EFFECTS OF CURCUMIN, RESVERATROL AND URSODEOXYCHOLIC ACID ON ETHINYLESTRADIOL AND CHLORPROMAZINE- INDUCED INTRAHEPATIC CHOLESTASIS IN RATS

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Summary

The present study was designed to examine the hepatoprotective effects of curcumin (CMN), resveratrol (RSV) and ursodeoxycholic acid (UDCA) against ethinylestradiol and chlorpromazine -induced intrahepatic cholestasis. Ethinylestradiol (EE) and chlorpromazine HCl (CPZ) were administered orally once daily simultaneously with the tested compounds for one week. At the end of the experimentation period, animals were sacrificed. Blood and liver samples were obtained, serum was used for the determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct& indirect bilirubin, gamma-glutamyltransferase (γ-GT) and cholesterol. Liver samples were used for hiatopathological examination. The results demonstrate that CMN showed marked reduction in all biochemical indices of cholestasis while RSV failed to ameliorate EE-CPZ-induced changes. UDCA produced limited protective effects against EE-CPZ-induced cholestatic injury. This study suggests that CMN has a potent anticholestatic effect in case of EE-CPZ-administration.

Keywords: ethinylestradiol, chlorpromazine, curcumin, resveratrol, cholestasis.

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Introduction

CHOLESTASIS constitutes one of the most common and severe manifestations of acquired or inherited liver disease which may result from a failure in bile secretion in hepatocytes or ductular cells, or from a blockade to the free bile flow **[1].** Intrahepatic cholestasis results from impairment of bile formation by liver cells **[2]** and it can be caused by drugs, sepsis, total parenteral nutrition, lymphomas, tuberculosis, sarcoidosis, and amyloidosis **[3].**

Estrogens are well known to cause intrahepatic cholestasis in susceptible women during pregnancy, administration of oral contraceptives and postmenopausal replacement therapy **[4].** Ethinylestradiol (EE) administration is known to cause hepatic cholestasis in experimental animals **[5] [6].** The effect of EE has been attributed to the endogenous estrogen metabolite estradiol 17β-glucuronide. This metabolite is one of a family of glucuronide conjugates of the estrogen D-ring that have been shown to reduce bile flow and bile acid secretion in the rat in a dose-dependent and reversible manner **[7].** The mechanism involved in the impairment of the bile flow is multifactorial. EE decreases sinusoidal uptake of bile acids, at least in part by inducing down-regulation of the expression of the sodium-taurocholate cotransporting polypeptide protein **[8].** Also, EE reduces hepatic capacity to excrete bile salts and organic anions **[9].** In addition, EE was shown to reduce bile salt synthesis, thus reducing the endogenous bile salt pool and, consequently, bile salt secretion **[10].** Moreover, EE and its 17β-glucuronide administrations increase tight-junctional permeability in rat liver. This increased paracellular permeability allows for the paracellular regurgitation of bile constituents into the blood **[2].**

Chlorpromazine (CPZ) is used for patients with psychiatric disorders, and this agent has been recognized as a cause of cholestatic hepatitis in humans, though the incidence is not more than 2 to 4% in patients receiving CPZ **[11].** CPZ is extensively metabolized by the liver and undergoes enterohepatic recirculation. About 77 metabolites have been recovered from the urine. N-demethylated and ring-hydroxylated products appear to be more toxic than the parent compound. In contrast, the sulfoxide metabolites are inert **[12].** CPZ and its metabolites produce multiple effects on hepatic ultrastructure and function. CPZ and its hydroxylated metabolites ,mainly 7,8-dihydroxychlorpromazine, cause irreversible inhibition of bile flow as they decrease Na^+/K^+-ATPase and Mg^{2+}-ATPase cation pumping in a dose-dependent fashion **[13].** Also, they inhibit the polymerization of soluble actin monomers to form fibrous strands that are essential structure of microfilaments. Moreover, GSH protects plasma membrane ion pumps from CPZ-mediated inactivation **[12].**

Curcumin (CMN, diferuloylmethane) is regarded as the most active constituent present in turmeric and exerts potent biological effects in vitro and in vivo **[14].** It possesses several functional groups that exhibit antioxidant activity **[15]** allowing it to modulate redox signalling pathways in cells. The preventive and improved effects of CMN on symptoms of liver diseases are shown to stem from its antioxidant effects **[16].** The free radical scavenging activity of CMN is beneficial to liver injury caused by a variety of hepatotoxic substances, including CCl_4, ethanol, pentobarbital and acetaminophen **[17] [18].**

Resveratrol (RSV, 3, 4′, 5-trihydroxystilbene) is a natural phytoalexin synthesized in a wide variety of plant species including grapes as a response to environmental stress or fungal infection. It constitutes one of the polyphenolic compounds of red wine and is responsible for the beneficial effect of regular wine consumption at moderate amounts **[19].** RSV possesses a variety of biological activities including antiinflammatory, anticarcinogenic, and antioxidative activities **[20].**

In the present study, we aimed to investigate the possible effect of curcumin and resveratrol, as an antioxidant agents, during the EE and CPZ treatment, which could prevent the drug induced cholestasis and compare their effects with ursodeoxycholic acid.

Materials and methods

Animals

Adult female Sprague-Dawley rats were purchased from Urology and Nephrology Center, Mansoura University, Egypt. Animals were adapted for one week to the laboratory before the onset of the experiments. Animals were kept in plastic cages, maintained under standard conditions of temperature about 30˚C with regular 12h light/12h dark cycle and allowed free access to standard laboratory chow (El Nasr Lab Chem. Co., Egypt) and tap water.

Drugs and chemicals

Curcumin (CMN) was purchased from Nanjing Tianshu Biological Eng. Co., Ltd., Nanjing, China. Resveratrol (RSV), Ursodeoxycholic acid (UDCA) and ethinylestradiol (EE) was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). They were suspended in 0.5 % CMC in distilled water. Chlorpromazine HCl was gifted from Misr Co. for Pharm Ind. S.A.E Cairo, Egypt. All other chemicals used in this study are of finest analytical grade. All drugs and chemicals were freshly prepared in distilled water just before use.

Experimental studies

Rats were divided randomly into five experimental groups, each group included eight rats. EE and CPZ were administered once daily for one week through oral gavages at a dose of 5 mg and 30 mg/kg of body weight, respectively. The doses were suitable for inducing a high incidence of jaundice while maintaining a low mortality rate in rats **[21].** The groups were treated according to the following schedule for one week:

Group 1: Normal control received the vehicle (0.5 % CMC).
Group 2: EE-CPZ control
Group 3: EE-CPZ and CMN (200 mg/kg/day, orally once daily).
Group 4: EE-CPZ and RSV (8 mg/kg/day, orally once daily).
Group 5: EE-CPZ and UDCA (25 mg/kg/day, orally once daily).

At the end of the experimental period, animals were sacrificed. Blood and liver samples were collected from each animal. Blood samples were immediately centrifuged (3000 rpm for 10 minutes). The serum was collected and used immediately for the determination of serum ALT, AST using Biomerieux kits (Marcy-l'Etoile, France), total and direct bilirubin, cholesterol and gamma-glutamyltransferase (γ-GT) levels using Biocon Diagnostik kits (Vöhl/Marienhagen, Germany). The liver samples were fixed in 10 % neutral-buffered formalin, embedded in paraffin wax, sectioned and stained with H&E. At least two different sections were examined per liver sample. The tissues were examined under a microscope in a random order and the pathologist was blind to the used animals and the treatment groups when assessing the histology.

Statistics

Data are expressed as mean ± standard error of the mean, S.E.M. Statistical comparisons between groups were performed using unpaired Student's t-test. Significance was calculated at $p<0.05$.

Results

Effect on biochemical parameters

As shown in Table (1), oral administration of EE and CPZ induced a significant increase in serum levels of ALT, about 2.4 fold, AST , about 2.2 fold, and total and direct bilirubin concentrations in rats as compared to the normal control group. CMN administration along with EE-CPZ for significantly decreased serum transaminases and bilirubin levels as compared to rats treated with EE-CPZ alone. On the other hand, concurrent administration RSV with EE-CPZ produced no significant change in serum transaminases and biliruibin levels compared to EE-CPZ control group.

Rats treated with UDCA concurrently with EE-CPZ showed no change in serum ALT and bilirubin levels while it caused significant reduction in serum AST level compared to EE-CPZ control group.

Table (1): Effect of curcumin, resveratrol or ursodeoxycholic acid on serum ALT, AST and bilirubin levels in EE-CPZ treated rats.

Treatment	ALT (IU/L)	AST (IU/L)	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)
Normal control	28.75 ± 3.37	56.25 ± 3.629	0.16 ± 0.05	0.04 ± 0.02
EE-CPZ control	68.75 ± 5.15 #	123.25 ± 7.95 #	0.74 ± 0.11 #	0.34 ± 0.05 #
EE-CPZ + CMN	52.50 ± 3.89 #*	78.75 ± 4.61 #*	0.41 ± 0.08 #*	0.14 ± 0.04 #*
EE-CPZ + RSV	79.38 ± 6.64 #	109.38 ± 6.84 #	0.68 ± 0.08 #	0.31 ± 0.06 #
EE-CPZ+ UDCA	57.50 ± 3.66 #	97.50 ± 6.27 #*	0.55 ± 0.9 #	0.28 ± 0.04 #

- Data are expressed as mean ± SEM of 8 rats.
significantly different from normal control group ($p<0.05$).
* significantly different from EE-CPZ control group ($p<0.05$).

As shown in figure (1), EE and CPZ produced a significant increase in serum γ-GT level compared to normal control group. CMN administration significantly attenuated the elevation in serum γ-GT level compared to EE-CPZ control group. RSV or UDCA acid treatment did not produce any significant change in serum γ-GT level compared to EE-CPZ control group.

Figure (2) illustrates that EE and CPZ caused a significant reduction in serum cholesterol level compared to normal control group. Simultaneous treatment of CMN with EE-CPZ produced significant increase in serum cholesterol level compared to EE-CPZ control group. Concurrent RSV treatment with EE-CPZ did not show any significant change in serum cholesterol level compared to EE-CPZ control group. UDCA treatment with EE-CPZ for one week resulted in significant increase in serum cholesterol level compared to EE-CPZ control group.

Effect on liver histology

Liver isolated from the normal control group showed normal hepatic architecture (figure 3A&B). Liver sections of EE-CPZ control group revealed marked proliferation of the bile ducts. Bile components were not observed in the hepatocytes or the bile canaliculi, and hepatic cell necrosis was seldom observed (figure 3C&D). Concurrent CMN administration with EE-CPZ prevented the proliferation of bile ducts. Liver sections showed normal liver structure with normal bile ducts (figure 3A). RSV administration did not prevent the proliferation of bile ducts (figure 3B). UDCA produced a decrease in the degree of proliferation of bile ducts compared to untreated control group (figure 3C).

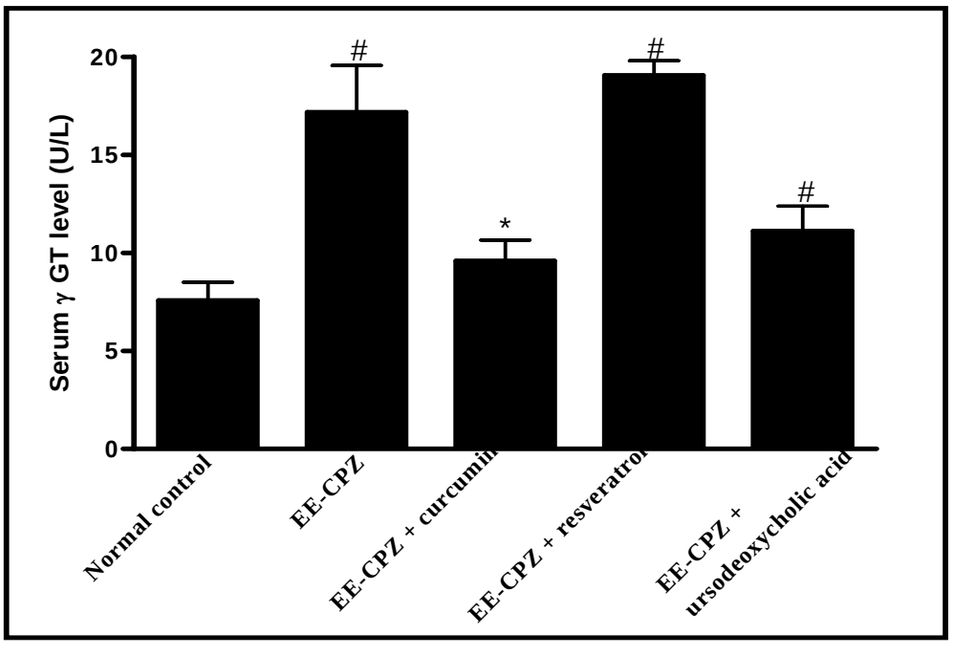

Figure (1): Effect of treatment with either curcumin, resveratrol or ursodeoxycholic acid on EE-CPZ induced changes in serum γ-GT in rats.

- Data are expressed as mean ± SEM of 8 rats.
significantly different from normal control group ($p<0.05$).
* significantly different from EE-CPZ treated group ($p<0.05$).

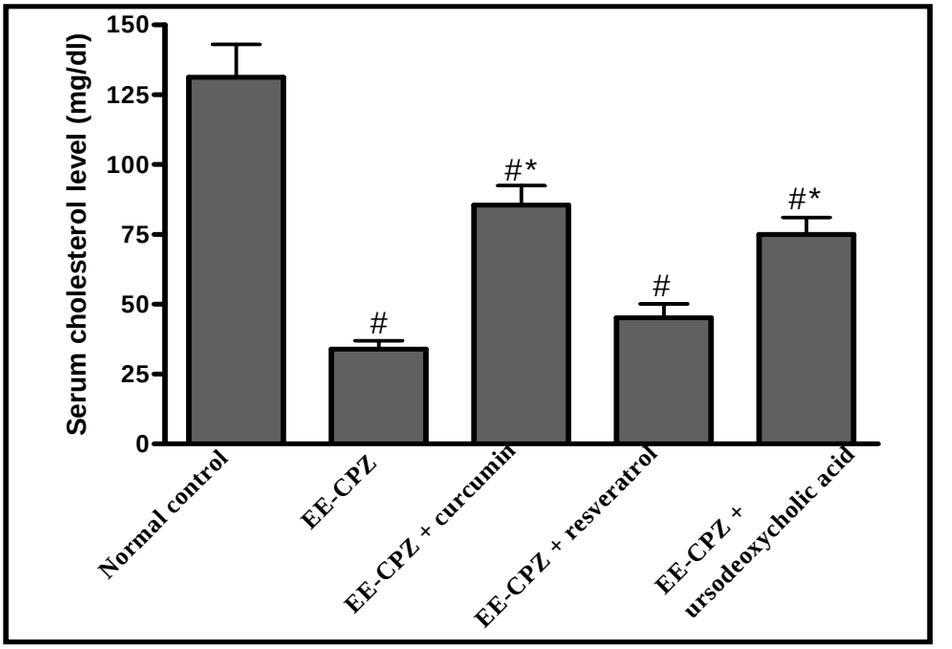

Figure (2): Effect of treatment with either curcumin, resveratrol or ursodeoxycholic acid on EE-CPZ induced changes in serum cholesterol in rats.

- Data are expressed as mean ± SEM of 8 rats.
significantly different from normal control group ($p<0.05$).
* significantly different from EE-CPZ treated group ($p<0.05$).

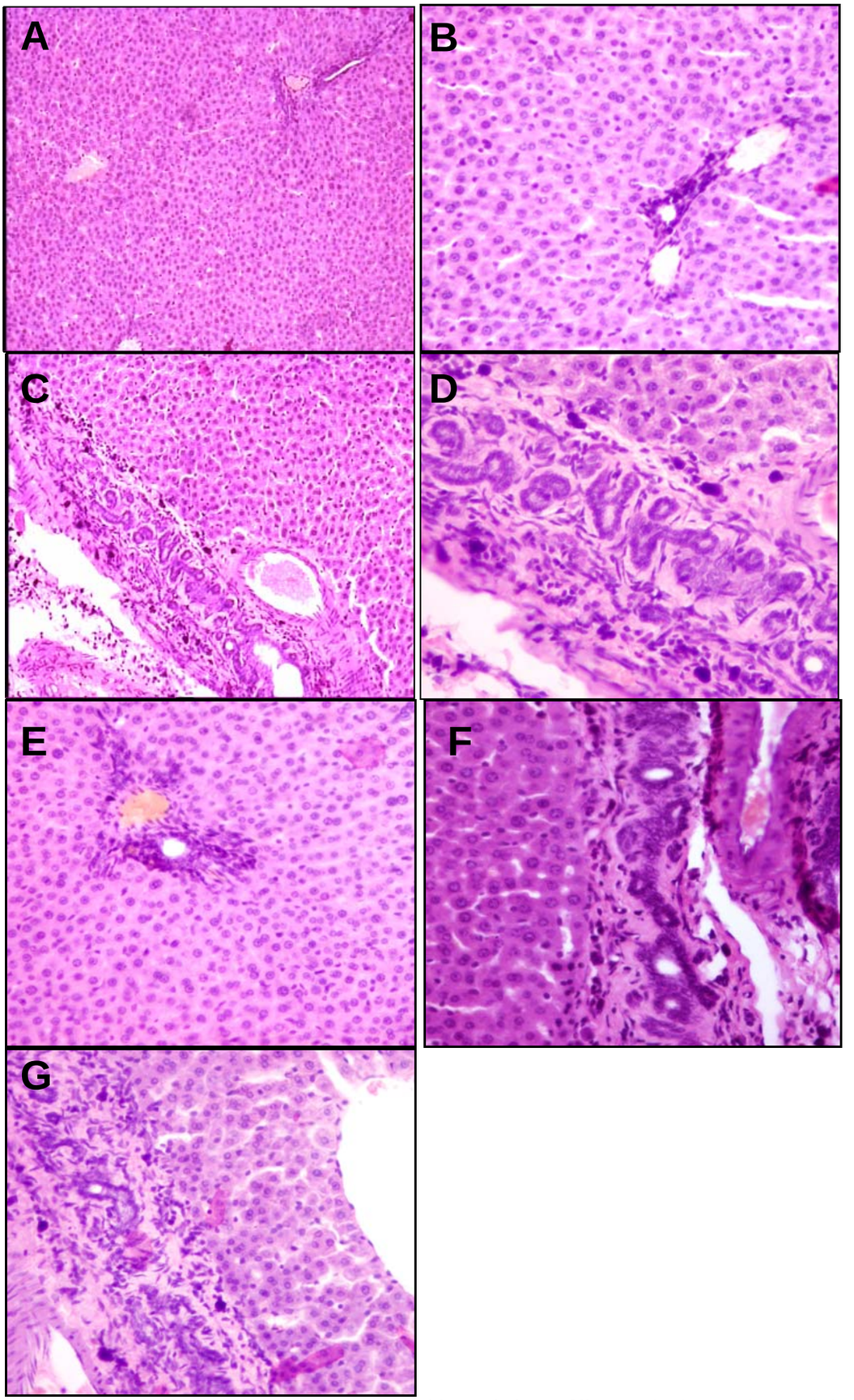

Figure (3): Effect of EE and CPZ treatment on the histopathology of liver. A & B) normal control showing normal bile duct and portal tract; C & D) EE-CPZ showing marked bile duct proliferation; E) EE-CPZ and curcumin showing normal bile ducts; F) EE-CPZ and resveratrol showing marked bile duct proliferation; G) EE-CPZ and ursodeoxycholic acid showing mild bile duct proliferation.

Discussion

Cholestasis was induced in the present study by oral simultaneous administration of EE and CPZ once daily for one week to adult female Sprague-Dawley rats. This treatment resulted in significant elevation in serum ALT, AST, direct & indirect bilirubin and γ-GT as compared to normal control group. Bilirubin and γ-GT are indicators of cholestatic injury **[22].** Moreover, simultaneous administration of EE and CPZ produced a significant decrease in serum cholesterol level as compared to normal control group. Previous studies have demonstrated that EE decreases serum cholesterol level which is accompanied with an increase in the cholesteryl ester content of the hepatocyte plasma membrane **[12].** This can be explained through the ability of EE to stimulate low density lipoprotein receptor activity and increase the binding of lipoproteins to liver plasma membrane **[23].** Also, EE causes an increase in hepatic catabolism of low density lipoprotein **[24].** Both mechanisms lead to marked hypocholesterolemia observed after EE administration. In addition, histopathological examination of excised livers revealed marked bile duct proliferation and rare hepatic cell necrosis. These findings are in agreement with the results of **Obata (1983),** using the same experimental model.

EE has already been shown to induce cholestasis through different mechanisms **[10] [25].** EE decreases profoundly the plasma cholesterol levels in rats due to a marked increase in the number of hepatic low-density-lipoprotein receptors and an increased hepatic clearance of plasma lipoproteins. This results in an increased cholesterol ester content of liver homogenate and a decreased fluidity of these membranes **[12] [13].** Previous investigators have proposed that the decreased membrane fluidity is responsible for the decrease in bile flow and Na^+/K^+-ATPase activity **[8].** EE has been shown to decrease the uptake of bile acids and other organic anions into isolated hepatocytes **[26].** In addition, EE alters bile acid composition, reduce bile salt synthesis, thus reducing the endogenous bile salt pool and, consequently, bile salt secretion **[10] [25].** Increased permeability across tight junctions has also been implicated in contribution to cholestasis **[27].**

Although bile flow decreases markedly in EE-treated rats, EE treatment does not produce jaundice. Therefore, this is not a suitable model for studying human intrahepatic cholestasis since jaundice is the most important and most frequent observed clinical sign in human cases of intrahepatic cholestasis. Hyperbilirubinemia and cholestasis in EE-treated rats can be induced by the simultaneous administration of another drug, chlorpromazine (CPZ), although CPZ alone does not cause elevation of serum bilirubin levels **[21].** CPZ has been reported to induce cholestatic hepatitis as it cause bile ducts to vanish (vanishing bile duct syndrome) and biliary cirrhosis to develop **[28].** The mechanism of CPZ-induced cholestasis can be explained by its detergent properties which enables CPZ to bind to membrane phospholipids. These properties are the most likely responsible for its ability to alter membrane fluidity and inhibit Na^+/K^+-ATPase activity **[11].** Moreover, the micelle formation of bile salt was disturbed due to the reduction in Na^+/K^+-ATPase activity **[13].** Also, CPZ affect the polymerization of actin in actin-containing microfilaments which are responsible for the canalicular contraction and mobility thus leading to inhibition of normal canalicular bile secretion **[27].**

Simultaneous administration of CMN with EE-CPZ produced significant decrease in the serum markers compared to animals treated with EE-CPZ alone. The histopathological examination of the excised liver revealed normal liver architecture and marked protection of EE-CPZ-induced lesions. This beneficial effect of CMN in correcting the serum cholestatic indices is in agreement with the results obtained from **[29],** using EE alone to induce cholestasis in rats. Also, **Deters et al., [30],** showed that CMN is able to stimulate bile flow and to reduce hypercholesterolemia leading to decrease in cyclosporine-induced cholestasis. The observed potent anticholestatic effect of CMN when given simultaneously with EE-CPZ can be explained by its ability to protect cells against the damaging effects of accumulated bile acids as it has a membrane stabilizing effect **[31].** Additionally, CMN was reported to lower intracellular levels of cholesterol

and cytotoxic bile acids **[32].** Moreover, CMN may exert a cytoprotective effect by inducing liver glutathione-S-transferase and liver cytochrome P_{450} **[33].**

On the other hand, RSV administration failed to ameliorate the cholestatic alterations induced by EE-CPZ administration. This result disagrees with the previous study of **Ara et al., (2005),** which demonstrated beneficial effect of RSV against extrahepatic cholestasis induced by bile duct ligation. This result indicates that RSV failed to exert any protective effect against EE-CPZ-induced cholestasis.

UDCA is an endogenous bile acid found in man in small quantities with more hydrophilic properties than other bile acids. UDCA has therapeutic usefulness in several cholestatic liver diseases. It can improve liver tests in patients with cholestatic disorders such as primary biliary cirrhosis **[34].** The beneficial effects of UDCA on EE-induced cholestasis have been demonstrated in rats **[25]** as UDCA increases bile flow and bile salt output, leading to an improvement of the biliary secretory function impaired by EE **[5].** The major beneficial effects of treatment with UDCA are protection against the damaging effects of toxic bile acids **[35]**, stimulation of hepatobiliary secretion, antioxidant activity due to an enhancement in glutathione levels and inhibition of liver cells apoptosis **[36].** In the present study, simultaneous administration of UDCA with EE-CPZ resulted in significant decrease in serum AST level and significant increase in serum cholesterol level compared to animals treated with EE and CPZ alone. However, UDCA did not show any significant reduction in the elevated serum direct and total bilirubin levels compared to untreated control group. In addition, histopathological examination showed mild degree of bile duct proliferation. Comparing the beneficial effect of CMN with that of UDCA in the present model of EE-CPZ induced cholestasis, CMN showed more anticholestatic activity than UDCA indicated by the measured biochemical parameters and the histopathological examination of liver. So, the present study revealed that CMN has a powerful anticholestatic effect in addition to its hepatoprotective, antioxidative action and free radicals scavenging activity.

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