

BENEFICIAL EFFECT OF PROPOLIS EXTRACT (BEE GLUE) AGAINST METHOTREXATE-INDUCED STRESS IN LIVER AND BRAIN OF ALBINO RATS

Fatma A. Khalil, Amal A. A. EL-Kirsh, Enas Ali Kamel and Nourhan Gamal EL-Rahmany
Biochemistry and Nutrition Department Faculty of Women for Arts, Science and Education Ain Shams University, Cairo, Egypt

Keywords:

Methotrexate, Propolis extract, Oxidative damage, Liver, Brain, Antioxidant enzymes, Rats

Abstract

Methotrexate (MTX) is an anti-folate drug that is widely used in the treatment of rheumatic disorders and malignant tumors. The efficacy of MTX is often limited by its severe side effects and toxic sequelae. Propolis (bee glue) is a natural bee product rich in polyphenolic compounds known for antioxidant activity. The present study was designed to evaluate the protective role of propolis (PP) extract against oxidative stress induced by methotrexate drug (MTX) on liver and brain of rats. The nutritive value and some main active polyphenolic compounds and antioxidant capacity of the propolis extract were evaluated. The total phenolic and flavonoid contents of propolis extract recorded in terms of mg gallic acid and catechin equivalent/100 ml extract were 1122.4 mg and 2885 mg respectively. In addition highly total antioxidant capacity presented as 178 mg ascorbic acid equivalent. Gas chromatography mass spectrometry (GC-MS) was performed for qualitative screening of phenolic compounds in the propolis. One hundred and twenty male Wistar albino rats with mean body weights $90 \text{ g} \pm 5 \text{ g}$ were classified into 5 groups. Rats were administered their respective doses of propolis extract and/or MTX for 3, 6 and 9 weeks intervals. Control groups (G1 and G2), propolis extract group (G3), MTX group (G4) and MTX plus propolis extract group (G5). Results suggested that co-administration of the PP extract with MTX reduced the deleterious effects of methotrexate, through the significant increase in concentration of reduced glutathione (GSH) and inhibition of lipid peroxidation as observed by significant decrease in malondialdehyde (MDA) level in brain tissue. As well as, significant increase in antioxidant enzyme activities (superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR)) in liver and brain tissues as compared to MTX group alone at $p < 0.05$ in a time dependent manner to approach the values found in control rats. Data showed that propolis has a powerful antioxidant capacity. It is capable of modulating the antioxidant enzyme activities and suppressing the oxidative damage induced by MTX drug in liver and brain tissues.

Propolis administration for 9 weeks proved to have remarkable protective effect against toxicity of MTX than 6 or 3 weeks.

Introduction

MTX is one of the earliest cytotoxic chemotherapeutic agent to treat various neoplastic diseases such as acute lymphoblastic leukemia, lymphoma, solid cancers. It is also one of the commonly used medications in the treatment of rheumatoid arthritis. It has the potential to generate free radicals, and such free radicals could precipitate oxidative stress especially in the presence of insufficient endogenous antioxidant enzymes within the biological system (*Mohamed and Metwally, 2009*). MTX is used in chemotherapy regimen in which it does not discriminate between normal and malignant cells and hence promotes even normal cells toward apoptosis (*Ali et al., 2014*).

Long-term drug administration can cause accumulation of MTX polyglutamates and decreased folate levels. The presence of higher levels of polyglutamates causes a longer intracellular presence of the drug and this has been suggested as a mechanism for MTX hepatotoxicity (*Jahovic et al., 2003*). The depletion of nucleic acid precursors seems to be responsible for the cytostatic and cytotoxic effects of methotrexate (*Neradil et al., 2012 and Rathaur, 2013*).

Methotrexate drug-induced oxidative stress is implicated as a mechanism of toxicity in numerous tissues and organ systems, including liver, kidney, cardiovascular and nervous systems. Metabolism of a drug may generate ROS, perturbations in oxidant homeostasis and mitochondrial dysfunction which contribute to clinically relevant drug side effects (*Deavall et al., 2012*). The drug could decrease the availability of NADPH in cells. This was the main reason for significant reduction in reduced glutathione level promoted by MTX and could lead to the reduction of antioxidant enzyme defense system effectiveness (*Vardi et al., 2010*).

In addition, deficits in neurological and cognitive function are complication of therapy that can persist in the years following treatment. MTX exerts anti-neoplastic effects by inhibiting folate-dependent thymidine and purine synthesis. This disruption of folate homeostasis within the nervous system may also explain its neurotoxic effects via inhibition of critical physiological processes, toxic substance accumulation and direct neuronal damage (*Li et al., 2010*).

There are many reasons which make nervous system organs susceptible to oxidative damage include; high oxygen uptake, neuronal membrane lipids rich in polyunsaturated fatty acids, modest antioxidant defense and several auto-oxidizable neurotransmitters (*Bhalla and Dhawan, 2009*). Brain is sensitive to lipid peroxidation-mediated injury because of its unique abundance of membrane lipids. ROS generation may cause abnormal cellular function, disrupt cell membrane integrity, increase blood brain barrier permeability and initiate apoptotic and necrotic cell death pathways (*Gadoth and Göbel, 2011*).

Supplementation with natural anti-oxidants may reduce the toxic effect of anticancer drugs. It does not only prevent free radicals from causing damage but repair any damages. Propolis is recently a most important dietary supplement as antioxidant compound, providing protection against free radicals adverse effects. Realizing this fact, propolis was taken into account as a natural product for emerging its protective efficacy (*Lushchak, 2012*). Propolis has attracted much attention in recent years as a useful substance for medicines and cosmetics, although it has been used in folk medicine since ancient times (*Wagh, 2013*).

Polyphenols (including flavonoids, phenolic acids and their esters) are considered to be the main pharmacologically active molecules in propolis. They inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility and the activity of enzymes including cyclooxygenase and lipoxygenase as well as simulate some hormones (*Tatli-Seven et al., 2009*). Propolis is rich of essential elements, including Zn^{+2} , Mg^{+2} , Cu^{+2} , Fe^{+2} , Mn^{+2} , Ni^{+2} and Ca^{+2} , that might also be responsible for reactivating antioxidant enzymes by providing optimum trace elements (*Farooqui and Farooqui, 2010*).

Materials and Methods

1. Materials:

Propolis (PP) was purchased from the faculty of agriculture, Cairo University, Giza, Egypt. Methotrexate drug (MTX) was imported from pharmaceutical company, Orion Corporation, Finland. Ethyl Alcohol (95 %) and dimethylsulphoxide (DMSO) of high analytical grade were supplied by El-Gomhoria Company for chemicals and drugs, Cairo, Egypt.

2. Preparation of Propolis extract:

Propolis extract was prepared by soaking 10 g in hydroalcoholic mixture (30% water and 70% ethanol) for three days in a dark place as described by *Mani et al. (2006)*. After filtration, the extraction solvent was evaporated under vacuum using a rotary evaporator at 40°C and the solvent was removed. The viscous residue was obtained.

3. Nutritive value analysis of propolis:

Protein, fat, carbohydrate and fiber content as well as ash and moisture, calcium, iron and sodium were measured according to (AOAC, 2000).

4. Phytochemical and antioxidant analysis of propolis extract:

Total phenolic content was determined by using Folin-Ciocalteu method according to *Kumazawa et al. (2004)*. Aluminium chloride colorimetric method was used for determination of total flavonoids according to *Gomez-Caravaca et al. (2006)*. The stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical was used for determination of free radical scavenging activity by spectrophotometric assay and represented as ascorbic acid equivalent as described by *El-Sohaimy and Masry (2014)*.

5. Gas chromatography-mass spectrometry (GC-MS) analysis of propolis:

Gas chromatography mass spectrometry (GC-MS) was performed for qualitative screening of phenolic compounds in the propolis used in the study. Hewlett Packard 6890 series II gas chromatograph, an Agilent 5973 mass spectrometer with electron ionization was used. The GC was performed using a HP%-MS capillary column (30 m x 0.25 mm, film thickness of 0.25 µm). Gas chromatography system equipped with mass detector was used for identification of compounds present in propolis sample. GC sample injection (1 µl) was performed in splitless mode. The GC oven temperature was programmed as follows: initial temperature 70°C for 2min, 10°C/ min up to 135°C and 4°C/min up to 220°C, 10°C/ min up to 270°C held for 4 min. Helium was used as carrier gas in constant flow mode at 1 ml/min. Relative percentage amounts of the separated compounds were calculated automatically from peak areas of the total ion chromatogram, using the Wiley 7 Nist05.L mass spectrometer data base to identify individual compounds.

6. Animals and experimental design:

In the present study one hundred and twenty male Wistar albino rats, weighting 90 ± 5 g were used. The animals were obtained from the animal house of Helwan-farm, Cairo, Egypt. After seven days of acclimatization, animals were housed individually in stainless-steel cages fitted with a wire mesh bottoms and fronts. The animals were randomly divided into five experimental groups of 24 rats (eight rats/weekly intervals, at 3, 6, and 9 consecutive weeks) and were provided with standard commercial diet according to *NRC (1995)* and water *ad libitum*. They maintained in an environmentally controlled animal house, temperature ($25 \pm 5^\circ\text{C}$) and relative humidity (50 ± 10) with 12 h light/dark cycle.

Rats were weighed weekly, and then the oral dose of propolis extract was calculated and dissolved in dimethylsulphoxide (DMSO) immediately before use. Propolis extract administered (Twice/week) at a dose of 200 mg kg⁻¹b.wt. (*Çetin et al., 2011*). The dose of propolis extract was given via intragastric intubation. Methotrexate (MTX) dose was calculated and dissolved in saline prior to use. It was intraperitoneally injected (Single dose/week) at a dose of 2.5 mg kg⁻¹b.wt. (*Rossato et al., 2014*) The groups were divided as the following:

Group 1 (control): Rats fed on standard commercial diet.

Group 2 (saline+ DMSO): Rats fed on standard commercial diet+ administered DMSO (p.o.) and injected with saline (i.p.).

Group 3 (PP group): Rats fed on standard commercial diet+ PP (p.o.).

Group 4 (MTX group): Rats fed on standard commercial diet+ injected with MTX (i.p.) to induce stress in liver and brain.

Group 5 (MTX+PP group): Rats fed on standard commercial diet+ administered PP extract (p.o.) and injected with MTX (i.p.).

At weeks 3, 6 and 9 following initiation of the experiment, eight rats from each group were sacrificed after 12 hour fasting under anaesthesia. Liver and brain were removed and washed twice with ice cold saline solution. Liver and brain tissues were stored at -20°C for estimation of various biochemical parameters.

In the brain homogenate reduced glutathione was determined using the colorimetric method described by *Beutler et al. (1963)* using kits supplied by the Biodiagnostic Company, Giza, Egypt. Malondialdehyde was determined in the brain according to the colorimetric method described by *Ohkawa et al. (1979)* using Ben Italy kits. Glutathione reductase and glutathione peroxidase activities were determined in liver and brain homogenates according to the colorimetric method described by *Goldberg and Spooner (1983)* & *Paglia and Valentine (1967)* respectively using

kits supplied by the Biodiagnostic Company, Giza, Egypt. Superoxide dismutase activity was determined in liver and brain homogenates according to the colorimetric method described by *Nishikimi et al. (1972)* using Ben Italy kits.

7. Statistical analysis:

Statistical analyses were performed using the SPSS software (version 17; SPSS Inc., Chicago, IL, USA). The results were expressed as means \pm standard deviation (SD). Differences between treatment groups were analyzed by least significant difference test (LSD) followed by Dunnett's multiple comparisons test *Levesque, (2007)*. The value of $P < 0.05$ was considered significant.

Results:

The present study was conducted to investigate the possible cumulative damage produced by MTX on liver and brain over the duration of administration (3, 6 and 9 weeks) and the possible ameliorative effect of propolis in alleviating the MTX induced oxidative damage in rats. There were no major differences recorded between control groups (negative control group, (G₁); Saline +DMSO group, (G₂) and propolis group, (G₃) at $p < 0.05$).

Chemical composition of propolis may vary substantially according to the origin of the samples and such differences are likely to affect its biological activities and consequently its clinical properties. Therefore, the assay of these components is of great importance.

The obtained data gives an overview on nutritive value and some main active phytochemicals of the propolis extract used in the current study. Results of nutritive value analyses showed that each 100 g of propolis contains 4 g protein, 2.11 g fat, 82.89 g carbohydrate, 10.5 g ash, 0.5g moisture, 1.98 mg iron, 15.93g calcium and 20.3 mg sodium. The values of total polyphenols measured in mg as gallic acid equivalent; GAE %, total flavonoids in mg as catechin equivalent; CE % and total antioxidant capacity as ascorbic acid content (mg %). Data showed that 100 ml of propolis extract contains 1122.4 mg GAE and 2885 mg CE. In addition highly total antioxidant capacity presented as 178 mg ascorbic acid equivalent.

Gas chromatography mass spectrometry (GC-MS) was performed for qualitative screening of phenolic compounds in the propolis used in the study. Results showed that propolis sample contains caffeic acid phenethyl ester, chrysin, galangin, quercetin, kaempferol, pinocembrin and tectochrysin. The values presented in table (1) showed glutathione reductase, glutathione peroxidase and superoxide dismutase activities in liver tissue homogenate. It is clear that there were a statistically significant reduction in the activities of GR and GPx in the MTX group (G₄) as compared to saline + DMSO group (G₂). The activities of GR and GPx recorded decrease by -21.84%, -32.75% and -48.26% for GR and by -38.97%, -43.27% and -54.95% for GPx at 3, 6 and 9 weeks respectively as compared to control group (G₂). The rats group consumed propolis extract with MTX drug (G₅) showed significant increment in GR activity by 5.71%, 25.91% and 81.27% and GPx activity by 27.61%, 46.88% and 102.99% in the three time intervals respectively compared to MTX group (G₄).

Also, there was a great reduction in the activity of SOD in rats injected with MTX drug (G₄) by -58.49%, -62.13% and -74.75% respectively compared to control group (G₂). While there was a great increment in the activity of SOD in rats co-administered propolis extract with MTX drug at 3, 6 and 9 weeks by 15.90%, 51.28% and 173.07% respectively as compared to MTX group.

Table (1): Glutathione reductase (GR) (U/g), glutathione peroxidase (Gpx) (U/g) and superoxide dismutase (SOD) (mg/g) activities in liver tissue of the studied rat groups:

| Parameters | weeks | Groups | | | | |
|------------|-------|-----------------------------|-------------------------------|-----------------------------|-----------------------------|-----------------------------|
| | | Healthy control (G1) | Saline + DMSO (G 2) | PP group (G 3) | MTX group (G4) | MTX + PP group (G5) |
| GR | 3 | 65.63 ± 0.86 ^{a,1} | 65.59 ± 1.14 ^{a,1} | 65.61 ± 0.39 ^{a,2} | 51.26 ± 0.60 ^{c,1} | 54.19 ± 0.80 ^{b,2} |
| | 6 | 65.91 ± 0.71 ^{a,1} | 65.76 ± 0.81 ^{a,1} | 66.30 ± 0.63 ^{a,1} | 44.22 ± 2.34 ^{c,2} | 55.68 ± 2.26 ^{b,2} |
| | 9 | 65.42 ± 0.54 ^{a,1} | 65.33 ± 0.65 ^{a,1} | 65.33 ± 0.65 ^{a,2} | 33.80 ± 1.14 ^{c,3} | 61.27 ± 1.14 ^{b,1} |
| Gpx | 3 | 53.17 ± 2.15 ^{a,1} | 53.04 ± 2.39 ^{a,b,1} | 51.32 ± 1.66 ^{b,2} | 32.37 ± 1.33 ^{d,1} | 41.31 ± 0.92 ^{c,3} |
| | 6 | 54.07 ± 1.11 ^{a,1} | 54.26 ± 1.68 ^{a,1} | 54.27 ± 1.07 ^{a,1} | 30.78 ± 1.10 ^{c,2} | 45.21 ± 1.37 ^{b,2} |
| | 9 | 54.00 ± 2.06 ^{a,1} | 53.44 ± 1.36 ^{a,1} | 54.19 ± 1.65 ^{a,1} | 24.07 ± 0.83 ^{c,3} | 48.86 ± 0.95 ^{b,1} |
| SOD | 3 | 1.05 ± 0.03 ^{a,1} | 1.06 ± 0.01 ^{a,1} | 1.04 ± 0.03 ^{a,1} | 0.44 ± 0.03 ^{c,1} | 0.51 ± 0.03 ^{b,3} |
| | 6 | 1.03 ± 0.02 ^{a,1} | 1.03 ± 0.03 ^{a,1} | 1.03 ± 0.03 ^{a,1} | 0.39 ± 0.03 ^{c,2} | 0.59 ± 0.01 ^{b,2} |
| | 9 | 1.03 ± 0.01 ^{a,1} | 1.03 ± 0.03 ^{a,1} | 1.03 ± 0.02 ^{a,1} | 0.26 ± 0.02 ^{c,3} | 0.71 ± 0.05 ^{b,1} |

GR, glutathione reductase; Gpx, glutathione peroxidase; SOD, superoxide dismutase.

- Values are expressed as mean ± S.D, n=8
- There was no significant difference between means have the same alphabetical superscripts (a, b, c) in the same row.
- There was no significant difference between means have the same numerical value superscripts (1, 2, 3) in the same column.

Table (2): Glutathione reductase (GR) (U/g), glutathione peroxidase (Gpx) (U/g) and superoxide dismutase (SOD) (mg/g) activities in brain tissue of the studied rat groups:

| Parameters | weeks | Groups | | | | |
|------------|-------|-----------------------------|-----------------------------|-------------------------------|------------------------------|------------------------------|
| | | Healthy control (G1) | Saline + DMSO (G 2) | PP group (G 3) | MTX group (G4) | MTX + PP group (G5) |
| GR | 3 | 68.84 ± 1.19 ^{a,1} | 68.15 ± 1.74 ^{a,1} | 68.86 ± 1.06 ^{a,1} | 55.53 ± 0.94 ^{c,1} | 58.04 ± 1.86 ^{b,3} |
| | 6 | 68.9 ± 1.08 ^{a,1} | 68.54 ± 0.83 ^{a,1} | 68.15 ± 1.14 ^{a,1} | 45.62 ± 2.41 ^{c,2} | 59.88 ± 2.06 ^{b,2} |
| | 9 | 68.22 ± 1.14 ^{a,1} | 67.54 ± 1.39 ^{a,1} | 67.79 ± 0.90 ^{a,1} | 37.35 ± 1.29 ^{c,3} | 63.67 ± 1.22 ^{b,1} |
| Gpx | 3 | 37.09 ± 1.49 ^{a,1} | 36.15 ± 1.27 ^{a,1} | 36.25 ± 1.43 ^{a,2} | 24.74 ± 1.84 ^{c,1} | 29.67 ± 1.38 ^{b,3} |
| | 6 | 36.59 ± 0.58 ^{a,1} | 37.08 ± 1.33 ^{a,1} | 37.02 ± 0.89 ^{a,1,2} | 21.57 ± 0.73 ^{c,2} | 30.88 ± 0.87 ^{b,2} |
| | 9 | 37.67 ± 1.19 ^{a,1} | 36.52 ± 1.21 ^{b,1} | 37.60 ± 1.14 ^{a,1} | 18.18 ± 0.91 ^{d,3} | 32.51 ± 0.71 ^{c,1} |
| SOD | 3 | 0.98 ± 0.01 ^{a,1} | 0.97 ± 0.03 ^{a,1} | 0.97 ± 0.02 ^{a,1} | 0.46 ± 0.01 ^{c,1} | 0.50 ± 0.01 ^{b,2,3} |
| | 6 | 0.98 ± 0.08 ^{a,1} | 0.98 ± 0.01 ^{a,1} | 0.97 ± 0.02 ^{a,1} | 0.40 ± 0.02 ^{c,2} | 0.52 ± 0.01 ^{b,2} |
| | 9 | 0.98 ± 0.02 ^{a,1} | 0.97 ± 0.02 ^{a,1} | 0.99 ± 0.01 ^{a,1} | 0.38 ± 0.02 ^{c,2,3} | 0.62 ± 0.04 ^{b,1} |

GR, glutathione reductase; Gpx, glutathione peroxidase; SOD, superoxide dismutase.

- Values are expressed as mean ± S.D, n=8
- There was no significant difference between means have the same alphabetical superscripts (a, b, c) in the same row.
- There was no significant difference between means have the same numerical value superscripts (1, 2, 3) in the same column.

Table (3): GSH (mg/g tissue) and MDA (nmole/mg tissue) concentration in brain in the studied rats groups:

| Parameters | weeks | Groups | | | | |
|------------|-------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| | | Healthy control (G1) | Saline + DMSO (G2) | PP group (G3) | MTX group (G4) | MTX + PP group (G5) |
| MDA | 3 | 3.16 ± 0.21 ^{c,1} | 3.11 ± 0.19 ^{c,1} | 3.21 ± 0.11 ^{c,1} | 14.93 ± 0.17 ^{a,3} | 10.97 ± 0.44 ^{b,1} |
| | 6 | 3.23 ± 0.21 ^{c,1} | 3.22 ± 0.18 ^{c,1} | 3.23 ± 0.23 ^{c,1} | 17.35 ± 0.15 ^{a,2} | 9.45 ± 0.25 ^{b,2} |
| | 9 | 3.28 ± 0.24 ^{c,1} | 3.19 ± 0.21 ^{c,1} | 3.21 ± 0.07 ^{c,1} | 26.50 ± 1.32 ^{a,1} | 7.72 ± 0.65 ^{b,3} |
| GSH | 3 | 74.92 ± 2.83 ^{a,1} | 74.70 ± 2.74 ^{a,1} | 76.05 ± 2.18 ^{a,1} | 55.82 ± 0.61 ^{c,1} | 60.22 ± 1.25 ^{b,3} |
| | 6 | 75.61 ± 2.20 ^{a,1} | 74.54 ± 1.69 ^{a,1} | 75.48 ± 2.13 ^{a,1} | 52.50 ± 2.14 ^{c,2} | 61.80 ± 2.73 ^{b,2} |
| | 9 | 74.21 ± 2.53 ^{a,1} | 73.70 ± 1.69 ^{a,1} | 74.89 ± 1.66 ^{a,1} | 44.84 ± 2.76 ^{c,3} | 67.27 ± 2.22 ^{b,1} |

MDA, malondialdehyde; GSH reduced glutathione.

- Values are expressed as mean ± S.D, n=8
- There was no significant difference between means have the same alphabetical superscripts (a, b, c) in the same row.
- There was no significant difference between means have the same numerical value superscripts (1, 2, 3) in the same column.

The results presented in tables (2&3) showed the effect of propolis and/or MTX treatment on glutathione reductase, glutathione peroxidase and superoxide dismutase activities and also, malondialdehyde (MDA) and reduced glutathione (GSH) concentration in brain tissue homogenate respectively. With respect to GR activity, it was significantly decreased in rats injected with MTX in the three time intervals as compared to saline + DMSO group (G₂). After nine weeks GR activity was significantly decreased by -44.69% as compared to control group (G₂). While propolis extract administration with MTX drug (G₅) significantly increased GR activity after nine weeks by (70.46%) as compared to MTX group.

Also, there was a significant decrease in GPx activity after 3, 6 and 9 weeks in MTX injected rats (G₄) as compared to control group (G₂). GPx activity significantly decreased in MTX injected rats by -50.21% after nine weeks as compared to control group (G₂) and significantly increased by 78.82% when propolis extract administered with MTX (G₅) as compared to MTX group (G₄). There was a significant reduction in SOD activity in MTX group by -53.06%, -59.18% and -60.82% respectively when compared to control group (G₂). When PP and MTX were administered simultaneously (G₅), SOD activity increased by 8.69%, 30% and 63.15% respectively as compared to MTX group.

With respect to MDA concentration, results showed that when rats administered propolis extract with MTX drug (G₅), marked reduction in the mean values of MDA concentration was observed in the three time intervals 10.97 ± 0.44 nmole/mg vs. 14.93 ± 0.17 nmole/mg, 9.45 ± 0.25 nmole/mg vs. 17.35 ± 0.15 nmole/mg and 7.72 ± 0.65 nmole/mg vs. 26.50 ± 1.32 nmole/mg respectively compared to MTX injected rats. Considering the results of GSH, values recorded in the control group (G₂) were 74.70 ± 2.74 mg/g, 74.54 ± 1.69 mg/g and 73.70 ± 1.69 mg/g. From these values it is clear that MTX injected rats showed significant reduction in GSH to reach 55.82 ± 0.61 mg/g, 52.50 ± 2.14 mg/g and 44.84 ± 2.76 mg/g respectively. In case of rats administered propolis extract with MTX drug there was a significant improvement in the values which were higher than those of MTX group which were 60.22 ± 1.25 mg/g, 61.80 ± 2.73 mg/g and 67.27 ± 2.22 mg/g respectively.

Discussion

It was found that the total protein content in ethanolic extract of propolis by *Kędzia (2009)* is about 2.8 %. Among other components present in propolis there are micro- and macroelements. About 30 elements have been discovered in propolis by *(Erenia and Tatiana, 2007)*, Calcium, manganese, zinc, copper and iron are present in the greatest amounts.

Among the compounds identified in propolis according to *(Kurek-Górecka et al., 2014)* are: phenolic acids, flavonoids, terpenes, lipid-wax substances, beeswax, bio-elements and other components such as vitamins, proteins, amino acids and sugars. Phenolic compounds constitute the most numerous group of propolis components with respect to the quantity and type.

The different flavonoids found in propolis by *El-Masry et al., (2011)* are: pinocembrin, acacetin, chrysin, rutin, catechin, naringenin, galangin, luteolin, kaemferol, apigenin, myricetin and quercetin, two phenolic acids, cinnamic and caffeic acid. Propolis contains acid derivatives such as benzoic-4- hydroxy benzoic which improves the digestive utilization of calcium *(Gómez-Caravaca et al., 2006)*.

Ibrahim (2011) found that the total flavonoid and phenolic contents in alcoholic extract of propolis were 23.87 % and 31.25 % respectively. Also, *Abd El-Hady et al. (2013)* found that the total phenolic and flavonoid contents of propolis extract recorded in terms of mg gallic acid equivalent/g and quercetin equivalent/g extract were 87.65 ± 0.5 mg GAE/g and 113.14 ± 0.86 mg QE/g extract respectively.

Results of *Wided et al. (2014)* indicated that propolis extract contain high concentrations of phenolic compounds 410.25 mg GAE/g and total flavonoid concentration is equal to 320.71 mg RE/g. *Osman and Tantaway (2013)* found that extract of propolis showed high phenol content was 157.99 ± 12.3 mg GAE/g. The total flavonoid content of propolis extract was 16.96 ± 1.48 mg/g. Moreover, results of *Mateia et al. (2004)* and *Dobrinas et al. (2006)* reported that ascorbic acid concentration in propolis sample was found to be 3.64 mg AA/g propolis.

In the present study MTX caused significant progressive decrease in the activities of GR, GPx and SOD enzymes in liver cells over the duration of administration (3, 6 and 9 weeks). One of the sources of oxidative stress induced by some drugs is their metabolism, which generates ROS and reactive metabolites. This metabolism occurs mainly in the liver, but the metabolites may reach other tissues through the circulatory system. MTX causes aggressive cellular damage to the liver cells with destruction of their membranes and the release of the enzymes into the blood stream.

These observations may be explained as; many chemotherapeutic drugs kill cancer cells oxidatively via the production of reactive oxygen species and the induction of either apoptosis or necrosis of tumorous cells; whereas others act on various components of cellular metabolism influencing activities of different enzymes needful for cell division. Since cytotoxicity not selective for cancer cells, it also affects normal cells *(Sak, 2012)*.

MTX is capable of generating potential toxic reactive oxygen species during its metabolism in the cell and thus the pro-oxidants-antioxidant balance that is usually maintained in a normal cell can be shifted towards the pro-oxidants following MTX metabolism *Oguntibeju and Coleshowers, (2012)* In addition, *Rossato et al., (2013)* reported that MTX and its toxic known metabolite accumulate in the liver tissue and its long-lasting effects can be attributed to MTX persistence in the cells and its strong affinity for cellular macromolecules and membranes.

Results of *Coleshowers et al. (2010)* were matched with the results of this study, who reported that oxidative stress commences approximately after one week of MTX administration and increases progressively. Results showed that MTX caused a significant progressive decrease in SOD and GR activities in the liver at week (2, 3, 4, 5 and 6, respectively) as compared to the control group.

Vardi et al. (2010) showed that MTX administration decreases the enzyme activities of SOD and GP-x capacity in liver. Furthermore, *Abdel-Ghaffar et al., 2013* reported that, MTX treated rats exhibited a significant decrease in liver antioxidant machinery represented by glutathione peroxidase, glutathione reductase and superoxide dismutase compared to control group.

In the current study, the oral administration of propolis extract to MTX injected rats (G₅) seems to significantly increased SOD, GR and GPx activities over the duration of administration time. The effect of propolis extract was more pronounced at 9 weeks. Propolis with its high concentration of flavonoids (polyphenolic compounds) possesses potent antioxidant and free radical scavenging activities. Therefore, it is capable of modulating the antioxidant enzymes activities and suppressing the oxidative damage. Such improvement supports the hepatoprotective effects of propolis. This might be due to the accelerated regeneration of hepatic parenchymal cells under the influence of various bioactive compounds of propolis like flavonoids and their esters (*Abu Aita et al., 2012 and EL-Mahalaway et al., 2015*).

Bhaduria, (2012) reviewed that propolis is helpful in absorption and utilization of various minerals due to the presence of organic acid derivatives in it, which in turn improved physiological functions by regulating the ion dependent enzymatic activities.

The inhibition of ROS formation by propolis and its components provides a potential molecular basis for the protective actions of propolis through the retardation of NF- κ B activation, inhibition of eicosanoid synthesis, and reduction in expression of various inflammatory cytokines in the nucleus, also through the inhibition of oxidative damage to proteins, lipids, DNA/RNA and carbohydrates (*Farooqui and Farooqui, 2010 and El-Masry et al., 2011*).

The present data is in consistent with that reported by *Çetin et al. (2011) and Badr et al. (2011)* they found that, co-administration of propolis extract with MTX, significantly increased SOD, GPx activities. Study by *Ali et al. (2014)* demonstrated the protective effect of chrysin as a component of propolis against MTX-induced hepatotoxicity. Chrysin co-administration reversed the decrease in GPx, GR, SOD activities.

It seems from the current study that, MTX injection significantly decreased the activities of GR, GPx and SOD and reduced GSH concentration in brain tissue. Oral administration of propolis reactivated the activities of GR, GPx and SOD and restored GSH concentration, and increased the detoxification of active metabolites of MTX. Also, MTX injection enhanced lipid peroxidation in brain tissue by increasing MDA concentration. While administration of propolis extract inhibited the generation of lipid peroxides that confirmed its anti-peroxidative effects against brain oxidative damage. The present findings demonstrate the neurotoxic effect of MTX.

The decreased activities of these enzymes in MTX-treated group (G₄) especially at 9 weeks indirectly showed an increase in ROS generation in brain cells. Confirm that long administration of MTX as long as 9 weeks may have adverse effects on antioxidant enzymes and cell integrity in the brain. Therefore cumulative effect of methotrexate could have played a role in this regard which could potentially lead to the release of more free radicals which interact with the cell membrane, causing membrane lysis and brain injury

MTX is an analog of folic acid, exerts anti-neoplastic effects by inhibiting folate-dependent thymidine and purine synthesis. This disruption of folate homeostasis within the CNS may also explain its neurotoxic effects via inhibition of critical physiological processes, toxic substance accumulation, and direct neuronal damage. It may lead to CNS morphological and functional alterations, an imbalance in some neurotransmitters or inhibit neurogenesis due to ROS production. Furthermore, an inflammatory response, initiated by cells in response chemotherapy, also affect cognitive function and trigger secondary neuronal injury (*Li et al., 2010*).

The present results are similar to that of *Oguntibeju and Coleshowers, (2012)* who found that MTX treatment caused a significant decrease in the activities of SOD and GR. In addition, *Vardi et al. (2012)* showed that MTX administration increases the level of MDA in the cerebellum and decreases the enzyme activity of SOD and GSH concentration. Also, *Abdu, (2013)* reported that MTX caused significant decrease in the level of SOD and increase in MDA concentration in the cerebellum.

The present data indicated that co-administration of propolis with MTX (G₅) caused reduction in the MDA level and increased the activities of the antioxidant enzymes (SOD, GPx and GR) and increased the level of GSH in the brain. Mechanisms of antioxidant action may include suppression of ROS formation, removal or inactivation of oxygen reactive species and up-regulation or protection of antioxidant defenses (*Montoro et al., 2005*). The results of *Newairy and Abdou, (2013)* reported that propolis was proved to be beneficial in decreasing the levels of free radicals and increasing the activities of the antioxidant enzymes.

The biological effects exhibited by propolis could be related to an overall effect of the phenolic compounds present in propolis (*El-Masry et al., 2011*). It was reported that MTX caused significant decrease of SOD activity in spinal cord and brain stem tissues (*Uzar et al., 2010*) in comparison to control group. CAPE administration with MTX injection caused a significant decrease in MDA level and increase in SOD when compared with the MTX group. *Kasai et al. (2011)* reported that propolis and its constituents (CAPE, chrysin and pinocembrin) protect against neuronal death at least partly by the mediation of their anti-inflammatory and antioxidant activities. Pinocembrin, a flavonoid abundant in propolis had potent neuroprotective effects by improving mitochondrial function, decreasing oxidative damage, reducing neuronal apoptosis, and inhibiting inflammatory responses (*Liu et al., 2012*).

Conclusion

Propolis is a natural product and has a powerful antioxidant capacity. It is capable of modulating the antioxidant enzyme activities and suppressing the oxidative damage induced by MTX drug in liver and brain tissues.

References

1. **Abdel-Ghaffar, F.R.; El-Elaimy, I.A.; El-DougDoug, K. and Nassar, H.I. (2013):** Protective and modulatory effects of curcumin and L-carnitine against methotrexate-induced oxidative stress in albino rats. *Res. J. Pharm. Biol. Chem. Sci.*4:744-754.
2. **Abd El-Hady, F.K.; El-Awdan, S.A. and Ibrahim, A.M. (2013):** Anti-ulcerative potential of Egyptian propolis against oxidative gastric injury induced by indomethacin in rats. *Asian J. Med. Pharm. Res.* 3(2): 35-42.
3. **Abdu, F. (2013):** Impact of *Physalis angulata* on methotrexate-induced neurotoxicity. *Biosci. Biotech. Res. Asia.* 10(2): 613-620.
4. **Abu Aita, N.A.; Hashesh, M.A. and Mohamed, A.H. (2012):** Clinicopathological and cytogenetic studies on the ameliorative effect of propolis against protenofos toxicity in rats. *G.V.* 9 (6): 669-682.
5. **Ali, N.; Rashid, S.; Nafees, S.; Hasan, S.K. and Sultana, S. (2014):** Beneficial effect of chrysin against mtx-induced hepatotoxicity via attenuation of oxidative stress and apoptosis. *Mol.cell.biochem.*385:215-223.
6. **AOAC, Association of official analytical chemists (2000):** Official method of analysis. 17th Edition, Washington D.C., USA.
7. **Badr, M.O.T.; Edrees, N.M.M.; Abdallah, A.A.M.; Hashem, M.A.; El-Deen, N.A.; Neamat-Allah, A.N.F. and Ismail, H.T.H. (2011):** Propolis protects against methotrexate induced hepato-renal dysfunctions during treatment of ehrlich carcinoma. *J. Am. Sci.* 7(12):313-319.
8. **Beutler, E.; Duron, O. and Kelly, B.M. (1963):** Improved method for the determination of blood glutathione. *J. Lab. Clin. Med.* 61:882-888.
9. **Bhadauria, M. (2012):** Propolis prevents hepatorenal injury induced by chronic exposure to carbon tetrachloride. *Evid. Based Complement. Alternat. Med.* 2012:1-12.
10. **Bhalla, P. and Dhawan, D.K. (2009):** Protective role of lithium in ameliorating the aluminium-induced oxidative stress and histological changes in rat brain. *Cell Mol. Neurobiol.* 29:513-521.
11. **Çetin, A.; Kaynar, L.; Eser, B. ; Karadag, C.; Saraymen, B.; Öztürk, A.; Koçyiğit, I.; Hacıoğlu, S.K.; Çiçek, B. and Silici, S. (2011):** Beneficial effects of propolis on methotrexate-induced liver injury in rats. *A.O.T.* 44:18-23
12. **Coleshowers, C.L; Oguntibeju, O.O; Ukpong, M. and Truter, E.J. (2010):** Effects of methotrexate on antioxidant enzyme status in a rodent model. *Med. Technol. SA.* 24 (1):5-9.
13. **Deavall, D.G.; Martin, E.A.; Horner, J.M. and Roberts, R. (2012):** Drug-induced oxidative stress and toxicity. *J. Toxicol.* 2012:1-13.

14. Dobrinas, S.; Matei, N.; Soceanu, A.; Birghila, S.; Popescu, V.(2006): Estimation of vitamin c and Cd, Cu, Pb content in honey and propolis. *J. Sci. Study and Res.*4:729-734.
15. El-Mahalaway, A.M.; Selim, A.A. and Mahboub, F.A.R. (2015): The potential protective effect of propolis on experimentally induced hepatitis in adult male albino rats. Histological and immunohistochemical study. *J. Histol. Histopathol.* 2(14):1-9.
16. El-Masry, T.A.; Emara, A.M. and El-Shitany, N.A. (2011). Possible protective effect of propolis against lead induced neurotoxicity in animal model. *J. Evol. Biol. Res.* 3(1):4-11.
17. El-Sohaimy, S.A. and Masry, H.D. (2014): Phenolic content, antioxidant and antimicrobial activities of Egyptian and Chinese propolis. *Agric. and Environ. Sci.* 14 (10): 1116-1124.
18. Erenia, N. and Tatiana, D. (2007): The content of micro- and macroelements in propolis. *Anim. Sci. Biotechnol.* 63/64:176–178.
19. Farooqui, T. and Farooqui, A.A. (2010): Molecular mechanism underlying the therapeutic activities of propolis. *Current Nutr. Food Sci.* 6:1-14.
20. Gadoth, N. and Göbel, H.H. (2011): Oxidative stress and free radical damage in neurology. Springer Science & Business Media, New york USA, PP.332.
21. Goldberg, D.M. and Spooner, R.J. (1983): In methods of enzymatic analysis (Bergmeyer, H.V. Ed.) 3rd ed. Vol. 3, Verlag Chemie, Deerfield beach, FI, PP. 258-265.
22. Gómez-Caravaca, A.M., Gómez-Romero, M.; Arráez-Román, D.; Segura-Carretero, A. and Fernández-Gutiérrez, A. (2006): Advances in the analysis of phenolic compounds in products derived from bees. *J. Pharma. Bio. Anal.* 41:1220–1234.
23. Ibrahim, A.M.S. (2011): Phytochemical composition of iraqi propolis and its effect on some microorganism. *Al-Anbar J. Vet. Sci.* 4 (2):13-25.
24. Jahovic, N.; Cevik, H.; Schirli, A.O.; Yegen, B.C and Sener, G. (2003): Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats. *J. Pineal. Res.* 34:282-287
25. Kasai, M.; Fukumitsu, H.; Soumiya, H. and Furukawa, S. (2011): Ethanol extract of chinese propolis facilitates functional recovery of locomotor activity after spinal cord injury. *J. Evid. Based. Complementary. Altern. Med.* 749627:1-9.
26. Kędzia, B. (2009): Chemical composition of polish propolis. Part 2. New study. *Post. Fitoter.* 2:122–128.
27. Kumazawa, S., Hamasak, T., Nakayama, T. (2004): Antioxidant activity of propolis of various geographic origins. *Food Chem.* 84: 329–339.
28. Kurek-Górecka, A.; Rzepecka-Stojko, A.; Górecki, M.; Stojko, M.; Sosada, M. and Świerczek-Zięba, G. (2014): Structure and antioxidant activity of polyphenols derived from propolis. *Molecul.* 19:78-101.
29. Levesque, R. (2007): SPSS programming and data management: A guide for SPSS and SAS user. 3rd edition, United States of America.
30. Li, Y.; Vijayanathan, V.; Gulinello, M. and Cole, P.D. (2010): Intrathecal methotrexate induces focal cognitive deficits and increases cerebrospinal fluid homocysteine. *Pharmacol. Biochem. Behavior.* 95:428–433.
31. Liu, R.; Wu, C.X.; Zhou, D.; Yang, F.; Tian, S.; Zhang, L. Zhang, T.T. and Guan-hua, D. (2012): Pinocembrin protects against b-amyloid-induced toxicity in neurons through inhibiting receptor for advanced glycation end products (RAGE)-independent signaling pathways and regulating mitochondrion-mediated apoptosis. *B.M.C. Med.*10 (105):1-21
32. Lushchak, V. (2012): Oxidative stress - environmental induction and dietary antioxidants, chapter 13: The effects of propolis in animals exposed oxidative stress. InTech. PP.267-288
33. Mani, F.; Damasceno, H.C.R; Novelli, E.L.B; Martins, E.A.M. and Sforcin J.M. (2006): Propolis: effect of different concentrations, extracts and intake period on seric biochemical variables. *J. Ethnopharmacol.* 105:95–98
34. Mateia, N.; Birghila, S.; Dobrinas, S. and Capotab, P. (2004): Determination of vitamin C and some essential trace elements (Ni, Mn, Fe, and Cr) in bee products. *Acta Chim. Slov.* 51:169–175.
35. Mohamed, A.M. and Metwally, N.S. (2009) Antiafla-toxicogenic activities of some plant aqueous extracts against aflatoxin-B₁ induced renal and cardiac damage, *J. Pharmacol. Toxicol.* 4(1):1-16.
36. Montoro, P.; Braca, A.; Pizz, C. and De-Tomasi, N. (2005): Structure-antioxidant activity relationships of flavonoids isolated from different plant species. *Food Chem.* 92: 349-355.

37. **Neradil, J.; Pavlasova, G. and Veselska, R. (2012):** New Mechanisms for an old drug; DHFR- and non-DHFR-mediated effects of methotrexate in cancer cells. *Klin Onkol. 25: 2S87– 2S92*
38. **Newairy, A.A. and Abdou, H.M. (2013):** Effect of propolis consumption on hepatotoxicity and brain damage in male rats exposed to chlorpyrifos. *Afr. J. Biotechnol. 12(33):5232-5243.*
39. **Nishikimi, M.; Roa, N.A. and Yogi, K. (1972):** Measurement of superoxide dismutase. *J. Biochem. Biophys. Res. Comm. 46: 849-854.*
40. **NRC, National Research Council (1995):** Nutrient requirements of laboratory animals. 4th revised edition. National Academy Press, Washington, D.C., chapter (2):PP. 11-79.
41. **Oguntibeju, O.O. and Coleshowers, C.L. (2012):** Modulation of brain antioxidant enzymes by methotrexate administration in animal model. *J. Food Agric. Environ. 10 (1): 223-226.*
42. **Ohkawa, H.; Ohishi, N. and Yagi, K. (1979):** Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. *J. Anal. Biochem. 95:351–358.*
43. **Osman, I.H. and Tantaway, A.A.H. (2013):** Antioxidant activity and protective effects of commercial propolis on gentamicin induced nephrotoxicity in rabbit's *in vitro* study. *Turk. J. Biochem. 38 (4):409–415*
44. **Paglia, D.E. and Valentine, W.N. (1967):** Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med. 70(1):158 – 169.*
45. **Rathaur, A.K. (2013):** Drugs responsible for bone marrow suppression and its herbal approaches: an overview. *R.J.P.B.C.S. 4: 547-556.*
46. **Rossato, L.G.; Costa, V.M.; Dallegrove, E.; Arbo, M.; Dinis-Oliveira, R.J.; Santos-Silva, A.; Duarte, J.A.; Bastos, M.D.L.; Palmeira, C. and Remiao, F. (2014):** Cumulative mitoxantrone-induced haematological and hepatic adverse effects in a subchronic *in vivo* study. *B.C.P.T.114: 254–262*
47. **Rossato, L.G.; Costa, V.M.; Villas-Boas, V.; de Lourdes Bastos, M.; Rolo, A.; Palmeira, C. and Remião, F. (2013):** Therapeutic concentrations of mitoxantrone elicit energetic imbalance in H_{9c2} cells as an earlier effect. *Cardiovasc. Toxicol. 13(4):413-25.*
48. **Sak, K. (2012):** Chemotherapy and dietary phytochemical agents. *Chemother. Res. Pract. 1-11.*
49. **Tatli-Seven, P.; Yilmaz, S.; Seven, İ.; Çerçi, İ.H.; Azman, M.A. and Yilmaz, M. (2009):** The effect of propolis on selected blood indicators and antioxidant enzyme activities in broilers under heat stress. *Acta. Vet. Brno.78: 75-83.*
50. **Uzar, E.; Koyuncuolu, H.R.; Yilmaz, H.R.; Uz, E.; Songur, A.; Sahin, O.; Yürekli, V.A.; Yilmaz, M.; Kilbas, S. and Kutluhan, S. (2010):** Ameliorating role of caffeic acid phenethyl ester (CAPE) against methotrexate-induced oxidative stress in the sciatic nerve, spinal cord and brain stem tissues of rats. *Turk. Norol. Derg.16:12-20.*
51. **Vardi, N.; Parlakpınar, H. and Ates, B. (2012):** Beneficial effects of chlorogenic acid on methotrexate-induced cerebellar purkinje cell damage in rats. *J. Chem. Neuroanat. 43:43–47*
52. **Vardi, N.; Parlakpınar, H.; Cetin, A.; Erdogan, A. and Ozturk, I.C. (2010):** Protective effect of β -Carotene on methotrexate induced oxidative liver damage: *Toxicol. Pathol. 38: 592-597*
53. **Wagh, V.P. (2013):** Propolis: A Wonder Bees Product and Its Pharmacological Potentials. *Adv. Pharmacol. Sci. 1-11*
54. **Wided, K.; Hassiba, R. and Mesbah, L. (2014):** Polyphenolic fraction of Algerian propolis reverses doxorubicin induced oxidative stress in liver cells and mitochondria. *Pak. J. Pharm. Sci. 27(6):1891-1897.*