The combined effect of honey and olive oil against methotrexate mediated hepatotoxicity in rats: A biochemical, histological and immunohistological study

Hani A. Alturkistani[1], Osama A.H. Abuzinadah[1],
Abdelhakeem M. Kelany[1], Gamal S. Abd El-Aziz[2] and Aziza R. Alrafiah[3]

[1]Department of Biological Sciences, Faculty of Science, [2]Department of Anatomy, Faculty of Medicine and [3]Department of Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, KSA

Summary. Background. Honey and olive oil are natural products that have high nutritional values, and therapeutic properties. Cytotoxic drugs, like methotrexate (MTX) are used to treat malignancies in tumour cells; however, these drugs also have serious side effects that could threaten the patient's life.

Aim. To evaluate the potential protective effects of honey and olive oil, administered alone or together, against MTX-induced hepatotoxicity in rats.

Methods. Adult male albino rats were divided: Group I: negative control (n=8); II: honey (daily by oral 1.2 g/kg bwt (n=8), III: olive oil (1 ml/day) (n=8), IV: single intraperitoneal injection of MTX (20 mg/kg bwt) (n=8), V: diluted honey for 3 days before injection of MTX (n=8), Group VI: olive oil for 3 days before injection of MTX (n=8), Group VII: both honey and olive oil for 3 days before injection of MTX (n=8). After treatment, rats were sacrified and blood samples were collected to determine liver function parameters, liver tissue used to measure the oxidative (malondialdehyde), antioxidative parameters (superoxide dismutase, catalase and glutathione peroxidase), histological and immunohistochemical techniques.

Results. The administration of honey and olive oil exerted a protective effect against MTX-induced hepatotoxicity, as demonstrated by the normalization of the liver enzymes, proteins and total bilirubin and by the histopathological and immunohistological changes observed in the livers. Both agents also reversed the oxidative damage in the liver by decreasing level of MDA levels and increasing the antioxidant related by enzymes in the liver homogenates compared to the control rats. These effects were more evident when the two agents were administered together.

Conclusion. The combined intake of honey and olive oil could be hepatoprotective. Co-administration of these agents might form an effective adjuvant therapy and minimize side effects of chemoherapy in cancerous patients.

Key words: Cancer, Chemoherapy, Methotrexate, Hepatotoxicity, Honey, Olive oil

Introduction

Chemotherapeutic drugs are widely used in the treatment of many types of malignancies. However, their use can entail high risks and may include many side effects in various organs and tissues; these side effects can be very challenging to manage (Moss, 2007; Chen et al., 2009; Minami et al., 2010). One chemotherapeutic drug is methotrexate (MTX) [4-amino-10-methylfolic acid (C20H22N8O5)], which is an anti-metabolite antifolate drug that disrupts cellular metabolism and blocks the synthesis of purines and pyrimidines, inhibiting cellular growth (Kose et al., 2012; Dalaklioglu et al., 2013). MTX is widely used to treat various illnesses where high doses are used in cancer therapy as it is the drug of choice for the treatment of acute

lymphocytic leukaemia, breast cancer, non-Hodgkin's lymphoma, testicular tumours and osteosarcoma. Small doses of MTX can effectively treat chronic and autoimmune diseases such as rheumatoid arthritis, sarcoidosis, psoriasis and Crohn's disease (Wessels et al., 2008; Cordero et al., 2010; Patane et al., 2013; Weinblatt, 2013; Daggulli et al., 2014). MTX is commonly used even though it is often associated with several organ toxicities; some of these are life-threatening, e.g. myelo-suppression, cardiotoxicity, nephrotoxicity and hepatotoxicity (Uraz et al., 2008; Dalaklioglu et al., 2013; Yuksel et al., 2017). Liver damage is one of the most significant side effects, especially when MTX is used chronically and in high doses, which restricts its clinical applications (Hytiroglou et al., 2004; Bayram et al., 2005; Sener et al., 2006; Vardi et al., 2008, 2010). Various suggestions have been offered to explain the mechanism of MTX toxicity (Uraz et al., 2008).

A number of experimental and clinical studies have confirmed that MTX triggered free radical formation along with decreased antioxidant defence activity; these effects are responsible for the side effects of MTX. In addition, MTX increases cells' vulnerability to ROS by inhibiting the antioxidant defence system. Hence, antioxidant treatment may help prevent MTX toxicity (Uraz et al., 2008; Guler et al., 2011; Dalaklioglu et al., 2013).

Recently, much attention has been given to the possible role of natural products with antioxidant properties in protecting against the toxic side effects of chemotherapeutic agents and improving the outcomes of such treatments (Hemeida and Mohafez, 2008; Gulgun et al., 2010; El-Sheikh et al., 2015). At the same time, honey and olive oil have been used more frequently to take advantage of their nutritional and health-promoting effects. The ability of these agents to treat and protect from a range of diseases is well established and is attributed to their rich phenolic compound content (Rodriguez-Malaver et al., 2009).

Honey is a natural product of honeybees; it is collected from nectars and plant (Mohd Zohdi et al., 2012). It contains a number of important biologically active compounds, including phenolic acids and flavonoids. It is abundant in proteins, carbohydrates, trace elements, vitamins, phenolic compounds and enzymes (Havsteen, 2002; Johnston et al., 2005). It also contains enzymes, vitamins, phenolic compounds and organic acids (Al-Waili and Boni, 2003). Therefore, honey is a promising agent. It has positive effects on the healing of wounds (Moghazy et al., 2010) and works as an antibacterial antioxidant; it also has antitumor and anti-inflammatory effects (Bilsel et al., 2002). The therapeutic role of honey in the treatment of various ailments has been receiving considerable attention recently, and its therapeutic value has been partly attributed to its antioxidant properties (Johnston et al., 2005; Lu et al., 2013). Honey has also been found useful in the treatment of cardiovascular, gastrointestinal and liver complications and of soft tissue infections and burns (Ezz El-Arab et al., 2006).

Olive oil (*Olea europaea*) has also been receiving increased attention over the past few years worldwide because of the nutritional and therapeutic value of its contents (Rafehi et al., 2012, Montano et al., 2016). Olive oil contains vital long-chain omega-3 polyunsaturated fatty acids, including eicosapentaenoic acid, docosahexaenoic acid, α-linoleic acid and oleuropein. These acids have antioxidant effects; they decrease oxidative stress and inhibit the formation of ROS (Lenihan-Geels et al., 2013; Patten et al., 2013). Historically, products made from olive oil have been used as aphrodisiacs, emollients, laxatives, nutritives and sedatives. Extra virgin olive oil (EVOO) has antioxidant properties and hypolipidemic, hypoglycaemic, cardiovascular, and nephro- and hepato-protective effects (Musumeci et al., 2014). It can also improve oxidative stress resulting from exhaustive exercise (Musumeci et al., 2014). Furthermore, EVOO is known for its antimicrobial and anti-inflammatory effects (Gilani et al., 2005; Lopez-Miranda et al., 2010; Omar, 2010). Several studies have shown the importance of olive oil on cartilage, muscles and liver. Investigators noted that the conjunction of EVOO enriched diet and of physical activity significantly improved articular cartilage recovery process in osteoarthritis disease (Musumeci et al., 2013; Szychlinska et al., 2019). Another study by Trovato et al. (2018) showed that high-fat extra-virgin olive oil-based diets could impair muscle metabolism and prevent damge (Trovato et al., 2018). Lastly, recent research supports the protective role of EVOO and vitamin D against the collagen I production in a rat model of non-alcoholic fatty liver disease (Trovato et al., 2018).

Hence, the present experiment was designed to elucidate the synergistic hepatoprotective effects of honey and olive oil as potent antioxidants against MTX-induced hepatotoxicity in rats.

Materials and methods

Drugs and chemicals

This study used methotrexate (MTX) in a 25 mg/ml solution designed for injection and produced by Hospira UK Ltd. This sterile, yellow solution for injection contains methotrexate sodium, equivalent to 25 milligrams of MTX, in clear glass vials. MTX solution was injected intraperitoneally at a single dose of 5 ml, equal to 20 mg/kg body weight. It was purchased from local scientific agents at Jeddah, KSA. The pure honey (Saudi Sidr variety) (*Ziziphus spina-christi*) used in this study was extracted from bees fed on Sidr plants; it and the extra virgin olive oil (*Olea europaea; family Oleaceae*) were purchased from local markets in Jeddah, KSA. Pure honey (Saudi Sidr variety) was purchased from an exclusive honey shop in Riyadh, Saudi Arabia.

Experimental design and animal groups

The current experiment used adult male Sprague-Dawley rats with body weights ranging between 180-200 gr. The rats were acquired from the animal house of the King Fahd Medical Research Centre (KFMRC). The research proceeded after consent was received from the Medical Research Ethics Committee, Faculty of Medicine, King Abdulaziz University, Jeddah, KSA (186-18 [HA-02-J-008]). The rats were accommodated in cages (four animals/cage) in a room with regulated temperatures (24±2°C), regular lighting (12h:12h light:dark) and humidity set at 55±5%. Water was supplied ad libitum, and the rats were fed standard Purina rat chow. During all the steps of the study, the animals were cared for in the KFMRC. The rats were observed daily for health status. After acclimatization, which lasted one week before the start of the experiment, the rats were divided into seven groups (n=8 for each group): Group No. (Group Name): Experimental treatment. Group I (Control group): The rats received distilled water only (2 ml/day) by oral gavage until the end of the study period. Group II (Honey group): The rats received diluted honey 50% (v/v) by oral gavage at a dose of 1.2 g/kg bwt/day diluted in 2 ml of dH2O until the end of the study period. Group III (Olive oil group): The rats received olive oil by oral gavage at a dose of 1.0 ml/day until the end of the study period. Group IV (MTX group): The rats were injected with intraperitoneal (ip) (20 mg/kg) only once on the third day of the experiment. The dose of MTX was selected according to the results of previous studies (Jahovic et al., 2004). This dose (20 mg/kg) approximates the MTX dose commonly used in human patients. Group V (MTX + honey group): The rats were given diluted honey as described above 3 days before injection of MTX. Group VI (MTX + olive oil group): The rats were given olive oil as described above 3 days before injection of MTX. Group VII (MTX + honey + olive oil group): The rats were given both honey and olive oil as described above 3 days before injection of MTX.

The rats in each group were observed daily for general condition, body weight and food intake. Following experiment (two weeks), the rats' body weight gain and food consumption were calculated. Then, the rats were sacrificed and blood samples were obtained using a cardiac puncture with a heparinized syringe. Samples were then centrifuged at 3000 rpm for ten min to separate the plasma, which was frozen and stored at -20°C for further analysis. Next, each rat's abdominal cavity was opened, and the entire liver was removed and weighed. Pieces of the livers were cut and immediately fixed with 10% neutral buffer formalin (NBF) for further histopathological and immunopathological studies. Other liver pieces were frozen in liquid nitrogen and stored at -70°C so the presence of oxidant and antioxidant markers could be determined.

Assessment of liver enzymes

Using an Olympus AU-2700 autoanalyzer (Olympus, Hamburg, Germany) and market kits, the levels of the following liver enzymes in the plasma samples from all the rats were assessed spectrophotometrically: aspartate amino transaminase (AST), alanine amino transaminase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), total bilirubin (TB) and total protein (TP).

Preparation of tissue homogenates and measurement of oxidative parameters

The frozen liver pieces were left to defrost and then mixed with 2 ml ice-cold Tris–HCl, pH 7.4, containing 1% protease inhibitor. The mixtures were homogenized at a speed of 16,000 rpm, and then buffer was added until the final volume was ten times the weight of the tissue. The levels of peroxidation markers in lipid and antioxidative enzyme activities were measured using a spectrophotometer (Pharmacia Biotech., Cambridge, England) as follows: The lipid peroxidation products were measured by malondialdehyde (MDA) according to the method used in Lopez-Miranda study (Mihara and Uchiyama, 1978). Superoxide dismutase (SOD) activity was assessed as by Sun et al. (1988). Catalase (CAT) activity was assessed as by Aebi (1984). Glutathione peroxidase (GPx) activity was assessed as by Koracevic et al. (2001).

Histological and immunohistochemical methods

The fixed liver samples were processed through graded alcohol and xylene and then embedded in paraffin blocks in an automatic processor. The sample blocks were cut into sections longitudinally. The sections were routinely stained with haematoxylin and eosin (H&E) to assess the liver architecture, with Masson's Trichrome (MT) to examine the fibrous tissue content, and histochemically with periodic acid schiff (PAS) to assess the glycogen content of the hepatocytes. For immunohistochemical staining, some other sections were deparaffinized, rehydrated and treated with 0.01 M citrate buffer (pH 6.0) for ten minutes to unmask antigens and then stained using a modified avidin-biotin peroxidase technique with primary antibodies monoclonal caspase3 (DAKO, Carpinteria, CA) diluted 1:100 in PBS + 1% normal goat serum (for the detection of apoptotic cells). Antibodies were detected using 0.05% diaminobenzidine as a chromogen and haematoxylin as a counterstain. Finally, each slide was photographed with an Olympus BX53 microscope equipped with a camera (Olympus, Tokyo, Japan) at different magnifications.

Morphometric studies

A modified scoring system was used to evaluate the

severity of hepatic injury in the H&E-stained sections according to the following parameters: 1) disruption in the radial arrangement, 2) dilatation and congestion of central veins, 3) sinusoidal dilatation, 4) inflammatory cell infiltration, 5) degenerative changes in hepatocytes (cytoplasmic vacuolization and swelling of hepatocytes), 6) nuclear changes in hepatocytes and 7) extent of fibrosis in the hepatic lobules. Hepatic damage parameters were scored as absent (0), mild or weak (1), moderate (2) or severe or strong (3). The scores and data were collected by two separate observers using Leica Qwin 500 image analyser software; six high-power fields under 100X or 200X magnification from each animal were observed.

Statistical analysis

Data was analysised using GraphPad Instat® Biostatistics version 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS) for Windows version 11.5 (SPSS, Chicago, IL, USA). The analysis of variance (ANOVA)-Tukey's multiple comparisons test was used for comparisons between more than two groups. The correlations between all variables were tested by Pearson's correlation coefficient. *p*-value of less than 0.05 ($p<0.05$) was considered statistically significant. The data are expressed as the mean ± SD.

Results

Morbidity and mortality

In the MTX-treated rats, loss of appetite, decreased activity, weakness and yellowish body hair were observed during the experimental period. Two cases of mortality were recorded, one on the fifth and one on the eighth day. The rats in the other groups appeared healthy and did not show clinical signs of toxicity; no mortality

was recorded for rats not treated with MTX during the duration of the experiment.

Effect of honey and olive oil on BW and intake of food following MTX

As shown, body weight gain Fig. 1, liver weight Fig. 2 and food intake Fig. 3 were more or less similar in the control, honey and olive oil groups, without any significant differences. In the MTX groups, these parameters decreased significantly (P<0001) compared to the control, honey and olive oil groups. However, the administration of honey and olive oil with MTX resulted in an improvement in all the values; this difference was highly significant when compared to MTX group, especially when honey and olive oil were administered together along with MTX (P<0001).

Estimation of liver function

The biochemical estimation of AST, ALT, ALP and LDH activity, as well as serum albumin and total bilirubin levels, were measured to evaluate liver function. The results (shown in Table 1) indicate that supplemented with either honey or olive oil did not show a noticeable change compared with control group, while MTX treatment resulted in a significant increase (P<0001) in these parameters and a decrease in serum albumin compared to the control group, indicating the hepatotoxic effect of MTX. Administration of honey or olive oil with MTX resulted in a significant improvement in hepatic profile compared to the MTX-only group; this difference was more prominent when only olive oil was administered with MTX than when only honey was administered (P<0001). However, co-treatment of the MTX group with both honey and olive oil resulted in a near-normalization of all parameters with a statistically significant difference to the results of the MTX-only group (P<0001). These results indicate the hepatoprotective effect of honey and olive oil.
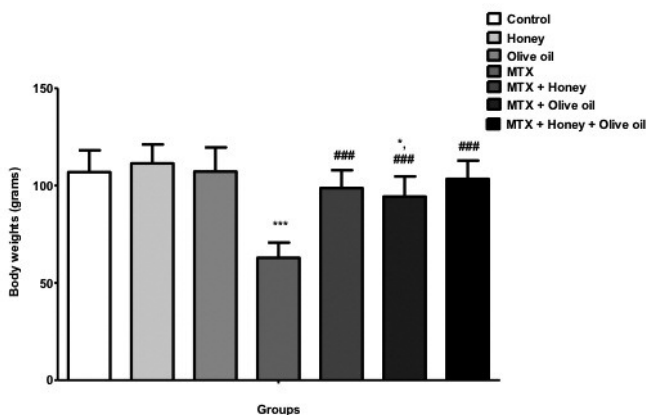

Fig. 1. Effect of MTX, honey and olive oil on body weight (gm) in different experimental groups. *: significance versus control; #: significance versus MTX group.
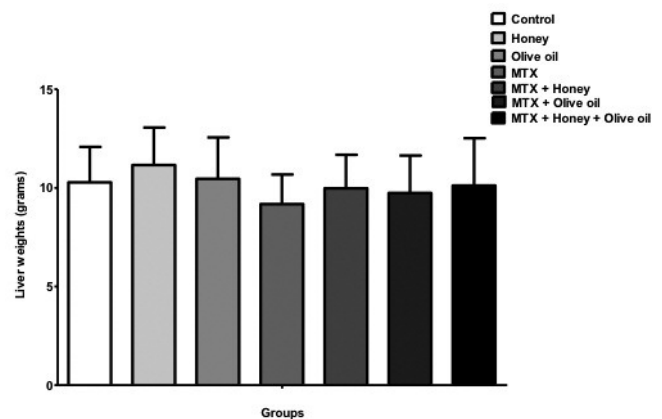

Fig. 2. Effect of MTX, honey and olive oil on liver weight (gm) in different experimental groups.

Results of oxidative/antioxidative status:

Effect of MTX, honey and olive oil of MDA, SOD, CAT and GPx in the rat's livers homogenates of different experimental groups

Fig. 4 shows the level of the final product of the lipid peroxidation. MDA was significantly elevated (P<0001) in the MTX group, whereas the antioxidant activities of SOD, CAT and GPx were significantly decreased compared to the control groups (P<0001). When the rats were fed honey or olive oil along with MTX, MDA decreased significantly and SOD, CAT and GPx increased compared to the MTX-only group, indicating the antioxidant roles of honey and olive oil. Furthermore, co-administration of both honey and olive oil with MTX resulted in a return of all parameters to nearly normal values.

Histopathogical morphometric analysis

Histopathological scoring of the liver changes in different groups

The results of the liver histopathological scoring for the different groups are summarized in Fig. 5. The MTX

treatment resulted in significant deterioration of the observed histopathological findings (P<0001) compared to the control, honey and olive oil groups. However, co-treatment of MTX with honey or olive oil resulted in improvement of all these scores, moving them towards
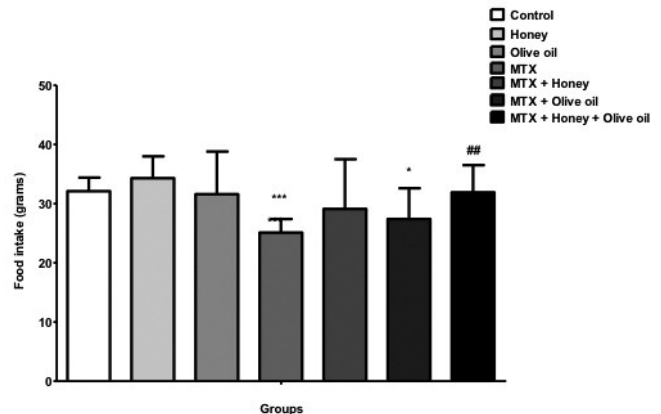
Fig. 3. Effect of MTX, honey and olive oil on food intake (gm) in different experimental groups.*: significance versus control; #: significance versus MTX group.

Table 1. Effect of MTX, honey and olive oil on the Mean ± SD of liver function parameters in different experimental groups.

Groups	AST / (U/ml)	ALT / (U/ml)	ALP (U/ml)	LDH (U/ml)	Serum albumin (gm/dl)	Total bilirubin (umol/ml)
Control	30.7±1.80	27.2±1.30	68.3±2.62	83.72±6.08	3.72±1.04	1.18±0.32
Honey	28.4±2.22	29.2±2.2	62.3±3.83	81.94±5.22	3.88±0.92	1.16±0.18
Significance	1P=0.055 1F value=1.521	1P=0.061 1F value=2.864	1P=0.005** 1F value=2.137	1P=0.568 1F value=1.357	1P=0.766 1F value=1.278	1P=0.888 1F value=3.160
Olive oil	33.2±1.88	30.3±1.9	71.3±4.73	87.56±6.82	3.66±0.86	1.2±0.24
Significance	1P=0.026* 1F value=1.091	1P=0.004** 1F value=2.136	1P=0.168 1F value=3.259	1P=0.288 1F value=1.258	1P=0.908 1F value=1.462	1P=0.841 1F value=1.778
MTX	96±4.64	71.5±3.70	138.7±11.43	236.92±14.7	2.77±0.79	5.57±1.77
Significance	1P=0.0001*** 1F value=6.645	1P=0.0001*** 1F value=8.101	1P=0.0001*** 1F value=19.03	1P=0.0001*** 1F value=5.846	1P=0.078 1F value=1.733	1P=0.0001*** 1F value=30.59
MTX + Honey	44±5.36	36±2.1	82.6±7.35	96.66±7.44	3.16±0.66	1.70±0.34
Significance	1P=0.0001*** 1F value=8.867 2P=0.0001*** 2F value=1.334	1P=0.0001*** 1F value=2.609 2P=0.0001*** 2F value=3.104	1P=0.0004*** 1F value=7.870 2P=0.0001*** 2F value=2.418	1P=0.004** 1F value=1.50 2P=0.0001*** 2F value=3.904	1P=0.252 1F value=2.483 2P=0.336 2F value=1.433	1P=0.012* 1F value=1.129 2P=0.0001*** 2F value=27.10
MTX + Olive oil	52±4.72	42±2.33	94.6±6.05	97.48±5.98	2.99±0.81	1.88±0.28
Significance	1P=0.0001*** 1F value=6.876 2P=0.0001*** 2F value= 1.035	1P=0.0001*** 1F value=3.212 2P=0.0001*** 2F value=2.522	1P=0.0001*** 1F value=5.332 2P= 0.0001*** 2F value=3.569	1P=0.001** 1F value=1.034 2P=0.0001*** 2F value=6.043	1P=0.169 1F value=1.649 2P=0.616 2F value=1.051	1P=0.0009*** 1F value=1.309 2P=0.0001*** 2F value=39.96
MTX + Honey + Olive oil	35.9±2.87	30±1.44	71.2±8.82	89.22±7.18	3.66±1.44	1.32±0.64
Significance	1P=0.002** 1F value=2.542 2P=0.0001*** 2F value=2.614	1P=0.0024** 1F value=1.227 2P=0.0001*** 2F value=6.602	1P=0.421 1F value=11.33 2P=0.0001*** 2F value=1.679	1P=0.148 1F value=1.395 2P= 0.0001*** 2F value=4.192	1P=0.930 1F value=1.917 2P=0.177 2F value=3.232	1P=0.614 1F value=4.000 2P=0.0001*** 2F value=7.649

Data are expressed as mean±SD. 1P: significance versus control; 2P: significance versus MTX group. Significance was made using unpaired student "t" test. *: P <0.05; **: P<0.010; ***P <0.001.

the control values, especially when the two agents were given together along with MTX.

Histological findings

Examination of the H&E-stained liver sections from the different groups showed that the hepatatic tissue of the control, honey and olive oil groups (Fig. 6A,B) had a nearly similar picture to the normal structure that was formed of classical lobules in which regular cords of hepatocytes were separated by slit-like blood sinusoids and radiated from the central vein towards the periphery. These cells have eosinophilic cytoplasm and contain one or two rounded nuclei. In the portal spaces, the small branches of the hepatic artery and portal vein and bile duct were observed. In the MTX groups (Fig. 6C-F), an array of histopathological changes could be seen, including disorganization of the hepatic cords with disturbed continuity and dilatation of the blood

sinusoids. The central veins were also markedly dilated and congested with detached endothelial linings. Dilation and inflammatory cellular infiltration were detected in the portal spaces. The shapes and nuclei of the hepatocytes also showed a range of changes; the hepatocytes appeared swollen and vacuolated with ill-defined boundaries, and the nuclei showed karyolysis or pyknosis. However, in the MTX-intoxicated rats that were co-treated with honey or olive oil (Fig. 6G,H) these histopathological changes were noticeably improved compared to the MTX group; particularly in the MTX and honey group. There was more organization of the hepatic cords and the blood sinusoids were narrower. However, slight congestion was observed in the central veins and portal venules. The hepatocytes displayed fewer degenerative cytoplasmic and nuclear changes. Furthermore, in the MTX rats co-treated with both honey and olive oil (Fig. 6I), there was remarkable improvement in the histopathological changes that
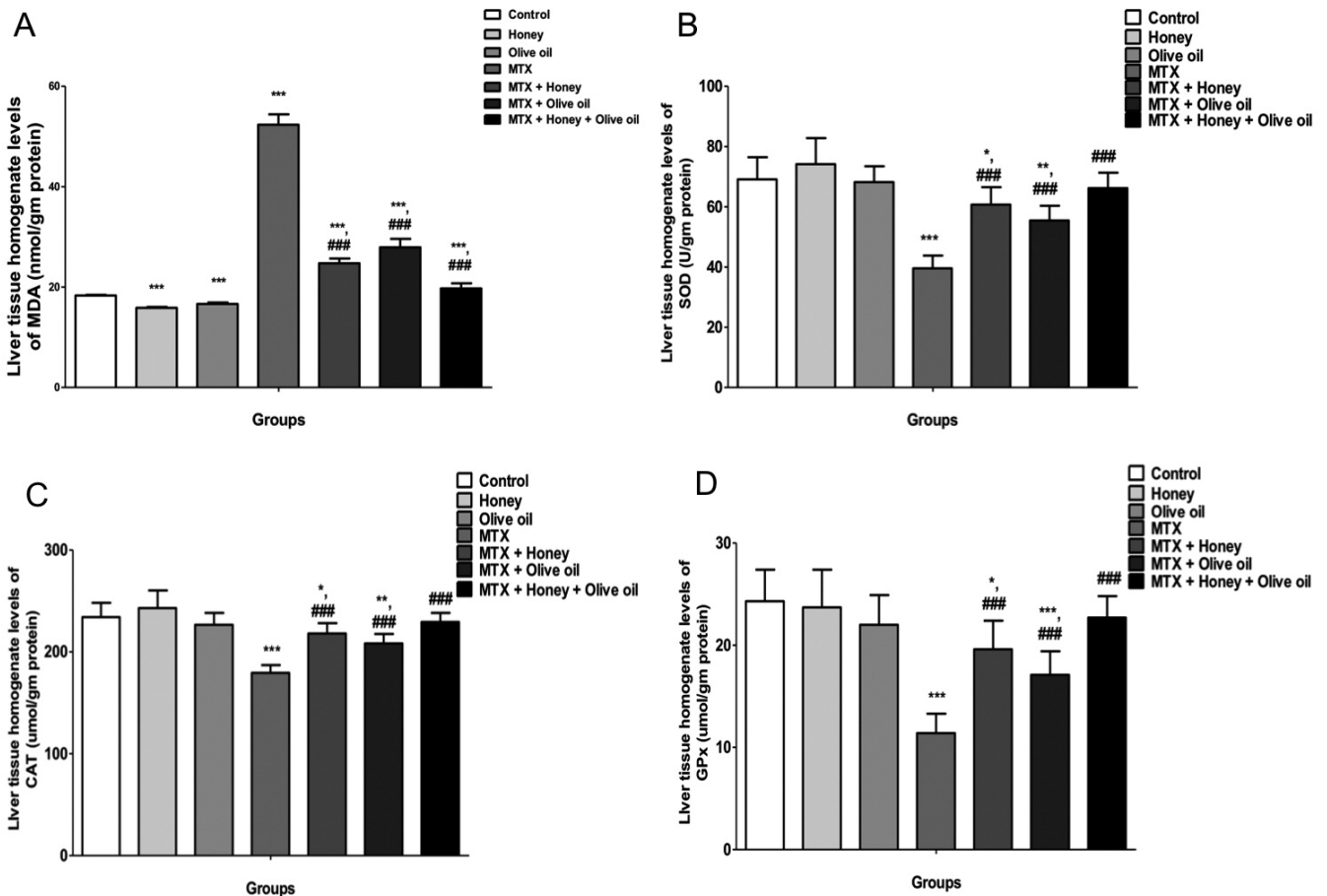

Fig. 4. SOD, CAT and GPx in the rat's livers homogenates of different experimental groups. **A.** Effect of MTX, honey and olive oil of MDA; Effect of MTX, honey and olive oil on MDA (nmol/gm tissue protein) in different experimental groups. **B.** Effect of MTX, honey and olive oil on SOD (U/gm tissue protein) in different experimental groups. **C.** Effect of MTX, honey and olive oil on CAT (umol/gm tissue protein) in different experimental groups. **D.** Effect of MTX, honey and olive oil on GPX (umol/gm tissue protein) in different experimental groups. *: significance versus control; #: significance versus MTX group.
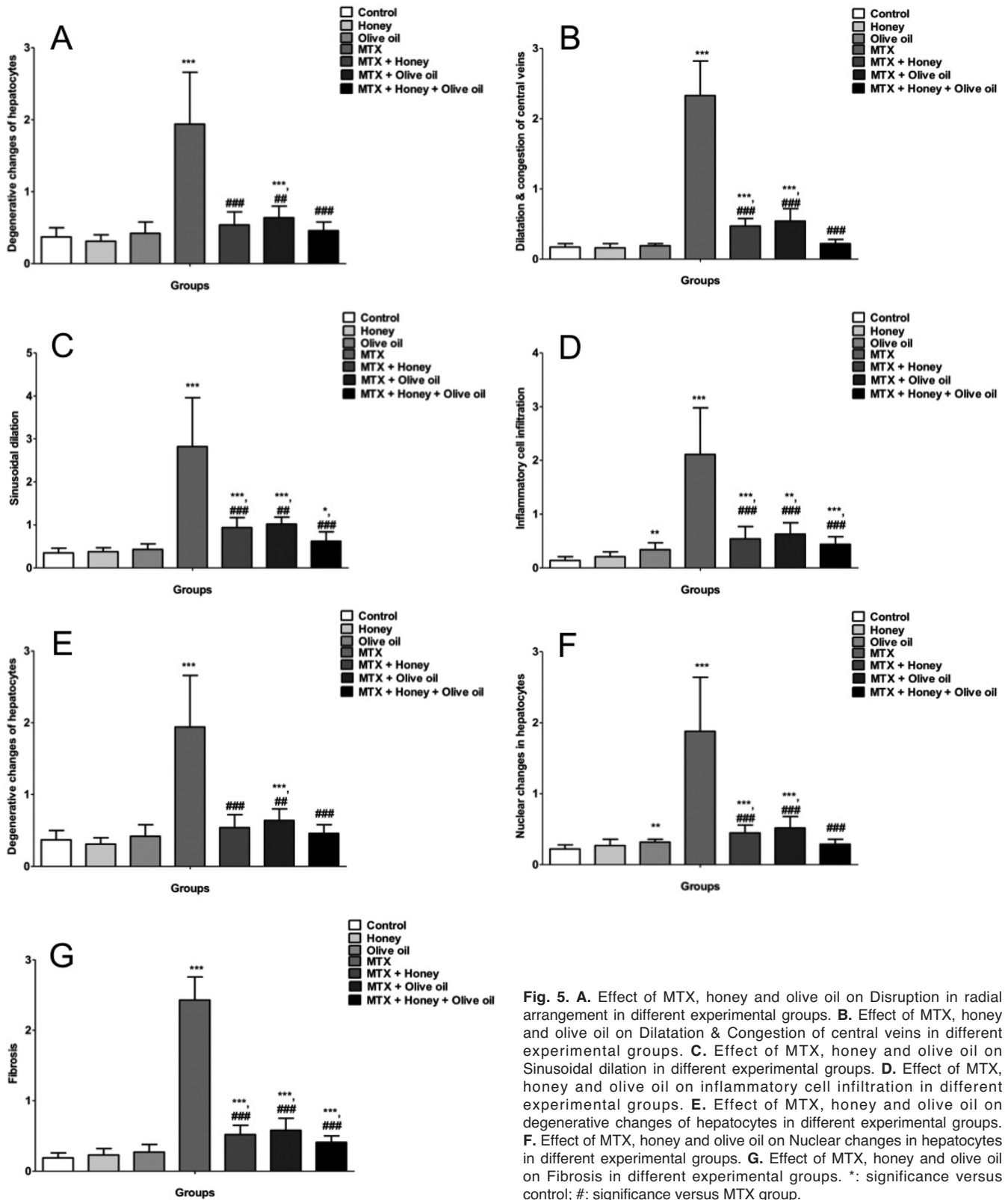
Fig. 5. A. Effect of MTX, honey and olive oil on Disruption in radial arrangement in different experimental groups. **B.** Effect of MTX, honey and olive oil on Dilatation & Congestion of central veins in different experimental groups. **C.** Effect of MTX, honey and olive oil on Sinusoidal dilation in different experimental groups. **D.** Effect of MTX, honey and olive oil on inflammatory cell infiltration in different experimental groups. **E.** Effect of MTX, honey and olive oil on degenerative changes of hepatocytes in different experimental groups. **F.** Effect of MTX, honey and olive oil on Nuclear changes in hepatocytes in different experimental groups. **G.** Effect of MTX, honey and olive oil on Fibrosis in different experimental groups. *: significance versus control; #: significance versus MTX group.

appeared almost like in the control group, with regular arrangements of the hepatic cords of the hepatocytes radiating from the central vein. Most of the hepatocytes appeared normal in shape and had euchromatic nuclei.

An examination of the MT-stained liver sections from the different groups showed that in the control, honey and olive oil groups (Fig. 7A) there were few connective tissue fibres (stained blue) in the portal spaces and around the central veins. In the MTX group (Fig. 7B), there was a marked increase in the amount of connective tissue in the portal areas, which appeared dilated around the central veins. However, in the MTX groups co-treated with only honey or olive oil (Fig. 7C,D), there was a noticeable decrease in the amount of connective tissue, especially in the portal areas and around the central veins, compared to the MTX group. Moreover, in the MTX group co-treated with both honey and olive oil (Fig. 7E), there was a marked decrease in the connective tissue as control.

An examination of the PAS-stained liver sections from different groups showed that the control rats (Fig. 8A) had a normal distribution of glycogen granules, which was seen as a strong positive magenta staining in the cytoplasm of most of the hepatocytes. In the MTX
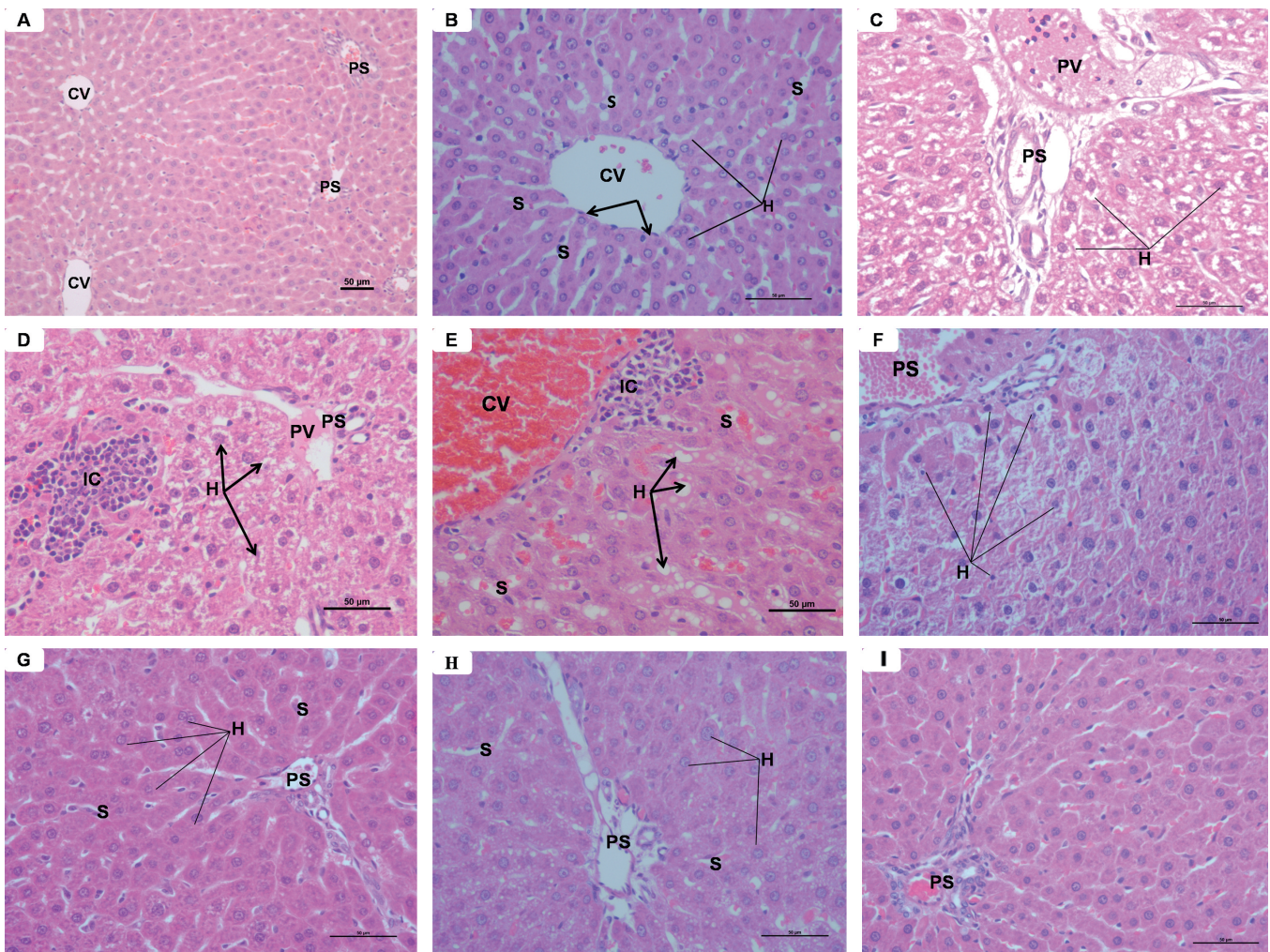

Fig. 6. Light photomicrographs of H&E-stained liver sections. **A, B.** (control groups): showing normal histological architecture of the liver including central vein (CV), portal spaces (PS) and narrow blood sinusoids (S), hepatocytes (H). **C.** (MTX group): showing periportal area with dilated portal space (PS) and marked congestion of portal vein (PV) with many vacuolated hepatocytes (H). **D.** (MTX group): showing periportal area with dilated portal space (PS) and inflammatory cell infiltration (IC) with many vacuolated hepatocytes (H). **E.** (MTX group): showing centrilobular area with marked congestion of the central vein (CV) and inflammatory cell infiltration (IC) and dilated congested sinusoids (S). **F.** (MTX group): showing periportal area with dilated portal space (PS) and hepatocytes with pyknotic nuclei (H). **G.** (MTX + honey group): showing improvement of the above changes with little portal spaces dilation. **H.** (MTX + olive oil group): showing improvement of the above changes with little portal space dilation. **I.** (MTX + honey + olive oil group): showing nearly normal hepatic architecture with normally appearing portal space (PS) and central veins (CV) and blood sinusoids (S). (H&E). A, x 200; B-I, x 400.

group (Fig. 8B); there was a marked decrease in glycogen in most of the hepatocytes compared to the control livers. In the MTX group co-treated with only honey or olive oil (Fig. 8C,D), the glycogen content of the hepatocytes was slightly more than in the MTX group, but still less than in the control livers. Moreover, in the MTX rats co-treated with both honey and olive oil (Fig. 8E), there was a noticeable increase in the amount of glycogen in the hepatocytes, but still less than in the control livers.

The immunohistochemical localization of caspase-3 in the liver sections from different groups showed that in the control rats (Fig. 9A) the hepatocytes were weakly stained; the MTX group (Fig. 9B) exhibited strong expression of caspase-3 in many hepatocytes compared to the control group, indicating the presence of many apoptotic hepatocytes. In the MTX groups co-treated with only honey or olive oil (Fig. 9C,D), the localization of caspase-3 was weakly expressed in the liver compared to the MTX group. Moreover, in the MTX groups co-

treated with both honey and olive oil (Fig. 9E), very weak localization of caspase-3 was seencontrol group.

Discussion

MTX is frequently used to treat various types of malignancies and chronic inflammatory diseases; however, its application is usually limited by its life-threatening side effects, the most severe of which are hematopoietic suppression, pulmonary toxicity and hepatotoxicity. The prevalence of MTX-induced liver damage in patients may be as high as 50% (Sener et al., 2006; Vardi et al., 2010; Fan et al., 2011; El-Sheikh et al., 2015).

We demonstrate that MTX treatment reduced body and liver weight and food intake. These results are supported by earlier findings by Pérez et al. (Yozai et al., 2005) and Cetinkaya et al. (Uraz et al., 2008), who found that injecting rats with MTX resulted in significantly decreased body weight gain, diet consumption and food
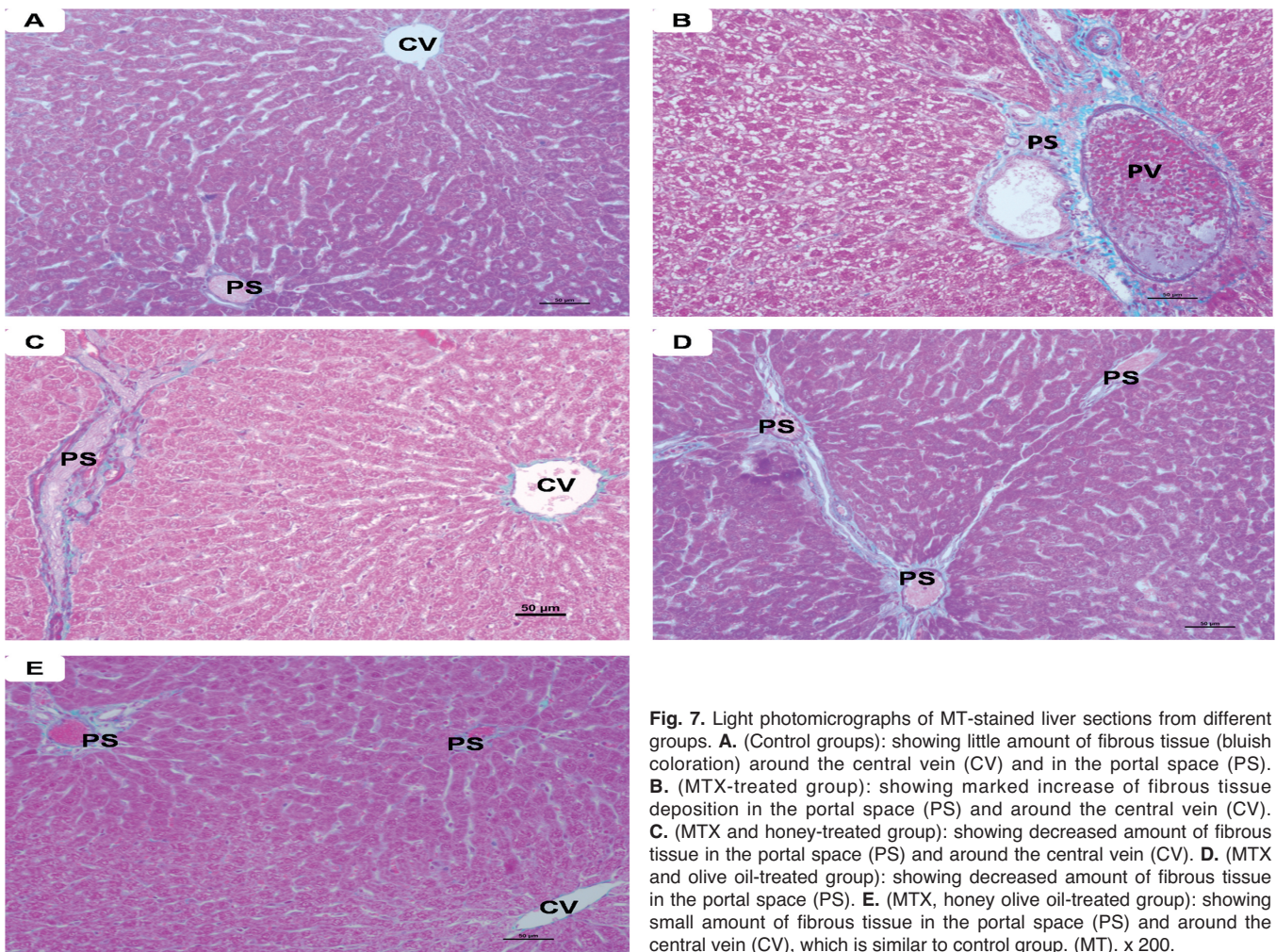

Fig. 7. Light photomicrographs of MT-stained liver sections from different groups. **A.** (Control groups): showing little amount of fibrous tissue (bluish coloration) around the central vein (CV) and in the portal space (PS). **B.** (MTX-treated group): showing marked increase of fibrous tissue deposition in the portal space (PS) and around the central vein (CV). **C.** (MTX and honey-treated group): showing decreased amount of fibrous tissue in the portal space (PS) and around the central vein (CV). **D.** (MTX and olive oil-treated group): showing decreased amount of fibrous tissue in the portal space (PS). **E.** (MTX, honey olive oil-treated group): showing small amount of fibrous tissue in the portal space (PS) and around the central vein (CV), which is similar to control group. (MT). x 200.

effectiveness ratio compared to the control group. These authors explained these results on the basis of gastrointestinal toxicity and diarrhoea after MTX administration. However, present work, co-treating rats co-treated with honey combined MTX showed good body weight gain and liver weight compared to rats treated with MTX alone. These results are supported by earlier studies finding that natural honey contains high-energy constituents in the form of carbohydrates and phenolic compounds (Havsteen, 2002). Similarly, Motallebnejad et al reports that patients treated with MTX and honey lost significantly less weight than patients treated with MTX alone (Motallebnejad et al., 2008). The current study also found that body weight gain and liver weight were improved substantially by the co-administration of olive oil with MTX compared to the MTX group. These findings correlate with those of Atakisi et al, who concluded that the omega-3 fatty acids in olive oil can have a significant prophylactic effect against the toxic effects of MTX (Atakisi et al., 2013). In

this study, the administration of MTX resulted in a highly significant increase in the levels of liver enzymes (ALP, AST and LDH) and bilirubin as well as a decrease in serum albumin. These results supported by Ali et al., who found that MTX treatment leads to severe hepatotoxicity, provoking a notable elevation in the serum activities of these enzymes, especially after a high dose or chronic use (Ali et al., 2014). Similar abnormal liver functions have been reported in other studies, in which dose adjustments were recommended during long-term treatment with MTX (Tunali-Akbay et al., 2010). Furthermore, these enzymes have been identified as sensitive biomarkers of liver injury; their increased activity in the serum indicate a leakage in the cell membrane, which is related in hepatocellular injury and dysfunction (Hafez et al., 2015). The serum bilirubin and albumin levels also correlate to the functions of hepatic cells; a rise in bilirubin level is one of the most important clinical indications of the severity of liver necrosis. Total serum protein levels can also provide
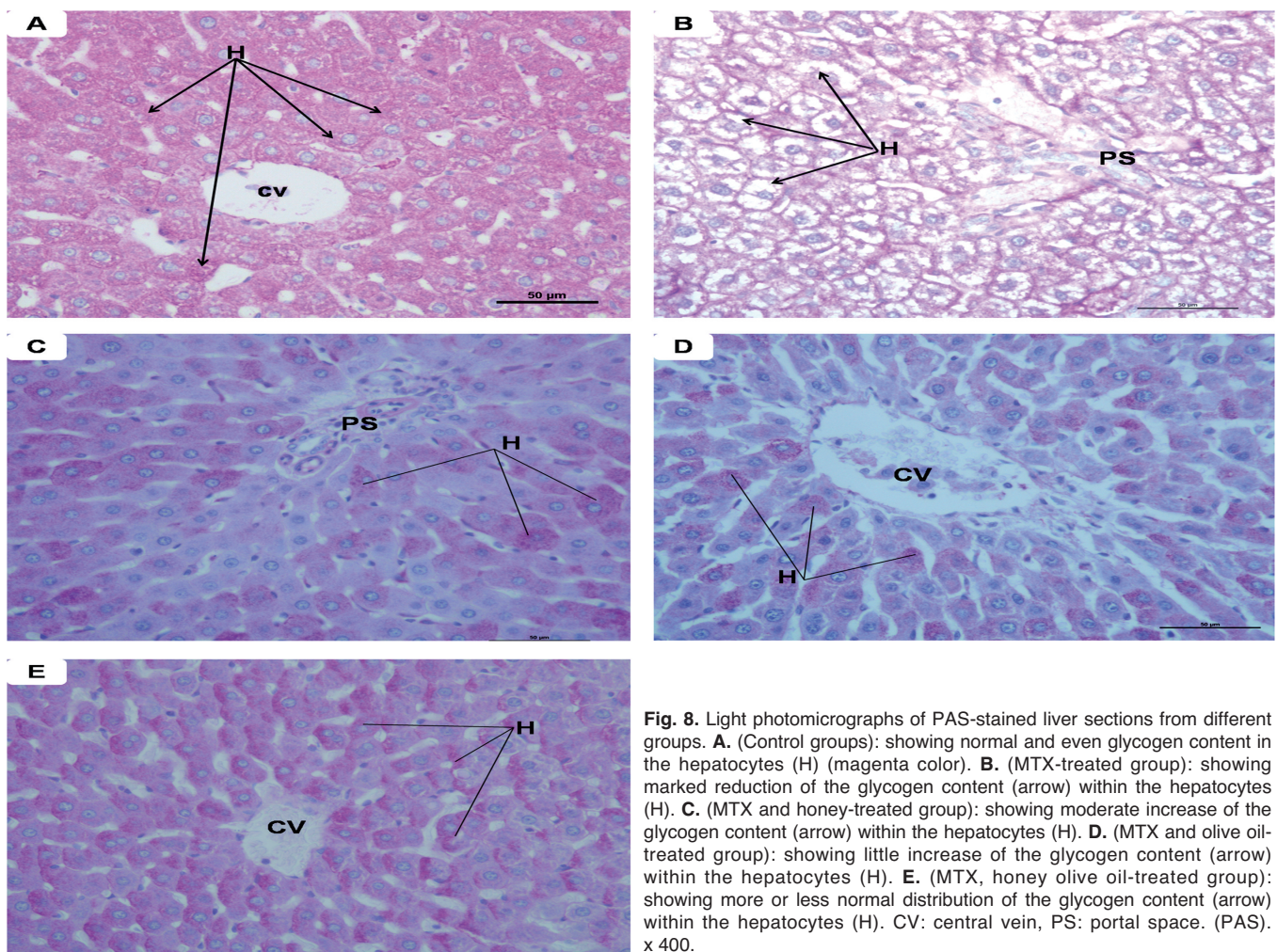

Fig. 8. Light photomicrographs of PAS-stained liver sections from different groups. **A.** (Control groups): showing normal and even glycogen content in the hepatocytes (H) (magenta color). **B.** (MTX-treated group): showing marked reduction of the glycogen content (arrow) within the hepatocytes (H). **C.** (MTX and honey-treated group): showing moderate increase of the glycogen content (arrow) within the hepatocytes (H). **D.** (MTX and olive oil-treated group): showing little increase of the glycogen content (arrow) within the hepatocytes (H). **E.** (MTX, honey olive oil-treated group): showing more or less normal distribution of the glycogen content (arrow) within the hepatocytes (H). CV: central vein, PS: portal space. (PAS). x 400.

information about the severity of liver diseases or necrosis.

Co-administration of honey along with MTX improved all of the above-mentioned parameters, indicating the honey's role in protecting the liver. In the present study, honey exhibited a hepatoprotective effect, evidenced by a decrease in ALT and AST and by a histopathological examination of the liver. This result is consistent with a previous study in which honey was observed to have a potent hepatoprotective action in oxidative stress and liver toxicity in rats, demonstrated by a significant decrease in serum ALP and AST and total bilirubin levels in rats with hepatotoxicity (Poudyal et al., 2010). It seems that one reason for the therapeutic properties of honeybee products is their function as an antioxidant. The administration of olive oil along with MTX also led to significant decreases in ALT, AST and ALP and total bilirubin levels associated with liver injury. These enzymes are used to assess the status of liver damage and are considered more sensitive

parameters to measure liver injury in rodents. Similarly, Poudyal et al, also reports the ability of olive oil to protect liver cells from damage by some toxic agents (Poudyal et al., 2010). Numerous mechanisms for MTX toxicity have been proposed, and inflammation, apoptosis and oxidative stress have all been suggested as contributing factors (Cakir et al., 2011; Vardi et al., 2013). The present study showed that MTX administration resulted in increased lipid peroxidation, which is a consequence of imbalance between prooxidant and antioxidant mechanisms. This was indicated by increased MDA levels as well as a decrease in SOD, CAT, GPx in the liver tissue. These findings are in accordance with numerous previous studies establishing that MTX caused free radicals in tissue as demonstrated by increased MDA levels and decreased SOD activity (Cetin et al., 2008; Uraz et al., 2008; Gautam et al., 2016). Similar results have been recorded before, showing that MTX significantly alters the balance of oxidants and antioxidants, which, in turn,
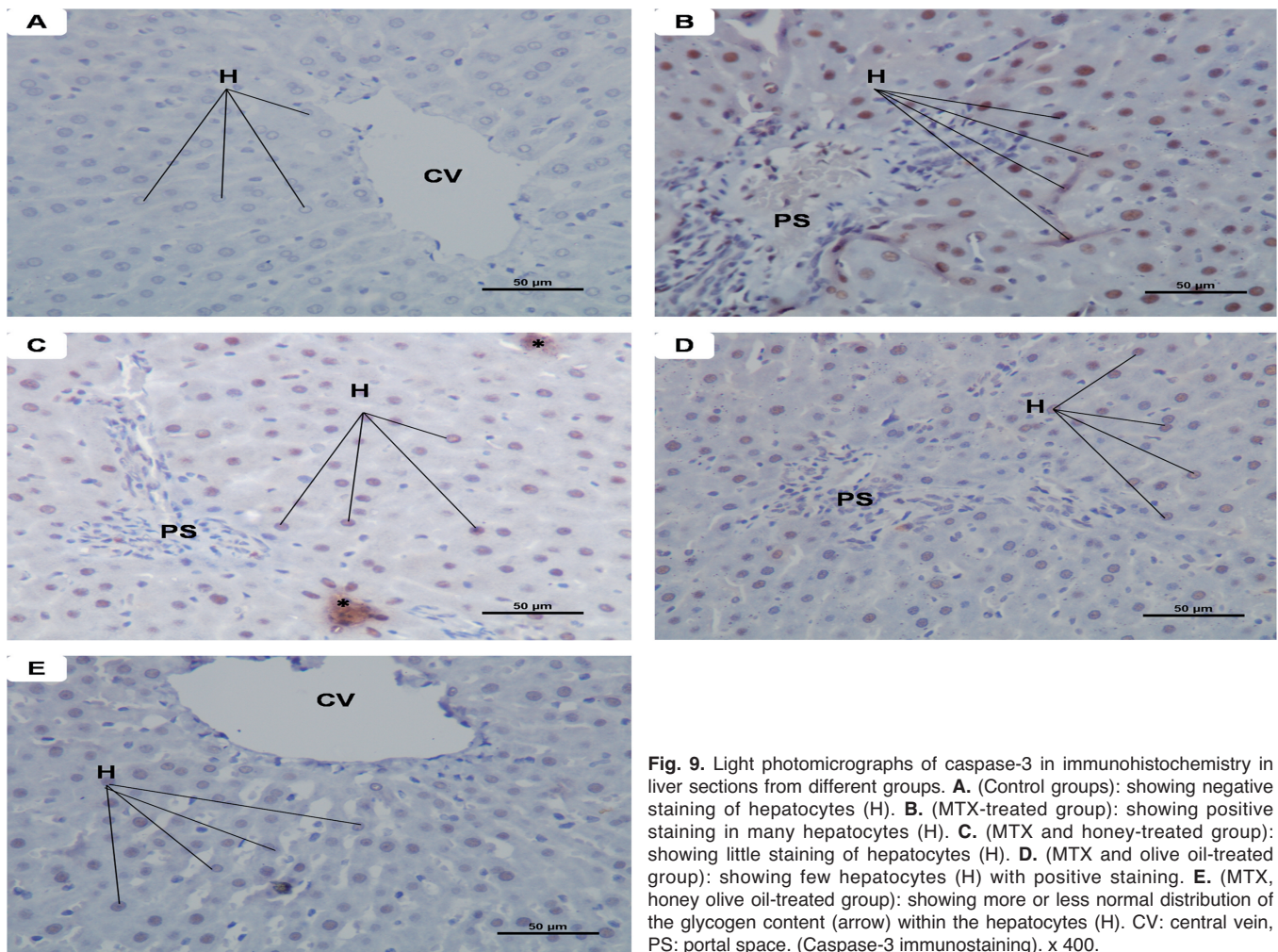

Fig. 9. Light photomicrographs of caspase-3 in immunohistochemistry in liver sections from different groups. **A.** (Control groups): showing negative staining of hepatocytes (H). **B.** (MTX-treated group): showing positive staining in many hepatocytes (H). **C.** (MTX and honey-treated group): showing little staining of hepatocytes (H). **D.** (MTX and olive oil-treated group): showing few hepatocytes (H) with positive staining. **E.** (MTX, honey olive oil-treated group): showing more or less normal distribution of the glycogen content (arrow) within the hepatocytes (H). CV: central vein, PS: portal space. (Caspase-3 immunostaining). x 400.

leads to overproduction of ROS, promoting the development and progression of hepatotoxicity (Weiss and Landauer, 2003; Jahovic et al., 2004). The increased ROS formation and decreased antioxidant protection causes oxidative stress, resulting in structural changes in bio-membranes, loss of liver integrity, decreased metabolic activity, and finally cell death (Ali et al., 2014).

In the present study, co-treatment of MTX with honey provided protection to the liver from LPO to a significant degree, as evidenced by reduced MDA levels. This protective role of honey corresponds to findings by Galal et al., stated that honey decreased the liver lesions induced by acetaminophen (Galal et al., 2012). Honey's powerful antioxidant properties could explain its notable protective effect in every parameter examined in the current study (Perez et al., 2006). Honey is full of enzymatic and non-enzymatic antioxidants (Bogdanov et al., 2008). Also, recent reports have demonstrated the antioxidant capacity of honey in in vivo and in vitro studies, concluding that honey contains natural antioxidants formed from phenolic compounds and flavonoids such as kaempferol, chrysin, pinobanksin, quercetin, luteolin, pinocembrin, apigenin, genistein, hesperetin and naringenin (Illam et al., 2017; Famurewa et al., 2018).

In the current study, the MTX group co-treated with olive oil showed a significant reduction in MDA levels, indicating a decreased rate of lipid peroxidation. Moreover, the anti-hepatotoxic effect of olive oil was evidenced by an amelioration of the activities of antioxidative enzymes SOD, CAT and GSH in the liver homogenate. Similarly, Chainy et al. found that olive oil could be used as an effective radical scavenger to protect the livers of experimental animals from chemical injuries (Chainy et al., 2000). According to Gorinstein et al., olive oil recovers lipid metabolism and rasies antioxidant potential (Gorinstein et al., 2002). The hepatoprotective effect of olive oil might be attributed due to phenolic compounds (Gorinstein et al., 2002). These phenolic compounds minimize oxidative stress and DNA fragmentation; the potency of these antioxidants contributes to the beneficial properties of olive oil (Covas et al., 2006; Al-Alawi et al., 2017).

The biochemical changes in this study were supported by histopathological changes and by the results of the morphometric analysis, which revealed marked hepatic injury in the MTX group compared to the control rats. The MTX group exhibited marked hydropic and vascular degeneration as well as nuclear changes in many hepatocytes that included inflammatory cell infiltration and congestion. The vacuolation of the liver cells could be explained by the accumulation of toxic metabolites, which damaged the cell membranes, leading to subsequent hydropic degeneration and vacuolation of the hepatocytes (Al-Ali et al., 2005). Consistent with these findings, Piggott et al. (2011) stated that MTX hepatotoxicity is the most important potential side effect of MTX treatment (Piggott et al.,

2011). Other studies have found similar results, especially with in vivo high doses or chronic administration of MTX (Hemeida and Mohafez, 2008; Uraz et al., 2008; Kose et al., 2012). In the current study, there was also a marked increase of fibrous tissue deposition in the portal spaces after MTX injection. This observation corresponds to those of Lopez-Miranda et al, suggested that perisinusoidal fibrosis occurs because of MTX toxic, and Hytiroglou et al has been reported hepatic fibrosis caused by MTX in patients and that this condition might progress to cirrhosis (Hytiroglou et al., 2004; Lopez-Miranda et al., 2010). Several other studies have also demonstrated the risk of MTX-induced hepatic fibrosis, especially in the portal and periportal areas (Chan and Cronstein; 2010, Tsai et al., 2013). In the current study, the glycogen content in the hepatocytes was reduced after MTX injections, which has also been reported previously, along with the explanation that glycogen reduction is due to the inhibition of mitochondrial metabolism and to the inability of the liver to store glycogen (Tsai et al., 2013).

The present study also found clear MTX-induced apoptosis, as shown by an increase in caspase-3 expression in the hepatocytes compared to the control livers. Caspase-3 has been main factor for triggering apoptosis in human cells (Salvesen and Dixit, 1997); it also plays a vital role in the treatment of apoptosis and is therefore a common topic of research (Salvesen and Dixit, 1997). Previous studies have demonstrated that MTX caused apoptosis through caspase activity in cancer cell lines (Chen et al., 2009; Fan et al., 2012, Wehner et al., 2013). It has also been reported that the caspase-3 activation indicating apoptosis than the detection of secondary processes (Duan et al., 2003). The increased oxidative stress caused by MTX may lead to changes in the shape and structure of the nucleus by causing DNA fragmentation and denaturation, both of which play critical roles in the initiation of apoptosis (van Swelm et al., 2013).

In conclusion, the present study is the first to examine the effects of combined usage of honey and olive oil for protecting against and ameliorating the toxic side effects of some chemotherapeutic drugs widely used in clinical practise where these two agents successfully improved the oxidative stress markers, liver function parameters and the histomorphological hepatic changes, especially when administered together. This effect is most probably due to the antioxidant phenolic compounds and other antioxidants present in honey and olive oil. These results provide new evidence for the hepatoprotective effects of honey and olive oil (especially when combined) on biochemical and structural MTX-induced liver damage, indicating that these two agents have a synergistic effect. Clinically, the application of these valuable natural products, honey and olive oil might be a useful adjuvant therapy alleviating the complications of chemotherapy in cancerous patients, which make them suffer even more than the primary lesions. Further studies will be needed to trace

the beneficial effect of these two products in other organ toxicity e.g., kidney, heart and immune organs. Further studies need to understand the molecular mechanisms of combined supplementation of honey and olive oil.

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