Advances in Bioscience and Biotechnology, 2014, 5, 252-260 **ABB**
http://dx.doi.org/10.4236/abb.2014.53032 Published Online February 2014 (http://www.scirp.org/journal/abb/)

Cytoprotectivity of the natural honey against the toxic effects of Doxorubicin in mice

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Received 5 December 2013; revised 16 January 2014; accepted 8 February 2014

ABSTRACT

The protectivity of the natural honey has been assessed against the toxicity of Doxorubicin (DOX) in liver tissues of 106 male Albino mice *Mus musculus* strain weighing 37 ± 3 gm. The body and liver weights, morphological behavior changes, liver function and pathological effects on liver were recorded. Toxicity study of DOX showed that the LD_{50} and LD were 20 and 30 mg/Kg, respectively. Intra-peritoneal (i.p.) injection of DOX induced significant ($p \leq 0.01$ - 0.001) pathological changes in the health, *i.e.* general weakness, a few morphological changes associated with bleedings, ulceration of skin, hair loss, dimorphism of limbs and bosselation. Daily ingestion of natural honey for seven weeks has led to significant ($p \leq 0.01$ - 0.001) improvement of these symptoms which appeared as increases in both body and liver weights in comparison with control animals. The natural honey had enhanced the function of liver in treated animals with DOX + honey and reduced the pathological effects of DOX on the above morphological symptoms as well as in the hepatocytes. It is concluded that the ingestion of natural honey has a protective potency against the toxic effects of DOX.

KEYWORDS

Toxicity; Doxorubicin (DOX); Protectivity; Natural Honey

1. INTRODUCTION

Doxorubicin or Doxil (DOX), a 14-hydroxylated version of daunorubicin which earlier was known as Adriamycin and hydroxyl-daunorubicin produced by a number of different wild type strains of *Streptomyces* is a drug used in cancer chemotherapy [1]. It is an anthracycline antibiotic closely related to the natural product daunomycin, and like all anthracyclines works by intercalating DNA, with the most serious adverse effect being life-threatening heart damage [2]. DOX, on the other hand, has also been known for its therapeutic potency against few clinical cases, e.g. ovarian and breast cancer, leukemia and Hodgkin's lymphoma [3]. The acute and chronic toxicity of DOX on general body tissues, particularly on cardiac muscles, has restricted its medical use [4]. Another common and potentially fatal complication of doxorubicin is Typhlitis, an acute life-threatening infection of the bowel [5]. There is some evidence for anti-malarial activity for DOX and similar compounds. Recently, a compound similar in structure to doxorubicin was found to inhibit plasmepsin-II, an enzyme unique to the malarial parasite *Plasmodium falciparum* [6]. The pharmaceutical company GlaxoSmithKline (GSK) later identified DOX in a set of compounds that inhibit parasite growth [7]. In 2011 and 2012, Doxil was unavailable for clinical use; however, as of 2013, Doxil is available as a generic version by FDA for clinical use, but in limited supply.

DOX affects both the normal and malignant cells inducing them to divide quicker than normal rate. Its cytotoxicity is due to its potency to produce free radicals [8,9] via two different mechanisms, *i.e.* aerobic by formation of a compound called *Semiquinone free radicals* [10] via a group of Oxidation-Reduction enzymes [11] where Oxygen ($-O_2$), Hydrogen peroxide (H_2O_2) and free Hydroxyl (−OH) radicals are produced [3]. Alternatively, DOX could react with ferrous ion (Fe^{3+}) to produce an Iron-doxorubicin complex which will involve in a series of chemical oxidation-reduction reactions to produce free radicals of both H_2O_2 and −OH [8]. These free radicals

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are believed to have a destructive role via provoking cells to produce H_2O_2 radicals which react with lipids forming lipid peroxides (LPO) which destroy the plasma membranes leading to many pathological and cellular changes in a few body parts, e.g. heart [12], kidneys [13], liver [14,15] and genital organs [16].

The natural honey has worldwide been known for its medical curing potency, e.g. antibacterial [17], antiseptic [18], antiviral [19] and has widely been used to treat wounds. The honey has also been used to treat ulcers, burns, various inflammations [20,21] as well as anti-tumor potency [22].

This research was designed to assess a range of DOX toxicity, *i.e.* lethal dose (LD), Sub-lethal does (Sub-LD), median lethal dose (LD_{50}) and to evaluate the curing potency of the natural honey in reducing the destructive effects of morphological and cellular changes of the DOX of liver in mice.

2. MATERIALS AND METHODS

Doxorubicin: The Doxorubicin was obtained as a Doxorubicin hydrochloride (EBEWE Arzneimittel Ges.m.b.h) from Saudi-Germany Hospital at Jeddah.

Honey: The "*Sidr*" honey (*Ziziphus spina-christi*) extracted from bees fed on Sidr plant was obtained from local market and "*Yahya*" honey from "*Yahya farm*" was 50% diluted using distilled water to facilitate absorption.

Only 106 of 3 months old healthy young sibling male Albino mice *Mus musculus* (MF1) 37 ± 3 gm weight obtained from the Medical Research Center at King Abdulaziz University, KSA were used. All animals were fed on diet contained vitamins, amino acids, fibers and salts *i.e.* K^+, Fe^{+3}, I^+, PO^+, Ca^{+2} in addition to tap water. Animals were divided into the following groups one week after settling in their new environment:

Group-A (n = 56) to test the toxicity subdivided into: 1). Control (n = 8) injected intra-peritoneally (i.p.) with physiologic neutral buffer; 2). Treated (n = 48) which was further subdivided into 6 mini-groups (n = 8 each) injected i.p. with six different doses of DOX (4, 8, 15, 20, 25, 30 mg/Kg body weight), all kept under continuous check up each 12 hours for a period of 7 weeks to record both the morphological and behavioral changes.

Group-B (n = 50) used to assess the potency of the natural honey against the destructive effects of DOX injected i.p. within a period of 7 weeks, were divided into: 1). DOX group (n = 20), weekly injected with dose of 4 mg/Kg body weight; 2). DOX + honey group (n = 20), weekly injected with dose of 4 mg/Kg body weight but fed orally with 5 mL/Kg of honey on daily basis for a period of 7 weeks; and 3). Honey group (n = 10) were fed only with 5 mL/Kg body weight.

Behavioral Measurements: Treated animals were monitored with naked eye during the experiments for their unbalanced movement and recorded as per animal. Percentages were taken as per total animals encountered in the experiments.

Measurement of Liver Function: The function of liver was measured using Aspertate Amino Transferase (AST) and Alanine Transferase (ALT) as µL in serum.

Histopathological Study: By the completion of the experiments all animals were dissected and the liver samples were immediately collected and processed using routine method and the slides were stained by heamtoxylene and Eosin (H&E) [Erwi, 2012].

Biostatistics: Sigma stat V2 biostatistics software was used to analyze the data while Student T-Test was used to analyze the differences in weights and other measurements *i.e.* enzymes between the control and treated groups. The Z-Test was used to compare the morphological changes between DOX treated only and those treated with DOX + honey. Percentages were calculated by measuring number of experimental demonstrated morphological alterations. Arithmetic means and the standard error (M ± SE) were calculated.

3. RESULTS

The results show that the toxicity of DOX is proportional with the doses being used *i.e.* 20 mg/Kg of DOX, the LD_{50}, 25 mg/Kg body weight represents the sub-lethal dose (sub-LD) while the dose 30 mg/Kg body weight represents the lethal dose (LD). Continuous monitoring of animals demonstrated that the DOX treated group exerted general weakness in activities (Table 1) and imbalanced movements with some morphological changes *i.e.* bosselation and tail atrophy while the clinical symptoms were represented in hair loss (alopecia) at chest and abdomen, lower jaw and hind limbs as well as some

Table 1. Morphological alterations assessed as proportion compared between mice group treated with DOX and DOX + honey expressed as percentages. All readings showed significant differences.

Morphological parameters	Changes DOX%	Changes DOX + Honey %
Red urination	30	5
Skin ulceration	100	60
Eye bleeding	90	35
Nose & Mouth bleeding	95	45
Hair loss (Alopecia)	95	20
Abnormal limbs	10	5
Tail atrophy	10	5
Unbalanced movement	45	10
Bosselation	100	55

clinical symptoms *i.e.* red urination (bleeding), skin ulceration, nose, mouth and eye bleeding (**Figures 1-3**). Significant differences were noticeable in the morphology, behavioral movements and clinical symptoms of those animals treated daily with 5 mg/Kg body weight DOX + honey while no morphological had seen on control and those treated with honey only.

The weekly ingestion of 4 mg/Kg of DOX for 7 weeks caused significant ($p \leq 0.01$) decline from 36.90 gm at the beginning of experiment towards the end 26.5 gm in the mean weight of mice (**Table 2**). A slight raise in the mean weight of mice was also noticeable in control. The mean weight of mice was increased in the daily honey fed group for seven weeks (from 37.9 gm to 46.9 gm) between the beginning and the end, respectively. The DOX treated group fed on honey showed significant ($p \leq 0.001$) decline in the mean weight of both mice (36.9 gm to 30.3 gm).

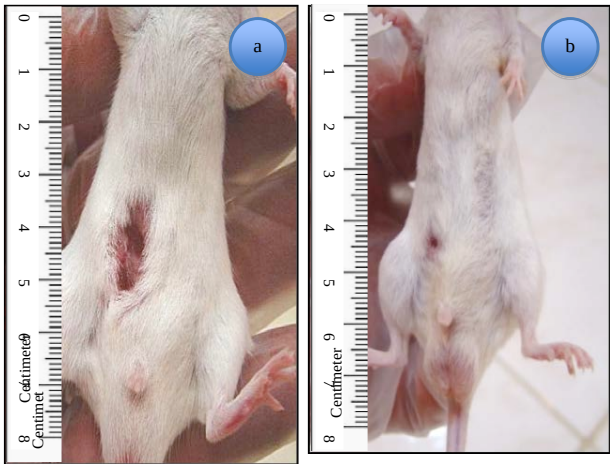

Figure 1. A comparison between the ulceration in DOX treated mice and treated with DOX + honey. Note the difference in size of the ulcers in both groups (a & b).

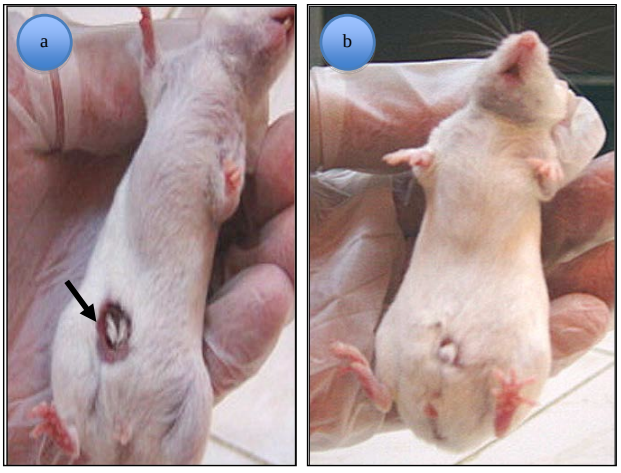

Figure 2. Growth of hair around the skin ulcer in treated mice group with DOX + honey at the first week of treatment (a) in comparison with those towards the end of experiment (b).

The mean biochemical values of ALT and AST enzymes of 4 different groups of mice in the weight proportion between the groups show significant differences ($p \leq 0.01$ - 0.001) as well as in their livers between the beginning and end of experiments (**Table 2**). The weight of liver represented almost 6% of the body weight in control but was declined to 4% in the treated group with DOX while its percentage was only 5% in other two groups (DOX + honey and only honey group) [**Table 3**].

The activity of feeding with honey to those mice treated with DOX shown over the clinical changes in symptoms and liver functions through measuring the concentrations of both ALT and AST in blood serum were illustrated in **Table 3**. The mean concentrations of both ALT and AST in control group reads (89.09 ± 5.144 uL) and (39.6 ± 3.17 to 5.0 uL) but they significantly increased to (141.02 ± 5.56 uL) and (122 ± 1.4 uL) respectively while the mean of ALT and AST in blood serum of DOX + honey or honey group were almost similar to those of control group.

4. HISTOLOGY DETAILS

The hepatic tissues in both control and the only honey groups showed no histological alterations (**Figure 4(a)**) while a few changes appeared in DOX group *i.e.* thrombosis in central veins accompanied with apoptosis and shrunken hepatocytes with increased intensity in stainability for acidophilic stains (acidophilic cells), small

Table 2. Weight differences in mice treated with 4 mg/Kg DOX, DOX + honey and Honey only between the beginning and end of experiments. (*): $p \leq 0.01$.

Groups	Mean weight ± Sd (gm) start of experiments	Mean weight (gm) ± Sd End of Experiments
Control	33.5 ± 0.47	37.7 ± 0.34
DOX	36.9 ± 0.37	26.5 ± 0.45*
DOX + honey	36.8 ± 0.74	30.3 ± 0.46*
Honey only	37.9 ± 0.46	46.9 ± 2.56*

Table 3. The arithmetic means of biochemical values of ALT and AST enzymes in 4 different groups of mice and the standard deviations. Changes in the weight proportion between the groups are represented in forth column as (*) refers to significant differences ($p \leq 0.01$) and (**) $p < 0.001$.

Groups	Mean ALT (µ/L)	Mean AST (µ/L)	Weight proportion %
Control	39.6 ± 3.17	89.1 ± 5.14	6
DOX	122.0 ± 1.44**	141.0 ± 5.56**	4
DOX + honey	50.3 ± 2.67*	94.0 ± 2.1*	5
Honey only	44.4 ± 1.3	67.2 ± 6.2	5

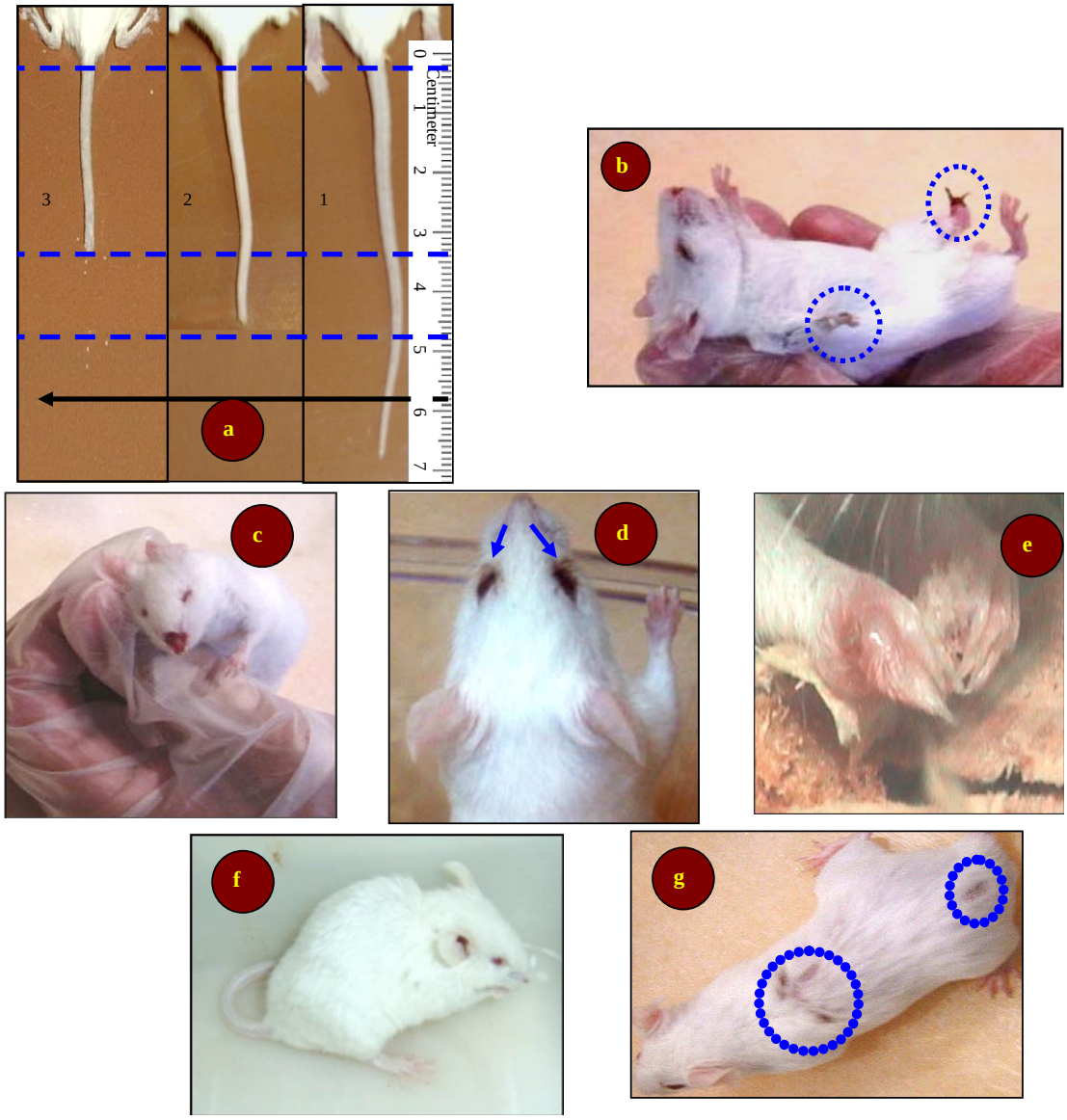

Figure 3. various morphological changes being developed in mice treated with DOX: (a) gradual atrophy of the tail along with the duration of the experiment; (b) dimorphism of the limbs (circles); (c) bleeding of nose; (d) bleeding in the eyes; (e) skin ulcers; (f) bosselation bendind in backbone and (g) hair loss (blue circles).

darker nuclei (pyknotic nuclei) (**Figure 4(b)**). Accumulation of inflammatory cells around the blood vessels had accompanied with deposition of fibrin at the central vein and hepatic synosoids was also noticeable (**Figures 4(c)** & **(d)**). A comprehensive necrosis of a part of hepatic tissue and degenerated dilation (ballooning degeneration) of hepatic tissue with loss of blood sinusoid was detected [**Figure 4(e)**]. DOX lead to development of both fatty droplets (steatosis) and macrovacuoes inside the cytoplasm of hepatocytes leading to displace the nuclei toward periphery of the cells involved abnormal shaped cells (amorphated cells) (**Figure 4(f)**). The cytoplasm of the hepatocytes around the central vein in treated mice with DOX + honey were either regular (unchanged) or rarely showed very few cellular structural changes. Synusoids had also persisted their regular organization and structures central vein around the central vein without development of fatty droplets, nor necrosis or steatosis in hepatocytes. However, precipitation blood within the central vein and aggregation of chromatin towards the periphery of nuclei of some hepatocytes and accumulation of inflammatory cells in some parts of hepatic tissues were noticeable (**Figures 4(d)** & **(h)**).

5. DISCUSSION

All mice injected i.p. with 30 mg/Kg body weight had died at LD_{50} 20 mg/Kg indicating the strong toxicity of

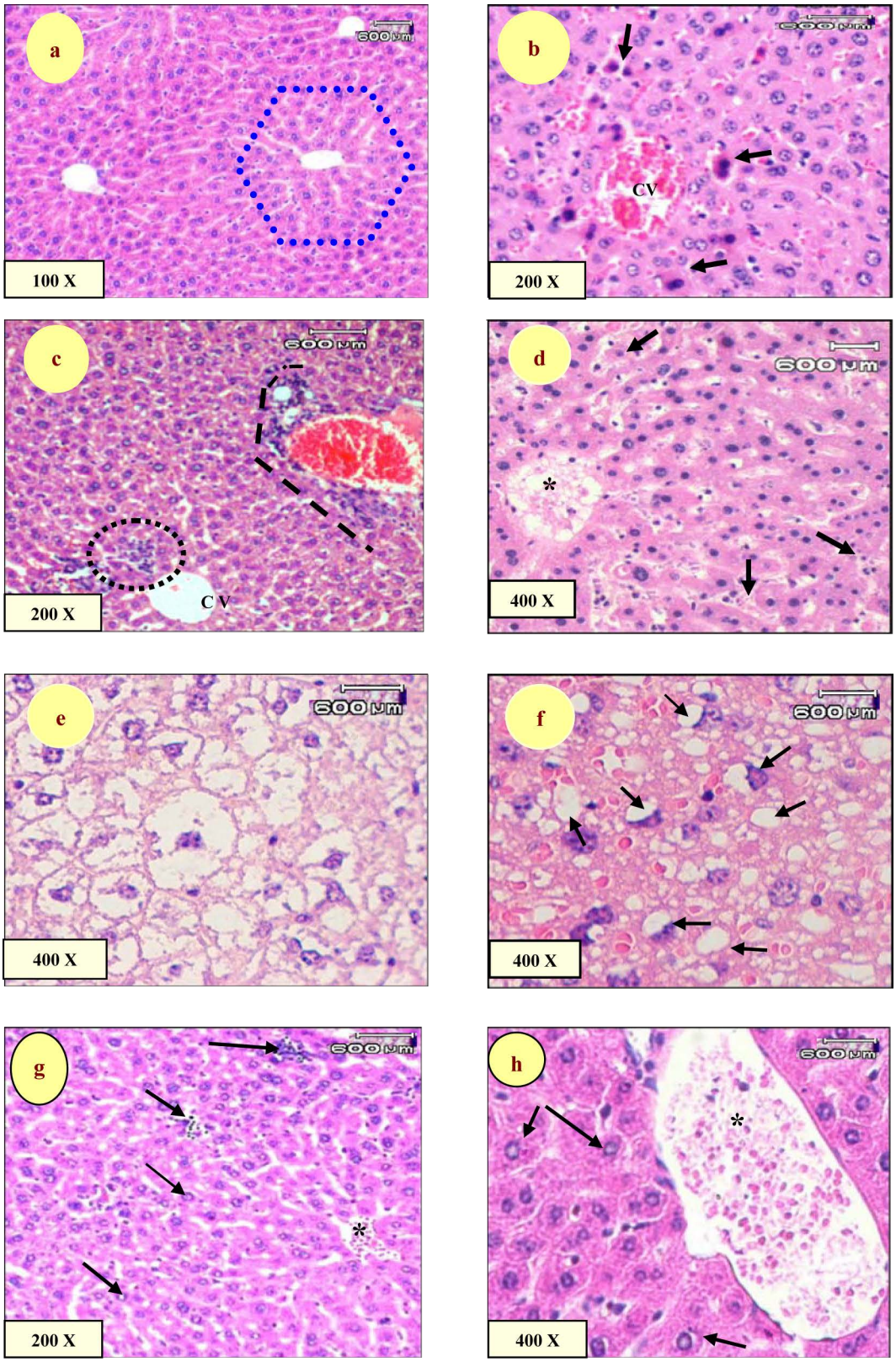

Figure 4. Sections through the liver: (a) irregularity in hepatic tissues in DOX treated groups *i.e.* atrophy of hepatocytes; (b) increase in blood vessels; (c) development of phagocytes; (d) accumulation of fibrin; (e) necrosis; (f) development of adipose (lipids); while improvement in cellular configuration in hepatice tissues treated with DOX + honey are clear in both G&H.

DOX. These results were similar to previous studies where LD_{50} was only 25 mg/Kg in female mice [23] at LD_{50} 20.8 mg/Kg [24]. This difference has been attributed to variations in methodology of each experiment [20]. The results showed general weakness in the activities of treated mice with DOX with development of some morphological and clinical alterations *i.e.* loss of hair (alopecia) from chest, abdomen parts, lower jaw and lower limb which are compatible to another study where only a dose of 7.5 mg/Kg DOX was injected into the vein of rats tail [21]. The DOX + honey had shown ameliorated morphological and clinical alterations in of this study and were concomitant with activity of the honey against hair dandruff (scaling), head itching and hair loss [25].

Bleeding of nose, eyes and mouth was the most evident symptoms in DOX treated mice. This could be interpreted that DOX can cause damage to blood platelets (thrombocytopenia) which are an important factor in blood clouting leading to bleeding. In addition, the DOX is an inhibitor to bone marrow (myelosuppression) to produce blood components [26]. The DOX could also cause blood cells to leak through the endothelial cells of the capillaries leading to red nose, eyes and soles of the feet [27].

Skin ulceration had accompanied the above symptoms as well as dermatitis around the injection spots of DOX which are concomitant with that of Asker *et al.* [28]. In addition, Van Vleet and Ferrans [29] had observed Lambness of ribs and fractures of bones at both fore and hind limbs of rabbits treated with 4 mg/Kg of DOX while in this study clinical symptoms and demorphation of limbs were seen in DOX treated mice. This could be interpreted as the effects of DOX and can extend to bones in treated animals too. There has been a significant difference in demorphism and activities of mice treated with DOX and between those treated with DOX + honey. This difference might refer to the potency of honey to protect or to minimize the deteriorating effects of DOX via reducing free H_2O_2 radicals [8,9] minimizing lipophosphate (LPO) which destroys the plasma membranes leading to many pathological and cellular changes [12-16]. Daily ingestion of honey therefore could protect body from many toxic effects of DOX or other chemicals.

Honey contains high concentrations of saccharides which provide a quick source of energy. Honey has also proved to have wound healing potency too and to cure the inflammatory effects of DOX in mice. This is concomitant with many previous researches which documented the curing power of honey in wound healing and against inflammatory symptoms [20-24]. Therefore, the honey could well be considered as an anti-inflammatory factor. The anti-inflammatory effects can be attributed to activation of immune white blood cells (WBC) particularly, the giant Monocytes which represent the first defense line of the body [30]. The natural honey could activate the giant Monocyte upto 50% in addition to increase the activity of lymphocytes and Acidophils [31]. Moreover, natural honey can also increase the immunity, remedy of eye infections, and inflammations caused by external wound [32]. All the results obtained in this research regarding the curing potency of the natural honey do approve the previous findings.

Significant decrease in the mean weight of Mice treated with DOX in comparison with those control was noticed. The results of the current research are in agreement with previous researches which involved most chemical compounds used in remedy of cancer leading to decrease in weight and loss of appetite (aborexia), constipation [33] and Dysphagia and dyspesia and gastrointestinalis [34]. The DOX could cause disintegrates the epithelia of the elementary canal [35] and inflammation of mucosa (macositis) which both strongly refer to the toxic effects of DOX leading to the loss in weight [36].

The histological changes in livers of mice developed following the weekly injection of 4 mg/Kg of DOX for seven weeks in this study. Similar results were obtained following treatment with DOX which has been interpreted as due to fatty infiltration of hepatic tissues [14] and ingestion of 100 mg/Kg of Aspartame [37]. The extensive cellular necrosis detected in the hepatic tissues of mice following DOX injection and the increase in inflammatory cells are concomitant with results of Samelis *et al.* [38]. Cellular necrosis had caused chronic lobular inflammation and accumulation of inflammatory cells. The thrombosis and the blood sinusoidal congestion detected in the current study could be attributed to the damage in tissues leading to coagulation of blood where anti-carcinogenic medicines cause disintegration of fibrin leading to clotting of blood within the blood vessels [39].

The cytoprotective potency of the natural honey in this study in minimizing the histopathological changes caused by DOX is in concomitant with the potency of the honey in treating the hepatic toxicity [40]. Foods rich in saccharides would increase the ability of hepatic cells for growth and renewal [41]. The natural honey, in general, is considered as one of the richest foods in its saccharide contents which could be around 70% - 80% [42].

Following the DOX injection, both ALT and AST of the serum recorded a significant increase in the arithmetic means of enzymes between the treated and control mice while DOX + honey group shown significant decrease in these enzymes in comparison with DOX only. Similar results of the current research are obtained in another study of Saad, *et al.* [43].

It is concluded that ingestion of honey following treatment of mice with various doses of DOX could decrease the deteriorating the pathological effects of it, protect the

hepatic tissue and the function of liver. These results, therefore do confirm the potency of honey in protecting the health as well as previous studies [44] which denoted the possession of honey to antioxidants *i.e.* flavonoids [45] and carotenoids as well as folic acids. Further research is necessary to explore the credence of the natural honey as an anti toxic agent against other chemicals.

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ABBREVIATIONS

ALT: Alanine Transferase.
AST: Aspertate Amino Transferase.
Ca^{+2}: Calcium.
DOX: Doxorubicin.
Fe^{+3}: Ferrose Ion.
GSK: GlaxoSmithKline.
H_2O_2: Hydrogen Peroxide.
I^-: Iodine.
i.p.: Intraperitoneal.
KSA: Kingdom of Saudi Arabia.
LD: Lethal dose.
LPO: Lipophosphate.
O_2: Oxygen.
PO_4: Phosphate ions.
P^+: Potassium ion.
LD_{50}: Sub lethal dose.
WBC: White blood cells.

