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Anti-tumor Effects of Bee Honey on PCNA and P53 Expression in the Rat Hepatocarcinogenesis

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ABSTRACT

In the present study, the bee honey was used as pharmaceutical agent on the carcinogenesis induced by Diethylnitrosamine (DEN) in liver of Sprague Dawley rats. Four groups of animals were used and fed *ad libitum*. The first group was a control; the honey group was fed *ad libitum* and given orally 2 g honey/rat/day. The 3rd group was intraperitoneally injected with a single dose of DEN 150 mg k⁻¹ b.wt. 4th group was intraperitoneally injected with DEN and after one week, the each rat was given 2 g honey until experiment termination. After six months all animals in different groups were sacrificed. The results of the present study observed that Honey treated rats showed normal liver histology, immunohistochemistry as seen in normal liver of control rats but DEN injected group produced a variety of lesions ranging from severe inflammatory reaction to liver carcinogenesis compared to the control groups. PCNA and P53 expression were significant nuclear positive staining in DEN group ($p < 0.05$). These results showed that supplementation of diet with honey has a protective effect against DEN-induced, inflammatory response and carcinogenesis in rat liver. So, the present study suggested that using honey is the useful therapeutic agents in hepatocarcinogenesis in rats.

Key words: Hepatocellular carcinoma, P53, PCNA, immunostaining, DEN

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently occurring forms of a solid tumor. It exhibits a high prevalence with 620,000 cases per year reported worldwide of which more than eighty percent of cases are reported from China, Africa and South East Asia (Bosch *et al.*, 2004). It is highly aggressive, as shown by the mortality of 595,000 cases per year that nearly matches the incidence of this tumor type. Hepatocellular carcinoma presents with limited therapeutic options. Hence, a thorough understanding of the biological bases of this malignancy might suggest new strategies for effective treatment (D'Alessandro *et al.*, 2007). Hepatocarcinogenesis induced by DEN is an ideal animal model to investigate liver tumor formation because it proceeds in stages similar to that of human liver cancer, i.e., formation of preneoplastic foci, neoplastic nodules and HCC nodules (Bruix *et al.*, 2004).

Much attention has recently been directed to the role of oxidative stress and apoptosis in the pathogenesis of HCC, being widely recognized as the central features of the disease (Hara *et al.*, 2006). Persistent oxidative stress in cancer explains part of the characteristic tumor biology of cancer such as activated transcription factors and proto-oncogenes, genomic instability, chemotherapy resistance, invasion and metastasis. Living organisms have developed complex

antioxidant systems to counteract Reactive Oxygen Species (ROS) and to reduce their damage. Thus, antioxidants protect against oxidative stress, prevent damage to cells and thus development of cancer (Pauwels *et al.*, 2007; Hassan *et al.*, 2010).

Honey is a product produced by bees (BH) from the nectar and other sugary substances derived from many plants. It has been traditionally used for centuries to promote health and fight disease, but the associated biochemical mechanisms for its possible protective and therapeutic effects are not yet clarified and remain an important challenge in research. It has been reported to contain about 181 active substances (Hassan *et al.*, 2010). Honey is a mixture of carbohydrates like fructose, glucose and sucrose and other carbohydrates. It also contains minerals and proteins, with a water content of about 17.2% (Doner, 1977; White, 1979). Honey contains flavonoids at low concentrations, acquired due to the contact with pollen which is rich in these secondary metabolites with high antioxidant activity (Almaraz-Abarca *et al.*, 2007), or propolis (El Denshary *et al.*, 2011). Recently, much attention has been directed to its anti-inflammatory, antioxidant, anti-proliferative, apoptotic and immunomodulatory properties. Recent studies by Gribel and Pashinskii (1990) indicated that honey possessed moderate antitumor and pronounced antimetastatic effects in 5 different strains of rat and mouse tumors. Furthermore, honey potentiated the antitumor activity of chemotherapeutic drugs, such as 5-fluorouracil and cyclophosphamide (Samarghandian *et al.*, 2011).

The aim of this study was to evaluate the protective effects of honey against induced hepatocellular carcinoma in rat.

MATERIALS AND METHODS

Experimental animals: Sixty healthy adult male of Sprague Dawley rats weighing about (90-100) at 50 days in age were used in this study. Rats were obtained from National Research center breeding farm, Cairo, Egypt. The animals maintained under standardized environmental conditions on 12 h light/dark cycle under a constant temperature of 25°+1°C with free access to rat chow and tap water. Rats were acclimated to laboratory conditions for one week prior to experiments.

Pure honey was available from the apiary of Faculty of Agriculture at Fayoum University, Egypt and Diethylnitrosamine (DEN) for hepatocarcinogenesis was the purest grades available Sigma (USA).

Animal grouping: Animals were randomly divided into four groups and treated as follows:

- **Untreated control group:** Animals of this group (15 rats) were fed on a standard diet and given tap water
- **Bee honey group:** Animals (15 rat) was given 2 g honey/rat/day orally till the time of sacrifice (Mabrouk *et al.*, 2002)
- **DEN group:** Animals (15 rat) were injected i.p. with 150 mg kg^{-1} b.wt. and after a week were given water contain 1% ethanol as the promoter
- **Bee honey treated group:** Animals (15 rat) were injected similarly with a single i.p. dose of DEN and after a weak was given 2 g of honey/rat/day orally and till the time of sacrifice (Mabrouk *et al.*, 2002)

Histological and immunohistochemical studies: At the end of six months, animals were fasted overnight, sacrificed in the morning and dissected to obtain the liver which fixed in 10% buffered formalin for histopathology. Sections 4 to 5 μm thick were prepared and put it on coated slides by microtome and stained with hematoxylin and eosin.

The selected paraffin blocks for immunohistochemical staining were sectioned at 4 μm and stained with two monoclonal antibodies (P53 and PCNA, all of them commercially purchased, LabVision, USA), using the avidin-biotin peroxidase method. Sections were dewaxed in xylene and rehydrated through graded alcohols. Antigen exposure was enhanced using a microwave technique. Sections were immersed in 0.01 M Citrate buffer (pH 6) and microwaved (Gibson's oven, USA) on full power until boiling, then allowed to cool for at least 10-20 min at room temperature. Endogenous peroxidase activity was then suppressed with 10 min. incubation in 3% hydrogen peroxide in phosphate buffer saline (PBS, 10 mM sodium phosphate, 140 mM Sodium chloride, pH 7.2). The slides were washed in three changes (2 min each) and started the immunostaining protocol.

Immunohistochemical staining was performed using streptavidin-biotin method by Histostain-plus kit (Zymed, USA) which contains 10% non-immune serum, biotinylated secondary antibody and streptavidin-peroxidase. Sections were washed several times with PBS and incubated with blocking serum for 10 min to block the unspecific binding which resulted from electrostatic or hydrophobic interaction between the antibody and tissue components. The excess of blocking serum was removed, then the sections incubated with primary antibodies (P53 and proliferating nuclear antigen, PCNA, in the proper dilution (1:50) for one hour at room temperature. After washing, the secondary antibody was used as a second layer for 10 min, after the sections were washed through three changes (5 min each) with PBS and streptavidin-peroxidase complex was applied for 10 min. Sections were washed in PBS and the peroxidase signal was developed in 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in Phosphate Buffer Saline (PBS). The sections were lightly counter stained in hematoxylin before dehydration and mounting with permount. Negative control slides were performed where substitution of the primary antibody by PBS was done on parallel slides. Also sections with normal positive reactivity of the antibodies (taken from the pamphlet) were used in the same run. Brown nuclear stain was seen in positive cases of P53 and PCNA expression.

Scoring for P53 and PCNA expressions: Each section was counted manually at high power (X400) after identifying at low power (X100) the representative areas with the highest concentration of stained cells according to the recommendation of Cohen *et al.* (1993). About 1000 cells/slide were counted in each of five microscopic fields from well-labeled areas to determine the average of P53 or PCNA labeling index (P53 and PCNA LI). P53 or PCNA LI was expressed as the number of labeled cells (positive staining) as a percentage of the total number of cells counted in each specimen. All identifiable staining was regarded as positive. The results are expressed as mean plus or minus standard deviation (P53 or PCNA LI = Mean+SD).

Statistical analysis: Statistical analysis for obtained results was carried out with the aid of the SPSS11 computer software program.

RESULTS

Changes in body weight gain: As shown in Fig. 1, rats fed on honey and showed a non-significant increase in the body weight gain respect to control rats. However, administration of
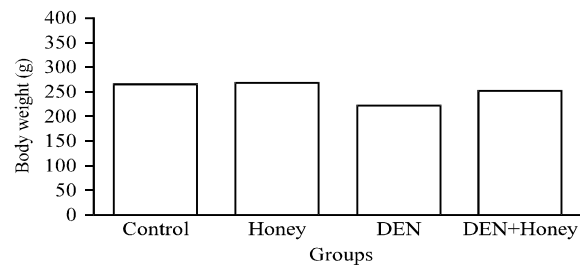
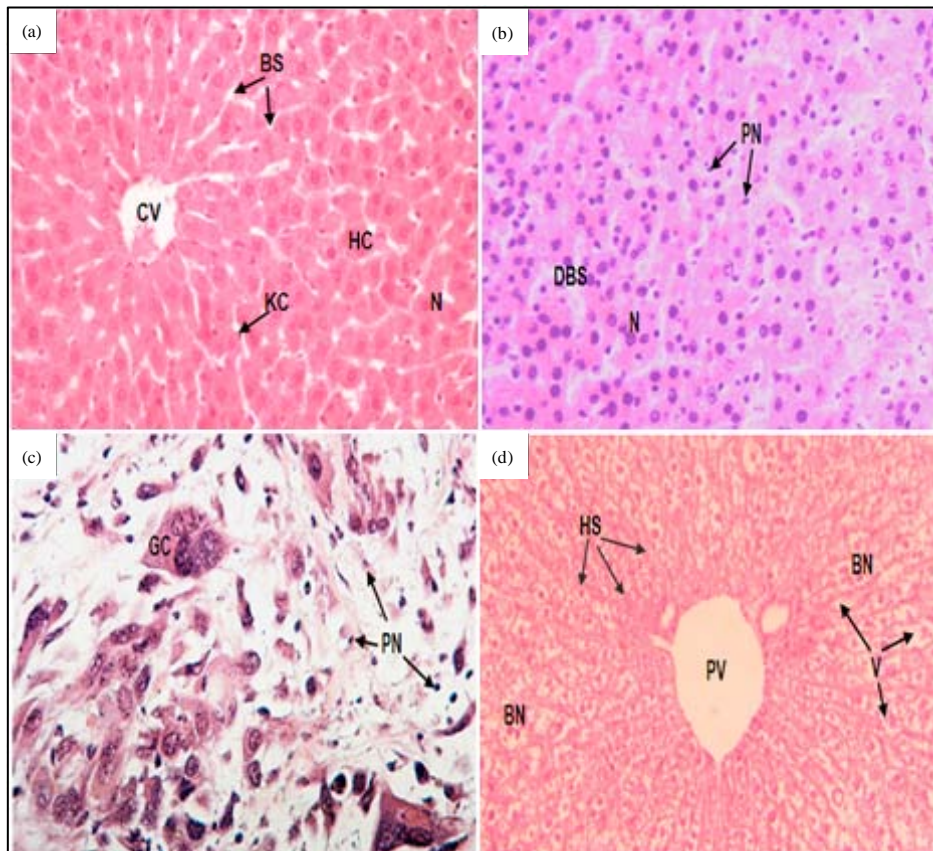Fig. 1: Body weight changes in different experimental animal groups

Fig. 2(a-d): A photomicrograph of liver sections obtained from, (a) Control rats, (b) and (c) Rat injected with DEN 150 mg kg^{-1} b.wt., (d) DEN injected rats treated with honey, hepatic cells (HC), centrally located nucleus (N), the blood sinusoids (BS), the Kupffer cells (KC) are shown with the central vein (CV), infiltration lymphocytes (IL), binucleated cells (BN), some vacuoles (V), undifferentiated anaplastic giant cells (GC), the hepatic strands (HS), portal vein (PV), H and E X 250

Diethylnitrosamine (DEN) precursors illustrated a significant decrease in the body weight gain with respect to control rats. Moreover, rats injected with DEN and treated with honey displayed a significant ($p<0.05$) increase in the body weight gain when compared with control animals.

Histological studies: The present results demonstrated the normal polyhedral hepatocytes with a noticeable granular cytoplasm in the control rats group (Fig. 2a). Also, Honey fed rats showed normal liver histology as previously seen in normal liver of control rats, so, the control groups indicate to these groups. Liver of rats injected with DEN (150 mg kg^{-1} b.wt. i.p.) showed obvious fatty degeneration with displacement of the nucleus. Hydropic degeneration (oedema) was also seen in severe injured hepatocytes. The nuclei of the hepatocytes were apparently hyperchromatic and displayed some features of pyknosis. Inflammatory lymphocytic infiltration was clearly visible in these rats. The neoplastic hepatic cells were polyhedral to round with dense, centrally located vesicular nuclei (Fig. 2b, c). Bee honey treated group; liver section showed nearly normal
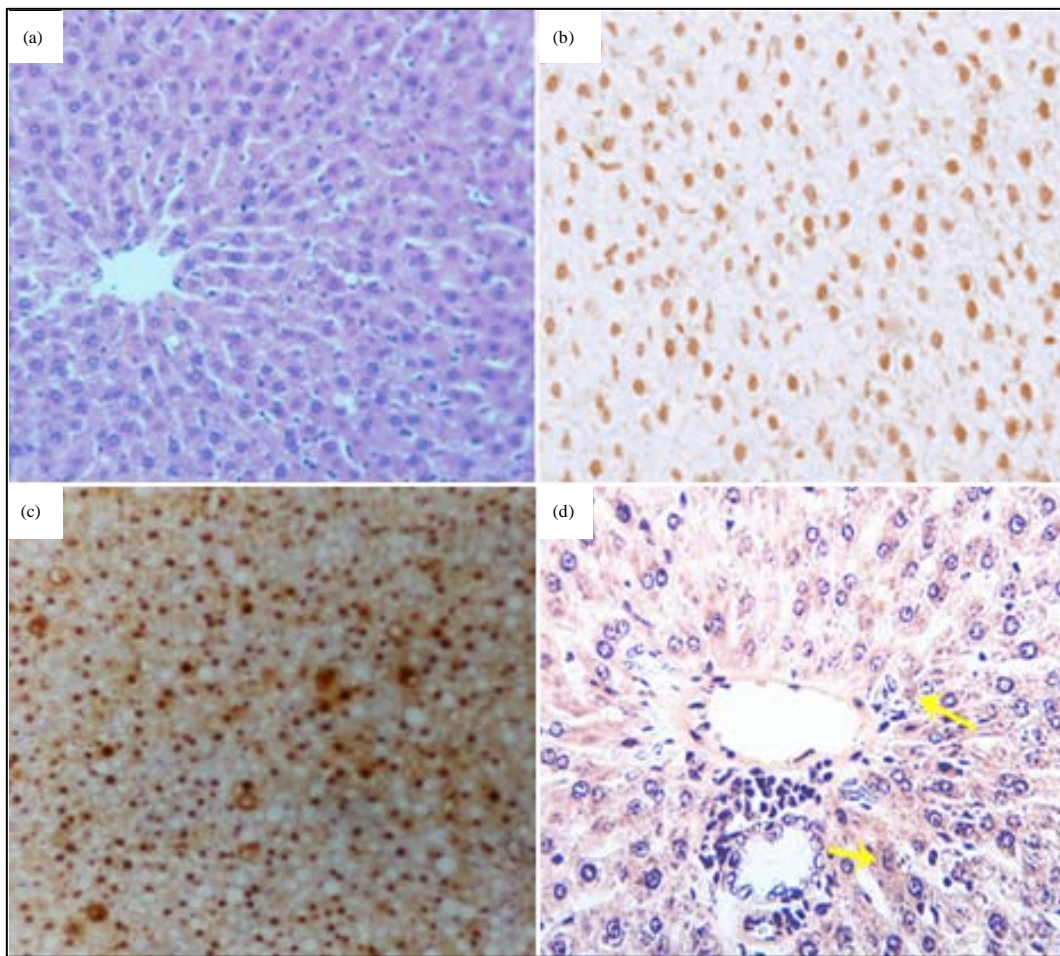

Fig. 3(a-d): (a) The section in normal liver of control rats immunostained with P53 antibody showing negative stained nuclei (less than 5% stained nuclei, brown color staining), (b and c) the section in liver of injected rats with DEN immunostained with P53 antibody showing strong positive stained nuclei and (d) A section in liver of injected rats with DEN and treated with bee honey immunostained with P53 antibody showing decrease the positive stained nuclei (arrows) and some lymphocyte's infiltration. Immunoperoxidase X 400

architectures tissue with normal pattern and normal hepatocytes, so honey act as the protective agent against DEN (Fig. 2d).

Immunohistochemical studies

P53 immunohistochemical expression: The hepatocytes of control (control and honey groups) rat's immunostained for p53 expression showed very weak positive (less than 5% stained nuclei, Fig. 3). In contrast, a large number of strong positive stained nuclei were observed in liver sections of rats given DEN as the liver carcinogen. In addition, the liver sections of rats injected with DEN and treated with honey showed the positive stain in some hepatocyte nuclei but less than that of the DEN carcinogen treated animals (Fig. 3).

Table 1: Liver P53 labeling index as the results of carcinogenicity of DEN and honey treatments

Groups	Mean±SD	p-value
Control groups	4.800±3.835	---
Honey group	4.478±2.899	---
DEN group	75.500±14.144	0.0001
DEN+honey group	15.200±4.177	0.045

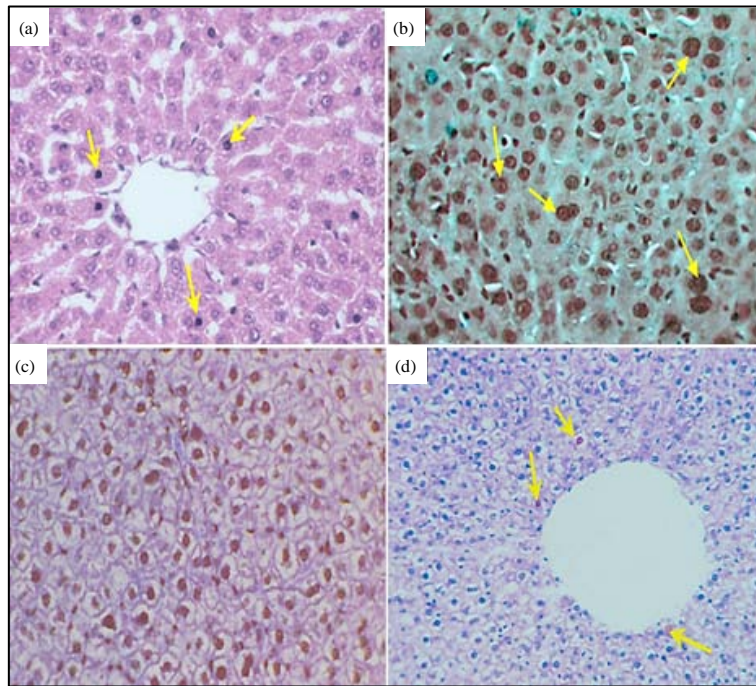Value are insignificant at p>0.05, Significant at p<0.05

Fig. 4(a-d): (a) The section in normal liver of control rats immunostained with PCNA antibody showing negative stained nuclei (less than 18% stained nuclei, arrows), (b) and (c) The section in liver of injected rats with DEN immunostained with PCNA antibody showing strong positive stained nuclei and (d) A section in liver of injected rats with DEN and treated with bee honey immunostained with PCNA antibody showing decrease the positive stained nuclei (arrows) and dilated central vein. Immunoperoxidase X 400

Table 2: Liver PCNA labeling index as the results of carcinogenicity of DEN and honey

Groups	Mean±SD	p-value
Control group	17.100±3.478	---
Honey group	17.213±2.998	---
DEN group	78.000±13.391	0.0001
DEN+honey group	19.000±3.197	>0.050

Value are insignificant at p>0.05, Significant at p<0.05

As shown in Table 1 rats fed on honey containing diet showed a non-significant increase, respectively in liver P53 expression when compared with control rats. The carcinogenicity of DEN on these animals without treatment caused a very high significant increase in P53 expression with respect to control animals.

PCNA immunohistochemical expression: As shown in Table 2 and Fig. 4a, the hepatocytes of control rats (control and honey groups) immunostained for PCNA expression showed weak positive (less than 18%) stained nuclei. On the other hand, a large number of strong positive stained nuclei were observed in liver sections of rats given DEN as the liver carcinogen (Fig. 4b, c). In addition, the liver sections of rats injected with DEN and treated with honey showed the positive stain in some hepatocyte nuclei but less than that of the DEN carcinogen treated animals (Fig. 4d).

DISCUSSION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer mortality worldwide (Witjes *et al.*, 2009).The enormous burden of hepatic cancer on society becomes clear by considering the fact that approximately 625,000 new cases of this cancer are diagnosed globally each year. Distressingly, the number of deaths is approximately 598,000 per year. Survival rates for liver cancer are only 3-5% globally. In the United States, 19,160 new cases of liver cancer and 16,780 deaths were reported for 2007 (Chen *et al.*, 2011).

Diethylnitrosamine (DEN) is widely used chemical carcinogen in models of carcinogenesis of liver and oesophagus (Macejova and Brtko, 2001). The liver is the first organ to receive blood from the intestinal tract. A primary function of the liver is the biotransformation, detoxification and excretion of xenobiotics, including carcinogens. The human liver is continually exposed to small doses of alkylnitrosamines, such as Dimethylnitrosamine (DMN). These compounds are present in ordinary foodstuffs (probably to a far greater extent) result from nitrosation of amines in the gut. At the same time, many people are exposed to powerful enzyme-inducing drugs, such as phenobarbitone and ethanol (Driver and McLean, 1986).

In the present study, the increase of the mean body weight of DEN treated rats during the experiment and when compared with control and treated rats with bee honey, where the animals marked with loss of appetite and loss of hair in some area of the body (Ha *et al.*, 2001). They stated that some animals receiving DEN lost considerable weight compared to the increase in the average weight of control rats. In the present study, the rats treated with bee honey showed non significant increase in the body gain, so they had the similar final body weight to control rats. DEN-treated rats showed a significant reduction (p = 0.02) with respect to control rats. However, DEN-treated group and fed with Honey displayed mild significant change in the body weight gain as compared to other groups (p-values = 0.045). Bee honey against DEN injected rats overcame the loss in the body weight.

The present study indicated that the Bee honey group showed the liver sections nearly with normal architectures, normal hepatocytes. However, the liver of rats injected with DEN (150 mg kg^{-1} b.wt., i.p.). The microscopic examination showed many of the lesions which manifested the characteristic of malignancy, sever damaged in hepatocytes with manifestations of extensive cytoplasmic vacuolization, hydropic degeneration (oedema). Similar findings have been reported by Bhathal and Hurley (1973) who carried out a comparative study of the carcinogenic activity of nitrosamines and found that dimethylnitrosamine produced tumors with a variety of histological characteristics in the liver, lung and kidney. Also, Rapp (1965) stated that hepatic carcinomas developed in 13 of 13 rabbits receiving diethylnitrosamine in their drinking water for the period ranging from 52 to 82 weeks. A sarcomas tumor of liver in rat and rabbit fed diethylnitrosamine was described by Rapp (1965).

The use of natural products as an alternative to conventional treatment in healing and treatment of various diseases has been on the rise in the last few decades. Honey is one of the oldest known medicines. Recent studies by Gribel and Pashinskii (1990) indicated that honey possessed moderate anti-tumor and pronounced anti-metastatic effects in five different strains of rat and mouse tumors. Furthermore, honey potentiated the anti-tumor activity of chemotherapeutic drugs such as 5-fluorouracil and cyclophosphamide (Wattenberg, 1986).

Nowadays, immunohistochemistry is a commonly used immunologic method. Immunohistochemical demonstration of the p53 tumor protein may be useful in predicting prognosis of several types of tumors. In the present study, liver cells of both control and honey groups did weak express p53 protein, p value non significant. The wild-type p53 protein cannot be detected by conventional immunohistochemical technique due to its shorter half life and hence does not accumulate in tumor cells (Wang *et al.*, 2003). However, liver sections of rats treated with DEN precursors showed the significant increase in p53 protein (p<0.0001). Expression levels of p53 were highly elevated in the liver of DEN-treated rats, Category (+++) was homogenous (more than 50% of the tumor nuclei are positive), however, liver sections of rats treated with honey showed Category (+) was heterogeneous (p = 0.045), so, p53 protein reduced by honey and this might be attributed to the anti-mutagenic effect of honey, which minimized DNA damage caused by DEN precursors. Another plausible explanation is that honey might have prevented high levels of mutant- p53 production in response to honey (Khan *et al.*, 2011).

This monoclonal anti-proliferating cell nuclear antigen is a useful tool for studying the proliferating cells in normal tissues and possible apparent expression in neoplasm indicating division and proliferation of the cells. Previous studies have demonstrated that PCNA labeling index increased sequentially from normal tissue through the premalignant stage to carcinoma of various tumors (Roy *et al.*, 2007). The hepatocytes of control and honey groups immunostained for PCNA expression showed very weak positive (less than 18% stained nuclei. In contrast, a large number of strong positive stained nuclei were observed in liver sections of rats given DEN as the liver carcinogen (p<0.0001 for DEN group). In addition, the liver sections of rats injected with DEN and treated with honey showed the positive stain in some hepatocytes nuclei but less than that of the DEN carcinogen treated animals (p<0.05).

In conclusion, in the present study show that DEN-induced severe liver injury and carcinogenesis in rat liver were prevented by bee honey suggesting that honey is a protective antioxidant against liver toxicity and an anti-tumor agent. Further pre-clinical and clinical trials are warranted to characterize the efficiency of bee honey with existing therapeutics for chemoprevention and chemotherapy of Hepatocellular carcinoma.

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