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THE PROTECTIVE EFFECT OF DAFLON AGAINST THE RENAL-HEPATIC TOXICITY INDUCED BY METHOTREXATE IN RABBITS

[1]Nael Mohammed Sarheed and Safa Azher Al-khalidy

College of Medicine, University of Al-Muthanna, Iraq. E. mail: [1]Nael.serheed@mu.edu.iq

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ABSTRACT

Methotrexate (MTX) is considered a folic acid antagonist, used frequently in the treatment of various types of diseases and has had a major role in the treatment of inflammation and suppressive material for immunity in Non – tumor diseases like acute arthritis and psoriasis. This study was designed to investigate the effect of methotrexate and pre-treatment with daflon in attenuation of its development for renal-hepatic toxicity. Twenty-four healthy, local, domestic rabbits were randomized divided into 4 groups: group 1 (control), group 2 (20mg\kg MTX group), group 3 (Daflon 100 + MTX) and group 4 (Daflon 200 + MTX). At the end of experiment, blood samples were collected from the heart then the liver and kidney tissues were excised. Plasma level of S.ALT, S.AST, ALP, total protein, bilirubin, urea, creatinine and oxidation parameters (GSH, and MDA) were measured. **The Results** show that the Compared with the control group, levels of S.ALT, S.AST, ALP, urea, creatinine and MDA were increased ($p<0.05$), while total protein and GSH were decreased ($p<0.05$) in MTX group with deterioration of liver and kidney tissues. While group 3 (Daflon 100 + MTX) and group 4 (Daflon 200 + MTX) showed decreased levels of S.ALT, S.AST, ALP, urea, creatinine and MDA while total protein and GSH were increased ($p<0.05$) in as compared with MTX group with improvement of liver and kidney structures. **Conclusions:** The results of the present study reveal that daflon has been shown to attenuate MTX induced hepato-renal toxicity.

Key words: Methotrexate, hepatotoxicity, renal toxicity, daflon, oxidative stress.

INTRODUCTION

Methotrexate (MTX), a folic acid antagonist, is a cytotoxic drug which is commonly used in the cancer therapy (El-Sheikh *et al*., 2015) and as anti -inflammatory and immunosuppressive agent in other diseases such as Reiter's syndrome, arthritis, biliary cirrhosis and psoriasis (Weinblatt, 1985). It has many therapeutic uses and it is well tolerated at low doses, however, when it has been used in high doses or for long duration, many serious side effects are resulted, including hepatorenal toxicity (Cronstein *et al*., 1991) which may be resulted from increased oxidative stress both through increased activity of reactive oxygen species and impairment of anti-oxidative defense mechanism via depletion of intra hepatic glutathione store (Jahovic *et al*., 2003). Nowadays, it is usual to use supplements rich with polyphenols, particularly flavonoids which have protective effects against reactive oxygen species (ROS) and also enhanced antioxi-dant defense system in the body (Pandey and Rizvi, 2009).Flavonoids are natural polyphenols appear in many plants and have antioxidant, anticancer, anti-atherogenic activity and the ability to scavenge any radicals may cause damage in the body (Erdogan *et al*., 2015).

Daflon (Micronized purified flavonoid fract-ion) is a drug similar to synthetic treatment includ-ed 10% flavonoids expressed as hesperidin and 90% a flavone derivative (Katsenis, 2005). It is a phlebotonic drug commonly used in chronic ven-ous or lymphatic insufficiency, and this effect of Daflon can be attributed to decrease the inflam-mation and antioxidant effects of the flavonoid that contain (Delbarre *et al*., 1995). The antioxi-dant effect is attributed to its ability to decrease the proportion of hydroxyl radicals (Rapavi *et al*., 2007), with increasing in concentration of free SH-group, and the scavenge activity that give it's important role (Mcginness *et al*., 1977). The aim of this study was to investigate the protective effect of daflon against to hepato-renal oxidative injury induced by MTX, supposing that free radical scavenging activity may have a protective role against drug which lead to the toxicity in liver and kidney cells (Amimoto *et al*., 1995).

MATERIALS AND METHODS

1. **Experimental Animals:** A 24 local rabbits used in the experiment, ages 2-3 months and their weights (1300-1500 g) were involved in this study. Before the study, the animals were allowed for acclimatization for 3 days in the conditions of the experiment and were kept on controlled tem-perature and environment with a 12 h. light/ dark cycle. All rabbits have to eat and drink.

2. **Preparation of drugs:** Methotrexate (EBE-WE pharma, AUSTRIA) was used in 20mg/kg per day (Asvadi et al., 2011, Swayeh *et al*., 2014). Daflon® (Servier, Paris, France) consisting of 90% diosmin and 10% hesperidin, was putting in isotonic solution (0.9% NaCl) immediately before they began. Daflon® was used in a dose of (100 and 200) mg/kg/ per day orally (Delbarre *et al*., 1995).

3. **Experimental design:** Rabbits were divided into 4 groups randomly, each group including 6 animals and treated orally for 7 days: Group 1 (control): Rabbits were treatment with normal saline only. Group 2 (group MTX): Animals were injectted methotrexate in intra-peritoneal (ip) with (20 mg/kg) in the fourth day of the experiment period continued to four consecutive days. Group 3 (Daflon 100+MTX): Animals were treated 100 mg/kg of daflon by mouth once daily for 7 days, while MTX was injected in (20mg/kg) in the fourth day of the experiment continued for four successive days. Group 4 (Daflon 200 + MTX): Rabbits were given 200 mg/kg of daflon orally once per day(for 7 days)and MTX was injected20 mg/kg daily in the fourth day from the experimental period (7 days) and continued to four days. At the end 8th day of the experiment, blood collection were done to all rabbits by inhalation of chloroform for anesthesia. The blood collected by direct puncture in the heart, than the blood centrifuged at 3000 rpm for 10 min. to get plasma which was stored at -20°C for determination of S.ALT, S.AST, ALP, total protein, TSB , urea, creatnine and oxidation parameters (GSH, and MDA). After scarification, prepared the sections by (Luna, 1978) then were examined by microscope.

4. **Statistical analysis:** All data were compared with an analysis of variance (ANOVA). T-test was used to identify the difference between two groups, Use $P < 0.05$ as Probability level were regarded as statistically significant (Dawood and Ilias, 1990).

RESULTSAND DISCUSSION

This study performed a histological and biochemical studies into whether daflon has a preventive and improved characteristics on the pathological effects induced on the tissue by methotrexate in rabbits. Methotrexate (MTX) is one of the folic acid synthesis inhibitor, commonly used in the treatment of many effects of changes (Tawfeeq *et al*., 2014). It is used in high doses for malignancies like leukemia, lung and breast carcinomas, while low doses used as medicines for inflammation conditions and in the therapy of many autoimmune disorders including psoriasis, juvenile idiopathic arthritis and rheumatoid arthritis (Funk *et al*., 2013). Toxicity induced by MTX appears to be a result of the interaction of many factors that include the duration of treatment, dosing schedule, type of disease, patient risk factors and the presence of genetic disease or molecular apoptotic factors (Cetinkaya *et al*., 2006).In present study, histopathological changes were examined to support the results of the parameters of liver and renal functions.

1. **Histology and physiology of liver:** The examination of liver tissue was demonstrate that liver structure in the normal state of control group with lobular structure, Central vein, Sinusoids and Hepatic cords are consisted of hepatocytes arranged radially (Figure 9A). While the treatment with use MTX led to congestion and dilatation of central vein, necrosis of hepatocytes and accumulation of inflammatory cells, in addition to extension in sinusoids(Figure 9B). As far the combined treatment of MTX and daflon in group3 showed normal hepatic structure with simple changes in the tissue as dilatation in hepatic sinusoids and cell death (Figure 9C), but the improvement was more significant in group 4 (Daflon200+MTX), where observed the tissue closer to the normal tissue in control group.

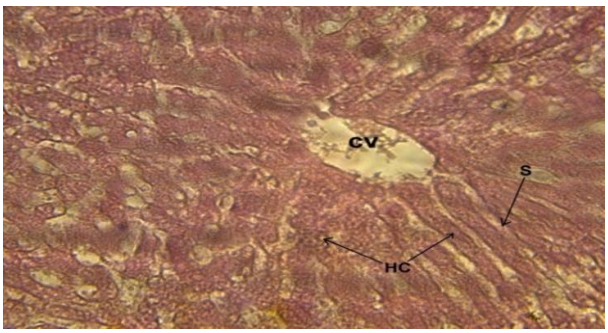

Figure 9A: Cross section of Liver in group1, (Control), Central vein (CV), Sinusoids(S), Hepatocytes (HC), (H&E40X).

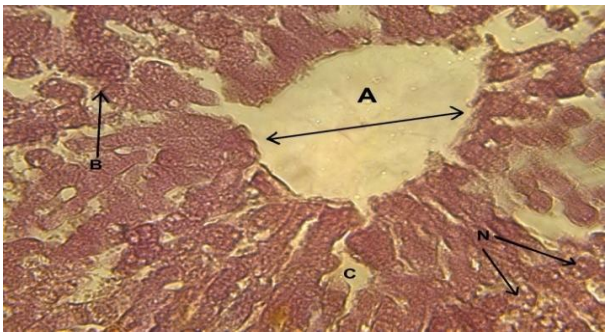

Figure 9B: Cross section of Liver in group2, Central vein dilatation (A), inflammatory cells (B), sinusoids extension(C), Necrosis(N), (H&E40X).

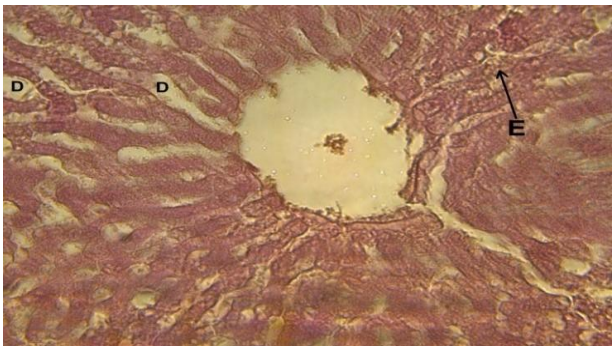

Figure 9C: Cross section of Liver in group 3, sinusoids dilatation (D), cell death (E)(H&E40X).

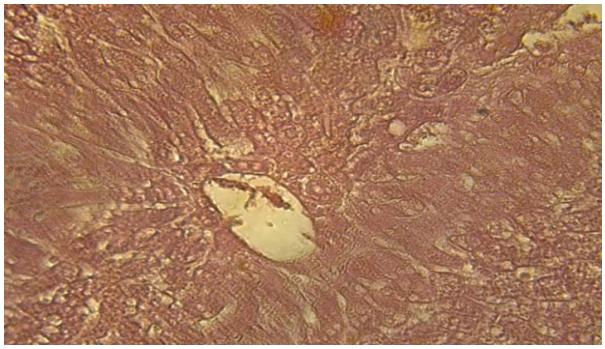

Figure 9**D:** Cross section of Liver in group 4, The tissue closer to the normal shape (H&E40X).

The functions enzymes tests have supported what appeared in this study that the treatment with MTX in rabbits induce hepatotoxicity, which reflected in a significant increased($P < 0.05$) in the rates of ALT, AST and ALP as shown when compared with control group, while treatment with daflon and MTX in group 3 showed a significant decrease in the levels of ALT, AST and ALP when compared with MTX group, but the reduction was higher significant in group 4 (Daflon 200 + MTX) when compared with group 3 (Daflon 100 + MTX)(Fig.1,2,3). As for total protein (T. protein) was significantly decreased of the second group (MTX) through the compared with control group. In contrast, this test was significantly increased in groups that treated with daflon and MTX when compared with MTX group, and the increase in total protein was significant in group 4 (Daflon 200 + MTX) when compared with group3 (Daflon 100 + MTX)(Fig.4).

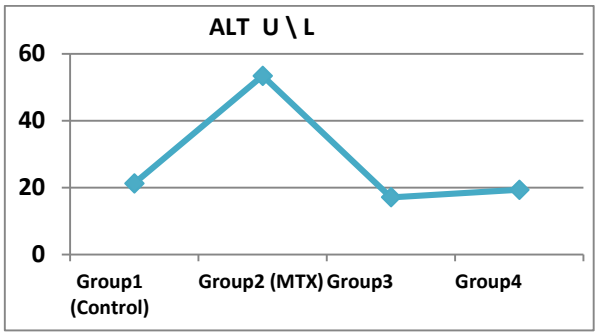

Figure 1: The differences in ALT test.

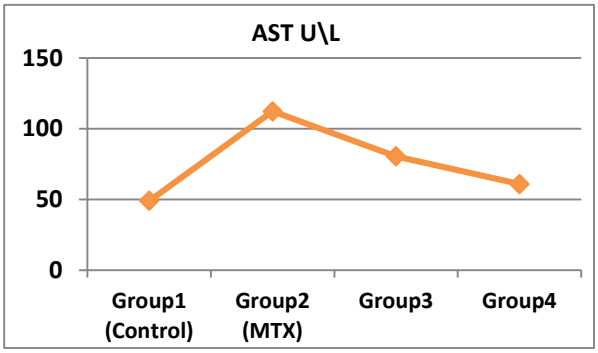

Figure 2: The differences in AST test.

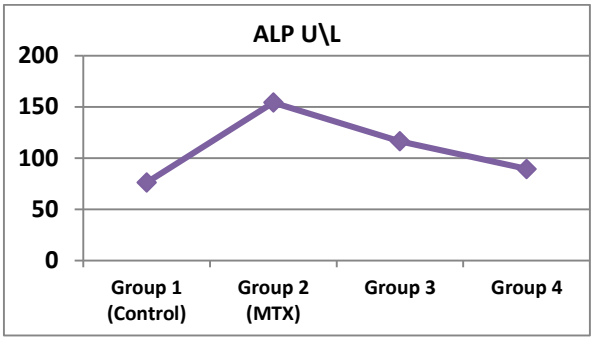

Figure 3: The differences in ALP test

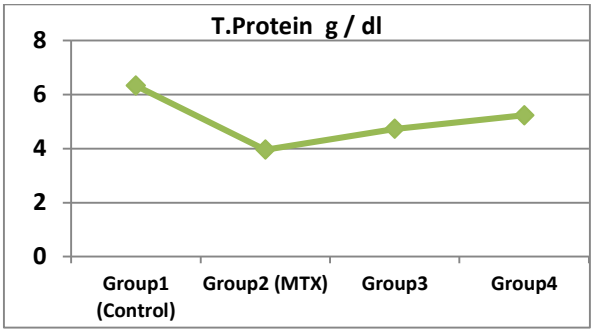

Figure 4: The differences in T. Protein.

Liver histopathological observation supported the results of liver function tests which showed that severe degree of damage in hepatic tissue. The present study agree with (Vardi *et al*., 2010),who observed that various of the side effects of methotrexate be associated with a presence of hydrogen peroxides and free oxygen radicals and these peroxides and radicals lead to cell death by binding between the large cellular molecules, especially the lipid molecules of cell membrane causing egression of AST and ALT from Inside the cell to serum which is the clear sign of hepatic injury, these results also are in agreement with the reports of other studies (Uraz *et al*., 2008, Walker *et al*., 2000) and this increased in ALT and AST enzymes are functional inefficiency of cell membranes and evidence of intracellular leakage in the liver (Drotman and Lawhorn,1978), that may be due to the pathological effects of methotrexate by the ability to discouragement of dihydrofolate reductase (DHFR) and Obstruction of thymidylate synthetase (which are important for DNA synthesis) and lead to restriction in nucleic acids industry (Alkhateeb *et al*., 2014). This disability in the formation of amino acids, DNA and RNA might lead to injury in the cell membranes and organelles of parenchymal cells in the liver and permiting to infiltration of enzymes (Hersh *et al*., 1966). Adding to this, may be occur changes in the structure and shape of nucleus by the increase in oxidative stress that a resulted from destruction and distortion of DNA, which have a key role in the development of apoptosis (Morcy *et al*., 2013), so

MTX caused with increase the oxidative stress in the renal and hepatic cells. While daflon prevents induced oxidative stress by methotrexate, because of their ability to effect against methotrexate injury in kidney and liver tissue by sweeping the free radicals formed (Amimoto *et al*., 1995). In the present study too, we observed that adding daflon to MTX was found to have hepato protective effect as demonstrated by a significant decrease in liver enzymes activities with improvement in liver histology. This result in agree with (Paysant *et al*., 2008, Kose *et al*., 2012, Erdogan and Ilgaz, 2015, Kalantar *et al*., 2017).

2. **Histology and physiology of Kidney:** Regarding the histological changes in kidney, The tissue presented with acute congestion and retrogression in glomerulus, the area of the Bowman capsule was expanding, tubular degradation and leakage of inflammatory cells in group2 (MTX group), when compared with control group (Figure F), which showed normal kidney histology, Glomerulus, Bowman capsule, Distal convoluted tubules and proximal convoluted tubules (Figure E). Treatment with daflon and MTX showed improved in the tissue of kidney, with some mild expansion of convoluted tubules and abnormal shape for tissue in group 3 (Figure G). While the improvement was more significant in group 4 (Daflon 200+ MTX) (Figure H) when compared with group 3 (Daflon 100+MTX).

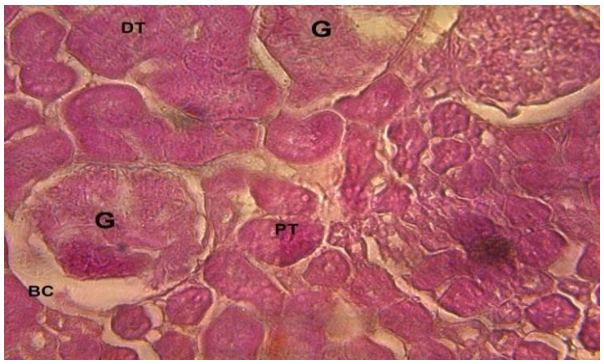

Figure 9E: Cross section of Kidney (Cortex) in group1 (Control), Glomerulus (G), Buman capsule (BC), DistalTubule (DT), Proximal tubule (PT) (H&E40).

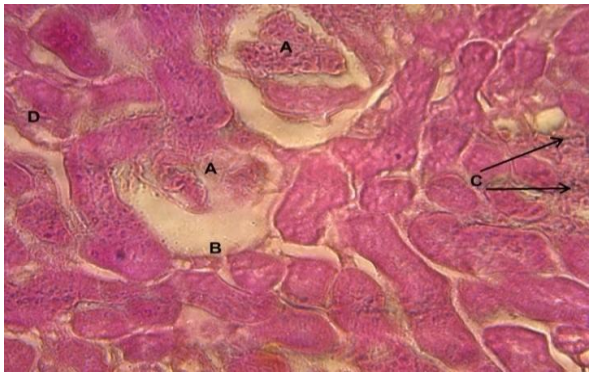

Figure 9F: Cross section of Kidney ingroup 2, (A) Glomerular congestion, (B) dilatation (C), Inflammatory Cell, (D) Tubular degeneration.

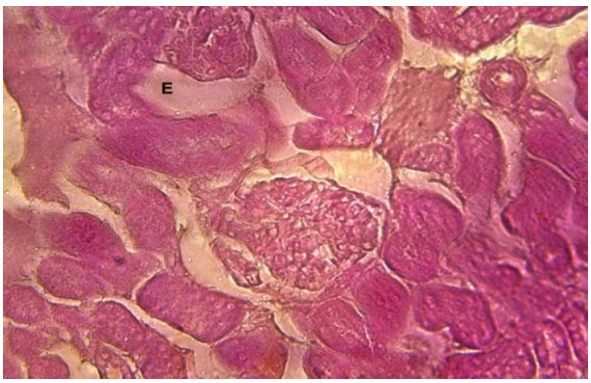

Figure 9G: Cross section of Kidney ingroup 3, Renal tubules dilatation (E) and abnormal shape for tissue (H&E40).

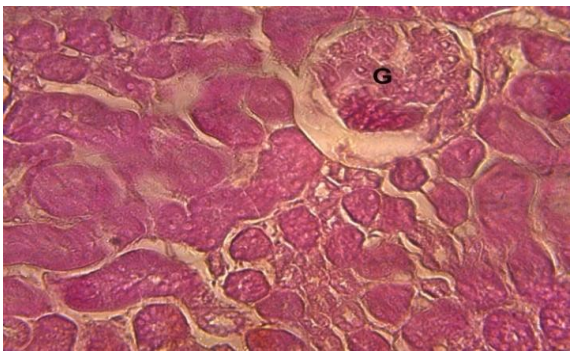

Figure 9**H:** Cross section of Kidney ingroup 4, (G) Semi normal shape, Glomerulus (H&E40).

Figure 9: histopathological changes in some organs (kidney and liver (H and E) 400X

Serum urea and creatinine levels were assessed as markers of renal functions which supported its results the histopathological effect in kidney of this study, Where this study showed the treatment with MTX alone in rabbits developed renal injury, that reflected by a significant increase ($P<0.05$) in the levels of urea and creatinine when the comparison with control group, while treatment with daflon and MTX revealed a significant decrease in the proportions of urea and creatinine in group 3 (Daflon 100 + MTX) and group 4 (Daflon 200 + MTX) when compared with control group, but the reduction was more significant in group 4 (Daflon 200 + MTX) when compared with group 3 (Daflon 100 + MTX) (Fig. 5, 6).

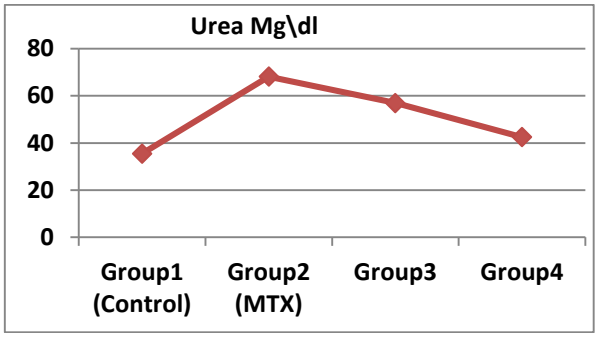

Figure 5: The differences in B. Urea test.

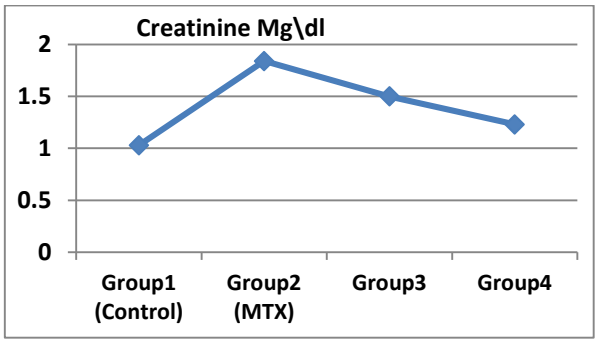

Figure 6: The differences in S. Creatinine.

Renal failure (acute stage) is one of the common pathological changes in the kidneys that results from the use of high doses of methotrexate continuously and which is found at 3-10% of treatment courses (Widemann *et al*., 2006). The most common patho-physiological cases of acute renal failure (ARF) is appearance of methotrexate in the urine and deposition of its metabolic products in the acidic environment with renal outcomes (Jahovic *et al*., 2003). In this study, our result is agree with (Kolli *et al*., 2008, Tousson *et al*., 2014) who reported that MTX increase urea and creatinine activities, which stimulate of toxicity in the kidney, in addition they are supported by kidney histopathological examination which shows severe renal injury in MTX treated group. On the contrary, the results are disagreement with (Chan *et al*., 2006). This study also showed that daflon has renal protective effect for the first time through reduction in urea and creatinine with improvement in kidney histopathological changes and here the study agree with (Asvadi *et al*., 2011, Morsy *et al*., 2013, El-Sheikh *et al*., 2015).

According to the results of this study, daflon has a dose dependent hepatorenal protective effect (increasing with dose increment). And here the improvement by daflon may be attributed to his role in anti-inflammation and antioxidant, as well as the protection from nephrotoxicity and hepatotoxicity (Amimoto *et al*., 1995, Delbarre *et al*., 1995)

3. **Oxidation parameters:** Concerning oxidation parameters, the result demonstrated that treatment with MTX leading to increasing with MDA level, which is lipid peroxidation product, and decreasing with GSH level which is considered as antioxidant enzyme in MTX group. Adding daflon to MTX in group 3 and group 4 significantly atteuated lipid peroxidation as evident by significant decreases in MDA level which associated with a significant increase in GSH level as shown in (Fig. 7, 8).

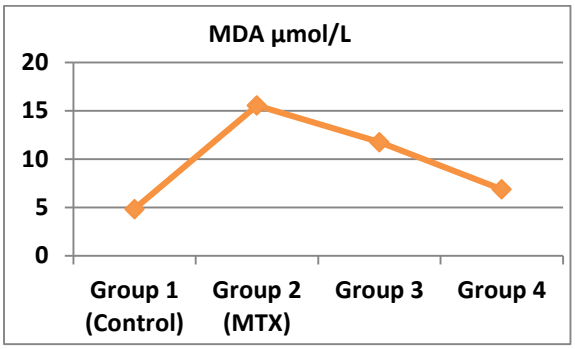

Figure 7: The differences in MDA test.

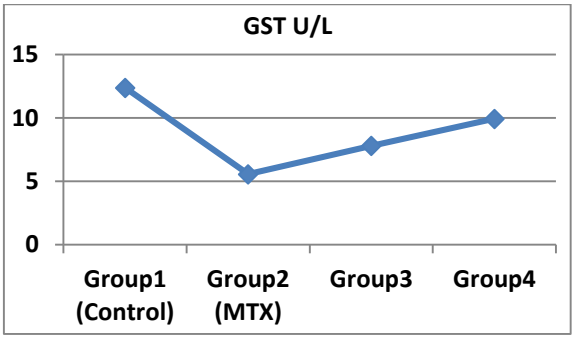

Figure 8: The differences in GST test.

Oxidative stress is a parameter used to denote the change in equilibrium between oxidizing agents and antioxidants which leads to injury or damage to a specific part of the body, If the equilibrium unable to continue in the tissues, there are many of histopathological and physiological changes will caused damage in the cell (Jahovic *et al*., 2003). The damage that resulted by oxidative stress with the common effects of peroxidation and generation of radicals are fixed sign of methotrexate pathological effect (Jahovic and Şener, 2004). It is the metabolic product to metabolize free radicals resulting from peroxidation of lipid (Kose *et al*., 2012). Its usefulness as an indicator of lipid release and peroxidation in saturated layers such as cell membranes, in addition to being a final outputs of irregular oxidation (Sahna *et al*., 2006). The lipid peroxidation may cause by Methotrexate via the clear increases in MDA and may be mediated by oxygen-free radicals which be an important pathological factor of distortion and fragmentation to the cell membranes and was suggested to be a contributing factor of the effect of MTX- mediated the

changes in the tissue (Kose *et al*., 2012). GSH is very important part for defense as antioxidant agents in living cells (Iyyaswamy and Rathinasamy, 2012). In the present experiment, MDA in the second group (MTX) was observed in high level, while the glutathione showed decreased when compared with control group. Our results agree with Delbarre *et al*., (1995), Ciralik *et al*., (2006) and Fu *et al*., (2008) who found that, the treatment with MTX causes clear decrease in GSH with increase in the level of MDA. Regarding daflon is an effecttive through the reduction in lipid peroxidation products and graduated increase in GSH in blood, suggesting that the active effect as anti-oxidant and the scavenge activity of the radicals by daflon may reduce the pathological changes by MTX (Mcginness et al., 1977, Rapavi et al., 2007).

REFERENCES

Al-khateeb, H.M., Barraj, A.H. and Z.A. Kadhim, Effect of methotrexate on mice embryo liver. Iraqi Journal of Science 55(2): 374-81 (214).

Amimoto, T., Matsura, T., Koyama, S.Y., Nakanishi, T., Yamada, K. and G. Kajiyama, Acetaminophen-induced hepatic injury in mice: the role of lipid peroxidation and effects of pretreatment with coenzyme Q10 and α-tocopherol. Free Radical Biology and Medicine 19(2): 169-176 (1995).

Asvadi, I., Hajipour, B., Asvadi, A., Asl, N.A., Roshangar, L. and A. Khodadadi, Protective effect of pentoxyfilline in renal toxicity after methotrexate administration. Eur. Rev. Med. Pharmacol. Sci. 15(9): 1003-109 (2011).

Cetinkaya, A., Bulbuloglu, E., Kurutas, E.B. and B. Kantarceken, N-acetylcysteine ameliorates methotrexate-induced oxidative liver damage in rats. Medical Science Monitor 12(8): BR 274-8 (2006).

Chan H., Evans W.E. and C.B. Pratt, Recovery from toxicity associated with high-dose methotrexate: Prognostic factors. Cancer Treatment Reports 61(5): 797 (1977).

Chan, E.S.L., Maria Carmen Montesinos, Patricia Fernandez, Avani Desai, David L Delano, Herman Yee, Allison B Reiss, Michael H Pillinger, Jiang-Fan Chen, Michael A Schwarzschild, Scott L Friedman and Bruce N Cronstein, Adenosine A2A receptors play a role in the pathogenesis of hepatic cirrhosis. British Journal of Pharmacology 148(8): 1144-1155 (2006).

Ciralik, H., Bulbuloglu, E., Cetinkaya, A., Kurutas, E.B., Celik, M. and A. Polat, Effects of N-acetylcysteine on methotrexate-induced small intestinal damage in rats. The Mount Sinai Journal of Medicine, New York. 73(8): 1086-1092 (2006).

Cronstein, B.N., Eberle, M.A., Gruber, H.E. and R.L. Levin, Methotrexate inhibits neutrophil function by stimulating adenosine release from connective tissue cells. Proceedings of the National Academy of Sciences 88(6): 2441-2445 (1991).

Dawood, K.M. and Z.A. Ilias, (1990). The statistical Methods to Agriculture Researches.AL-Musel University.

Delbarre, B., Delbarre, G. and F. Calinon, Effect of Daflon 500 mg, a flavonoid drug, on neurological signs, levels of free radicals and electroretinogram in the gerbil after ischemia-reperfusion injury. International Journal of Microcirculation 15(Suppl. 1): 27-33 (1995).

Drotman, R.B. and G.T. Lawhorn, Serum enzymes as indicators of chemically induced liver damage. Drug and Chemical Toxicology 1(2): 163-171 (1978).

El-Sheikh, A.A., Morsy, M.A., Abdalla, A.M., Hamouda, A.H. and I.A. Alhaider, Mechanisms of thymoquinonehepatorenal protection in methotrexate-induced toxicity in rats. Mediators of Inflammation Article ID 859383, 12 pages (2015) http//: dx.doi.org/10.1155/2015.

Erdogan, E., Ilgaz, Y., Gurgor, P.N., Oztas, Y., Topal, T. and E. Oztas, Rutin ameliorates methotrexate induced hepatic injury in rats. Acta Cirurgica Brasileira. 30(11): 778-784 (2015).

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

Funk, R.S., van, H. L., Becker, M.L. and J.S. Leeder, Low-dose methotrexate results in the selective accumulation of aminoimidazole carboxamideribotide in an erythroblastoid cell line. Journal of Pharmacology and Experimental Therapeutics 347(1):154-163 (2013).

Hersh, E.M., Wong, V.G., Henderson, E.S. and E. J. Freireich, Hepatotoxic effects of methotrexate. Cancer 19(4): 600-606 (1966).

Iyyaswamy, A. and S. Rathinasamy, Effect of chronic exposure to aspartame on oxidative stress in brain discrete regions of albino rats. Journal of Biosciences 37(4): 679-688 (2012).

Jahovic, N. and G. Şener, Teritis by melatonin in rats. Cell Biochemistry and Function 22(3): 169-178 (2004).

Jahovic, N., Çevik, H., Şehirli, A.O., Yegen, B.Ç. and G. Şener, Melatonin prevents methotrexate-induced hepatorenal oxidative injury in

rats. Journal of Pineal Research 34(4): 282-287 (2003).

Kalantar M., Shirali S., Hasanvand A., Valizadeh M., Tavakoli R., Asadi M. and M. Goudarzi, Ameliorative Effects of Hydroalcoholic Extract of Lavandulaofficinalis L. on Methotrexate-Induced Oxidative Stress in Rats. Pharmaceutical Sciences 23(1):18-26 (2017)

Katsenis, K., Micronized purified flavonoid fraction (MPFF): A review of its pharmacological effects, therapeutic efficacy and benefits in the management of chronic venous insufficiency. Current Vascular Pharmacology 3(1): 1-9 (2005).

Kolli, V.K., Abraham, P. and S. Rabi, Methotrexate-induced nitrosative stress may play a critical role in small intestinal damage in the rat. Archives of Toxicology 82(10): 763-70 (2008).

Kose, E., Sapmaz, H.I., Sarihan, E., Vardi, N., Turkoz, Y. and N. Ekinci, Beneficial effects of montelukast against methotrexate-induced liver toxicity: a biochemical and histological study. The Scientific World Journal Article ID 987508 (2012) doi: 10.1100/2012/987508.

Luna, L.G. (1978). Manual of histological staining methods of the armed force institute of pathology. 3rd ed. MC grow hill book. Londen.

Mcginness, J.E., Proctor, P.H., Demopoulos, H.B., Hokanson, J.A. and D.S. Kirkpatrick, Amelioration of cis-platinum nephrotoxicity by orgotein (superoxide dismutase). Physiological Chemistry and Physics 10(3): 267-277 (1977).

Morcy, M.A., Ibrahim, S.A., Amin, E.F., Kamel, M.Y., Rifaai, R.A. and M.K. Hassan, Curcumin ameliorates methotrexate-induced nephrontoxicity in rats. Advances in Pharmacological Sciences Article ID 387071, 7 pages (2013) https://doi.org/10.1155/2013/387071.

Pandey, K.B. and S.I. Rizvi, Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity 2(5): 270-278 (2009).

Paysant, J., Sansilvestri-Morel, P., Bouskela, E. and T.J. Verbeuren, Different flavonoids present in the micronized purified flavonoid fraction (Daflon® 500 mg) contribute to its anti-hyperpermeability effect in the hamster cheek pouch microcirculation. International Angiology 27(1): 81 (2008).

Rapavi, E., Kocsis, I., Fehér, E., Szentmihályi, K., Lugasi, A., Székely, E. and A. Blázovics, The effect of citrus flavonoids on the redox state of alimentary-induced fatty liver in rats. Natural product research 21(3): 274-281 (2007).

Sahna, E., Parlakpinar, H., Cihan, O.F., Turkoz, Y. and A. Acet, Effects of aminoguanidine against renal ischaemia–reperfusion injury in rats. Cell Biochemistry and Function 24(2): 137-141 (2006).

Şener, G., Ekşioğlu-Demiralp, E., Çetiner, M., Ercan, F. and B.C. Yeğen, β-glucan ameliorates methotrexate-induced oxidative organ injury via its antioxidant and immunomodulatory effects. European Journal of Pharmacology 542(1): 170-178 (2006).

Swayeh, N.H., Abu-Raghif, A.R., Qasim, B.J. and H.B. Sahib, The protective effects of Thymus vulgaris aqueous extract against Methotrexate-induced hepatic toxicity in rabbits. Int. J. Pharm. Sci. Rev. Res. 29: 187-193 (2014).

Tawfeeq, A.A. and S.M. Taifoor, Histopathological effects of mthotrexate in mice livers. Kirkuk Uni J. Sci Studies 9(1): 18-27 (2014).

Tousson, E., Atteya, Z., El-Atrash, A. and O.I. Jeweely, Abrogation by Ginkgo Byloba leaf extract on hepatic and renal toxicity induced by methotrexate in rats. Journal of Cancer Research and Treatment 2(3):44-51 (2014).

Uraz, S., Tahan, V., Aygun, C., Eren, F., Unluguzel, G., Yuksel, M., Senturk, O., Avsar, E., Haklar, G., Celikel, C. and S. Hulagu, Role of ursodeoxycholic acid in prevention of methotrexate-induced liver toxicity. Digestive Diseases and Sciences 53(4): 1071-1077 (2008).

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

Walker, T.M., Rhodes, P.C. and C. Westmorel, The differential cytotoxicity of methotrexate in rat hepatocyte monolayer and spheroid cultures. Toxicology in Vitro 14(5): 475-485 (2000).

Weinblatt, M.E., Toxicity of low dose methotrexate in rheumatoid arthritis. The Journal of Rheumatology. Supplement 12: 35-39 (1985).

Widemann, B.C and P.C. Adamson, Understanding and managing methotrexate nephrotoxicity. The Oncologist 11(6):694-703 (2006).