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Sequential analysis and staging of a diethylnitrosamine induced hepatocellular carcinoma in male Wistar albino rat model

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Abstract

Hepatocellular carcinoma (HCC) is one of the most life-threatening cancers. The present study was designed to chronologically analyze the HCC chemically induced by diethylnitrosamine (DEN) in male Wistar rats during 27 weeks. DEN was given to rats in drinking water (100 mg/L) to induce HCC. In the present study, the DEN administered groups recorded dramatic results in the tumor markers, oxidative stress, lipid profile, liver function and hematological parameters at all intervals when compared to their corresponding values in the control groups. In addition, the morphometric analysis of livers of the DEN administered groups (from 9 to 27 weeks) showed gradual enlargement and several grayish white nodules and foci on the peripheral surface of the liver as the features of HCC. In conclusion, the present sequential model chronologically analyzes all steps of hepatocarcinogenesis and presents new staging system for classification of HCC that may be valuable for investigating the effects of anti-carcinogenic compounds at varying stages of hepatocarcinogenesis *in vivo*.

Keywords: DEN; Hepatocarcinogenesis; tumor markers; staging system; oxidative stress; hematology; lipid profile.

Introduction

The primary liver cancer or hepatocellular carcinoma (HCC) is the fifth most common cancer and is the second most common cause of cancer mortality worldwide (Ferlay et al. 2014 and Stewart & Wild 2014). It also disproportionately influences males, with HCC the second leading cause of cancer mortality in men and the ninth leading cause of women (Lozano et al. 2012).

Nitrosamines are compounds formed by the combination of amines and nitrates or nitrites. Studies have shown that nitrosamines can be formed in the human stomach by a process commonly referred to as endogenous nitrosation. The bacteria in the mouth chemically reduce nitrate found in many vegetables to nitrite, which in turn can form nitrosating agents. Many foods that contain amines can react with these nitrosating agents in the acidic environment of the stomach to form nitrosamines (Jakszyn and Gonzalez 2006). Diethylnitrosamine (DEN) is a representative chemical of a family of carcinogenic N-nitroso compounds and well-known hepatocarcinogen; forming DNA adducts in the liver and inducing HCC (Singer and Cruderger 1984).

DEN is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals (IARC 1987). The routes of human exposure to DEN are ingestion, inhalation and dermal contact. There is some potential for occupational exposure of laboratory, copolymer, lubricant and pesticide workers. The general population may possibly be exposed to unknown quantities of DEN present in preserved foods, beverages, tobacco smoke, herbicides, pesticides, drinking water and industrial pollution (Tricker & Preussmann 1991). The present study analyzed and staged the HCC induced by DEN in rats.

Materials and methods

Chemicals

Diethylnitrosamine was purchased from Sigma-Aldrich (St. Louis, MO, USA). DEN was given to rats in drinking water (100 mg/L). The DEN solution was prepared as a fresh solution every other day and administered to rats in dark bottles. Other chemicals and reagents were of high analytical grade and were purchased from standard commercial suppliers.

Experimental animals

A patch of 240 adult mature male Wistar albino rats obtained from the Egyptian Holding Company for Biological Products and Vaccines (VACSERA, Giza, Egypt) averaged weight 150-170 g were allowed to acclimatize in the laboratory and distributed into 16 groups of 15 rats each (8 control groups and 8 DEN administered groups) as demonstrated in **Fig. (1)**. Rats were maintained under standard laboratory conditions at the animal center, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. They were kept in a temperature-controlled environment (20-25°C) and 45%–55% relative humidity with an alternating 12 h light-dark cycle. Five rats were placed into each cage and provided with standard diet pellets and drinking DEN or tap water *ad libitum*.

Ethics statement

The *in vivo* animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (**National Research Council 1996**) and the use of animals was reviewed and approved by an independent ethics committee of the National

Research Centre in Egypt. Samples were collected from each animal under anesthesia and all efforts were made to minimize suffering.

Collection of samples

At the end of the experiment, blood samples were collected from each animal from the retro-orbital venous plexus puncture. One part of the blood was collected in EDTA tubes for hematological study. The other part of the blood was left to clot at room temperature for 15 minutes. Sera were separated by centrifugation at 3000 rpm at 20°C for 15 minutes where the clear serum was obtained and kept frozen at -80°C for various biochemical analyses. After blood sampling, animals were sacrificed and livers were isolated and washed with isotonic ice-cold saline, dried on filter paper and weighed immediately.

Morphometric analysis

The nodules in nodule-bearing livers were counted and classified into three categories according to size; the nodular size in millimeter was measured by the use of an official standard caliper.

Biochemical study

A portion of each animal liver tissue was taken from all animal groups. Each tissue was homogenized in ice-cold Tris-HCl lysis buffer, pH 7.4 containing 1% protease inhibitor cocktail (Cell Signaling Technology, Inc., MA, USA) using Potter-Elvehjem rotor-stator Homogenizer, fitted with a Teflon pestle (Omni International, Kennesaw, GA, USA). The tissue homogenates were centrifuged under cooling at 3000 rpm for 20 min., supernatants were subsequently aliquoted and stored at -80°C until used for determination of hepatic oxidative stress parameters.

Hepatic oxidative stress parameters

Lipid peroxidation product (TBARS) (**Yoshioka et al. 1979**), Advanced oxidation protein products (AOPPs) (**Witko-Sarsat et al. 1996**), nitric oxide (NO) (**Montgomery and Dymock 1961**), reduced glutathione (GSH) (**Beutler et al. 1963**), superoxide dismutase (SOD) (**Minami and Yoshikawa 1979**) and catalase (CAT) (**Aebi 1984**) levels were estimated using kits from bio-diagnostic Co., for research kits, Egypt.

Liver function tests

The serum levels of transaminases, AST and ALT (**Bergmeyer et al. 1986**), alkaline phosphatase (ALP) (**the German Society for Clinical Chemistry 1972**), total protein (TP) (**Gornal et al. 1949**), albumin (**Doumas et al. 1971**), and total bilirubin (TBIL) (**Scherwin and Thompson 2003**) were estimated using kits from Elitech diagnostic Co., France. The globulin was determined by subtracting the albumin from the TP for each rat.

Lipid profile

The serum lipid profile levels of triglycerides (TG) (**Fossati and Principe 1982**), total cholesterol (TC) (**Allain et al. 1974**) and high-density lipoprotein cholesterol (HDL-C) (**Burstein et al. 1970**) were estimated using kits from Elitech diagnostic Co., France. (LDL-C) was calculated according to the formula of **Wieland and Seidel (1982)**.

Serum tumor markers

Serum gamma-glutamyl transferase (γ -GT) (**Szasz 1969**) activity was determined kinetically (Elitech diagnostic Co. France). Serum alpha-L-fucosidase (AFU) (WUHAN EIAAB SCIENCE CO., LTD., CHINA), alpha-fetoprotein (AFP) (LifeSpan BioSciences,

Inc. Seattle, WA, USA), AFP-L3 (Alpha-Fetoprotein Lens Culinaris Agglutinin 3) (Elabscience Biotechnology Co., Ltd. Wuhan, China), glypican-3 (GPC3) (LifeSpan BioSciences, Inc. Seattle, WA, USA), Golgi protein 73 (GP73) (MyBioSource, Inc. San Diego, USA), Serum vascular endothelial growth factor (VEGF) (LifeSpan BioSciences, Inc. Seattle, WA, USA) levels were assayed by ELISA technique.

Hematological parameters

The erythrocyte count, total and differential leukocyte count, platelet count, hematocrite percentage, and hemoglobin concentration were estimated in the blood by using a CBC analyzer (Sino thinker. sk9000, U.S).

Western blot assessments

The Western blot assessments of glypican-3 and Golgi protein 73 were conducted according to the procedure described by **Elsadek et al. (2017)**.

Statistical analysis

The statistical analysis of the results was performed by using statistical package for social sciences SPSS/PC computer program (version 19). All values were expressed as mean $\pm$ SE and the results were analyzed using one-way analysis of variance (ANOVA) test followed by least significant difference (LSD) test for multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

Results

The body weight and the mortality rate

The change in body weight during the period of the experiment (27 weeks) in the control and diethylnitrosamine (DEN) administered groups illustrated in **Fig. (2)** and demonstrated that, in the first weeks following DEN administration, the rats began to show a slow growth and along with the DEN administration, the mean weight decreased gradually as compared to the control rats. The marked decrease in body weight was recorded start from week 12 to week 18 of DEN administration. The body weight became nearly steady with no marked changes from week 18 to the end of the experiment. The present study showed no **mortality** in the control groups during the experiment (27 weeks). On the other hand, the mortality rate of DEN administered groups was 0%, 0%, 6.67%, 13.33%, 20.00%, 33.33%, 46.67% and 60.00% **during** 6, 9, 12, 15, 18, 21, 24 and 27 weeks, respectively.

The liver weight

The DEN administered groups at all intervals recorded a significant decrease ($p < 0.05$) of the **final body weight** and **body weight gain** when compared to their corresponding values in the control groups. On the contrary, a significant increase ($p < 0.05$) in **liver weight** and **relative liver weight** were recorded at all DEN administered intervals except for the interval of 6 weeks, which recorded a significant decrease ($p < 0.05$) when compared to their corresponding values in the control groups (**Table 1**).

Morphometric analysis of the liver

The morphometric analysis of the liver in the control groups reveals smooth surface with normal morphology, without any sub capsular nodules through the 27-week experimental period. Similarly, the livers in the DEN administered group at the end of 6 weeks also, appeared with smooth surface without any nodules. On the contrary, the macroscopic gross appearance of livers in the DEN administered groups (from 9 to 27 weeks interval) showing gradual enlargement and several grayish white nodules and foci on the peripheral surface of the liver as the features of HCC (**Fig. 3**).

The incidence of nodules in DEN administered groups were zero%, 30%, 70%, 80% at the end of 6, 9, 12 and 15 weeks, respectively, and 100% at the end of 18, 21, 24 and 27 weeks. The average number of nodules at nodule-bearing liver in DEN administered groups showed an insignificant increase at the first three time points (6, 9 and 12 weeks) as compared to each others. On the other hand, the other five time points (15, 18, 21, 24 and 27 weeks) recorded a significant increase ($p < 0.05$) in the average number of nodules at nodule-bearing livers when compared to each others in an ascending manner and as any of them being compared to any of the first three time points (**Table 1**). In the present study, the number of nodules with size less than 1 mm in diameter at nodule-bearing liver in DEN administered groups recorded a significant increase ($p < 0.05$) in an ascending manner. Moreover, the time points 18, 21, 24 and 27 weeks recorded a significant increase ($p < 0.05$) in the number of hypoplastic nodules with size from 1-3 mm in diameter at nodule-bearing livers when compared to each others in an ascending manner. Furthermore, the time points 21, 24 and 27 weeks recorded a significant increase ($p < 0.05$) in the number of hyperplastic (neoplastic) nodules with size more than 3 mm in

diameter at nodule-bearing livers when compared to each others in an ascending manner (**Table 1**).

Oxidative stress parameters

The DEN administered groups recorded a significant increase ($p < 0.05$) in TBARS and AOPPs throughout the experimental period when compared to their corresponding control values, except for the TBARS at the 6 weeks it recorded insignificant change as compared to the corresponding value in the control group. In addition, the DEN administered groups recorded a significant decrease ($p < 0.05$) in NO, SOD, CAT and GSH at the end of 6, 9, 12, 15, 18, 21, 24 and 27 weeks respectively, when compared to their corresponding values in the control groups (**Table 2**).

Liver function tests

The DEN administered groups recorded a significant increase ($p < 0.05$) in AST, ALT, ALP, TBIL, TP & globulin in contrast to a significant decrease ($p < 0.05$) in albumin & A/G ratio at the end of 6, 9, 12, 15, 18, 21, 24 and 27 weeks respectively, when compared to their corresponding values in the control groups. Except for TBIL at 6 weeks, it recorded an insignificant increase as compared to the corresponding control value (**Table 3**).

Hepatocellular carcinoma tumor markers

The DEN administered groups recorded a significant increase ($p < 0.001$) in GGT, AFU, AFP, AFP-L3 & GP73 at the end of 6, 9, 12, 15, 18, 21, 24 and 27 weeks respectively, in DEN administered groups when compared to their corresponding values

in the control groups. In addition, the DEN administered groups recorded an insignificant increase in GPC3 in the first four time intervals (6, 9, 12 & 15 weeks) and a significant increase ($p < 0.001$) throughout the remaining experimental periods (18, 21, 25 & 27 weeks) when compared to their corresponding control values. However, the DEN administered groups recorded an insignificant increase in VEGF in the first two time intervals (6 & 9 weeks) and a significant increase ($p < 0.001$) throughout the remaining experimental periods (12, 15, 18, 21, 24 & 27 weeks) when compared to their corresponding control values (**Table 4 and Fig. 4**). Moreover, the DEN administered groups recorded over-expressions of GPC3 and GP73 in the liver tissue homogenates as indicated by the Western blot assessments (**Fig. 5**).

Lipid profile

The DEN administered groups recorded a significant increase ($p < 0.05$) in TG, TC, LDL-C, TG/HDL-C, TC/HDL-C & LDL-C/HDL-C risk ratios and a significant decrease ($p < 0.05$) in HDL-C at the end of 6, 9, 12, 15, 18, 21, 24 and 27 weeks respectively when compared to their corresponding values in the control groups (**Table 5**).

Hematological parameters

The DEN administered groups recorded a significant decrease ($p < 0.05$) in the RBC count, Hb concentration, Hct percentage, platelet count, WBC count and lymphocyte percentage at the end of 6, 9, 12, 15, 18, 21, 24 and 27 weeks respectively, when compared to their corresponding control values. Except for lymphocyte percentage at 6 weeks, it recorded insignificant change as compared to the corresponding values in the control groups. On the other hand, the DEN administered groups recorded a significant

increase ($p < 0.05$) in neutrophil percentage and monocyte percentage at the end of 6, 9, 12, 15, 18, 21, 24 and 27 weeks, respectively, when compared to their corresponding control values, except for the neutrophils percentage at 6 weeks and monocytes percentage at 6 and 9 weeks they recorded insignificant change as compared to the corresponding values in the control groups (**Table 6**).

Hepatocellular carcinoma staging

Bashandy, Mansour & Fathy (BMF) staging system in the present study includes an assessment of liver disease (HCC) and tumor extension through physical, oxidative stress, hematological, biochemical and specific tumor marker parameters for HCC following treatment with DEN. The BMF staging system might be an appropriate classification system for HCC induced in rat model using diethylnitrosamine. In addition, based on the intensive investigation of the current data, the BMF staging system is classified into an early (6-12 weeks), intermediate (15-18 weeks), advanced (18-21 weeks) and late stages (24-27 weeks) of the disease. All stages recorded a significance difference ($p < 0.05$) in all tested parameters from each other's and as any of them being compared to the control rats. The notable feature of the BMF staging system is the assignment of treatment recommendations for each stage based on the best treatment options currently available, and this system has been updated according to the results of our investigations that have incorporated strong evidence. The BMF staging system and treatment allocations are summarized in (**Table 7**).

Discussion

Hepatocellular carcinoma is a highly heterogeneous disease with highly variable etiological factors, including viruses with a DNA (HBV) or RNA (HCV) genome, chemicals (diethylnitrosamine and aflatoxins), and inborn and acquired metabolic diseases. In addition, HCC undergoes a dynamic process changing morphological features as it advances. Therefore, mechanisms of hepatocellular carcinogenesis may vary depending on different factors. The most prominent ones including loss of cell cycle control, escape from senescence control, resistance to cell death, phenotypic plasticity, motility, invasion, and metastasis (**Brian 2016**).

A correlation was noted between the raised incidence of HCC and obesity. Overweight and obese individuals had an increased risk of developing HCC (**Clark et al. 2006**). It was determined that the non-alcoholic fatty liver disease (NAFLD) is related to obesity, diabetes and metabolic syndrome (MS) (**Scalera and Tarantino 2014**). NAFLD, the abnormal accumulation of fat in the liver is the strict clinical and patho-physiological links with obesity, insulin resistance and type 2 diabetes mellitus (T2DM) have led to the suggestion that NAFLD can be considered a new criterion of MS (**Tarantino and Finelli 2003**). An increased risk of developing HCC was demonstrated not only in T2DM patients, but also in obese subjects and those with the criteria of MS (**Duan et al. 2013**). The relationships between T2DM, MS and HCC seem to be closely related to NAFLD development. The majority of obese patients or those with MS develop NAFLD (**Neuschwander-Tetri and Caldwell 2003**). The increased incidence of HCC in obese and diabetic animals has led to the necessity to characterize the role of MS in liver carcinogenesis (**Obara et al. 2017**). Glucose intolerance, hyperglycemia, T2DM, obesity,

hypertension and dyslipidemia are the key components in MS (**Scalera and Tarantino 2014**).

Chemicals like diethylnitrosamine (DEN) contribute to cancer development. In agreement with the present study, perturbations in single cells lead to the focal outgrowth of putatively preneoplastic lesions. The altered areas can evolve into nodular hyperplasia, focus in nodule pathology and areas of malignancy (**Rochen and Carl-McGrath 2001**). Since the liver is the primary site for cancer induction in the bioassays used for carcinogen testing, there is a need for extrapolation of animal neoplasms that arise at this site to man. The utility of defining common biomarkers for the conversion of benign to malignant transition will assist in developing appropriate interspecies extrapolation as demonstrated in the present work. In light of the present investigated tumor markers, several studies have combined various biomarkers and in general, shown improved detection performance (**Hadziyannis et al. 2013; Wang et al. 2013; Johnson et al. 2014**).

In view of our data, the identification of a carcinogenic potential for an agent delineates the conditions of exposure (dose, time and duration) under which the agent may induce cancer. Animals are surrogate models of humans since they possess similar physiology and biochemistry. Several factors are important for cancer development, including a loss of normal growth control as recorded in the present study with contributions from inhibition of apoptosis and enhanced, but altered proliferation control (**Hanahan and Weinberg 2011**).

Various researchers confirmed that the attenuation of tumor expansion *via* natural products (**Pradeep et al. 2007; Jagan et al. 2008**). Few of researchers observed that the decreased content of nutrition might be connected with reduced tumor volume. Recently, it has been proven that caloric restriction protocols and restricted feeding schedules are protective in experimental carcinogenic animal models and the time-caloric restriction prevents fibrosis from progressing into cirrhosis, thus avoiding chronic inflammation and regenerative processes. It also prevents, probably through circadian entrainment and caloric restriction, the neoplastic transformation of tumoural lesions induced by diethylnitrosamine (**Molina-Aguilar et al. 2017**).

Various studies proved that there is a low content of the reactive oxygen species (ROS), during the normal cell physiological progression. Several factors are directly responsible for increasing the ROS production and affect the cellular macromolecules (nucleic acid, protein and lipids) as environmental factors, carcinogenesis and stressful conditions (**Ghosh et al. 2012**).

Diethylnitrosamine (DEN) is a representative chemical carcinogen with the potential to cause tumors in various organs, including the liver, skin, gastrointestinal tract, kidney and respiratory system (**IARC, 1987**). Specifically DEN has been widely used to induce experimental hepatocellular carcinoma (**Brian 2016**). Chemical carcinogenesis is strongly associated with the formation of ROS. Consequently, excessive generation of oxygen-derived radicals stimulates the oncogenesis *via* modulation of the redox signal pathways. ROS also generates the cell death *via* DNA fragmentation, lipid peroxidation and protein oxidation (**Bishayee et al. 2010**).

In agreement with the present study, oxidative stress can be considered a key event in the progression from fatty liver to steatohepatitis. An increase in the production of ROS and RNS, mainly derived from mitochondrial free fatty acids oxidation, leading to dyslipidemia and increased supply of free fatty acids to the liver that combined with increased *de novo* lipogenesis determines an abnormal intrahepatic triglyceride accumulation (Tarantino et al. 2011; Salomone et al. 2013). The absence of efficient endogenous scavenging activity like in the present study, leads to increased levels of free radicals which may trigger hepatocyte injury and activation of inflammatory and fibrogenic signaling in the liver (Gambino et al. 2011).

The unconjugated bilirubin is a metabolic end product of heme breakdown into the reticular-endothelial system (Ryter et al. 2006). Several experimental evidences suggest that the unconjugated bilirubin is the main endogenous lipid antioxidant and is cytoprotective in different tissues and organs (Stocker 2004; Salomone et al. 2013). Stocker et al (1987) first recognized the antioxidant effect of bilirubin in an *in vitro* experiment showing that bilirubin is able to scavenge peroxy radicals in liposomes even more efficiently than alpha-tocopherol, the best characterized molecule counteracting lipid peroxidation. Consistently, the unconjugated bilirubin suppresses the oxidation of lipids and lipoproteins, especially LDL cholesterol (Wu et al. 1996).

The lipid peroxidation products are considered as the classical marker of oxidative stress. Several incidences confirmed the possible mechanism for damage of the red blood corpuscle in lipid peroxidation, which is generally induced by the free radicals. DEN-administered rats in the present work demonstrated high levels of liver enzymes (AST & ALT) and augmented content of total bilirubin and ALP in serum; all are considered as

the hepatic toxicity markers. The high concentration of total bilirubin in the present study may attributed to the high conjugated bilirubin which confirmed the increased degradation rate of red blood cells, while modulation of ALP content directly showed the pathological changes in biliary flow (**Zhao et al. 2015**).

During the oxidative stress, constant generation of oxygen radicals reacts with the unstable fatty acids and forms products such as peroxy fatty acid radical and the produced radical continues to react with another free radical and forms the unpredictable chain of lipid peroxide. Altered lipid profile in malignant tissue like in the present work is the critical parameter, due to an effect on the fluidity, membrane integrity and circulation cellular process, which is directly related to the cell survival and growth (**Tarantino et al. 2011; Thangavel and Vaiyapuri 2013**).

In light of the present work, during the DEN-induced carcinogenesis, the lipid peroxidation products (TBARS) such as 4-hydroxy nominal and MDA react with the various free radicals and initiate the oxidative stress, which causes the carcinogenic effect (**Bansal et al. 2005; Verma et al. 2017**). The increased content of the lipid peroxidation products (TBARS) and protein oxidation products (AOPPs) during DEN administration in the present study provoked the generation of free radicals, which confirmed by reduced oxidative stress markers of enzymatic endogenous antioxidant (SOD & CAT), non-enzymatic antioxidant (GSH) and nitric oxide (NO) concentrations.

In line with the present work, spontaneous liver cancer in both humans and animals occurs predominantly in males. Early studies on rodents exposed to carcinogens indicated that male rodents are more likely to develop liver tumors and the incidence in males

being marginally greater in the Wistar strain (**McKillop et al. 2006**). In liver cancer research with experimental animals, DEN is used either as a complete carcinogen or as an initiator in multistage models. When used as a tumor initiator, DEN is usually given as a single dose of 200 mg/kg to induce pronounced liver necrosis and presumably certain gene mutations in some hepatocytes (**Goldsworthy and Hanigan 1986**). When used as a complete carcinogen as in the present study, DEN is usually given for several weeks (**Elsadek et al. 2017**). The sequential administration of DEN for several weeks has been demonstrated to induce HCC in rodents (**Chuang et al. 2000**).

Liver cancer induction and progression has been extensively examined in the rodent. The induction process represented by a population of initiating hepatocytes in the rat liver and this is true for several types of genotoxic carcinogens including diethylnitrosamine in a dose dependent manner of administration that results in the formation of bulky DNA adducts (**Saeter et al. 1989**). Some subset of these cells will grow into colonies of hepatocytes and by the definition of some of those that will progress into hepatic neoplastic nodules and HCC (**Dragan et al. 1994**). The promotion stage of cancer development has been operationally defined as the clonal expansion of the initiated cell population (**Saeter et al. 1989**). The stage of progression encompasses the spectrum of changes that occur in the conversion of preneoplastic cells into malignant neoplasia (**Pitot 2007**). In agreement with the present work, morphologically, the focus in nodule configuration is the earliest endpoint for detection of progression in the liver (**Pitot 2007; Zhao et al. 2013**).

Because of the high recurrence rate and poor prognosis, the prognostic assessment and selection of treatment strategy in HCC patients are quite important and a precise

stratification system for the prognosis of HCC patients is required (**Llovet et al. 2004; Llovet et al. 2008**). In patients with HCC, the prediction of prognosis is complex compared with most solid tumors. It is well known that the prognosis and treatment of HCC depend on the tumor burden in addition to patient's underlying liver disease and liver functional reserve (**Bruix & Sherman 2011**). Therefore, staging systems based on information regarding both tumor factors and host factors such as liver function has been required to accurately classify HCC patients undergoing various therapeutic options (**Duseja 2014; Kinoshita et al. 2015**). Staging systems aim to direct patients to different treatment arms, ensuring optimal utilization of resources. Different staging systems and survival prediction models have been suggested for HCC, mainly to ensure appropriate treatment allocation. The most widely used in human is the Barcelona Cancer Liver Clinic (BCLC) staging system (**Pons et al. 2005; Bruix & Sherman 2011**).

In the present study the Bashandy, Mansour & Fathy (BMF) staging system for HCC induced in rat model using DEN is in agreement with the BCLC staging system in human in that, it links staging with treatment modalities and with an estimation of the mortality rates. It identifies early HCC that may benefit from curative therapies and provide excellent long-term survival, those at intermediate or advanced disease stage that may benefit from treatment therapies, as well as those at the end stage with a very poor life expectancy. Other staging systems are less frequently used. Some offer only clinical staging with no prognostic value while others are very population specific and not applicable to all HCC patients. The BMF staging system developed based on the combination of data from several parameters representing different disease stages. It includes variables related to tumor stage and liver functional status.

Various chemo-protective and epidemiologic investigations already proved that the regular intake of fruit and vegetable has been reducing the risk factor of cancer in animal and in human. Several shreds of evidence have accentuated that biologically active products present in functional food may be exploited as a complementary treatment for hepatic cancer (**Butler 2008; Verma et al. 2017**).

Compounds that have potential chemopreventive and therapeutic effects on the liver and working by different mechanisms include acyclic retinoid (ACR), capsaicin, caffeine, cinnamaldehyde, curcumin, diallyl sulfide (DAS), eicosapentaenoic acid (EPA), epigallocatechin-3-gallate (EGCG), genistein, resveratrol, lycopene, sulforaphane (SFN) and silymarin (**Okano et al. 2011**). In addition, various forms of the foods and drinks confirm the presence of triterpenoids which have been recently scrutinized as a potential treatment of various inflammatory and cancer disease through down-regulation of the oxidative stress, inflammation and pathology *via* NF- κ B pathway (**Verma et al. 2017**).

The HCC model using DEN and the BMF staging system may be utilized for investigating the beneficial effects of anti-carcinogenic compounds and for the compounds used for the treatment of HCC *in vivo* and may be valuable for investigating the effects of compounds at varying stages of hepatocarcinogenesis.

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Legends

Fig. (1): The experimental schedules of diethylnitrosamine (DEN) administered and control (C) groups during 27 weeks.

Fig. (2): The chronological changes of body weight during 27 weeks in the control and diethylnitrosamine (DEN) administered groups.

Fig. (3): The macroscopic gross appearance of livers at the end of the experimental periods in the control and DEN administered groups (Arrows indicate nodules).

Fig (4): percentage of AFP-L3 (Lens culinaris agglutinin-reactive fraction of AFP) to total AFP at the end of the experimental periods in the control and DEN administered groups.

Fig (5): Western blot analysis of GPC3 (A) and GP73 (B) expressions in liver tissues at the end of experimental periods in the control and DEN administered groups.

Draft

Table (1): The physical parameters of the DEN and control groups.

W	Groups	Final body wt. (g)	Gain in body wt. (g)	Liver Wt. (g)	Relative liver Wt. (g)	Nodule incidence %	Average no. of nodules/nodule bearing liver	Relative nodular size (% of total numbers)					
								<1 mm	%	1-3 mm	%	>3 mm	%
6	C	261±1.56 ^a	97.3±2.80 ^a	6.54±0.07 ^a	2.49±0.01 ^{ab}	0	0±0 ^a	0±0 ^a	0.00	0±0 ^a	0.00	0±0 ^a	0.00
	DEN	244±1.63 ^b	76.6±3.18 ^{bc}	5.84±0.12 ^a	2.39±0.05 ^b	0	0±0 ^a	0±0 ^a	0.00	0±0 ^a	0.00	0±0 ^a	0.00
9	C	302±1.20 ^c	144±2.10 ^d	8.66±0.08 ^b	2.86±0.01 ^a	0	0±0 ^a	0±0 ^a	0.00	0±0 ^a	0.00	0±0 ^a	0.00
	DEN	278±1.25 ^d	117±2.61 ^e	9.38±0.23 ^{bc}	3.36±0.08 ^c	30	1.2±0.62 ^a	1.2±0.62 ^{ab}	100.00	0±0 ^a	0.00	0±0 ^a	0.00
12	C	347±0.90 ^e	188±2.31 ^f	9.73±0.03 ^c	2.80±0.00 ^a	0	0±0 ^a	0±0 ^a	0.00	0±0 ^a	0.00	0±0 ^a	0.00
	DEN	306±1.02 ^e	145±1.78 ^d	12.22±0.20 ^d	3.99±0.06 ^d	70	7.33±1.54 ^{ab}	5.2±1.21 ^b	78.79	1.4±0.45 ^{ab}	21.21	0±0 ^a	0.00
15	C	384±1.18 ^f	217±1.78 ^g	10.76±0.04 ^e	2.80±0.01 ^a	0	0±0 ^a	0±0 ^a	0.00	0±0 ^a	0.00	0±0 ^a	0.00
	DEN	323±1.36 ^g	162±1.30 ^h	14.82±0.66 ^f	4.57±0.18 ^e	80	17.2±3.31 ^b	11.4±2.10 ^c	66.28	4.9±1.08 ^b	28.49	0.9±0.23 ^a	5.23
18	C	425±2.11 ^h	261±2.39 ⁱ	11.80±0.03 ^d	2.77±0.01 ^a	0	0±0 ^a	0±0 ^a	0.00	0±0 ^a	0.00	0±0 ^a	0.00
	DEN	242±2.25 ^b	82.4±2.62 ^c	20.75±0.39 ^g	8.57±0.14 ^f	100	38.3±6.12 ^c	23.9±3.36 ^d	62.40	12.2±2.45 ^c	31.85	2.2±0.53 ^a	5.74
21	C	473±1.62 ⁱ	312±2.30 ^j	12.92±0.03 ^d	2.72±0.00 ^{ab}	0	0±0 ^a	0±0 ^a	0.00	0±0 ^a	0.00	0±0 ^a	0.00
	DEN	234±1.34 ^j	73.6±2.02 ^b	27.52±0.75 ^h	11.73±0.30 ^g	100	75±7.40 ^d	37.7±3.45 ^e	50.27	25.3±3.10 ^d	33.73	12±1.17 ^b	16.00
24	C	510±1.21 ^k	350±1.77 ^k	14.32±0.05 ^f	2.80±0.00 ^a	0	0±0 ^a	0±0 ^a	0.00	0±0 ^a	0.00	0±0 ^a	0.00
	DEN	242±1.26 ^b	83.9±1.84 ^c	29.30±0.45 ⁱ	12.07±0.13 ^g	100	93.5±6.62 ^e	42.9±2.16 ^f	45.88	32.3±2.85 ^e	34.55	18.3±2.28 ^c	19.57
27	C	529±1.08 ^l	377±2.49 ^l	14.85±0.05 ^f	2.80±0.00 ^a	0	0±0 ^a	0±0 ^a	0.00	0±0 ^a	0.00	0±0 ^a	0.00
	DEN	234±1.13 ^j	72.8±1.99 ^b	30.31±0.63 ^j	12.91±0.30 ^h	100	114±9.32 ^f	48.5±3.50 ^g	42.43	40.7±3.33 ^f	35.61	25.1±2.95 ^d	21.96

Results are expressed as mean±SE: standard error; Values not sharing common superscript are significant with each other at p<0.05; W: weeks; C: control; DEN: diethylnitrosamine; wt: weight.

Table (2): The oxidative stress markers in the liver tissue of the DEN and control groups.

W	Groups	TBARS (nmole/g tissue)	AOPPs (nmole/g tissue)	NO (μ mol/g tissue)	SOD (U/mg tissue)	CAT (U/mg tissue)	GSH (nmol/g tissue)
6	C	194 $\pm$ 1.08 ^a	195 $\pm$ 0.94 ^a	40.96 $\pm$ 0.48 ^a	74.32 $\pm$ 1.20 ^a	1.55 $\pm$ 0.01 ^a	2.94 $\pm$ 0.01 ^a
	DEN	197 $\pm$ 0.98 ^a	200 $\pm$ 0.78 ^b	37.21 $\pm$ 0.62 ^b	69.26 $\pm$ 1.38 ^b	1.32 $\pm$ 0.04 ^b	2.33 $\pm$ 0.01 ^b
9	C	193 $\pm$ 0.96 ^a	194 $\pm$ 0.61 ^a	41.29 $\pm$ 0.77 ^a	74.59 $\pm$ 1.40 ^a	1.54 $\pm$ 0.01 ^a	2.96 $\pm$ 0.02 ^a
	DEN	214 $\pm$ 1.51 ^b	219 $\pm$ 1.78 ^c	34.68 $\pm$ 0.60 ^c	64.24 $\pm$ 1.47 ^c	1.11 $\pm$ 0.02 ^c	2.18 $\pm$ 0.01 ^c
12	C	195 $\pm$ 1.09 ^a	195 $\pm$ 1.09 ^a	41.05 $\pm$ 0.89 ^a	73.85 $\pm$ 1.59 ^a	1.54 $\pm$ 0.01 ^a	2.94 $\pm$ 0.01 ^a
	DEN	251 $\pm$ 1.65 ^c	254 $\pm$ 1.24 ^d	32.36 $\pm$ 0.90 ^d	59.86 $\pm$ 1.67 ^d	0.97 $\pm$ 0.02 ^d	2.03 $\pm$ 0.01 ^d
15	C	195 $\pm$ 1.03 ^a	194 $\pm$ 0.93 ^a	41.06 $\pm$ 0.80 ^a	73.80 $\pm$ 1.73 ^a	1.54 $\pm$ 0.01 ^a	2.95 $\pm$ 0.01 ^a
	DEN	270 $\pm$ 1.18 ^d	275 $\pm$ 1.05 ^e	25.99 $\pm$ 0.92 ^e	54.88 $\pm$ 1.43 ^e	0.87 $\pm$ 0.01 ^e	1.94 $\pm$ 0.01 ^e
18	C	194 $\pm$ 1.30 ^a	194 $\pm$ 0.85 ^a	41.91 $\pm$ 0.79 ^a	74.12 $\pm$ 1.69 ^a	1.55 $\pm$ 0.01 ^a	2.95 $\pm$ 0.01 ^a
	DEN	315 $\pm$ 1.93 ^e	314 $\pm$ 1.20 ^f	20.48 $\pm$ 0.80 ^f	51.27 $\pm$ 1.71 ^e	0.76 $\pm$ 0.01 ^f	1.84 $\pm$ 0.02 ^f
21	C	196 $\pm$ 1.06 ^a	194 $\pm$ 0.73 ^a	40.40 $\pm$ 0.64 ^a	73.98 $\pm$ 1.62 ^a	1.54 $\pm$ 0.01 ^a	2.95 $\pm$ 0.01 ^a
	DEN	339 $\pm$ 1.78 ^f	344 $\pm$ 1.16 ^g	16.95 $\pm$ 0.68 ^g	45.32 $\pm$ 1.25 ^f	0.66 $\pm$ 0.01 ^g	1.76 $\pm$ 0.01 ^g
24	C	195 $\pm$ 1.22 ^a	194 $\pm$ 0.90 ^a	41.90 $\pm$ 0.94 ^a	74.24 $\pm$ 1.46 ^a	1.54 $\pm$ 0.01 ^a	2.95 $\pm$ 0.01 ^a
	DEN	358 $\pm$ 2.02 ^g	359 $\pm$ 1.39 ^h	14.04 $\pm$ 1.35 ^h	38.69 $\pm$ 1.59 ^g	0.57 $\pm$ 0.01 ^h	1.61 $\pm$ 0.01 ^h
27	C	194 $\pm$ 1.07 ^a	194 $\pm$ 0.77 ^a	41.97 $\pm$ 0.81 ^a	73.72 $\pm$ 1.49 ^a	1.54 $\pm$ 0.01 ^a	2.95 $\pm$ 0.01 ^a
	DEN	378 $\pm$ 2.06 ^h	382 $\pm$ 0.82 ⁱ	11.09 $\pm$ 0.79 ⁱ	28.91 $\pm$ 1.64 ^h	0.50 $\pm$ 0.01 ⁱ	1.51 $\pm$ 0.01 ⁱ

Results are expressed as mean $\pm$ SE: standard error; Values not sharing common superscript are significant with each other at $p < 0.05$; W: weeks; C: control; DEN: diethylnitrosamine; TBARS: thiobarbituric acid reactive substances; AOPPs: advanced oxidation protein products; SOD: superoxide dismutase; CAT: catalase; GSH: reduced glutathione; NO: nitric oxide.

Table (3): The serum liver function parameters of the DEN and control groups.

W	Groups	AST (U/L)	ALT (U/L)	ALP (U/L)	TBIL (mg/dl)	TP (g/dl)	ALB (g/dl)	GLOB (g/dl)	A/G ratio (g/dl)
6	C	71.1±1.63 ^a	65.32±1.49 ^a	114±1.97 ^a	0.37±0.03 ^a	6.35±0.05 ^a	4.06±0.03 ^a	2.28±0.08 ^a	1.80±0.07 ^a
	DEN	80.5±1.56 ^b	78.07±1.60 ^b	136±2.20 ^b	0.40±0.01 ^a	6.73±0.02 ^b	3.84±0.07 ^b	2.89±0.08 ^b	1.34±0.06 ^b
9	C	70.9±1.56 ^a	64.62±1.50 ^a	114±1.83 ^a	0.35±0.03 ^a	6.26±0.06 ^a	4.07±0.04 ^a	2.19±0.08 ^a	1.88±0.08 ^a
	DEN	105±2.08 ^c	85.25±2.31 ^c	155±1.97 ^c	0.80±0.03 ^b	6.91±0.01 ^c	3.62±0.04 ^c	3.29±0.04 ^c	1.10±0.02 ^c
12	C	71.2±1.50 ^a	64.27±1.69 ^a	113±2.24 ^a	0.35±0.04 ^a	6.27±0.06 ^a	4.05±0.05 ^a	2.22±0.08 ^a	1.86±0.11 ^a
	DEN	134±2.01 ^d	107.8±2.39 ^d	176±1.68 ^d	1.03±0.03 ^c	7.16±0.03 ^d	3.42±0.01 ^d	3.73±0.03 ^d	0.91±0.01 ^d
15	C	70.3±1.29 ^a	64.40±1.28 ^a	114±2.22 ^a	0.36±0.03 ^a	6.32±0.05 ^a	4.09±0.04 ^a	2.22±0.04 ^a	1.84±0.04 ^a
	DEN	182±1.90 ^e	138.2±2.22 ^e	216±3.08 ^e	1.24±0.03 ^d	7.31±0.02 ^e	3.23±0.02 ^e	4.08±0.03 ^e	0.79±0.01 ^{de}
18	C	70.1±1.40 ^a	65.18±1.39 ^a	115±1.66 ^a	0.37±0.04 ^a	6.29±0.07 ^a	4.01±0.03 ^a	2.28±0.07 ^a	1.77±0.07 ^a
	DEN	241±2.26 ^f	158.4±2.34 ^f	257±2.98 ^f	1.45±0.03 ^e	7.40±0.02 ^e	3.00±0.04 ^f	4.4±0.04 ^f	0.68±0.01 ^{ef}
21	C	70.7±1.48 ^a	64.61±1.37 ^a	114±1.42 ^a	0.35±0.03 ^a	6.26±0.07 ^a	4.06±0.04 ^a	2.20±0.08 ^a	1.87±0.08 ^a
	DEN	298±2.38 ^g	185.9±2.23 ^g	304±2.43 ^g	1.73±0.02 ^f	7.60±0.01 ^f	2.78±0.01 ^g	4.82±0.02 ^g	0.57±0.00 ^{fg}
24	C	70.7±1.42 ^a	64.43±1.40 ^a	114±1.73 ^a	0.36±0.03 ^a	6.35±0.04 ^a	4.04±0.04 ^a	2.30±0.07 ^a	1.77±0.08 ^a
	DEN	353±2.22 ^h	200.4±2.09 ^h	353±2.21 ^h	1.91±0.02 ^g	7.85±0.02 ^g	2.58±0.02 ^h	5.27±0.02 ^h	0.48±0.00 ^g
27	C	71.8±1.69 ^a	64.71±1.36 ^a	115±1.51 ^a	0.35±0.04 ^a	6.27±0.06 ^a	4.04±0.04 ^a	2.23±0.08 ^a	1.84±0.09 ^a
	DEN	375±2.31 ⁱ	218.5±2.45 ⁱ	379±2.93 ⁱ	2.21±0.06 ^h	7.9±0.02 ^g	2.33±0.02 ⁱ	5.56±0.03 ⁱ	0.42±0.00 ^g

Results are expressed as mean±SE: standard error; Values not sharing common superscript are significant with each other at p<0.05; W: weeks; C: control; DEN: diethylnitrosamine; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; TBIL: serum total bilirubin; TP: total protein; ALB: albumin; GLOB: globulin; A/G: albumin-globulin ratio.

Table (4): Hepatocellular carcinoma tumor markers in the DEN and control groups.

W	Groups	GGT (U/L)	AFU (pg/ml)	AFP (ng/ml)	GPC3 (ng/ml)	GP73 (ng/ml)	VEGF (ng/L)
6	C	2.51±0.12 ^a	34.24±0.94 ^a	5.01±0.25 ^a	0±0 ^a	203±1.87 ^a	85.24±1.40 ^a
	DEN	5.06±0.32 ^b	42.90±1.40 ^b	14.01±0.80 ^b	0±0 ^a	233±9.24 ^b	86.35±1.52 ^a
9	C	2.53±0.12 ^a	34.81±1.02 ^a	4.96±0.28 ^a	0±0 ^a	203±1.76 ^a	85.96±1.74 ^a
	DEN	7.92±0.33 ^c	53.28±2.34 ^c	24.67±1.06 ^c	0.21±0.01 ^a	270±2.60 ^c	86.57±0.42 ^a
12	C	2.50±0.10 ^a	33.97±0.95 ^a	4.75±0.36 ^a	0±0 ^a	201±1.28 ^a	84.56±1.26 ^a
	DEN	14.2±0.47 ^d	76.54±1.89 ^d	43.25±1.24 ^d	0.50±0.02 ^a	300±2.30 ^d	95.41±1.22 ^b
15	C	2.55±0.11 ^a	33.45±0.85 ^a	4.76±0.18 ^a	0±0 ^a	203±1.52 ^a	84.53±1.47 ^a
	DEN	45.4±0.87 ^e	101.32±1.68 ^e	63.80±1.51 ^e	8.46±0.53 ^a	351±10.6 ^e	107.17±1.15 ^c
18	C	2.55±0.12 ^a	33.16±0.82 ^a	4.60±0.25 ^a	0±0 ^a	202±1.81 ^a	85.87±2.54 ^a
	DEN	76.8±0.68 ^f	124.95±1.74 ^f	96.27±2.38 ^f	61.78±3.94 ^b	387±3.46 ^f	123.3±1.62 ^d
21	C	2.49±0.11 ^a	34.37±0.93 ^a	4.66±0.18 ^a	0±0 ^a	202±1.27 ^a	84.43±1.33 ^a
	DEN	109.±0.99 ^g	173.30±1.78 ^g	153.78±2.26 ^g	243.05±11.3 ^c	450±2.68 ^g	143.02±1.52 ^e
24	C	2.49±0.12 ^a	34.34±0.89 ^a	4.70±0.22 ^a	0±0 ^a	202±1.65 ^a	85.38±1.40 ^a
	DEN	130.±0.80 ^h	205.61±2.10 ^h	181.86±2.22 ^h	473.54±3.59 ^d	489±2.37 ^h	155.35±1.72 ^f
27	C	2.54±0.10 ^a	34.49±0.92 ^a	4.63±0.14 ^a	0±0 ^a	202±1.38 ^a	84.12±1.62 ^a
	DEN	149.±0.86 ⁱ	242.28±1.68 ⁱ	199.54±2.56 ⁱ	867.35±4.38 ^e	527±2.05 ⁱ	176.87±2.44 ^g

Results are expressed as mean±SE: standard error; Values not sharing common superscript are significant with each other at p<0.001; W: weeks; C: control; DEN: diethylnitrosamine; GGT: γ-glutamyl transferase; AFU: alpha-1-fucosidase; AFP: alpha-fetoprotein; GPC3: glypican-3; GP73: Golgi protein 73; VEGF: vascular endothelial growth factor.

Table (5): The serum lipid profile and lipid risk ratios in the DEN and control groups.

W	Groups	TG (mg/dl)	TC (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	TG/HDL-C risk ratio (mg/dl)	TC/HDL-C risk ratio (mg/dl)	LDL- C/HDL-C risk ratio (mg/dl)
6	C	81.53±1.44 ^a	98.21±1.23 ^a	65.91±1.59 ^a	15.99±1.70 ^a	1.24±0.04 ^a	1.49±0.03 ^a	0.24±0.03 ^a
	DEN	91.44±1.72 ^b	111.59±1.19 ^b	60.77±1.56 ^b	32.52±2.25 ^b	1.51±0.05 ^{ab}	1.84±0.05 ^{ab}	0.54±0.04 ^{ab}
9	C	80.91±1.54 ^a	97.20±1.46 ^a	64.89±1.65 ^{ab}	16.13±1.99 ^a	1.25±0.04 ^a	1.50±0.03 ^a	0.25±0.03 ^a
	DEN	102.02±1.64 ^c	124.82±1.36 ^c	53.21±1.58 ^c	51.19±2.05 ^c	1.93±0.08 ^b	2.36±0.08 ^b	0.97±0.06 ^b
12	C	81.69±1.52 ^a	98.46±1.58 ^a	64.37±1.52 ^{ab}	17.75±2.51 ^a	1.27±0.03 ^a	1.53±0.04 ^a	0.28±0.04 ^a
	DEN	113.99±1.44 ^d	137.06±1.14 ^d	45.91±1.52 ^d	68.34±2.30 ^d	2.50±0.07 ^c	3.01±0.11 ^c	1.51±0.10 ^c
15	C	81.14±1.50 ^a	97.83±1.43 ^a	64.03±1.50 ^{ab}	17.56±2.36 ^a	1.27±0.03 ^a	1.53±0.05 ^a	0.28±0.04 ^a
	DEN	125.98±1.46 ^e	150.61±1.54 ^e	39.56±1.54 ^e	85.85±2.11 ^e	3.22±0.12 ^d	3.85±0.15 ^d	2.21±0.13 ^d
18	C	80.05±1.50 ^a	97.59±1.52 ^a	64.07±1.63 ^{ab}	17.50±2.04 ^a	1.25±0.04 ^a	1.53±0.04 ^a	0.27±0.03 ^a
	DEN	131.08±1.65 ^f	165.29±1.85 ^f	33.08±1.53 ^f	105.98±2.81 ^f	4.02±0.16 ^e	5.10±0.26 ^e	3.29±0.22 ^e
21	C	80.43±1.48 ^a	97.09±1.78 ^a	65.16±1.66 ^a	15.83±2.57 ^a	1.23±0.02 ^a	1.49±0.04 ^a	0.25±0.04 ^a
	DEN	142.85±1.52 ^g	184.10±2.21 ^g	29.86±1.56 ^f	125.66±3.11 ^g	4.89±0.24 ^f	6.33±0.37 ^f	4.35±0.32 ^f
24	C	80.52±1.54 ^a	98.36±1.08 ^a	65.09±1.57 ^a	17.16±2.22 ^a	1.24±0.04 ^a	1.52±0.04 ^a	0.27±0.03 ^a
	DEN	161.60±1.71 ^h	198.51±1.57 ^h	23.94±1.60 ^g	142.25±2.68 ^h	7.00±0.43 ^g	8.63±0.56 ^g	6.22±0.48 ^g
27	C	80.97±1.52 ^a	97.01±1.78 ^a	64.26±1.55 ^{ab}	16.55±2.28 ^a	1.26±0.03 ^a	1.51±0.04 ^a	0.26±0.03 ^a
	DEN	179.81±1.93 ⁱ	212.76±1.81 ⁱ	18.02±0.54 ^h	158.76±2.05 ⁱ	10.0±0.26 ^h	11.9±0.40 ^h	8.89±0.36 ^h

Results are expressed as mean±SE: standard error; Values not sharing common superscript are significant with each other at $p<0.05$; W: weeks; C: control; DEN: diethylnitrosamine; TG: triglycerides; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

Table (6): The hematological values in the DEN and control groups.

W	Groups	RBC count ($\times 10^6/\text{mm}^3$)	Hb concentration (g/dl)	HCT (%)	Platelet count ($10^3/\text{mm}^3$)	WBC count ($10^3/\text{mm}^3$)	Differential leukocytic count		
							Lymphocytes (%)	Neutrophils (%)	Monocytes (%)
6	C	8.63±0.05 ^a	15.74±0.07 ^a	47.2±0.12 ^a	938±2.80 ^a	9.69±0.05 ^a	72.6±0.33 ^a	19.6±0.16 ^a	4.1±0.23 ^a
	DEN	7.86±0.03 ^b	14.33±0.06 ^b	43.10±0.21 ^b	889±2.04 ^b	8.91±0.03 ^b	72.8±0.24 ^a	19.9±0.23 ^a	4.2±0.2 ^a
9	C	8.58±0.07 ^a	15.67±0.10 ^a	47.22±0.24 ^a	935±2.76 ^a	9.69±0.04 ^a	72.4±0.22 ^a	19.5±0.16 ^a	4±0.25 ^a
	DEN	7.47±0.04 ^c	13.59±0.08 ^c	40.81±0.20 ^c	842±2.23 ^c	8.42±0.03 ^c	70.6±0.16 ^b	20.5±0.22 ^b	4.3±0.15 ^a
12	C	8.55±0.08 ^a	15.55±0.14 ^a	47.53±0.23 ^a	933±3.49 ^a	9.68±0.04 ^a	72.6±0.37 ^a	19.5±0.16 ^a	4.1±0.17 ^a
	DEN	7.12±0.03 ^d	12.94±0.07 ^d	39.01±0.15 ^d	730±2.23 ^d	7.70±0.02 ^d	68.3±0.21 ^c	21.5±0.16 ^c	5.2±0.35 ^b
15	C	8.64±0.04 ^a	15.72±0.08 ^a	47.13±0.20 ^a	935±2.88 ^a	9.74±0.04 ^a	72.6±0.33 ^a	19.4±0.16 ^a	4.2±0.24 ^a
	DEN	6.53±0.05 ^e	11.88±0.10 ^e	35.60±0.31 ^e	642±2.57 ^e	6.85±0.02 ^e	66.8±0.24 ^d	22.6±0.16 ^d	5.6±0.22 ^{bc}
18	C	8.66±0.06 ^a	15.73±0.10 ^a	47.07±0.28 ^a	934±2.77 ^a	9.75±0.03 ^a	72.4±0.33 ^a	19.6±0.16 ^a	4.1±0.17 ^a
	DEN	5.99±0.08 ^f	10.92±0.14 ^f	32.48±0.30 ^f	550±3.67 ^f	6.13±0.03 ^f	65.9±0.23 ^e	23.5±0.16 ^e	5.6±0.30 ^{bd}
21	C	8.50±0.07 ^a	15.47±0.12 ^a	47.11±0.16 ^a	933±3.17 ^a	9.70±0.03 ^a	72.5±0.26 ^a	19.4±0.16 ^a	4.2±0.2 ^a
	DEN	5.37±0.05 ^g	9.77±0.09 ^g	29.37±0.30 ^g	463±2.48 ^g	5.27±0.02 ^g	64.8±0.24 ^f	24.3±0.21 ^f	5.9±0.23 ^{cd}
24	C	8.52±0.05 ^a	15.49±0.10 ^a	46.93±0.12 ^a	934±3.42 ^a	9.75±0.04 ^a	72.2±0.35 ^a	19.5±0.16 ^a	4±0.25 ^a
	DEN	5.16±0.06 ^h	9.40±0.10 ^h	28.19±0.22 ^h	377±2.40 ^h	4.82±0.02 ^h	63.5±0.16 ^g	24.9±0.23 ^g	6.6±0.26 ^e
27	C	8.60±0.05 ^a	15.63±0.09 ^a	47.02±0.12 ^a	937±2.70 ^a	9.76±0.04 ^a	72.5±0.34 ^a	19.5±0.16 ^a	4±0.25 ^a
	DEN	4.72±0.04 ⁱ	8.57±0.06 ⁱ	25.56±0.25 ⁱ	289±2.25 ⁱ	4.50±0.06 ⁱ	62.9±0.23 ^g	24.9±0.23 ^g	7.2±0.32 ^e

Results are expressed as mean±SE: standard error; Values not sharing common superscript are significant with each other at p<0.05; W: weeks; C: control; DEN: diethylnitrosamine; RBC: red blood corpuscle; Hb: hemoglobin; Hct: hematocrit; WBC: white blood cell.

Table (7): Bashandy, Mansour & Fathy (BMF) staging system for prognosis and treatment allocation of hepatocellular carcinoma (HCC) in diethylnitrosamine induced male Wistar albino rat model.

Parameters	HCC stages				Reference (6-27 weeks)
	Early stage (6-12 weeks)	Intermediate stage (15-18 weeks)	Advanced stage (18-21 weeks)	Late stage (24-27 weeks)	
The body weight gain (%)	31.32%-47.60%	50.16%-33.99%	33.99%-31.38%	34.57%-31.00%	37.16%-71.31%
The relative liver weight (g)	2.39-3.99	4.57-8.57	8.57-11.73	12.07-12.91	2.49-2.80
Nodule incidence (%)	0%-70%	80%-100%	100%	100%	0%
Nodules <1 mm in diameter	0%-100%	66.28%-62.40%	62.40%-50.27%	45.88%-42.43%	0%
Nodules 1-3 mm in diameter	0%-21.21%	28.49%-31.85%	31.85%-33.73%	34.55%-35.61%	0%
Nodules >3 mm in diameter	0%	5.23%-5.74%	5.74%-16.00%	19.57%-21.96%	0%
The average no. of nodules	0-7.33	17.2-38.3	38.3-75	93.5-114	0
The mortality rate (%)	0%-6.67%	13.33%-20.00%	20.00%-33.33%	46.67%-60.00%	0%
GGT (U/L)	5.06-14.2	45.4-76.8	76.8-109.0	130.0-149.0	2.49-2.54
AFU (pg/ml)	42.90-76.54	101.3-124.9	124.9-173.3	205.6-242.3	33.16-34.81
AFP (ng/ml)	14.01-43.25	63.80-96.27	96.27-153.8	181.9-199.5	4.60-5.01
AFP-L3 (ng/ml)	0.57-12.80	21.37-33.69	33.69-70.44	91.49-119.5	0.05-0.07
GPC3 (ng/ml)	0-0.50	8.46-61.78	61.78-243.0	473.5-867.3	0
GP73 (ng/ml)	233-300	351-387	387-450	489-527	201-203
VEGF (ng/L)	86.35-95.41	107.2-123.3	123.3-143.0	155.3-176.9	84.12-85.96
TBARS (nmole/g tissue)	197-251	270-315	315-339	358-378	193-196
AOPPs (nmole/g tissue)	200-254	275-314	314-344	359-382	194-195
NO (μmol/g tissue)	37.21-32.36	25.99-20.48	20.48-16.95	14.04-11.09	40.96-41.91
SOD (U/mg tissue)	69.26-59.86	54.88-51.27	51.27-45.32	38.69-28.91	73.72-74.59
CAT (U/mg tissue)	1.32-0.97	0.87-0.76	0.76-0.66	0.57-0.50	1.54-1.55
GSH (nmol/g tissue)	2.33-2.03	1.94-1.84	1.84-1.76	1.61-1.51	2.94-2.96
AST (U/L)	80.5-134	182-241	241-298	353-375	70.1-71.8
ALT (U/L)	78.07-107.8	138.2-158.4	158.4-185.9	200.4-218.5	64.27-65.32
ALP (U/L)	136-176	216-257	257-304	353-379	113-115
TBIL (mg/dl)	0.40-1.03	1.24-1.45	1.45-1.73	1.91-2.21	0.35-0.37
Total protein (g/dl)	6.73-7.16	7.31-7.40	7.40-7.60	7.85-7.9	6.26-6.35
Albumin (g/dl)	3.84-3.42	3.23-3.00	3.00-2.78	2.58-2.33	4.01-4.09
Globulin (g/dl)	2.89-3.73	4.08-4.4	4.4-4.82	5.27-5.56	2.19-2.30
A/G ratio (g/dl)	1.34-0.91	0.79-0.68	0.68-0.57	0.48-0.42	1.77-1.88
TG (mg/dl)	91.44-113.99	125.98-131.08	131.08-142.85	161.60-179.81	80.05-81.69
TC (mg/dl)	111.59-137.06	150.61-165.29	165.29-184.10	198.51-212.76	97.01-98.46
HDL-C (mg/dl)	60.77-45.91	39.56-33.08	33.08-29.86	23.94-18.02	64.03-65.91
LDL-C (mg/dl)	32.52-68.34	85.85-105.98	105.98-125.66	142.25-158.76	15.83-17.75
TG/HDL-C, TC/HDL-C & LDL-C/HDL-C risk ratios	Risk	Risk	Risk	Risk	Not risk
RBC count (X10 ⁶ /mm ³)	7.86-7.12	6.53-5.99	5.99-5.37	5.16-4.72	8.50-8.66
Hb concentration (g/dl)	14.33-12.94	11.88-10.92	10.92-9.77	9.40-8.57	15.47-15.73
Hct percentage	43.10%-39.01%	35.60%-32.48%	32.48%-29.37%	28.19%-25.56%	46.93%-47.22%
Platelet count (10 ³ /mm ³)	889-730	642-550	550-463	377-289	933-938
WBC count (10 ³ /mm ³)	8.91-7.70	6.85-6.13	6.13-5.27	4.82-4.50	9.68-9.76
combined modality treatment	Chemoprevention or phytotherapeutic agent or hepatic resection, reversible, curative stage	Phytotherapeutic agent & radiofrequency ablation or trans-arterial therapies (chemoembolization)	Phytotherapeutic Agent & radiofrequency ablation and/or trans-arterial or targeted therapies	Symptomatic treatment, irreversible, incurative stage	

GGT: γ-glutamyl transferase; AFU: alpha-L-fucosidase; AFP: alpha-fetoprotein; GPC3: glypican-3; GP73: Golgi protein 73; VEGF: vascular endothelial growth factor; TBARS: thiobarbituric acid reactive substances; AOPPs: advanced oxidation protein products; SOD: superoxide dismutase; CAT: catalase; GSH: reduced glutathione; NO: nitric oxide; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; TBIL: serum total bilirubin; A/G: albumin-globulin ratio; TG: triglycerides; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; RBC: red blood corpuscle; Hb: hemoglobin; Hct: hematocrit; WBC: white blood cell.

Weeks	Groups				DEN (100 mg/L)	Sterile tap water	
6	C			6W			
	DEN	4W	2W				
9	C			9W			
	DEN	6W	3W				
12	C			12W			
	DEN	8W	4W				
15	C			15W			
	DEN	10W	5W				
18	C			18W			
	DEN	12W	6W				
21	C			21W			
	DEN	12W	9W				
24	C			24W			
	DEN	12W	12W				
27	C			27W			
	DEN	12W	15W				

The experimental schedules of diethylnitrosamine (DEN) administered and control (C) groups during 27 weeks.

76x26mm (600 x 600 DPI)

Draft

The chronological changes of body weight during 27 weeks in the control and diethylnitrosamine (DEN) administered groups.

111x57mm (300 x 300 DPI)

The macroscopic gross appearance of livers at the end of the experimental periods in the control and DEN administered groups (Arrows indicate nodules).

143x122mm (300 x 300 DPI)

percentage of AFP-L3 (Lens culinaris agglutinin-reactive fraction of AFP) to total AFP at the end of the experimental periods in the control and DEN administered groups.

89x37mm (300 x 300 DPI)

Western blot analysis of GPC3 (A) and GP73 (B) expressions in liver tissues at the end of experimental periods in the control and DEN administered groups.

116x62mm (300 x 300 DPI)