EFFECTS OF VITAMIN E, L-CARNITINE AND MELATONIN ON CARBON TETRACHLORIDE/DIABETES INDUCED HEPATIC OXIDATIVE STRESS

Mohamed E. Shaker [a,*], Maha E. Houssen [b], Ekbal M. Abo Hashem [c], Tarek M. Ibrahim [a]

[a] Department of Pharmacology and Toxicology, Faculty of pharmacy, University of Mansoura, Mansoura, Egypt.
[b] Department of Biochemistry, Faculty of Pharmacy, University of Beni Sueif, Beni Sueif, Egypt.
[c] Department of Clinical Pathology, Faculty of Medicine, University of Mansoura, Mansoura, Egypt.

Summary

This study aimed to investigate whether I.P. treatments with vitamin E (100 mg/kg), L-carnitine (300 mg/kg) and melatonin (10 mg/kg) can protect against CCl_4/diabetes induced hepatic oxidative stress in the same manner or not. It was performed in Wistar rats as a comparison between two different models by 50% v/v CCl_4 (1 ml/kg, 3 days, I.P.) in the first model and by STZ (40 mg/kg, I.P.) induced diabetes mellitus in the second model over 6 weeks. The results obtained were surprising and antioxidants showed different responses in the two models. In CCl_4-treated rats, melatonin was found to be the most effective treatment, while vitamin E was found to be the least effective treatment. In STZ-treated rats, vitamin E showed significant attenuation than CCl_4-treated rats, followed by melatonin and L-carnitine as indicated by the changes in liver function tests, malondialdehyde (MDA) content, reduced glutathione (GSH) content, superoxide dismutase (SOD) activity in liver, and serum total antioxidant capacity (TAC) level. In conclusion these data indicate the beneficial effects of antioxidants, especially melatonin against oxidative stress and hepatic disorders induced by CCl_4 and to less extent diabetes. Moreover, the potent effect of vitamin E in reducing hepatic oxidative stress induced by diabetes, which can be linked not only to the antioxidant actions of vitamin E, but also to the superior effect in reducing diabetes-induced hyperglycemia. Finally, same antioxidants can have varying responses in different models of oxidative stress.

Key words: Vitamin E; L-carnitine; Melatonin; Carbon Tetrachloride; Diabetes.

Introduction

Oxidative stress has been implicated in the etiology of different diseases including cardiovascular diseases, cancer, neuro-degenerative diseases, ischemia-reperfusion injury, rheumatoid arthritis, aging, and has recently been shown to be a major contributor to liver damage in many situations such as liver fibrosis and diabetes mellitus. Oxidative stress is characterized by excess formation and insufficient removal of highly reactive species that can be damaging for liver at high concentration [1].

Carbon tetrachloride (CCl_4) has been used drastically to induce liver injury and fibrosis in various experimental models and to elucidate the mechanisms behind hepatotoxicity. The mechanism underlying the CCl_4 hepatoxicity involves oxidative stress induced by CCl_4-derived reactive free radical metabolites [2]. Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and insufficiency of action or secretion of endogenous insulin. Increased glucose flux both enhances oxidant production and impairs antioxidant defenses by multiple interacting pathways, such as, glucose autooxidation, protein glycosylation, polyol pathway, activation of protein kinase C and hexosamine pathway [3].

Vitamin E is the most important lipid soluble antioxidant in biological systems. The major store of membrane bound vitamin E is in the inner mitochondrial membrane, where it is used for electron transport and prevents oxidation of various compounds such as polyunsaturated fatty acids [4]. L-carnitine is an essential cofactor in the transport of long-chain fatty acids from the cytosol to mitochondria for subsequent β-oxidation and production of cellular energy, and was also reported to possess antioxidant properties [5]. Melatonin is a lipophilic indoleamine derived from tryptophan. It is principally secreted at night and is centrally involved in sleep regulation and is often called the hormone of darkness. Melatonin, as well as its metabolites possesses redox properties due to the presence of an electron rich system allowing these molecules to act as electron donors [6].

The aim of the present study whether treatments with vitamin E (100 mg/kg), L-carnitine (300 mg/kg) and melatonin (10 mg/kg) can protect against CCl_4 and diabetes induced hepatic oxidative stress in the same manner or not.

Materials and Methods

Drugs and Chemicals

STZ was purchased from MP Biochemicals (Irvine, CA, USA). Melatonin (N-acetyl-5-methoxytryptamine), Vitamin E (DL-α-Tocopherol acetate) and L-carnitine (β-hydroxy-γ-N-trimethyl ammonium- butyrate) were purchased from Sigma-Aldrich (St Louis, MO, USA). Other chemicals were of the highest quality available.

Animals

Adult male Wistar albino rats (170-230 g) were purchased (Research Institute of Ophthalmology, Giza, Egypt) and kept under constant conditions for food, water, light and temperature throughout the experimental period. All animals were acclimatized for 1 week prior to experimentation and received humane care in compliance with the national institutes of health criteria for care of laboratory animals.

Experimental Design

Rats were randomly divided into 10 groups (20 rats per group) except for CTRL and CTRL2 (10 rats per group). Intraperitoneal route was used in all administrations and any drug treatment was given daily at 5:00 pm. In the first model , Liver cell injury and fibrosis were induced by injecting 50% v/v CCl_4 in olive oil (1ml/kg) every 3 days at 11:00 am. In the second model, experimental diabetes was induced by injection of STZ (40 mg/kg) prepared in cold citrate buffer (0.1 M, pH: 4.5). After 3 days, rats with blood glucose levels greater than 300 mg/dL were accepted to be diabetic.

1-CTRL: Rats were injected with olive oil (1 ml/kg) every 3 days.
2-CCl_4: Received CCl_4 alone.
3-CCl_4 + E: Received CCl_4 + vitamin E (100 mg/kg) dissolved in olive oil.
4-CCl_4 + LC: Received CCl_4 + L-carnitine (300 mg/kg) dissolved in physiological saline.
5-CCl_4 + M: Received CCl_4 + melatonin (10 mg/kg) suspended in 1% v/v absolute ethanol and 1% w/v gum tragacanth in physiological saline to prolong its absorption as it is rapidly metabolized.
6-CTRL 2: Control non-diabetic rats received citrate buffer and after 3 days, physiological saline (1 ml/kg).
7- STZ: Untreated diabetic rats + physiological saline (1 ml/kg).
8-STZ + E: Diabetic rats + vitamin E (100 mg/kg).
9-STZ + LC: Diabetic rats + L-carnitine (300 mg/kg).
10-STZ + M: Diabetic rats + melatonin (10 mg/kg).

After 6 weeks, rats were fasted overnight and at the following morning, rats were anesthetized by thiopental (70 mg/kg) and blood samples were withdrawn for serum preparation. Liver was isolated and homogenized (20 mM Tris, 1mM EDTA, HCl pH 7.4) using a glass homogenizer. Homogenate was centrifuged (6000 r.p.m, 4 ºC, 15 minutes) and supernatant was used immediately for assay of MDA, GSH and SOD.

Biochemical Analysis

Serum glucose, alanine aminotransferase (ALT)/aspartate aminotransferase (AST), alkaline phosphatase (ALP) and Protein in liver homogenate were determined according to the methods described respectively [7,8,9,10]. Hepatic MDA content was measured by the thiobarbituric acid method [11].

Hepatic GSH was determined according to DTNB method that is based on reduction of Ellman's reagent by GSH [12]. Hepatic SOD activity was determined according to the method that is based on the fact that one unit of SOD can inhibit the oxidation of pyrogallol by 50% [13]. Serum TAC was assay based on elimination of certain amount of the provided H_2O_2 by antioxidants present in the sample and reaction of residual H_2O_2 with 3,5-dichloro-2-hydroxy benzene sulfonate and 4-aminoantipyrine in the presence of peroxidase enzyme producing a colored chromogen that can be measured at 505 nm and is inversely proportional to TAC concentration [14].

Statistical Analysis

Data were expressed as means ± SEM for the animals in each experimental group. Statistical evaluation of the results was carried out by means of one way analysis of variance, followed by Tukey-Kramer multiple comparison test, when appropriate. Statistical tests were performed with GraphPad Instat V 3.05 (GraphPad Software Inc, San Diego, CA, USA).

Results

Table 1. Effects of vitamin E, L-carnitine and melatonin treatments on serum glucose Concentration, ALT, AST and ALP activities in model I

Remaining	**CTRL** (n=10/10)	**CCl_4** (n=8/20)	**CCl_4 + E** (n=10/20)	**CCl_4 + LC** (n=10/20)	**CCl_4 + M** (n=13/20)
Glucose (mg/dL)	130.4 ± 4.84	116.4 ± 7.63	115.4 ± 7.20	103.5 ± 3.79*	117 ± 4.76
ALT (U/ml)	24.59 ± 3.23	193.2 ± 11.30***	138.9 ± 16.92^{***,a}	98.82 ± 17.51^{***,a}	67.99 ± 8.21^{*,a,3}
AST (U/ml)	62.06 ± 4.73	474.8 ± 29.09***	237.2 ± 20.10^{***,a}	127.6 ± 8.31^{*,a,3}	151 ± 13.94^{**,a,2}
ALP (IU/L)	145.7 ± 14.98	289.5 ± 27.20***	202.4 ± 23.04^{a}	186.7 ± 25.85^{a}	137.2 ± 8.77^{a}

- *, ** and *** Significantly different from **CTRL** at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- ^{a}, ^{b} and ^{c} Significantly different from **CCl_4** at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- 1, 2 and 3 Significantly different from **CCl_4+E** at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- 4 Significantly different from **CCl_4+LC** at $P < 0.01$.

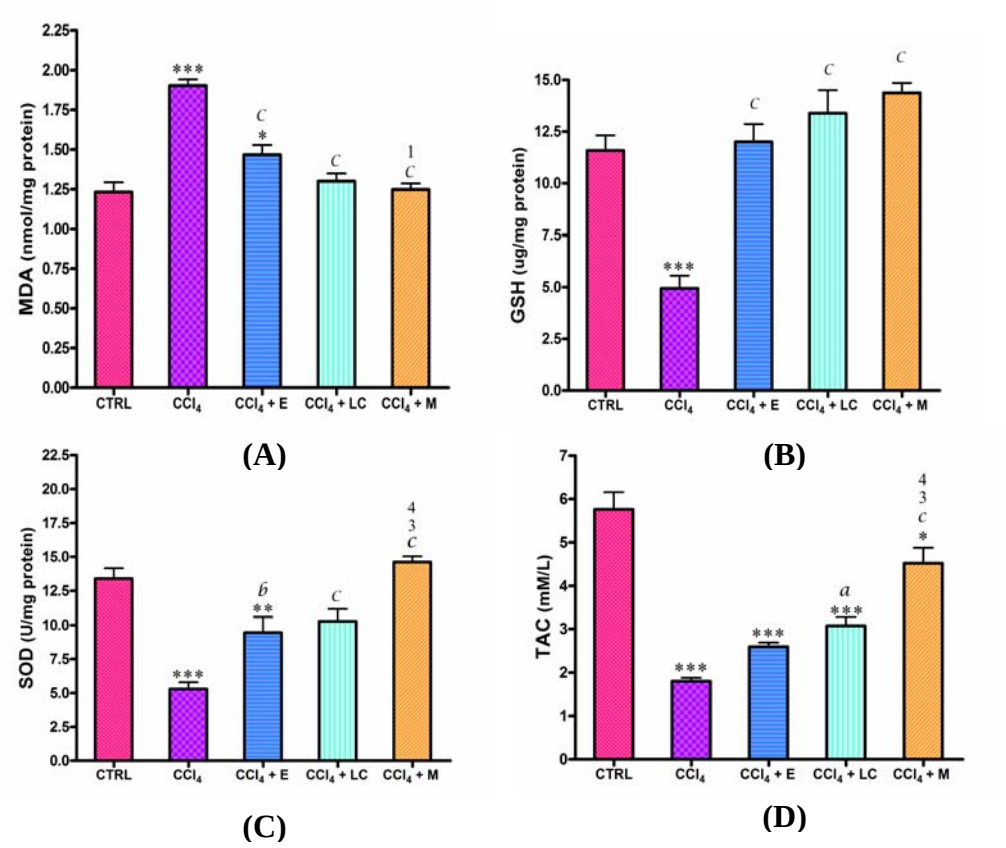

Fig. 1. Effects of vitamin E, L-carnitine and melatonin treatments on hepatic MDA content (A), hepatic GSH content (B), hepatic SOD activity (C) and serum TAC level (D) in model I

- *, ** and *** Significantly different from **CTRL** at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- *a*, *b* and *c* Significantly different from **CCl_4** at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- 1, 2 and 3 Significantly different from **CCl_4+E** at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- 4 Significantly different from **CCl_4+LC** at $P < 0.01$.

Table 2. Effects of vitamin E, L-carnitine and melatonin treatments on serum glucose Concentration, ALT, AST and ALP activities in model II

Remaining	**CTRL 2** (n=10/10)	**STZ** (n=7/20)	**STZ + E** (n=11/20)	**STZ + LC** (n=10/20)	**STZ + M** (n=10/20)
Glucose (mg/dL)	101 ± 7.35	374.3 ± 36.67***	232.5 ± 20.38**,ℓ	254.2 ± 25.7***,ℓ	298.1 ± 23.52***
ALT (U/ml)	21.33 ± 2.25	157 ± 15.97***	22.91 ± 2.42ℓ	92.34 ± 10.01***,ℓ,3	48.80 ± 5.07ℓ,4
AST (U/ml)	63.78 ± 3.76	196.7 ± 13.04***	97.5 ± 6.44ℓ	126.4 ± 10.84***,ℓ	113.5 ± 13.37**,ℓ
ALP (IU/L)	169.8 ± 10.54	448.8 ± 45.54***	303.3 ± 28.31**,ℓ	368 ± 27.79***	263.7 ± 23.49ℓ

- *, ** and *** Significantly different from CTRL 2 at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- ℓ, ℓ and ℓ Significantly different from STZ at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- 1, 2 and 3 Significantly different from STZ+E at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- 4 Significantly different from STZ+LC at $P < 0.01$.

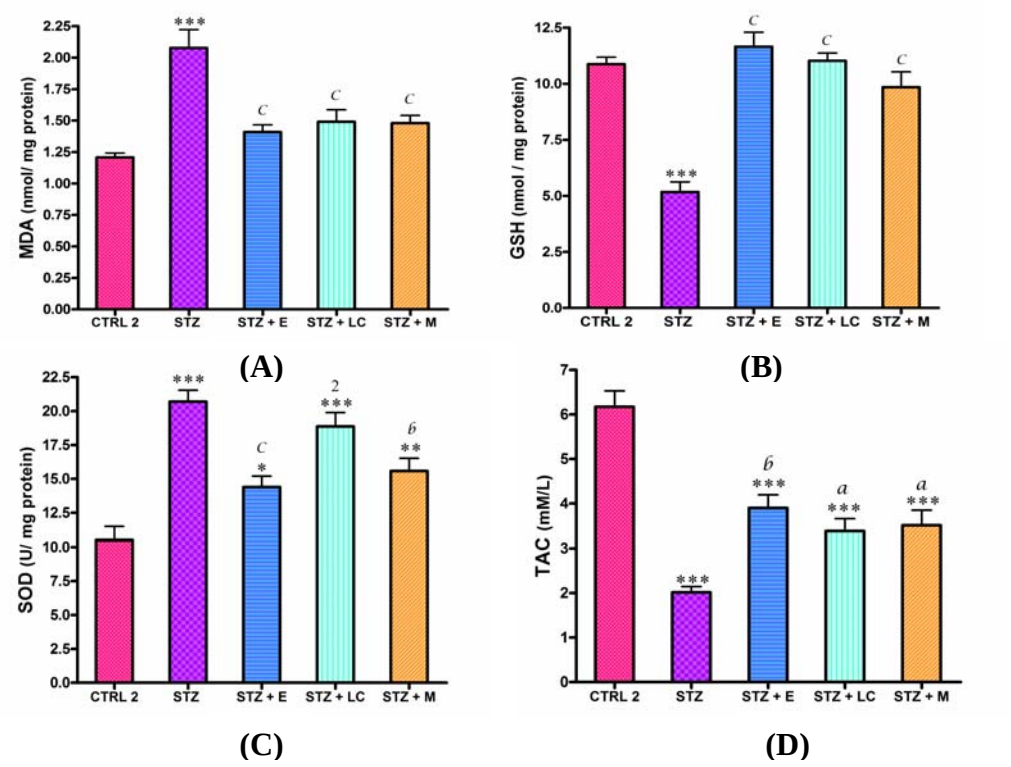

Fig. 2. Effects of vitamin E, L-carnitine and melatonin treatments on hepatic MDA content (A), hepatic GSH content (B), hepatic SOD activity (C) and serum TAC level (D) in model II

- *, ** and *** Significantly different from **CTRL 2** at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- *a*, *b* and *c* Significantly different from **STZ** at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- 1, 2 and 3 Significantly different from **STZ**+**E** at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.
- 4 Significantly different from **STZ**+**LC** at $P < 0.01$.

Discussion

Although, many studies have investigated the effects of antioxidants on solitary models, there is no study compared between certain antioxidants effects in many concomitant models until now. In CCl_4 model, the activities of liver function tests (Table 1) and hepatic MDA content (Figure 1A) were highly elevated as a result of CCl_4 metabolism to highly toxic radicals such as trichloromethyl radical ($^{\bullet}CCl_3$) and trichloromethyl peroxyl radical ($^{\bullet}OOCCl_3$) [2].

$^{\bullet}CCl_3$ is responsible for the covalent binding to cell components resulting in inhibition of lipoprotein secretion, steatosis and cancer, while $^{\bullet}OOCCl_3$ is responsible for lipid peroxidation resulting in impairment of cellular functions dependent on membrane integrity, loss of calcium homeostasis, apoptosis and cell death [15]. Vitamin E treatment slightly decreased the elevated activities of liver function tests and hepatic MDA content. It was reported that vitamin E did not react at comparable rates with carbon-centered radicals and did not block $^{\bullet}CCl_3$-induced covalent binding resulting in inhibition of secretion of lipoproteins [15,16]. Cardioprotective effects of L-carnitine treatment decreased AST more pronouncedly than other treatments [17]. L-carnitine decreases lipid peroxidation by stimulating β-oxidation in an early phase and hinders the effect of CCl_4 on mitochondrial functions [18]. Melatonin is a potent trap of $^{\bullet}OOCCl_3$ [19]. Accordingly, it decreased elevation of liver function tests and hepatic MDA content greater than vitamin E and L-carnitine treatments.

GSH content in liver homogenate, an index of non-enzymatic antioxidants, was found to be decreased as a result of CCl_4 administration (Figure 1B). $^{\bullet}CCl_3$ reacts with sulfhydrl groups resulting in depletion of GSH and protein thiols [20]. In addition, hepatic transulphuration pathway is impaired in fibrosis hindering the conversion of methionine to cysteine, whichis necessary for GSH synthesis [21]. L-carnitine treatment showed a significant elevation in hepatic GSH content, which can be attributed to the energy enhancing action of L-carnitine through increasing ATP needed for synthesis of GSH [22]. Melatonin treatment showed superior elevation in hepatic GSH content than other treatments due to stimulation of γ-glutamylcysteine synthase and potentiation of GSH recycling by increasing GSH-reductase [23].

SOD activity in liver homogenate, one of the indices of enzymatic antioxidants, was found to be decreased as a result of injection of CCl_4 (Figure 1C). Depletion of hepatic SOD content can be attributed to the burst of oxygen-free radicals (OFRs) that occurs during the hepatic damage development, which lead to consumption of SOD [24]. Melatonin treatment showed superior increase in hepatic SOD activity than vitamin E and L-carnitine treatments suggesting that melatonin has indirect antioxidant actions through stimulating the activities of antioxidant enzymes, such as SOD, GSH-peroxidase and GSH-reductase and direct through dismutation of superoxide anion radical ($O_2^{\bullet -}$) [25].

In STZ-induced diabetes model, vitamin E treatment of diabetic rats showed a significant reduction in serum blood glucose level indicating that vitamin E may have a role in preventing hyperglycemia (Table 2). It has been proposed that vitamin E may have a role in modulating insulin action [26]. Moreover, vitamin E administration resulted in protein kinase C inhibition due to the direct interaction between α-tocopherol and protein kinase C in the cell membrane [27].

L-carnitine treatment showed a slight reduction in serum glucose level of diabetic rats. L-carnitine improves pyruvate dehydrogenase activity, decreases acetyl CoA/CoA ratio and activates phosphofructokinase in the glycolytic pathway resulting in glucose flux increase [28,29]. Serum transaminases in diabetic rats were found to be highly elevated in comparison with non-diabetic rats. This can be attributed to the excessive free fatty acids formation, which is known to be directly toxic to hepatocytes [30]. It is also hypothesized that the elevation in ALT, a gluconeogenic enzyme whose gene transcription is suppressed by insulin, could indicate impairment in insulin signaling rather than purely hepatocyte injury. In contrast, AST activity was unrelated to changes in hepatic insulin action [31]. The present study showed that Vitamin E treatment significantly reduced serum ALT activity in diabetic rats when compared to L-carnitine and melatonin. Accordingly, the decrease in serum glucose due to vitamin E treatment may provide improvement in hepatocellular function, hepatic insulin resistance and glucose output.

MDA in liver of diabetic rats used in this study was found to be elevated (Figure 2A). This can be attributed to the overproduction of OFRs which could be due to their increased production or decreased destruction. Glucose can increase OFRs through glucose autoxidation and through non-enzymatic protein glycation. The oxidative degradation of these oxidants could participate in the formation of lipid peroxidation products [32]. Vitamin E treatment showed a significant reduction in serum activities of liver function tests and hepatic MDA content in model II greater than model I. Vitamin E prevents chain reaction of lipid peroxidation by reacting with peroxyl radical and trapping $LOO^{\bullet}$, the primary product of peroxidation [33].

GSH in liver of diabetic rats was found to be decreased as shown in Figure 2B. Since, the hexose monophosphate shunt is impaired in diabetes, NADPH availability is reduced and the ability to recycle GSSG to GSH is decreased [34]. Alternatively, the decrease in GSH content in liver during diabetes is probably due to its increased utilization by the hepatic cells and this may be an attempt by the hepatocytes to counteract the increased formation of lipid peroxides. Vitamin E restored hepatic GSH content by trapping $LOO^{\bullet}$ that leads to GSH utilization [35].

Our results showed a significant elevation in SOD activity in liver of diabetic rats (Figure 2C). The stimulation of SOD activity mainly Cu/Zn-SOD might occur by a cytosolic factor produced through glycolysis or the pentose phosphate pathway, which may function as an important mechanism to prevent the toxic effects of high glucose concentrations [36]. According to our results, vitamin E showed significant decrease in hepatic SOD activity of diabetic rats, which was more than melatonin and L-carnitine, knowing that vitamin E was the most significant in decreasing serum glucose level.

TAC is a sensitive and reliable marker to detect changes of *in vivo* oxidative stress that cannot be detected through the measure of a single antioxidant [37]. TAC results confirm that CCl_4 and diabetes caused a significant depletion of antioxidants in the face of reactive species (Figure 1D and Figure 2D respectively). According to treatments efficacy, the order was melatonin, L-carnitine and vitamin E for CCl_4 model and vitamin E, melatonin and L-carnitine for diabetes model. In conclusion these data indicate that low dose of melatonin is more effective than high doses of vitamin E and L-carnitine in reducing hepatic oxidative stress induced by CCl_4 and to less extent diabetes-induced hyperglycemia. Moreover, failure of vitamin E to protect against CCl_4, and its potency in reducing hepatic oxidative stress induced by diabetes can be linked not only to the antioxidant actions of vitamin E, but also to the superior effect in reducing diabetes-induced hyperglycemia. Finally, same antioxidants can have varying responses in different models of oxidative stress.

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*Corresponding author at: Department of Pharmacology and Toxicology, Faculty of pharmacy, University of Mansoura, Mansoura 35516, Egypt, Tel.: +20502246253, Fax: +20502247496. Email address: mshaker2222@yahoo.com (Mohamed E. Shaker)