Does glycogen synthase kinase-3 β signaling pathway has a significant role in date palm pollen cancer therapy?

Mai O. Kadry[a], Rehab M Abdel Megeed[a], Hassan Z. Ghanem[a], Ahmed S. Abdoon[b], Abdel-Hamid Z. Abdel-Hamid[a]

Departments of [a]Therapeutic Chemistry, [b]Animal Reproduction & A.I., Veterinary Research Division, National Research Centre, Cairo, Egypt

Correspondence to Rehab Abdel Megeed, Department of Therapeutic Chemistry, Pharmaceutical and Drug Industries Division, National Research Center, El Buhouth St., Dokki, Cairo, 12622, Egypt.
Tel: 00201010075907; fax: (00202) 33370931; e-mail: rehabzenbaa@gmail.com

Received 21 January 2019
Accepted 18 April 2019

Egyptian Pharmaceutical Journal 2019, 18:208–215

Background and objective
Palm pollen (PP) has attracted much attention for its wide applications as an anticancer natural product due to their high phenolic and flavonoid contents. In the current of PP to inhibit the progression of hepatocellular carcinoma was assessed in a rat model.
Materials and methods
First, high-performance liquid chromatography analysis was performed to identify the active constituents in PP. Diethyl nitrosamine as a hepatocarcinogenic agent was administered at a dose of 4 gm/kg body weight intraperitoneally for 4 months sequenced by PP treatment orally (200 mg/kg) daily for 3 weeks. Biochemical and molecular analyses were estimated.
Results and discussion
HPLC analysis showed the presence of chlorogenic acid, quercetin, coumaric acid, caffeine, vanillin, and ferulic acid in PP. Diethyl nitrosamine significantly elevated serum lipid peroxide, nitrite/nitrate levels, and decreased glutathione level. In addition to an obvious alternation of tumor necrosis factor-α1, nuclear factor kappa-β, cellular oncogene-Fos, and glycogen synthase kinase-3β genes expression. Meanwhile, PP improved all the previously deviated biochemical parameters reflecting great antioxidant, anticancer, and anti-inflammatory index. These findings were confirmed histopathologically.

Keywords:
cellular oncogene-Fos, glycogen synthase kinase-3β, hepatocellular carcinoma, nuclear factor kappa-β, palm pollen

Egypt Pharmaceut J 18:208–215

1687-4315

Introduction

Cancer is an economic burden worldwide. The suggested cause of cancer may be attributed to metabolic disturbance and genetic alteration [1]. The novel trend of different diseases therapy depends on synthetic remedies which are costly, induce adverse side effects, and disturb genetic pathways. Thus, a more secure approach is required to avoid the progression of the disease. In this regard, natural products are a good regimen for cancer therapy and lack side effects. Palm pollen (PP) acts as a potent antioxidant, antitumor in addition to being an anti-inflammatory agent and cures various diseases [2].

Diethylnitrosamine (DEN) is the most commonly used hepatocarcinogen in animal models. Nitrite and nitrate can result in the endogenous formation of carcinogenic nitroso compounds [3].

Glycogen synthase kinase-3β (GSK-3β) regulates glycogen synthesis and is implicated in cancer, noninsulin-dependent diabetes mellitus, and Alzheimer's disease [4].

GSK-3β regulates the activity of nuclear factor kappa-β (NFκB), activation of protein 1, cellular oncogene-Fos (c-Fos), and C-Jun implicated in cancer. It can also phosphorylate NFκB [4].

c-Fos expressing livers display necrotic foci, immune cell infiltration, and altered hepatocyte morphology. NFκB plays an important role in the development of hepatocellular carcinoma (HCC) [5]. Owing to the therapeutic implications of PP on DEN induced HCC through their antioxidant, anti-inflammatory, antiapoptotic, and anticancerous characterization. The main objective of the present study is to evaluate the therapeutic index of PP on chronically induced HCC.

Materials and methods

Chemicals

Diethyl nitrosamine (DEN) was purchased from Sigma-Aldrich Co. (St Louis, Missouri, USA). Kits

used for the determination of liver function and oxidative stress biomarkers were obtained from Randox Company (Antrim, UK). Primers for tumor necrosis factor-β (TNF-β), NFκB, GSK-3, and c-Fos, used in real-time PCR were purchased from Qia Gene (Hilden, Germany). All other chemicals are of highest analytical grade.

Characterization of palm pollen active constituents

High-performance liquid chromatography (HPLC) analysis was carried out using an Agilent 1260 series. The separation was carried out using C18 column (4.6 mm×250 mm i.d., 5 μm). The mobile phase consisted of water (A) and 0.02% trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (80% A), 0–5 min (80% A), 5–8 min (40% A), 8–12 min (50% A), 12–14 min (80% A), and 14–16 min (80% A). The multiwavelength detector was monitored at 280 nm.

Animals

Twenty-four male albino western rats, weighing 180–190 g, from the National Research Center animal house were utilized in this study. The animals were housed in cages kept at standardized conditions. They were allowed free access to water and standard chow diet.

All procedures relating to animal care and the ethical procedures approved by the Animal Care and Use Committee of National Research Center and US National Institute of Health were strictly adhered to.

Experimental design

One-week postacclimatization, the animals were divided into three groups (eight rats each). Group 1, animals that received saline and served as a normal control group. Group 2, animals that received DEN for 4 months and were left untreated. Group 3, the DEN-intoxicated animals were treated with a daily oral dose of PP (200 mg/kg) for 30 concessive days.

Blood sampling and liver tissue preparation

After 5 months, the rats were weighed and blood samples were collected from the sublingual vein. Sera were separated by centrifugation at 5000 rpm for 15 min and were kept at −80°C for subsequent assessment of biochemical parameters.

The animals were then sacrificed by cervical dislocation and liver tissues were carefully separated and kept in 10% formaldehyde for subsequent histopathological inspection.

Estimated parameters

Serum alanine and aspartate aminotransferases activities

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were estimated spectrophotometrically using commercially available kits provided from Randox Company [6].

Serum lipid peroxide level

Malondialdehyde (MDA), as an index of lipid peroxidation (LPOO), was measured using the kit provided by Randox Company [7].

Serum total nitrite–nitrate level

NO was measured according to the method of Miranda *et al.* [8] using a kit provided by Randox Company.

Serum glutathione level

Serum glutathione (GSH) level was estimated using a kit provided by Randox Company [9].

Quantitative real-time-polymerase chain reaction for the analysis of serum tumor necrosis factor-α1, nuclear factor kappa-β, glycogen synthase kinase-3, and cellular oncogene-Fos mRNA expression

TriPure Isolation Reagent (Roche, Basel, Switzerland) was used for total RNA isolation according to the manufacturer's instructions. Complementary DNA was generated using Superscript Choice systems (Life Technologies, Breda, the Netherlands) according to the manufacturer's instructions. To assess the mRNA expression of GSK-3, c-Fos, NFκB, and TNF-α1, quantitative real-time PCR was performed using SYBR green PCR Master Mix (Applied Biosystems, Foster City, California, USA) as described by the manufacturer. The sequences of primers are described in Table 1 [10].

Histopathological examination

Deparaffinized sections of 4 μm were stained with hematoxylin and eosin and examined under a light microscope [11].

Statistical analysis

Data were expressed as means±SEM. Statistical analysis was performed using InStat-3 computer program (Graph Pad Software Inc., San Diego, California, USA). One-way analysis of variance by SPSS 12 program followed by post-hoc test was used to determine the differences between means of different groups. The level of significance was set at a P value of less than 0.05 using Tukey's test.

Results

High-performance liquid chromatography characterization of date palm pollen

The major identified phenolic compounds separated with HPLC are illustrated in Fig. 1 and Table 2. The major compound was identified as chlorogenic acid with a concentration of 81133.051 μg/g extract, followed by querecetin (19 138.54 μg/g extract), and then caffeine (6561.18 μg/g extract) proceeded by ferulic acid (2973.19 μg/ g extract).

Table 1 Primer sequence used in real-time-polymerase chain reaction analysis

Gene	Sequence
GSK-3β forward	5′-GGAACTCCAACAAGGGAGCA-3′
GSK-3β reverse	5′-TTCGGGGTCGGAAGACCTT A-3′
c-Fos forward	5′-GGGACAGCCTTTCCTACTACC-3′
c-Fos reverse	5′-GATCTGCGCAAAAGTCCTGT-3
TNF-α1 forward	5′-CCAGACCCTCACACTCAGATCA-3′
TNF-α1 reverse	5′-TCCGCTTGGTGGTTTGCTA-3′
NFκB forward	5′-CATGAAGAGAAGACACTGACCATGGAAA-3′
NFκB reverse	3′-TGGATAGAGGCTAAGTGT AGACACG-5′

c-Fos, cellular oncogene-Fos; GSK-3β, glycogen synthase kinase-3β; NFκB, nuclear factor kappa-β; TNF, tumor necrosis factor.

Modulation of diethyl nitrosamine – liver damage

Diethyl nitrosamine (DEN) significantly increased serum ALT and AST, with regard to the normal values. Nevertheless, PP reduced liver function enzymes (Fig. 2).

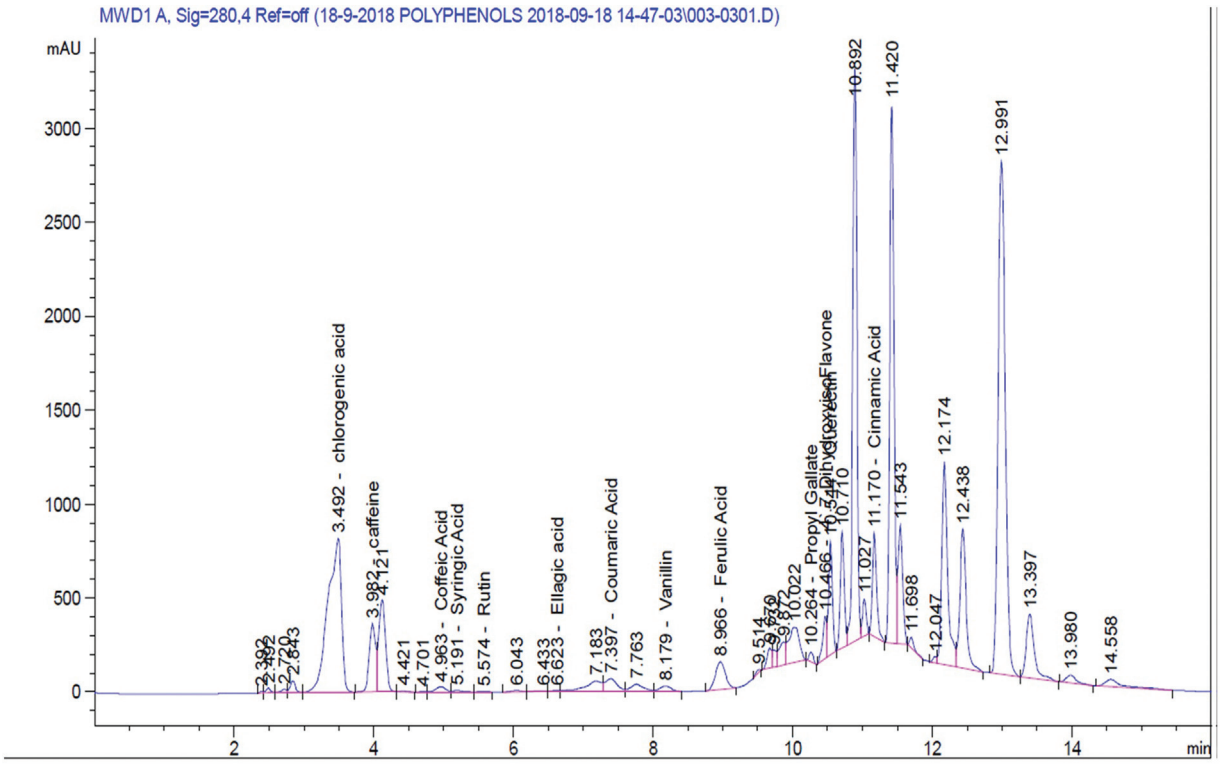
Modulation of oxidative stress biomarkers

Diethyl nitrosamine decreased GSH and increased MDA and nitrite–nitrate levels with regard to the

Table 2 High-performance liquid chromatography of date palm pollen

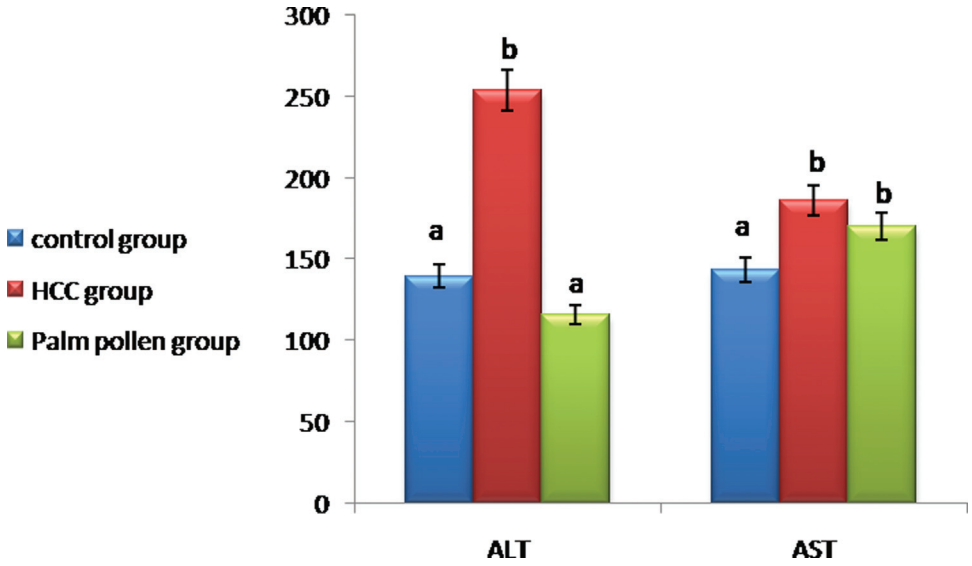
	Sample (10.2 mg/ml)	
	Concentration (μg/g)	Concentration (mg/g)
Chlorogenic acid	81 133.05	81.13
Caffeine	6561.18	6.56
Caffeic acid	918.67	0.92
Syringic acid	282.99	0.28
Rutin	251.68	0.25
Ellagic acid	426.65	0.43
Coumaric acid	1831.21	1.83
Vanillin	1132.21	1.13
Ferulic acid	2973.19	2.97
Propyl gallate	554.05	0.55
4′.7-Dihydroxyisoflavone	2292.91	2.29
Querectin	19 138.54	19.14
Cinnamic acid	1927.42	1.93

Figure 1



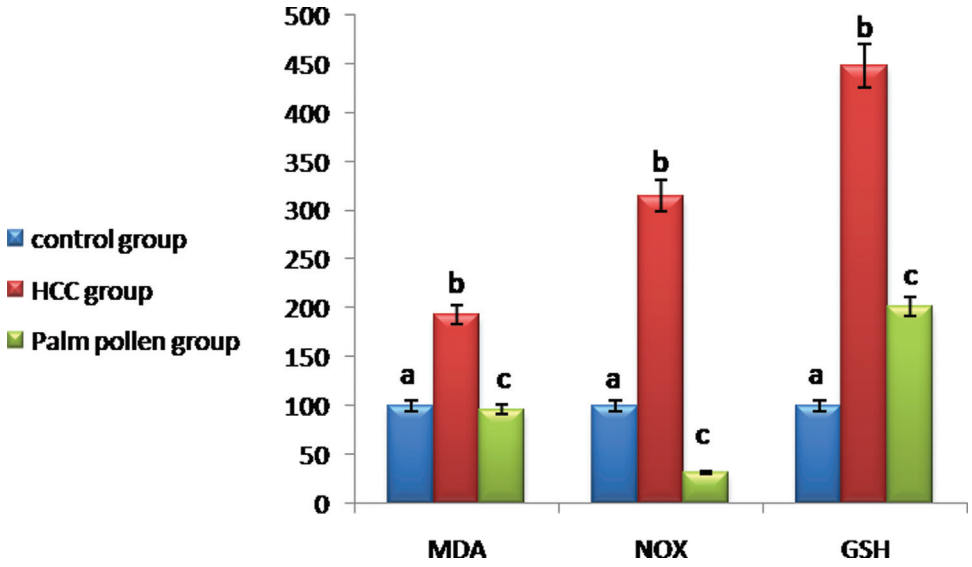
HPLC analysis of polyphenols and flavonoids in date palm pollen. HPLC, high-performance liquid chromatography.

Figure 2



Effect of palm pollen treatment on serum ALT and AST in DEN-induced hepatocellular carcinoma. Data are expressed as means±SEM (*n*=10). A *P* value less than 0.05 is considered significant. Groups having the same letter are not significantly different from each other, while those having different letters are significantly different. ALT, alanine aminotransferase; AST, aspartate aminotransferase; DEN, diethyl nitrosamine.

Figure 3



Effect of palm pollen treatment on serum MDA, NOX, and GSH in DEN-induced hepatocellular carcinoma. Data are expressed as means±SEM (*n*=10). A *P* value less than 0.05 is considered significant. Groups having the same letter are not significantly different from each other, while those having different letters are significantly different.

normal values. Meanwhile, PP increased GSH and decreased MDA and nitrite–nitrate levels with regard to the DEN values (Fig. 3).

Impact of palm pollen on nuclear factor kappa-β

Diethyl nitrosamine significantly elevated NFκB, TNF-α1 genes with regard to the normal values, while mice treated with PP altered the level of NFκB and TNF-β near normal (Fig. 4).

Impact of palm pollen on glycogen synthase kinase-3β

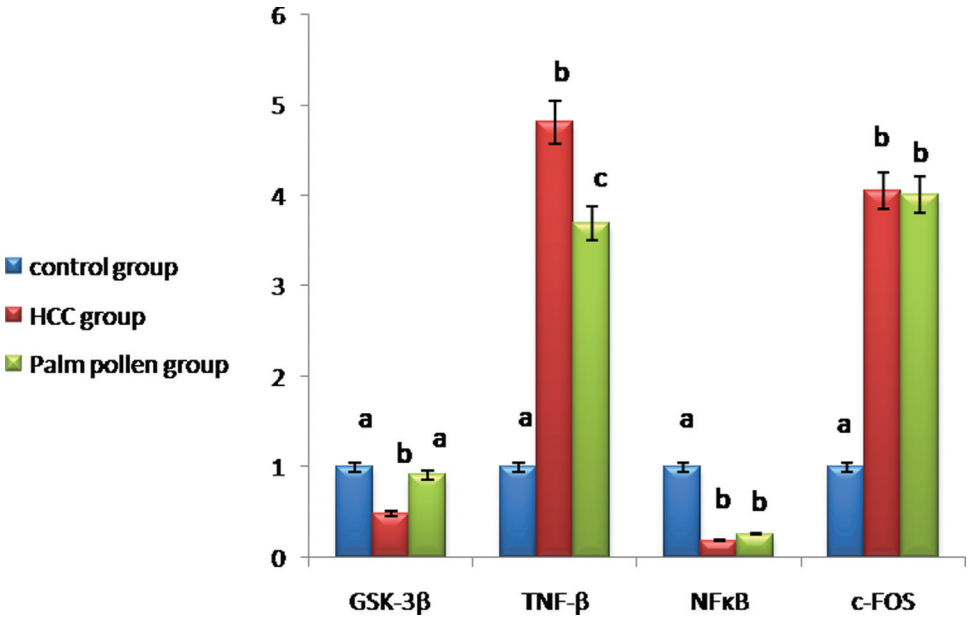
Diethyl nitrosamine elevated c-Fos and reduced GSK-3β genes with regard to the normal values.

Palm pollen reversed these alterations (Fig. 4).

Histopathological findings

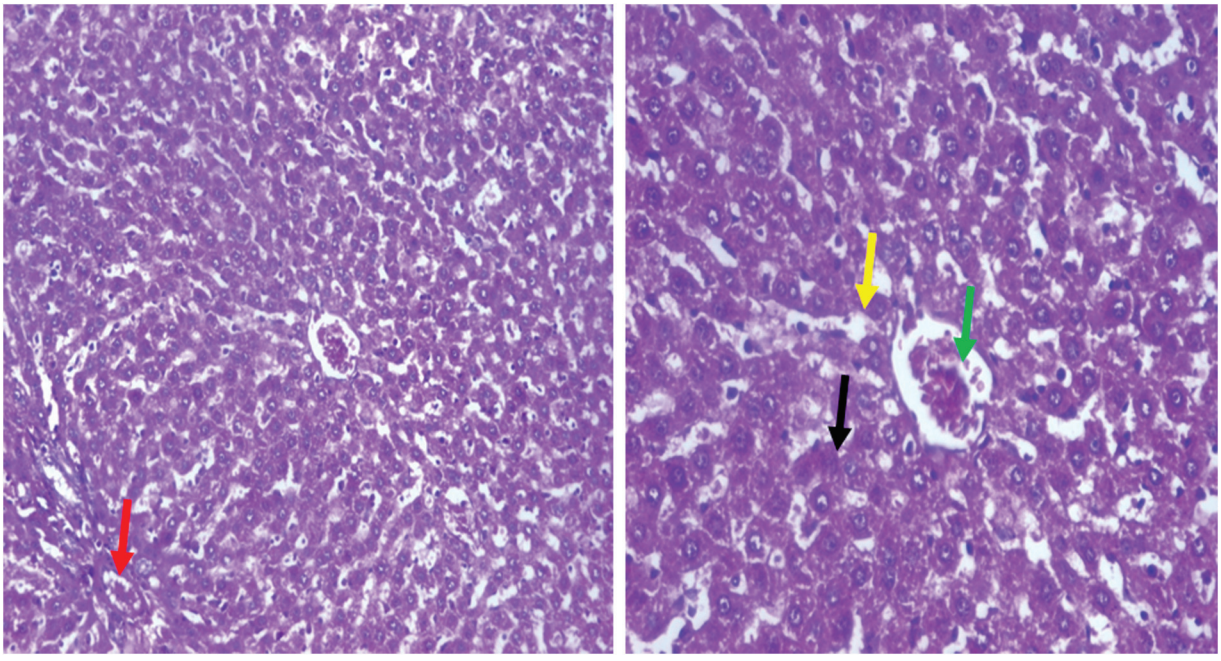
As demonstrated in Fig. 5, normal liver tissue declared normal structure and architecture;

Figure 4



Effect of palm pollen treatment on mRNA expression of GSK, TNF-β, NFκB, and c-Fos following hepatocellular carcinoma induction. GAPHD was used as an internal control for calculating mRNA fold changes. Data are expressed as means±SEM (n=10). A P value less than 0.05 is considered significant. Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other. C-Fos, cellular oncogene-Fos; GSK, glycogen synthase kinase; NFκB, nuclear factor kappa-β; TNF, tumor necrosis factor.

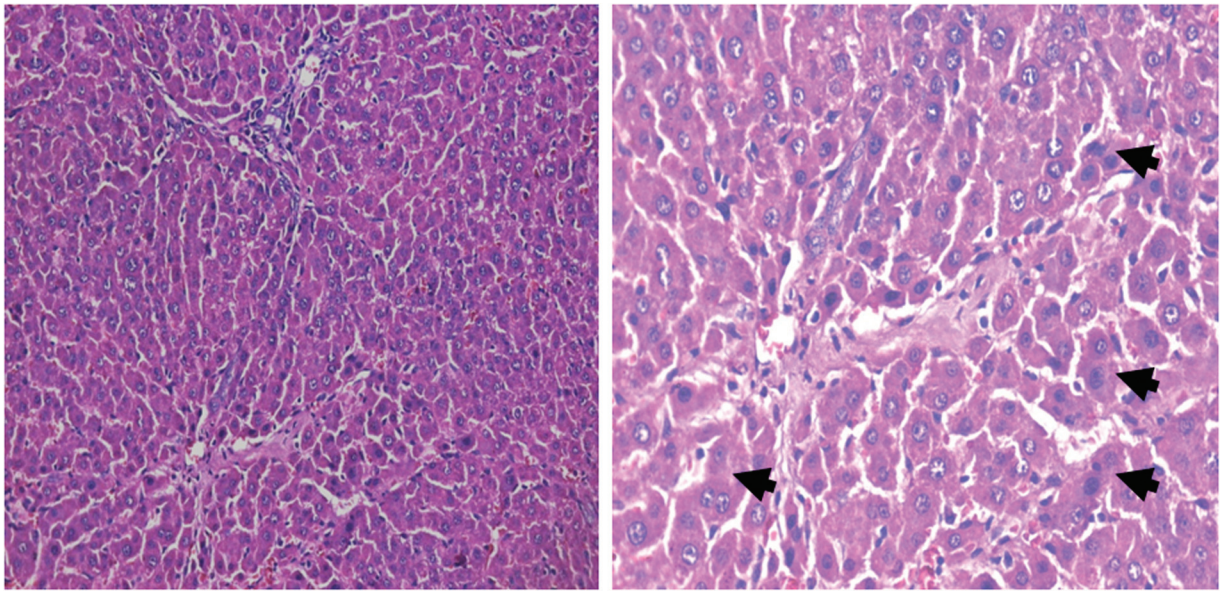
Figure 5



Liver section from the normal control group showed hepatic tissue with normal structure and architecture; hepatocytes arranged in thin plates (black arrow) and sinusoids (yellow arrow); portal tracts contain one bile duct, one artery, and one vein (red arrow), congested central vein (green arrows) (hematoxylin and eosin, ×200, ×400).

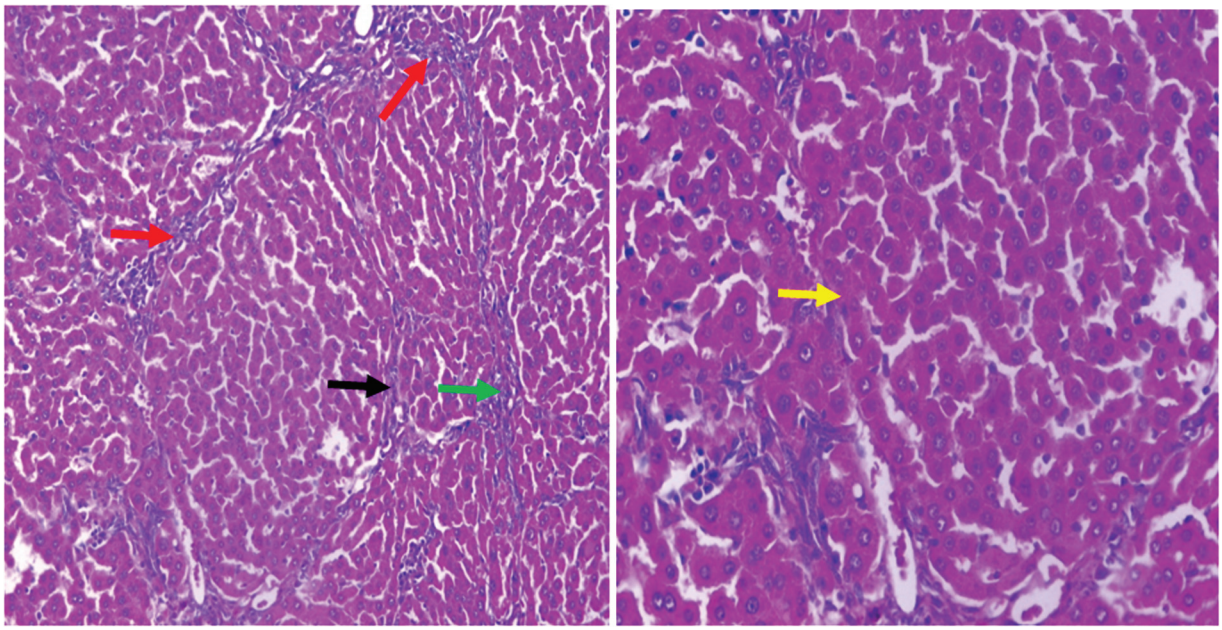
hepatocytes are arranged in thin layers and sinusoids. However, the Diethyl nitrosamine group displayed mononuclear cellular infiltration of hepatocytes as represented in Fig. 6. Adminstration of PP declared regeneration of normal hepatocytes as show in Fig. 7.

Figure 6



DEN group showing malignant hepatocytes showing large polyhedral cells with eosinophilic cytoplasm and enlarged nuclei arranged in cords and acinar pattern (arrows).

Figure 7



Liver section of palm pollen showed hepatic tissue with loss architecture (cirrhotic pattern, variable size nodules); hepatocytes arranged in thick plates (yellow arrows) and sinusoids (yellow arrows); portal tracts contain one bile duct, one artery, and one vein (red arrow); infiltration of hepatocytes in portal tracts and in between hepatocytes (black and green arrow) (hematoxylin and eosin, ×200, ×400).

Discussion

A growing body of evidence deduced that HCC is widespread worldwide and the second leading cause of death. The proliferation of HCC cells is the primary cause of death [1].

Diethyl nitrosamine is transformed to potent ethyl radical that combines with DNA leading to mutation and cancer [3].

Palm pollen serves as a beneficial antioxidant and may be considered as a functional food. PP lowers the

incidence of cancers, especially liver cancer due to antitumor activity or antimutagenic properties, and boosts the immune system [2].

High-performance liquid chromatography analysis declared an obvious large percent of phenolic compounds and flavonoids including chlorogenic acid, quercetin, caffeine, ferulic acid, cinnamic acid, coumaric acid, vanillin, and 4′,7-dihydroxy isoflavone.

Chlorogenic acid is the most abundant polyphenol and ester of caffeic acid and quinic acid that is present in coffee, palm date pollen, and black tea, has potent antioxidant and chemopreventive activities through scavenging free radicals that inhibits DNA damage and prevents carcinogenesis, upregulates the genes activating the immune system, and activates and proliferates cytotoxic macrophages and T-lymphocytes [12].

The second flavonoid was quercetin. It protected the rat liver against oxidative stress by neutralizing the products of LPOO and increasing GSH, limited the cytotoxicity, DNA damage, and lowers the degree of inflammatory processes. Quercetin lowers TNF-α and, hence, lowers the risk of cancer; TNF-α encourages the propagation of various tumor cells. Quercetin causes apoptosis (cell death) of HCC cells by inhibiting the NFκB-signaling pathway [13].

Ferulic acid is an antioxidant phenolic compound similar to SOD. It performs this action via donating one hydrogen atom from its phenolic OH group to ROS, leading to strong anti-inflammatory effects [14].

The current work declared that DEN significantly increased ALT and AST serum activities with regard to the normal value. However, PP significantly improved liver function.

Increased liver function enzymes were induced due to the proliferation of the liver cell membrane. Histopathologically, necrosis in hepatocytes of DEN confirmed these results [3].

Palm pollen is valuable in various disease cure and hepatic toxicity [15].

The pathogenic mechanism of DEN is dominated by oxidative stress, inflammation, apoptosis, and DNA damage [16]. In this regard, the current results deduced that DEN significantly increased serum MDA and NOx levels. Meanwhile, GSH level was significantly reduced although PP significantly alleviated this action [4].

Diethyl nitrosamine is converted to free ethyl radical that binds the DNA leading to cancer and mutation.

Various studies on date fruit deduced a direct relation between antioxidant activity and phenolic content. PP serves as a beneficial antioxidant and lowers the incidence of cancers, especially pancreatic cancer due to antitumor activity or antimutagenic properties, and boosts the immune system [5]. Antioxidant and anti-inflammatory activities of PP is related to its phenolic components (p-coumaric, ferulic, and sinapic acids), flavonoid glycosides (luteolin, quercetin, and apigenin) in addition to procyanidins [17,18].

The current study elucidated that DEN produced a significant elevation in inflammatory markers NFκB and TNF-α1 with regard to the normal. However, PP significantly downregulated this effect [19]. NFκB is essential in inflammation and innate immunity crosslink is mediated by GSK-3 [19].

In harmony, PP showed anti-inflammatory response and reduce cytokines, iNOS, and TNF-α1 due to its polyphenolic and flavonoid constituents [2]. GSK-3β transports the phosphate group from ATP to the target tissue which controls cell signaling, apoptosis, and intracellular communication. GSK-3 may also contribute to cancer progression [20].

In addition, the current work deduced that DEN significantly increased C-Fos and decreased GSK-3 gene. However, PP improved these genes.

c-Fos contributes to premalignant transformation of hepatocytes, to HCCs [21–23].

Meanwhile, PP extract had antiapoptotic effect in murine intestinal Eimeria papillata infection [24].

Conclusion

Palm date pollen, elucidated a significantly modulatory effect on proinflammatory cytokines, oxidative stress, and apoptotic markers and it may be recommended as a promising agent in cancer therapy.

Acknowledgements

Th authors acknowledge Dr Olfat Ali Hammam, Professor of pathology Theodor Bilharz Research Institute for histopathological aid.

This research was financially supported by the National Research Centre, in house-projects (Proj ID: 11010314), National Research Center.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References

1 Rahmani A, Alzohairy M, Mandal AK, Rizvi MA. Expressional evaluation of androgen receptor in transitional cell carcinoma of urinary bladder patients. Br J Med Med Res 2011; 1:233–238.

2 Marwat SK, Khan MA, Rehman F, Bhatti IU. Aromatic plant species mentioned in the Holy Qura'n and Ahadith and their ethnomedicinal importance. Pak J Nut 2009; 8:1472–1479.

3 Sadik NAH, EL-Maraghy SA, Ismail MF. Diethylnitrosamine-induced hepatocarcinogenesis in rats: possible chemoprevention by blueberries. Afr J Biochem Res 2008; 2:81–87.

4 Gao C, Holscher C, Liu Y, Li L. GSK3: a key target for the development of novel treatments for type 2 diabetes mellitus and Alzheimer disease. Rev Neurosci 2012; 23:1–11.

5 Nault JC, Zucman-Rossi J. Genetics of hepatobiliary carcinogenesis. Semin Liver Dis 2011; 31:173–187.

6 Reitman S, Frankle S. A colorimetric method for determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. Am J Clin Path 1957; 28:56–63.

7 Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95:351–358.

8 Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 2001; 5:62–71.

9 Moron MS, Depierre J, Mannervik B. Levels of glutathione, glutathione reductase andglutathione-S-transferase activities in rat lung and liver. Biochem Biophys Acta 1979; 582:67–78.

10 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C (T) method. Methods 2001; 25:402–408.

11 Bancroft JD, Stevens A. Theory and practice of histological techniques. 4th ed. London: Churchill Livingstone; 1996. 163.

12 Granado-Serrano AB, Martin MA, Izquierdo-Pulido M, Goya L, Bravo L, Ramos S. Molecular mechanisms of (−)-epicatechin and chlorogenic acid on the regulation of the apoptotic and survival/proliferation pathways in a human hepatoma cell line. J Agr Food chem 2007; 55:2020–2027.

13 Kahraman A, Çakar H, Köken T. The protective effect of quercetin on long-term alcohol consumption-induced oxidative stress. Mol Biol Rep 2012; 39:2789–2794.

14 Kikuzaki H, Hisamoto M, Hirose K, Akiyama K, Taniguchi H. Antioxidant properties of ferulic acid and its related compounds. J Agric Food Chem 2002; 50:2161–2169.

15 Abdu SB. The protective role of Ajwa date against the hepatotoxicity induced by Ochratoxin A. Egyp J Nat Tox 2011; 8:1–15.

16 Asuku O, Atawodi SE, Onyike E. Antioxidant, hepatoprotective and ameliorative effects of methanolicextract of leaves of grewiamollisjusson carbon tetrachloride-treated albino rats. J Med Food 2011; 15:83–88.

17 Mansouri A, Embarek G, Kokkalou E, Kefalas P. Phenolic profile and antioxidant activity of the Algerian ripe date palm fruit (Phoenix dactylifera). Food Chem 2005; 89:411–420.

18 Bilgari F, Alkarkhi AFM, Easa AM. Antioxidant activity and phenolic content of various date palm (Phoenix dactylifera) fruits from Iran. Food Chem 2008; 107:1636–1641.

19 Papa S, Bubici C, Zazzeroni F, Franzoso G. Mechanisms of liver disease: the crosstalk between the NF-κB and JNK pathways. Biol Chem 2009; 390:965–976.

20 Woodgett JR. Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J 1990; 9:2431–2438.

21 Bakiri L, Wagner EF. Mouse models for liver cancer. Mol Oncol 2013; 7:206–223.

22 Abdel-Megeed RM, Hamed AR, Matloub AA, Kadry MO, Abdel-Hamid AZ. Regulation of apoptotic and inflammatory signaling pathways in hepatocellular carcinoma via Caesalpinia gilliesi galactomannan. Mol Cell Biochem 2019; 451:173–184.

23 Kadry MO, Abdel-Megeed RM, El-Meliegy E, Abdel-Hamid AZ. Crosstalk between GSK-3, c-Fos, NFκB and TNF-α signaling pathways play an ambitious role in Chitosan Nanoparticles Cancer Therapy. Toxicol Rep 2018; 5:723–727.

24 Mahmoud SM, Mohamed AD, Saleh A-Q. Anti-coccidial and anti-apoptotic activities of palm pollen grains on Eimeria papillata-induced infection in mice. Biologia 2014; 69:254–259.