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Possible Hepatoprotective Effects of Pentoxifylline-Glycyrrhizin Combined Treatment Against CCl_4-Induced Liver Injury in Rats

[1]Nahed M. A. Hassanein, [2]Helmy M. Sayd Elahl, [1]Amany M. Hegab and[1]Eman S. G. Hassan.

[1]Developmental Pharmacology Department, National Organization for Drug Control and Research. Cairo, Egypt.
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Egypt

ABSTRACTS

Background: The present study was designed to investigate whether the combination of glycyrrhizin (GL) and pentoxifylline (PTX) could offer better hepatoprotective and antifibrotic effects against CCl_4 induced hepatotoxicity, as compared to their separate effects. Methods: Adult male albino rats were randomly assigned into 10 groups (10 rats each) namely, non-treated control, saline control, olive oil control, CCl_4 group (1 ml CCl_4 in olive oil (1:3, v/v)/kg twice weekly/6 weeks), the remaining other 6 groups were administered PTX (100mg/kg/daily/6 weeks), GL (50 mg/kg/daily/6 weeks) and their combination with and without CCl_4. The following parameters: serum ALT and AST, hepatic tissue SOD, MDA and total nitrate/ nitrite content, serum and tissue IL-6 were assessed, along with the histopathology of the liver. Results: The results revealed that CCl_4 caused significant elevation in all biochemical parameters and significant reduction in SOD. Liver histopathology revealed fibrosis (stage3) with severe necrosis. Administration of GL or PTX prior to CCl_4 resulted in significant elevation of tissue SOD as well as significant reduction of other biochemical parameters and the histopathology revealed reduction in the grade of fibrosis and necrosis. The co-administration of GL and PTX prior CCl_4 could restore the biochemical parameters to nearly their normal values. Histopathological examination showed great improvement in the grade of fibrosis, where, hepatocytes showed normal architecture. Conclusion: The investigated combination proved to have a potent hepatoprotective and antifibrotic activity against CCl_4 induced liver fibrosis which is better than the effect of each one alone.

Key words: Glycyrrhizin; Pentoxifylline; CCl_4; Liver fibrosis; Hepatotoxicity

Introduction

Chronic liver diseases are major global health problems causing approximately 800,000 deaths per year worldwide (Bataller and Brenner, 2005). Liver fibrosis is the common pathologic process of all chronic liver diseases; it results from chronic damage to the liver in conjunction with the progressive accumulation of fibrillar extracellular matrix protein (Gressner, 1995, Lieber, 1999). The main causes of hepatic fibrosis in humans include infection by hepatitis B and C, alcohol abuse and non-alcohol steatohepatitis; and experimentally, liver fibrosis can be induced by carbon tetrachloride (CCl_4) (Brattin et al., 1985, Boulton *et al.,* 1998). On the other hand, CCl_4 is a well-known hepatotoxin that is widely used to induce toxic liver injury in a range of laboratory animals. Carbon tetrachloride -induced hepatotoxicity is believed to involve two phases, the initial phase involves the metabolism of CCl_4 by cytochrome P450 to the trichloromethyl radical (CCl_3*) and trichloromethyl-peroxy radical (CCl_3OO*) which leads to lipid peroxidation and oxidative stress as well as the depletion of endogenous anti-oxidant enzymes , such as superoxide dismutase (SOD) and catalase (CAT) (Edwards *et al.,* 1993). The second phase of CCl_4-induced hepatotoxicity involves the activation of Kupffer cells, which is accompanied by the production of proinflammatory and profibrotic mediators, such as transforming growth factor B (TGF-B), reactive oxygen species (ROS), interleukin-6 (IL-6) and other effecting factors (Boulton *et al.,* 1998, Badria *et al.,* 2011). These pro-fibrotic factors play key roles in hepatic fibrosis (De Minicis *et al.,* 2006).

In the search for novel drugs that can alleviate hepatocyte injury and reduce fibrosis, pentoxifylline (PTX) has received considerable interest (Windmeier, and Gressner, 1997, Wu and Zern, 2000). PTX is a methylxanthine derivative and a phosphodiesterase inhibitor with rheologic and vasodilating properties. By increasing the red blood cell flexibility, the drug leads to increased tissue perfusion and improved regional microcirculation (Ward and Clissold, 1987). Apart from its effect on blood cell rheology, PTX has, in addition, an anti-inflammatory (Abdel-Salam *et al.,* 2005) and immune-modulating properties (Weiss *et al.,* 1995). There is also accumulating experimental evidence to suggest that PTX might be of therapeutic value in chronic hepatic disorders characterized by excessive hepatic fibrosis (Raetsch *et al.,* 2002). In different studies, PTX showed variable effects on hepatocyte injury from failure to alter indices of cell necrosis and cholestasis (Lee *et al.,* 1997, Demir and Inal-Erden, 1998), preventing liver damage. Additionally, it has been also reported that PTX prevents liver fibrosis via exerting antiproliferation and antifibrogenic effects on hepatic stellate cells (HSCs) although the precise mechanism has not yet been elucidated (Preaux *et al.,* 1997).

Corresponding Author: Nahed M. A. Hassanein, Developmental Pharmacology Department, National Organization for Drug Control and Research. Cairo, Egypt.
E-mail: nahed_hassanein2001@yahoo.com

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Moreover, glycyrrhizin (GL), a triterpene glycoside and a conjugative compound of enoxolone and glucuronic acid as an active component of licorice (*Glycyrrhiza glabra*) root, it has a variety of pharmacological actions including anti-inflammatory, anti-viral, antioxidative, anti-livercancer, immunomodulatory, hepatoprotective and cardioprotective activities (Song *et al.,* 2013). It has been reported that GA can treat chronic hepatitis C by inhibiting the type I collagen gene transcription (Moro *et al.,* 2008). It can be used to treat liver fibrosis by decreasing the collagen deposition and down regulation of the type I pro-collagen (Zhang *et al.,* 2005). Additionally, it was found that glycyrrhizin may dose dependently inhibit CCl_4-induced liver fibrosis (Qu *et al.,* 2012). These effects could be attributed to suppression of the proliferation and activation of HSCs and induction of apoptosis of HSCs following GL treatment by blocking of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) translocation which is responsible for induction of apoptosis (Qu *et al.,* 2012).

Based on the aforementioned data, it was reported that GL and PTX are ancillary drugs that used clinically for protection of liver function. Therefore, this study was designed to investigate whether the combined GL and PTX treatment can offer better hepatoprotective and antifibrotic effects against CCl_4 induced hepatotoxicity, as compared to their separate effects.

Materials and Methods

Animals

Adult male albino rats of Sprague-Dawely strain, weighing 180±30 gram were used along the study period. The rats used were obtained from the animal house facility at the National Organization for Drug Control and Research (NODCAR). The animals were housed as ten rats per cage and kept one week at the laboratory for accommodation under standard laboratory animal housing conditions and fed standard diet and water *ad libitum*. The experiments were conducted on basis of the international guidelines regarding use of laboratory animals.

Chemicals

Glycyrrhizin, pentoxifylline, thiobarbituric acid (TBA), n-butanol, vanadium III chloride (VCl_3), sulfanilamide and N- (1-naphthyl) ethylenediamine were obtained from Sigma-Aldrich (Sigma- Aldrich Inc., Saint Louis, U.S.A). Carbon tetrachloride (CCl_4) was obtained from the Egyptian company for chemicals and pharmaceuticals (ADWIA). Alanine amino-trranseferase (ALT), aspartate amino transferase (AST) and SOD kits were obtained from Bio-diagnostic Co., Giza, Egypt. Interleukin-6 (IL-6) ELISA kit was obtained from Invitrogen (Invitrogen, Camarillo, CA, USA).

Experimental Design:

After an acclimation period of 7 days, 100 rats were enrolled in the experiment and equally randomized into 10 groups as the following: control non treated, saline control that administered (2.5 ml of normal saline/kg/daily/6weeks/i.p.), olive oil control that received (2ml olive oil /kg/twice a week/6weeks/i.p.), CCl_4 group that administered (1 ml of CCl_4/kg, diluted in a ratio of 1:3 with olive oil via i.p. route twice weekly/6 weeks). The other remaining 6 groups, were administered either PTX (100mg/kg/daily/6weeks/i.p.), GL (50 mg/kg/daily/6 weeks/i.p.) or their combination treatments with and without CCl_4.

After 24 hr of last dosing, rats were euthanized under ether anesthesia, blood was withdrawn from the orbital sinus of each animal and subsequent assays of serum ALT, AST and IL-6. Animals were then decapitated; livers were rapidly excised weighted for assessment of relative liver weight. Fixed weight of each animal liver was frozen at -80ºC for estimation of alteration in lipid peroxidation indices. malondialdehyde (MDA), an antioxidant enzyme superoxide dismutase (SOD), total nitrite/nitrate content and IL-6. Fixed small parts of the livers were fixed in 10% formalin for histopathological examination.

Homogenization of liver tissue samples

The fixed liver weight of samples were homogenized in an ice container at a concentration of 10% of potassium chloride solution and were centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was used for estimation of MDA, SOD.

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Biochemical Assays:
Estimation of serum ALT and AST:

Serum ALT and AST were determined colorimetrically according to Reitman and Frankel method (1957), using a double beam Thermo Spectronic Helios^{TM}- UV-Vis spectrophotometer (Thermo Scientific, North Carolina, USA) and the absorbance was measured at 505 nm (490-520 nm).

Estimation of Hepatic Malondialdehyde (MDA):

Hepatic lipid peroxidation was assessed by determining the MDA content of liver homogenates by using a colorimetric assay, as previously described by Uchiyama and Mihara, (1978). Briefly, to 0.5 ml liver homogenate, 1 ml of 20% trichloroacetic (TCA) for precipitating the protein, 3ml of 1% orthophosphoric acid (1% H_3PO_4) and 1 ml of 0.6% thiobarbituric acid (0.6 TBA) was added and then incubated in a boiling water bath. After cooling, the samples were extracted with n-butanol and centrifuged. The absorbance of samples was determined at 520 and 535 nm. 1, 1, 3, 3-tetraethoxypropane was used as a standard. Concentrations of MDA were expressed as nmol/g tissue.

Estimation of Hepatic superoxide dismutase (SOD) activity:

Activity of SOD was determined calorimetrically according to method described by Nishikimi *et al.,* (1972). This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. The increase in absorbance over a period of 5 minutes, of the control ($\Delta A_{control}$) and sample solutions (ΔA_{sample}) was then measured at 25 ^{o}C and at wavelength 560 nm.

Estimation of Liver Nitrite and Total Nitrite/nitrate Contents

Total nitrate/nitrite accumulation in liver was performed according to Miranda *et al*., (2001) as an indication of nitric oxide (NO) production. Before NO estimation, liver homogenate were de-proteinized by adding absolute ethanol in double volume of the sample. Experiments were performed by adding equal volumes of sample, saturated solution of VCl_3 (200 mg VCl_3 (Sigma-Aldrich) in 25 ml of1 M HCl), Griess reagents (1:1 mixture of 0.1% N-(1-naphthyl) ethylene diamine in de-ionized H2O and 2% sulfanilamide (Sigma-Aldrich) in 5% HCl and premixed immediately prior application). The absorbance at 540 was measured using a spectrophotometer (He□ios- thermospectonic).

Estimation of liver and serum interleukin-6 (IL-6) content:

IL-6 levels were determined in liver homogenate and serum by enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Invitrogen), and read at 450 nm using ELISA reader (BioTEk Instruments Inc., ELx 808, USA).

Histopathological Examination

The fixed liver samples were trimmed, dehydrated, embedded in paraffin, sectioned, mounted on glass slide, stained with hematoxylin and eosein and examined by light microscopy. Grading and staging of the histopathological lesions and fibrosis of liver tissues were performed according to histological activity scoring system (HAI) of Ishak (Ishak, 1994, Ishak *et al.,* 1995).

Histological Grading:

Ishak's activity scoring system; for scoring the degree of necrosis and inflammation are given scores from 0-18. The stage A0, scoring no necro-inflammatory activity, while stage A1 scoring 8/18 refer to mild necro-inflammatory activity. On the other hand, the stages A2 and A3 indicating moderate and severe necro-inflammatory activity and were scored 9-12/18 and 13-18/18, respectively.
Detection of fibrosis was done according to Ishak's fibrosis staging system where F0 indicating absence of fibrosis. While detection of fibrosis on some but not all portal tracts was scored as stage F1, but when detected on most or all portal tracts with occasionally porto-portal bridging, it was scored as F2. As fibrosis of most portal tracts with porto-portal and porto-central bridging, with occasionally nodules (incomplete cirrhosis) was detected, this indicated the stage F3, while the detected cirrhosis indicating stage F4.

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Statistical analysis:

Statistical analysis was performed using SPSS 20.0 statistical software (SPSS Inc, Chicago, IL, USA). Arithmetic means were used and data were analyzed by one-way analysis of variance (ANOVA). If the test showed a significant difference, the least significant difference test was used as a Post hoc Tukey's test for multiple comparisons. The differences were considered significant if the probability was associated with $p \leq 0.05$.

Results

Effects of CCl_4 and GL, PTX and their combined pre-treatment on relative liver weight:

As shown in (Fig. 1), the administration of CCl_4 has significantly increased the relative liver weight by 39 % ($P \leq 0.001$) as compared to the non-treated control. Treatment with either PTX or GL prior to CCl_4 has significantly decreased the relative liver weight by 12 % ($P \leq 0.001$) and 16% ($p \leq 0.001$), respectively as compared to CCl_4 (Fig. 1). On the other hand, PTX-GL combined treatment prior CCl_4 significantly decreased the relative liver weight by 25 % ($P \leq 0.001$) as compared to CCl_4 non-treated group (Fig. 1).

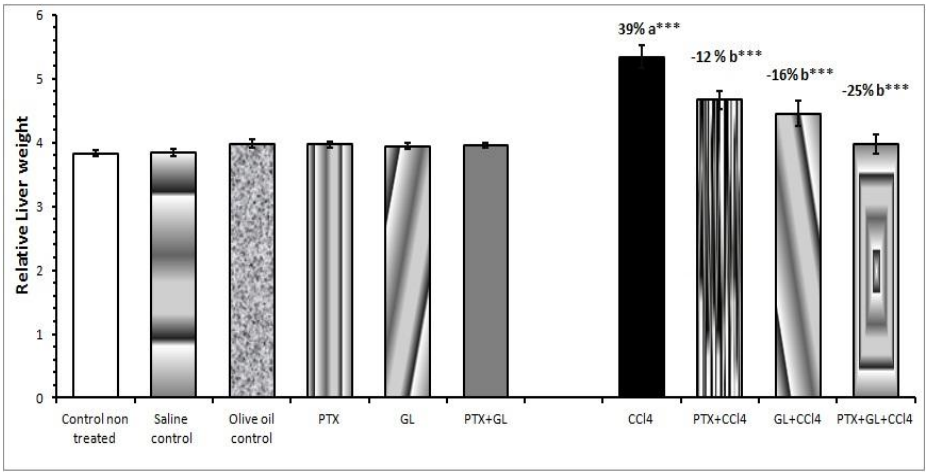

Fig.1: Effects of CCl_4, GL, PTX and their combined pre-treatment on relative liver weight (Values are mean ±SEM of ten animals)
(*=$p \leq 0.05$, **= $p \leq 0.01$and ***= $p \leq 0.001$)
a significantly different from non-treated control group
b significantly different from CCl_4 group

Effects of CCl_4 and GL, PTX and their combined pre-treatment on serum ALT and AST:

Administration of CCl_4 twice a week for 6 weeks exhibited significant increase in serum ALT and AST activities by 325% ($p \leq 0.001$) and 329% ($P \leq 0.001$), respectively as compared to the control non treated group (Fig.2). While administration of PTX prior to CCl_4 induced reduction in serum ALT and AST by 22% ($p \leq 0.001$) and 24 %($p \leq 0.001$), respectively, administration of GL induced more reduction in serum ALT and AST by 41% ($p \leq 0.001$) and 40% ($p \leq 0.001$), respectively as compared to CCl_4 group (Fig.2). On the other hand, GL-PTX combined pretreatment induced pronounced reduction in serum ALT and AST by 70% ($p < 0.001$) as compared to CCl_4 group, restoring serum ALT and AST activities to nearly their normal values (fig. 2a,b).

Effects of CCl_4 and GL, PTX and their combined pre-treatment on hepatic tissue MDA level:

Intraperitoneal injection of CCl_4 for 6 weeks resulted in a significant increase in tissue MDA level by 821% ($p \leq 0.001$) as compared to the control group (Fig.3). Pre-treatment with either PTX, GL or their combined treatment for 6 weeks prior to CCl_4 reduced significantly MDA level by nearly comparable values 69% ($p \leq 0.001$), 67% ($p \leq 0.001$) and 68%, respectively as compared to CCl_4 group (Fig.3).

Effects of CCl_4 and GL, PTX and their combined pre-treatment on hepatic tissue SOD:

Hepatic tissue SOD activity under the influence of CCl_4 administration exhibited marked decrease by 38% ($p < 0.001$) as compared to the control group (Fig. 4). While PTX or GL pre-treatment prior CCl_4 induced

significant increase in hepatic SOD activity by 35% (P≤0.001) and 47%% (p<0.001), respectively, their combined pre-treatment caused restricted additive increase in SOD activity by 54% (P≤0.001) as compared to CCl_4 (Fig. 4).

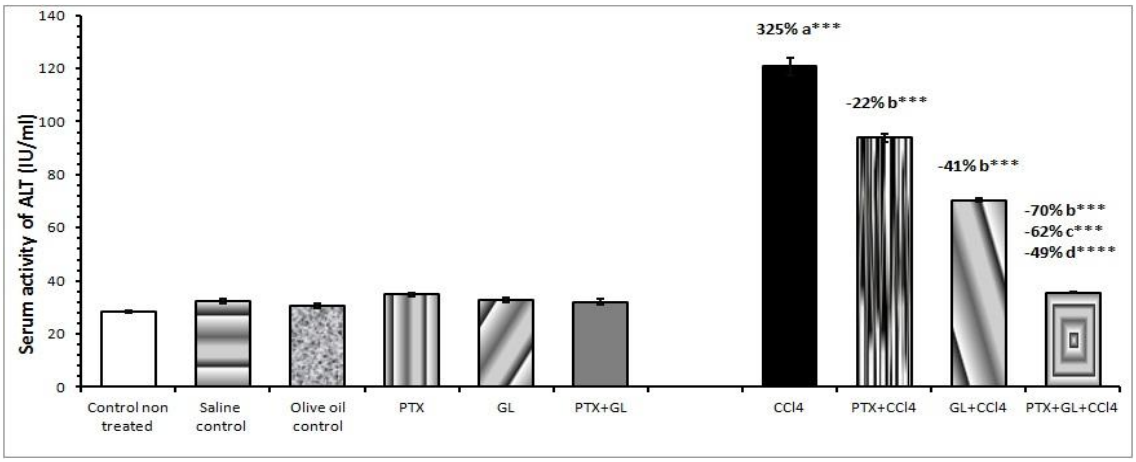

Fig.2 A: Effects of CCl_4, GL, PTX and their combined pre-treatment on serum ALT (Values are mean ±SEM of ten animals)
(*=p≤0.05, **= p≤0.01and ***= p≤0.001)
a significantly different from non-treated control group
b significantly different from CCl_4 group
c significantly different from PTX+ CCl_4 group
d significantly different from GL+ CCl_4 group

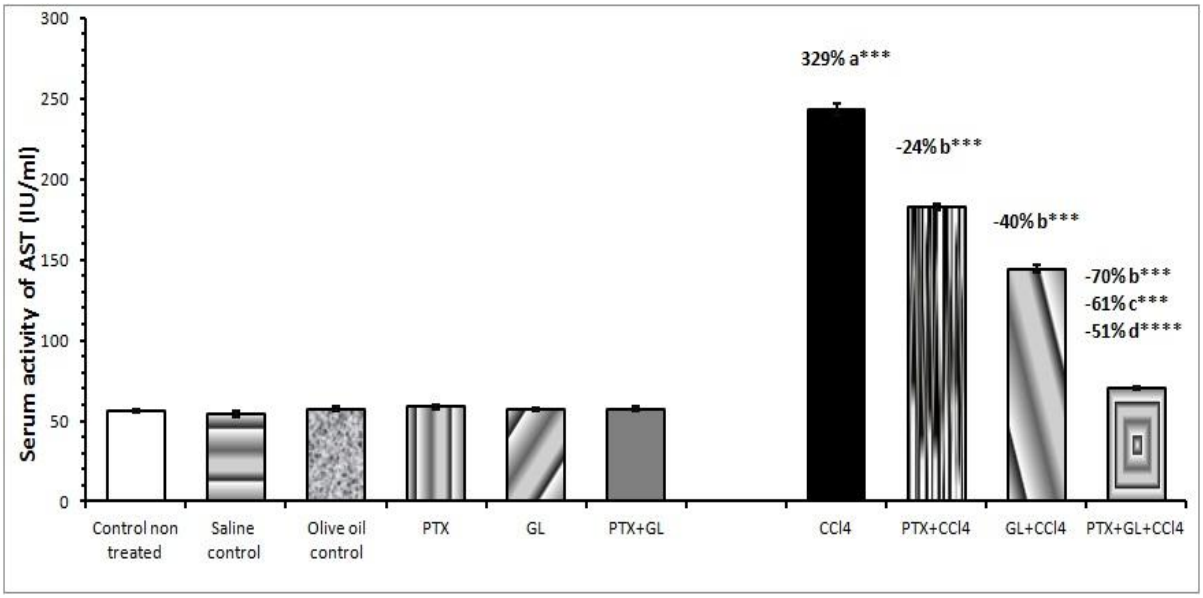

Fig.2 B: Effects of CCl4, GL, PTX and their combined pre-treatment on serum AST (Values are mean ±SEM of ten animals)
(*=p≤0.05, **= p≤0.01and ***= p≤0.001)
a significantly different from non-treated control group
b significantly different from CCl4 group
c significantly different from PTX+ CCl4 group
d significantly different from GL+ CCl4 group

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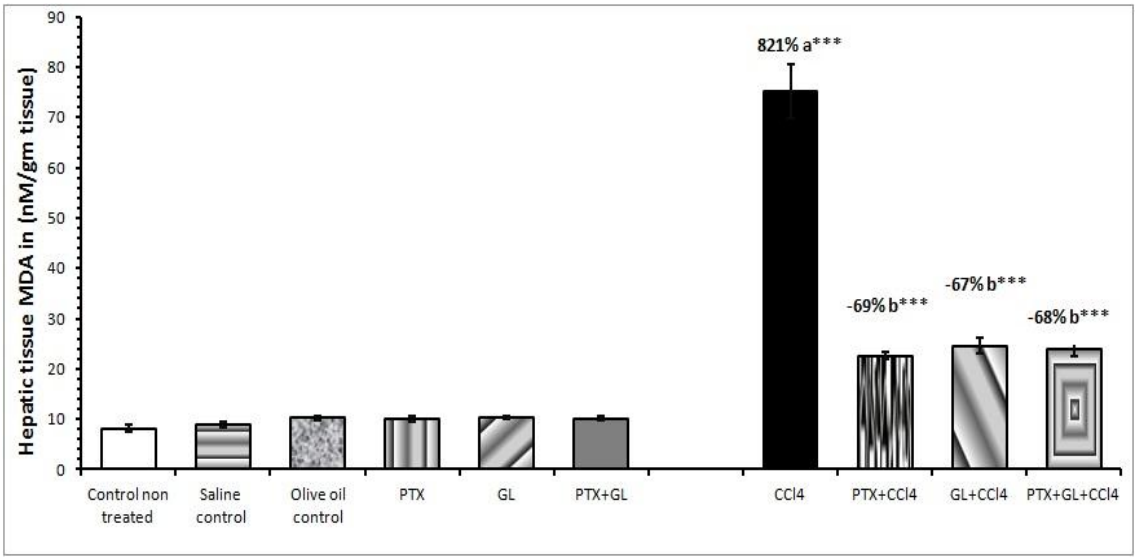

Fig. 3: Effects of CCl_4, GL, PTX and their combined pre-treatment on hepatic tissue MDA (Values are mean ±SEM of ten animals)
(*=p≤0.05, **= p≤0.01and ***= p≤0.001)
a significantly different from non-treated control group
b significantly different from CCl_4 group

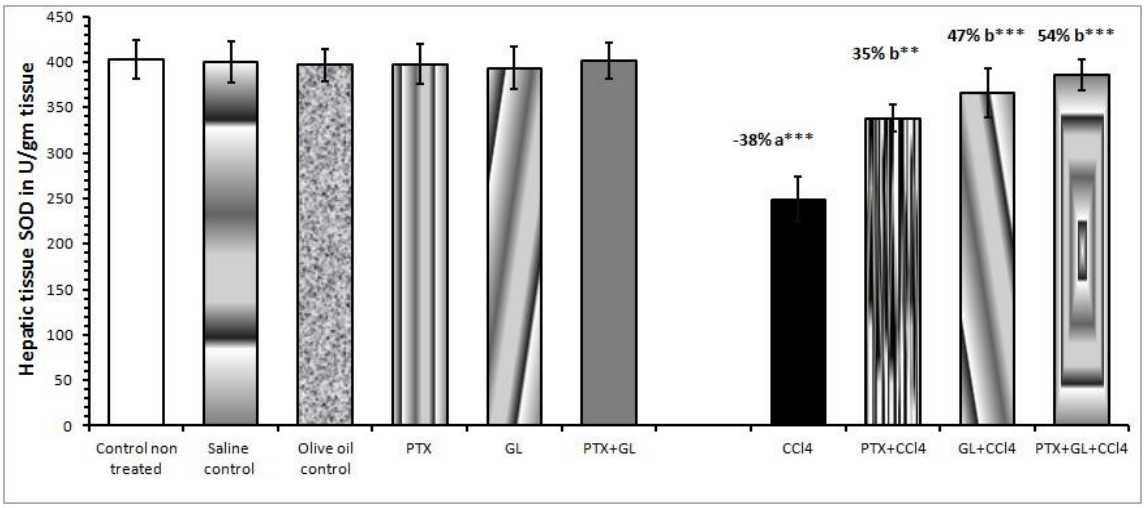

Fig. 4: Effects of CCl_4, GL, PTX and their combined pre-treatment on hepatic tissue SOD (Values are mean ±SEM of ten animals).
(*=p≤0.05, **= p≤0.01and ***= p≤0.001)
a significantly different from non-treated control group
b significantly different from CCl_4 group

Effect of CCl_4 and GL, PTX and their combined pre-treatment on liver total nitrate/nitrite content:

The intraperitoneal administration of CCl_4 induced a significant increase in the total liver nitrate/nitrite content by 551% (P≤0.001) as compared to the control group (Fig. 5). Statistical analysis revealed that, the pre-treatment with either PTX or GL alone prior to CCl_4 caused a significant decrease in the total nitrate/nitrite content by 58% (P≤ 0.001) and 69% (p<0.001), respectively as compared to CCl_4 (Fig. 5). The combined pre-treatment induced additive decrease in total liver nitrate/nitrite content by 79% (P≤ 0.001) as compared to CCl_4, group (Fig. 5).

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Effect of CCl_4 and GL, PTX and their combined pre-treatment on serum and hepatic tissue interleukin 6 (IL-6):

The results of this study, revealed that the administration of CCl_4 induced significant increase in serum and hepatic tissue IL-6 content by 367 % ($p<0.001$), and 383 % ($p<0.001$), respectively as compared to the control group (Fig. 6). Statistical analysis revealed that, both serum and tissue IL-6 content exhibited significant decrease by 28% and 29% ($P\leq 0.001$), respectively, post PTX pretreatment, and 31% and 32%, respectively, ($P\leq 0.001$) post GL pre-treatment as compared to CCl_4 group (Fig. 6). Additionally, PTX-GL combined pre-treatment induced a significant additive decrease in both serum and hepatic IL-6 content by 67%, ($P\leq 0.001$), as compared to CCl_4. Such decrease was better than that induced by PTX pre-treatment alone (54% ($P\leq 0.001$) and that induced by GL pre-treatment alone (51% and 52% ($P\leq 0.001$) as compared to PTX and GL-treatment alone, respectively, (Fig. 6).

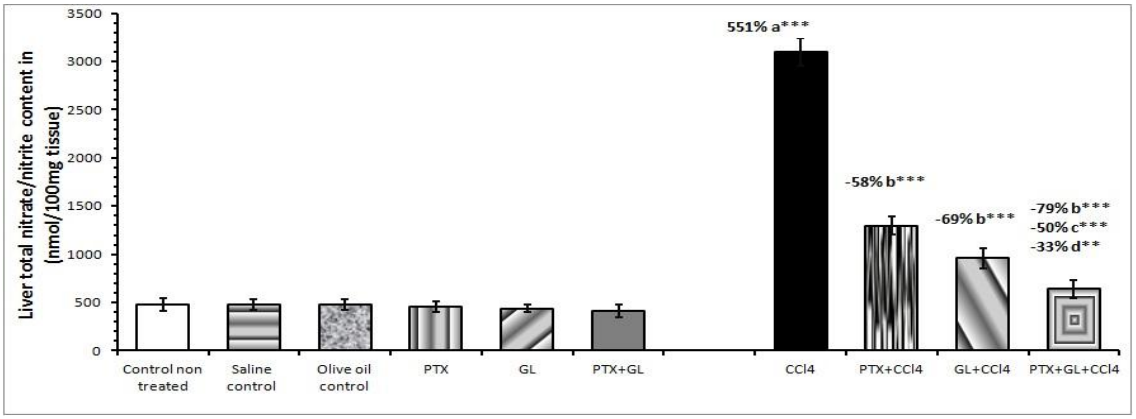

Fig. 5: Effects of CCl_4, GL, PTX and their combined pre-treatment on liver total nitrate/nitrite content (Values are mean ±SEM of ten animals)
(*=p≤0.05, **= p≤0.01and ***= p≤0.001)
a significantly different from non-treated control group
b significantly different from CCl_4 group
c significantly different from PTX+ CCl_4 group
d significantly different from GL+ CCl_4 group

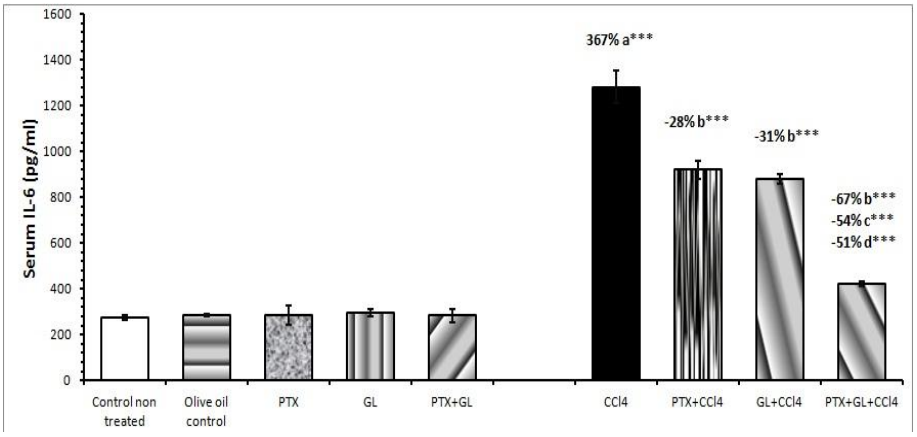

Fig.6 A: Effects of CCl_4, GL, PTX and their combined pre-treatment on serum IL-6 (Values are mean ±SEM of ten animals)
(*=p≤0.05, **= p≤0.01and ***= p≤0.001)
a significantly different from non-treated control group
b significantly different from CCl_4 group
c significantly different from PTX+ CCl_4 group
d significantly different from GL+ CCl_4 group

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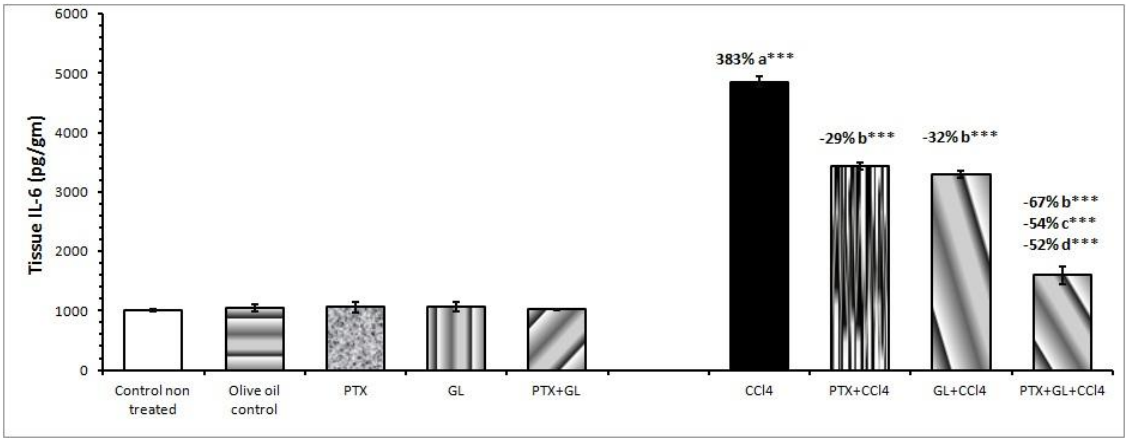

Fig. 6 B: Effects of CCl_4, GL, PTX and their combined pre-treatment on hepatic tissue IL-6 (Values are mean ±SEM of ten animals)
(*=p≤0.05, **= p≤0.01and ***= p≤0.001)
a significantly different from non-treated control group
b significantly different from CCl_4 group
c significantly different from PTX+ CCl_4 group
d significantly different from GL+ CCl_4 group

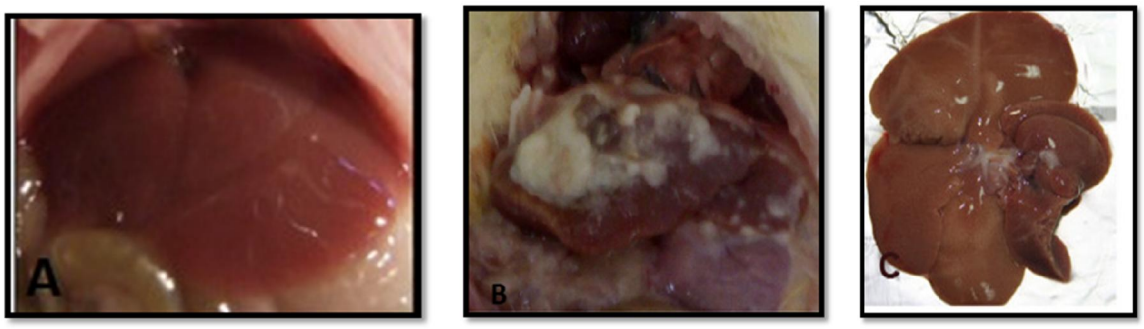

Fig. 7: Macroscopic changes of rat livers. (A) Control livers; (B) livers of rat intoxicated with CCl_4 (1.0 mL/kg twice a week for 6 weeks); (C) liver of rat intoxicated with CCl_4 (1.0 mL/kg twice a week for 6 weeks) after pre-treatment with GL (50 mg/kg/d) and PTX (100 mg/kg/d).

Histopathological Results:

Histological examination showed that the normal liver architecture (A0F0) of non-treated control group (Fig. 8). The examined liver tissues of rats administered CCl_4 revealed distortion of liver architecture with lobular nodulation accompanied by vacuolated hepatocytes with ballooning in nests more in subcapsular area (fatty-degeneration). Additionally, fibrosis of most portal tracts with porto-portal and porto-central bridging with shifted central vein were seen (F3). Dense portal inflammatory infiltrates mainly lymphocytes with confluent necrosis and porto-portal and porto-central necrosis with porto-central bridging (A3) with dilated congested portal veins and sinusoids were also observed (Fig.9). Pre-administration of PTX prior to CCl_4 decreased the severity of fibrosis as compared to CCl_4, where in this group moderate portal fibrous expansion with porto-portal fibrous septa was only observed (F2). However, the dense portal inflammatory infiltrates mainly lymphocytes with confluent necrosis and porto-portal and porto-central necrosis (A3) was observed in this group like that in CCl_4 (Fig. 10). The livers of rats treated with GL prior to CCl_4 revealed distinct porto-portal fibrous tissue septa with widening of the portal fields (peri-portal fibrosis with fine strands at periphery), therefore, there was a decrease in the incidence and severity of fibrosis as compared to CCl_4 (F2). Also, pre-treatment with GL showed a decrease in the inflammatory response, where mild inflammatory activity in the

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portal tract (A2) was only observed (Fig. 11). Liver section of combined pre-treatment animals group prior CCl4, showed signs of improvement in liver compared to PTX or GL pre-treatment alone prior CCl_4. Such improvement was illustrated in the normal lobular pattern of the liver with wide areas of intact hepatocytes and occasionally vacuolated fatty degenerative. Additionally, normal appearing of portal tract and portal veins with only mild fibrous septa in some but not all portal tract and mild portal inflammatory infiltration (A1F1) were observed (Fig. 12).

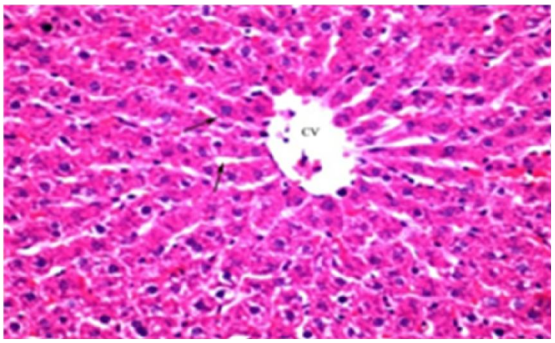

Fig. 8: Liver section of control animal group shows: normal liver architecture, intact central veins. hepatocytes shows vesicular nuclei (arrow), (CV) central vein A0F0. H&E (400X).

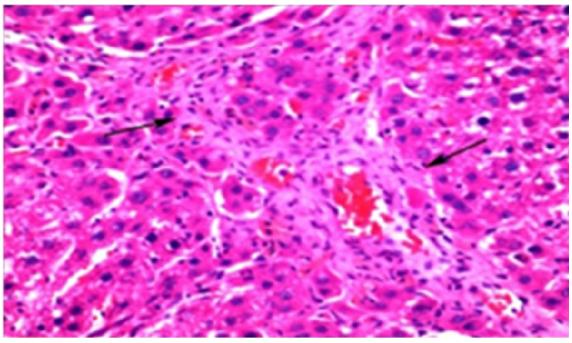

Fig. 9: Liver section of CCL4 animal group shows: fibrous bands in the portal tract (arrow), ballooning in hepatocytes with degenerative changes. A3F3 . H&E (400X).

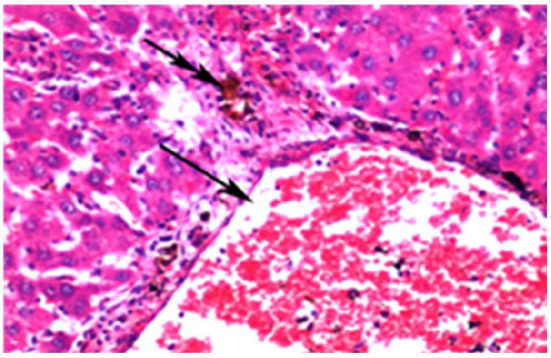

Fig. 10: Liver section of animal treated with PTX+CCl4 shows: congested dilated portal tract with inflammation and fibrosis (arrow), bile pigment deposition due to bile stagnation (double arrow) A3F2. H&E (400X).

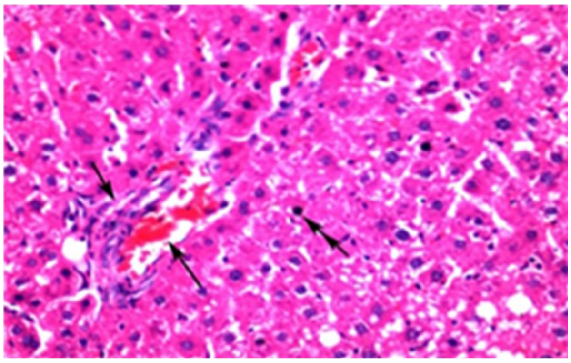

Fig. 11: Liver section of animal treated with GL+CCl4 shows: Dilated fibrosed portal tract (arrow), pyknotic nuclei (double arrow). A2F2. H&E (100X).

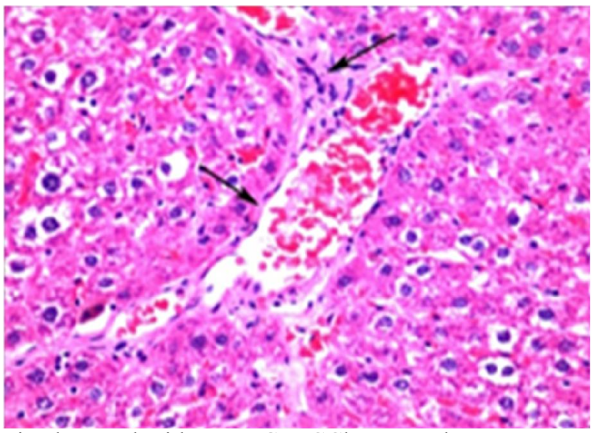

Fig. 12: Liver section of animal treated with PTX+GL+CCl_4 group shows: congested dilated portal tract with mild inflammation and fibrosis. A1F1 H&E (400X).

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Discussion

The profound results of this study revealed that, there were significant increases in the relative liver weight of those rats administered CCl_4 to nearly two folds, as compared to the control group, which may be referred to the hepatootoxicity of CCl_4 as well as the decrease in appetite of those rats that led to a decrease in the body weight and therefore, increase the relative liver weight (Uzma *et al.,* 2011).

Although GL pre-treatment had better effect on the relative liver weight than PTX, their combined treatment prior CCl_4 could restore relative liver weight to the normal value, providing a better protection against CCl4 toxicity than single treatment and these results could refer to a hepatoprotective effect which were confirmed by the tested biochemical parameters as well as liver histolopathological examination.

Serum ALT and AST enzymes activities are very sensitive markers and can be quantified to assess the type and extent of liver injury (Basu, 2003, Wang *et al.,* 2012). In this concern, the result of the present study revealed that CCl_4 administration caused significant elevation of serum liver enzymes ALT and AST activities as a result of altering the hepatocellular membrane integrity (Jeong *et al.,* 2002, Wang *et al.,* 2012, Moreira *et al.,* 2014), therefore, the liver enzymes that normally present in the cytosol are released into the blood stream. The obtained results showed that PTX pre-treatment alone significantly attenuated the elevation in ALT and AST activities induced by CCL4 -to some extent- as compared to CCl_4 (Abdel-Salam *et al.,* 2005, Al-Zahra *et al.,* 2009). Moreover, GL administration alone prior CCl_4, could attenuate the effect of CCl_4 on liver ALT and AST activities, and this effect might be due to the ability of GL to alter the membrane fluidity and increase the integrity of the hepatocyte membrane, and/or due to the inhibition of CCl_4-induced membrane lipid peroxidation (Nakamura *et al.,* 1985, Lee *et al.,* 2007, Chen *et al.,* 2013).

Surprisingly, our results also revealed that the combined pre-treatment could nearly restore the liver enzymes to their normal values and had a better effect on liver protection than single treatment, probably due to the synergistic reaction of the two drugs on alleviating the increased serum AST and ALT activities induced by CCL4.

Another approach for the detection of hepatic injury involves measurement of lipid peroxides, MDA, the end product of poly-unsaturated fatty acid peroxidation, which is a marker of free radical mediated lipid peroxidation injury (Del Rio *et al.,* 2005). In the current study, MDA exhibited significant increase in rat liver after CCl_4 administration as a result of increase in lipid peroxidation (Aleynik *et al.,* 1997, Wang *et al.,* 2012, Chen *et al.,* 2013, Moreira *et al.,* 2014). Pre-treatment with PTX significantly could attenuate CCL4-induced elevation in hepatic MDA. This effect may be explained by the ability of PTX to act as agonist for both adenosine receptors, A1 and A2; in addition to its property as an inhibitor of phosphodiesterase enzymes and increase of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels, these effects leading to inhibition polymorphneuclear cells response (Tajima *et al.,* 2001, Noyan *et al.,* 2006, Al-Zahra *et al.,* 2009). Regarding the GL pre-treatment prior CCl_4, the hepatic tissue MDA exhibited significant decrease as compared to CCL4-administered group. This effect may be attributed to GL's antioxidant activity, and its ability to scavenge free radicals that helps in lipid peroxidation inhibition (Ju *et al.,* 1990, Huo *et al.,* 2011, Chen *et al.,* 2013). It is worth noted that combined treatment failed to induce any additive effect on hepatic MDA level than monotherapy.

The antioxidant enzymes are depleted by lipid peroxides or reactive oxygen species (ROS), that generated by CCl_4 toxicity as a result of oxidative damage of these proteins (Gabele *et al.,* 2009, Yang *et al.,* 2008, Chen *et al.,* 2013). In the present study, SOD activity exhibited significant decrease in the liver tissue in response to CCl_4 compared to control rats, implying increased oxidative damage to the liver Gabele *et al.,* 2009, Augustyniaket *et al.,* 2005, Yang *et al.,* 2008, Chen *et al.,* 2013).

On the other hand, each of PTX and GL pretreatment could significantly attenuate CCL4-induced decrement in SOD activity as compared to CCl_4 group, suggesting that PTX and GL has the ability to restore this enzyme activity in CCl_4 damaged liver. This effect may probably be illustrated by the ability of PTX to block TNF-alpha expression; reduces the activation of NF-Kappa B and reduce the oxidative stress (Ji 2004; Al-Zahra *et al.,* 2009; Chen *et al.,* 2013). While the effect of GL on SOD level may be to its anti-oxidant activities (Huo *et al.,* 2011; Chen *et al.,* 2013). It is worth mentioning that, combined pretreatment had an unambiguous influence on SOD level, it could restore the hepatic SOD level to its normal value. This result may be attributed to their action via different mechanisms which enable these two compounds to act concurrently to increase tissue SOD and potentiate the effect of each other.

Certain evidences have indicated that hepatic damage may be caused by excessive NO production, our results confirmed that CCl_4 treatment induced a significant increase by about 6 folds in the total nitrate/nitrite level (the stable metabolite of NO) in liver as compared to control group (Tipoe *et al.,* 2006; Domitrovic *et al.,* 2011; Huang *et al.,* 2012). The present results indicated that each of PTX and GL pre-treatment significantly attenuated the elevated liver total nitrate/nitrite content induced by CCL4. This result may be due the down regulatory effect of PTX on the inflammatory response, which was assessed in previous studies by significant decrease in NF-kappa B-positive staining in hepatocytes and Kupffer cells, as well as iNOS immunostaining in Kupffer cell (Taye 2009). On the other hand, the most pronounced effect of GL pre- treatment on hepatic

nitrite/nitrate content than PTX-pre-treatment may due to the direct effect of GL to suppress iNOS protein secretion and/or enhances the degradation of their protein (Lee *et al.,* 2007). Interestingly, combined treatment induced profound and remarkable effect attenuating CCl4-induced elevation in liver total nitrate/nitrite content, indicating a synergistic effect of the combined treatment.

Interleukin- 6 (IL-6) has diverse biological functions on different types of cells. It was found that activated Kupffer cells, T-cells and damaged hepatocytes release the inflammatory cytokines (TNF-α, interferon γ (INF-γ), IL-6), free radicals and growth factors like, platelet-derived growth factor (PDGF) after hepatic injury (Salazar-Montes *et al.,* 2000). Since the cytokines are closely related with fibrogenesis, they possibly could be used as biomarkers for liver fibrosis (Chakraborty *et al.,* 2012; Seki *et al.,* 2007&2012). In this study, CCl_4 caused a significant increase in serum and hepatic IL-6 content compared to control group, this was attributed to CCL4-induces chronic hepatic inflammation accompanied by up regulation of pro-inflammatory and chemotactic mediators, including TNF-α, IL-6, IL-8, MCP-1, and TGF-β (Seki *et al.,* 2007&2012). On the other hand, PTX and GLX monotherapy exhibited comparable effect on attenuating IL-6 rise in serum and hepatic tissue induced by CCL4, such effect of PTX may be attributed to the ability of PTX to down regulate the inflammatory response, through the inhibition of phosphodiesterase which up regulates intracellular cAMP (Neuner *et al.,* 1994; Rodrigo *et al.,* 2011). While the effect GL may be explained by neutralizing anti-HMGB1 antibodies (Mollica *et al.,* 2007). Where high mobility group box 1 (HMGB1) is not only released in response to pro-inflammatory stimuli, but induces the production of multiple inflammatory mediators by a variety of cells (Erlandsson-Harris and Andersson 2004, Sims *et al.,* 2010). The profound results revealed that, the combined treatment was found to have a better effect on attenuating CCl_4-induced elevated IL-6, than monotherapy indicating a synergistic effect of these combined treatment.

The macroscopical and histological examination, in the current study, it was clear that CCl_4 caused alteration on the liver morphology, liver injury and fibrosis (F3), where there was a marked steatosis and necrosis (A3) compared to control groups (Chuan-Tao *et al.,* 2012; Ai *et al.,* 2013). Concerning the effect of PTX-pretreatment on liver, according to Ishak's activity scoring the degree of necrosis and inflammation as well as scoring fibrosis staging system, PTX decreased the severity of fibrosis and necrosis (A3F2) as compared to CCl_4. However, there was distortion of liver architecture with no lobular pattern. This results is consistent with Abdel-salam *et al.*, (2005). Furthermore, the histopathological examination showed that, the livers of rats treated with GL prior CCl_4 revealed a decrease in the incidence and severity of fibrosis (F2) and mild inflammatory activity (A2) as compared to CCl_4. This profound result was in consistent with the finding of Qu *et al.,* (2012). On the other hand, the combined treatment showed a remarkable hepatoprotective and antifibrotic effects, where, it alleviated the macroscopical changes of liver that induced by CCl_4, and neither necrosis nor steatosis had been observed. Additionally, the histopathological findings of our study revealed signs of improvement in liver profile (A1F1) compared to CCl_4, where only mild fibrous septa were observed as well as normal lobular pattern of the liver. In conclusion: The profound results showed that, the administration of the combined treatment of PTX and GL attenuated the CCl_4 induced liver toxicity and fibrosis in rats and had better effects in protecting the liver than did GL or PTX alone.

Abbreviations

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CCl_4: Carbon tetrachloride; GL: Glycyrrhizin; HSCs: Hepatic stellate cells; IL-6: Interleukin-6; MDA: Malondialdehyde; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; PTX: Pentoxifylline; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TGF-B: Transforming growth factor B

Disclosure

The authors declare that there is no conflict of interest that would prejudice the impartiality of the reported research.

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