

Ameliorative Effect of Propolis Against Hepatorenal Alterations Induced by Methotrexate: Morphohistopathological Study

Efecto Protector del Propóleo en las Alteraciones Hepatorrenales Inducidas por el Metotrexato: Estudio Morfohistopatológico

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SUMMARY: Methotrexate (MTX) is widely used in the treatment of some forms of cancer but having severe side effects. The present work aimed to investigate the protective role of propolis treatment against alterations induced by MTX on the hepatic and renal tissues. Rabbits were exposed to MTX (0.25 mg/kg), with or without propolis (50 mg/kg) while hepatic and renal biopsies were examined for histological and histochemical abnormalities. Methotrexate induced hydropic degeneration, pyknosis, sinusoidal dilatation and bile duct hyperplasia in the liver together with renal tubular degeneration, glomerular shrinkage and hyaline droplet precipitation. While propolis partially ameliorated some of the morphometric and biochemical alterations, none of the hepatic alterations induced by MTX was protected by propolis treatment. Nevertheless glomerular shrinkage and renal tubule degeneration were partially protected in animals received both MTX plus propolis. It is concluded that propolis treatment has little or no ameliorative effect in protecting the hepatic and renal tissues from MTX toxicity.

KEY WORDS: Histological alterations; Kidney; Liver; Methotrexate toxicity; Propolis.

INTRODUCTION

Methotrexate is invested in treatment of several disorders including some types of malignancy (Bennett & Brown, 2003; Watanabe *et al.*, 2011). This drug inhibits DNA synthesis, slows down the growth of rapidly dividing cells and reduces the activity of several enzymes including dihydrofolic acid reductase. However, MTX is known to cause hepatotoxicity and renal damage by inducing oxidative stress and weakening the antioxidant defense (Novaes *et al.*, 1996; van Ede *et al.*, 1998; Hopwood & Nyfors, 2004). Methotrexate was reported to induce hepatocytes fatty changes and sinusoidal lining cells, mild necrosis and inflammation (Maskaleris *et al.*, 1998; Hemeida & Mohafez, 2008). Several previous studies indicated that hematological, hepatic and renal toxicity are frequent with MTX treatment (Sotoudehmanesh *et al.*, 2010; Gaies *et al.*, 2012).

Propolis is natural resinous material of different plants consisting of many natural compounds and collected by honeybees (Khalil, 2006; Valadares *et al.*, 2008). This crude has been invested for long time by different nations in

compacting many diseases due to its antioxidants content (Lee *et al.* 2000; Ota *et al.*, 2001; de Almeida & Menezes, 2002). In addition, propolis is being used as immune system stimulant agent due to its ability as free radicals scavenger (Urgur & Arslan, 2004). Moreover, this natural product was reported to help in tissue regeneration and blood capillaries strengthening (Lejeune *et al.*, 1988).

Propolis contribution as food and therapy supplement makes many people justify that this natural product can be used as supplement for cancer patients and can treat or prevent cancer (Galvao *et al.*, 2007). It is not unusual to see patients using propolis and posting information about its ability in diseases fighting and cancer prevention (De Almeid & Menezes 2002; Galvo *et al.*, 2007; Li *et al.*, 2010; El-Mazoudy *et al.*, 2011). In addition, some investigators intrigued by propolis potential to give better results in controlling the adverse effects of cancer therapy (Badr *et al.*, 2011; Watanabe *et al.*). Caffeic acid phenethyl ester, an active ingredient of propolis could slow tumor growth by

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singling networks that control the growth of the cancer cells in breast, bladder and prostate cancers (Chuu *et al.*, 2012). Moreover, propolis flavonoids showed cytotoxic activity against fibrosarcoma and lung adenocarcinoma (Li *et al.*). In addition, some studies reported that propolis could exert anticarcinogenic properties effects by immunomodulating action (Orsolich *et al.*, 2005; Ozkul *et al.*, 2006; Ishihara *et al.*, 2009; Sforcin & Bankova, 2011).

The present work was carried out to find out if propolis can ameliorate hepatorenal histological and histochemical alterations induced by MTX.

MATERIAL AND METHOD

Experimental Subjects. Thirty-two healthy adult male rabbits weighing 950-1050g, were used in the present study. The animals had free access to water and pellets.

Drugs. Methotrexate injections (Product of Ebewe Pharma, Austria) was invested in the present study.

Propolis. Commercial water soluble propolis crude in the form of capsules (1000 mg) manufactured by Marnys Spanish Company (Spain) and legally imported by Saudi Arabian Dug Store Ltd. (Saudi Arabia) was used. Its active ingredients were identified by the quality control of the manufacturer to contain the following: Phenolic acids (caffeic acid, tocopherol, sinapic acid, cinnamic acid, coumaric acid and ferulic acid) and flavonoids (quercetin, kaempferol, rutin and apigenin) together with amino acids and vitamins.

Propolis dissolve was prepared immediately before use by dissolving capsules content in distilled water (50 mg/ml).

Experimental Protocol. Thirty-two rabbits were divided into four groups and were exposed to the used drugs as follows:

Group 1 (Control group): Each member of this group received a daily single i.m. injection of one ml normal saline for 35 days.

Group 2 (MTX group): Each member of this group received a daily single i.m. injection of MTX (0.25 mg/kg) for 35 days.

Group 3 (Propolis-MTX group): Each member of this group received a single a daily i.m. injection of MTX (0.25 mg/kg) plus oral dose of propolis (50 mg/kg) for 35 days.

Group 4 (Propolis only group): Each member of this group received daily 50 mg/kg propolis during the period of experimentation.

All experiments were carried out according to protocol approved by the ethical committee of King Saud University. Moreover, all animals were treated and handled according to the international guidelines for care and use of laboratory animals.

Morphometric Observation. Daily observation throughout the study was made for mortality, general well being and behavior patterns of all animals under study.

Body Weight Monitoring: The rabbits body weight was monitored at the beginning of treatment and after 35 days of treatment.

Percentage Absolute Liver Weight and Liver Index: Rabbits were euthanized and the liver together with the kidneys were taken from each rabbits immediately for the determination of the percentage absolute liver weight. The grade of change in the organ index induced by methotrexate doses was calculated according to the following equation:

$$\text{Organ index} = \frac{\text{Average weight of the experimental organs/}}{\text{Average weight of the experimental animals}}$$

$$\frac{\text{Weight of the control organs/weight of the control animals}}$$

Biochemical Analysis. Blood samples were collected from all rabbits under study. Serum was separated by centrifugation and were analyzed for the following biochemical parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, and creatinine.

Histological Processing. Fresh portions from the right kidney including the cortex and medulla together with portions from the median lobe of the liver of each rabbit were taken and used for histological processing. Tissue processing was carried out by automatic Tissue Processor (Thermo Shandon Company). The samples were fixed in 10% neutral buffered formalin, dehydrated with ethanol, cleared with chloroform, wax impregnated, embedded and blocked with melted paraffin wax. Paraffin sections (4-5 μm) from all experimental rabbits of all groups were applied for hematoxylin and eosin (H&E) stain together with some special stains (Jarrar & Taib, 2008). Stained histological sections were tested and viewed for histological abnormalities in the hepatic and renal tissues of all rabbits used in the present study.

Statistical Analysis. Statistical analysis was done by using independent t-test and the results of the morphometric and biochemical analysis were expressed by the average \pm sd. The p-value < 0.05 was considered statistically significant.

RESULTS

No mortality occurred in any of the experimental groups of the present study. In comparison with the control animals, rabbits exposed to MTX (0.25 mg/kg) for 35 days demonstrated diarrhea, reddening of the skin, dark urine and tarry fecal matter.

Morphometric Alterations. Alterations in the body weight of rabbit exposed to MTX for 35 days are seen in Table I. One may observe that MTX exposure affected significantly (p value <0.05) the body weight gain, while propolis administration didn't influence the body weight gain. On the other hand, animals subjected to MTX treatment only did not demonstrate body weight gain.

The liver average weight and the percentage absolute liver weight to control one together with the change in the liver index are seen on Table II. There was no significant difference (p value > 0.05) in the liver and kidney index between MTX, propolis and the control group.

Biochemical Alteration. As seen in Table III and in comparison with the control rabbits and the ones exposed to propolis only, MTX treatment for 35 days lowered total proteins but elevated creatinine, AST and ALT (p value < 0.001-0.05). Levels of ASL, ALT and creatinine were higher in MTX plus propolis rabbits than the control ones. Moreover, ASL, ALT in rabbits exposed to MTX plus propolis were less significant (p value <0.05) in comparison with those treated with MTX only where partial amelioration was demonstrated by propolis combination with the drug.

Table I. Change on the average body weight (g) of rabbits subjected to overdoses of MTX for 35 days.

Dose	Starting Weight	Weight after 35 days	P-value
Control group (Normal saline)	1068 ± 98	1217 ± 112	* 0.013
Received MTX 0.25 mg/kg)	1077 ± 109	1042 ± 114	0.54
Received MTX and propolis (0.25 mg/kg MTX, 50 mg/kg propolis)	1082 ± 119	1074 ± 117	0.89
Received propolis only (50 mg/kg)	1093 ± 122	1242 ± 118	*0.026

“*” Represents statistically significant (p-value < 0.05) using student t test.

Table II. Change on the relative ratio of liver and kidneys weight to body weight of rabbits subjected to overdoses of MTX for 35 days.

Dose	Average organ weight (g)	Average body Weight (g)	The percentage absolute organ weight	Organ index
Control group (Normal saline)	Average liver weight(g)	59.9±2.6	4.92±0.53 %	Lx 1
	Average kidneys weight(g)	29.1±0.9	2.39±0.32 %	Kx 1
Received MTX 0.25 mg/kg)	Average liver weight(g)	51.1± 3.3	4.90±0.53 %	Lx 0.99
	Average kidneys weight(g)	26.8±1.2	2.57±0.32 %	Kx 1.07
Received MTX and propolis (0.25 mg/kg MTX, 50 mg/kg propolis)	Average liver weight(g)	54.5±2.2	5.07±0.55 %	Lx 1.03
	Average kidneys weight(g)	25.3±1.7	2.35±0.41 %	Kx 0.98
Received propolis only (50 mg/kg)	Average liver weight(g)	63.7±2.1	5.12±0.64 %	Lx 1.04
	Average weight of both kidneys (g)	28.2±0.9	2.27±0.35 %	Kx 0.94

Table III. Biochemical analysis of the rabbits under study.

Biochemical test	Control rabbits	Rabbits received MTX	Rabbits received MTX plus propolis	Rabbits received propolis
AST (IU/l)	23.4±4.4	***70.5±9.7	†*36.3±5.2	18.8±2.8
ALT	11.4±2.9	33.8±6.7***	*19.3±4.7†	9.07±1.9
Total Protein (g/dl)	8.6±1.05	***4.58±0.65	7.2±0.76	8.37±0.97
Creatinine (mg/dl)	0.54±0.11	***1.73±0.26	1.42±0.19**	0.62±0.12

“*”, “**” and “***” represent statistically significant p-value < 0.05, <0.01 and <0.001, respectively, in comparison with control group.

“†” represents statistically significant p-value < 0.05, in comparison with the MTX group.

Hepatic Histological Alteration. Examination of the hepatic tissues of all rabbits used in the present study showed the followings:

Liver of the Control Rabbits. All members of the control group demonstrated normal hepatic structures concerning the hepatocytes, lobules architecture and the portal spaces (Fig. 1 a-b).

Liver of Rabbits Treated with MTX. The following abnormalities were seen in the hepatic tissues of rabbits exposed to MTX (0.25 mg/kg) for 35 days:

Hepatocytes Cytoplasmic Vacuolation: The liver of this group of rabbits demonstrated hepatocytes vacuolation with swelling and cytoplasmic clearings (Fig. 2a).

Hydropic Degeneration: Hepatocytes hydropic degeneration were seen in all rabbits of this group (Fig. 2b).

Sinusoidal Dilatation: All members of this group of animals showed sinusoidal dilatation together with Kupffer cell activation and enlargement. In addition, this alteration was accompanied by widening of capillaries lining the hepatic strands (Fig. 2c).

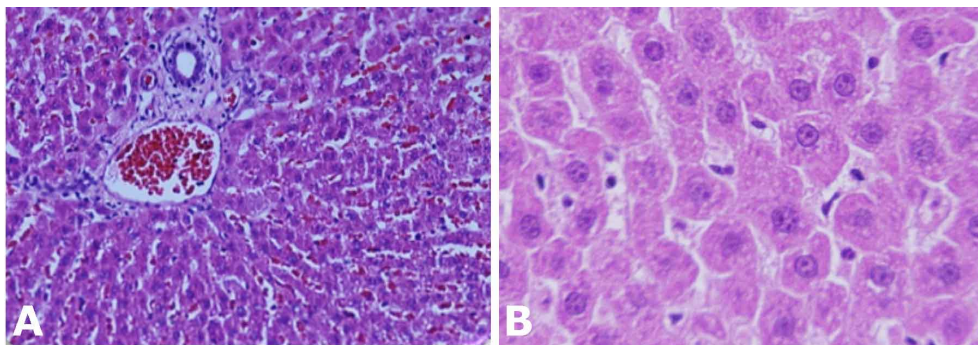


Fig. 1. Microphotograph sections in the liver of control rabbits demonstrating: (a) Normal hepatic architecture (b) Normal hepatocytes.

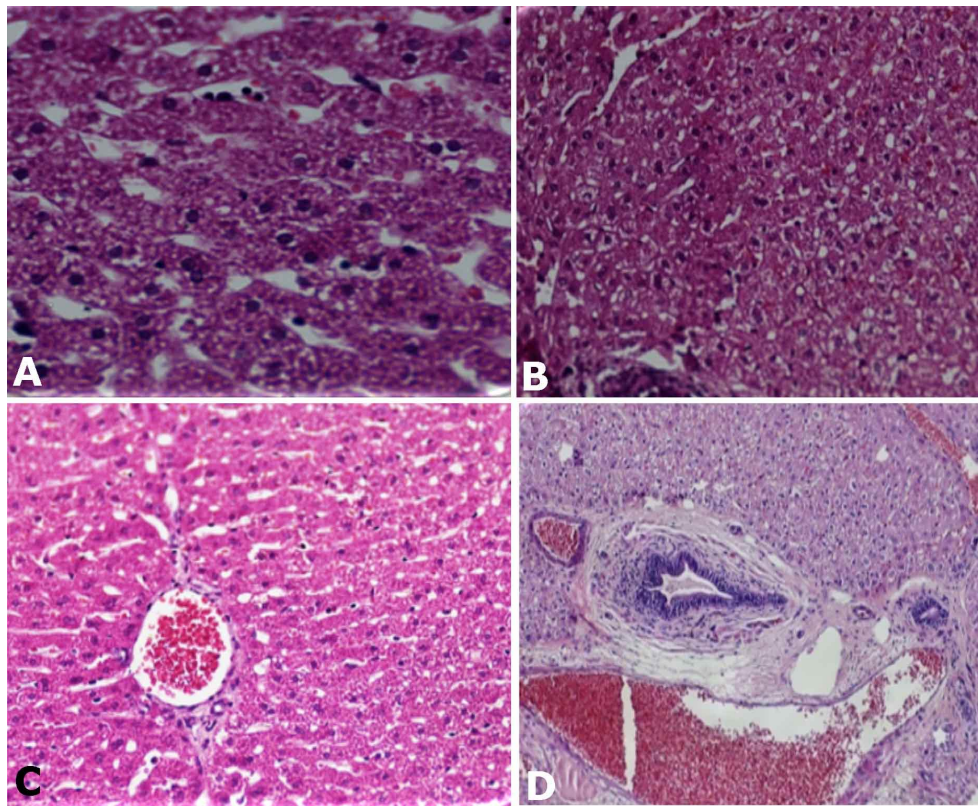


Fig. 2. Microphotograph sections in the liver of rabbits treated with MTX (0.25 mg/kg) demonstrating: (a) Hepatocyte cytoplasmic vacuolation together with pyknotic hepatocytes, H&E stain. (b) Hepatocytes hydropic degeneration, H&E stain. (c) Sinusoidal dilatation and pyknotic nuclei, H&E stain. (d) Bile duct hyperplasia and proliferation, H&E stain.

Karyopyknosis: Some hepatocytes showed condensed nuclei with irregular nuclear margin (Fig. 2c).

Bile Duct Hyperplasia: Hyperplasia and proliferation was identified in the bile ducts of all members of MTX treated rabbits (Fig. 2d).

Liver of Rabbits Treated with MTX Plus Propolis

The following abnormalities were detected in the liver of rabbits treated with MTX (0.25 mg/kg) plus propolis (50 mg/kg) for 5 weeks.

Hydropic Degeneration: The hepatocytes of the rabbits received MTX plus propolis showed almost hydropic degenerative cytoplasmic vacuolation similar to that seen in the animals received MTX only (Fig. 3a).

Karyopyknosis: The hepatocytes of this group of rabbits showed no protection from pyknosis that was seen in the hepatocytes of rabbits treated with MTX only (Fig. 3b).

Bile Duct Proliferation: Proliferation and hyperplasia of the bile duct together with peribiliary mononuclear inflammatory aggregation was observed in the liver of this group of rabbits. Less sinusoidal dilatation was seen in the rabbits of this group in comparison with the liver of animals received MTX only (Fig. 3c).

Liver of Rabbits Treated with Propolis Only. The hepatic lobular architecture and zonal accentuation of rabbits subjected to propolis (50 mg/kg) only were well preserved and kept intact and demonstrated normal hepatocytes, sinusoids and hepatic portal triads (Fig. 4).

Renal Histological Alterations

Kidneys of the Control Rabbits. Both cortex and medulla of the control rabbits kidneys showed normal glomeruli together with normal proximal and distal renal tubules (Fig. 5a-b). Loops of Henle and the collecting tubules also showed normal pattern.

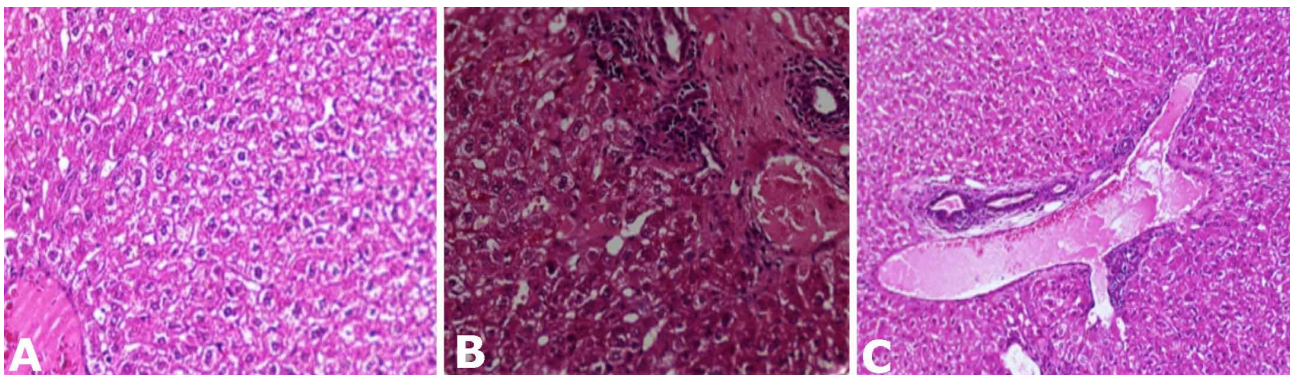


Fig. 3. Microphotograph of sections in the liver of rabbits treated with MTX (0.25 mg/kg) plus propolis (50 mg/kg) demonstrating: (a) Hydropic degeneration, H&E stain. (b) Karyopyknosis and cytoplasmic vacuolation, H&E stain. (c) Hyperplasia of the bile duct and proliferation together with peribiliary mononuclear inflammatory aggregation, H&E stain.

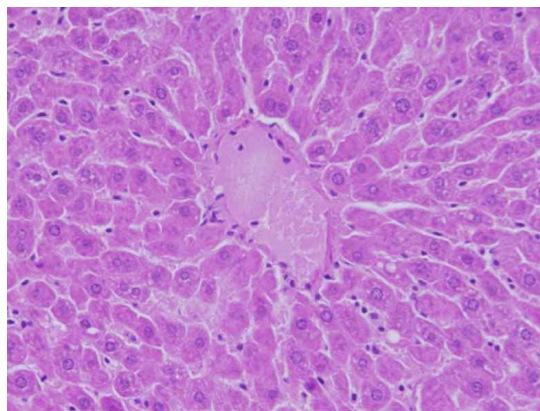


Fig. 4. Microphotograph of section in the liver of rabbit subjected to propolis demonstrating normal lobular architecture and normal hepatocytes, H&E stain.

Kidneys of Rabbits Exposed to MTX

Rabbits received MTX (0.25 mg/kg) for 5 weeks showed the following renal histological abnormalities:

Glomerular Shrinkage: Glomerular atrophy was visible in renal tissue of rabbits of this group where the shrunken tuft occupied small portion of Bowman's space (Fig. 6a).

Renal tubular Degeneration: Renal tubules mild degeneration was demonstrated with proximal tubules were more affected than the distal ones (Fig. 6b).

Hyaline Droplets Precipitation: Hyaline droplets formation was visible in the epithelial lining of some distal nephritic tubules (Fig. 6c).

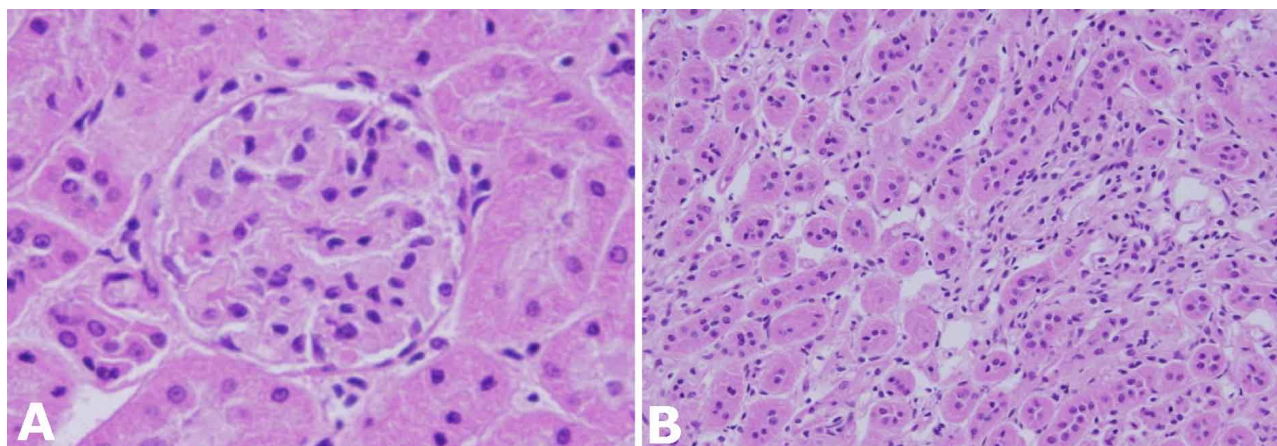


Fig. 5. Microphotograph sections in the kidney of control rabbits demonstrating: (a) Normal glomerular and normal interstitial tissues, H&E stain. (b) Normal renal tubules, H&E stain.

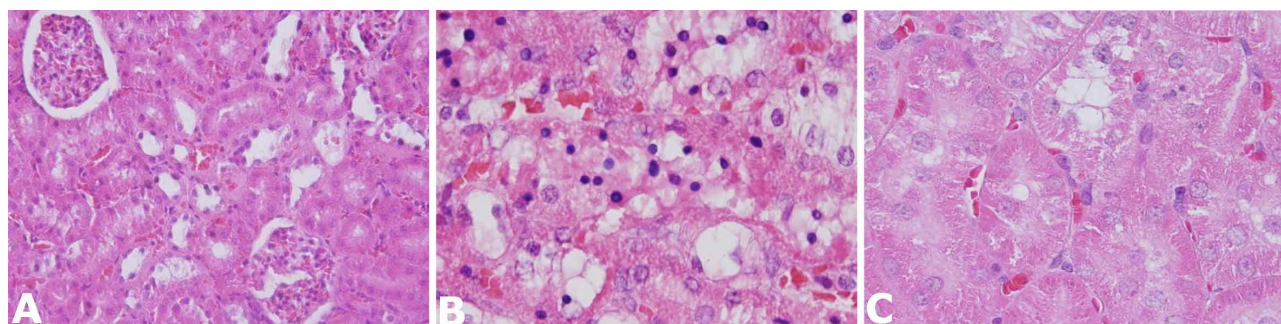


Fig. 6. Microphotograph of sections in the kidney of rabbits treated with MTX showing: (a) Shrinkage of glomerular tuft with renal degeneration, H&E stain. (b) Tubular necrosis, H&E stain. (c) Hyaline droplets formation, H&E stain.

Kidneys of Rabbits Treated with MTX Plus Propolis. Less prominent glomerular shrinkage was demonstrated in the kidneys of this group of rabbits in comparison with those treated with only MTX (Fig. 7a). Prominent renal tubules necrosis and degeneration were also observed in the renal tissues of this group (Fig. 7b).

Kidneys of Rabbits Treated with Propolis Only. No glomerular, tubular or interstitial alterations were seen in the renal tissues of rabbits received propolis (50 mg/kg) for 5 weeks (Fig. 8).

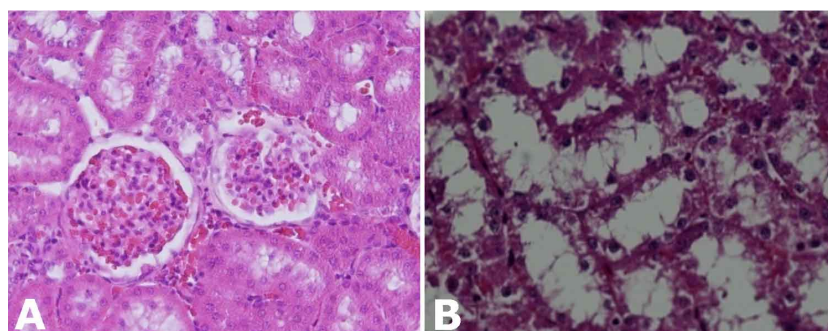


Fig. 7. Microphotograph of sections in the kidney of rabbits treated with MTX plus propolis exhibiting: (a) Mild glomerular shrinkage, H&E stain. (b) Tubular degeneration and necrosis, H&E stain.

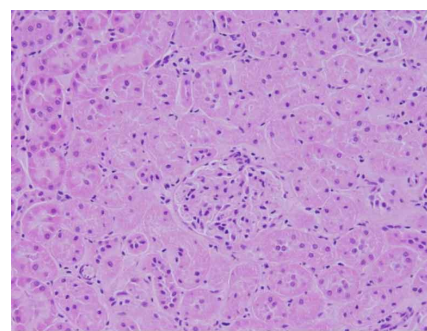


Fig. 8. Microphotograph of section in the kidney of rabbit treated with propolis only exhibiting normal glomerular, tubular and interstitial tissues, H&E stain.

DISCUSSION

Methotrexate is used in the treatment of several malignancies and inflammatory disorders (Badr *et al.*; Gais *et al.*, 2012). This chemotherapeutic agent can suppress DNA synthesis by inhibiting dihydrofolate reductase, the key role player in methionine and purine synthesis (Galivan, 1980; Gaies *et al.*). However, it was found that MTX could cause oxidative stress in hepatic and renal tissues (van Ede *et al.*; Walker *et al.*, 2000).

The morphometric findings of the present work showed a decline in body weight due to MTX exposure while propolis failed to ameliorate this alteration. This might indicate an oxidative stress on the pathway of the metabolic enzymes and the immunological status induced by MTX while propolis showed no protective amelioration from these alterations.

The findings of the present investigation indicated that MTX treatment lowered total proteins but elevated creatinine, AST and ALT. The concentration of the liver enzymes ALT and AST is used as an indicator for liver functions and in detecting hepatocytes injury, while ALT is primarily released during liver damage and hepatocellular necrosis (Singh *et al.*, 2011). In addition, blood total proteins concentration is often reduced slightly during hepatocellular damage (Thapa & Walia, 2007; Singh *et al.*). Furthermore, elevation of creatinine level indicates a deficiency in the filtering function of the kidney. The findings of this study showed that propolis combination with MTX reduced significantly the elevation in AST and ALT and normalized significantly the total protein level. Moreover, propolis showed little amelioration to creatinine level induced by MTX but failed to reach statistical significant (p value > 0.05). These biochemical findings may indicate that propolis could partially ameliorate the biochemical alterations induced by MTX.

The findings of the present study showed that MTX could induce hepatic hydropic degeneration, pyknosis, sinusoidal dilatation and bile duct hyperplasia together with renal tubular degeneration, glomerular shrinkage and hyaline droplet precipitation. Some of these alterations were reported by other investigators where Roenigk *et al.* (1988) observed hepatorenal fatty infiltration, nuclear changes, inflammation and necrosis. The induced histopathological alterations might be resulted from folate stores depletion, one of the main side effect of MTX in the hepatic tissues. In addition, the seen renal tubular degeneration induced by MTX might be resulted from

precipitation of this drug and its metabolites in the epithelial lining of the tubules due to their relatively insolubility in acidic urine (Donehower *et al.*, 1979; Smeland *et al.*, 1996).

The results of the present study indicated that the severity in the liver damage and to lesser extent in the renal tissues of rabbits exposed to MTX plus propolis were still evident. This may indicate that the protective activity of propolis could not compensate the toxicity induced by MTX in the hepatic and renal tissues. Some investigations indicated that propolis could exhibit hepatoprotective effects by protecting mitochondria against oxidative damage (Guimarães *et al.*, 2012).

Propolis contains a group of antioxidants including flavonoids, phenolic acid esters, terpenes, cinnamic acid and others. The antioxidant activity of propolis showed evidences of protective role against oxidative stress caused by free radicals oxidative stress under normal conditions. Propolis exhibits capability of combating against lipid peroxidation that can impair cellular structure and functions due to its scavenging free radicals protection role (Bergamini *et al.*, 2004; Yousef & Salama, 2009). This crude can also activate superoxide dismutase and catalase that have capability against free radicals (Jasprica *et al.*, 2007). Some previous studies found that the phenolic contents of propolis had an ability to inhibit membrane free radical formation (Russo *et al.*, 2006; Kanbur *et al.*, 2009). The results of the present work indicated that propolis could not protect the renal tissue from injury induced by MTX. Some studies reported that the antioxidant properties of propolis could involve in the protection of renal tissues from damage by some toxicants (Conde de la Rosa *et al.*, 2006). In addition, some reports indicated that propolis has therapeutic potential and protective role against the toxicity of several chemical compounds such as aluminum chloride and environmental pollutants (Sarabia *et al.*, 2009; Yousef & Salama; Yousef *et al.*, 2010; El-Mazoudy *et al.*). Moreover, propolis was reported to ameliorate the MTX-induced ileum injury and biochemical alterations towards normal and showed slight protection in the hepatorenal histological alterations induced by MTX during treatment of Ehrlich carcinoma (Badr *et al.*; Abdul-Hamid & Salah, 2016). In addition, propolis extract was found to have protective effect against doxorubicin and cypermethrin induced toxicity (Eraslan *et al.*, 2008; Singla *et al.*, 2014). Furthermore, some previous studies reported an inhibitory activity to the crude of Egyptian propolis (El-khawaga *et al.*, 2003).

CONCLUSION

One can conclude from the finding of the present study that bee propolis has little or no ameliorative role in modulating the hepatic and renal injury induced by MTX toxicity.

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ALMANSOUR, M. I.; JARRAR, Y. B.; ALOYAIDY, K. A. & JARRAR, B. M. Efecto protector del propóleo en las alteraciones hepatorenales inducidas por el metotrexato: estudio morfohistopatológico. *Int. J. Morphol.*, 35(2):756-764, 2017.

RESUMEN: El metotrexato (MTX) es ampliamente utilizado en el tratamiento de algunas formas de cáncer, pero tiene efectos secundarios graves. El presente trabajo tuvo como objetivo investigar el papel protector del tratamiento con propóleo frente a las alteraciones inducidas por el MTX en los tejidos hepático y renal. Se expusieron conejos a MTX (0,25 mg / kg), en grupos con y sin propóleo (50 mg / kg), y se realizaron biopsias hepáticas y renales, que fueron examinadas buscando anomalías histológicas e histoquímicas. El metotrexato indujo la degeneración hidrópica, picnosis, dilatación sinusoidal e hiperplasia del conducto biliar en el hígado, junto con la degeneración tubular renal, la contracción glomerular y la precipitación hialina. Mientras que el propóleo parcialmente mejoró algunas de las alteraciones morfo métricas y bioquímicas, ninguna de las alteraciones hepáticas inducidas por MTX fue protegido por el tratamiento con propóleo. Sin embargo, la contracción glomerular y la degeneración de los túbulos renales fueron parcialmente protegidos en animales que recibieron MTX más propóleo. Se concluye que el tratamiento con propóleo tiene poco o ningún efecto mejorador en la protección de los tejidos hepáticos y renales sometidos a la toxicidad de MTX.

PALABRAS CLAVE: Alteraciones histológicas; Riñón; Hígado; Toxicidad del metotrexato; Propóleos.

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