Ameliorative Effect of Propolis Extract on Hepatotoxicity Induced by Methotrexate in Mice

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ABSTRAC--- ***-Propolis, rich in natural antioxidant flavonoids. It is produced by honey bee (Apis melifera ligutica). Our study aimed to evaluate the ameliorative effects of propolis water extract on liver toxicity induced by methotrexate. The study used thirty-five male albino mice randomly divided into five groups. The first is control (C); the second, Methotrexate-induced hepatotoxicity (MTX, single dose 20 mg/kg IP); Third, Propolis water extract (PWE, 100 mg/kg PO for 7 days) and both fourth and fifth groups are Proplis Before/After Methotrexate, PBM and PAM; respectively). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyle transferase (γ-GT), C-reactive protein (CRP) and hepatic histopathology estimated the protective effect of propolis against the liver injury induced by MTX. Catalase (CAT) and superoxide dismutase (SOD) evaluated the antioxidant activities in liver tissue. In additions, the immune efficacy of Propolis was determined by liver interleukins levels (IL4, IL10, IL1 and IL6). The results revealed that propolis treatment before injection of methotrexate have significant hepatoprotective, antioxidant and enhancing immune liver response compared to PAM group. The study, assumes in line with the fact that propolis is a natural, low cost and nontoxic product possesses ameliorative activities against hepatic alterations induced by MTX.***

Keywords---- Propolis, Methotrexate, liver, antioxidants, cytokines

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1. INTRODUCTION

The clinical efficacy of the widely used chemotherapeutic drug methotrexate (MTX) is limited due to its associated liver toxicity. The liver organ, is a vital organ which maintain the homeostasis and control many critical functions of the body (Kivity et al., 2014). Different natural flavonoids include rutin, quercetin, galangin and caffeic acid phenethyl ester in propolis have protective effect on chemotherapy-induced toxicity and reduce the adverse effects on normal tissues (Roth and Ganey, 2011, Hassan et al., 2015 and Akyol et al., 2016). Therefore, our study used flavonoids-rich propolis against methotrexate-induced hepatotoxicity in mice.

Propolis is a natural product which is collected and used produced by honeybees (Rizk, et al., 2014). It has been used for years in folk medicine due to its therapeutic and pharmacological properties (Hassan et al., 2015, Wali et al., 2015 and Akyol et al., 2016). Propolis contain hundreds of polyphenols, terpenoids, steroids, amino acids and vitamins, these constituents have numerous pharmacological effects, especially anti-inflammatory, antioxidant, antitumor and anticancer activities (Erdogan et al., 2011 and El Sohaimy and Masry, 2014). Caffeic acid phenethyl ester is one of major compounds in propolis that has immunomodulatory and hepatoprotective activities (Akyol et al., 2012 and Akyol et al., 2016). Propolis has free radical scavenging properties can induce the activation the antioxidant enzyme system (Talas et al., 2012 and Sönmez et al., 2016). These medicinal properties of propolis are directly related to its polyphenols which have been proved to scavenge free radicals and act as a strong cytoprotective natural product against any exogenous toxicity (Rizk et al., 2014 and Campos et al., 2015). It was observed that all propolis exhibited enhancing immune activities (El-Aidy et al., 2015 and Salas et al., 2016).

Methotrexate, a structural analogue of folic acid, is used to treat malignancies and some autoimmune disease, but at high doses or long used preferred as a cytotoxic chemotherapeutic agent (Barker et al., 2011 and Duman et al., 2013). MTX is associated with a risk of hepatotoxicity and tissue fibrosis, which looks to be related to its metabolic pathway in the liver (Arenaa et al., 2012). MTX belongs to the antimetabolite group, which mechanism is involved in folate

antagonism. Its major toxicity side effects are neutropenia, anemia and stomatitis (Swelm et al., 2013). The metabolite of MTX in the liver is 7 hydoxy methotrexate which can induce liver stress and toxicity (Chladek et al., 1997). Moreover, methotrexate depletes NADPH as a result of inhibition of the enzymes NAD (P) dependent dehydrogenases and NADP malic enzyme (Johovic et al., 2003). Depletion of NADPH by methotrexate decreases the sensitivity of hepatocytes against oxidative stress there by the propensity to cause hepatotoxicity and fibrosis. (Sener et al., 2006 and Schmiegelow, 2009). The histological effects of MTX-induced hepatic toxicity characterized by fatty changes inflammations, hepatocytes necrosis, fibrosis and cirrhosis (Arenaa et al., 2012).

In the present study, we are reporting the hepatoprotective, antioxidants potential and immune-enhancing effects of propolis water extract against the side effects of methotrexate-induced liver damage.

2. MATERIAL AND METHODS

The present study was carried on (35) Swiss male adult albino mice, weighing (45±50g). Animals housed in cages with free access to water and food, at temperature of 22 ± 2°C, on a 12-hour light/dark cycle. The experiment was performed in accordance with the Guidelines for Ethical Care and Use of Experimental Animals by the Ethical Committee of Science of King Faisal University methotrexate powder was purchased from Sigma, St Louis, MO, USA. Pure propolis powder capsules each contain 500 mg, California Gold Nutrition, USA.

2.1 Preparation of PWE:

Propolis extract was prepared according to Oršolić & Bašić, (2005) method. Under sterile conditions 5 g of powder of propolis was dissolved in 500 ml distilled water and well mixing for 10 min forming suspension which was centrifuged at 1,000 rpm for 10 min at room temperature. The supernatant was collected and stored under freezing condition at −20 °C until use. Each animal was given 0.5 ml PO for 7 days.

2.2 Experimental Design.

First group: Control group (C): free water

Second group: Methotrexate-induced hepatotoxicity group (MTX): Animals intraperitoneally injected with single dose of methotrexate 20 mg/kg b.w. according to Moghadam et al., 2015.

Third group: Proplis Water Extract group (PWE): Animals treated with oral 100mg/kg b.w. of PWE for 7 days according to El-Naggar et al., 2015.

Fourth and Fifth groups: Propolis Before/After Methotrexate; PBM/PAM: Animals treated with aforementioned doses.

2.3 Biochemical Assay. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes determined according to Retiman and Frankel (1975) as described by the manufacturer's manual (Randox Laboratories Ltd). Serum Alkaline phosphatase activity (ALP) was determined using clorimetric method according to Belfield and Goldberg (1971) and γ- glutamyl transferase (γ- GT) activity by using Pointe, Scientific, INC, USA of Whitfield et al. (1973) method. Serum mouse Creative reactive protein (CRP) Therm Fisher Scientific Catalog number (ERCRP).

2.4 Antioxidant Assay: Different pieces of liver tissue was homogenized in a solution containing 5% tricolor acetic acid and 5 mM EDTA at 4 ºC using Glass-Teflon homogenizing tube and centrifuged at 2500 r/min for 10 min for estimation Superoxide Dismutase (SOD) following the method of Minami and Yoshikawa (1979) and Catalase (CAT) activity acoording to Aebi (1983) method.

2.5 Cytokine Assay. Estimation of liver cytokines levels of IL-1β, IL-6, IL-4 and IL-10 in mouse by colorimetric ELISA (TMB substrate) in a clear 96-well strip plate. Absorbance at 450nm minus absorbance at 550nm, Using Mouse ELISA Kits, Thermo Fisher Scientific according to the manufacturer's instructions. Assay IL-6 (mouse interleukin-6) Catalg NO. EM2IL6, Assay IL- beta (mouse interleukin-1 beta) Catalog NO. KMC0011, Assay IL-4 (mouse interleukin-4) Catalog NO. EMIL45 and Assay IL-10 (mouse interleukin-10) Catalog NO.EM2IL10.

2.6 Histopathological examination:

A small pieces of liver fixed in 10% neutral buffered formalin for 24 hours using then embedded in paraffin and sectioned at 3 and 5 µm thick and stained with haematoxylin and eosin (H&E) according to the method of Bancroft and Gamble (2008). The tissue sections were then blindly examined by a light Olympus microscope provided with camera at magnification 100 (BX 51, USA).

2.7 Statistical Analysis

Data expressed as means ± SE. Statistical analysis evaluated by one-way ANOVA. Once a significant F test obtained, LSD comparisons performed to assess the significance of differences among various treatment groups ($P < 0.05$). Statistical Processor System Support "SPSS" for Windows software, Release 20.0 (SPSS, Chicago, IL) was used.

3. RESULTS AND DISSCUSSION

3.1 Serum AST- ALT-ALP and γ-GT values:

In Table 1. The present data showed Male Swiss albino mice exposed to MTX (20 mg/kg body weight) exhibited distinct markers of toxicity such as increased serum activities of enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ-glutamyle transpeptidase (γ-GT). While treatment with

propolis (PBM and PAM) was modulated the effect of MTX. AST and ALT are the major critical liver enzymes involved in the metabolism of amino acids and electron transport chain (Maiti et al., 2004 and Wali et al., 2015). Elevation in serum enzymes activities in MTX treated group, reflecting leakage in plasma membrane permeability of hepatocytes. The present data is in harmony with (Fu et al., 2008 and De et al., 2015). The results of the most recent studies indicated that flavonoids are biologically active compounds that have significantly effect on reducing the serum levels of ALT, AST and γ-GT (Madrigal-Santillán et al., 2014). MTX treatment increase the intracellular hepatocytes polyglutamate amount and decrease the folic acid amount causing liver damage (Sener et al., 2006). Flavonoids are the major pharmacological polyphenols constituents give propolis its hepatoprotective activity (Babatunde et al., 2015). The study explored the protective role of Propolis extract (PWE) in ameliorating MTX-induced hepatic damage. Significant increase in the level serum CRP level was represented in table.1 in MTX group and PAM groups as compared to control, PWE and PBM groups. A prototypical acute C-reactive protein (CRP) is a sensitive and nonspecific systemic inflammatory marker synthesized and released by the liver under the stimulation of cytokines such as TNF- α and IL-6 during inflammation (Pepys and Hirschfield, 2003).

3.2 Liver Antioxidants (Catalse and Superoxide dismutase):

Superoxide dismutase (SOD) and catalase (CAT) are the common antioxidant enzymes in liver tissue, which are involved in ROS scavenging. Where, SOD converts superoxide radical to hydrogen peroxide, which converted into H_2O and O_2 by catalase (CAT) enzyme (Johansen et al., 2005). In our study, in table 1., the data showed reduction in SOD and CAT activities in the group treated with methotrexate compared to healthy group, on the other hand, elevation occurred in all groups treated with (PBM) compared to both MTX and PAM groups. The present data are agreement with (Jasprica et al., 2007 and Hemeida and Mohafez, 2008). Methotrexate increase the free radicals in the cells, which inhibit antioxidant immune systems and reduce SOD and CAT levels, and so the oxidative stress may harm the lipids, proteins, carbohydrates and DNA (Sönmez et al., 2016). The antioxidant activities of propolis dut to its polyphenolic components, which are related to its ability to scavenge Reactive Oxygen species (ROS) and to play an important role as hepatoprotective (Wided et al., 2014, Mossa et al., 2015 and El-Naggar et al., 2015). The major principal toxicity of MTX is believed to its stored metabolite polyglutamated form in hepatocytes. High levels of polyglutamates causes intracellular accumulation of MTX, which suggested being as a mechanism for hepatotoxicity (Hemeida and Mohafez, 2008 and Kose et al., 2012).

Table 1. Serum and liver markers showing the effect of propolis (100 mg/kg for 7 days, PO) on methotrexate (20 mg/kg/ single dose, IP)-induced hepatotoxicity in mice.

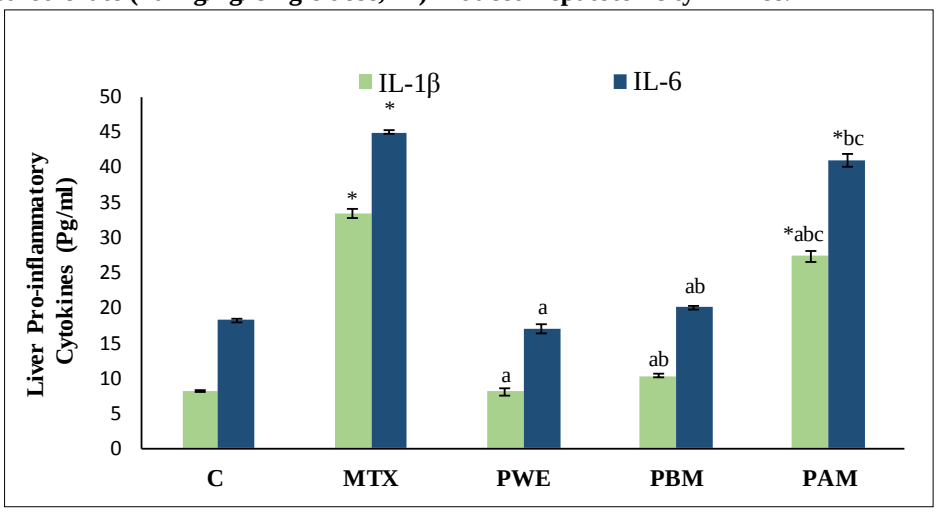
Tissue	Groups / Item	C	MTX	PWE	PBM	PAM
Serum	ALT (U/L)	23.12±1.41	43.71±1.06*	24.03±1.14^{a}	28.37±0.44^{a}	33.03±1.22^{*ab}
	AST (U/L)	42.33±2.22	77.40±1.10*	42.54±2.11^{a}	54.36±1.4^{*ab}	63.93+1.44^{*abc}
	ALP (U/L)	31.23±0.33	50.22±0.55*	30.06±0.54^{a}	35.44±0.12^{a}	42.21±0.13^{*bc}
	γ- GT (U/L)	43.0±1.81	98.96±1.74*	45.31±2.41^{a}	44.86 ± 0.11^{ab}	64.43±1.22^{*abc}
	CRP (ng/ml)	2.08±0.13	8.32±0.23*	2.22±0.45^{a}	3.44±0.86^{a}	5.32±0.95^{*bc}
Liver	SOD (U/mg protein)	5.33±0.46	1.44±0.33*	5.2±0.55^{a}	3.55±0.37^{*ab}	1.21±0.03^{*bc}
	CAT (U/mg protein)	1.2±0.93	0.3±0.37*	1.0±0.66^{a}	0.9±0.78^{a}	0.2±0.84^{*bc}

Data expressed as mean ± SE (n=7). Significance ($p < 0.05$) between groups represented by superscripts as (*) significant as compared to control (C) group, (^{a}) significant as compared to Methotrexate induced hepatotoxicity (MTX) group, (^{b}) significant as compared to propolis water extract (PWE) group, (^{c}) significant as compared to propolis before methotrexate (PBM) group.

3.3 Liver pro inflammatory (IL-1β-IL-6) and anti-inflammatory interleukins (IL-4-IL-10):

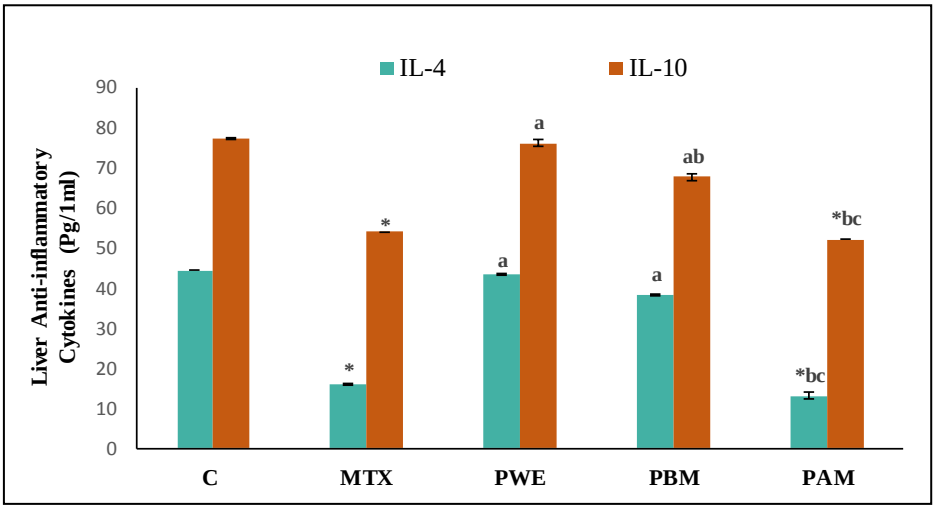
Liver immune response in propolis treated mice was significantly stimulated the secretion anti-inflammatory interleukins IL-4 and IL-10 at dose 100 mg/kg/body weight. Moreover, propolis could modulated the injured hepatic immune function in methotrexate-treated mice. These results (Figure 1 and Figure 2) showed elevation in IL-4, IL-10 and reduction in IL-1β and IL-6 levels in all groups treated with propolis compared to MTX group, suggesting that propolis possessed immunomodulating in liver and systemic immune system (Campos et al., 2015). These results are agreed with inflammatory response indicators; plasma TNF-α and IL-1β levels were also increased due to MTX administration (Lu et al., 2012 and Aslaner et al., 2015). 7 hydoxy methotrexate is reactive metabolite of Methotrexate. It modifies cellular macromolecules, which mediated intracellular oxidative stress and cytokine-mediated response that are important components in the pathophysiology of hepatotoxicity. Toxicity decrease adaptive response of cells leading to cell death. Furthermore, the intracellular stress pathways cause the sensitization of the proinflammatory and inhibition of anti-inflammatory cytokines (Fredriksson et al., 2014). Methotrexate can reduce the blood immunoglobulins on the other hand, it cause elevation of the serum liver enzymes and increase inflammation (Banji et al., 2011).

Figure 1. Liver proinflammatory cytokines IL-1β and IL-6 (Pg/ml) showing the effect of propolis (100 mg/kg for 7 days, PO) on methotrexate (20 mg/kg/ single dose, IP)-induced hepatotoxicity in mice.



Data were expressed as mean ± SE (n=7). Significance ($p < 0.05$) between groups represented by superscripts as (*) significant as compared to control (C) group, (^{a}) significant as compared to methotrexate induced hepatotoxicity (MTX) group, (^{b}) significant as compared to propolis Water Extract (PWE) group, (^{c}) significant as compared to propolis before methotrexate (PBM).

Figure 2. Liver anti-inflammatory cytokines IL-4 and IL-10 (Pg/ml) showing the effect of propolis (100 mg/kg for 7 days, PO) on methotrexate (20 mg/kg/ single dose, IP)-induced hepatotoxicity in mice.



Data were expressed as mean ± SE (n=7). Significance ($p < 0.05$) between groups represented by superscripts as (*) significant as compared to control (C) group, (^{a}) significant as compared to methotrexate induced hepatotoxicity (MTX) group, (^{b}) significant as compared to propolis water extract (PWE) group, (^{c}) significant as compared to propolis before methotrexate (PBM).

3.4 Liver histolopathology:

The histological changes induced by MTX treatment were confirmed biochemically. Liver enzymes release is considered a marker associated with cell death and necrosis (Rosen and Keeffe, 2000). Methotrexate (M)-induced sever pathological hepatocellular damage showing necrosis, vacuolar degeneration of hepatocytes, sinusoidal dilatation, congestion of central vein, lymphocyte infiltration associated with focal bleeding (Fig 3 B and C). The present investigations are in line with Ali et al., 2014 and Aslaner et al., 2015. In Fig 3 D, Light micrograph in propolis before methotrexate group (PBM) showing healing hepatocytes appearance and in Fig 3 E and F, Light micrograph in propolis after methotrexate group (PAM) showing vacuolar degeneration of hepatocytes and vascular congestion with mild hepatic hemorrhage. Treatments with propolis significantly modulated the toxic effects of MTX. Propolis polyphenols are of huge health benefits and known to possess remarkable antioxidant properties against MTX-cell death (Wided et al., 2014). Also, MTX reduce the blood immunoglobulins on the other hand, it elevate release the serum liver enzymes resulted to the damage of hepatocytes, and this was associated with central and lobular necrosis, and inflammatory cellular infiltrations (Nikolova et al., 1994 and Gowri Shankar et al., 2008). Moreover, MTX inhibits the folic acid metabolism resulting in impairing of DNA synthesis and leads to necrosis of hepatocytes (Tian and Cronstein, 2007 and Banji et al., 2011).

Figure 3. Light micrographs of liver sections showing the effect of Propolis (100 mg/kg for 7 days, PO) on methotrexate (20 mg/kg/single dose, IP)-induced hepatotoxicity in mice.

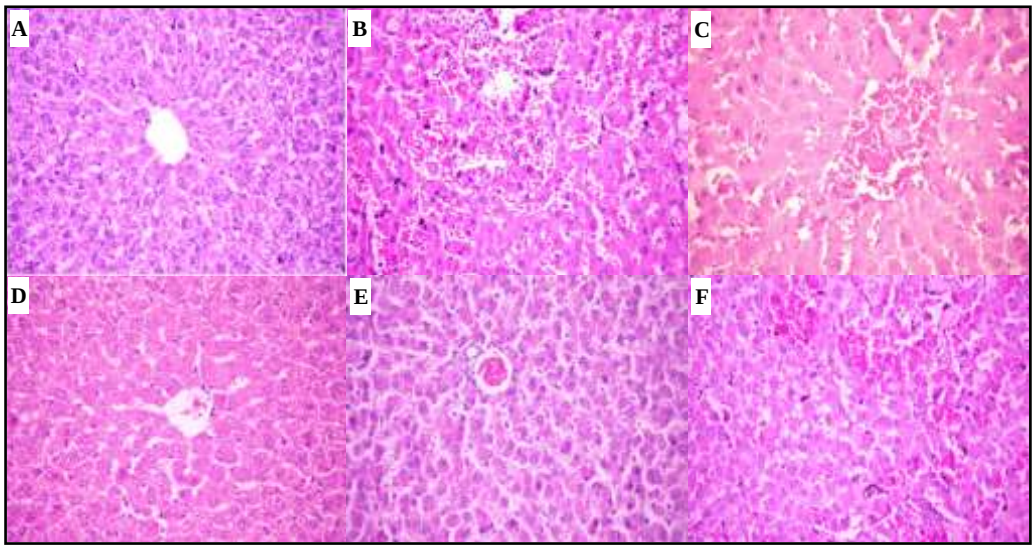


Fig A: Light micrograph in Control group (C) showing normal histological appearance of hepatic lobules. Fig (B and C): Light micrograph in Methotrexate (M)-induced hepatocellular damage showing necrosis, vacuolar degeneration of hepatocytes, sinusoidal dilatation, congestion of central vein, lymphocyte infiltration associated with focal bleeding. Fig D: Light micrograph in propolis before methotrexate group (PBM) showing healing hepatocytes appearance. Fig (E and F): Light micrograph in propolis after methotrexate group (PAM) showing vacuolar degeneration of hepatocytes and vascular congestion with mild hepatic hemorrhage H&E x10.

4. CONCLUSION

Propolis may be a potential adjuvant drug in MTX chemotherapy to reduce the hepatic side effects.

5. CONFLICT OF INTEREST

The author has declared that no conflict of interests exists.

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