RESEARCH

Trigonelline: a novel natural hepatoselective fibrosuppressive agent

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ABSTRACT

Background and Aim: Trigonelline (L-proline analogue) has been commonly used as traditional food and medicine. This study was done to evaluate the possible prophylactic effect of trigonelline on CCL_4 treated rats as a model for liver fibrosis.

Methods: 24 adult female rats weighing (200-250 gm) were divided into four groups; the first (negative control), second (CCL_4), third (CCl_4 + trigonelline) and the fourth (CCl_4 + silymarin; positive control). After eight weeks of treatment, blood and liver samples were taken for the assessment of liver functions.

Results: In comparison with the CCl_4 group; trigonelline group showed a decrease in the serum level of ALT and AST, increase in the manual platelet count, a significant decrease in the APRI index, relatively preserved liver architecture with the appearance of signs of liver regeneration with a highly significant decrease in the staging of fibrosis. In the treatment group, a few collagen fibers were found without the formation of fibrous septa by masson trichrome stain, a decrease in the area occupied by collagen using image analysis and a few α-SMA positive cells. In comparison with the silymarin group; trigonelline group showed decreased area occupied by collagen in image analysis, highly significant decrease in the staging of fibrosis and a significant decrease in APRI index.

Conclusion: Trigonelline appears to be an effective hepatoprotective drug against liver fibrosis in CCl_4 induced model of fibrosis.

KEYWORDS: Trigonelline; Liver fibrosis, CCL4; Hepatoprotective

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INTRODUCTION

Liver fibrosis is the final pathway for most chronic liver diseases. Initially, it was thought to be a passive process. Recently, fibrosis was redefined by the World Health Organization (WHO) as ''the presence of excess collagen due to new fiber formation'' (Gressner et al., 2016). It was proved that experimental hepatic fibrosis induced by carbon tetrachloride is due to increased collagen synthesis. Not only does the rate of collagen synthesis increase, but there is a parallel increase in the level of hepatic prolyl hydroxylase (Guzman, 1998). The synthesis of hydroxyproline (Hyp) residues by the enzyme, prolyl 4-hydroxylase (P4H), is considered to be the most important step in the intracellular collagen processing (Prockop 1979). They permit the sharp twisting of the collagen helix, in a special arrangement of collagen Xaa-Yaa-Gly triad (where Xaa and Yaa are any amino acids), a proline occupying the Yaa position is hydroxylated to give a Xaa-Hyp-Gly sequence (Brinckmann et al., 2005).

Trigonelline is considered as one of the proline analogues as both of them belong to betaines, which when incorporated into proteins in place of proline, result in non-folding of the newly synthesized polypeptides of procollagen. If a critical number of prolyl residues have been replaced by this analogue, the net production of extracellular collagen is decreased (Gorres & Raines, 2010; Zeisel et al., 2003). It is an alkaloid with chemical formula $C_7H_7NO_2$. (Zeiger & Tice 1997). Fenugreek and coffee are the commonly occurring and widely known substances containing trigonelline and it has since been found in many other plant species including pea, soybean, and potato. Fenugreek (*Trigonella foenum-graecum*), a plant which contains trigonelline, was proved to have a role in the inhibition of carbon tetra chloride (CCl_4) - induced liver fibrosis (Mishkinsky et al., 1974; Snehlata & Payal, 2012; Thaakur et al., 2007). Trigonelline exhibited anticarcinogenic (cervix and liver), antimigraine, antiseptic, hypocholesterolemic, and hypoglycemic activities (Duke et al., 1997).

The present study was designed to investigate the possible protective effect of natural L-proline analogue (Trigonelline) against carbon tetrachloride (CCl_4)-induced liver injury in comparison to silymarin as a positive control in such cases. Silymarin is a flavonolignan that has been introduced fairly recently as a hepatoprotective agent. It is extracted from the seeds and fruit of *Silybummarianum* (Compositae). Silymarin is a complex mixture of four flavonolignan isomers, namely silybin, isosilybin, silydianin, and silychristin. Among the isomers, silybin is the major and most active component and represents about 60-70 percent (Saller et al., 2001).

MATERIALS AND METHODS

Twenty-four adult female albino rats, weighing 200-250gm aged 12-16 weeks were purchased from Mansoura Experimental Research Center (MERC), Egypt, and kept in stainless steel mesh cages under temperature (23ºC±3), and relative humidity with ad libitum access to food and water and fixed 12:12-hours light/dark cycle.

Study design

1st group (-ve control): (n=6), rats were fed on the basal diet and injected intraperitoneally with corn oil for 8 weeks.

2nd group: (n=6), rats were injected intraperitoneally with 50% CCL_4 in corn oil (2 ml/ kg b.w.) for 8 weeks (Janakat & Merie 2002).

3rd group: (n=6), rats were injected intraperitoneally with 50% CCL_4 in corn oil (2 ml/ kg b.w.) with simultaneous administration of 50 mg/Kg/day (trigonelline) via gastric lavage for 8 weeks (Thaakur et al., 2007).

4th group (+ve control): (n=6), the rats were injected intraperitoneally with 50% CCL_4 in corn oil

(2ml/kg b.w.) with simultaneous administration of 50 mg/Kg/day (silymarin) via gastric gavage for 8 weeks with addition of drops of TWEEN 80 (Sigma) to increase solubility of Silymarin (Boigk et al., 1997).

By the end of the experiment (8 weeks); the animals were anesthetized by thiopental injection, sacrificed and cardiac blood samples were obtained for biochemical study. The liver was dissected out and cut into 2-3mm^3 pieces, which were immersed immediately in 10% formol saline fixative and in absolute alcohol and finally processed into 5-6 µm paraffin sections for the light microscopic examination.

Biochemical Study

The levels of serum ALT and AST were measured by colorimetric method using the commercial diagnostic kits produced by Randox Co., United Kingdom (Reitman & Frankel 1957).

Manual platelet count of whole blood (PLT)

It was performed by using diluent of 1% aqueous solution of ammonium oxalate in which the red cells were lysed (Boigk et al., 1997).

Histological examination

Liver sections were stained with hematoxylin and eosin (H&E), Masson trichrome stains and immune stain by alpha smooth muscle actin (α-SMA) (Lewis et al., 1079; Ronis et al., 2004; Korourian et al., 1999).

Image analysis of the area occupied by collagen fibers

Slides were photographed using Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor, using 40 X objective. The result images were analyzed on Intel® Core I3® based computer using VideoTest Morphology® software (Russia) with a specific built-in routine for immunohisto staining analysis and % area of fibrosis.

Fibrosis grade

The histopathological scoring of liver sections was done as follows (Yoshiji et al., 2000).

Stages	Categorical description
0	There is no fibrosis
1	mild fibrosis that is started and extended from hepatic central venules
2	central –central fibrotic septa were formed
3	multiple central –central fibrotic septa that aren't incompletely reached and divided hepatic lobule
4	central –central septa connected with each other completely that divide the hepatic lobule into rectangle-like pseudo-lobule, portal tract located in the center usually (early stage of cirrhosis)
5	moderate central –portal bridging septa that divide pseudo-lobule into smaller sized nodules less than 50% (incomplete cirrhosis)
6	Multiple C–P bridging septa divided pseudo-lobule further, the number of smaller sized nodules more than 50% (complete cirrhosis)

Simple noninvasive index

APRI (AST to platelet ratio index) ratio was calculated as suggested by Zhao et al. 2008 (Zhao et al., 2008).

Fibrotic index - Liver fibrosis staging correlation

Correlation between histological fibrosis staging and each biochemical fibrotic index was evaluated statistically by Spearman's rho coefficient ® test which correlates different parameters.

Statistical analysis

The computer program SPSS (Statistical package for social science) version 17.0 was used for tabulating, coding and analyzing data. Mean and Standard deviation (±SD) were used for the calculation of Descriptive statistics. For the statistical comparison, between more than two groups (ANOVA (analysis of variance) was used for (parametric) data followed by post-hoc Tukey for multiple comparisons and Kruskal Wallis test for (non-parametric) data followed by Mann-Whitney for multiple comparisons).

A P-value <0.05 was considered statistically significant. And a P value <0.0001 was considered highly significant in all analyses.

RESULTS

As shown in (Tables 1,2,3,4), there was a highly significant increase in the serum levels of GPT, GOT, PLT, AST/ALT & APRI (84.33±10.60, 338.00±72.70, 390.00±48.89, 4.03±.93 and.8834±.2230), respectively, in the CCL4 treated group as compared to the other groups.

In trigonelline group: As shown in Table 1, the serum levels of ALT (40.00±6.00) showed a highly significant decrease as compared to the animals in the CCl_4 group, but this level was still significantly high as compared to the -ve control value. As compared to silymarin, the treated group showed no significant difference.

According to the serum level of AST (128.33±11.45), trigonelline group showed highly significant decrease as compared to the animals that received CCl_4 only and in comparison to the animals of +ve control (silymarin) group. However, this level showed no significant difference as compared to the –ve control value (Table 2).

As shown in (Table 3), the serum levels of PLT (861.50±114.16) showed a highly significant increase as compared to the animals of CCl_4 group, but this level was still significantly high as compared to the –ve control value. As compared to the silymarin treated group, there was no significant difference.

As regard with APRI index, there was a significant difference between animals that received CCl_4 injection only relative to those which received silymarin and trigonelline groups (Table 4). In animals of trigonelline group, APRI index showed a significant decrease in comparison with animals that received CCl_4 alone. However, there was no significant difference between animals of trigonelline and (-ve control & silymarin) groups (Table 4).

Haematoxylin and Eosin (Hx & E) stained sections

The negative control group demonstrated the normal histological appearance of the liver with hepatocytes arranged in cords radiating from the central vein and the hepatic sinusoids with their lining cells (Fig. 1A).

In the CCL^4 (2^{nd}) group, the liver sections showed distortion of their architecture, the hepatocytes showed zone1 macrovesicular steatosis 10%, microvesicular steatosis, areas of focal necrosis limited to scattered cells within the hepatic lobules, in relation to which the inflammatory cells had been attracted and aggregated, Portal and parynchmal inflammation in most portal tracts was moderate (Fig. 1B). In trigonelline group, hepatocytes showed no steatosis, Portal and parynchmal inflammation was mild in most portal tracts with inflammatory cell infiltrate (mainly lymphocyte without neutrophils) could be seen in the portal areas and hepatic sinusoids. Regenerating hepatocytes (dark blue nucleus with deeply eosinophilic cytoplasm) with increased number of binucleated hepatocytes suggest regeneration (Fig. 1C). silymarin group showed both macro- and micro-vesicular steatosis, mild portal and parynchmal inflammation (mononuclear inflammatory infiltrate) was seen in the portal areas and in the hepatic sinusoids (Fig. 1D).

Table. 1: Comparisons between different groups as regard to ALT level.						
	Control	CCL_4	CCL_4+silymarin	CCL_4+trigonelline	ANOVA	
	Mean±SD	Mean± SD	Mean± SD	Mean± SD	F	P
ALT	21.50±5.75	84.33±10.60	50.17±6.59	40.00±6.00	74.3	<0.001
P1		<0.001	<0.001	.002		
P2			<0.001	<0.001		
P3				0.120		

Table. 2: Comparisons between different groups as regard to AST level.						
	Control	CCL_4	CCL_4+silymarin	CCL_4+trigonelline	ANOVA	
	Mean±SD	Mean± SD	Mean± SD	Mean± SD	F	P
AST	92.67±7.69	338.00±72.70	195.17±12.32	128.33±11.45	50.04	<0.001
P1		<0.001	0.0010	0.376		
P2			<0.001	<0.00		
P3				0.027		

Table. 3: Comparisons between different groups as regard to platelet count.						
	Control	CCL_4	CCL_4+silymarin	CCL_4+trigonelline	ANOVA	
	Mean±SD	Mean± SD	Mean± SD	Mean± SD	F	P
PLT	578.50±111.21	390.00±48.89	756.67±121.93	861.50±114.16	24.2	<0.001
P1		.023	.034	.001		
P2			<0.001	<0.001		
P3				0.322		

Masson's trichrome-stained sections

The negative control group showed few collagen fibers that surround the central veins, the hepatocytes and sinusoidal endothelial cells of the liver lobules (Fig. 2A). On induction hepatic lesion in CCL4 (2nd) group, there was marked an increase of the collagen fibers in the portal tracts and around the central veins, thick well-developed septa could be seen throughout the sections which extend between central veins connecting them together (centro-central septa) (Fig. 2B). In the trigonelline (3rd) group, the amount of

collagen fibers around central veins and in the portal areas was within the normal amount (Fig. 2C). In silymarin group(4th) showed decrease in the amount of collagen fibers around central venules and portal tracts as compared to the group that treated by CCl4 only but is still high as compared to animals of trigonelline group with short incomplete septa could be seen radiating in-between central veins into the surrounding parenchyma (Fig. 2D).

Table. 4: Comparisons between different groups as regard to APRI ratio.

	Control	CCL_4	CCL_4+silymarin	CCL_4+trigoneline	ANOVA	
	Mean±SD	Mean± SD	Mean± SD	Mean± SD	F	P
APRI	.1632±.0218	.8834±.2230	.2636±.0450	.1526±.0361	54.5	<0.001
P1		<0.001	.454	.999		
P2			<0.001	<0.001		
P3				.369		

Table. 5: Comparisons between different groups as regard to image analysis of the area occupied by collagen fibers.

	Control	CCL_4	CCL_4+silymarin	CCL_4+trigonelline	ANOVA	
					F	P
Mean±SD	1.34±.23	9.62±1.61	4.07±1.09	1.75±.43	143.8	<0.001
P1		<0.001	<0.001	.8		
P2			<0.001	<0.001		
P3				<0.001		

(SD: standard deviation; P: Probability P1: significance relative to control group; P2: significance relative to CCL_4 group; P3: significance relative to CCL_4+silymarin group)

Immunohistochemistry for α-SMA

In the negative control group, α-SMA positive reaction was detected in the muscle layer of the portal veins and hepatic arteries within the portal tracts. (Fig.3A). Alpha-SMA positive cells were almost markedly diminished in all sections of the livers of the animals of trigonelline group as compared with the animals that received CCl_4 alone, Alpha-SMA positive cells appeared in the wall of the portal venule, hepatic arteriole and central venule without sinusoidal Alpha-SMA positive cells (Fig. 3C). In the silymarin group, Alpha-SMA positive stain was diminished in all liver sections as compared with the animals that received CCl_4 alone. Alpha-SMA expressing cells

were seen in the portal, perivenular areas and adjacent perisinusoidal spaces (Fig. 3D).

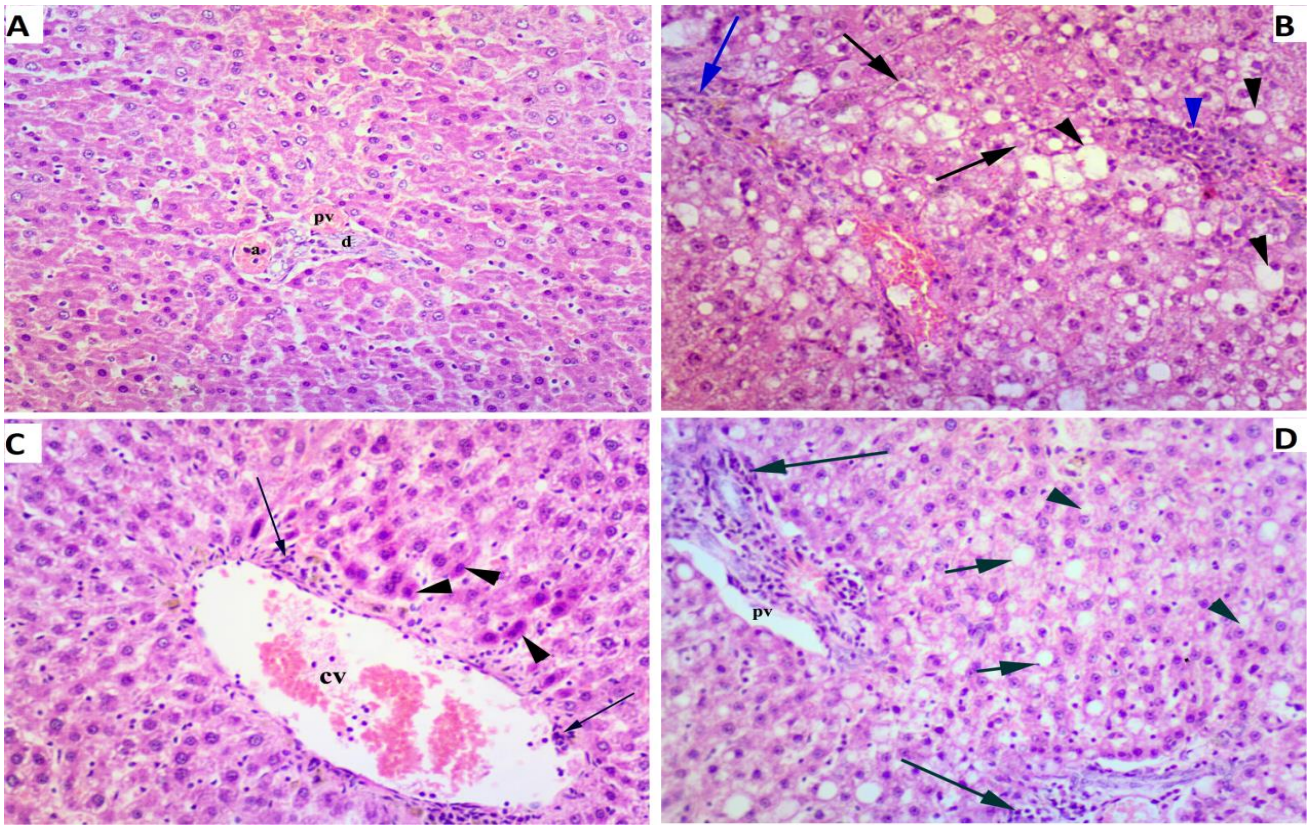

Figure 1. A: A photomicrograph of liver section of control group showing normal histological architecture of the liver. Hepatic lobules formed of cords of hepatocytes with flat, anastomosing plates separated by hepatic sinusoids shows portal tract (triad) which consists of a portal venule (PV), hepatic arteriole (a) and bile ductule (d). (Hematoxylin & eosin; ×100). B: A photomicrograph of liver section of CCL_4 group showing area of loss of hepatic architecture; the hepatocytes around the central venule appeared small and with pyknotic nuclei (black arrow) with multiple microvacules in the cytoplasm with condensed nuclei. Other hepatocytes had macrovacules occupying the whole cytoplasm with eccentric condensed nuclei giving the hepatocyte signet ring appearance (black arrow head). Inflammatory cell infiltrate were distributed mainly around central (blue arrow) & area of necrosis (blue arrow head) (Hematoxylin & eosin ×;100). C: A photomicrograph of a liver section of rat receive trigonelline simultaneously with CCL_4 for 8 weeks showing relatively preserved hepatic architecture in which showing central venule (cv) lined by cells with flat nuclei with little inflammatory cell infiltrate (arrow) compare with CCL_4 treated group. There are regenerating hepatocytes (cells with dark blue nucleus with deeply eosinophilic cytoplasm) around it (Hematoxylin & eosin; ×100). D: A photomicrograph of a liver section of rat receive silymarin simultaneously with CCL_4 for 8 weeks showing inflammatory cell infiltrate (black arrow) in the portal tract. Macrosteatosis (short arrow) and macrosteatosis of hepatocytes (arrow head). (Hematoxylin& eosin; ×100).

Image analysis of the area occupied by collagen fibers (fibrosis area)

As shown in (Table 5), image analysis of Masson trichrome stained liver sections revealed that the area occupied by collagen fibers in the –ve control

group was 1.34±.23%. In the CCL_4 treated group, there was a highly significant increase (9.62±1.61%) as compared to all other groups (Fig. 4).

In the animals of trigonelline group, the area occupied by collagen fibers was 1.75±.43 %. This area was highly significant decreased as compared with that of the animals that received CCl_4 alone and silymarin group. However, there was no significant difference as compared with the –ve control value (Table 5) The area occupied by collagen fibers was increased in all animals of silymarin group (4.07±1.09%) as compared to the –ve control and trigonelline treated groups but was highly significant decreased as compared to the CCl4 group (Table 5).

The grade of liver fibrosis:

According to Staging criterion for experimental hepatic fibrosis in rats, Median (range) of liver fibrosis was 0(0-1.00) (Table 6). In the animals that received CCl_4 alone, the grade of liver fibrosis showed a highly significant increase (4.00(3.00-4.00)) as compared to the other groups (Fig. 5).

The animals of trigonelline group showed a highly significant decrease (Median (range) of the grade of liver fibrosis was 1.00(1.00-2.00) as compared to that of the animals CCl_4 group (Table 6). However, the decrease in the grade of liver fibrosis was not significant as compared to the –ve control and silymarin groups (Fig. 5).

The animals of silymarin group showed a highly significant decrease (Median (range) of the grade of liver fibrosis was 2.00 (1.00-2.00) as compared to that of CCl_4 and the –ve control groups (Table 6). However, the decrease in the grade of liver fibrosis was not significant as compared with trigonelline treated group (Fig. 5).

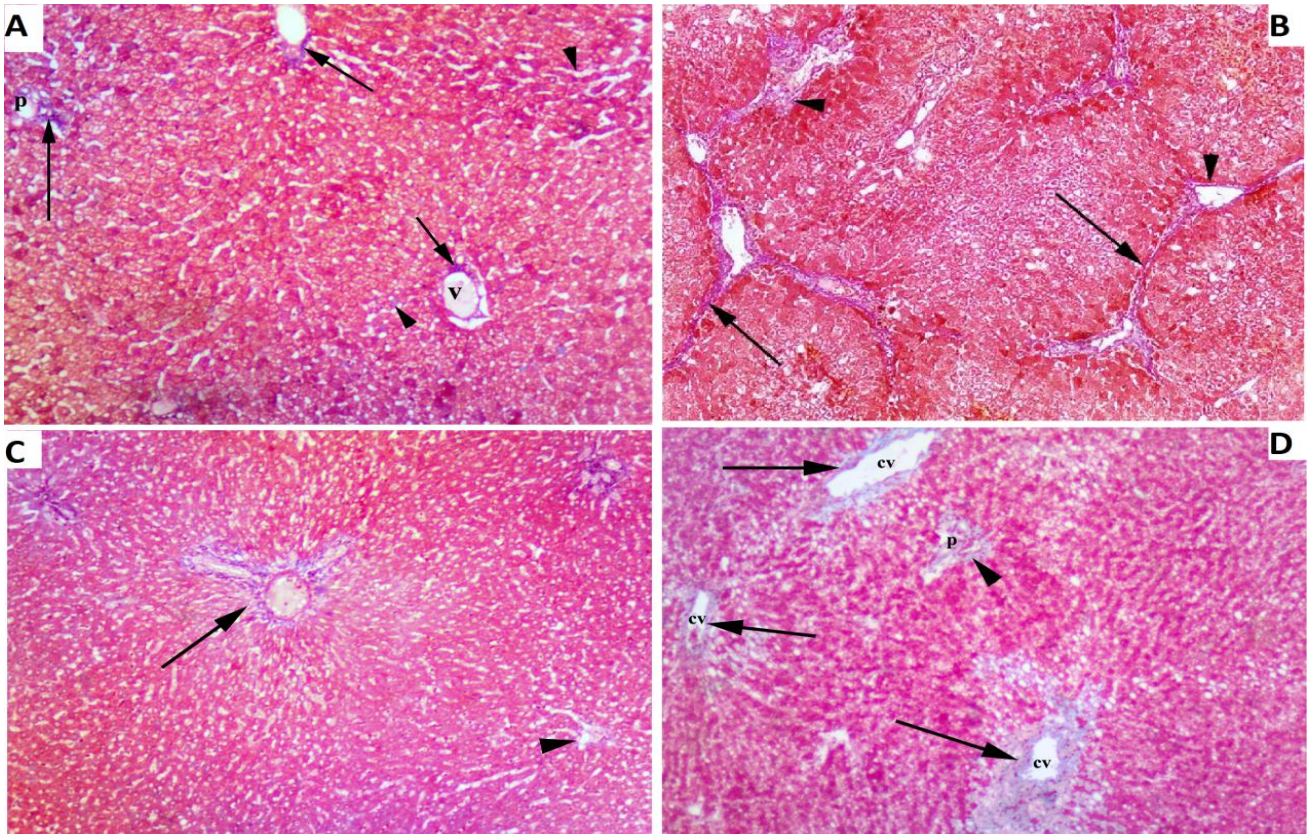

Figure 2. A: A photomicrograph of a liver section of control albino rat showing few collagen fibers (arrow) around central venule (V) and in the portal tract (p). (Masson's trichrome; ×40). B: A photomicrograph of a liver section of CCL_4 treated rat for 8 weeks showing a marked increase in the amount of collagen fibers

around the central venule (cv) (arrow head). Thick well-developed septa could be seen throughout the section. These septa are extending between the central veins (arrow). (Masson's trichrome ×40). C: A photomicrograph of liver section of rat receive trigonelline simultaneously with carbon tetra chloride for 8 weeks showing collagen fibers within normal amount in the portal tract (p) (arrow) and around the central venule (cv) (arrow head) in compare with CCL_4 treated group. Preservation of liver architecture without pseudolobular formation. (Masson's trichrome ×40) D: A photomicrograph of liver section of rat receives silymarin simultaneously with carbon tetra chloride for 8 weeks showing collagen fibers in the portal tract (p) (arrowhead). Extensive necrosis and increased collagen fibers around the central vein (arrow) in comparison with trigonelline treated group. Collagen fibers are seen around portal tract (p). (Masson's trichrome;×40).

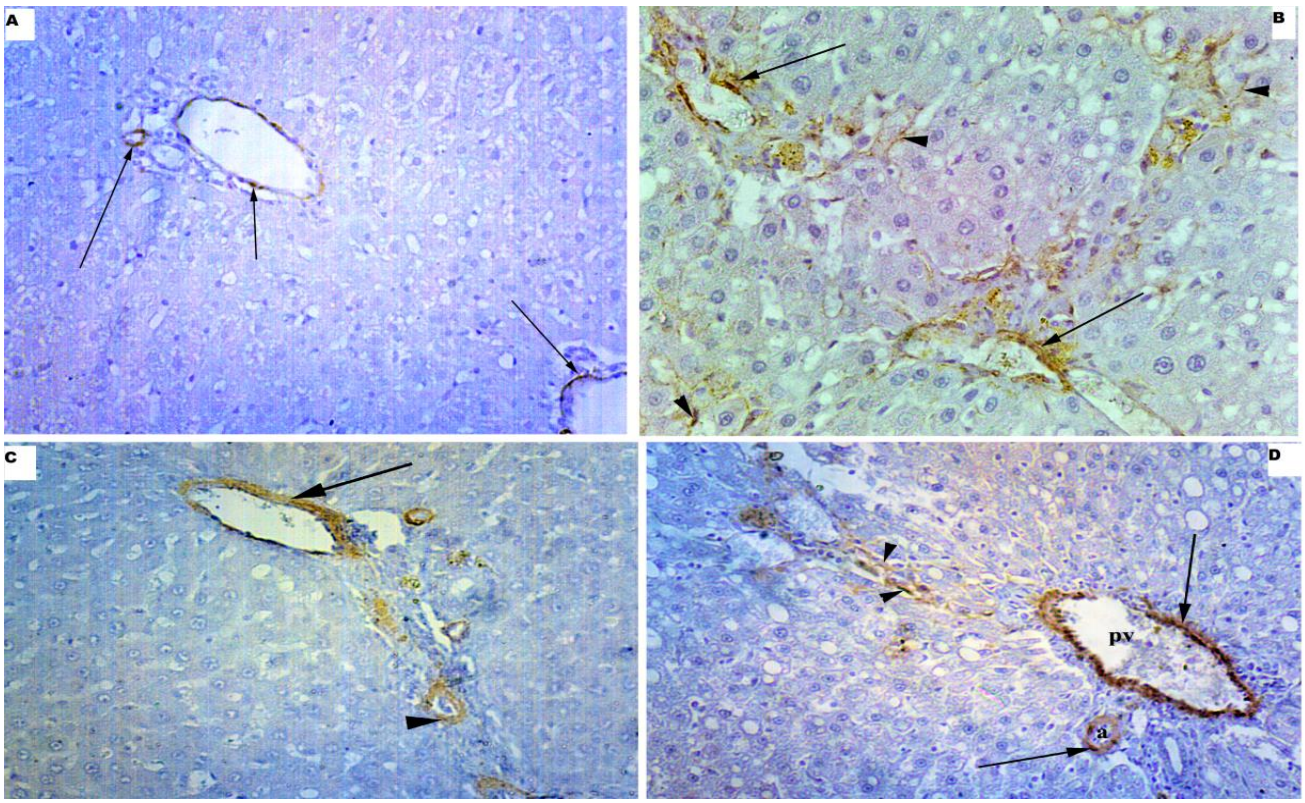

Figure 3. A: A photo micrograph of liver section of control albino rat stained with an immune stain by alph smooth muscle actin (showing α-SMA positive stain (brown) in the muscle layer of portal venule and hepatic arteriole within the portal tract. (α-SMA stain;×100). B: A photomicrograph of liver section of CCL_4 group stained with an immune stain by alph smooth muscle actin (α-SMA) 48 showing α-SMA positive cells are distributing in the wall of portal venule and hepatic arteriole (black arrow). Alpha-SMA positive cells were distributed in perisinusoidal spaces (arrow head). (α-SMA stain;×100). C: A photomicrograph of liver section section of rat receive trigonelline group showing α-SMA positive cells appear in the wall of portal venule and hepatic arteriole (arrow). (α-SMA stain;×100). D: A photomicrograph of liver section section of rat receive silymarin group showing α-SMA positive stain (brown) in the muscle layer of the portal venule, hepatic arteriole & central venule (arrow) in the perisinusoidal space (arrow head). (α-SMA stain;×100).

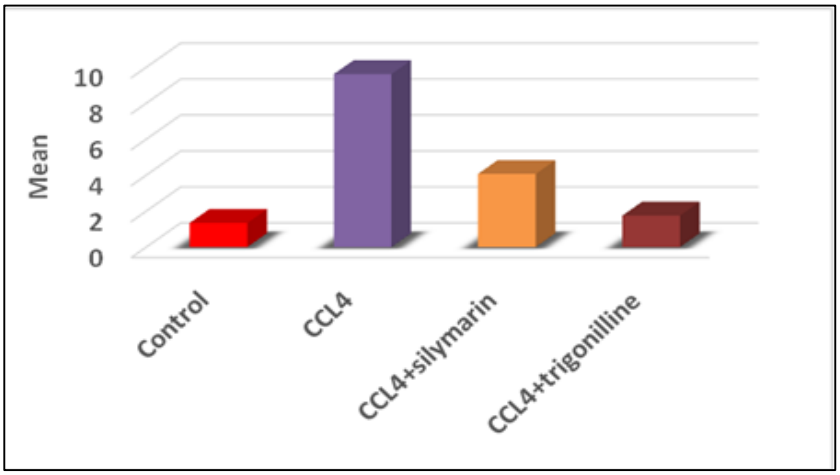

Figure 4. The area occupied by collagen fibers in all group (control, CCL_4 treated group, silymarin+CCL_4 treated group & trigonelline treated group+ CCL_4).

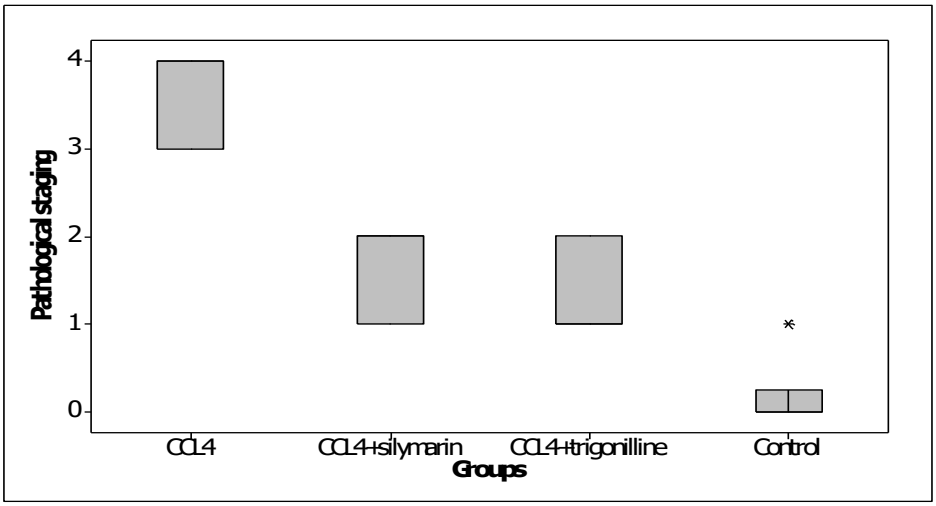

Figure 5. Pathological staging in all groups (control, CCL_4 treated group, silymarin+ CCL_4 treated group & trigonelline treated group+ CCL_4).

Table. 6: Comparisons between different groups as regard to Pathological staging.

	Control	CCL_4	CCL_4+silymarin	CCL_4+trigonelline	P
	Median(range)	Median(range)	Median(range)	Median(range)	
Pathological staging	0(0-1.00)	4.00(3.00-4.00)	2.00(1.00-2.00)	1.00(1.00-2.00)	<0.001
P1		<0.001	.002	.009	
P2			<0.001	<0.001	

P3				.8	

Table.7: Correlation between staging scores, image analysis, APRI ratio & AST/ ALT ratio.

			Pathological staging	Image analysis	AST/ALT
Spearman's rho	APRI	r	.671**	.743**	.219
		P	.000	.000	.304
		N	24	24	24
	Pathological staging	r		.905**	.007
		P		.000	.973
		N		24	24
	Image analysis	r			-.100-
		P			.642
		N			24

r: Spearman's rho correlation coefficient P: Probability**: High significance

DISCUSSION

Liver fibrosis is a common sequel of many hepatic diseases. Even with the treatment of the primary cause, the reversibility of the already formed cirrhosis can occur only if it is still in the early stages. It may take years for complications to occur. This is why liver fibrosis or cirrhosis should be treated separately apart from the primary cause and even it is better to be prevented from the start (Wai et al., 2003).

Trials aim to select materials, which are economic and safe with applying the principle of a combined drug therapy to make use of the possible synergistic effect and to reduce the possible toxicity (Badria et al., 2001; Marwan et al., 2003; Badria & Attia, 2007; Badria et al., 2003; Badria et al., 2005).

It has been demonstrated that silymarin improves hepatic fibrosis *in vivo* in rats subjected to complete occlusion of the biliary duct and when administered at a dosage of 50 mg/kg/day for 6 weeks, was able to reduce fibrosis by 30 to 35% as compared with controls (Kanter et al., 2005; Mourelle et al., 1989; Muriel & Mourelle, 1990; Favari & Pérez-Alvarez, 1996). In the current study, we used silymarin (50 mg/kg per day) as a +ve control to compare its hepatoprotective effect of trigonelline.

Several well-established chemical substances were found to induce experimental liver fibrogenesis. One of them is carbon tetrachloride (CCl_4) either in mice or rats (Domenicali et al., 2009). Liver changes resulting from CCl_4 toxicity closely mimic that of viral hepatitis B and C in humans. This is why this model is currently highly recommended in this field of study (Constandinou et al., 2005).

In the present study, chronic administrations of CCl_4 for 8 weeks distortion in hepatic architecture with the formation of fibrous septa incompletely divide the hepatic tissue into many pseudolobules. These results are in agreement with *Luo et al. (2004)*, who reported that the treatment of rats with CCl_4 for 8 weeks induced liver fibrosis, in which liver exhibited a marked increase in the content of extracellular matrix (ECM) and displayed bundles of collagen surrounding the lobules distorting tissue architecture (Luo et al., 2004). However, *Lin et al. (1999), Germano et al. (2001)* and *Al Gamdi, (2003)* reported that the treatment of rats with CCl_4 induced hepatic lesions including fatty changes, ballooning degeneration, cell necrosis, and centrilobular inflammatory infiltrate (Lin et al., 1999; Germano et al., 2001; Al-Ghamdi, 2003). Turkdogan et al. (2003) stated that in CCl_4

treated group, necrosis and hydropic degeneration were marked in periacinar regions associated with fibrosis. The knowledge of the stages of liver fibrosis is essential for prognosis and decisions on antiviral treatment (Dienstag, 2002; Türkdoğan et al., 2003;). The current study respected the fact that the concept of fibrosis staging criteria for humans should not be applied blindly to experimental fibrosis in animals. In rodents, it starts around the central venule area followed by central to central bridging fibrosis (Yoshiji et al., 2000). It was found that there was a significant difference between –ve control and CCl_4, silymarin & trigonelline groups. There was also a significant difference between CCl_4 and silymarin & trigonelline groups, but no significant difference between silymarin and trigonelline treated groups was found.

Image analysis measures the area of collagen and the total area of tissue, producing a fibrosis ratio to represent a relative amount of collagen (Standish et al., 2006). In the current study, there was a significant difference between -ve control and CCL_4 & silymarin groups regarding the measured surface area of collagen, so trigonelline was more superior in preventing liver fibrosis than silymarin regarding the measured surface area of collagen. Such simplified morphometric measurement, however, is unable to reflect changes in the histological structure such as architecture distortion and bridging fibrosis, which are hallmarks of fibrosis and ideally should be directly described, so histologic examination of the liver was done (Wright at al., 2003). In the present study, oral administration of CCl_4 treated rats with trigonelline showed improvement in the pathological changes in comparison to the CCl_4 and silymarin groups in the form of diminution of fibrosis, preserved liver architecture without pseudolobular formation with little inflammatory cell infiltrate and fewer number of micro steatotic vesicle, regenerating hepatocytes and increase in the number of binucleated hepatocytes.

The expression of α-SMA in the liver tissues is considered as a reliable marker of activated hepatic stellate cells, which precede fibrous tissue deposition (Nouchi et al., 1991; Carpino et al., 2005; Parsons et al., 2004). In both groups which received Trigonelline and Silymarin simultaneously with CCl_4, α-SMA positive cells were less than that of the group treated with CCl_4 only. Trigonelline was superior to Silymarin in amelioration of liver fibrosis indicated by the lower level of α-SMA expression. The results of this work were in agreement with *Iredale et al. (1998)*. It was suggested that this ameliorative effect of both trigonelline and silymarin is through preventing fibrogenesis and proliferation of hepatic stellate cells.

In this current study, both silymarin and trigonelline groups showed a significant decrease in comparison with the CCL_4 group regarding the levels of both ALT and AST. With regard to AST serum level, trigonelline was significantly superior to silymarin. However, there was no significant difference between the two groups regarding ALT. This indicates that trigonelline is superior to silymarin as hepatoprotective therapy. The relation between platelets and liver diseases is a growing area of investigation (Witters et al., 2008; Badria et al., 2009; Gebo et al., 2002). In this study, both silymarin and trigonelline showed significant differences in relation to CCL_4 treated group regarding platelet count. However, there was no statistically significant difference between the two groups. This indicates the therapeutic effect of trigonelline on liver fibrosis. Wai et al. (2003) devised a novel index, called the AST to platelet ratio index (APRI): APRI = AST level (/ULN)/ Platelet counts (109/L) ×100.45 APRI is currently considered accurate in predicting both significant fibrosis and cirrhosis (Zhao et al., 2008; Viana et al., 2009).

In this study, it was found that there was a significant difference between CCL_4 and –ve control, silymarin and trigonelline groups. However, there wasn't significant difference between trigonelline and silymarin groups, indicating supremacy of trigonelline over silymarin as a hepatoprotective agent.

Recommendations and future perspectives

In this study, two natural drugs (silymarin and trigonelline) were used. Trigonelline showed good results, superior to silymarin due to its good oral bioavailability and less toxic effects. Although silymarin is one of the most commonly used hepatoprotective agents, yet it doesn't prove actually good hepatoprotective effect in the hepatic patient, which may be attributed to its poor solubility. Therefore, it is recommended to use silymarin in early stages of liver fibrosis and it should be in conjugation with substances that increase its oral bioavailability. Regarding trigonelline, we recommend researchers to pay attention to its power of preventing extracellular fibers deposition. Wide range of experiments is required in order to evaluate this preventive power on a large scale, longer periods and different doses and monitor any possible side effects to ensure the safety of this natural product.

The trial to use trigonelline as antifibrotic therapy in advanced fibrosis and cirrhosis stages and its possible synergetic effect with other antifibrotics in prevention and treatment of extracellular fibers deposition is still to be evaluated. Although none of the currently available noninvasive markers of fibrosis is an ideal test to accurately differentiate between disease stages, the combination of serum markers and imaging appear to have good predictive values in excluding patients with cirrhosis. The most reliable fibrotic index is APRI that is recommended for use during further research on experimental liver fibrosis.

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Conflict of interest statement

The author has declared that no competing or conflict of interests exists. The funders had no role in study design, writing of the manuscript and decision to publish.

Authors' contributions

Farid A. Badria: Suggested the protocol, work design, and submission of the manuscript
Marwa M. Hassan: All laboratory work and writing the draft of the manuscript
Moustafa M. Abd-Al Moneim: Supervising Dr. Marwa during all over the research work

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