

Protective Effect of Ca- Folate and Ginseng in Male Rats Treated with Methotrexate

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Abstract: The objective of this study was to assess the prevalence of neuron and hepatotoxicity of Methotrexate (MTX) and prophylactic response to Ca- Folate (Ca-F) and Ginseng (G). Forty two of male albino rat were divided to seven groups six rats for each. 1st control group received 1ml saline i.p daily for 4 weeks (C), 2nd group treated with Ca-F (5 mg /kg b.wt i.p.) for 4 weeks. 3rd group treated with G (25 mg /kg b.wt orally) for 4 weeks. 4th group treated with MTX (0.45 mg /kg b.wt i.p.) for 4 weeks. 5th group treated with MTX + Ca-F (0.45 mg/kg and 5 mg/ kg i.p.) for 4 weeks, respectively. 6th group treated with MTX + G (0.45 mg/kg and 25 mg/ kg i.p.) for 4 weeks, respectively. 7th group treated with MTX + Ca-F + G (0.45 mg/kg, 5 mg/kg and 25 mg/ kg i.p) for 4 weeks, respectively. In this study, aspartic acid (ASP), glutamic acid (GLU), ALT, AST, γ -GT, MDA, GSSG and NO were increase ($P < 0.05$) in MTX group comparing with control group in rat brain. Glycine (GLY), γ amino butyric acid (GABA), Norepinephrine (NE), Dopamine (DA), serotonin (5TH) and GSH were decrease ($P < 0.05$) in MTX group comparing with control group in rat brain. All these parameters were improved with G, F and their combination comparing with MTX group. Histopathological examination showed ameliorates effect for all treatments against MTX pathogenesis. It could be concluded that F and G influences and regeneration of neurons amino acids and catecholamine's activity and decrease liver enzymes and oxidative stress markers.

Keywords: Methotrexate, CA-folate, Ginseng, neurotransmitters and Amino acids.

I. Introduction

Methotrexate (MTX) is an anti-metabolite widely used in the treatment of neoplastic disorders, Rheumatoid arthritis and psoriasis. The basis for its therapeutic efficacy is the inhibition of dihydrofolate reductase (DHFR), which a key enzyme in the folic acid (FA) metabolism. MTX may induce severe toxicity during treatment of both human and experimental animals (Li *et al.*, 1956).

Folinic acid (FNA) is the beneficial effect might include reduced risk of vascular disease, anemia, neuropathy and sub-acute combined degeneration of the spinal cord. Folate may aggravate epilepsy, inflammatory arthritis, familial hyper lipidemia, hypercholesterolemia, hyper homocysteinemia and diabetes mellitus. FNA reduces both toxicity and side effects of MTX in human compromising the idiopathic arthritis (Kelly, 1998).

Ginseng extract is the dried roots of the Korean *Panax ginseng* which contain a combination of pharmacological important ingredients including ginsenosides (saponin glycosides), oil, phytosterols, carbohydrates, sugars, amino acids, peptides, vitamins, minerals and certain enzymes (Xun and Zhou, 2003). Ginseng exerts has a wide range of pharmacological activities on oral administration. It improves the psychomotor functions with evident anti-fatigue and anti-stress properties. In addition, the combined components of ginseng extract act directly on body cells and promote DNA and protein synthesis and increase in the specific activities of enzymes associated with cellular metabolism (Saito *et al.*, 1977).

The aim of this work to study how to decrease the side effect of MTX (toxicity) which used in treatment of cancer and rheumatoid arthritis by adding CA-folinic acid (Ca-F) and ginseng to ameliorate the neurotoxicity and hepatotoxicity of MTX in brain and liver in rats.

II. Materials and Methods

Chemicals

Aspartic acid, GLU, GLY, GABA, NE, DA, 5HT, MDA, GSH, GSSG and NO standard for High Performance Liquid Chromatography (HPLC) was purchased from SIGMA Company and MTX was purchased from Glacso Company. AST, ALT and γ GT were determined by spectrophotometric method by bio diagnostic kit which purchased from Bio-diagnostic Company.

Animals and Experimental Design:

The present study was carried out using adult male albino rats obtained from the animal house of National organization of Drug Control and Research (NODCAR) their average weights ranged from 180-200 g.

The animals were housed in standard conditions, animals were allowed to access standard diet and water *ad libitum*. Animals were cared in accordance with standard guidelines (**Olfert et al., 1993**).

Animals were divided into 7 groups namely

1. Control group, animal were received 1ml saline i.p daily for 4weeks.
2. Ca-folate (Ca-F) treated group animals were received Ca-F in a dose (5 mg /kg) b.wt i.p. for 4 weeks.
3. Ginseng (G) in a dose (25mg/kg) orally for 4weeks.
4. MTX -treated group animals were received MTX in a dose (0.45 mg/kg) b.wt i.p. for 4weeks.
5. **MTX+ Ca-F** group, animals were received MTX in a dose 0.45 mg/kg b.wt i.p. + Ca-folate in a dose (5mg /kg) b.wt i.p. for 4 weeks.
6. **MTX + G** group, animals were received MTX in a dose (0.45 mg/kg) b.wt i.p. + Gn in a dose (25 mg/kg) orally for 4weeks.
7. **MTX + Ca-F + G** group, animals were received MTX in a dose (0.45 mg/kg) b.wt i.p. + Ca-F in a dose (5mg /kg) b.wt i.p. for 4 weeks + G in a dose (25 mg/kg) orally for 4weeks.

Serum, Brain and Liver were taken after 4 weeks. Brain was homogenate in 70% methanol then centrifuge at 5000 r.p.m. Supernatant was taken for determination of neurotransmitter NE, DA, 5HT and amino acids ASP, GIY, GLU and GABA by HPLC. Liver oxidative stress marker (MDA, GSH, GSSG and NO) were determined by HPLC and histopathological examination were taken from rats in different groups and fixed in 10% formal saline for twenty four hours.

Determination of Brain Amino Acids:

Each brain tissue was weighed and homogenized in 75% aqueous HPLC grade methanol (10% w/v) (**Arafa et al., 2010**). The homogenate was spun at 4000 r.p.m. for 10 min. and the supernatant was divided into two halves; the first was dried using vacuum (70 Millipore) at room temperature and used for amino acids ASP, GLU, GLY and GABA (gammaamino- butyric acid, whereas the second half was used for monoamine determination. Brain amino acids were detected by High Performance Liquid Chromatography (HPLC) using the precolumn PITC derivatization technique according to the method of **Heinrikson and Meredith (1984)**.

Determination of the brain monoamines concentrations by HPLC method

The HPLC system Agilent 1200 series USA consisted of quaternary pump; a column oven, Rheodine injector and 20 μ l loop, UV variable wavelength detector. The report and chromatogram taken from chemstation program. The sample was immediately extracted from the trace elements and lipids by the use of solid phase extraction chromabond column NH2 phase cat. No.730031. the sample was then injected directly into an AQUA column 150 54.6 mm 5 μ C18, purchased from phenomenex, USA under the following conditions: mobile phase 20mM potassium phosphate, pH 2.5, flow rate 1.5ml/min, UV 190 nm. The resulting chromatogram identified each monoamine position and concentration from the sample as compared to that of the standard, and finally, the determination of the content of each monoamine as μ g / gram brain tissue (**Pagel et al., 2000**).

Assessment of Liver Function: Serum was used for the determination alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ -GT activities by spectrophotometric method using Bio-diagnositc from Bio-diagnositc Company

Evaluation of Liver Biomarkers of Oxidative Stress:

Determination of MDA by HPLC

Preparation of the standard solution

MDA standard was prepared by dissolving 25 μ L 1,1,3,3 tetraethoxypropane (TEP) in 100 ml of water to give a 1 mM stock solution. Working standard was prepared by hydrolysis of 1 ml TEP stock solution in 50 ml 1% sulfuric acid and incubation for 2 h at room temperature. The resulting MDA standard of 20 nmol/ml was further diluted with 1% sulfuric acid to yield the final concentration of 1.25 nmol/ml to get the standard for the estimation of total MDA (**Karatepe, 2004**).

HPLC Analysis

The samples were analyzed on an Agilent HP 1100 series HPLC apparatus (USA). The analytical column Supelcosil C18 (5 μ m particle and 80 \AA pore size) (250 x 4.6 ID). Mobile phase consists of 30 mmol KH₂PO₄ and methanol (65%-35%, H₃PO₄ by pH 4), and the mobile phase at a 1.5 ml/ min flow rate, wavelength 250 nm. According to the method of **Karalas et al., 2002**.

Determination of GSH and GSSG by HPLC: The thiols compounds of oxidized and reduced glutathione were detected by HPLC using the method of **Jayatilleke and Shaw (1993)**. Glutathione (oxidized and reduced)

reference standard purchased from Sigma Chemical Co. Dissolved in 75% methanol in stock 1mg/ml and diluted before application to HPLC.

The HPLC system of Agilent consisted of quaternary pump, a column oven, Rheodine injector and 20 μ l loop, UV variable wavelength detector. The report and chromatogram taken from Chemstation program purchased from Agilent. Synergi RP Max column 3.9 at wavelength 210 nm with flow rate 2ml/min was used. Pot. Phosphate buffer - acetonitrile at PH 2.7 was used as an isocratic mobile phase.

Determination of nitrites and nitrates by HPLC: Nitrites and nitrate was determined according to the method of Papadoyannis *et al.*, (1999) by HPLC

Preparation of the standard solution: Sodium nitrite and sodium nitrate used for the reference standard preparation with stock concentration 1mg/ml. A standard mixture of nitrite and nitrate was used to determine the retention times and separation of the peaks. Nitrite and nitrate concentrations were equal in the mixture solution.

HPLC Analysis: The samples were analyzed on an Agilent HP 1200 series HPLC apparatus (USA). The analytical column was anion exchange PRP-X100 Hamilton, 150 x 4.1 mm, 10 μ m. The mobile phase was a mixture of 0.1 M NaCl - methanol, at a volume ratio 45:55. The flow rate of 2 mL/min, wavelength adjusted to 230 nm.

Histopathological Examination:

Autopsy samples were taken from the liver of rats in different groups and fixed in 10% formol saline for twenty four hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin & eosin stain for examination through the light electric microscope (Bancroft *et al.*, 1996).

Statistical Analysis:

Statistical package for social science SPSS (version 18) statistical program (SPSS Inc., Chicago, IL, USA) was used to carry out a one-way analysis of variance (ANOVA) on our data. When significant differences by ANOVA were detected, analysis of differences between the means of the treated and control groups were performed by using Duncan's test.

III. Results

Table (1): Effect of Ca-folate, ginseng and their combined treatments on serum levels of ASP, GLU, GLY and GABA (μ g/gm tissue) in rats treated with Methotrexate (MTX).

Groups	Parameters (Brain amino acids)			
	ASP	GLU	GLY	GABA
C	2.15 \pm 0.168	2.319 \pm 0.129	2.699 \pm 0.26	2.299 \pm 0.215
Ca-F	3.95 \pm 0.314ab	2.759 \pm 0.187ab	2.94 \pm 0.269b	2.779 \pm 0.253ab
G	3.93 \pm 0.264ab	2.807 \pm 0.264ab	2.747 \pm 0.202b	2.802 \pm 0.225ab
MTX	6.945 \pm 0.474a	4.831 \pm 0.481a	0.706 \pm 0.053a	1.125 \pm 0.077a
MTX + Ca-F	4.312 \pm 0.332a	3.239 \pm 0.26ab	1.191 \pm 0.08ab	1.818 \pm 0.181ab
MTX + G	4.026 \pm 0.36a	2.463 \pm 0.206ab	1.914 \pm 0.136ab	1.33 \pm 0.133a
MTX + Ca-F + G	2.604 \pm 0.174b	2.528 \pm 0.207ab	1.526 \pm 0.127ab	1.83 \pm 0.128ab

- a significant from control group with one way ANOVA at P < 0.05.
- b significant from M group with one way ANOVA at P < 0.05.

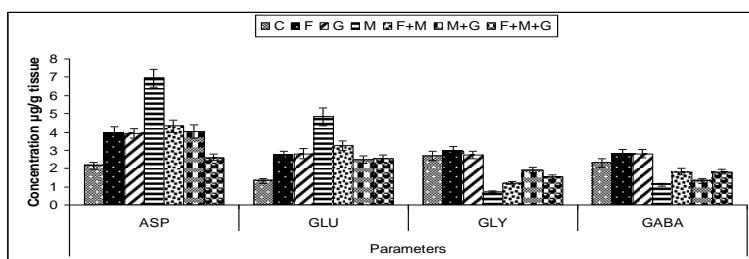


Figure (1): Effect of Ca-folate, ginseng and their combined treatments on serum levels of ASP, GLU, GLY and GABA (μ g/gm tissue) in rats treated with Methotrexate (MTX).

There were an increase in ASP and GLU in groups treated with MTX p (<0.05) in comparing with control. While there were a decrease in GLY and GABA in groups treated with MTX comparing with control. Also there was a decrease in ASP and GLU in groups treated with Ca-F + MTX, Gn + MTX and with Ca-F +Gn +MTX in comparing with MTX. Also there were an increase in GLY and GABA in groups treated with Ca-F +MTX, G n +MTX and with Ca-folinate +MTX+G in comparing with MTX. Table (1) and fig (1).

Table (2): Effect of Ca-folinate, Ginseng and their combined treatments on monoamines levels in brain of rats treated with Methotrexate (MTX).

Groups	Parameters (Brain monoamines)		
	NE	DA	5HT
C	0.668 ± 0.016	1.259 ± 0.0294	0.530 ± 0.012
Ca-F	0.507 ± 0.011b	1.073 ± 0.025b	0.496 ± 0.009ab
G	0.822 ± 0.019ab	1.291 ± 0.29b	0.575 ± 0.015b
MTX	0.571 ± 0.017a	0.732 ± 0.074a	0.295 ± 0.007a
MTX + Ca-F	0.543 ± 0.013b	1.101 ± 0.025b	0.441 ± 0.010b
MTX +G	0.662 ± 0.015b	1.037 ± 0.029b	0.472 ± 0.014b
MTX + Ca-F +G	0.681 ± 0.016b	1.231 ± 0.031b	0.503 ± 0.015b

- a significant from control group with one way ANOVA at P < 0.05.
- b significant from M group with one way ANOVA at P < 0.05.

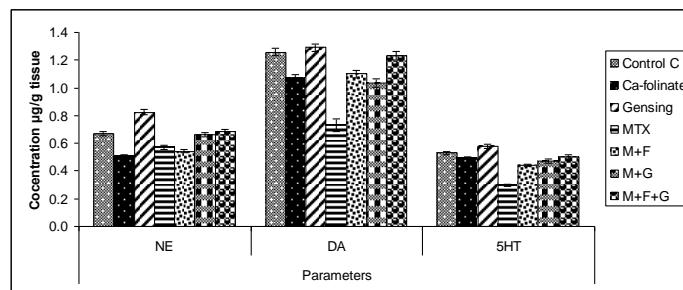


Figure (2): Effect of Ca-folinate, Ginseng and their combined treatments on monoamines levels in brain of rats treated with Methotrexate (MTX).

There were a decrease in NE, DA and 5HT in groups treated with MTX (p <0.05) in comparing with control. While there were an increase in NE, DA and 5HT in groups treated in groups treated with Ca-F + MTX, Gn+MTX and with Ca-F +MTX +Gn in comparing with MTX. Table (2) and fig (2).

Table (3): Effect of Ca-folinate, ginseng and their combined treatments on serum AST, ALT and γ GT levels (U/ml) in rats treated with Methotrexate (MTX).

Groups	Parameters (Serum liver function)		
	ALT U/mI	AST U/mL	γ GT U/m ^l
C	26.28 ± 0.497	73.63 ± 1.734	21.03 ± 0.494
Ca-F	27.87 ± 0.662	69.67 ± 1.64	21.34 ± 0.476
G	25.3 ± 0.596	64.8 ± 1.461	28.67 ± 0.677
MTX	94.8 ± 2.155a	165.58 ± 3.743a	64.97 ± 1.484a
MTX + Ca-F	45.51 ± 1.033ab	116.39 ± 2.725ab	57.01 ± 1.275ab
MTX +G	54.87 ± 1.249ab	102.16 ± 2.357ab	49.63 ± 1.168ab
MTX + Ca-F +G	52.06 ± 1.233ab	84.53 ± 1.986ab	37.4 ± 0.854ab

- a significant from control group with one way ANOVA at P < 0.05.
- b significant from M group with one way ANOVA at P < 0.05.

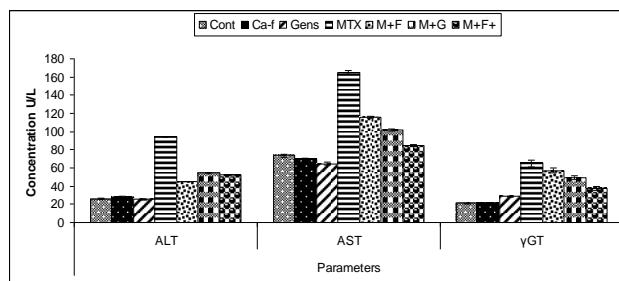


Figure (3): Effect of Ca-folinate, ginseng and their combined treatments on serum AST, ALT and γ GT levels (U/ml) in rats treated with Methotrexate (MTX).

Treatment rats with MTX showing increase in ALT, AST and γ -GT ($p < 0.05$) comparing with control. In contrary there was decrease in ALT, AST and γ -GT in groups treated with Ca-folate+ MTX, Gn +MTX and Ca-F + Gn+ MTX in comparing with MTX. . Table (3) and fig (3).

Table (4): Effect of Ca-folate, Ginseng and their combined treatments on MDA (nmol/g tissue), GSH μ mol/g tissue, GSSG μ mol/g tissue and NO (μ mol/g tissue) in rats treated with Methotrexate (MTX).

Groups	Parameters (Liver oxidative stress marker)			
	MDA nmol / g Liver	GSH μ mol/g Liver	GSSG μ mol/g Liver	NO μ mol/g Liver
C	6.254 \pm 0.143	55.654 \pm 1.302	12.054 \pm 0.282	0.354 \pm 0.008
Ca-F	6.187 \pm 0.141	53.369 \pm 1.251	11.269 \pm 0.264	0.378 \pm 0.009
G	6.987 \pm 0.157	56.547 \pm 1.334	12.258 \pm 0.289	0.348 \pm 0.008
MTX	13.658 \pm 0.319a	22.984 \pm 0.519a	17.369 \pm 0.392a	0.984 \pm 0.022a
MTX + Ca-F	9.587 \pm 0.224ab	32.694 \pm 0.756ab	14.684 \pm 0.34ab	0.654 \pm 0.015ab
MTX + G	7.329 \pm 0.173ab	37.152 \pm 0.852ab	13.725 \pm 0.315ab	0.601 \pm 0.014ab
MTX + Ca-F + G	6.984 \pm 0.156b	38.215 \pm 0.898ab	12.884 \pm 0.303ab	0.418 \pm 0.01ab

- a significant from control group with one way ANOVA at $P < 0.05$.
- b significant from M group with one way ANOVA at $P < 0.05$.

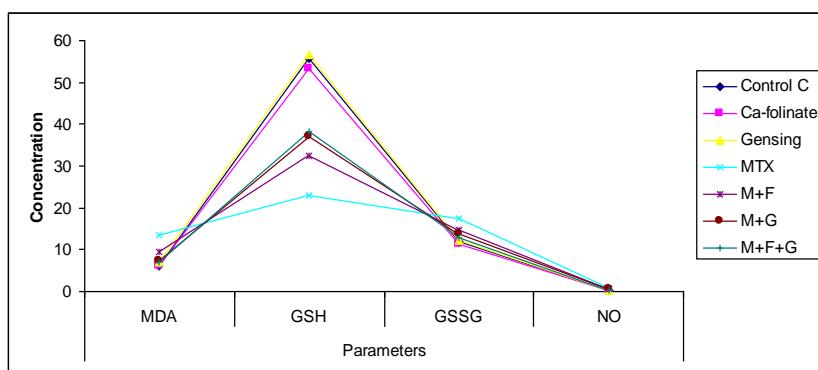


Figure (4): Effect of Ca-folate, Ginseng and their combined treatments on MDA (nmol/g tissue), GSH μ mol/g tissue, GSSG μ mol/g tissue and NO (μ mol/g tissue) in rats treated with Methotrexate (MTX).

There were an increase in MDA, GSH and GSSG in groups treated with MTX ($p < 0.05$) in comparing with control. While there was a decrease in NO in the same group treated with MTX and comparing with control. Also, there were a decrease in MDA, GSSG and NO in groups treated with Ca-F + MTX, G +MTX and Ca-F+ G+ MTX in comparing with MTX while there was an increase in GSH in the same groups comparing with MTX . Table (4) and fig (4).

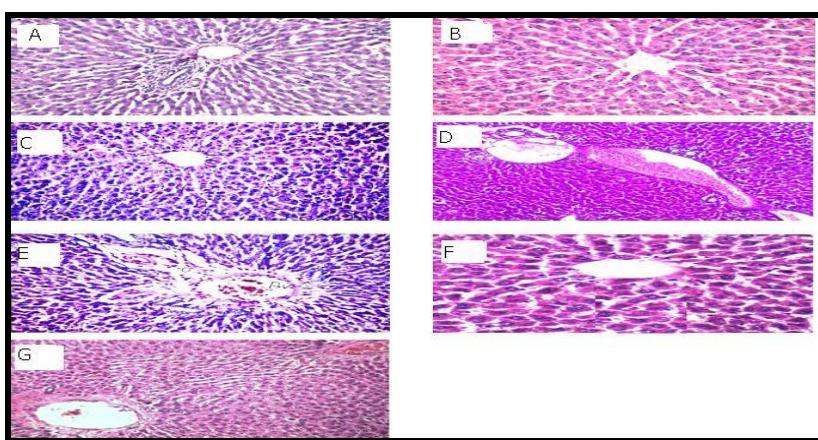


Figure 5: Histological examination of liver tissue

Figure 5 (A, B and C) for control, Ca-F and G groups respectively, didn't show any histopathological alteration in the central vein and surrounding hepatocytes in the hepatic parenchyma. Figure 5 (D) for MTX group showed dilatation in the central and portal veins associated with inflammatory cells infiltration and congestion in the portal area. Figure 5 (E, F and G) for MTX+Ca-F, MTX+G and MTX+Ca-F+G respectively, Showed ameliorative effect in liver tissue and few inflammatory cells infiltration in the portal area surrounding the bile ducts.

IV. Discussion

MTX is widely used as a cytotoxic chemotherapeutic agent in the treatment of various malignancies as well as various inflammatory diseases (**Uzar, et al., 2006**). The efficacy of this agent is often limited by its toxicity which causes severe side effects affecting the hematopoietic system and hepatocytes (**Weinblatt and Fraser, 1989**). Neurotoxicity of MTX was partly due to its direct toxic action by increasing reactive oxygen species production; MTX causes oxidative stress by reducing the activities of superoxide dismutase, catalase and glutathione reductase (**Coleshawers et al., 2010**). **Gressier et al. (1994)** demonstrated that MTX increases the amount of hydrogen peroxide and other free radicals which may lead to toxicity thus accelerating the rate of cellular damage. Our work highlights the importance of ginseng and ca-folate in combination with chemotherapy aiding in reducing the MTX induced side effects. The results showed an increase in Aspartate and glutamate and decreasing in glycine and GABA after treatment with MTX these results may be due to oxidative stress which leads to change in amino acids in brain tissues. Also treatment with Ca-folate and ginseng causing decrease in Aspartate and glutamate and increasing in glycine and GABA this is due improve in rats behavior and amino acids and decrease neurotoxicity of MTX. This results agree with **Leskiewicz et al. (2008); Mauricio et al. (2014)** whom decided that oxidative stresses lead to imbalance and disturbance between excitatory and inhibitory amino acids. Hence, the Neuroprotective effects of these drugs were mediated by modulation of target in GABA/GLYcinergic and glutamatergic pathway.

Proposed neurotoxicity by methotrexate is due to interference in supply of neurotransmitters. Our study has confirmed with **Netter et al. (1991)** reported that, the involvement of neurotransmitters in methotrexate induced learning and memory deficits. In which the level of NE, DA and 5HT were significantly decreased ($P<0.05$). Thus our present study claims that methotrexate induced neurotoxicity is due to impaired synthesis of amines, rather than cytotoxic effect. **Millot et al., 1995** and **Netter et al. (1991)** supported this view, claiming that altered biopterine metabolism is blocking the synthesis of these amines in brain. The treatment with MTX showed decrease in NE, DA and 5HT and there was increase in NE, DA and 5HT after treatment with Ca-folate and ginseng and their combination this results agree with **Bottiglieri et al., 2000** who decided that Altered monoamine neurotransmitter metabolism has been identified in psychiatric patients with folate deficiency , Also, low levels of serotonin metabolites were observed in the CSF of patients suffering from folate-responsive neuropsychiatric disease (**Botez et al., 1982**). The 5-HIAA/5-HT ratio is considered a sensitive index of utilization of 5-HT (**Shannon et al., 1986**).

The results of the studies indicate that MTX causes oxidative tissue damage by increasing lipid peroxidation in the liver tissue and decreasing the level of antioxidant enzymes. These lead to increasing AST, ALT and GGT values, biochemical indicators of liver damage, and histopathological findings supported this conclusion. In our study, after MTX injection, AST, ALT and GGT levels, known indicators of hepatic damage, had increased compared with the control group. It is known that in the use of drugs causing liver toxicity transaminase levels increase in the plasma depending upon the degree of hepatic damage. **Kuvandik et al. (2008)** reported that the levels of AST and ALT in plasma serum have increased significantly in acetaminophen hepatotoxicity; and **Uzar et al. (2006)** reported that MTX also increased ALT levels. In our study; the level of AST and ALT in the group supplied with ginseng and Ca-F was statistically lower than in those animals given MTX alone. **Sugiura et al. (2006)** reported that flavonoides may prevent increases of serum aminotransferase increase in the early periods of liver damage in hyperglycemic patients.

The mechanisms involved in MTX-induced hepatic damage were investigated and our results showed that MTX induced oxidative stress, evident by decreased GSH level and increased lipid peroxidation product, MDA, GSSG and NO which was in concurrence with previous studies (Morsy et al ., 2013). In the current study, it was found that ginseng improved antioxidants' levels and decreased MDA, GSSG, and NO levels in the treated group when compared to control group. In agreement, **Liu et al. (2003)** found that ginseng extracts scavenge oxidative species; also, **Surh et al. (2001)** indicated that ginseng extracts attenuate lipid peroxidation. Ameliorative effect of ginseng may be due to saponins which play a major role in antioxidant activities. In addition, ginsenosides which are important components heavily present in ginseng production of powerful antioxidant activities other than radical scavenging activities by stimulating gene expression of antioxidant enzymes and enhancing their activities. These results were in agreement with **Kim et al. (2003)** who reported that, ginseng saponins have been proposed as possible candidates in the research of therapeutic modulation of stress related disorders, for their inhibitory effect on the level of stress.

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