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Tenofovir/Lamivudine/Efavirenz-Induced Hepatotoxicity: Cucurmin as a Potential Protective Agent

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Abstract: Tenofovir/Lamivudine/Efavirenz (TLE) is a first-line agent used for the treatment of human immunodeficiency virus (HIV). Its use has decreased HIV progression, but the occurrence of hepatotoxicity is a serious burden. Curcumin (CUM) is a polyphenol compound which has potential therapeutic health benefits. This study assessed its protective effect against TLE-induced hepatotoxicity in Wistar rats. Forty adult male Wistar rats (180-200 g) grouped into 8 of n = 5 were used. Group I (Control) was orally administered with normal saline (0.2 mL) daily. Groups II-IV were orally administered with CUM (50, 100 and 200 mg kg^{-1}) daily. Group V was orally administered with TLE (300/300/600) mg kg^{-1}. Groups V1-VIII; were orally administered with CUM (50, 100 and 200 mg kg^{-1}) before the administration of TLE (300/300/600) mg kg^{-1} daily. The rats were treated for 30 days. After treatment, the rats were anesthetized and blood samples were collected and assessed for serum liver function markers. Liver samples were excised and assessed for oxidative stress markers and histology. Serum aminotransferases, bilirubin, lactate dehydrogenase alkaline phosphase, gamma-glutamyl transferase, liver malondialdehyde levels and liver weight were significantly ($p<0.001$) increased in TLE administered rats when compared to control. Body weight, liver catalase, glutathione (GSH), superoxide dismutase and GSH peroxidase levels were significantly ($p<0.001$) decreased in TLE-treated rats when compared to control. TLE caused hepatocytes necrosis. Interestingly, CUM supplementation abrogate TLE-induced hepatotoxicity at 50 mg kg^{-1} ($p<0.05$), 100 mg kg^{-1} ($p<0.01$) and 200 mg kg^{-1} ($p<0.001$) when compared to TLE. CUM may clinically prevent TLE related hepatotoxicity.

INTRODUCTION

Highly active antiretroviral therapy (HAART) comprises three or more antiretroviral drugs used to treat human immunodeficiency virus (HIV). HIV related socio-economic problems have decreased in recent years after the introduction of HAART[1]. Despite this achievement, HAART causes undesirable effects including hepatotoxicity.

HAART related hepatotoxicity is a cause of morbidity, mortality and medication change in HIV-infected people. The mechanisms of HAART-induced hepatotoxicity are poorly defined. Possible hepatotoxic mechanisms seem to be multiple including hypersensitivity reactions, direct toxicity, mitochondrial toxicity and immune reaction associated with hepatitis B or C co-morbidities[2].

HAART comprising Tenofovir/Lamivudine/Efavirenz (TLE) is a first-line agent for the treatment of HIV. Its use has decreased HIV progression, but the occurrence of hepatotoxicity is a serious burden. Various forms of liver disorders including jaundice, hepatitis, hepatic encephalopathy and fulminant liver failure may occur with the use of TLE. The occurrence of hepatotoxicity has been attributed to EFV, a Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI)[3].

Hepatotoxicity caused by TLE may occur within 100-168 days (14-24 weeks) of therapy[4, 5]. Liver biomarkers including transaminases, alkaline phosphatase and bilirubin are elevated whereas serum protein level is decreased in TLE associated hepatotoxicity[6]. Alteration in liver histology including inflammatory cell infiltrations may occur.

The infiltration of the liver by inflammatory cells shows that immune-mediated mechanism may play a vital role in hepatotoxicity caused by TLE. Some studies suggested oxidative stress as a possible mechanistic factor[7].

Curcumin (CUM) is a polyphenol compound which has been shown to target multiple signaling molecules. It has demonstrated significant activity at the cellular level which supports its multiple health benefits[8]. CUM is used worldwide for health conditions including inflammation, metabolic syndrome, pain and degenerative eye conditions[9].

Anti-inflammatory and antioxidant effects have been associated with most of its health benefits[9]. Its antioxidant activity includes scavenging and neutralization of free radicals, inhibition of lipid peroxidation (LPO) and up-regulatory action on the functions of endogenous antioxidants[10]. Its anti-inflammatory effect includes the inhibition of Nuclear Factor (NF)-kb and increased anti-inflammatory gene expression[11]. CUM has been declared safe by US Food and Drug Administration and is available in several forms including tablets, capsules and ointments[8]. CUM has shown promising protective effects against some toxicities induced in animals[12, 13]. Available information shows no study on its protective effect against TLE-induced hepatotoxicty in animal models which the current study assessed in Wistar rats.

MATERIALS AND METHODS

Drug and chemicals: Tenofovir/lamivudine/efavirence (Mylan Labolatories Limited, India) Curcumin (Sigma Chemicals Corp, St. Louis, MO, USA). Other chemicals used are of purest quality.

Experimental animals and treatment: Forty male adult Wistar rats (180-200 g) housed for 2 weeks before the initiation of the experiment were used. The rats were kept at 23±2°C and 50-60% humidity with access to chow and water *ad libitum*. The rats were randomized into 8 groups of n = 5. The rats were treated as follows: Group I (Control) was orally administered with normal saline (0.2 mL) daily. Groups II-IV were orally administered with CUM (50, 100 and 200 mg kg^{-1}) daily[14]. Group V was orally administered with TLE (300/300/600) mg kg^{-1} daily[15]. Groups V1-VIII were orally administered with CUM (50, 100 and 200 mg kg^{-1}) before the administration of TLE (300/300/600) mg kg^{-1} daily. Piperine (20 mg/kg/p.o) was added to CUM to improve bioavailability[16]. The rats were treated (using intragastric feeding needle) for 30 days. After, the last dose, the rats were fasted over night; anesthetized and blood samples were collected from the heart. The collected blood samples were left to clot and sera were collected through centrifuging (4000 rpm for 5 min) and assessed for liver function markers. Liver samples were excised, rinsed in cold saline and homogenized in phosphate buffer (0.1 M, pH 7.4). The homogenates were centrifuged (3000 rpm for 15 min) and the supernatants collected and assessed for oxidative stress markers. The rats were handled based on the regulation on the use of laboratory animals by National Research Council 8th Edition.

Determination of liver function markers: Serum Total Bilrubin (TB), Alanine Aminotransferase (ALT), Conjugated Bilirubin (CB), Aspartate Aminotransferase (AST), Gama-Glutamyl Transferase (GGT), Alkaline Phosphatase (ALP) and Lactate Dehydrogenase (LDH) were assayed using an auto analyzer.

Table 1: Effects of curcumin on body and liver weights of tenofovir/lamivudine/efavirenz-treated rats

Dose (mg kg^{-1})	FBW (g)	ALW (g)	RLW (%)
Control	260.6±15.6	5.23±0.34	2.01±0.05
CUM 50	255.4±16.5	5.19±0.45	2.03±0.03
CUM 100	250.5±14.7	5.20±0.27	2.07±0.01
CUM 200	260.2±16.0	5.22±0.56	2.01±0.08
TLE	160.8±15.4#	9.99±0.47#	6.21±0.78#
CUM 50+TLE	200.3±14.5	8.91±0.52	4.44±0.16*
CUM 100+TLE	230.6±17.7*	6.50±0.68*	2.81±0.33**
CUM 200+TLE	245.5±16.8*	5.90 ±0.11**	2.40±0.41**

CUM: Curcumin; TLE = Tenofovir/lamivudine/efavirenz; FBW = Final Body Weight; ALW = Absolute Liver Weight; RLW = Relative Liver Weight; Data as mean±SEM (Standard error of mean), n = 5, #p<0.01 in comparison to control, *p<0.05 when compared to TLE **p<0.01 when compared to TLE

Histological examination: Liver tissues were excised and fixed in 10% neutral buffered formalin for 24 h. Liver tissues were dehydrated in ethyl alcohol and embedded in paraffin wax. Sections (5-µm) were produced, paraffin removed and stained with Hematoxylin and Eosin (H&E). Stained sections were examined using light microscopy for histological changes.

Determination of liver oxidative stress markers: Malondialdehyde (MDA) was measured as reported by Buege and Aust[17]. Catalase (CAT) was measured as described by Aebi. Superoxide Dismutase (SOD) was assayed according to Sun and Zigman[18]. Reduced Glutathione (GSH) was measured as described by Sedlak and Lindsay[19]. Glutathione Peroxidase (GPx) was measured as described by Rotruck *et al*.[20].

Statistical analysis: Values are expressed as mean ± standard error of mean (SEM) of n = 5. Variations between groups were determined by one-way analysis of variance (ANOVA) and post hoc testing using Tukey's test with the aid of Graph Pad Prism 5 software. Significance was determine at p<0.05, <0.01 and <0.001.

RESULTS

Effects of curcumin on body and liver weights of tenofovir/lamivudine/efavirenz-treated rats: Body and liver weights were not different (p>0.05) in CUM administered rats when compared to control. But decrease (p<0.01) in body weight with increase (p<0.01) in liver weights were observed in TLE administered rats when compared to control (Table 1). However, CUM supplementation significantly increased body weight, but significantly decreased liver weight at 50 mg kg^{-1} (p<0.05), 100 mg kg^{-1} (p<0.01) and 200 mg kg^{-1} (p<0.01) when compared to TLE (Table 1).

Effect of curcumin on serum biochemical markers of tenofovir/lamivudine/efavirenz-treated rates: Normal (p>0.05) serum AST (30.7±4.00), ALT (35.6±4.16), ALP (27.9±3.55), GGT (0.27±0.09) TB (3.90±0.40), CB

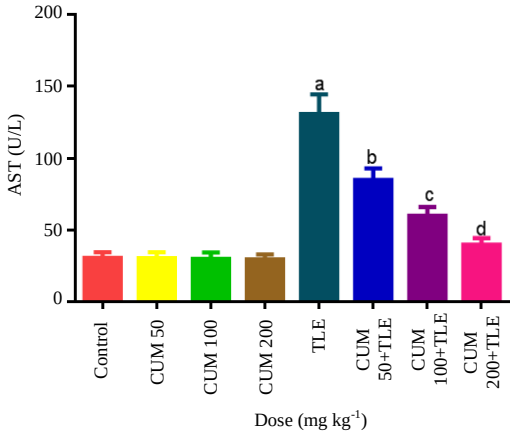

Fig. 1: Effect of curcumin on serum aspartate aminotransferase of tenofovir/lamivudine/efavirenz-treated rats; CUM: Cucumin, TLE: tenofovir/lamivudine/efavirenz, AST: Aspartate aminotransferase, n = 5, data as mean+SEM (Standard error of mean), a p<0.001 when compared to control, b p<0.05 when compared to TLE, c p<0.01 when compared to TLE, d p<0.001 when compared to TLE

(2.32±0.67) and LDH (28.8±4.74) levels were observed in CUM-administered rats. TLE administration produced significant (p<0.001) elevations in serum AST (130.9±13.6), ALT (135.0±17.2), ALP (100.8±11.0), GGT (1.10±0.08) TB (15.6±2.55), CB (11.6±1.32) and LDH (112.7±15.5) when compared to control. But supplementation with CUM produced significant decreases in serum AST, ALT, ALP, GGT, TB and LDH levels at 50 mg kg^{-1} (p<0.05), 100 mg kg^{-1} (p<0.01) and 200 mg kg^{-1} (p<0.001) when compared to control (Fig. 1-7). Supplementation with CUM (200 mg kg^{-1}) produced the following decreases in serum AST (40.1±4.54), ALT (42.0±5.65), ALP (31.6±5.35), GGT (0.35±0.06) TB (4.22±0.28), CB (3.00±0.18) and LDH (33.6±4.03) levels (Fig. 1-7).

Effects of curcumin on liver oxidative stress markers and histology of tenofovir/lamivudine/efavirenz-treated rats: Liver antioxidants (GSH, SOD, CAT and GPx) and MDA levels were normal (p>0.05) in CUM-administered rats and when compared to control (Table 2). The administration of TLE significantly (p<0.001) increased liver antioxidant levels and significantly (p<0.001) decreased MDA levels when compared to control (Table 2). However, supplementation with CUM caused significant increases in liver antioxidant levels with significant decreases in liver MDA levels at 50 mg kg^{-1} (p<0.05), 100 mg kg^{-1} (p<0.01) and

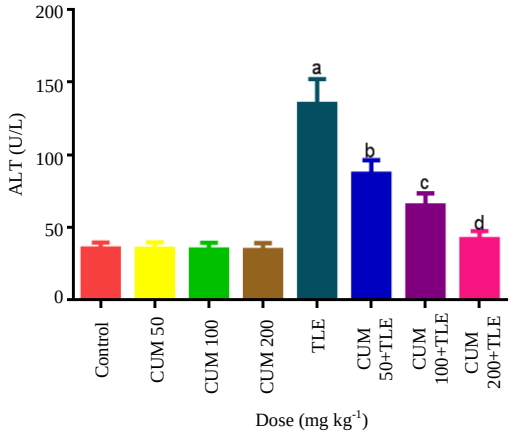

Fig. 2: Effect of curcumin on serum alanine aminotransferase of tenofovir/lamivudine/efavirenz-treated rats; CUM = Cucumin, TLE = Tenofovir/Lamivudine/Efavirenz, ALT = Alanine aminotransferase, n = 5, Data as mean+SEM (Standard error of mean), a p<0.001 when compared to control, b p<0.05 when compared to TLE, c p<0.01 when compared to TLE, d p<0.001 when compared to TLE

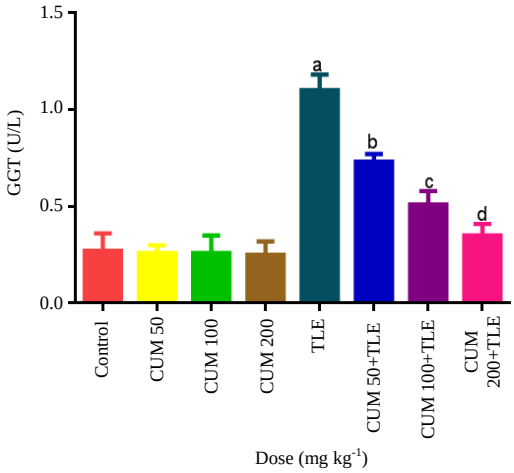

Fig. 4: Effect of curcumin on serum gamma-glutamyl transferase of tenofovir/lamivudine/efavirenz-treated rats; CUM = Curcumin, TLE = tenofovir/lamivudine/efavirenz, GGT = Gamma-glutamyltransferase, n = 5, Data as mean+SEM (Standard error of mean), a p<0.001 when compared to control, b p<0.05 when compared to TLE, c p<0.01 when compared to TLE, d p<0.001 when compared to TLE

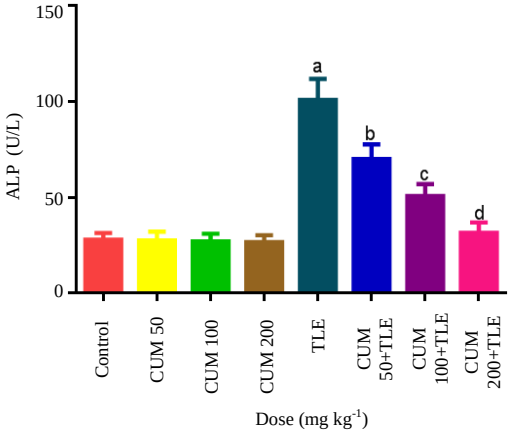

Fig. 3: Effect of curcumin on serum alkaline phosphatase of tenofovir/lamivudine/efavirenz-treated rats; CUM = Curcumin, TLE = Tenofovir/Lamivudine/Efavirenz; ALP = Alkaline Phosphatase, n = 5, Data as mean+SEM (Standard error of mean), a p<0.001 when compared to control, b p<0.05 when compared to TLE, c p<0.01 when compared to TLE, d p<0.001 when compared to TLE

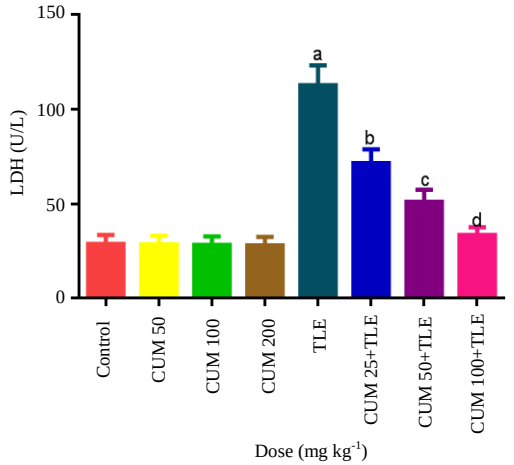

Fig. 5: Effect of curcumin on serum lactate dehydrogenase of tenofovir/lamivudine/efavirenz-treated rats; CUM = Curcumin; TLE = Tenofovir/Lamivudine/Efavirenz, LDH = Lactate dehydrogenase, n = 5, Data as mean+SEM (Standard error of mean), a p<0.001 when compared to control, b p<0.05 when compared to TLE, c p<0.01 when compared to TLE, d p<0.001 when compared to TLE

200 mg kg^{-1} (p<0.001) when compared to TLE (Table 2). The liver of control rat (Fig. 8a) and the liver of CUM administered rat (Fig. 8b) showed normal histology. The liver of TLE administered rat shows hepatocyte necrosis (Fig. 8c) whereas the liver of rats supplemented with CUM (50 mg kg^{-1}) (Fig. 8d), (100 mg kg^{-1}) (Fig. 8e) and (200 mg kg^{-1}) (Fig. 8f) showed normal histology.

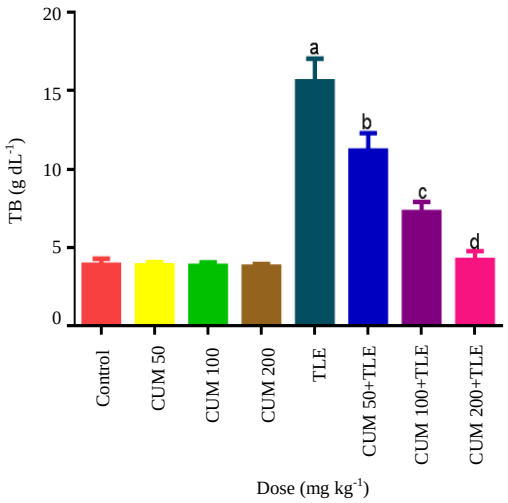

Fig. 6: Effect of curcumin on serum total bilirubin of tenofovir/lamivudine/efavirenz-treated rats; CUM = Curcumin; TLE = Tenofovir/Lamivudine/ Efavirenz; TB = Total Bilitubin , n = 5, Data as mean+SEM (Standard error of mean), a $p<0.001$ when compared to control, b $p<0.05$ when compared to TLE, c $p<0.01$ when compared to TLE, d $p<0.001$ when compared to TLE

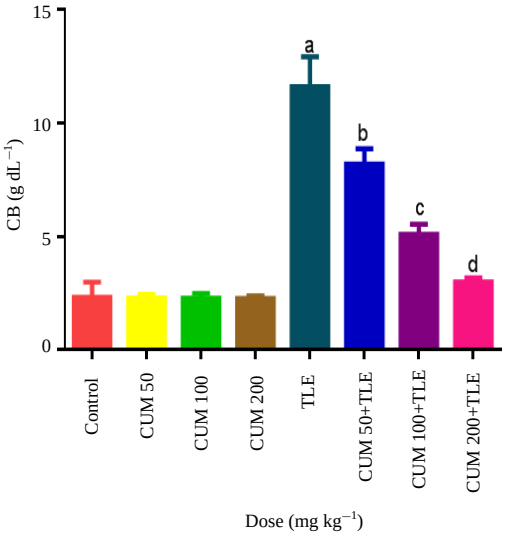

Fig. 7: Effect of curcumin on serum conjugated bilirubin of tenofovir/lamivudine/efavirenz-treated rats; CUM = Curcumin; TLE= Tenofovir/ Lamivudine/ Efavirenz; CB = Conjugated Bilirubin, Data as mean+SEM (Standard error of mean), a $p<0.001$ when compared to control, b $p<0.05$ when compared to TLE, c $p<0.01$ when compared to TLE, d $p<0.001$ when compared to TLE

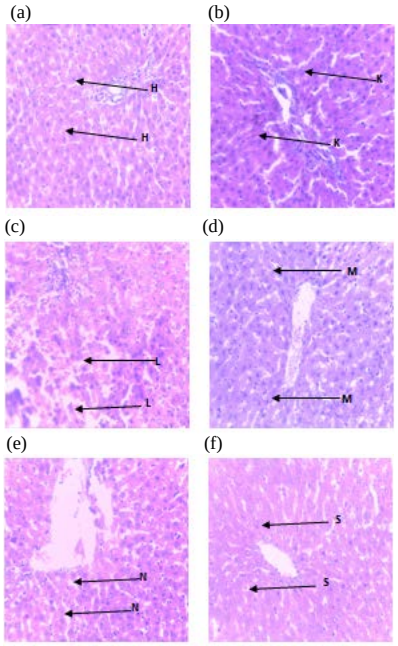

Fig. 8(a-f): (a) Liver of control rat showing normal histology (H), (b). Liver of CU (200 mg kg^{-1}) treated rat showing normal hepatocytes (K), (c). Liver of tenofovir/lamivudine/efavirenz treated rat showing hepatocyte necrosis (L), (d). Liver of rat supplemented with CU (50 mg kg^{-1}) showing normal hepatocytes (M), (e). Liver of rat supplemented with CU (100 mg kg^{-1}) showing normal hepatocytes (N), (f). Liver of rat supplemented with CU (2000 mg kg^{-1}) showing normal hepatocytes (S)

Table 2: Effect of curcumin on liver oxidative stress markers of tenofovir/lamivudine/efavirenz-treated rats

Treatment (mg kg^{-1})	SOD (u mg^{-1} protein)	CAT (u mg^{-1} protein)	GSH (µg mg^{-1} protein)	GPx (u mg^{-1} protein)	MDA (nmol mg^{-1} protein)
Control	33.3±4.00	40.1±5.88	17.4±2.11	21.5±4.33	0.15±0.08
CUM 50	33.7±4.22	41.0±5.76	17.6±2.32	22.0±4.00	0.14±0.09
CUM 100	33.8±3.99	41.7±5.19	18.1±2.44	22.7±4.17	0.13±0.04
CUM 200	35.1±4.52	43.1±5.98	19.0±2.78	24.0±4.27	0.12±0.07
TLE	10.0±1.43[a]	14.1±2.90[a]	5.54±0.11[a]	6.47±0.18[a]	0.80±0.07[a]
CUM 50+TLE	13.8±2.55[b]	20.6±4.41[b]	8.61±0.12[b]	9.51±0.27[b]	0.52±0.05[b]
CUM 100+TLE	19.7±2.00[c]	29.3±4.48[c]	11.7±1.56[c]	12.7±2.52[c]	0.30±0.06[c]
CUM 200+TLE	28.9±3.67[d]	37.4±5.84[d]	15.8± 2.60[d]	18.9±2.27[d]	0.17±0.08[d]

SOD = Superoxide dismutase; CAT = Catalase; GSH = Glutathione; MDA = Malondialdehyde; Gpx = Glutathione Peroxidase; CUM = Curcumin; TLE = Tenofovir/Lamivudine/Efavirenz, n = 5, Data as mean±SEM (Standard error of mean), [a]$p<0.001$ when compared to control, [b]$p<0.05$ when compared to TLE, [c]$p<0.01$ when compared to TLE, [d]$p<0.001$ when compared to TLE

DISCUSSION

Drug-induced hepatotoxicity is an important clinical issue. It is the cause of 5% of most hospital admissions and 50% of most cases of acute liver failure[21]. TLE, an antiretroviral drug combination has been associated with

hepatotoxicity marked by mild elevations in aminotransferases to fulminant liver failure[22]. Generally, treatment of hepatotoxicity is challenging due to few preventive or treatment methods[21], hence, the search for new therapeutic methods is imperative. Reports in animal studies showed that supplementations with natural products could serve as excellent therapeutic strategies for many diseases[23]. CUM (diferuloyl methane), a small-molecular weight compound extracted from *Curcuma longa L* is used traditionally for medicinal and dietary purposes[24]. This study was undertaken to assessed if CUM supplementation could prevent TLE-induced hepatotoxicity in Wistar rats. Current liver tests include the assessments of plasma markers of injury (AST, ALT, GGT and ALP) and markers of liver function (bilirubin). Among the injury markers, ALT and AST are commonly used[25]. These aforementioned markers are usually elevated in cases of acute hepatotoxicity, mild hepatocellular injury and extrahepatic obstruction[26]. In this study, serum GGT, CB, TB, AST, ALT ALP and LDH levels were normal in CUM administered rats, but were elevated in TLE-administered rats. This observation in TLE-administered rat is consistent with similar reports[23]. This may be due to the leakage of these markers from the liver cytosol into the blood stream as a result of hepatic membrane damage. TLE might have impaired the biosynthetic and the regulatory capacity of the liver on these markers and also altered hepatic membrane permeability. According to Gaskill *et al*.[27] the release of liver markers from liver cytosol may be secondary to hepatic necrosis. In the current study, supplementation with CUM prevented the hepatotoxic impact of TLE in a dose-dependent fashion by restoring the serum levels of the aforementioned markers. Antioxidants including SOD, GSH, GPx and CAT are substances that even at low concentrations can significantly delay or inhibit oxidative damage of biomolecules caused by ROS[28]. SOD catalyses the dismutation of superoxide radicals with hydrogen peroxides (H_2O_2) and molecular oxygen as byproducts[29]. GPx works in tandem with glutathione-S-transferase (GST) to decompose H_2O_2 and other organic hydroperoxides to non-toxic products[30]. CAT catalyses the reduction of hydrogen peroxides and protects cells from hydroxyl radicals[31]. GSH is a direct scavenger of free radicals and co-substrate for peroxide detoxification in conjunction with GPx[32]. These antioxidants work in tandem to forestall cellular damage caused by oxidative stress, but could be consumed and depleted in the presence of an overwhelming oxidative stress[33]. This study observed normal antioxidants (SOD, GSH, GPx and CAT) in CUM administered rats, but depleted liver antioxidant concentrations was observed in TLE administered rats which is an evidence of oxidative stress. However, liver antioxidants were up-regulated in a dose-dependent fashion in CUM supplemented rats. LPO, the reaction of oxygen with poly unsaturated lipids produces a variety of oxidation products including Malondialdehyde (MDA), propanal, hexanal and 4-hydroxynonenal[34]. MDA has been widely used as a convenient biomarker for LPO because of its reaction with thiobarbituric acid (TBA)[35]. In the present study, MDA levels were normal in CUM administered rats. However, MDA level was increased in TLE administered rats which is an indication of LPO. Studies showed that LPO can disrupt hepatic membrane function, thus, increasing fluidity, permeability and incapacitating receptor functions[36]. However, CUM supplementation caused notable decreases in LPO characterized by low hepatic MDA levels in a dose-dependent fashion. The mechanisms by which TLE causes hepatotoxicity are poorly defined. But studies suggest multiple mechanisms based on metabolism and/or direct cell toxicity. TLE has been associated with endoplasmic reticulum stress, mitochondrial dysfunction and oxidative stress[37].

The current study shows that supplementation with CUM produced substantial protective effect against TLE-induced hepatotoxicity in Wistar rats. This finding may be due to the numerous pharmacological activities of CUM primarily antioxidant and anti-inflammatory activities. CUM performs its effect as an antioxidant through a number of mechanisms as explained. It can scavenge free radicals including ROS and nitrogen species (RNS)[38]; it can upregulate the activities of endogenous antioxidants in neutralizing free radicals[39]. CUM can also inhibit ROS-generating enzymes such as xanthine hydrogenase/oxidase and lipoxygenase/ cyclooxygenase[39]. It is a lipophilic compound, making it an efficient scavenger of peroxyl radicals and a chain-breaking antioxidant[40]. In addition, oxidative stress can initiate an intracellular signaling cascade that stimulates pro-inflammatory gene expression and inflammation. CUM can suppress inflammation through many different mechanisms including the inhibition of nuclear factor- κB (NF-κB)(11).

CONCLUSION

CUM may clinically protect against TLE-related hepatotoxicity.

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