Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 5,

Chemopreventive, apoptotic, antiangiogenic efficacy of Hesperidin via mitigation of epigenetic alterations of global DNA methylation and targeting microRNA in a rat model of hepatocellular carcinoma

A'laa E. Al-Semelawy[1*], Samy A. Hussein[1], Hussein A. Ali[1], Yakout A. EL-Senosi[1], Afaf D. Abdel Magid[1], Shawky A. Mostafa[2]

[1]*Department of Biochemistry and molecular biology, Faculty of Veterinary Medicine, Benha University, Egypt.*
[2]*Department of Pathology, Faculty of Veterinary Medicine, Benha University, Egypt.*

ARTICLE INFO

Recieved: 16 January 2024

Accepted: 01 February 2024

***Correspondence:**

Corresponding author: A'laa E. Al-Semelawy
E-mail address: alaaalsemelawy0123@gmail.com

Keywords:

Hepatocellular carcinoma
Hesperidin
Epigenetics
Angiogenesis
Apoptosis

ABSTRACT

Hepatocellular carcinoma (HCC) accounting about 75% of hepatic neoplasia, making it the most common kind of liver cancer worldwide. So, this study was planned to evaluate the beneficial chemopreventive efficacy of hesperidin (Hes) in experimental model of Diethyl nitrosamine (DEN) / Carbon tetrachloride (CCl_4) –induced HCC in rats. Thirty male rats were divided into 3 equal groups. Group 1 (normal control): rats didn't receive any treatment. Group 2 (HCC): HCC was induced in rats by injection of DEN (200mg/kg b.w/i.p), then 2 weeks later of DEN injection rats received 3 weekly successive doses of CCl_4 (3ml/kg b.wt/ orally) at 1:1 dilution in corn oil as a promoter of carcinogenic effect. DEN and CCl_4 administration were repeated once again after 5 weeks. Group 3 (HCC+ hesperidin): 15 weeks after HCC induction, rats treated with Hes (150 mg/kg b.wt), orally and continued for 6 weeks. A significant increase in serum ALT, AST and ALP activities were observed in HCC-induced rats. However, significant downregulation of liver Nrf2, Caspase-3, Bcl-2 and MicroRNA-34a with upregulation of FGF-2 and MicroRNA-221 with Global DNA hyper-methylation were observed in HCC group. Hesperidin treatment exhibited downregulation of microRNA-221 and FGF-2 with upregulation of Nrf2, Bcl-2, caspase 3 gene and Global DNA hypo-methylation. Interestingly, improvement of liver histopathological alterations supported the chemopreventive activity of Hesperidin. Conclusively, Hesperidin ameliorates the progression of HCC and has promising chemopreventive, and antiangiogenic activity, inhibiting growth promoting oncogene and initiation of gene regulating apoptosis and protects the liver from oxidative damage and inflammation.

Introduction

Cancer Statistics 2021 states that of the four most common malignancies around the world is hepatocellular carcinoma (HCC) which also known as liver cancer represent the first one among them (Supraja *et al*., 2022). In Egypt, Hepatitis C virus is the primary cause of HCC, which is the fourth most prevalent kind of cancer. The highest global prevalence averages are seen in Asia and Africa (Rashed *et al*., 2020; Petrick *et al*., 2020).

Due to HCC diverse etiology and molecular gene mutations, it has become more well-known in the field of oncology. These comprise oncogenes, tumor suppressor genes, mutations, and epigenetic changes to different genes linked to cell cycle control (Supraja *et al*., 2022). Usually, viral infections and/or exposure to carcinogens cause inflammatory hepatic disorders, which are followed by HCC. Any type of cirrhosis raises the risk of HCC, with a frequency of 2–4% per year. The risk varies based on the cause, region, age, sex, and severity of liver injury (McGlynn *et al*., 2021).

The liver is an essential organ that helps the body detoxify and metabolize chemicals that come from outside and inside sources. It is, however, vulnerable to harm from both natural and chemical toxin (Gao *et al*., 2023). Diethyl nitrosamine (DEN) is a significant chemical that poses a risk to human health because it produces reactive oxygen species (ROS), which can induce oxidative stress and disrupt the processes that repair nucleic acids (Khan *et al*., 2017). HCC can be reliably induced with combination of DEN and Carbon Tetrachloride (CCl_4) (Thiele *et al*., 2017).

Patient nutrition is associated with cancer prevention, development, progression, and treatment. Reduced risk of cancer is associated with higher consumption of fruits and vegetables in the diet (Soerjomataram *et al*., 2010). Flavonoids, such as hesperidin, hesperetin, tangerine, erodcyol, nobiletin, naringin, ,and naringenin, have been shown in recent studies to exhibit a variety of therapeutic effects on liver damage, including mechanisms that prevent oxidative stress, cytotoxicity, inflammation, fibrosis, and tumors (Gao *et al*., 2023). Hesperidin, a flavonoid commonly prevalent in citrus plants such as blood orange, orange, lemon, and lime, provides considerable economic benefits (Wabitsch *et al*., 2022). Hesperidin functions in a variety of cancer cells by regulating several pathways that include DNA repair, anti-angiogenic, anti-metastatic, cell cycle arrest, and apoptosis. Also, numerous molecular targets linked to carcinogenesis, including drug transporters, cell cycle mediators, transcription factors, reactive oxygen species, reactive nitrogen species, and inflammatory cytokines, have all been shown to be altered by hesperidin (Pandey and Khan, 2021). Consequently, the potential chemopreventive impact of hesperidin on alterations of epigenetic and molecular markers as well as histopathological examination of liver tissues were evaluated in experimental model of HCC in rats.

Materials and methods

Experimental Animals

Thirty white male albino rats, 4-5 week's old with 100– 150g weight were used. Animals were provided with a constant supply of standard pellet diet and fresh clean drinking water-libitum. Rats were left for 15 days before the experiment for adaptation. The Experimental protocol was conducted according to the guide for Institutional Animals Care and Use Committee approved by Research Ethics Board, Faculty of Veterinary Medicine, Benha University (BUFVTM 12-02-23).

ISSN: 2090-6277/2090-6269/

Chemicals and natural agents

Diethylnitrosoamine (DEN) with common name (N-Nitrosodiethylamine): N-nitrosodiethylamine presents in a clear yellow liquid form (1g/1ml vial) was purchased from Sigma Aldrich Company for Trading Chemicals, Medicines and Medical Appliances, Egypt. DEN was freshly prepared in normal saline and intraperitoneally (i.p) injected to rats at a dose of (200 mg/kg b.wt) (Singh *et al*., 2009).

Carbon Tetrachloride (CCl_4): Carbon tetrachloride present in colourless liquid form and purchased from El Gomhouria Company for Trading Chemicals and Medical Appliances, Egypt. CCl_4 was freshly prepared in corn oil at (1:1) dilution and orally administered to rats at a dose of (3ml/kg b.wt) as a promoter of carcinogenesis (Hassan *et al*., 2014).

Hesperidin: Hesperidin (95%) HSP with M.W 610.56 was purchased from International Company for Scientific and Medical Supplies, Egypt. Hesperidin was prepared by dissolving firstly in 2ml of Dimethyl sulfoxide (DEMSO) due to its poor solubility then completed by distilled water to make suspension. Hesperidin administered orally at a dose of (150 mg/kg body weight/day) (Zaghloul *et al*., 2017).

Experimental design

Rats were divided into three main equal groups each one contained 10 rats as follow:

Group 1 (Normal control): Rats were fed with ordinary diet only without any treatment during the entire experimental period of 21 weeks.

Group 2 (HCC): HCC was induced in rats by i.p injection of DEN in normal saline (200 mg/kg b.wt), 2 weeks later rats received 3 weekly successive dose of CCl_4 (3ml/kg b.wt) orally as mentioned above. DEN and CCl_4 injections were repeated once again after 5 weeks from first DEN injection.

Group 3 (HCC + Hesperidin): Rats were received DEN and CCl_4 as in group II and post-treated with Hesperidin (150 mg/kg b.wt/day), orally after 15 weeks from the administration of DEN and CCl_4 for 6 weeks.

N.B: During the experimental period, the dosage was adjusted every week according to any change in body weight to maintain similar dose per kg body weight of rat over the entire period of study for each group.

Sampling

Blood samples

Blood samples for serum separation were obtained at the end of the experiment (21 weeks). Serum was separated by centrifugation at 2500 rpm for 15 minutes. The separated serum was stored in a deep freeze at -20°C until used for determination of liver marker enzymes [Alanine transaminase (ALT), Aspartate transaminase (AST) and Alkaline phosphatase (ALP)].

Tissue specimens

At the end of experiment (21 weeks), and after blood samples collection, rats were euthanized according to Animal Ethics Committees and abdomen was opened, then liver were removed. The liver tissue specimens were dissected out and divided into 2 parts. First part flushed with sterile physiological saline to remove any blood cells and clot then, put in Eppendorf tubes and immediately kept in liquid nitrogen and stored at -80°C till RNA extraction for determination of gene expression by reverse transcription polymerase chain reaction (RT-PCR). The second part of liver was placed in 10% formalin solution for histopathological examination.

Analysis

Biochemical analysis

Serum ALT and AST activities were determined according to the kinetic method described by Schumann *et al*. (2002) and ALP activity by the method of Tietz *et al*. (1983).

Molecular analysis

Real-time quantitative polymerase chain reaction analysis (real-time qPCR) was used to assess the mRNA expression levels of Caspase-3, B-cell lymphoma 2 (Bcl-2), nuclear factor erythroid 2–related factor 2 (Nrf2) and Fibroblast Growth factor-2 (FGF-2) in the rat livers. Pure RNA was extracted from liver tissues using total RNA Purification Kit following the manufacturer protocol (Thermo Scientific, Fermentas, #K0731). Revert Aid TM First Strand CDNA synthesis kit (#EP0451, Thermo Scientific, Fermentas, USA) was used to reverse transcribe each cDNA sample. Then, Real-time PCR with SYBR Green was used to measure gene expression by following the manufacturer protocol (Thermo scientific, USA, # K0221) and gene specific primers. Using the $2^{-\Delta\Delta Ct}$ method, the target gene was normalized with glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).

miR-34a and miRNA-221 Using U6 as an internal control, the expression of miRNAs in the liver was measured using Real-time PCR and SYBR Green. Thermo Scientific, USA, # K0221), a miRNA-specific forward primer (Table 2), and a universal reverse primer supplied with the Quanti-Mir RT kit were used to amplify the extracted cDNA in accordance with the manufacturer's instructions.

Table 1. Forward and reverse primers sequence for genes that used in qPCR.

Gene	Forward primer (/5 ------ /3)	Reverse primer (/5 ------ /3)
Caspase3	GGTATTGAGACAGACAGTGG	CATGGGATCTGTTTCTTTGC
Bcl-2	ATCGCTCTGTGGATGACTGAGTAC	AGAGACAGCCAGGAGAAATCAAAC
Nrf2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
FGF-2	GAACCGGTACCTGGCTATGA	CCGTTTTGGATCCGAGTTTA
GAPDH	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA

Table 2. Universal reverse primers sequence for primers used in qPCR.

Gene	Primer sequence (/5 ------ /3)
MiRNA-34a	TGGCAGTGTCTTAGCTGGTTGT
MiRNA-221	AGCTACATTGTCTGCTGGGTTTC
U6	TGACACGCAAATTCGTGAAGCGTTC
Universal reverse primer	CCAGTCTCAGGGTCCGAGGTATTC

Evaluation of the degree of global DNA methylation according to the method described by (Colorimetric) Base Catalog # P-1030. The level of global DNA methylation was evaluated using a MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Epi Gentek, Farmingdale, NY, USA) and as previously described (Li *et al*., 2018).

Histopathological examination

Histopathological analysis of liver' tissue specimens treated in neu-

tral buffered formalin solution at 10% according to Bancroft and Gamble (2008). After proper fixation, the samples were dehydrated in ascending grades of ethyl alcohol, then cleared in xylol, embedded in paraffin and finely blocking occurred. These samples were sectioned at 5 µm in thickness and stained with hematoxylin and eosin (H & E) for microscopical examination.

Statistical analysis

All the data were expressed as means ±SEM. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS, 18.0 software, 2011 and the individual comparisons were obtained by Duncan's multiple range test (DMRT). Values were considered statistically significant when $p<0.05$.

Results

A significant increase in serum ALT, AST and ALP activities were observed in DEN/ CCl_4 induced HCC in rats when compared with normal control group (Table 3). Treatment with hesperidin to HCC induced rats exhibited a significant decrease in serum ALT, AST and ALP activities as compared with untreated group.

Table 3. Effect of hesperidin treatment on serum ALT, AST and ALP activities in DEN+ CCL_4-induced HCC in rats.

Parameters	Animal groups		
	Control (G1)	HCC non –treated (G2)	HCC + Hes. treated (G3)
ALT (U/L)	67.22 ± 2.40^{c}	194.50 ± 4.53^{a}	121.05 ± 6.50^{b}
AST (U/L)	78.26 ± 3.45^{c}	232.66 ± 9.15^{a}	160.00 ± 6.07^{b}
ALP (U/L)	116.28 ± 5.80^{c}	315.61 ± 16.17^{a}	203.84 ± 12.05^{b}

Data are presented as (Mean ± SEM). SEM = Standard error of mean.Mean values with different superscript letters in the same row are significantly different at ($P \leq 0.05$)

Table 4 showed a significant downregulation of Bcl-2, Nrf2 gene expressions and upregulation of FGF-2 gene expression in HCC-induced rats when compared with normal control group, while Caspase-3 showed non-significant upregulation compared with normal group. Conversely, treatment with hesperidin to HCC -induced rats showed a significant upregulation Caspase-3, Bcl-2, Nrf2 and downregulation of FGF-2 gene expression as compared with HCC non-treated group.

Table 5 showed significant downregulation of miR-34a and upregulation of miRNA-221 gene expressions of liver tissue in DEN/ CCl_4 -induced HCC rats when compared with normal control group. Treatment with hesperidin to HCC-induced rats exhibited a significant upregulation of miR-34a and downregulation of miRNA-221 gene expression when compared with non-treated rats with liver cancer.

Table 5 showed a significant hyper methylation of global DNA in DEN/ CCl_4 -induced HCC when compared with normal control group. However, Treatment with hesperidin to HCC-induced in rats exhibited a significant global DNA hypo methylation as compared with HCC non-treated group.

Histopathological findings

The liver of control group showed normal histological appearance of hepatic architecture (Fig. 1A), portal areas, central veins, sinusoids and hepatocytes which appeared as large polygonal cells with eosinophilic (pink) cytoplasm, round nuclei, and prominent nucleoli (Fig. 1B). Meanwhile, livers of DEN-treated group revealed marked hydropic and fatty degeneration of hepatocytes with distorted, dilated and congested sinusoids (Fig. 1C). Multifocally, hydropic degeneration characterized by swollen and vacuolated hepatocytes with cytoplasm rarefaction, whereas lipid-type degeneration showed few large, discrete, clear, cytoplasmic vacuoles that displace the nucleus. There were occasional moderate strands of fibrous tissue proliferation rich in blood vessels (Fig. 1D) inbetween the degenerated hepatocytes. Rarely, there was coagulative necro sis of few hepatocytes characterized by loss of cellular detail, shrunken hepatocytes with hypereosinophilic cytoplasm and pyknotic nuclei. Multifocally, there were clear cell foci of cellular alteration composed of hypertrophied hepatocytes that merge imperceptibly with surrounding hepatic parenchyma without separation or encircling by fibrous connective tissue (Fig. 2A). The hepatic cells within the foci were enlarged, polygonal, with pale pink vacuolated cytoplasm, enlarged centrally located nucleus, and prominent nucleolei; occasionally surrounded by dilated and congested sinusoids (Fig. 2B). The liver of DEN- Hesperidin treated group revealed maintained normal hepatic architecture with mild cytoplasmic vacuolation of hepatocytes, biliary hyperplasia (Fig. 2C) and lymphocytic infiltration in portal areas (Fig. 2D).

Discussion

Hepatocellular carcinoma (HCC) ranks second in terms of the causes of cancer-related deaths and is the seventh most common cancer globally, with a growing incidence (McGlynn *et al*., 2021). Herbal remedies provide an abundant source for identifying natural items with beneficial and less harmful side effects, so presenting a unique idea in modern drug research (SP *et al*., 2020). According to several research, the citrus flavonoid hesperidin may have chemo-preventive properties (Siddiqi *et al*., 2015). An additional research demonstrated an inverse correlation between the content of Hes (flavonoids) and the risk of inducing cancer (Roohbakhsh *et al*., 2015).

The results of current study showed a significant increase in serum ALT, AST and ALP activities in DEN/ CCl_4 induced HCC in rats. This is con-

Table 4. Effect of hesperidin treatment on liver tissue Caspase-3, Bcl-2, Nrf2 and FGF-2 gene expressions in DEN/ CCl_4-induced HCC in rats.

Parameters Animal groups	Caspase-3 Fold change mean	Bcl-2 Fold change	Nrf2 Fold change	FGF-2 Fold change
G1: Control	1.00 ± 0.00^{b}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{c}
G2: HCC non –treated	1.24 ± 0.12^{b}	0.05 ± 0.003^{c}	0.03 ± 0.002^{c}	13.18 ± 0.72^{a}
G3: HCC +Hes treated	9.38 ± 0.42^{a}	0.68 ± 0.03^{b}	0.11 ± 0.01^{b}	2.03 ± 0.12^{b}

Data are presented as (Mean ± SEM). SEM = Standard error of mean. Mean values with different superscript letters in the same column are significantly different at ($P \leq 0.05$)

Table 5. Effect of hesperidin treatment on liver tissue miRNA-34a, miRNA-221 gene expressions and Global DNA Methylation in DEN+ CCL_4-induced HCC in rats.

Parameters Animal groups	miR-34a Fold change	miR-221 Fold change	Global DNA Methylation 5-mC (%)
G1: Control	1.00 ± 0.00b	1.00 ± 0.00c	1.24 ± 0.09c
G2: HCC non –treated	0.53 ± 0.02c	16.56 ± 0.85a	6.62 ± 0.43a
G3: HCC +Hes treated	2.97 ± 0.12a	4.20 ± 0.23b	3.50 ± 0.15b

Data are presented as (Mean ± SEM). SEM = Standard error of mean. Mean values with different superscript letters in the same column are significantly different at ($P \leq 0.05$)

sistent with Lyngdoh *et al.* (2023) who reported that a significant increase in serum ALT, AST and ALP activities in DEN induced HCC in mice. Also, the delivery of DEN led to an increase in the serum stress-specific enzymes (ALT, AST, and ALP), suggesting that DEN has a toxic effect on liver tissue, causing membrane permeability and subsequent enzyme leakage into the circulation (PRADEEP *et al.*, 2010).

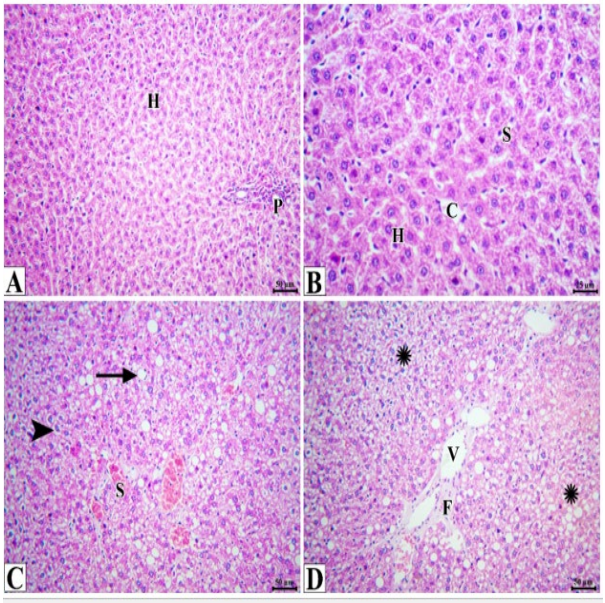

Fig. 1. Liver sections of rats from control (A, B) and DEN-treated (C, D) groups. (A) Normal histological appearance of hepatic architecture, portal area and hepatocytes. (B) Normal central vein, sinusoids and hepatocytes appeared as large polygonal cells with eosinophilic cytoplasm, round nuclei, and prominent nucleoli. (C) Marked hydropic (arrowhead) and fatty (arrow) degeneration of hepatocytes with distorted, dilated and congested sinusoids. (D) Moderate strands of fibrous tissue proliferation rich in blood vessels; note degenerated hepatocytes (asterisk). H: hepatocytes, P: portal area, S: sinusoids, C: central vein, F: fibrous tissue and V: blood vessel.

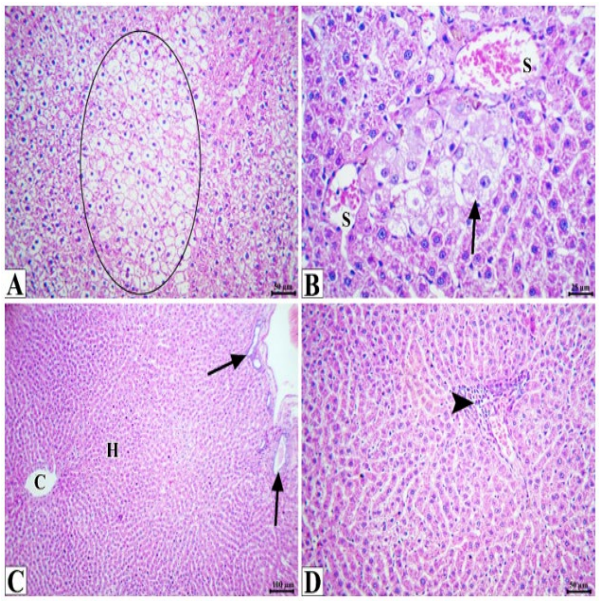

Fig. 2. Liver sections of rats from DEN (A, B) and DEN- Hesperidin (C, D) treated groups. (A) Clear cell focus of cellular alteration (black circle) composed of hypertrophied hepatocytes that merge imperceptibly with surrounding hepatic parenchyma without separation or fibrous capsule. (B) Clear cell focus surrounded by dilated and congested sinusoids; note enlarged hepatocytes (arrow), within the focus, with pale pink vacuolated cytoplasm. (C) Maintained normal hepatic architecture with mild cytoplasmic vacuolation of hepatocytes and biliary hyperplasia in DEN- Hesperidin treated group. (D) Mild lymphocytic infiltration in portal area. H: hepatocytes, S: sinusoids and C: central vein.

Treatment with hesperidin to HCC induced rats exhibited a significant decrease in serum liver markers enzyme as compared with non-treated group. These outcomes be similar to Mahmoud *et al.* (2017) who established that hesperidin treatment (50 or 100 mg/kg.b.wt) lowered the activity of serum ALT, AST and ALP in DEN/CCl_4-induced HCC in rats. Other studies shown that hesperidin have hepatoprotective ability against many harmful substances (El-Sisi *et al.*, 2017) . This results were supported by the results of histopathological findings that shown structure to liver tissues. In this study, the hepatoprotective efficacy of hesperidin in HCC treated rats were supported by histopathological findings of liver tissues that revealed maintained normal hepatic architecture with mild cytoplasmic vacuolation of hepatocytes.

The caspase-3 gene in DEN/CCl_4-induced HCC in current study shown non-significant up-regulation of apoptotic liver caspase-3 gene expression level when compared to normal control group. However, (Mohamed *et al.*, 2021) reported that the relative quantity of caspase3 mRNAs was significantly reduced in liver tissue of the DEN treated rats.. Hesperidin suppresses proliferation by inducing apoptosis via cell cycle arrest and e6ndoplasmic reticulum stress mechanisms (Siddiqi *et al.*, 2015). Conversely, treatment with hesperidin to HCC -induced rats showed a significant upregulation Caspase-3 of gene expression. Similarly, Mo'men *et al.* (2019) reported that, there was a major rise in caspase-3 level in hesperidin treated DEN group.

In the current study a significant downregulation of liver Bcl-2 gene expression was observed in HCC-induced rats. Exploring the role of the anti-apoptotic gene Bcl-2 in the development of liver cancer, previous study done by Pierce *et al.* (2002) who revealed that injection of new born mice at 15 day of age with DEN whose carcinogenic effect can exacerbate by transforming growth factor alpha (TGF-α) in TGF-α / Bcl-2 double transgenic mice, TGF-α and Bcl-2 single transgenic, and wild type mice showed DEN-induced liver carcinogenesis was reduced by Bcl-2 expression, which also mitigated TGF-α promoting effect. As well as, in the early phases of carcinogenesis, Bcl-2 reduced cell proliferation in proliferative foci and delayed their expansion. However, treatment with hesperidin to HCC -induced rats showed a significant upregulation of Bcl-2 gene as compared with HCC non-treated group. Similarly, Abdelaziz *et al.* (2020) reported that, the increased expression of Bcl-2 mRNA was detected after Hesperidin administration in Methotrexate (MTX)-induced hepatotoxicity that might be help to explain its anti-apoptotic effects.

An antioxidant response elements (ARE)-nuclear factor E2-related factor 2 (Nrf2)-Keap1-like ECH-associated protein pathway shields the cell from oxidative stress (Taguchi *et al.*, 2011). Numerous genes that control redox state, detoxification of toxins, and drug transport are regulated by Nrf2. Therefore, cancer and other chronic disorders can be treated by specifically modulating Nrf2. In contrast, the ongoing upregulation of Nrf2 might potentially encourage the survival and spread of cancer (Cuadrado *et al.*, 2018). The achieved results showed significant down-regulation of liver Nrf2 gene expressions in HCC-induced rats, this outcome was consistent with Mahmoud *et al.* (2017) who stated that even though ROS activate Nrf2, the rats that were given DEN/CCl_4 showed less Nrf2 expression. Excessive and prolonged generation of ROS leads to down-regulation of the Nrf2 pathway. In addition, Rachakonda *et al.* (2010) indicated that depletion of Nrf2 accelerated the chemically induced development of HCC. Conversely, rats treated with hesperidin exhibit significant upregulation of Nrf2 in liver tissues. This was supported by the result of Mahmoud *et al.* (2017) who demonstrate that hesperidin-induced up-regulation of Nrf2 signaling pathway in the liver of DEN/CCl_4-induced rats. Also, Abdelaziz *et al.* (2020) reported that Hes upregulate Nrf2 in a rat liver model of oxidative stress, lipid peroxidation, inflammation, and apoptosis caused by MTX . Likewise, their result showed that hepatoprotective effect of hesperidin could be attributed to its ability to positively regulate Nrf2 and Bcl-2.

The molecular pathophysiology of HCC is known to include the FGF signaling pathway (Sandhu *et al.*, 2014). In the current study a significant upregulation of FGF-2 gene expression was observed in HCC-induced rats. This results was consistent with the study of Hassan *et al.* (2021) who reported that there was significant FGF expression in the HCC –induced rats. FGF2 is mostly expressed in HCC cells and is hardly seen in non-cancerous liver tissue or non-parenchymal cells. FGF2 is essential for HCC invasion and the induction of angiogenesis. It also promotes HCC proliferation through an autocrine mechanism (Kin *et al.*, 1997). Meanwhile, treatment with hesperidin to HCC-induced rats showed significant downregulation of liver FGF-2 gene expression level. This come in agreement with Roohbakhsh *et al.* (2015) who reported that flavonoids such as Hesperidin have anti-proliferative properties so, they can regulate cancer angiogenesis through basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), progression, migration, and metastasis of endothelial cells. Also, Yeh *et al.* (2009) was demonstrated the anti-metastatic potential of Hesperidin in HepG2 human hepatocellular carcinoma cells.

It has been demonstrated that deregulation of epigenetic alteration occurs during HCC development, having a significant effect on cell differentiation, proliferation, and function. DNA methylation is a significant epigenetic alteration that adds methyl groups to cytosines without altering the DNA sequence. Methylated DNA markers have been discovered in studies to be specific for HCC identification (Fu *et al.*, 2023).

Regarding to epigenetic miRNA the obtained results showed significant downregulation of miR-34a and upregulation of miRNA-221 gene

expressions in liver tissue of DEN/ CCl_4 -induced HCC in rats. Notably, Ren *et al*. (2018) reported that in human cancer, particularly HCC, reduced miR-34a expression. whereas, hesperidin treatment to HCC-induced rats exhibited a significant upregulation of miR-34a and downregulation of miRNA-221 gene expression as compared to rats with liver cancer. Likely, Lou *et al*. (2015) proved that HepG2 cells treated with quercetin which is a flavonoid compound showed an upregulation of miR-34a and displaying wild-type p53 thus, the antitumor effects of quercitin in HCC are significantly influenced by miR-34a. Enforced miR-34a expression greatly inhibit both Hep3B and SNU-449 cells growth, migration, and invasion. Also, miR-34a treatment of these cell lines showed an increase in caspase-3 activity and this suggested that miR-34a can modulate expression of caspase-3 thus, accelerating the apoptosis of liver cells with cancer (Han *et al*., 2019). MicroRNA-221 belongs to the miRNA family which is expressed abnormally in liver cancer (Fornari *et al*., 2017). MiR-221 was discovered to bind to many targets, which contributed to the activation of multiple tumor signaling pathways. Also, it exhibits promise as an HCC prognostic marker (Liu *et al*., 2021). In liver tumors, miR-221 functioned as a proto-oncogene. Patients with HCC expressed more miR-221 than either liver cirrhosis patients or healthy individuals (Braconi and Patel, 2008). This outcome consistent with our current study which reported significant increase of miR-221 in chemical induced HCC in rats. Moreover, Biersack (2016) noted that consuming dietary natural products is essential for both inhibiting the activity of oncogenic miRNAs and inducing the activity of tumor suppressor miRNAs, which are expected to inhibit the proliferation and progression of tumorogenesis.

The obtained data showed a significant hyper methylation of global DNA in DEN/ CCl_4 -induced HCC when compared with normal control group. Nearly similar results was stated by Hlady *et al*. (2014) who recorded that, as cirrhosis progresses to early and more severe neoplastic lesions, changes in DNA methylation significantly increase, and many of these changes are retained in fully established HCCs. Hesperidin administration to HCC-induced group showed significant hypo-methylation of global DNA. These outcomes resemble to the data of Fernández-Bedmar *et al*. (2017) who found that Hesperidin treatment initiated significantly global demethylates repetitive regions in LINE-1 and ALU-M2 on leukemia model cells. Likewise, Li and Tollefsbol (2010) reported that, Hesperidin flavanol and other common dietary phenolic substances decrease the activity of DNA methyltransferase (DNMT) in vitro, changing gene expression patterns and eventually inhibiting cancer.

Conclusion

In conclusion, results indicated that Hesperidin ameliorates the progression of HCC and has promising chemopreventive, apoptotic and antiangiogenic activity, inhibiting growth promoting oncogenic miRNA-221 and induction of gene regulating apoptosis miRNA 34a as well as suppression of cancer cell proliferation and angiogenesis growth factors (FGF-2) in liver. Also, Hesperidin could be possessing hepatoprotective and antioxidant properties protects the liver against oxidative damage and inflammation. Also, these results indicated that epigenetic alterations of DNA methylation and expressions of non -coding miRNAs play a significant role in the regulation of numerous genes related to HCC. But, we need more research on the potential mechanisms of hesperidin to enhancing chemoprevention and chemotherapeutic strategies.

Acknowledgments

Authors would like to thank all staff members of Biochemistry and Molecular Biology Department, Faculty of Veterinary Medicine, Benha University, Egypt.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abdelaziz, R.M., Abdelazem, A.Z., Hashem, K.S., Attia, Y.A., 2020. Protective effects of hesperidin against MTX-induced hepatotoxicity in male albino rats. Naunyn-Schmiedeberg's Archives of Pharmacology 393, 1405–1417. https://doi.org//10.1007/s00210-020-01843-z

Bancroft, J.D., Gamble, M., 2008. Theory and practice of histological techniques. 6th Edition, Churchill Livingstone Elsevier, China.

Biersack, B., 2016. Non-coding RNA/microRNA-modulatory dietary factors and natural products for improved cancer therapy and prevention: Alkaloids, organosulfur compounds, aliphatic carboxylic acids and water-soluble vitamins. Non-Coding RNA Research 1, 51–63. https://doi.org/10.1016/j.ncrna.2016.09.001

Braconi, C., Patel, T., 2008. MicroRNA expression profiling: a molecular tool for defining the phenotype of hepatocellular tumors. Hepatology 47, 1807–1809. https://doi.org/10.1002/hep.22326

Cuadrado, A., Manda, G., Hassan, A., Alcaraz, M.J., Barbas, C., Daiber, A., Ghezzi, P., León, R., López, M.G., Oliva, B., 2018. Transcription factor NRF2 as a therapeutic target for chronic diseases: a systems medicine approach. Pharmacological Reviews 70, 348–383. https://doi.org/10.1124/pr.117.014753

El-Sisi, A.E.-D.E.-S., Sokar, S.S., Shebl, A.M., Mohamed, D.Z., 2017. Antifibrotic effect of diethylcarbamazine combined with hesperidin against ethanol induced liver fibrosis in rats.Biomedicine & Pharmacotherapy 89, 1196–1206.https://doi.org/10.1016/j.biopha.2017.03.013

Fernández-Bedmar, Z., Anter, J., Alonso-Moraga, A., Martín de las Mulas, J., Millán-Ruiz, Y., Guil-Luna, S., 2017. Demethylating and anti-hepatocarcinogenic potential of hesperidin, a natural polyphenol of Citrus juices. Molecular Carcinogenesis 56, 1653–1662. https://doi.org/10.1002/mc.22621

Fornari, F., Pollutri, D., Patrizi, C., La Bella, T., Marinelli, S., Casadei Gardini, A., Marisi, G., Baron Toaldo, M., Baglioni, M., Salvatore, V., Callegari, E., Baldassarre, M., Galassi, M., Giovannini, C., Cescon, M., Ravaioli, M., Negrini, M., Bolondi, L., Gramantieri, L., 2017. In Hepatocellular Carcinoma miR-221 Modulates Sorafenib Resistance through Inhibition of Caspase-3–Mediated Apoptosis. Clinical Cancer Research 23, 3953–3965. https://doi.org/10.1158/1078-0432.CCR-16-1464

Fu, S., Debes, J.D., Boonstra, A., 2023. DNA methylation markers in the detection of hepatocellular carcinoma. In European Journal of Cancer 191, 112960. https://doi.org/10.1016/j.ejca.2023.112960.

Gao, S., Chen, X., Yu, Z., Du, R., Chen, B., Wang, Y., Cai, X., Xu, J., Chen, J., Duan, H., Cai, Y., Zheng, G., 2023. Progress of research on the role of active ingredients of Citri Reticulatae Pericarpium in liver injury. Phytomedicine 115, 154836. https://doi.org/10.1016/j.phymed.2023.154836

Han, R., Chen, X., Li, Y., Zhang, S., Li, R., Lu, L., 2019. MicroRNA-34a suppresses aggressiveness of hepatocellular carcinoma by modulating E2F1, E2F3, and Caspase-3. Cancer Management and Research 11, 2963–2976. https://doi.org/10.2147/CMAR.S202664

Hassan, H.M., El-Kannishy, S.M.H., Alattar, A., Alshaman, R., Hamdan, A.M., Al-Gayyar, M.M.H., 2021. Therapeutic effects of blocking β-catenin against hepatocellular carcinoma-induced activation of inflammation, fibrosis and tumor invasion. Biomedicine & Pharmacotherapy 135, 111216. https://doi.org/10.1016/j.biopha.2021.111216

Hassan, S.K., Mousa, A.M., Eshak, M.G., Farrag, A., Badawi, A., 2014. Therapeutic and chemopreventive effects of nano curcumin against diethylnitrosamine induced hepatocellular carcinoma in rats. Int. J. Pharm. Pharm. Sci 6, 54–62

Hlady, R.A., Tiedemann, R.L., Puszyk, W., Zendejas, I., Roberts, L.R., Choi, J.-H., Liu, C., Robertson, K.D., 2014. Epigenetic signatures of alcohol abuse and hepatitis infection during human hepatocarcinogenesis. Oncotarget 5, 9425–9443. https://doi.org/10.18632/oncotarget.2444

Khan, F., Khan, T.J., Kalamegam, G., Pushparaj, P.N., Chaudhary, A., Abuzenadah, A., Kumosani, T., Barbour, E., Al-Qahtani, M., 2017. Anti-cancer effects of Ajwa dates (Phoenix dactylifera L.) in diethylnitrosamine induced hepatocellular carcinoma in Wistar rats. BMC Complementary and Alternative Medicine 17, 418. https://doi.org/10.1186/s12906-017-1926-6

Kin, M., Sata, M., Ueno, T., Torimura, T., Inuzuka, S., Tsuji, R., Sujaku, K., Sakamoto, M., Sugawara, H., Tamaki, S., Tanikawa, K., 1997. Basic fibroblast growth factor regulates proliferation and motility of human hepatoma cells by an autocrine mechanism. Journal of Hepatology 27, 677–687. https://doi.org/10.1016/S0168-8278(97)80085-2

Li, W., Li, Z., Li, S., Wang, X., Wilson, J., Huang, G., 2018. Periconceptional Folic Acid Supplementation Benefit to Development of Early Sensory-Motor Function through Increase DNA Methylation in Rat Offspring. Nutrients 10, 292. https://doi.org/10.3390/nu10030292

Li, Y., Tollefsbol, T.O., 2010. Impact on DNA Methylation in Cancer Prevention and Therapy by Bioactive Dietary Components. Current Medicinal Chemistry 17, 2141–2151. https://doi.org/10.2174/092986710791299966

Liu, W., Hu, K., Zhang, F., Lu, S., Chen, R., Ren, Z., Yin, X., 2021. The prognostic significance of microRNA-221 in hepatocellular carcinoma: An updated meta-analysis. The International Journal of Biological Markers 36, 40–47. https://doi.org/10.1177/17246008211032689

Lou, G., Liu, Y., Wu, S., Xue, J., Yang, F., Fu, H., Zheng, M., Chen, Z., 2015. The p53/miR-34a/SIRT1 Positive Feedback Loop in Quercetin-Induced Apoptosis. Cellular Physiology and Biochemistry 35, 2192–2202. https://doi.org/10.1159/000374024

Lyngdoh, A., Baruah, T.J., Sharan, R.N., Kma, L., 2023. Inhibitory Potential of Apium graveolens L. Extract on Inflammation in Diethylnitrosamine-induced Hepatocellular Carcinoma in Mice. Pharmacognosy Magazine 19, 736–750. https://doi.org/10.1177/09731296231170931

Mahmoud, A.M., Mohammed, H.M., Khadrawy, S.M., Galaly, S.R., 2017. Hesperidin protects against chemically induced hepatocarcinogenesis via modulation of Nrf2/ARE/HO-1, PPARγ and TGF-β1/Smad3 signaling, and amelioration of oxidative stress and inflammation. Chemico-Biological Interactions 277, 146–158. https://doi.org/10.1016/j.cbi.2017.09.015

McGlynn, K.A., Petrick, J.L., El-Serag, H.B., 2021. Epidemiology of Hepatocellular Carcinoma. Hepatology 73, 4–13. https://doi.org/10.1002/hep.31288

Mo'men, Y.S., Hussein, R.M., Kandeil, M.A., 2019. Involvement of PI3K/Akt pathway in the protective effect of hesperidin against a chemically induced liver cancer in rats. Journal of Biochemical and Molecular Toxicology 33, 1–8. https://doi.org/10.1002/jbt.22305

Mohamed, D.A.W., Elazeem, H.M.A., Al-Hameid, H.A., Mohamed, R.H., Abdel-Salam, M.F., Abuelela, S., Abozeid, A.A., Mohamad, M.I., 2021. Low dose pterostilbene-mediated hepatic chemoprevention in diethylnitrosamine-treated rats: Modulation of Ppar alpha, Nrf2 and Capase3 expression. Gene Reports 25, 101353. https://doi.org/10.1016/j.genrep.2021.101353

Pandey, P., Khan, F., 2021. A mechanistic review of the anticancer potential of hesperidin, a natural flavonoid from citrus fruits. Nutrition Research 92, 21–31. https://doi.org/10.1016/j.nutres.2021.05.011

Petrick, J.L., Florio, A.A., Znaor, A., Ruggieri, D., Laversanne, M., Alvarez, C.S., Ferlay, J., Valery, P.C., Bray, F., McGlynn, K.A., 2020. International trends in hepatocellular carcinoma incidence, 1978–2012. International Journal of Cancer 147, 317–330. https://doi.org/10.1002/ijc.32723

Pierce, R.H., Vail, M.E., Ralph, L., Campbell, J.S., Fausto, N., 2002. Bcl-2 expression inhibits liver carcinogenesis and delays the development of proliferating foci. American Journal of Pathology 160, 1555–1560. https://doi.org/10.1016/S0002-9440(10)61101-7

PRADEEP, K., RAJ MOHAN, C.V., GOBIANAND, K., KARTHIKEYAN, S., 2010. Protective effect of Cassia fistula Linn. on diethylnitrosamine induced hepatocellular damage and oxidative stress in ethanol pretreated rats. Biological Research 43, 113–125. https://doi.org/10.4067/S0716-97602010000100013

Rachakonda, G., Sekhar, K.R., Jowhar, D., Samson, P.C., Wikswo, J.P., Beauchamp, R.D., Datta, P.K., Freeman, M.L., 2010. Increased cell migration and plasticity in Nrf2-deficient cancer cell lines. Oncogene 29, 3703–3714. https://doi.org/10.1038/onc.2010.118

Rashed, W.M., Kandeil, M.A.M., Mahmoud, M.O., Ezzat, S., 2020. Hepatocellular Carcinoma (HCC) in Egypt: A comprehensive overview. Journal of the Egyptian National Cancer Institute 32, 5. https://doi.org/10.1186/s43046-020-0016-x

Ren, F.-H., Yang, H., He, R., Lu, J., Lin, X., Liang, H.-W., Dang, Y.-W., Feng, Z.-B., Chen, G., Luo, D.-Z., 2018. Analysis of microarrays of miR-34a and its identification of prospective target gene signature in hepatocellular carcinoma. BMC Cancer 18, 12. https://doi.org/10.1186/s12885-017-3941-x

Roohbakhsh, A., Parhiz, H., Soltani, F., Rezaee, R., Iranshahi, M., 2015. Molecular mechanisms behind the biological effects of hesperidin and hesperetin for the prevention of cancer and cardiovascular diseases. Life Sciences 124, 64–74. https://doi.org/10.1016/j.lfs.2014.12.030

Sandhu, D.S., Baichoo, E., Roberts, L.R., 2014. Fibroblast growth factor signaling in liver carcinogenesis. Hepatology 59, 1166–1173. https://doi.org/10.1002/hep.26679

Schumann, G., Bonora, R., Ceriotti, F., Férard, G., Ferrero, C.A., Franck, P.F.H., Gella, F.-J., Hoelzel, W., Jørgensen, P.J., Kanno, T., Kessner, A., Klauke, R., Kristiansen, N., Lessinger, J.-M.,

Linsinger, T.P.J., Misaki, H., Panteghini, M., Pauwels, J., Schiele, F., Schimmel, H.G., 2002. IFCC Primary Reference Procedures for the Measurement of Catalytic Activity Concentrations of Enzymes at 37°C. Part 4. Reference Procedure for the Measurement of Catalytic Concentration of Alanine Aminotransferase. Clinical Chemistry and Laboratory Medicine 40, 718–724. https://doi.org/10.1515/CCLM.2002.124

Siddiqi, A., Hasan, S.K., Nafees, S., Rashid, S., Saidullah, B., Sultana, S., 2015. Chemopreventive efficacy of hesperidin against chemically induced nephrotoxicity and renal carcinogenesis via amelioration of oxidative stress and modulation of multiple molecular pathways. Experimental and Molecular Pathology 99, 641–653. https://doi.org/10.1016/j.yexmp.2015.11.012

Singh, B.N., Singh, B.R., Sarma, B.K., Singh, H.B., 2009. Potential chemoprevention of N-nitrosodiethylamine-induced hepatocarcinogenesis by polyphenolics from Acacia nilotica bark. Chemico-Biological Interactions 181, 20–28. https://doi.org/https://doi.org/10.1016/j.cbi.2009.05.007

Soerjomataram, I., Oomen, D., Lemmens, V., Oenema, A., Benetou, V., Trichopoulou, A., Coebergh, J.W., Barendregt, J., de Vries, E., 2010. Increased consumption of fruit and vegetables and future cancer incidence in selected European countries. European Journal of Cancer 46, 2563–2580. https://doi.org/10.1016/j.ejca.2010.07.026.

SP, N., KANG, D.Y., KIM, D.H., YOO, J.-S., JO, E.S., RUGAMBA, A., JANG, K.-J., YANG, Y.M., 2020. Tannic Acid Inhibits Non-small Cell Lung Cancer (NSCLC) Stemness by Inducing G 0 /G 1 Cell Cycle Arrest and Intrinsic Apoptosis. Anticancer Research 40, 3209–3220. https://doi.org/10.21873/anticanres.14302

Supraja, G., Maheswari, K.C., Pamarthy, D., Saritha, K.V.R., 2022. Phytochemicals for hepatocellular carcinoma therapy: from in vitro to clinic. Theranostics and Precision Medicine for the Management of Hepatocellular Carcinoma 3, 109–132. https://doi.org/https://doi.org/10.1016/B978-0-323-99283-1.00006-9

Taguchi, K., Motohashi, H., Yamamoto, M., 2011. Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. Genes to Cells 16, 123–140. https://doi.org/10.1111/j.1365-2443.2010.01473.x

Thiele, N.D., Wirth, J.W., Steins, D., Koop, A.C., Ittrich, H., Lohse, A.W., Kluwe, J., 2017. TIMP-1 is upregulated, but not essential in hepatic fibrogenesis and carcinogenesis in mice. Scientific Reports 7, 714. https://doi.org/10.1038/s41598-017-00671-1

Tietz, N.W., Burtis, C.A., Duncan, P., Ervin, K., Petitclerc, C.J., Rinker, A.D., Shuey, D., Zygowicz, E.R., 1983. A reference method for measurement of alkaline phosphatase activity in human serum. Clinical Chemistry 29, 751–761.

Wabitsch, S., McCallen, J.D., Kamenyeva, O., Ruf, B., McVey, J.C., Kabat, J., Walz, J.S., Rotman, Y., Bauer, K.C., Craig, A.J., Pouzolles, M., Phadke, I., Catania, V., Green, B.L., Fu, C., Diggs, L.P., Heinrich, B., Wang, X.W., Ma, C., Greten, T.F., 2022. Metformin treatment rescues CD8+ T-cell response to immune checkpoint inhibitor therapy in mice with NAFLD. Journal of Hepatology 77, 748–760. https://doi.org/10.1016/j.jhep.2022.03.010

Yeh, M.-H., Kao, S.-T., Hung, C.-M., Liu, C.-J., Lee, K.-H., Yeh, C.-C., 2009. Hesperidin inhibited acetaldehyde-induced matrix metalloproteinase-9 gene expression in human hepatocellular carcinoma cells. Toxicology Letters 184, 204–210. https://doi.org/10.1016/j.toxlet.2008.11.018

Zaghloul, R.A., Elsherbiny, N.M., Kenawy, H.I., El-Karef, A., Eissa, L.A., El-Shishtawy, M.M., 2017. Hepatoprotective effect of hesperidin in hepatocellular carcinoma: Involvement of Wnt signaling pathways. Life Sciences 185, 114–125. https://doi.org/10.1016/j.lfs.2017.07.026