin, mg/dL	0.4 ± 0.0	0. 7 ± 0.1	0.61 ± 0. 1

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DEN, diethylnitrosamine.
Values are expressed as mean ± SE.
*Statistically significant in comparison with the control group at $P < 0.05$.
#Statistically significant in comparison with the DEN group at $P < 0.01$.
###Statistically significant in comparison with the DEN group at $P < 0.001$.

2.6 | Measurement of caspase-3 concentration by enzyme-linked immunosorbent assay

The quantitative determination of caspase-3 level in the liver tissue homogenates was done using (Rat Cysteinyl Aspartate Specific Proteinase [CASPASE-3] ELISA Kit; MyBiosource). Briefly, liver tissue homogenates were added to the wells of a microtiter plate with anti-caspase-3 antibody and then were incubated with caspase-3-HRP conjugate for 1 hour. The color intensity was measured at 450 nm by a plate reader and it was inversely correlated with caspase-3 concentration. Using a standard curve, the caspase-3 concentration was calculated and expressed as (ng/mg tissue). The sensitivity of the kit is 0.1 ng/mL.

2.7 | Statistical analysis

Data analysis was done using SPSS (IBM SPSS Statistics version 22, SPSS Inc, Chicago, IL). Data are expressed as mean ± SE. Statistical comparisons were evaluated by one-way analysis of variance, followed by Tukey's post hoc test and the significance was set at $P < 0.05$.

3 | RESULTS

3.1 | Effect of hesperidin on DEN-induced hepatotoxicity

The results showed that serum ALT, AST, ALP activities, and total bilirubin concentration were significantly elevated in the DEN group in comparison with the control group at $P < 0.05$. Also, the results showed a statistically significant difference in ALT and ALP activities between hesperidin + DEN and DEN groups at $P < 0.05$, while there was no statistically significant reduction in serum AST activity and total bilirubin between both groups (Table 1). In addition, there was no statistically significant difference in the albumin level among the studied groups. These results indicated that DEN was a hepatotoxic agent and hesperidin effectively protected against DEN-induced hepatotoxicity.

3.2 | Effect of hesperidin on DEN-induced macroscopic and microscopic liver damage

The macroscopic examination of the livers from the DEN group showed the appearance of nodules as markers of liver carcinoma. However, the gross appearance of HCC was minimally noticed on livers of hesperidin + DEN group in comparison with the DEN group (Figure 1A).

The microscopic examination of the liver tissue sections by HE stain revealed that DEN group showed severe degenerative changes, necrotic hepatocytes death, fibrous connective tissue proliferation and HCC formation (moderately differentiated HCC, stage II) when compared with the control group. Interestingly, the hesperidin + DEN group showed no blood vessel invasion with minimal lymphatic vessels invasion as well as mild congestion and hepatocytomegally. Hesperidin attenuated the HCC development since the hesperidin + DEN group showed stage I of HCC development (Table 2 and Figure 1B). These results indicated that hesperidin could largely protect the liver from the macroscopic and microscopic features of DEN-induced liver carcinogenesis.

3.3 | Effect of hesperidin on DEN-induced elevation of AFP

To confirm our histopathological findings, we measured the serum AFP level among the studied groups. Our results showed a

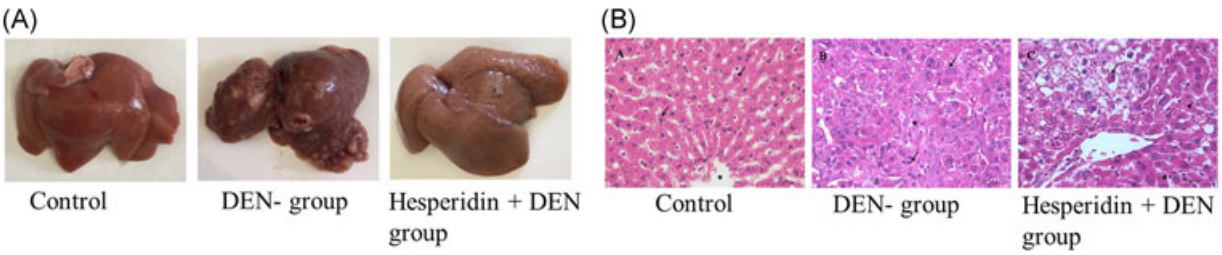

FIGURE 1 Effect of hesperidin on the macroscopic and microscopic features of liver damage. A, Macroscopic examination of livers from control, DEN, and hesperidin + DEN groups. B, Images of liver sections stained by HE. A, Control group showed a normal histological structure of hepatocytes (arrows) and central vein (*); B, DEN-A group showed hepatocytomegaly with mitotic figures of neoplastic cells (arrow) and proliferation of spindle shape fibrocytes (*); C, Hesperidin + DEN group showed regenerative changes of hepatocytes (*) with degenerated and necrotized hepatocytes (arrow); ×100 magnification. DEN, diethylnitrosamine; HE, hematoxylin and eosin [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Scores of the hepatic lesions among the studied groups

Lesion	Control group	DEN group	Hesperidin + DEN group
Degenerative changes	−/+	+++	+++
Fatty changes	−/+	++	++/+++
Necrosis	−	+++	+/++
Hepatocytomegally	−	++	+
FCT proliferation	−	+++	++
HCC formation	−	+++	+/++
Mitotic figure	−	+++	+/++
BV invasion	−	+	−
LV invasion	−	++	−/+
Van Kupffer cell proliferation	−/+	+++	++
Congestion	−	++	+
Leukocytic proliferation	−	+++	++
HCC stage	−	Stage II	Stage I

Abbreviations: BV, blood vessel; DEN, diethylnitrosamine; FCT, fibrous connective tissue; HCC, hepatocellular carcinoma; LV, lymphatic vessel. −, not present, −/+, minimal; +, mild, ++, moderate, +/++, mild to moderate, +++, severe.

significant increase in serum AFP level in the DEN group in comparison with the control group (7.84 ± 0.92 vs 0.902 ± 0.072, respectively, at $P < 0.001$). Interestingly, there was a significant decrease in serum AFP level in the hesperidin + DEN group when compared with DEN group (2.7 ± 0.38 vs 7.84 ± 0.92, respectively, at $P = 0.004$) (Figure 2).

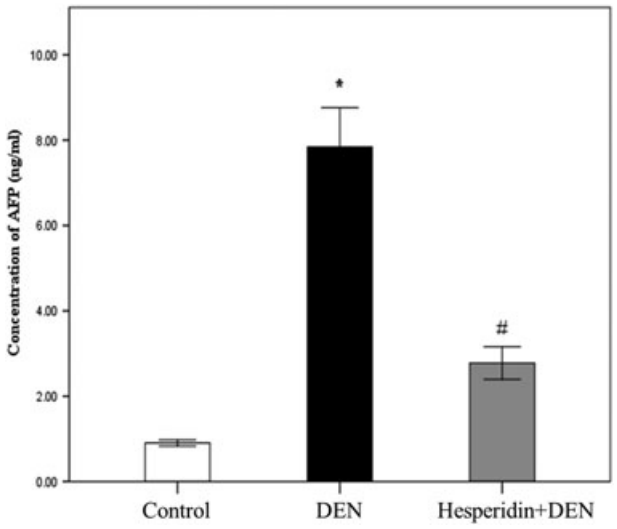

FIGURE 2 Effect of hesperidin on the serum α-fetoprotein level. Column chart shows a significant decrease in serum α-fetoprotein (AFP) level of the hesperidin + DEN group in comparison with the DEN group. Values are presented as mean ± SE. *Statistically significant in comparison with the control group at $P < 0.05$; #Statistically significant in comparison with DEN group at $P < 0.05$. DEN, diethylnitrosamine, GPx, glutathione peroxidase; MDA, malondialdehyde; NO, nitric oxide

3.4 | Effect of hesperidin on DEN-induced oxidative stress

The results showed a significant increase in the MDA and NO levels of the DEN group in comparison with the control group at $P < 0.001$ (Figure 3 A&B respectively). Additionally, the DEN group showed significantly lower CAT and GPx activities when compared with the control group at $P < 0.001$ (Figure 3 C&D respectively). Interestingly, the results showed a significant decrease in the MDA and NO levels of the hesperidin + DEN group in comparison with the DEN group at $P < 0.001$. Additionally, the hesperidin + DEN group showed significantly higher CAT and GPx activities in comparison with the DEN group at $P < 0.001$. These results indicated that hesperidin was protective against DEN-induced oxidative damage.

3.5 | Effect of hesperidin on PI3K, Akt, and CDK2 proteins expression

The results of western blot analysis showed a significant increase in PI3K and phospho-Akt proteins concentrations in the DEN group when compared to the control group. Additionally, the results showed a significant decrease in these protein concentrations in the hesperidin + DEN group in comparison with the DEN group (Figure 4A and 4B). These results indicated that hesperidin protected against DEN-induced hepatocarcinogenesis by down-regulation of the PI3K/Akt signaling pathway. Furthermore, the results showed a significant increase in CDK2 protein concentration in the DEN group when compared with the control group. Similarly, CDK2 protein concentration was significantly decreased in the hesperidin + DEN group in comparison with the DEN group (Figure 5A and 5B).

3.6 | Effect of hesperidin on caspase-3 level

The results showed a significant increase in the caspase-3 level of the DEN group in comparison with the control group (3.5 ± 0.5 vs 0.9 ± 0.08, $P < 0.05$). Interestingly, there was a significant increase in caspase-3 level in the hesperidin + DEN group when compared with the DEN group (6.6 ± 0.7 vs 3.5 ± 0.5, $P < 0.05$) (Figure 6).

4 | DISCUSSION

In this study, we demonstrated the protective effect of hesperidin against DEN-induced HCC via targeting the PI3K/Akt pathway as a possible mechanism.

The current findings demonstrated that DEN rats showed elevated serum ALT, AST, and ALP activities as well as total and indirect bilirubin concentrations. Additionally, redox imbalance was observed since CAT and GPx activities decreased, while NO and

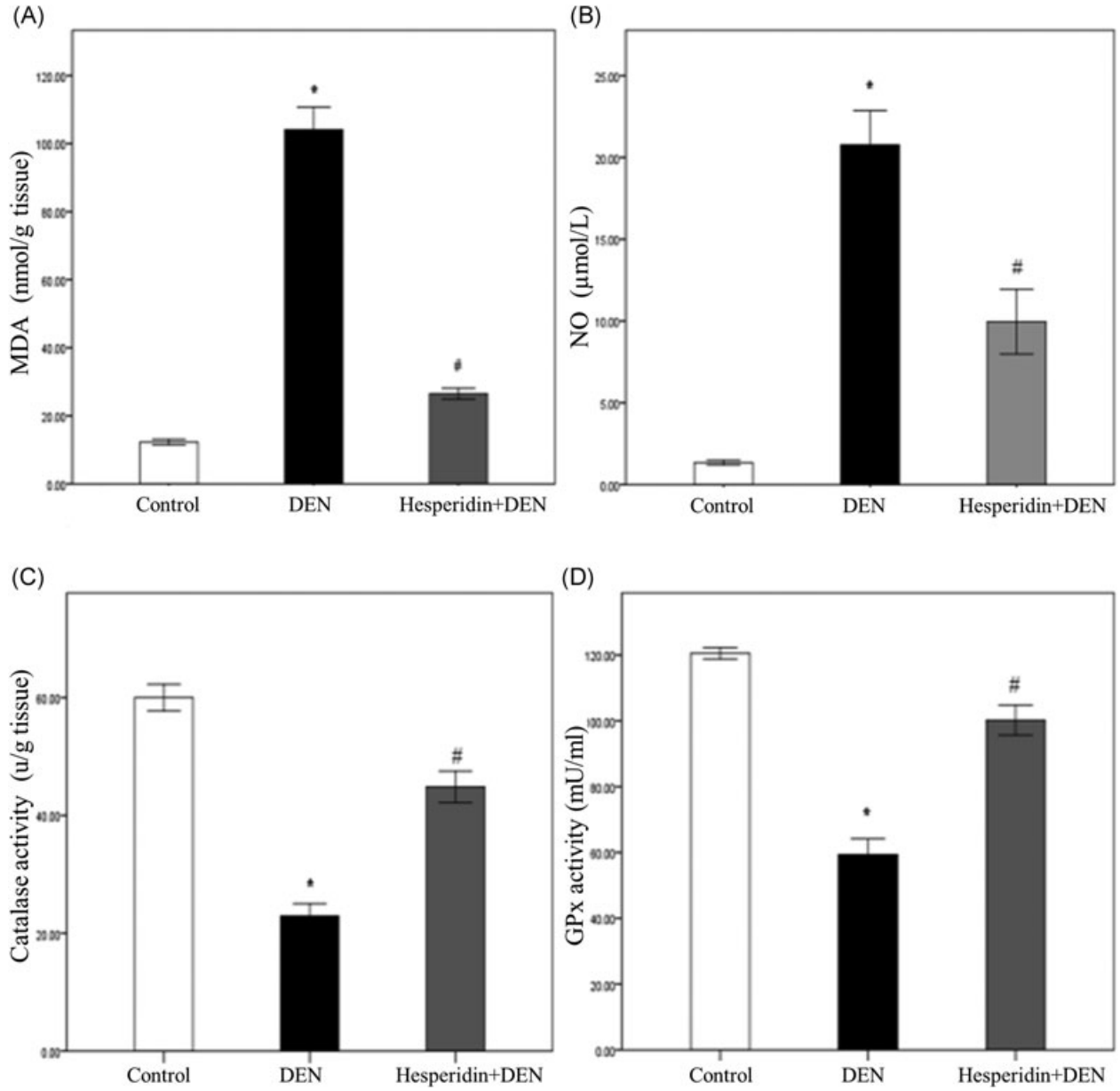

FIGURE 3 Effect of hesperidin on the oxidative stress biomarkers. Column chart shows a significant decrease in malondialdehyde (A) and nitric oxide (B) levels, and a significant increase in activity of catalase (C) and glutathione peroxidase (D) in hesperidin + DEN group in comparison with the DEN group. Values are presented as mean ± SE. *Statistically significant in comparison with the control group at $P < 0.05$: #Statistically significant in comparison with DEN group at $P < 0.05$. DEN, diethylnitrosamine

MDA levels increased in DEN rats. These results were confirmed by the histopathological examination in which fatty and degenerative changes in liver tissues with necrotic hepatocytes death were observed in DEN rats. Our results are consistent with other previous studies[34–36] and can be explained as DEN is a genotoxic hepatocarcinogen and its bioactivation in the liver is accompanied with increased ROS formation.[37,38] Interestingly, we found that hesperidin (200 kg b.wt.) efficiently prevented the increase in serum ALT and ALP activities as well as the redox imbalance which are consistent with other research.[20,39–41] However, our results indicated that hesperidin did not protect against DEN-induced oxidative hemolysis that was observed previously[42] as an elevated serum AST activity, total bilirubin, and indirect bilirubin. Importantly, hesperidin protected against DEN-induced elevation of serum AFP level and it alleviated the histomorphological features of HCC such as mitotic figures, pseudolobular formation as well as stromal and vascular invasion. Altogether, our results are consistent with the previous studies that proved the anticancer activity of hesperidin.[36,43]

Interestingly, hesperidin was found to protect against DEN-induced hepatocarcinogenesis via targeting the PI3K/Akt signaling pathway since it downregulated the expression level of PI3K

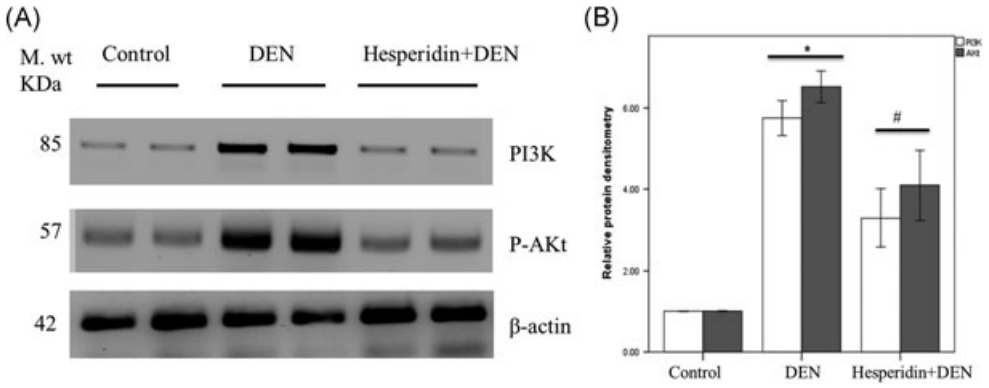

FIGURE 4 Effect of hesperidin on the PI3K/Akt signaling cascade. A, Immunoblots of PI3K and phospho-Akt in liver tissues of experimental groups. B, Column chart shows the quantification of the protein concentrations where hesperidin prevented the DEN-induced elevation in the levels of PI3K and p-Akt. Values are presented as mean ± SE. *Statistically significant in comparison with the control group at $P < 0.05$; #Statistically significant in comparison with the DEN group at $P < 0.05$. Akt, serine/threonine kinase; DEN, diethylnitrosamine; PI3K, phosphatidylinositol 3-kinase

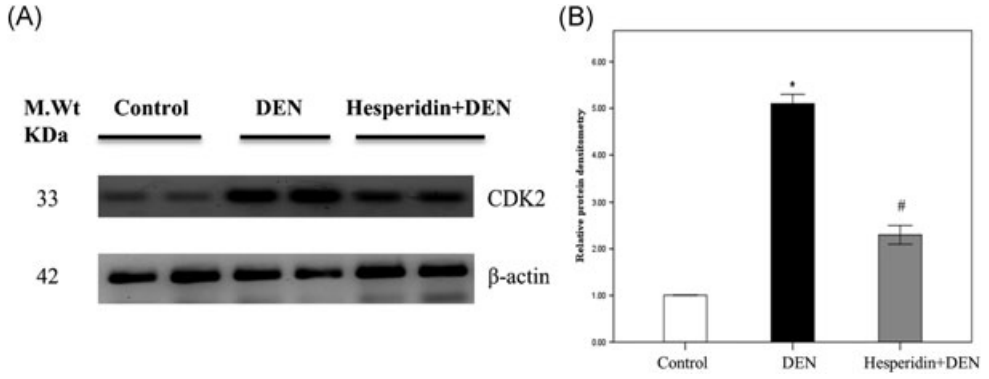

FIGURE 5 Effect of hesperidin on the expression level of CDK2. A, Immunoblots of CDK2 in the liver tissue homogenates among the studied groups. B, Column chart shows the quantification of CDK2 protein concentration where hesperidin prevented the DEN-induced elevation in CDK2 levels. Values are presented as mean ± SE. *Statistically significant in comparison with the control group at $P < 0.05$; #Statistically significant in comparison with the DEN group at $P < 0.05$. DEN, diethylnitrosamine

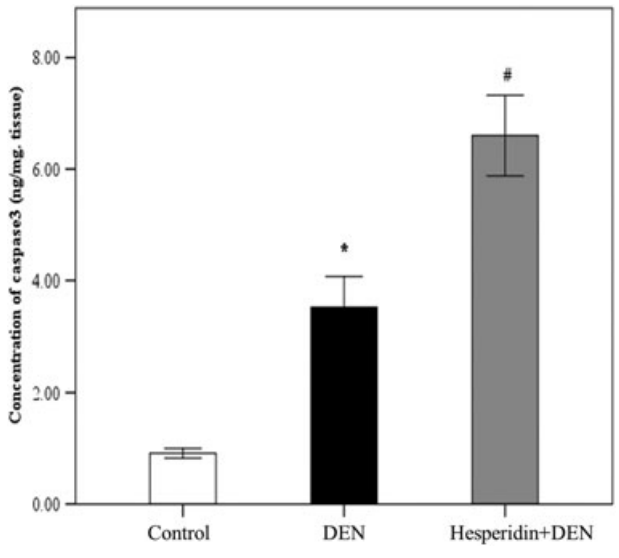

FIGURE 6 Effect of hesperidin on the caspase-3 level. The column chart shows the level of caspase-3 measured by ELISA among the studied groups. Values are presented as mean ± SE. *Statistically significant in comparison with the control group at $P < 0.05$; #Statistically significant in comparison with the DEN group at $P < 0.05$. DEN, diethylnitrosamine

and Akt proteins. This result is compatible with that of Saiprasad et al[44] whereas hesperidin suppressed the prosurvival PI3K/Akt pathway that was upregulated in colon cancer and activated many apoptotic and autophagic proteins. Additionally, hesperidin targeted the same pathway to inhibit the survival of other cancer types, particularly the lung carcinoma cells.[45] Interestingly, hesperidin was effective in targeting other pathways to protect against HCC formation such as wingless/int-1 signaling pathway that is responsible for HCC cell proliferation and angiogenesis[36] in addition to Nrf2/ARE/HO-1, PPARγ, and TGF-β1/Smad3 signaling, which are responsible for oxidative stress and inflammation.[43] Hence, our findings confirm that downregulation of the PI3K/Akt pathway by hesperidin is one of its mechanisms by which it could protect against HCC development.

CDK2 is a protein kinase responsible for cell cycle progression and G1/S transition. CDK2 was upregulated in DEN-induced HCC to increase the proliferation of dysplastic hepatocytes and hence its inhibition exhibited an anti-HCC effect.[46–49] Additionally, CDK2 is a downstream target of the PI3K/Akt pathway and the dual targeting of PI3K and CDK2 was found to be a chemopreventive and

therapeutic strategy for colorectal, breast, and prostate cancers.[50–54] Moreover, hesperidin was found to inhibit the proliferation of cervical cancer cells by CDK2 downregulation, which is consistent with our findings.[55]

In this study, the hesperidin + DEN group showed a significant increase in caspase-3 level in comparison with the DEN group. The apoptotic effect of hesperidin can be attributed to its ability to inhibit the activated PI3K/Akt pathway. This result is in agreement with the previous studies whereas the inhibition of the PI3K/Akt pathway was associated with the activation of the apoptotic marker, caspase-3, in colon cancer cells.[36,44]

5 | CONCLUSION

Hesperidin effectively protected from DEN-induced HCC in rats via its antioxidant activity and downregulation of the PI3K/Akt pathway.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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How to cite this article: Mo'men YS, Hussein RM, Kandeil MA. Involvement of PI3K/Akt pathway in the protective effect of hesperidin against a chemically induced liver cancer in rats. *J Biochem Mol Toxicol*. 2019;e22305. https://doi.org/10.1002/jbt.22305