Bee Honey Modulates the Oxidant-Antioxidant Imbalance in Diethyl nitrosamine-Initiated Rat Hepatocellular Carcinoma

Naima Zayed Mohamed¹*, Hanan Farouk Aly¹, Hatem Abdel moneim El-Mezayen², Hadeer E. El-Salamony¹

¹Therapeutic Chemistry Department, Pharmaceutical and Drug Industries Division, National Research Centre (NRC), Giza, Egypt. ²Biochemistry Department, Faculty of Science, Helwan University, Cairo, Egypt.

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ABSTRACT

Oxidative stress reflects the mechanism that contributes to initiation and progression of hepatic injury in a variety of liver disturbance. From here, there is a great demand for the expansion of agents with a potent antioxidant effect. The aim of this work is to approximate the efficiency of bee honey as a hepatoprotective and antioxidant agent versus diethyl nitrosamine (DEN) motivate hepatocellular damage. The single intrapritoneal (IP) management of diethyl nitrosamine (50mg/kg followed by 2ml/kg CCl₄) to rats, referred for the histopathological examination of liver sections of rats after induction and before treatment with honey showed that many well differentiated tumor cells were formed in the liver of rats also, the examined sections showed disorganization of hepatic lobular architecture and obvious cellular damage. A significant lift in the enzymatic activity of liver functions (AST, ALT, ALP), and gamma glutamyltransferase (GGT) which is a signal of hepatocellular damage. DEN stimulates oxidative stress, which was assured by increase lipid peroxidation level and hindrance in antioxidant enzymes (SOD, CAT, GPx, and GST) activities in the liver. The position of non-enzymatic antioxidants comparable reduced glutathione (GSH) was likewise set up to be slimmed down significantly in DEN inoculated rats. Also, we have studied the underlying mechanism and/or(s) of the therapeutic role of bee honey as hepatocarcinogenesis remediation through investigation the inflammatory biomarkers; α-fetoprotein (AFP) and α-fucosidase (AFU). The current results clearly showed that bee honey demonstrates good ameliorative and antioxidant capacity toward diethyl nitrosamine induced hepatocellular damage in rats.

INTRODUCTION

Hepatocellular carcinoma (HCC, also called malignant hepatoma) is one of the most prevalent cancers and deadly diseases in the world (Salim et al., 2009). The present manipulation assays, including partial hepatectomy and liver transplantation, have been set up to be rendered inefficient (Bishayee et al., 2010). So, in that location is an evident another strategy the chemoprevention dealings with HCC.

Oxidant-antioxidant imbalance as well as immune response has been incriminated in the hepatic neoplasia development (Bishayee et al., 2010). Natural phytochemicals and compounds appear powerful antioxidant and anti-inflammatory characters which consider a fresh approach to deny and dominate HCC (Bishayee et al., 2010). Hepatocellular carcinoma submits with bounded curative options. Hence, an inclusive comprehension of the biological bases of this malignancy suggests itself to novel method for therapeutic approach of patient (D’Alessandro et al., 2007). DEN-induced hepatocarcinogenesis in rats is considered a suitable model to examine the process of carcinogenesis due to it exhibits steps match to what happen in human liver cancer (Bruix et al., 2004).
Oxidative stress and apoptosis playing the principle role in the HCC pathogenesis and considered as the primary factor responsible for the disease (Hara et al., 2006). Oxidative stress is a leader cause in cancer and declared the transcription factors activation and proto-oncogenes, genomic instability, chemotherapy resistance, invasion and metastasis. Completed antioxidant system is required to neutralize and minimize Reactive Oxygen Species (ROS) damage (Pauwels et al., 2007; Hassan et al., 2010).

Evidence suggested that honey can produce many powerful health effects as gastric protective Gharzouli et al., 2002; Jones, 2009), hepato-protective (Al-Waili et al., 2006) antioxidant (Erejuwa et al., 2010), antihypertensive (Al-Waili, 2003) and anti-inflammatory (Kassim et al., 2010). Several enzymes were detected in honey content such as glucose oxidase, diastase, invertase, catalase and peroxidase (Bogdanov et al., 2008). In addition to, other active ingredients, organic acids, ascorbic acid, trace elements, vitamins, amino acids, and proteins (Bogdanov et al., 2008). This study was therefore planned to examine the ameliorative efficacy of antioxidant-rich bee honey following intraperitoneal injection of diethyl nitrosamine in rats.

MATERIALS AND METHODS

Chemicals

All chemicals in the present study were of analytical grade, product of Sigma (US), Merck (Germany) and BDH (England).

Pure honey was available from the apiary of Faculty of Agriculture Cairo University, Egypt and diethyl nitrosamine (DEN) for hepatocarcinogenesis was the purest grades available Sigma Chemical Company (USA).

Experimental animals

Sixty healthy adult male Wister albino rats weighting about (150±30 gm) were used in this study. Rats were obtained from National Research Centre breeding farm, Cairo, Egypt. All animals maintained under standardized environmental conditions on a 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 two weeks prior to the experiment. Anesthetic procedures and handling with animals complied with the ethical guidelines of the Medical Ethical Committee of National Research Centre in Egypt (Approval no: 13/90).

Experimental design

Sixty rats were randomly divided into four groups, each of fifteen rats as follow:

Control group

Normal animals were fed on a standard diet and given tap water.

Bee honey group

Normal animals were orally administered two gram honey/rat/day for six months (El-Kott, 2012).

DEN group

Animals were intraperitoneally (IP) injected single dose 50 mg kg⁻¹ b. Wt. of DEN followed (2 weeks later) with a single dose of CCl₄ 2 ml/kg (i.p.) as 1:1 dilution in corn oil (Cayama et al., 1978; Salim et al., 2009), and sacrificed after six months.

Bee honey treated group

Animals (15 rats) were injected similarly with a single IP dose of DEN and CCl₄ and after a week were given 2 g of honey/rat/day orally till the time of sacrifice (Cayama et al., 1978) (six months).

Liver specimen’s collection

At the end of six months, rats were fasted overnight. In the morning each rat was weighed and then the anesthesia is done by diethyl ether. The abdominal cavity was opened up through a midline abdominal incision to expose the liver. Then the liver was excised and trimmed of all fat. The liver of each animal was weighted and evaluated.

Histopathological examination

A portion of the median lobe of the liver was dissected and fixed at 10% formalin-saline for histological examination. Sections 4 to 5 µm thick were prepared and put it on coated slides by microtome and stained with Hematoxylin and Eosin (Suzuki and Suzuki, 1998).

Biochemical analysis

The remaining parts of the liver were frozen quickly in dry ice and stored at -4°C for biochemical analysis.

Serum sample

Blood collected from each animal by puncture the sublingual vein in clean and dry test tube, left 10 min to clot and centrifuged at 3000 rpm (4°C) for serum separation. The separated serum was stored at -80°C for further determinations of liver function enzymes, cholestatic biomarkers and serum protein. Liver tissue was homogenized in normal physiological saline solution (0.9% NaCl) (1:9 w/v). The homogenate was centrifuged at 4°C for 5 min at 3000 rpm. The supernatant was used for estimation of liver marker enzymes and the antioxidant parameters.

Assay of liver enzymatic antioxidants

Catalase (CAT) activity

Catalase activity was measured according to the method of Aebi (1983). The 0.1 ml of the liver homogenate (supernatant) was a pipette into the cuvette containing 1.9 ml of 50 mM phosphate buffer, pH 7.0. The reaction was started by the addition of 1.0 ml of freshly prepared 30% (v/v) hydrogen peroxide (H₂O₂). The rate of decomposition of H₂O₂ was measured...
spectrophotometrical method from the changes in absorbance at 240nm. Activity of the enzyme was expressed as unit’s mg⁻¹ protein.

**Superoxide dismutase (SOD) activity**

Superoxide dismutase activity was measured according to the method of Nishikimi et al. (1972). The principle of the assay was based on the ability of SOD to inhibit the reduction of Nitro-blue tetrazolium (NBT). Briefly, the reaction mixture contained 1.8 ml of 0.1M sodium pyrophosphate buffer, pH 8.3, 0.5 ml of 0.3 mM of Nitro-blue tetrazolium solution, 0.5 ml of 0.47mM of nicotinamide adenine dinucleotide, reduced form (NADH) and 20 μL liver homogenate of 20% concentration. The absorbance was measured at 560nm. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction of NBT by 50 % under the specific condition. It was expressed as μM min⁻¹ mg⁻¹ protein.

**Glutathione peroxidase (GPx) activity**

Glutathione peroxidase (GPx) activity was assayed according to Paglia and Valentine (1967). To assay GPx a tissue homogenate is added to a solution containing glutathione, glutathione reductase and NADPH. The enzyme reaction is initiated by adding the substrate, hydrogen peroxide and the absorbance at 340 nm is recorded. The rate of decrease at 340 nm is directly proportional to the GPx activity in the sample.

**Glutathione-s-transferase (GST) activity**

Glutathione-S-transferase (GST) activity was determined according to the procedure of Habig et al. (1974). The formation of conjugate or adduct between GSH and CDNB was spectrophotometrically measured at 340nm. A reaction solution of 100mM or 1ml of phosphate buffer at pH 7.4 contained 1mM or 0.1ml of 1-chloro-2,4-dinitrobenzen, 1mM or 0.1ml of GSH, 1.7ml of distilled water and 0.05 ml of supernatant. Blank contained no CDNB. Absorbance was read for 5minutes at 1 min. intervals. The GST activity was expressed as μMol of CDNB conjugated/min/mg protein.

**Assay of liver non-enzymatic antioxidants**

**Reduced glutathione (GSH) concentration**

Reduced glutathione (GSH) concentration was determined using Beutler et al. (1963). The method based on the reduction of 5, 5’ dithiobis (2-nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound which is directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

**Lipid peroxidation analysis.**

Lipid peroxidation measured by the thiobarbituric reactive species (TBARs) assay, which measures the production of malondialdehyde (MDA) that reacts with thiobarbituric acid (Ohkawa et al., 1979). Absorption was measured at 535 nm in a spectrophotometer and a molar extinction coefficient of 1.56x10⁵ M⁻¹ cm⁻¹ was used to determine the concentration of TBARs. 1 mM EDTA was added to 0.5ml of the supernatant and was mixed with 1.0 ml cold 10% (M/V) trichloroacetic acid (TCA) to precipitate the protein. The solution was mixed and centrifuged for 10mins at 5,000xg.

The supernatants from the TCA extract were combined with the same volume of TBA and heated in boiling water for 15mins. Control sample contained water instead of supernatant.

**Estimation of liver function enzymes**

Serum ALT, AST (Rietman and Frankle, 1957), and ALP (Gond and Khadabadi, 2008; Belfield and Goldberg, 1971) activities were investigated as biochemical markers for the early hepatic damage using commercial Kits (Biodiagnostic, ARE). G-glutamayltransferase (GGT) is usually most significantly elevated by obstructive disease and has good specificity for the liver was estimated by spectrum kit (Saw et al., 1983).

**Inflammatory biomarkers**

**Serum Alpha-Fetoprotein (AFP)**

Serum α-fetoprotein (AFP) was determined according to the method of (Abelov, 1974; Chan and Miao, 1986; Uotila et al., 1981) using ELISA Biotech kits (USA) following the instructions of the manufacturer.

**α-L-Fucosidase (AFU)**

The AFU assay is based on the enzymatic cleavage of the synthetic substrate p-Nitro phenyl α-L- fucopyranoside to α-L- fucoside and 4- nitro phenol. The yellow color of p- nitro phenol in an alkaline medium can be measured quantitatively at 405 nm (El-Houseini et al., 2005).

**Statistical analysis**

Differences between obtained values (mean ± SD, n = 15) were carried out using SPSS (version 7) computer program, one way analysis of variance (ANOVA) followed by the Co-state computer program, where unshared letter is significant at P value ≤0.05.

**RESULTS**

**Body weight changes**

Rats fed on honey demonstrated an unsignificant gain in the body weight regarding to normal control rats. Nevertheless, the administration of diethyl nitrosamine (DEN) precursors illustrated a significant reduction in the elevated body weight comparing to control rats. Moreover, rats injected with DEN and treated with honey, displayed a significant (p<0.05) increase in the body weight comparing to normal control rats (Fig.1).
Liver histopathology

Histopathological examination of the normal control liver revealed normal liver architecture of the hepatic lobule (Fig. 2A). While Fig. 2B, declared slight congestion of hepatic sinusoids in normal rats administered honey. In addition, Fig. 2 (C&D) clearly indicated karyomegaly of hepatocytic nuclei, fine strands of collagen fiber deposition and proliferation of oval cells in rats’ I.P. injected with DEN. However, hepatocarcinogenic rats treated with honey, showed improvement in the histopathological picture demonstrated as necrosis of sporadic hepatocytes and proliferation of oval cells (Fig. 2E&F).

Liver oxidative stress

Activities of liver enzymes-superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-s-transferase (GST)

Treatment with honey alone caused an insignificant change in liver SOD activity, whereas, DEN-injected rats showed a statistically significant reduction in SOD activity as compared to control animal. Treatment of carcinogenic rats with honey significantly increased the liver SOD activity as compared to carcinogenic rats (Fig. 3A). Normal rats administered honey declared insignificant change in catalase enzyme activity as compared to normal control untreated one (Fig. 3B). However, DEN-injected rats resulted in a statistically significant inhibition in liver CAT activity as compared to control rats. Treatment of carcinogenic rats with honey significantly enhanced CAT activity, where it declared insignificant change as compared to normal control value. Moreover, GPx showed insignificant changes in normal rats post honey administration as compared to control untreated rats. Diethyl nitrosamine injected rats, however, markedly decreased the enzyme activity as compared to control values. Treatment of DEN –injected rats with honey significantly improved GPx activity in liver tissue as compared to normal control and carcinogenic untreated rats (Fig. 3C). Also, GST activity demonstrated an insignificant change in normal control rats administered honey as compared to untreated control one. Whereas, DEN- injected rats showed statistically significant decrease in GST activity as compared to control rats. Treatment of carcinogenic rats with honey significantly improved liver GST activity as compared to normal control and carcinogenic rats (Fig. 3D).
Liver content of glutathione (GSH) and malondialdehyde (MDA)

Treatment of normal control rats with honey markedly declared an unsignificant change in GSH level as compared to control group. A remarkable significant reduction in GSH content was detected in hepatocarcinogenic rats comparing to control one. Treatment of carcinogenic rats with honey significantly enhanced GSH content respecting to diseased untreated rats (Fig.4A).

As shown in Fig.4B, honey had no effect on the liver content of lipid peroxides (MDA) as compared to normal untreated control rats. DEN however, significantly elevated the liver MDA by about two folds as compared to the control value. Treatment of carcinogenic rats with honey exhibited a notable decrease in MAD level comparing to carcinogenic and normal control rats.

The activities of serum hepatic biomarker enzymes

As shown in Fig.5, DEN-injected rats significantly elevated serum enzyme activities, AST, ALT, ALP and GGT levels in rats, which indicated severe liver damage. Treatment of carcinogenic rats with honey improved liver function enzymes, where a significant reduction of these liver biomarkers near to control values was observed.

Serum Alpha-Fetoprotein (AFP) and α-L-Fucosidase (AFU) Concentration

Fig.6 demonstrated significant increase in serum AFP and AFU levels of DEN-intoxicated rats regarding to normal one (P≤0.05). Amelioration signs were noticed post treatment of intoxicated rats with honey, as they exhibited significant reduction in serum AFP and AFU comparing to carcinogenic rats.
DISCUSSION

The limited progress achieved by cancer therapy in the last three decades has increased the interest of researchers in cancer chemoprevention (Bruix and Llovet, 2002; Befeler and Di Bisceglie, 2002; Llovet, 2003; Jennifer et al., 2008; López-Lázaro, 2008; Gupta et al., 2011), especially using nutraceuticals derived from nutritional sources which are naturally multi-targeting, less expensive, safer and immediately available (Gupta et al., 2011).

DEN plays a significant effect in DEN-initiated hepatocarcinogenesis. The high production of ROS and minimal antioxidant enzymes in organs has been associated with DEN in most experiments (Kweon et al., 2003; Ramakrishnan et al., 2006; Yadav and Bhatnagar, 2007; Sivaramakrishnan et al., 2008). Honey is characterized by its highly selectivity as it appears cytotoxicity against cancer cells while it is non-cytotoxic to normal one (Omotayo et al., 2014). It able to suppress the initiation and progress the cancerogenesis process at the level of molecular mechanisms (Omotayo et al., 2014). This work concentrates on the use of honey in amelioration liver carcinogenesis growth and development. Investigation of liver at the cellular level declared HCC in DEN-induced rats. This finding was documented by many authors (Bendong et al., 2012; Omotayo, 2014; Zhao et al., 2014). The hepatocellular examination revealed the therapeutic role of bee honey in suppressing hepatocarcinogenesis in a dose dependent relationship (Salim et al., 2009; Zhao et al., 2014). It is recognized that the AST, ALT, ALP, GGT serum levels are suggestive of hepatic biomarkers, and their elevation is related to hepatic damage (Yao et al., 2004; Sreepriya and Bali, 2006; Al-Rejaie et al., 2009; Zhao et al., 2014). Hepatic injury induced by DEN is linked to the membrane of hepatocytes pores damage resulting in leakage of these enzymes into circulation. While, ALP is related to the membrane lipid in canalicular ducts, where its elevation attributed to injury in the biliary function (Zhao et al., 2014). Also, the present results demonstrated an increase in GGT activity in carcinogenic rats which may be explained on the basis of its diffusion from the cell membrane into the circulation suggesting damage in cellular membrane integrity by DEN (Al-Rejaie et al., 2009). The inhibition in ALT, AST, ALP and GGT
enzyme activities in carcinogenic rats administered bee honey may be assigned to the improvement in cellular membrane architecture (Al-Rejaie et al., 2009; Mohamed et al., 2010). Previous works ascertained that, DEN produces failure in the complex antioxidant defense systems due to it induces overproduction of reactive oxygen species as well as membrane lipid peroxidation that in turn leads to bio-membranes damage (Kausshik and Kaur, 2003; Suboudhi and Chainty, 2010; Ismail et al., 2011). In the present study, DEN-injected rats experienced a substantial reduction in the SOD, CAT, GPx, GST activities and GSH level, while a significant gain in MDA level was detected (Zhao et al., 2014). In a parallel results, many researches declared that, the therapeutic efficacy of bee honey is attributed to its ability to neutralize and scavenge free radicals due to its powerful antioxidant character as well as its ability to enhance glutathione content (Crane, 1975; Mobarok Ali and al-Swayeh, 1997; Chen et al., 2000; Hassan et al., 2012). Regarding to alpha fetoprotein (AFP) is the best diagnostic biomarker for HCC because its serum level is elevated paralleling with tumor size (Wen-Jun et al., 2013). In this concern, Soresi et al. (2003) detected elevated AFP level in DEN -injected rats. The current research indicated that, bee honey has promising therapeutic effect on AFP level in carcinogenic rats. On the other hand, Deugnieret et al. (1984) demonstrated α-fucosidase (AFU) high level in patients with HCC, while its values were not related to tumor size and were poorly detected in hepatocarcinogenic early cases (Zhou et al., 2006). Tangkijvanich et al.(1999) showed that the sensitivity and specificity of AFU were about 80% and 70%, while 40% and 100%, for AFP respectively. Thus AFU and γ-glutamyltransferase could help AFP in HCC early detection.

CONCLUSION

The present study indicates that the increased oxidative stress leads to an incident liver cancer. Moreover bee honey supplementation showed protective effects against DEN induced carcinogenic liver rats. This is corroborated by the suppression of lipid peroxidation marker, malonaldehyde. Also honey supplementation restored the activities of superoxide dismutase and catalase levels, although glutathione peroxidase and glutathione transferase activities remained up-modulated. Our information showed that honey has not undesirable’s effect on antioxidant enzymes and also oxidative stress markers in non-carcinogenic liver. Additionally more studies are appropriate to solve up the mechanism and/or (s) by which bee honey protects the liver against oxidative stress. In conclusion, the therapeutic value of bee honey is possible to be in part due to its antioxidant and anti-inflammatory effects.

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