Journal of Cancer Research and Treatment, 2014, Vol. 2, No. 3, 44-51
Available online at http://pubs.sciepub.com/jcrt/2/3/1

DOI:10.12691/jcrt-2-3-1

Abrogation by *Ginkgo Byloba* Leaf Extract on Hepatic and Renal Toxicity Induced by Methotrexate in Rats

Ehab Tousson*, Zeinab Atteya, Afaf El-Atrash, Ola I. Jeweely

Zoology Department, Faculty of Science, Tanta University, Tanta, Egypt
*Corresponding author: toussonehab@yahoo.com

Received September 26, 2014; Revised October 10, 2014; Accepted October 14, 2014

Abstract Methotrexate (MTX) is used as a chemotherapeutic agent and its anti-oxidant activity is used to treat many cancer types. The present study aimed to examine the possible modifying effects of Ginkgo biloba leaf extract (GLE) against hepatic and renal toxicity induced by MTX in rats. A total 60 male albino rats were equally divided into six groups; the first and second groups were the control and GLE groups respectively while the 3rd group was MTX rat group; the 4th and 5th groups were Co- and post treated MTX rat with GLE respectively and the 6th group was MTX self treated rat group. Serum GPT, GOT, urea, creatinine, uric acids and MDA levels in MTX group showed a significant increase when compared with control group, in contrast, MTX-treated group also exhibited a significant decrease in liver antioxidant machinery represented by GSH, catalase, SOD and total protein. Administration of GLE combined with MTX improved the liver and kidney damages induced by MTX. Histopathological and evidence, together with observed CD68 immunoreactivity, supported the detrimental effect of MTX and the ameliorating effect of GLE on liver and kidney toxicities. GLE possessed various protective mechanisms against MTX-induced liver and kidney toxicity throughout Co- and post- treatment. We can conclude that Co-treatment with GLE has beneficial properties and can reduce the liver and kidney damages and toxicity induced by MTX.

Keywords: *Methotrexate, Gingko biloba leaf extract, oxidative damage, antioxidants, CD68 immunoreactivity, liver, kidney*

Cite This Article: Ehab Tousson, Zeinab Atteya, Afaf El-Atrash, and Ola I. Jeweely, "Abrogation by *Ginkgo Byloba* Leaf Extract on Hepatic and Renal Toxicity Induced by Methotrexate in Rats." *Journal of Cancer Research and Treatment*, vol. 2, no. 3 (2014): 44-51. doi: 10.12691/jcrt-2-3-1.

1. Introduction

Methotrexate (MTX, 4-amino-N^{10}-methyl folic acid), a folic acid reductase inhibitor, affects primarily the tissues that are growing most rapidly. There are other synonyms for Metatrexate such as Mexate, Abitrexate, Antifolan, Amethoptrin, Metatraxan, Folex, Tremetex, Rheumatrex, Trexall. Toxicity studies with methotrexate highlight the role of oxidative stress in causing toxicity on the most of body organs [1]. With the widespread use of MTX, although hepatic and renal toxicity is the most important potential major side effect [2,3,4,5]. Levels of both enzymatic and nonenzymatic anti-oxidants are inhibited and the levels of oxidants increase in the liver, kidney, heart, testes, and gut tissues of laboratory animals given methotrexate [5-11].

Herbal medicine is increasingly gaining acceptance from the public and medical professionals due to advances in the understanding of the mechanisms by which herbs positively influence health and quality of life [12,13,14,15]. *Ginkgo biloba* (maidenhair tree) is one of the oldest herbal medicines that have been used as a therapeutic agent in modern pharmacology. Standardized extracts from dried ginkgo leaves take also important place in modern medicine [13]. *Ginkgo biloba* leaf extract (GLE) is standardized to contain approximately 24% flavone glycosides and 6% terpene lactones. These compounds are extracted from the tree's healthy green leaves and is believed to provide beneficial effects in memory impairment, edema, inflammation, and vaso oclusive disorders [1-23]. Based on these evidences, the present study was conducted to examine the possible modifying effects of GLE against hepatic and renal toxicity induced by methotrexate in male rats. This could be fulfilled through the histological, immunohistochemical and biochemical analysis of liver and kidney tissues.

2. Materials and Methods

2.1. Animals

The experiments were performed on 60 male albino rats (*Rattus norvigicus*) weighing 140-150 g and of 9-10 week's age. The rats were kept in the laboratory for one week before the experimental work and maintained on a standard rodent diet (20% casein, 15% corn oil, 55% corn starch, 5% salt mixture and 5% vitaminzed starch; Egyptian Company of Oils and Soap Kafr-Elzayat Egypt)

and water available *ad libitum*. The temperature in the animal room was maintained at 23±2°C with a relative humidity of 55±5%. Light was on a 12:12 hr light -dark cycle. The experimental protocol was approved by Local Ethics Committee and Animals Research.

2.2. *Ginkgo biloba* Extract (GLE) Preparation

The extraction procedure for *Ginkgo biloba* leaves was carried out as reported by Sener et al [24].

2.3. Animal Treatments

The rats were randomly and equally divided into six groups (6 animals each).

G_1: Control group in which animals did not received any treatment.

G_2: GLE or positive control group in which animals received orally *Gingko biloba* by stomach tube a dose of (80 mg /kg body weight /twice a week) for four week.

G_3: MTX group in which rats were injected intraperitoneally with MTX administration (0.5 mg /kg body weight/ twice a week) for four weeks according to Yozai et al [25].

G_4: Co-treated group in which animals injected intraperitoneally with MTX administration (0.5 mg /kg body weight/ twice a week) and also received orally GLE (80 mg/Kg body weight/ week) for four weeks.

G_5: Post treated group in which animals injected intraperitoneally with MTX administration (0.5 mg /kg body weight/ twice a week) for four weeks and then treated orally with GLE (80 mg/Kg body weight/ week) for another four weeks.

G_6: Self treated rat group in which rats were injected intraperitoneally with MTX administration (0.5 mg /kg body weight/ twice a week) for four weeks and self treated without drugs for another four weeks.

At the end of the experimental period (8 week), Animals were euthanized with intraperitoneal injection with sodium pentobarbital and subjected to a complete necropsy.

2.4. Sample Preparation

Animals were fasted overnight and for clinical chemistry blood samples from each rat were collected from the eyes by retro-orbital puncture using blood capillary tubes without heparin as per requirement under mild ether anesthesia Blood samples were incubated at room temperature for 10 minutes and left to clot then centrifuged at 3000 r.p.m for 10 min and the serum were collected, serum was separated and kept in clean stopper plastic vial at –80°C until the analysis of serum parameters. After animals were sacrificed, the liver and kidney were instantly removed, washed three times in ice cold saline and blotted on filter paper, then used for preparation of tissue homogenates for estimation of tissue MDA, total protein, Reduced glutathione (GSH) and catalase enzymes..

2.5. Biochemical Analysis

Serum GPT and GOT [26], albumen [27], urea [28], and creatinine [29] were estimated.

2.6. Preparation of Tissue Homogenates

Tissue homogenates were prepared as reported by Sakeran et al [15]. Briefly, specimens were separated into two parts. Each piece was weighed and homogenized separately with a Potter Elvenhjem tissue homogenizer. The crude tissue homogenate was centrifuged at 11,739 rcf, for 15 min in a cold centrifuge, and the resultant supernatant was used for the different estimations.

2.7. Enzymatic and Non-enzymatic Antioxidant Assays

Malondialdehyde (MDA) in liver and kidney were detected by the method of Mesbah et al [30]. The catalase (CAT) and superoxide dismutase (SOD) activites in liver and kidney were detected by the method of Sakeran et al.[15] Reduced glutathione (GSH) in liver and kidney were detected by the method of Beutler et al [31]. The total protein concentration in liver and kidney were detected by the method of lowery et al. [32] as modified by Tsuyosh and James [33].

2.8. Histopathological Examination

Immediately after decapitation animals were dissected, liver and kidney from different groups were quickly removed and fixed in 10 % neutral buffered formalin. After fixation, specimens were dehydrated in an ascending series of alcohol, cleared in two changes of xylene and embedded in molten paraffin (mp. 50–58°C). Sections of 5 microns thickness were cut using rotary microtome and mounted on clean slides. Sections were stained with Ehrlich's haematoxylin and counterstained with eosin as a routine method after Bancroft and Stevens [34]. All stained slides were viewed by using Olympus microscope and images were captured by a digital camera (Cannon 620).

2.9. Immunohistochemical Detection of CD68 Expression

Expression of CD68 proteins was detected using avidin Biotin Complex (ABC) method [1]. Paraffin sections (5µm thick) of fixed rat liver and kidney that mounted on gelatin chromalum–coated glass slides were dewaxed and rehydrated sections were washed in distilled water for 5 min, rinsed in PBST for 10 min and incubated with 10% normal goat serum for 15 min to reduce non-specific background staining. Then, the sections were incubated with anti-rat CD68 (Dako, 1:100) for 1-2 hours at room temperature. The sections after 5 baths in PBST were incubated with biotinylated goat anti rat immuoglobulin (Nichirei, Tokyo, Japan). The sections after 5 baths in PBST were further incubated with Avidin Biotin Complex (ABC: Nichirei, Tokyo, Japan) for 1 hour at room temperature. The reaction was developed by using 20 mg 3-3µ diaminobenzidine tetra hydrochloride (DAB, Wako pure chemical industries, (Ltd) in 40 ml PBST, pH 7.2 containing 10 ml of hydrogen peroxide (H_2O_2) for 7-9 min at a dark room followed by distilled water then dehydrated and mounted.

The criterion for a positive reaction confirming the presence of CD68 is a dark, brownish, intra cytoplasm precipitate. For the negative control, the primary antibody

was omitted to guard against any false positive results which might develop from a non-specific reaction. Negative control sections were done by substituting CD68 primary antibodies by normal serum of rat. Sections of liver and kidney from different groups immunohistochemicaly proven to be CD68 positive were used a positive control with each run. All stained slides were viewed by using Olympus microscope and images were captured by a digital camera (Cannon 620). Brightness, contrast were adjusted using Adobe Photoshop software. Image analysis was adjusted using PAX-it image analysis software.

2.10. Statistical Analysis

Data were expressed as mean values ± SE and statistical analysis was performed using one way ANOVA to assess significant differences among treatment groups. The criterion for statistical significance was set at $p<0.05$ for the biochemical data. All statistical analyses were performed using SPSS statistical version 16 software package (SPSS® Inc, USA).

3. Results

3.1. *In vivo* Hepatic and Renal Protective Effects of GLE Extract.

The data summarized in Table 1 indicates that a significant ($P<0.01$) elevation in GPT, GOT, urea, creatinine and uric acid in MTX group (G_3) when compared with control (G_1) and GLE (G_2) groups, this elevation decreased in treated group with GLE (G_4&G_5) and increased in MTX self treated group (G_6) when compared with MTX group (G_3). GPT, GOT, urea, creatinine and uric acid levels in Co- treated MTX group with GLE group (G_4) were significantly decreased when compared with post treated MTX group with GLE group (G_5). Total protein and albumin were significantly decreased in MTX (G_3) and in MTX self treated (G_6) groups when compared with control (G_1) and GLE (G_2) groups. In contrast, total protein and albumin were significantly increased in treated group with GLE (G_4&G_5) when compared with G_3 and G_6 groups (Table 1).

Table 1. Changes in the serum GPT (U/l), GOT (U/l), total protein, urea (mg/dl), creatinine (mg/dl) and uric acid (mg/dl) levels in different groups under study

	GPT	GOT	Total protein	Albumin	Urea	Creatinine	Uric acid
G1	22.43±0.29[a]	34.50±0.2[a]	6.26±0.14[a]	3.63±0.31[a]	21.07±0.6[a]	0.65±0.02[a]	5.90±0.37[a]
G2	22.83± 0.60[a]	29.57±0.7[a]	6.50±0.26[a]	3.70±0.36[a]	19.05±0.6[a]	0.59±0.02[a]	5.73±0.37[a]
G3	118.62±11.81[b]	93.18±4.1[b]	3.21±0.23[b]	2.05±0.27[b]	56.91±4.7[b]	1.13±0.01[b]	9.46±0.39[b]
G4	63±5.41[ab]	62±5.97	4.64±0.56[ab]	3.05±0.42[a]	33.62±2.4[ab]	0.85±0.04[a]	6.94±0.40 [ab]
G5	90.20±4.43[b]	79±4.13	4.20±0.29[ab]	2.58±0.52[b]	41.12±3.76	1.02±0.06[b]	8.06±0.25[ab]
G6	145.3±8.61[b]	98.82±8.2[b]	2.30±0.21[b]	1.68±0.18[b]	65.25±2.0[b]	1.28±0.09[b]	9.55±0.55[b]

Data are expressed as mean ± SE of 10 observations. Superscripts of different letters differ significantly ($p<0.01$) from each other. G_1, control group; G_2, GLE group; G_3, MTX group; G_4, Co- treated group with GLE; G_5, Post treated group with GLE; G_4, MTX self treated group.
[b]Significantly different from MTZ (G_3) group. [a]Significantly different from control (G_1) group.

Table 2. Changes in MDA (nmole/g tissue), Glutatione (GSH; mg/tissue), catalase (mmole/min./g tissue), total protein (mg/g) and SOD (mmole/min/g tissue) levels on liver and kidney tissues in different groups under study

		G1	G2	G3	G4	G5	G6
Liver	MDA	9.46±0.42 [b]	7.50 ±0.28 [b]	21.65 ±0.8 [a]	13.84 ±0.3 [b]	18.24 ±0.39 [ab]	24.95±0.44 [a]
	GSH	17.37±0.40 [b]	18.08±0.47 [b]	9.75± 0.23 [a]	13.46±0.42 [ab]	11.96± 0.22 [b]	7.70± 0.56 [a]
	Catalase	30.73±1.69 [b]	31.50±0.64 [b]	13.94±0.91 [a]	26.27±1.10	22.91±0.63 [ab]	12.03±0.52 [a]
	SOD	14.06±0.52 [b]	14.57±0.37 [b]	8.18 ±0.38 [a]	11.66±0.38 [ab]	10.72 ±0.28 [ab]	6.45±0.24 [a]
	Total protein	77.33±4.33 [b]	80.50±4.17 [b]	41.45±2.55 [a]	60.43±4.25 [ab]	54.31± 4.22	31.75±0.85 [a]
Kidney	MDA	10.50±0.28 [b]	8.37 ± 0.23 [b]	38.38±1.76 [a]	21.94 ±1.3	31.56 ±3.38 [ab]	42.84 ±1.68 [a]
	GSH	53.83± 0.83 [b]	52.60± 0.94 [b]	17.80± 0.93 [a]	44.45± 0.38	37.65± 0.54	13.30± 0.81 [a]
	Catalase	66.63±1.51[b]	70.25±0.85 [b]	40.66±1.80 [a]	58.68±1.34	53.28±0.52	31 ±0.91 [a]
	SOD	27.43 ± 0.83[b]	28.66 ±0.40 [b]	17.50 ±0.42 [a]	22.74 ± 0.55 [ab]	19.86 ±0.46 [ab]	14.82±0.31 [a]
	Total protein	74.06± 1.77 [b]	76.90± 2.78 [b]	39.95± 0.85 [a]	65.50± 1.77 [ab]	57.45± 0.55	34.50±1.15 [a]

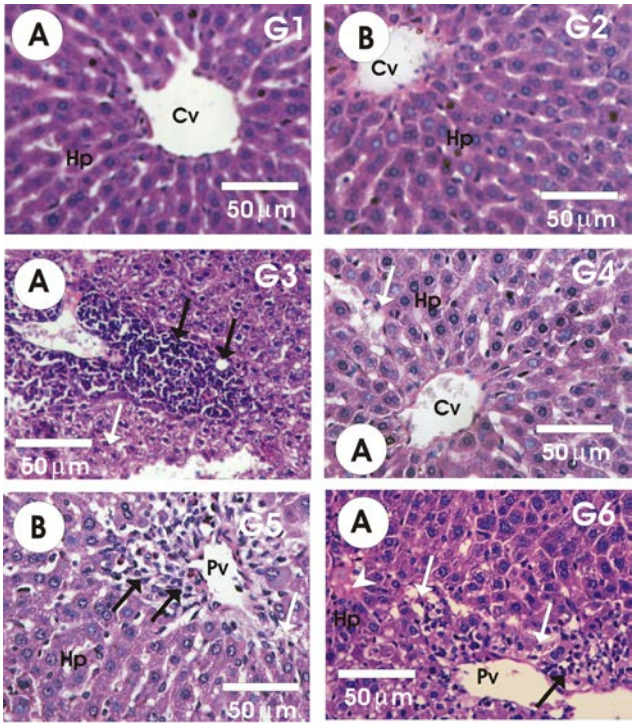
Data are expressed as mean ± SE of 10 observations. Superscripts of different letters differ significantly ($p<0.01$) from each other. G_1, control group; G_2, GLE group; G_3, MTX group; G_4, Co- treated group with GLE; G_5, Post treated group with GLE; G_4, MTX self treated group. [b]Significantly different from MTX (G_3) group. [a]Significantly different from control (G_1) group.

3.2. GLE Effects on Oxidative Stress Markers in MTX Toxicity

The data summarized in Table 2 indicates that, a significant ($P<0.05$) increased in the liver and kidney MDA of MTX (G_3) and MTX self treated (G_6) groups as compared with the control and GLE (G_1&G_2) groups. In the same time, data declared significant decreased ($P<0.05$) in the liver and kidney GSH, SOD, catalase and total protein levels of the MTX (G_3) and MTX self treated (G_6) groups as compared with the control and GLE (G_1&G_2) groups. As well, the importance of treatment with GLE groups (G_4&G_5) has been shown significant ($P<0.05$) decreased in the MDA and significant increased in the liver and kidney GSH, SOD, catalase and total protein levels when compared to the MTX (G_3) and MTX self treated (G_6) groups (Table 2). Table 2 shows that; Co-treatment MTX with GLE groups (G_4) has been shown significant ($P<0.05$) increased in the MDA and significant decreased in the liver and kidney GSH, SOD, catalase and total protein levels when compared to the post treatment with GLE groups (G_5) group.

3.3. Effect of GLE on Liver Histopathology

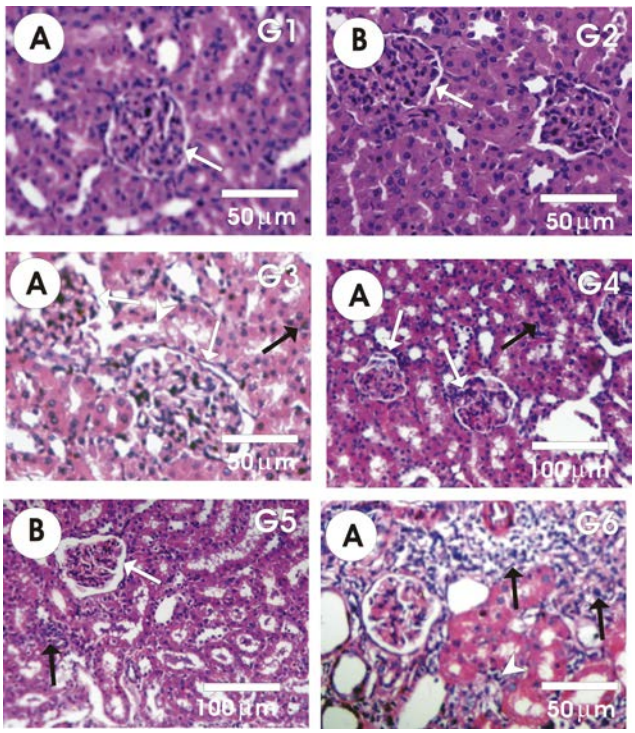
Liver sections in control (G_1) and GLE (G_2) groups showing normal structure of hepatocytes where the hepatocytes are polygonal in shape with eosinophilic granular cytoplasm and vesicular basophilic nuclei (Figure 1A&1B). Liver sections in MTX only group showed moderate to severe lose of liver architecture, disturbance of the hepatocytes radially arranged cords, congestion in central veins, atrophied and mild vacuolated hepatocytes (Figure 1C). In some slides inside the lobule many foci of apoptopic cells were detected, also shrinkage and inflection in some of hepatocytes nucleus were observed in the nuclear contour, and condensation in the structure of chromatin was also observed (Figure 1C). Liver sections in Co-treated MTX with GLE group (G_4) shows a moderate degree of improvement in hepatocytes where a few vacuolated hepatocytes and mild congestion in central veins were observed, also the apoptotic cells were not observed in this group. (Figure 1D). Liver sections in post treated with GLE showed mild disturbance of the hepatocytes radially arranged cords, a few atrophied, infiltrations, congestion in central veins and focal necrosis (Figure 1E). Liver section in self treated showed severe lose of liver architecture, marked disturbance of the hepatocytes and strong marked hepatocellular vacuolation (Figure 1F).



Figures 1A-1F. Photomicrographs of rat Liver sections in the different experimental groups stained with Haematoxylin & Eosin. **A:** Rat Liver sections in control group revealed normal structure of hepatocytes. **B:** Rat Liver section in GLE group revealed normal structure of hepatocytes (hp). **C:** Liver sections of MTX group showed moderate to severe lose of liver architecture, disturbance of the hepatocytes, congestion (Black arrows) in central veins (CV), atrophied, and vacuolated hepatocytes (White arrows). **D&E:** Liver sections in co-treated and post treated rat with GLE groups revealed a moderate to mild degree of improvement in hepatocytes where a few vacuolated hepatocytes (White arrows) and mild congestion in central veins were observed. **F:** Liver section in self treated showed severe lose of liver architecture, marked disturbance of the hepatocytes and strong marked hepatocellular vacuolation (White arrows)

3.4. Effect of GLE on Kidney Histopathology

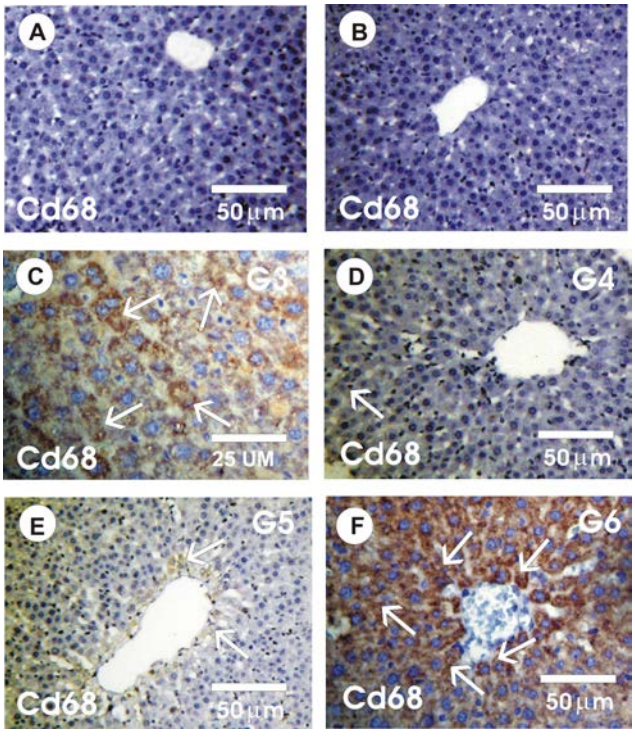
Normal structure of the cortex and medulla was observed in the rat kidney of control (G_1) and GLE (G_2) groups (Figure 2A&2B). kidney sections in MTX group showed variable pathological changes in glomeruli and some parts of the urinary tubules (Figure 2C). The most severe changes were in the malpighian corpuscles lost their characteristic configuration and cell infiltration atrophied and mild vacuolated (Figure 2C). Kidney sections of Co-treated MTX with GLE revealed a good degree of improvement glomerular damage with minimal vacuolization in tubular cells (Figure 1D). Kidney sections of post treated group with GLE showed moderate organized tubular and glomerular structures with well-established epithelia which resembled that of the control group except mild inflammatory infiltration (Figure 2E). Kidney sections of MTX self treated showed severe lose of kidney architecture, marked disturbance in glomeruli seemed to have lost their attachments and mesangial stroma and others were atrophied with dilatation in the sub capsular space (Figure 2F), The tubular epithelia were exfoliated from their underlying basement membrane and their lining cells exhibited cytoplasmic vacuolation and pyknotic nuclei (Figure 2F).



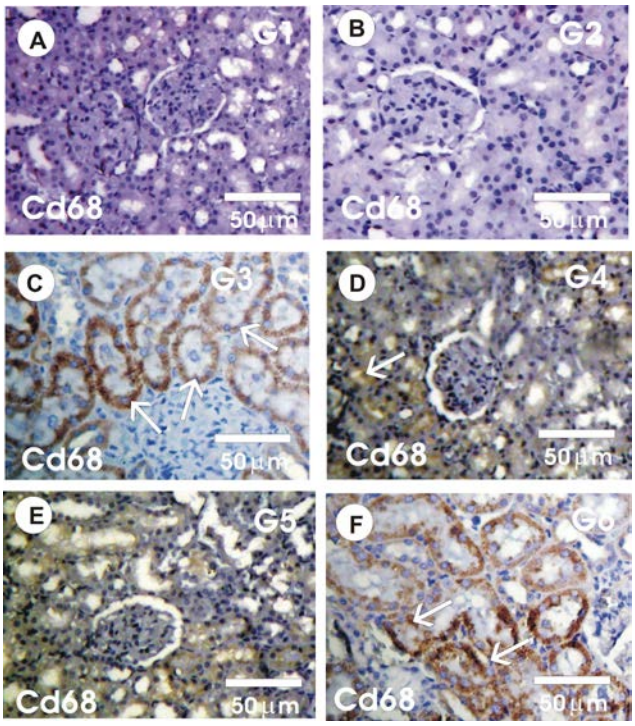
Figures 2A-2F. Photomicrographs of rat kidney sections in the different groups under study stained with Haematoxylin & Eosin. **A&B:** Rat kidney sections in control and GLE groups respectively showed normal structure of renal cortex which comprised renal corpuscles (White arrows), proximal and distal convoluted tubules. **C:** Rat kidney sections in MTX group showed glomerular damage (White arrows), vacuolization in tubular cells focal necrosis and cell infiltration (Black arrows). **D:** Kidney section of co-treated group with GLE revealed a good degree of improvement glomerular damage with minimal vacuolization(Arrow heads) in tubular cells. **E:** Rat kidney section in post treated group with GLE showed moderate organized tubular and glomerular structures except mild inflammatory infiltration (Black arrows). **F:** Kidney section of MTX self treated showed severe lose of kidney architecture, marked disturbance in glomeruli with strong inflammatory infiltration (Black arrows)

3.5. Effect of GLE on Liver and Kidney CD68 Immunoreactivity

The detection and distribution of CD68 immunoreactivity (CD68-ir) in liver and kidney sections in the different groups under study were revealed in Figure 3 & Figure 4 and Table 3. Liver sections in control and GLE groups showed negative reaction for CD68-ir (grade 0) (Figure 3A & Figure3B). Strong positive reactions for CD68-ir (grade 4) were detected in the liver sections in MTX (grade 4) and MTX self treated (grade 5) groups (Figure 3C&3F). The intensity of CD68-ir in methotrexate rat liver was significantly decreased when compared with control rat in liver. However, mild to moderate positive reactions for CD68-ir were observed in co-treated (grade 2) and post treated (grade 3) rats with GLE (Figure 3D & Figure 3E). While the intensity of CD68–ir were stay strong positive reaction in MTX self treated in liver section when compared with Co- and post treated MTX with GLE groups (grades 2&3 respectively). Kidney sections in control and GLE groups showed negative reaction for CD68-ir (grade 0) (Figure 4A & Figure 4B). Moderate to strong positive reactions for CD68-ir (grade 4) were detected in the kidney tubules in MTX (G_3) and also in MTX self treated (G_6) groups, while the malpighian corpuscles showed negative reaction against CD68 (Figure 4C & Figure 4F respectively). Mild positive reactions for CD68-ir (grade 2) were observed in Co-treated and post treated MTX with GLE (Figure 4D & Figure 4E).



Figures 3(A-F). Photomicrographs of rat Liver sections in the different experimental groups stained with CD68-ir. **A&B**: Negative CD68-ir reaction in control and GLE respectively. **C:** Severe positive reactions for Cd68-ir in MTX group. **D&E:** Mild to moderate positive reactions for CD68-ir in Co-treated and post treated rats with GLE respectively. F: Strong positive reaction for CD68-ir in MTX self treated rats



Figures 4(A-F). Photomicrographs of rat kidney sections in the different experimental groups stained with CD68-ir. **A&B**: Negative CD68-ir reaction in control and GLE respectively. **C:** Severe positive reactions for Cd68-ir in MTX group. **D&E:** Moderate to mild positive reactions for CD68-ir in Co-treated and post treated MTX with GLE respectively. F: Strong positive reaction for CD68-ir on the kidney in MTX self treated rats

Table 3. Changes in CD68 expressions in liver and kidney sections in different groups under study

	CD68	
	Liver	Kidney
G1	0	0
G2	0	0
G3	5	4
G4	2	3
G5	2	3
G6	5	4

Data are expressed as mean ± S.E.M of 5 observations. G1, Control group; G2, GLE group; G3, MTX group; G4, Co- treated group with GLE; G5, Post treated group with GLE; G4, MTX self treated group. bSignificantly different from MTX (G3) group. aSignificantly different from control (G1) group. where 0, Negative reaction; 1, light reaction; 2, mild reaction; 3 moderate reaction; 4, strong reaction; 5, very strong.

4. Discussion

This study conducts a biochemical, histopathological and immunohistochemical investigation into whether *Ginkgo biloba* Extract (GLE) has a protective and ameliorated effect on methotrexate - induced hepatic and renal toxicity in male rats. Methotrexate is an antimetabolite and an analogue of folic acid that used to treat autoimmune diseases such as psoriasis, rheumatoid arthritis and Crohn's disease and as a chemotherapeutic agent to treat many cancer types such as breast, skin, head, neck, lung, lymphoma, osteosarcoma and leukemia [11,35,36]. Methotrexate enters the cell via active transport across the reduced folate carrier [37,38]. It is effluxed from the cell by several of the ATP-binding cassette (ABC) transporters, especially ABCC1-5 and ABCG2 [39]. Levels of both enzymatic and non-enzymatic anti-oxidants are inhibited and the levels of

oxidants increase in the testes, heart, liver, kidney, and gut tissues of laboratory animals given methotrexate [1,5,6,8,9,10,11] inside the hepatic cells, MTX is stored in a polyglutamated form.

In liver, the conversion of MTX to its major extracellular metabolite, 7-hydroxy methotrexate, takes place where it is oxidized by a soluble enzymatic system. In the assessment of liver injury the analysis of enzyme levels such as GPT and GOT is largely used and located in the cytosol of hepatocytes. They are involved in the breakdown of amino acids into α-keto acids [40]. Necrosis or membrane damage releases the enzyme into the circulation and hence it can be measured in the serum. Clinical diagnosis of disease and damage to the structural integrity of liver is commonly assessed by monitoring the status of serum GPT and GOT activities.

In the current study, a significant elevation in GPT and GOT in MTX group however, this elevation decreased in treated group with GLE and increased in self treated when compared with MTX group. A significant decreased in GPT and GOT levels in Co- treated methotrexate group with GLE group when compared with post treated group.

Our results agreed with Fu et al. [8], Hemeida and Omar [41] and Vardi et al. [42] who reported that Serum GPT and GOT were significantly increased in MTX induced liver damage. Elevated levels of serum GPT and GOT enzymes are indicative of cellular leakage and loss of functional integrity of cell membranes in the liver [43,44,45]. The estimation of these enzymes in the serum is a useful quantitative marker for the extent and type of hepatocellular damage [46]. Also, our results agreed with Kadikoylu et al. [47] Klukowska et al.48 and Saad et al. [49] who reported that, MTX administration induced significant increase in serum GPT and GOT levels. The ability of MTX to cause alterations in the activity of these enzymes could be a secondary event following MTX-induced liver damage with the consequent leakage from hepatocytes. The authors reported that presence of higher level of MTX poly glutamates inside liver cells causes a longer intracellular presence of the drug, and this has been suggested as a mechanism for MTX hepatotoxicity [6]. Our histological observations basically supported the results obtained from serum enzyme assays. There was a significant (P<0.01) restoration of these enzyme levels on administration of the *Gingko biloba* leaf extract. The reversal of increased serum enzymes in MTX induced liver damage by the *Gingko biloba* leaf extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes [50].

In the current study, a significant (P<0.01) elevation in urea, creatinine and uric acid in MTX group when compared with control, this elevation decreased in treated group with GLE and increased in methotrexate self treated when compared with MTX group. This result is in harmony with the previous studies which reported that MTX increased urea and creatinine activities [51]. On the other hand, our results are disagreement with Cetiner et al [53].

Oxidative stress is an indicator of the damage that results from a change in the balance between oxidants and anti-oxidants in favour of oxidants. If the delicate balance between oxidants and anti-oxidants cannot be maintained in tissues, many pathological changes extending to cellular damage occur. In the current study, MDA levels in MTX group were significantly increased unlike glutathione, catalase, SOD and total protein levels which were significantly decreased when compared with control group. So, MTX increased MDA level accompanied with decreased GSH content and catalase activities. Similar results were previously reported by other investigators Jahovic et al. [4] and ALL et al. [6]. Oxidative stress or oxidative cellular damage with its dual of free radical generation and profound lipid peroxidation are hallmarks of MTX toxicity [52]. MTX induces oxidative stress in tissues as demonstrated by increasing MDA levels [7]. The mechanisms of MTX-induced renal toxicity however, free radicals are expected to play a role in MTX induced renal toxicity. In our study we used MDA levels to show damage to the kidney caused by lipid peroxidation. Elevated observed MDA levels suggest that lipid peroxidation, mediated by oxygen free radicals, which is believed to be an important cause of destruction and damage to cell membranes, was an important contributing factor to the development of MTX-mediated tissue damage [54].

In the current study; a significant (P<0.05) decreased in the MDA and significant increased in GSH, SOD, catalase and total protein levels in co- and post treated MTX with GLE in rat liver and kidney when compared to the MTX and MTX self treated groups. Also, a significant (P<0.05) increased in the MDA and significant decreased in the liver and kidney GSH, SOD, catalase and total protein levels in Co-treatment with GLE when compared to post treatment with GLE groups. The high level of MDA in the MTX and self treated groups in liver and kidney tissues indicates that methotrexate gives rise to oxidative stress in hepatic tissue. Our current results agree with Vardi et al.[55] who reported that, oxygen radicals and hydrogen peroxides have been associated with the many side effects of MTX and these free radicals trigger cell damage through binding to cellular macromolecules, particularly membrane lipids leading to releasing of GPT and GOT from cells to serum.

The results of this study indicate that MTX causes oxidative tissue damage by increasing lipid peroxidation in the liver and kidney tissues and decreasing the level of antioxidant enzymes. Catalase acts as a preventative antioxidant whereas it catalyses the reduction of H_2O_2 and plays an important role in protection against the deleterious effects of lipid peroxidation, ROS and hydroxyl radicals caused by MTX administration [9]. SOD is one of the most vital enzymes in the enzymatic antioxidant defense system. Decrease in the enzyme activity of SOD is a sensitive index of hepatocellular damage and is the most sensitive enzymatic index in liver injury. It scavenges the superoxide anion to form H_2O_2, thus diminishes the toxic effect caused by this radical. GLE extract ameliorate and protected the liver against MTX induce hepatotoxicity and restored the levels of SOD. The function of SOD is to catalyse the dismutation of O2 and to protect the tissue against the harmful effects of toxic oxygen radicals [56]. GSH is considered to be one of the most very important components of the antioxidant defense of living cells. The reduced tri-peptide GSH is a hydroxyl radical and singlet oxygen scavenger, and

participates in a wide range of cellular functions [11]. Recent study reported that GSH forms the first line of defense against oxidative stress, by direct interaction of its sulfhydryl group with ROS and/or it can be involved in the enzymatic detoxification reaction of ROS as a cofactor or as a coenzyme. Our current results agree with Johovic et al. [52]; Ciralik et al. [57]; Sener et al. [54]; Vardi et al. [55] and Tousson et al. [1] who reported that, MTX administration induced significant increase in GSH levels.

Our data confirmed the concept that oxidative stress plays a role in MTX – induced tissue damage, whereas GSH reduction was accompanied by reduction in the antioxidant enzyme defense system represented as depletion in the levels of SOD and CAT. This is in agreement with several studies demonstrated that MTX induces oxidative stress in tissues companied with decreased antioxidants levels [9,58].

In the current study; decrease in the activities of antioxidant enzymes can be explained either with their induction during the conversion of free radicals into inactive metabolites or secondarily with the direct inhibitory effect of MTX on enzymes activities. In our microscopic investigations in the livers of rats given MTX, hepatic nuclei were larger and karyolemma contours were irregular; and in some areas, apoptopic hepatocytes could be seen. MTX acts as adihydrofolic acid analogue that binds to the dihydrofolic acidreductase enzyme by inhibiting the synthesis of tetrahydrofolate, which is required for DNA synthesis [59]. Increased oxidative stress may cause shape and structural changes of the nucleus by causing DNA fragmentation and denaturation, which play a critical role in the initiation of apoptosis [60]. Our histopathological result is agreed with O'Rourke and Eckert and Ros et al. [61,62] who stated that such hepatic fibrosis seemed to be due to direct toxic effects of MTX which induced proliferation of the hepatic fibrous connective tissue. Also, Hytiroglou et al. [63] Found that the methotrexate is known to cause hepatic fibrosis in some patients, which might progress to cirrhosis. Our results showed that; treatment with GLE exhibited decreased MDA contents, anti-oxidant effects not only on the non enzymatic defense system (GSH), but also on the enzymatic one such as catalase and SOD activities compared to MTX self treated animals.

Antioxidants have been shown to prevent severe increases in AST, ALT, urea, creatinine, uric acid and antioxidant parameters in liver and kidney injury. In the MTX groups (G_3&G_6), in which liver and kidney function activities and oxidant parameters were higher, there were apoptotic bodies, focal necrosis and intense inflammation in the interstitial areas. In contrast, in the GLE groups (G_4&G_5) there were only a few necrotic cells. Previous studies have also shown that methotrexate (MTX) intensifies apoptosis [64]. Methotrexate led to oxidative stress in the rat liver and kidney, while GLE significantly prevented methotrexate- induced oxidative stress. Data so far obtained from this study would suggest that administration of GLE after MTX challenge may have beneficial effects that could possibly be ascribed, in part, to its regulation of the oxidant/anti-oxidant balance. So, it is therefore possible that GLE could scavenge free radicals and produce beneficial effects against MTX damage in liver and kidney. This shows that the desired dose of methotrexate can safely be used with GLE in the treatment of cancer and non-cancer diseases.

References

[1] Tousson E, Hafez E, Zaki S, Gad A. P53, Bcl-2 and CD68 expression in response to Amethopterin-induced lung injury and ameliorating role of L-carnitine. Biomed and pharmacology 2014.

[2] West SG. Methotrexate hepatoxicity. Rheum Dis Clin North Am 1997; 23: 883-915.

[3] Yozai K, Shikata K, Sasaki M, Tone A, Ohga S, Usui H. Methotrexate prevents renal injury in experimental diabetic rats via anti-inflammatory actions. J Am Soc Nephrol 2005; 16: 3326-3338.

[4] ALL C, Ertan B, Ergul BK, Bulent K. N-acetylcysteine ameliorates methotrexate-induced oxidative liver damage in rats. Med Sci Monit 2006; 12 (8): 247-248.

[5] Ozogula B, Kisaoglua A, Turanb M.I, Altunerc D, Senerd E, Cetine N, Ozturk C. The effect of mirtazapine on methotrexate-induced toxicity in rat liver. Science Asia 2013; 39: 336-356.

[6] Johovic N, Cevik H, Sehirli OA, Yegen BÇ, Şener G. Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats. *Journal of Pineal Research 2003*; 34, 282-287.

[7] Cetin A, Kaynar L, Kocyigit I, Hacioglu S.K, Saraymen R, Ozturk A, Chan ES, Montesinos MC, Fernandez P. Adenosine A (2A) receptors play a role in the pathogenesis of hepatic cirrhosis. Br J Pharmacol 2006; 148 (8): 1144-1155.

[8] Hemeida A.R, Omar M.M. Curcumin Attenuates Methotraxate-Induced Hepatic Oxidative Damage in Rats. Journal of the Egyptian Nat Cancer Inst 2008; 20 (2): 141-148.

[9] Vardi N, Parlakpinar H, Ozturk F, Ates B, Gul M, Cetin A, Erdogan A, Otlu A. Potent protective effect of apricot and β-carotene on methotrexate induced intestinal oxidative damage in rats. *Food and Chemical Toxicology 2008;* 46*:* 3015-*3022*.

[10] Vardi N, Parlakpinar H, Ates B, Cetin A, Otlu A. Anti apoptotic and antioxidant effects of beta-carotene against methotrexate-induced testicular injury. *Fertil Steri 2009;* 92*:* 2028-*2033*.

[11] Iyyaswamy A, Rathinasamy S. Effect of chronic exposure to aspartame on oxidative stress in the brain of albino rats. J Biosci 2012; 37: 679-688.

[12] Stickel F, Schuppan D. Herbal medicine in the treatment of liver diseases. Dig Liver Dis 2007; 39: 230-293

[13] Abad MJ, Bedoya LM, Bermejo P. An update on drug interactions with the herbal medicine Ginkgo biloba. Current Drug Metabolism 2010; 11: 171-181.

[14] Sakr SA, Abo-El-Yazid SM. Effect of fenugreek seed extract on adriamycin-induced hepatotoxicity and oxidative stress in albino rats. Toxicology and Industrial Health 2012; 28 (10): 876-885.

[15] Sakeran MI, Zidan N, Rehman H, Aziz AT, Saggu S. Abrogation by Trifolium alexandrinum root extract on hepatotoxicity induced by acetaminophen in rats. Redox Rep 2014; 19 (1): 26-33.

[16] Elsabagh S, Hartley D.E, Ali O, Williamson E.M, File S.E. Differential cognitive effects of Ginkgo biloba after acute and chronic treatment in healthy young volunteers. Psychopharmacology 2005; 179: 437-446.

[17] Altiok N, Ersoz M, Karpuz V, Koyuturk M. Ginkgo biloba extract regulates differentially the cell death induced by hydrogen peroxide and simvastatin. Neurotoxicology 2006; 27: 158-163.

[18] Kalisz O, Wolski T, Gerkowicz M. Miłorząb japoński (Ginkgo biloba) i jego preparaty wterapii zabur zeńkrążenia mózgowego i obwodowego [Ginkgo biloba (*Ginkgo biloba*) and its preparations in therapy of cerebral and peripheral circulation disorders]. Ann. Univ. Mariae Curie-Skłodowska 2006; 61 (2): 11-17.

[19] Abdel-Kader R, Hauptmann S, Keil U, Scherping I, Leuner K, Eckert A, Müller WE. Stabilization of mitochondrial function by Ginkgo biloba extract. Pharmacol Res 2007; 56 (6): 493-502.

[20] Liu JJ, Ching LM, Goldthorpe M, Sutherland R, Baguley BC, Kirker JA, McKeage MJ. Antitumour action of 5,6-dimethylxanthenone-4-acetic acid in rats bearing chemically induced primary mammary tumours. Cancer Chemother Pharmacol 2007; 59: 661-669.

[21] Mahadevan S, Park Y. Multifaceted therapeutic benefits of Ginkgo biloba L.: chemistry, efficacy, safety, and uses. J Food Sci 2008; 73 (R) 14-19.

[22] Kobus J, Flaczyk E, Siger A, Nogala-Kalucka M, Korczak J, Pegg R.B. Phenolic compounds and antioxidant activity of extracts of

Ginkgo leaves. Eur J Lipid Sci Technol 2009; 111 (11): 1150-1160.

[23] Cha´vez-Morales RM, Jaramillo-Jua´rez F, Posadas del Rı´o FA, Reyes-Romero MA, Rodrı´guez-Va´zquez ML, Martı´nezSaldan˜a MC. Protective effect of Ginkgo bilobaextract on liver damage by a single dose of CCl4in male rates. Hum Exp Toxicol 2011; 30: 209-216.

[24] Sener G, Eksioglu-Demiralp E, Cetiner M, Ercan F, Yegen BC. Beta-glucan ameliorates methotrexate-induced oxidative organ injury via its antioxidant and immunomodulatory effects. Eur J Pharmacol 2006; 542 (33): 170-178.

[25] Yozai K, Shikata K, Sasaki M, Tone A, Ohga S, Usui H. Methotrexate prevents renal injury in experimental diabetic rats via anti-inflammatory actions. J Am Soc Nephrol,2005; 16: 3326-3338.

[26] Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Amer J Clin Pathol 1957; 28: 56-63.

[27] Doumas BT, Watson WA, Biggs HG. Albumin standards and the measurement of serum albumin with bromocresol green. Clin Chim Acta 1971; 31: 87-96.

[28] Fawcett J.K, Scott J.E. A rapid and precise method for the determination of urea. J Clin Path 1960; 113: 156.

[29] Bowers LD, Wong ET. Kinetic serum creatinine assays. II. A critical valuation and review. Clin Chem 1980; 26 (5): 555-561.

[30] Mesbah L, Soraya B, Narimane S, Jean PF. Protective effect of flavonoides against the toxicity of vinblastine cyclophosphamide and paracetamol by inhibition of lipid- peroxidation and increase of liver glutathione. Haema 2004; 7: 59-67.

[31] Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963; 61: 882-888.

[32] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J. Biol. Chem. 1951; 193: 265-275.

[33] Tsuyosh P, James KB. A simplified method of quantitating Protein using the Buiret and phenol reagents, Anal Biochem 1978; 86: 193-200.

[34] Bancroft JD, Stevens A. Theory and Practice of Histological Technique. 3rd Ed. Churchill Living stone. Edinburgh, London benefits in Stage +2 postmenopausal women after 6 weeks of treatment with Ginkgo biloba. J Psychopharmacol 1990; 19: 173-181.

[35] Balk RA. Methotrexate-induced lung injury. UpT o Date, 2006. Available at: www.uptodate.com. Accessed 8 May 2006.

[36] Kremer JM. Major side effects of methotrexate. UpToDate, 2006. Available at: www.uptodate.com. Accessed 8 May 2006.Laboratory Medicine. Chapter 66. Lexi-Comp Inc.

[37] Genestier L, Paillot R, Quemeneur L, Izeradjene K, Revillard JP. Mechanisms of action of methotrexate. Immunopharmacology 2000; 47: 247-257.

[38] Walling J. From methotrexate to pemetrexed and beyond. A review of the pharmacodynamic and clinical properties of antifolates. Investigational New Drugs 2006; 24: 37-77.

[39] Wielinga P, Hooijberg JH, Gunnarsdottir S. The human multidrug resistance protein MRP5 transports folates and can mediate cellular resistance against antifolates. Cancer Res 2005; 65: 4425-4430.

[40] Maiti R, Jana D, Das U.K, Ghosh D. Antidiabetic effect of aqueous effect of seed of Tamarindus indica in streptozotocininduced diabetic rats. J Ethnopharmacol 2004; 92: 85-91.

[41] Fu Y, Zheng S, Lin J, Ryerse J, Chen A. Curcumin protects the rat liver from CCl4-caused injury and fibrogenesis by attenuating oxidative stress and suppressing inflammation. Mol Pharmacol 2008; 73 (2): 399-409.

[42] Vardi A, Bosviel R, Rabiau N, Adjakly M, Satih S, Dechelotte P. Soy phytoestrogens modify DNA methylation of GSTP1, RASSF1A, EPH2 and BRCA1 promoter in prostate cancer cells. *In Vivo 2010;* 24: 393-400.

[43] Drotman R, Lawhan G. Serum enzymes are indications of chemical induced liver damage. Drug Chem Toxicol 1978; 1: 117-163.

[44] Sakeran M.I, Zidan N, Rehman H, Aziz AT, Saggu S. Abrogation by Trifolium alexandrinum root extract on hepatotoxicity induced by acetaminophen in rats. Redox Rep 2014; 19 (1): 26-33.

[45] Saggu S, Kumar R. Modulatory effect of seabuckthorn leaf extract on oxidative stress parameters in rats during exposure to cold, hypoxia and restraint (C-H-R) stress and post stress recovery. J Pharm Pharmacol 2007; 59 (12): 1739-1745.

[46] Jadon A, Bhadauria M, Shukla S. Protective effect of Terminalia belerica Roxb. and gallic acid against carbon tetrachloride induced damage in albino rats. J Ethnopharmacol 2007; 109: 214-218.

[47] Kadikoylu G, Bolaman Z, Demir S, Balkaya M, Akalin N, Enli Y. The effects of desferrioxamine on cisplatininduced lipid peroxidation and the activities of antioxidant enzymes in rat kidneys. Hum Exp Toxicol 2004; 23: 29-34.

[48] Klukowska L, Nadulska A, Dyba S. The influence of cisplatinum and goserelinum on the magnesium and calcium level in rat serum. Ann Univ Mariae Curie Sklodowska [Med]. 2001; 56: 483-486.

[49] Saad MF, Greco S, Osei K, Lewin AJ, Edwards C, Nunez M, Reinhardt RR. Ragaglitazar improves glycemic control and lipid profile in type 2 diabetic subjects: a 12-week, double-blind, placebocontrolled dose-ranging study with an open pioglitazone arm. Diabetes 2004; 27: 1324-1329.

[50] Thabrew M, Joice P. A comparative study of the efficacy of Pavetta indica and Osbeckia octanda in the treatment of liver dysfunction. Planta Med 1987; 53 (3): 239-241.

[51] Kolli VK, Abraham P, Rabi S. Methotrexate-induced nitrosative stress may play a critical role in small intestinal damage in the rat. *Archives of Toxicology* 2008; 82 (10): 763-770.

[52] Jahovic N, Sener G, Cevic H, Ersoy Y, Arbak S, Yegen BC. Amelioration of methotrexate-induced enteritis by melatonin in rats. Cell Biochem Function 2004; 22-28.

[53] Cetiner M, Sener G, Sehirli AO, Eksioglu-Demiralp E, Ercan F, Sirvanci S, Gedik N, Akpulat S, Tecimer T, Yegen BC. Taurine protects against methotrexate-induced toxicity and inhibits leucocyte death. Toxicol Appl Pharmacol 2005; 209 (1): 39-50.

[54] Sener G, Eksioglu-Demiralp E, Cetiner M, Ercan F, Yegen BC. Beta-glucan ameliorates methotrexate-induced oxidative organ injury via its antioxidant and immunomodulatory effects. Eur J Pharmacol 2006; 542 (33): 170-178.

[55] Vardi A, Bosviel R, Rabiau N, Adjakly M, Satih S, Dechelotte P. Soy phytoestrogens modify DNA methylation of GSTP1, RASSF1A, EPH2 and BRCA1 promoter in prostate cancer cells. *In Vivo.* 2010; 24: 393-400.

[56] Fridovich I. Superoxide radical and superoxide dismutases. Annu Rev Biochem 1995; 64: 97-112.

[57] Ciralik H, Bulbuloglu E, Cetinkaya A, Kurutas E.B, Celik M, Polat A. Effects of N acetylcysteine on methotrexate induced small intestinal damage in rats. The Mount Sinai Journal of Medicine. 2006; 73: 1086-1092.

[58] Lone I.A, Kaur G, Athar M, Alam M.S. Protective effect of Rumex patientia (English Spinach) roots on ferric nitrilotriacetate (Fe-NTA) induced hepatic oxidative stress and tumor promotion response. Food Chem Toxicol 2007; 45: 1821-1829.

[59] Babiak RM, Campello AP, Carnieri EG, Oliveira M.B. Methotrexate: pentose cycle and oxidative stress. Cell Biochem Function 1998; 16: 283-293.

[60] Prahalathan C, Selvakumar E, Varalakshmi P. Protective effect of lipoic acid on adriamycin-induced testicular toxicity. *Clinica Chimica Acta 2005*; 360: 160-*166.*

[61] O'Rourke RA, Eckert GE. Methotrexate-induced hepatic injury in an adult A case report. Arch Int Med 1964; 113: 191-194.

[62] Ros S, Juanola X, Condom E, Canas C, Riera J, Guardiola J, Del Blanco J, Rebasa P, Valverde J, Roig-Escofet O. Light and electron microscopic analysis of liver biopsy samples from rheumatoid arthritis patients receiving long-term methotrexate therapy. Scand J Rheumatol 2002; 31 (6): 330-336.

[63] Hytiroglou P, Tobias H, Saxena R, Abramidou M, Papadimitriou CS, Theise ND. The canals of hering might represent a target of methotrexate hepatic toxicity. Am J Clin Pathol 2004; 121 (3): 324-329.

[64] Horie T, Li T, Ito K, Sumi S, Fuwa T. Aged garlic extract protects against methotrexate-induced apoptotic cell injury of IEC-6 cells. J Nutr 2006; 53: 239-241.