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Evaluation of hepatoprotective effect of Nebivolol and sodium copper Chlorophyllin on CCL4-induced hepatotoxicity in mice

E.S. SAID[1,2], A.H. MOHAMMED[3], H.M. ALI[2,4], A.Y. BABIKER[3], R. ALNUGHAYMISHI[5], N.Z. ALTHAQEEL[5], A.S. AHMED[2,6]

[1]Department of Clinical Pharmacology, Faculty of Medicine, Fayoum University, Fayoum, Egypt
[2]Department of Pharmacology and Toxicology, College of Pharmacy, Qassim University, Qassim, Saudi Arabia
[3]Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University, Qassim, Saudi Arabia
[4]Department of Biochemistry, Faculty of Medicine, Al-Azhar University, Assiut, Egypt
[5]College of Pharmacy, Qassim University, Qassim, Saudi Arabia
[6]Hormones Department, Medicine and Clinical Studies Research Institute, National Research Centre, Giza, Egypt

Abstract. – OBJECTIVE: In this study, the protective effect of sodium copper chlorophyllin and nebivolol was evaluated in a mice model of CCL4 induced hepatotoxicity. Silymarin was used as a traditional hepatoprotective drug.

MATERIALS AND METHODS: Thirty (30) mice were used as they were divided into five groups: the first group was the control group which received distilled water + olive oil, the second group which received 1.5 ml/kg of CCl4 diluted in olive oil three times a week, the third group which received CCl4 + Silymarin 50 mg/kg/day, the fourth group which received CCl4 + nebivolol 4 mg/kg/day, and the fifth group which received 1.5 ml/kg of CCl4+ Cu-chlorophyllin 50 mg/kg/day. The drugs were given by intraperitoneal route for 5 weeks. The detection, quantification of CCl4 induced hepatotoxicity and possible protective effect of either silymarin, nebivolol, or sodium copper chlorophyllin were assessed using biochemical analysis of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, lipid profile, an assay of oxidants and antioxidants, assay of interleukin 6 (IL6) and tumor necrosis factor-alpha (TNF-α), and histopathological examination.

RESULTS: The administration of carbon tetrachloride (CCl4) produced pronounced liver impairment. It significantly increased ALT, AST, ALP, malondialdehyde, and serum nitric oxide levels compared to normal control group besides a decrease in total protein, serum catalase, tissue SOD, and GSH levels. IL-6 and TNF-α levels were significantly higher while total cholesterol was significantly lower in mice receiving CCL4 compared to the normal control group. CCL4 induced severe hyperemia and congestion inside the portal area with leukocytic infiltration, hepatic degeneration, and bridge fibrosis.

CONCLUSIONS: Co-administration of either silymarin, nebivolol, or sodium copper chlorophyllin with CCl4 was able to ameliorate up to almost contradict CCl4 induced hepatic injury through their anti-inflammatory and antioxidant activities.

Key Words:
Carbon tetrachloride, Hepatotoxicity, Silymarin, Nebivolol, Sodium copper chlorophyllin.

Abbreviations
CCl4: Carbon Tetrachloride, Cu-Chl-Na: Sodium Copper Chlorophyllin, GSH: Glutathione, HDL: High Density Lipoprotein, HSCs: Hepatic Stellate Cells, IP: Intraperitoneal, LDL: Low Density Lipoprotein, MDA: Malondialdehyde, TG: Triglyceride.

Introduction

Liver is the main target tissue tangled in responding to different sorts of oxidative stresses[1]. Hepatotoxicity is a consequence of overwhelming mediators such as chemical agents used in laboratories and industries (i.e., carbon tetrachloride (CCl4), thioacetamide and paracetamol) or metabolic reactions. This is usually due to excessive free radical formation[2,3]. Risk factors for hepatotoxicity include idiosyncrasy, alcohol drinking, smoking, age, gender and drug addiction, and en-

Corresponding Author: Eman Sayed Said, MD; e-mail: Emanabdelmohti@gmail.com; e.abdelmoti@qu.edu.eg; ess02@fayoum.edu.eg

vironmental and genetic factors[4]. Hepatotoxicity leads to serious outcomes ranging from metabolic disorders to even death[5].

CCI4 is an essential commercial chemical. It is used as a solvent to create other chemicals, a dry-cleaning agent, and in laboratories for induction of hepatotoxicity model. It causes injury to liver, kidney, central nervous system, gastrointestinal system and the lungs[6,7]. CCl4 is used as a model of hepatotoxicity through numerous mechanisms[8].

Management of liver diseases is still a great challenge to the modern medicine. Only a few hepatoprotective drugs from natural sources are available and there is lack of effective allopathic medication available for treatment of liver disorders[9]. More attention has been paid to the preventive effects of natural antioxidants against drug-induced toxicities. Plant-based hepatoprotective drugs with their safety, efficacy, and cost effectiveness caused a sharp rise in the use of medicinal plants in latest years[10].

Silymarin is a traditional antioxidant herb constituting of a mixture of four flavonolignan isomers, namely, silybin, isosilybin, silydianin, and silychristin[11]. It is extracted from seeds of the milk thistle, and it is used in the treatment of hepatitis, liver cirrhosis, and chemical or drug produced liver injury. It has antineoplastic action. It is an antioxidant, antidiabetic, anti-inflammatory, antifibrotic, and cytoprotective agent[12]. Its antioxidant activity is at least ten times more powerful than that of vitamin E guarding the liver against toxins[13,14].

Nebivolol is a third generation beta-1 blocker, used as an antihypertensive drug. It is a racemic mixture of a D- and an L-isomer. The L-isomer of nebivolol causes the stimulation of endothelial nitric oxide synthase (eNOS) leading to NO production that enhances endothelial vasodilation and liver cell regeneration[15,16]. Beta-blockers decrease portal blood flow and portal hypertension in cirrhotic patients so effectively to reduce the incidence of variceal hemorrhage and related mortality[17].

Chlorophyllin, derived from chlorophyll, has been widely used as a green pigment in the food industry. Sodium Copper Chlorophyllin (Cu-Chl-Na) is used in cosmetics, personal care products, a dentifrice colorant and deodorant agent. Cu-Chl-Na has a higher antioxidant activity than that of natural chlorophylls due to the presence of the chelated metal in the porphyrin ring and of Mg-free derivatives[18].

Chlorophyllin is 410-fold more potent as a phase$_2$ enzyme inducer than chlorophyll. It has extra detoxification properties since it is much more water-soluble than chlorophyll. Cu-Chl-Na acts as a hydrogen donor to break the chain reaction due to the porphyrin in its chemical structure. It has antidiabetic and anti-dyslipidemic activity, so it prevents cardiovascular disorder. It reduces fever, increases the level of hemoglobin and ferritin, increases breast milk production, and enhances non-specific immune responses[18,19].

This study aimed to clarify and compare the modulating effects of sodium copper chlorophyllin (natural product) and nebivolol (synthetic drug) on hepatotoxicity induced by carbon tetrachloride in mice. Silymarin was used as a traditional hepato-protective drug.

Materials and Methods

Ethical Statement

All producers conducted along with ethical strategies of the medical ethics committee of Qassim University. It followed the international ethics and regulations for animal research in laboratory applications[20]. The research ethics committee approved our animal protocol with approval number (19-14-08). Animals were treated according to the guide for care and use of laboratory animal by national research council[21].

Drugs and Chemicals

Carbon tetrachloride was provided from Merck Company, Germany (BDH Chemicals, Poole, UK). Silymarin was obtained from MADAUS GmbH, 51101 Koin Company, Germany. Nebivolol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium Copper Chlorophyllin was obtained from Unicity International, Inc, USA, and was dissolved in distilled water.

Animals and Housing

Thirty (30) male mice were used in this experiment, their weights ranged from 30 to 35 gm. They were kept in the animal house of the College of Pharmacy, Qassim University. They were separated, based on their groups, in cages containing bedding material as husk at room temperature. Mice fed with usual laboratory diet and free access to water, 12-h daily light-dark cycles.

Experimental Design

Thirty mice were randomly divided into 5 groups (6 mice each)

Group 1: Control group; mice received intraperitoneally 0.5 ml distilled water and 0.5 ml olive oil for 5 weeks.

Group 2: CCl4 group; mice received 1.5 ml/kg of CCl4 diluted in olive oil (1:7) by intraperitoneal route three times a week for 5 weeks[22].

Group 3: CCl4+Sily group; mice received CCl4 diluted as formerly stated + Silymarin 50 mg/kg/day by intraperitoneal route daily for 5 weeks[23].

Group 4: CCl4+Nebi group; mice received CCl4 diluted as previously mentioned + Nebivolol 4 mg/kg by intraperitoneal daily[24].

Group 5: CCl4+ Cu-Chl-Na group; mice received CCl4 diluted as described before + Cu-chlorophyllin 50 mg/kg by intraperitoneal route daily for 5 weeks[25].

Sample Collection

At the end of the experiment, the mice were anesthetized using an I.P injection of thiopental sodium 50 mg/kg. Blood was extracted from the facial veins of all mice. Sera were separated from all groups and stored at −80°C until analyses. Before assay, samples were thawed at room temperature. Liver tissues, from sacrificed rats, were fixed in 10% neutral buffered formalin solutions for 48 hr.

Preparation of Liver Tissue Homogenate

Prior to dissection, the liver tissue was perfused with phosphate buffer saline, pH 7.4 contains 0.16 mg/ml heparin to remove any red blood cells and clots. The tissue was homogenized in 10 ml cold 50 mM potassium phosphate buffer, pH 7.5 per gram tissue and centrifuged at 4000 rpm for 15 min. The supernatant was removed and stored at −80°C until assay.

Assay of Liver Function Tests

The kinetic method was used to analyze serum level of alanine transferase (ALT), aspartate transferase (AST), and alkaline phosphatase (ALP), while the total protein was assayed by colorimetric method. All kits were purchased from the Crescent Diagnostics Test (KSA) according to the method of Thefeld et al[26] for ALT and AST, whereas ALP level was established using the Belfield and Goldberg's method[27] and total protein was assessed according to the Weichselbaum's method[28].

Assay of Lipid Profile

Crescent Diagnostics Test (KSA) kits were used to assay the serum total cholesterol and triglycerides (TG) levels according to the colorimetric methods suggested by Trinder et al[29,30], respectively, whereas high density lipoprotein (HDL) level was measured by the colorimetric method of Ramadan et al[31]. The concentration of low density lipoprotein (LDL) was calculated following Friedewald et al[32] equation:

LDL- C (mg/dl) = Total cholesterol – HDL-C – TG/5

Assay of Oxidants and Antioxidants

Lipid peroxide, malondialdehyde (MDA) was assayed in serum by the colorimetric method as a result of reaction between MDA and thiobarbituric acid in acidic medium at the temperature of 95°C for 30 min. This produced thiobarbituric acid pink products, which were measured at 534 nm[33]. Nitric oxide was assayed in serum by colorimetric determination of nitrite which represented one of the final products of nitric oxide *in vivo* in addition to nitrate. The addition of Griess reagents converts nitrite into a deep purple azo compound that measured at 540 nm[34]. The catalase enzymatic activity was assayed in serum by the colorimetric method where the catalase reacts with a known amount of hydrogen peroxide in the presence of peroxidase. The remaining hydrogen peroxide reacted with 3,5 dichloro-2-hydroxybenzene sulfonic acid and 4-amino phenazone to form a chromophore with a color that is measured at 520 nm[35]. SOD activity in liver tissue was assayed by colorimetric method, which relied on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitobluetetazolium dye. The increase of the absorbance was measured at 560 nm for 5 min for control and tissue samples[36]. The reduced glutathione was assayed in liver tissue samples by colorimetric method based on the reduction of dithiobis- 2 ntrobenzoin acid with reduced glutathione to produce a yellow color that was measured at 405 nm[37]. All oxidants and antioxidants reagents were provided by the Biodiagnotic Company, Egypt.

Assay of Interleukin 6 (IL6) and Tumor Necrosis Factor alpha (TNF-α)

The measurement of IL6 and TNFa was assayed in both serum and liver tissues homogenate samples by enzyme linked immunosorbent assay (ELISA) kits according to instructions of the manufacturer. The reagents were provided by Cloud Clone Corp Company, USA. The microplates were measured at 450 nm filter by microplate reader.

Liver Sectioning and Staining

Standard techniques were processed for paraffin wax sectioning by dehydration in grading of alcohol, cleared by xylene and embedded in par-

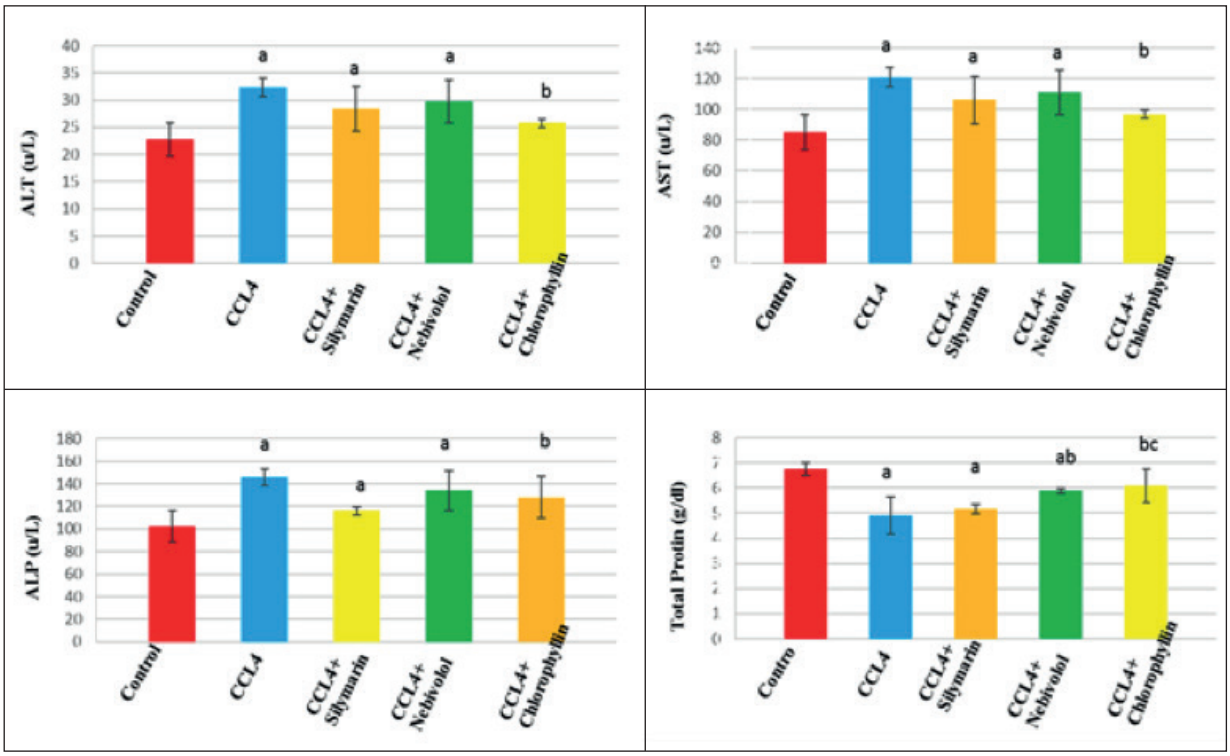

Figure 1. Effect of silymarin, nebivolol, and chlorophyllin on ALT, AST, ALP, and total protein level. Values are expressed as mean ± SD of 6 mice for each group. [a]Significant difference from the control group. [b]Significant difference from the CCL4 group. [c]Significant difference from the CCL4 + silymarin group. *p*-values ≤ 0.05 were considered significant.

affin wax blocks, then sectioned by rotary microtome (Leica) 5 µm thickness, stained with Mayer's hematoxylin and eosin (H&E), and examined under a light microscope. The liver sections were graded numerically to assess the degree of histopathological topographies of hepatic injury. The number of foci of inflammation was counted in 10 randomized non-overlapping fields for each section. The assessment of hepatic inflammation was performed by a scoring system, as grade 0: No foci; grade 1: <2 foci per field; grade 2: 2-4 foci per field; grade 3: >4 foci per field. Masson's trichrome stain was used for assessment of liver collagen fibres. The extent of hepatic fibrosis was scored as grade 0: no fibrosis; grade 1: Perisinusoidal or periportal; grade 2: Both perisinusoidal and portal/periportal; grade 3: Bridging fibrosis; grade 4: Cirrhosis[38].

Statistical Analysis

Statistical analysis was performed using R studio v 3.6.1. Descriptive statistics were performed using mean ± standard deviation (SD). One-way ANOVA was used to test the null hypothesis of equality of means between the five groups. Post-hoc pairwise comparisons were performed using unpaired *t*-test with Tukey correction. Bar plots were used to visualize the distribution (mean ± standard deviation) of the dependent variables across the five groups. Pearson's correlation was used to assess the association between oxidative stress biomarkers and inflammatory biomarkers. Hypothesis testing was performed at 5% level of significance.

Results

Impact of Silymarin, Nebivolol, and Chlorophyllin on Liver Function Tests

The statistical analysis showed a significant difference in ALT, AST, ALP, and total protein between the five groups ($p < 0.001$ for all parameters). ALT, AST, and ALP were significantly higher in mice that received CCL4, CCL4 + silymarin, and CCL4 + nebivolol compared to the control group. There was insignificant difference between mice that received CCL4 + chlorophyllin and the control group ($p > 0.05$). However, the levels of ALT, AST, and ALP were significantly lower in mice that received CCL4 + chlorophyllin than mice that received only CCL4 (Figure 1).

Table I. Impact of silymarin, nebivolol, and chlorophyllin on lipid profile (n=6).

	Control	CCL4	CCL4+ silymarin	CCL4+ nebivolol	CCL4+ chlorophyllin	*p*-value
Total Cholesterol (mg/dl)	138 ± 0.88	96.2 ± 4.16[a]	130 ± 8.43[b]	126 ± 6.79[b]	155 ± 19.1[abcd]	<0.001
TG (mg/dl)	164 ± 38.1	130 ± 2.52	141 ± 18.0	138 ± 15.3	164 ± 30.8	0.062
HDL-C (mg/dl)	67.2 ± 10.5	57.6 ± 7.01	62.4 ± 8.76	60.8 ± 10.5	70.4 ± 11.4	0.196
VLDL-C (mg/dl)	32.8 ± 7.62	25.9 ± 0.50	28.2 ± 3.61	27.5 ± 3.07	32.8 ± 6.16	0.062
LDL-C (mg/dl)	38.4 ± 2.02	12.7 ± 2.34[a]	39.5 ± 3.94[b]	37.9 ± 6.79[b]	51.4 ± 2.36[abcd]	<0.001

Data are summarized using mean ± SD

[a] Significant difference from the control group. [b] Significant difference from the CCL4 group. [c] Significant difference from the CCL4 + silymarin group. [d] Significant difference from the CCL4 + nebivolol group. *P* values ≤ 0.05 were considered significant.

The total protein level was significantly lower in mice that received CCL4, CCL4 + silymarin, and CCL4+ nebivolol compared to the control group. There was a significant increase of protein in mice that received CCL4 + nebivolol and CCL4 + chlorophyllin compared to mice that received only CCL4. The average total protein was significantly lower in mice that received CCL4 + silymarin than mice that received CCL4 + chlorophyllin. There was insignificant difference between mice that received CCL4+ chlorophyllin and the control group (Figure 1).

Impact of Silymarin, Nebivolol, and Chlorophyllin on Lipid Profile

There was a statistically significant difference in the average total cholesterol and LDL-C between groups ($p < 0.001$). No significant difference was observed in TG, HDL-C, and VLDL-C levels between groups ($p > 0.05$). The current data showed that the total cholesterol was significantly lower in the mice that received CCL4 than those in the control group. There was a significant increase of total cholesterol in the mice that received any of the three therapies compared to CCL4 group. Additionally, total cholesterol was higher in the mice that received CCL4 + chlorophyllin than mice that received CCL4 + silymarin or the mice that received CCL4 + nebivolol. Similar results were observed comparing the LDL-C levels (Table I).

Impact of Silymarin, Nebivolol, and Chlorophyllin on Oxidative Stress Biomarkers

Statistical analysis revealed a significant difference in the distribution of all oxidative stress markers between groups ($p < 0.001$). The serum catalase was significantly lower in the mice that received CCL4 compared to the control group. On the other hand, catalase was significantly elevated in all treated groups compared to the CCL4 group (Figure 2).

Serum MDA was significantly higher in the mice that received CCL4 than in the control group. However, there was a significant decrease of MDA in the mice that received CCL4 + chlorophyllin compared to the control group. MDA was lower in the latter three groups compared to the mice that received CCL4 only (Figure 2).

Regarding tissue SOD, it was lower in all groups than in the control group. SOD was higher in the latter three groups than the mice that received CCL4. No significant difference was observed between the latter three groups (Figure 2).

Adding nebivolol or chlorophyllin resulted in superior performance concerning the level of tissue reduced glutathione (GSH). GSH level was higher in the mice that received CCL4 + chlorophyllin or CCL4 + nebivolol than all other groups, and the former even showed a better performance compared to the latter. There was a significant decrease of GSH in the mice that received CCL4 and CCL4 + silymarin compared to the control group (Figure 2).

Serum nitric oxide (nitrite) level was not significantly different between mice that received CCL4 + silymarin or CCL4 + chlorophyllin and the control group. Nitric oxide was significantly higher in the mice that received CCL4 and CCL4 + nebivolol than the control group. Furthermore, there was a significant decrease of nitrite in all the treated groups compared to CCL4 group. There was a statistically significant increase of nitrite in CCL4 + nebivolol group compared to both CCL4 + silymarin and CCL4 + chlorophyllin groups (Figure 2).

Impact of Silymarin, Nebivolol, and Chlorophyllin on Inflammatory Biomarkers

The results showed that all inflammatory parameters were significantly different between

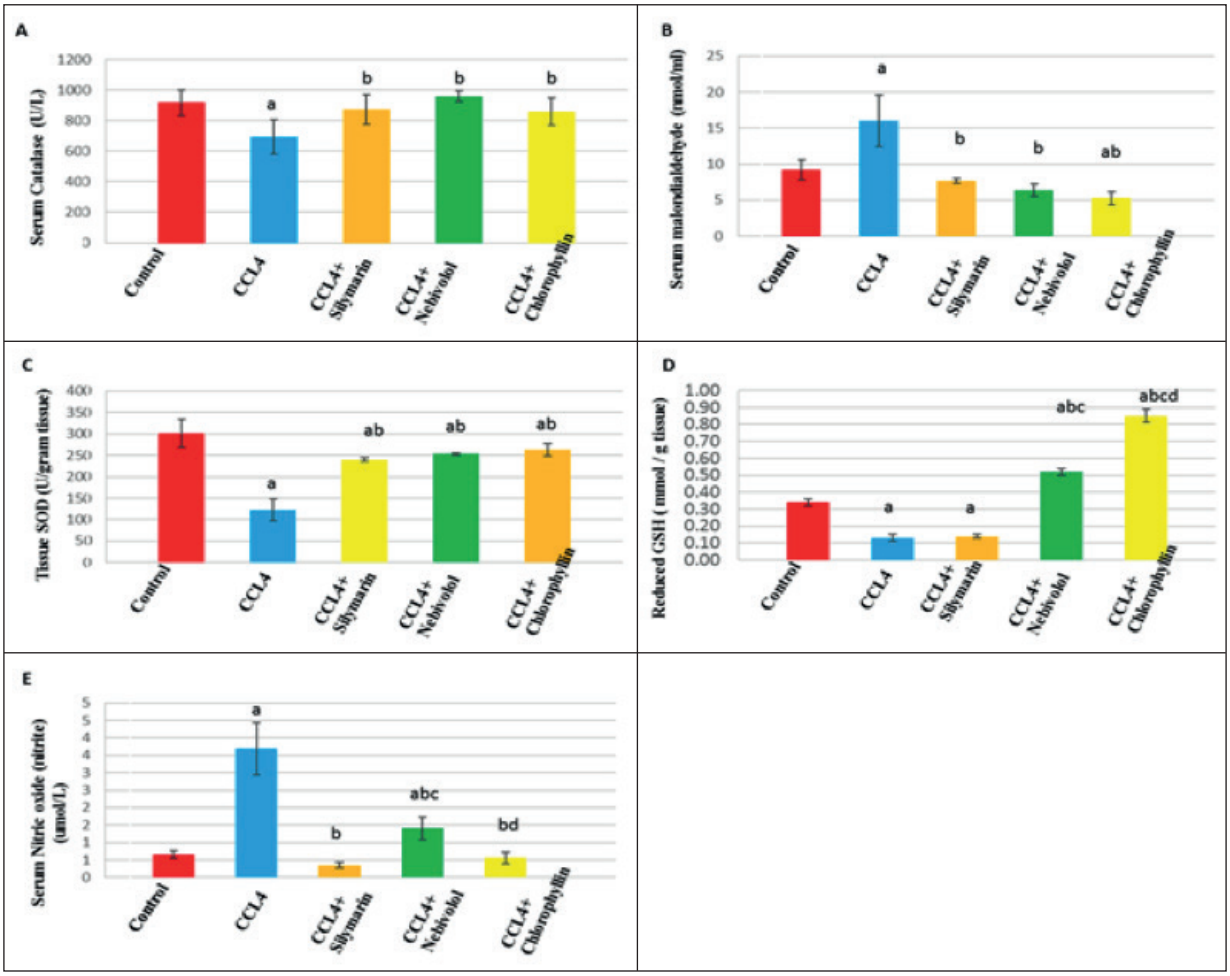

Figure 2. Effect of silymarin, nebivolol, and chlorophyllin on serum catalase, malondialdehyde, tissue SOD, GSH, and serum nitric oxide (nitrite) levels. Values are expressed as mean ± SD of 6 mice for each group. SOD: superoxide dismutase; GSH: reduced glutathione. [a]Significant difference from the control group. [b]Significant difference from the CCL4 group. [c]Significant difference from the CCL4 + silymarin group. [d]Significant difference from the CCL4 + nebivolol group. *p*-values ≤ 0.05 were considered significant.

groups ($p < 0.001$). Serum as well as tissue IL-6 and TNF-α levels were significantly higher in the mice that received CCL4 than those in the control group. All the three treated groups with silymarin or nebivolol or chlorophyllin had significantly lower tissue TNF-α, and serum IL-6 and TNF-α levels compared to the mice that received CCL4. The tissue IL-6 level was significantly lower in the CCL4 + chlorophyllin group compared to that in the CCL4, CCL4 + silymarin and CCL4 + nebivolol groups (Table II).

Correlation between Oxidative Stress Biomarkers and Inflammatory Biomarkers

The results showed a statistically significant positive correlation between serum MDA and all inflammatory biomarkers. The correlation was higher for serum IL-6 than tissue IL-6. No statistically significant association was observed between serum catalase and tissue IL-6, while a statistically significant negative correlation was observed between serum catalase and all remaining inflammatory biomarkers. A statistically significant negative correlation was observed between tissue GSH and all inflammatory biomarkers with the highest correlation observed between tissue IL-6 and tissue GSH. No significant correlation was observed between serum nitric oxide (nitrite) and tissue IL-6, while a strong positive correlation was observed between serum nitric oxide and all other inflammatory biomarkers (Table III).

Histological Examination

The main histological findings in hepatic section in this work were swelling, degeneration and necrosis of hepatocytes, infiltration with immune

Table II. Impact of silymarin, nebivolol, and chlorophyllin on inflammatory biomarkers (n=6).

	Control	CCL4	CCL4+ silymarin	CCL4+ nebivolol	CCL4+ chlorophyllin	*p*-value
Serum IL-6 (pg/ml)	18.9 ± 2.82	149 ± 1.55[a]	21.3 ± 4.60[b]	21.6 ± 4.07[b]	17.7 ± 2.06[b]	<0.001
Tissue IL-6 (pg/ g tissue)	518 ± 88.1	1782 ± 375[a]	1730 ± 174[a]	1715 ± 25.7[a]	588 ± 33.0[bcd]	<0.001
Serum TNF-α (pg/ml)	19.8 ± 2.67	79.7 ± 13.2[a]	18.7 ± 4.03[b]	19.0 ± 2.70[b]	18.5 ± 0.70[b]	<0.001
Tissue TNF-α (pg/g tissue)	1891 ± 140	7055 ± 44.8[a]	1803 ± 26.2[b]	1837 ± 33.5[b]	1914 ± 6.67[b]	<0.001

Data are summarized using mean ± SD.
IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha.
[a] Significant difference from the control group. [b] Significant difference from the CCL4 group. [c] Significant difference from the CCL4 + silymarin group. [d] Significant difference from the CCL4 + nebivolol group. *p*-values ≤ 0.05 were considered significant.

cells, proliferation of mesenchymal cells, along with healing and repair by collagen fiber (fibrosis). Fibrosis is a response to inflammation and degeneration. Evaluation of hepatic fibrosis was done via examination of H&E and Masson Trichrome stained hepatic sections (Figures 3 and 4). By scoring for inflammation and fibrosis, there was a significant difference between groups ($p < 0.001$) except for CCL4 + silymarin which was comparable to normal group. Both inflammation and fibrosis were significantly higher in mice that received CCL4 than those in the control group. All the three treated groups with silymarin or nebivolol or chlorophyllin had significantly lower scores compared to mice receiving CCL4. Inflammation and fibrosis grades were significantly lower in CCL4 + chlorophyllin group compared to that in CCL4, CCL4 + silymarin and CCL4 + nebivolol groups (Table IV).

Discussion

In the present study, CCL4 significantly increased ALT, AST, ALP, MDA, and serum nitric oxide levels compared to normal control group besides decrease in total protein, serum catalase, tissue SOD and GSH levels.

These results are comparable to numerous studies that demonstrated the effect of CCl4 in rats and stated that hepatic oxidative stress persuaded by CCl4 intoxication and the reactive intermediate products trichloromethyl radical (CCl3) generated during its bio-activation caused a decline in the activities of SOD and GSH[38-40].

Oxidative stress plays a prominent causative role in many diseases including liver damage. Oxidative stress is the state of imbalance between antioxidants and free radical levels. The radicals bind to nucleic acids, proteins, and lipids then impairs vital cellular processes such as lipid peroxidation, potentially resulting in necrosis, degeneration of fats, fibrosis, cirrhosis, cell death and cancer, relative to the dose and exposure time resulting in elevated levels of ALT, AST and ALP enzymes into the blood circulation[40-42].

The current data shows that the total cholesterol level was significantly lower in the mice that received CCL4 than those in the control group. These outcomes are in accordance with the findings of Ishikawa et al[43] observations. These findings could be explained by the toxic injury of CCl4 that diminishes the ability of liver cells to produce VLDL and HDL, which resulted in decrease of the triglycerides and cholesterol esters

Table III. Correlation between oxidative stress biomarkers and inflammatory biomarkers.

Oxidative stress biomarkers / *Inflammatory biomarkers*	Serum catalase (U/L)	Serum malondialdehyde (nmol/ml)	Tissue SOD (U/ gram tissue)	Tissue GSH (mmol/g tissue)	Serum nitric oxide (nitrite) (umol/L)
Serum IL-6 (pg/ml)	-0.684***	0.905***	-0.964***	-0.560**	0.918***
Tissue IL-6 (pg/g tissue)	N.S.	0.572**	-0.440*	-0.817***	N.S.
Serum TNF-α (pg/ml)	-0.775***	0.803***	-0.936***	-0.525**	0.917***
Tissue TNF-α (pg/g tissue)	-0.701***	0.905***	-0.959***	-0.528**	0.917***

Results are expressed as correlation coefficients (r). SOD: superoxide dismutase; GSH: reduced glutathione. N.S.: non-significant correlation.
* $p< 0.05$,** $p< 0.01$, *** $p< 0.001$

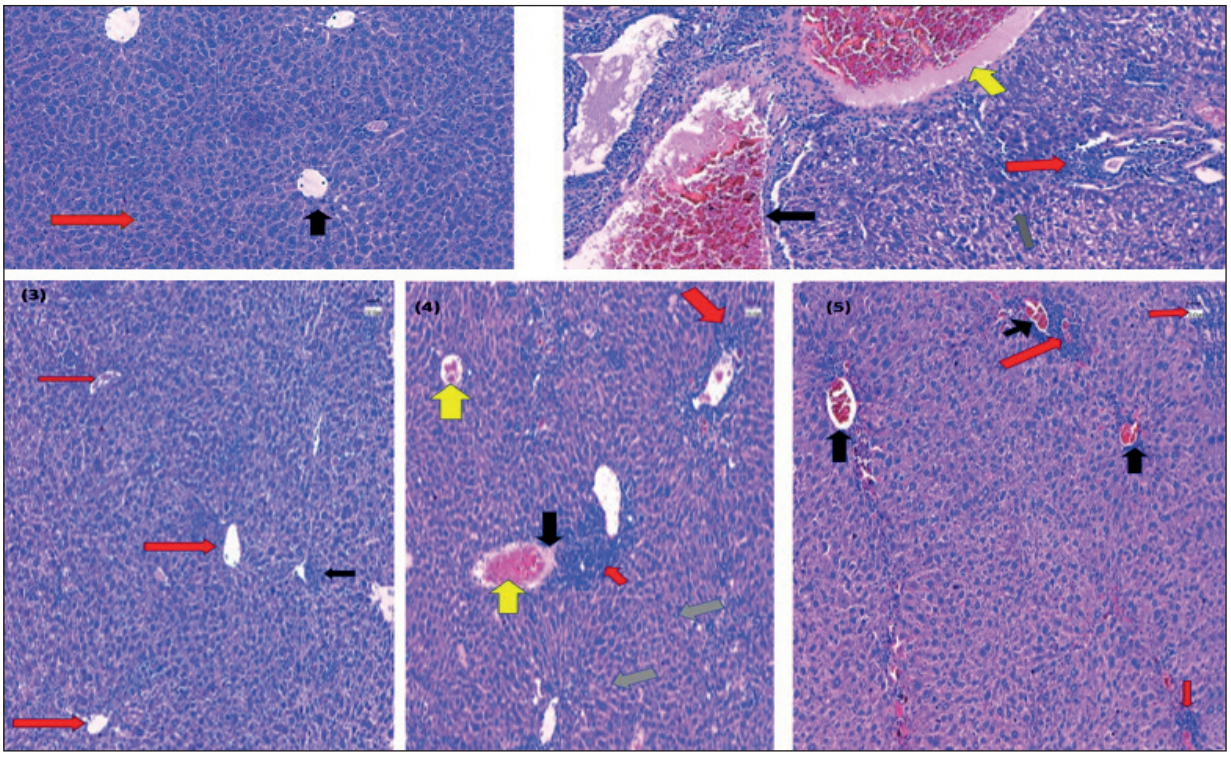

Figure 3. A photomicrograph of H&E-stained sections of hepatic tissue.
1- Normal control group showing normal hepatic architecture, (Red arrow) hepatocyte with their normal nuclei. (Black arrow) central vein and sinusoids. **2-** CCL4 group, (Black arrow) show a large portal tract with sever hemorrhages, indicate Hyperemia and congestion inside portal area. (Yellow arrow) show swelling of blood vessels. (Red arrow) perivascular and advance leukocytic infiltration mainly neutrophils cellular. (Grey arrow) hepatocyte with degeneration and necrosis are seen. **3-** CCL4+Silymarin group, liver sections show with overall preserved lobular architecture. (Black arrow): Hepatocytes are continuously renewed with proliferation near portal space with Normal Sinusoidal. (Red arrow) show central vein within normal limits, new blood vessels establish blood circulation in the healing area. **4-** CCL4+Nebivolol group, (Black arrow) show portal tracts and central vein are dilatation (Yellow arrow) show Hyperemia and congestion inside blood vessels, (Red arrow) leukocytic cells infiltration with lobular congestion indication sign of inflammation (Grey arrow) mild degeneration is seen. **5-** CCL4+Chlorophyllin group, section show liver tissue with overall preserved lobular architecture. he hepatic architecture is normal. (Black arrow) show Portal tracts are swelling and mild congestion is seen. (Red arrow) show less leukocytic cells infiltration. (H&E, X100).

secretion[8]. On the other hand, a previous study[44] examined the hepatoprotective effect of methanolic tanacetum parthenium extract on CCl4-induced liver damage in rats and stated that CCl4 significantly increased all lipids parameters since oxidative stress increase level of hydroperoxides and impair the activity of lipolytic enzymes.

The present study shows that serum and tissue IL-6 and TNF-α levels were significantly higher in the mice that received CCL4 than those in the control group. This goes in hand with Zhang et al[45] who approved that the levels of TGF-b1, TNF-a and IL-6 were higher in fibrogenesis group (CCl4) than in the control group. TNF-α and IL-6 are deliberated major hepatotoxicity mediators in several experimental models of liver injuries as they activate hepatic stellate cells (HSCs) and sinusoidal endothelial cells (SECs) to secrete cytokines which mediate the liver fibrogenesis, besides its effect on extracellular matrix (ECM) production leading to fibrosis[45].

The data of the present work show a hepatoprotective effect of silymarin, nebivolol and chlorophyllin as evident by significant decrease of MDA and serum nitric oxide levels in addition to increase in total protein, serum catalase, tissue SOD and GSH levels in their groups compared to CCl4 group. Hepatic GSH signifies an enzyme reserve of the liver, which is accountable for reducing the hepatotoxicity made by the active metabolites of CCl4[3].

A previous experimental study[42] investigating the role of silymarin in hepatocellular carcinoma is in agreement with the existing work. According to its results, silymarin improved hepatic functions by restoration of hepatic architecture, ame-

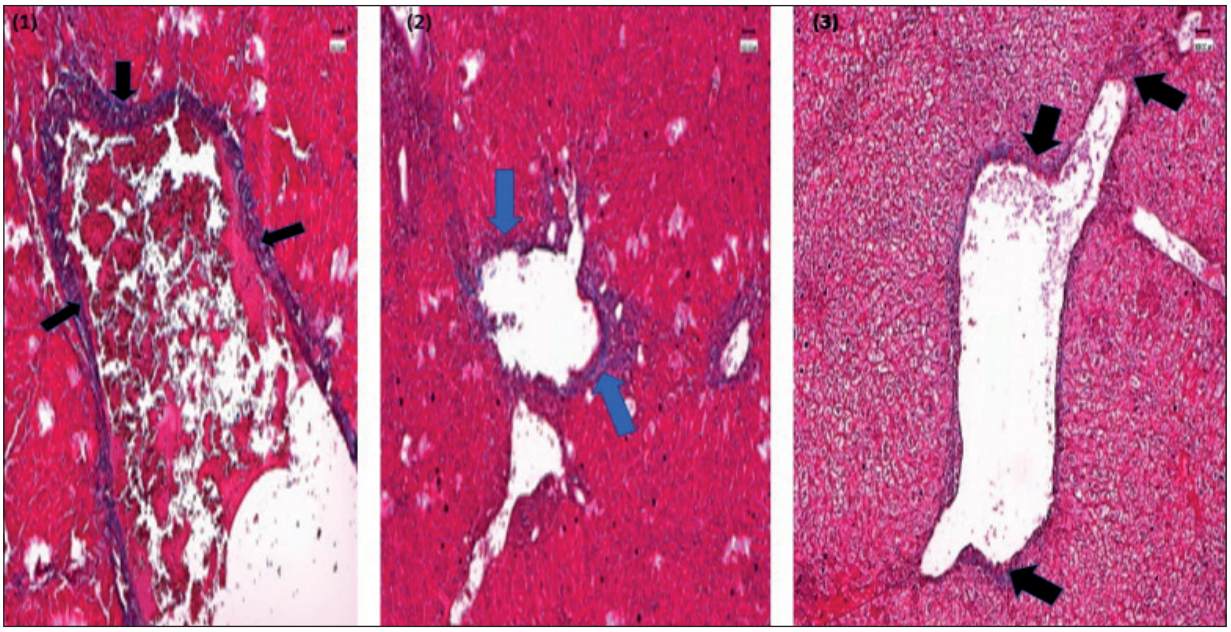

Figure 4. Masson Trichrome stained sections of liver tissues (bluish coloration).
1- CCL4 group, (Black arrow) show portal tract with marked increase in collagen fibers extending across lobules, between portal areas, and between portal areas and central veins. as response of inflammation which consider as sign of healing and repair, collagen fibers deposition around the portal tract.**2-** CCL4+Nebivolol group, (Purple arrow) Shows normal distribution of collagen fibers stained blue around a portal tract and central vein, sign of healing and repair. **3-** CCL4+Chlorophyllin group, (Black arrow) shows minimal degree of collagen fibers around the portal tract (Masson Trichrome – X100). Hepatic sections with no fibrosis (normal & Silymarin groups) did not take Masson Trichrome stain.

liorating oxidative stress, normalizing ALT, AST and tissue nitric oxide levels. Silymarin is used as a standard liver support drug[8]. Silymarin protects liver against acetaminophen-induced liver damage[46]. The antioxidant effect of silymarin was confirmed by decreased levels of lipid peroxidation, increased level of glutathione in the liver and decreased binding of hepatotoxin to hepatocyte, restoring the enzymatic and non-enzymatic antioxidants of the liver nearer to normal[47].

Silymarin decreased the expression of the pro-inflammatory chemokine MCP-1, the pro-fibrogenic cytokine TGF-beta along with collagen I in isolated human HSCs, which considered the key effector cells of hepatic fibrosis[7].

Regarding nebivolol, the results of the prevailing study are in line with Refaie et al[48] who assessed the role of nebivolol in cadmium-induced hepatotoxicity and found that nebivolol decreased liver ALT, AST, total cholesterol levels, MDA, iNOS immunoexpression and TNF-α gene expression nevertheless significantly increased SOD and total antioxidant capacity[48]. Nebivolol decreases blood pressure, preserves vascular endothelium and maintains normal blood supply plus cellular integrity so protects from cell damage[16,48].

Chlorophyllin reduced the expression of IL-6 and TNF-α in the fibrotic liver induced by CCl4 in mice. It also inhibits NF-κB pathway via Ikk-phosphorylation suppression[49]. Hepatic-protective effect of chlorophyllin was confirmed from the decreased activity of AST, ALT and ALP in liver of streptozotocin administered mice. The antioxidant activity of chlorophyllin was comparable with the standard ascorbic acid[25]. Cu-Chl-Na possesses a unique combination of anti-inflammatory, antibacterial, and antioxidant activities[18].

In the present study, Carbon tetrachloride induced severe hyperemia and congestion inside portal area with leukocytic infiltration, hepatic degeneration and bridge fibrosis. Similar to our finding, other studies[3,22] found that the exposure of rats to CCl4 resulted in a marked elevation of hepatic fibrotic changes due to inflammation, hemorrhage and continuous hepatocyte degeneration. Our observations coincide with Shankar et al[41] who stated that CCl4 persuades liver cell necrosis with inflammatory cellular infiltrate and fibrosis. CCl4 causes severe hepatic damage by inducing a state of oxidative stress. Exposure to CCl4 results in centrilobular hepatic necrosis and fibrosis, which is a common pathological condi-

Table VI. The effect of silymarin, nebivolol and sodium Copper Chlorophyllin on hepatic inflammation and fibrosis in mice (n=6).

	Control	CCL4	CCL4+ silymarin	CCL4+ nebivolol	CCL4+ chlorophyllin	*p*-value
Hepatic inflammation	0.0	2.97± .18[a]	.383 ± .23[b]	1.98 ± .23 abc	1.13 ± .30[abcd]	<0.001
Hepatic fibrosis	0.0	2.88 ± .21	.167 ± .12	1.67± .33	1.00 ± .33	<0.001

Data are summarized using mean ± SD
[a] Significant difference from the control group. [b] Significant difference from the CCL4 group. [c] Significant difference from the CCL4 + silymarin group. [d] Significant difference from the CCL4 + nebivolol group. *p*- values ≤ 0.05 were considered significant.

tion that occurs in most conditions associated with chronic liver injury[50]. Our results were in accord with previous studies representing hepatotoxicity of CCl4 in rats[1,40,51].

The data of the present study show that the three treated groups with silymarin or nebivolol or chlorophyllin had significantly lower scores regarding inflammation and fibrosis compared to the mice receiving CCL4. These findings are congruent with Sokar et al[22] who found that silymarin ameliorated CCL4 induced liver fibrosis in rats. Silymarin protects from the CCl4-induced hepatic injury in a dose dependent manner[7]. Nebivolol increases regeneration after partial hepatectomy in rats[16]. Nebivolol improved the hepatocellular damage caused by cadmium toxicity in rats[48]. In contrast to our findings, a previous study[52] discussed effects of nebivolol on liver fibrosis in rats demonstrated that nebivolol did not seem to have a strong beneficial effect on liver fibrosis as feature of inflammation, necrosis, and hemorrhage were still present.

Chlorophyllin significantly ameliorated necro-inflammation and liver injury, as well as improved mortality in fibrosis induced by CCl4 in mice[49]. Chlorophyllin had the ability to restore morphological and cellular alterations as detected in STZ-induced diabetic mice[25]. Impaired enterohepatic circulation and gut dysbiosis may indirectly lead to the liver fibrogenesis[53]. Zheng et al[49] speculated that chlorophyllin could work as a prebiotic and that it can modulate gut microbiota consequently, protecting the liver from fibrosis. Another mechanism for hepatoprotection, the flat ring structure of chlorophyllin directly inserts into endotoxin and decreases the binding to TLR4 on the membrane.

From the overall results, it was concluded that either silymarin, nebivolol or sodium copper chlorophyllin exhibits anti-inflammatory and antioxidant activities, which could have a valuable effect against oxidative liver injury induced by CCl4. This supports the use of nebivolol and chlorophyllin in the prevention of chemically induced toxicity resembling the standard hepatoprotective, silymarin.

Conclusions

Co-administration of silymarin, nebivolol or sodium copper chlorophyllin with CCl4 was able to ameliorate up to almost contradict CCl4 induced hepatic injury due to their antioxidant and anti-inflammatory effects. So, either chlorophyllin or nebivolol should merit thoughtful consideration as an adjudicative therapy to protect against hepatotoxicity induced by CCL4. The significant findings and superior effect of chlorophyllin upon nebivolol, even better than the traditional hepatoprotective drug (Silymarin) in some measured parameters indicates the potential of this natural compound in the prevention and treatment of hepatic fibrosis in patients with chronic liver disease. Further experimental and clinical studies are required to illuminate the exact functional mechanisms of chlorophyllin to support its use as a potent therapeutic agent.

Conflicts of Interest

The authors declare that they have no conflict of interests.

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ORCID ID

Eman S. Said: https://orcid.org/0000-0002-7456-1906.

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