Therapeutic effects of curcumin and ursodexycholic acid on non-alcoholic fatty liver disease

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ABSTRACT

Fatty liver disease is commonly associated with inflammation, oxidative stress and apoptosis of hepatocytes. This study was designed to investigate the combinational therapeutic effects of curcumin (CMN) and Ursodeoxycholic acid (UDCA) on non-alcoholic fatty liver disease (NAFLD). Male Wistar rats were divided into 8 groups: NAFLD-induced rats, NAFLD-induced rats + CMN, NAFLD-induced rats + UDCA, and NAFLD-induced rats that received CMN + UDCA. CMN (200 mg/kg) and UDCA (80 mg/kg) was administered orally for 14 and 28 consecutive days. Biochemical and histopathological analysis were conducted in all the groups. It was seen that co-administration of CMN and UDCA significantly reduced fatty degeneration, cellular necrosis, edema, and immune cell infiltration compared to non-treated NAFLD-induced rats. Whereas, combinational therapy caused a significant decrease in levels of SGOT and SGPT enzymes and expression of p53, caspase III, iNOS and bcl-2 mRNA and proteins, in variant with the treatment of CMN and UDCA, respectively. Co-administration of CMN and UDCA was also associated with the restoration of the levels of serum TG and HDL-C however, had no effect on LDL-C. It also resulted in an in TAC, GSH- PX, and SOD and decrease in MDA level. Our study concludes that combinational therapy of CMN and UDCA is effective for the treatment of NAFLD, as compared to their solo treatment.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), one of the most common causes of chronic liver injury, encompasses liver damage from steatosis to nonalcoholic steatohepetitis [1,2]. NAFLD affects 2.8–24% of the general population [3,4] and is associated with obesity, type 2 diabetes, and hyperlipidaemia [5,6]. The main etiology of disease is still unknown however, high rate of hepatocyte apoptosis, mitochondrial dysfunction, fat accumulation, inflammation, increased oxidative stress, and amino acid imbalance are some of the commonly reported underlying mechanisms [7,8]. Oxidative stress due to decreased antioxidant defenses and increased generation of reactive oxygen species is observed in NAFLD [9,10]. Increased oxidative stress is associated with a high rate of apoptosis in hepatocytes [11] whereas, imbalance in the ratio of pro- and antiapoptotic proteins can influence the cell survival.

Curcumin (CMN) is the active compound of perennial plant Curcuma longa, which is generally known as turmeric. CMN is an anti-inflammatory and anti-oxidative compound. It is known to inhibit the activity of reactive oxygen-generating enzymes such as xanthine dehydrogenase/oxidase, lipoxygenase/cyclooxygenase, and inducible nitric oxide synthase [12]. Several animal-based studies have shown that CMN ameliorates liver injury and cirrhosis [13–17].

Ursodeoxycholic acid (UDCA) is clinical used for the treatment of cholestatic liver diseases and recently, has been seen to treat non-cholestatic liver diseases [18]. UCDA has effective therapeutic potencies in alcoholic liver injury and can prevent mitochondrial damage in chronic alcohol intoxication [19].

High-fat diet (HFD) is extensively exploited recently to produce nutritional animal models for NAFLD [20–22]. It induces insulin resistance, hepatic steatosis, liver damage, and hypertriglyceridemia, conditions which are characteristics of NAFLD.

This study is designed to determine therapeutic effects of CMN and UDCA against NAFLD. For this purpose, mRNA and protein levels of p53, bcl-2, caspase III and iNOS were determined. In addition, its effects

on serum levels of glutamic oxaloacetic transaminase (SGOT), glutamic pyruvic transaminase (SGPT), lipid profile, tissue concentrations of total antioxidant capacity (TAC), malondialdehyde (MDA) and nitric oxide (NO) as well as activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were also assessed in the liver of NAFLD-induced rats.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma (Sigma–Aldrich, St. Louis, MO), unless specified otherwise.

2.2. Animals and induction of Non-alcoholic fatty liver disease

Male Wistar rats (200 ± 10 g) were kept under room temperature 23 ± 2°C with a 12-h light and dark cycle and humidity of 50 ± 6%. The procedure of animal care was accepted by the Animal Care Ethics Committee of Urmia University.

Rats had free access to water and a commercial pelleted diet. NAFLD was induced by feeding HFD containing 35% fat (31.6% saturated fat and 3.2% unsaturated fat), 57% of the metabolizable energy, for 14 days [23].

2.3. Experimental design

Rats were divided into 5 groups of 6: control group of NAFLD-induced rats; NAFLD-induced rats receiving CMN (200 mg/kg/day, orally) for 14 and 28 consecutive days; NAFLD-induced rats receiving UDCA (80 mg/kg/day, orally) for 14 and 28 consecutive days; NAFLD-induced rats receiving CMN + UDCA (200 and 80 mg/kg/day, orally, respectively) for 14 and 28 consecutive days.

Fasting serum levels of SGOT, SGPT, triglycerides (TG), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) as well as tissue concentrations of TAC, MDA, and NO and the activity of GSH-Px and SOD were measured on day 14 and day 28 of treatment. mRNA and protein levels of p53, bcl-2, and caspase III and only mRNA levels of iNOS were measured 14 days and 28 days after treatment, in all the groups.

2.4. Biochemical analysis and tissue parameters

After overnight fasting, blood samples taken were centrifuged at 5000 g for 10 to extract the serum. Serum levels of SGOT, SGPT and TG were determined using commercial kits (Pars Azmoon, Tehran, Iran). Serum HDL-C and LDL-C concentrations were measured using a spectrophotometric assay with commercial kits (Titr Azmoon, Tehran, Iran). Intra-assay coefficient of variations (CVs) for SGOT, SGPT, TG, HDL-C, and LDL-C were 0.42%, 0.38%, 0.57%, 0.50%, and 0.58%, respectively whereas, those of inter-assay were 0.96%, 0.67%, 0.72%, 0.81%, and 0.89%, respectively.

Liver sample was washed in saline and homogenized in the ratio 1:10 (w:v) with ice-cold 150 mM KC1 for MDA, SOD, GSH-Px, TAC, NO, and protein examination. MDA levels were measured using the thiobarbituric acid reaction as explained earlier [24]. Ferric reduction antioxidant power assay was used to determine TAC levels [25]. Whereas, NO was evaluated based on Köroğlu et al., method [26]. Total protein of tissue was evaluated using Freitas method [27]. The activities of SOD and GSH-px were measured using commercial kits (Rondaxlab, Crumlin, BT 29, UK).

2.5. Assessment of carbohydrates accumulation in hepatocytes

Periodic acid–Schiff (PAS) staining technique was used for the analysis of carbohydrate accumulation in hepatocytes. Briefly, the paraffin embedded tissue sections (5 μm) were deparraffinized and hydrated, which were then oxidized in 5% periodic acid solution for 5 min. Following rinsing with distilled water, the sections were placed in Schiff reagent for 15 min and then washed with lukewarm water for 5 min. Finally, they were incubated with Borax's solution and counterstained with Meyer's hematoxylin for 1 min.

2.6. Immunohistochemical staining

Formalin-fixed paraffin embedded tissue sections (5 μm) were deparaffinized and rehydrated in phosphate-buffered salin (PBS). Antigen retrieval process was performed in sodium citrate buffer (10 mM, pH 7.2). Endogenous peroxide was blocked with respective agents (0.03% hydrogen peroxide containing sodium azide) for 5 min. Tissue sections were washed gently with washing buffer and subsequently incubated with p53 (1:500), bcl-2 (1:600), and caspase III (1:500) biotinylated primary antibodies (Rabbit anti-mouse) for 15 min After gentle rinse with washing buffer and holding them in buffer bath, slides were then placed in a humidified chamber with the necessary amount of streptavidin–HRP (streptavidin conjugated to horseradish peroxidase in PBS containing an anti-microbial agent) and were incubated for 15 min A diaminobenzidine-substrate-chromogen was added to the tissue sections following incubated for 5 min, washing and counterstaining with hematoxylin for 5 s. These sections were immersed in weak ammonia (0.037 M/L) 10 times, rinsed with distilled water and cover slip was placed. Positive immunohistochemical staining was observed as brown stains under a light microscope and results were studied as cells distribution per one mm^2 of the tissue.

2.7. RNA isolation, cDNA synthesis and RT- PCR

Total RNA was extracted from the liver cells using the RNA extraction kit (Fermentase, Cinnagen Co. Iran). To each liver, 1 ml of Tri Reagent was added and 10 mg of liver was then homogenized in homogenizer Precellys (Bertin Technologies, Aix-en-Provance, France). Samples were processed according to the manufacturer's instructions. The isolated RNA was stored at -70°C until used further. Quality and purity of the extracted RNA were measured (260 nm and A260/280 = 1.8–2.0) using NanoDrop-1000 spectrophotometer (Thermo Scientific, Washington, USA).

cDNA was synthesized in a 20 μl reaction mixture containing 1 μg RNA, oligo (dT) primer (1 μl), 5× reaction buffer (4 μl), RNAse inhibitor (1 μl), 10 mM dNTP mix (2 μl) and M-MuLV Reverse Transcriptase (1 μl) as instructed by the manufacturer (Fermentas, GmbH, Germany). The cycling protocol for 20 μl reaction mix was 5 min at 65°C, followed by 60 min at 42°C, and 5 min at 70°C to terminate the reaction.

Total volume of 25 μl with PCR master mix (12.5 μl), forward and reverse specific primers (each 0.75 μl) and cDNA as a template (1 μl) and nuclease free water (10 μl) was used to run PCR. For amplification of bcl-2, caspase III, p53, iNOS, and β-Actin forward and reverse primers, respectively, were designed by Cinnagen Co. Iran. (The forward and reverse (5′-3′) are represented in Table 1). PCR was executed under following program: denaturation at 95°C for 3 min, 1 cycle, followed by 40 cycles at 95°C for 20 s; annealing temperature (62°C for bcl-2, 58°C for p53, 60°C for caspase III, 57°C for iNOS, and 63°C for β-Actin) for 45 s; elongation at 72°C for 1 min and final elongation at 72°C for 5 min. Final PCR products formed were analyzed using 1.5% agarose gel electrophoresis and densitometric analysis of the bands was accomplished through PCR Gel analyzing software (ATP, Tehran, Iran). The control was set at 100% and experimental samples were compared to the control (Table 1).

2.8. Statistical analysis

Analyses were done using Graph Pad Instat@ software (GraphPad

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Table 1
The names and sequences of the primers used for RT-PCR.

Primer	Forward	Reverse
P53	5'-GAG GAGATGATGCTGCTGAG-3	5'-TGCTGCTGCTGCTATTACC-3'
Bcl-2	5-CTGGTG GACAACATCGCT CTG-3	5-GGT CTG CTGACCTCACTTGTG-3
Caspase III	5-TACCCTGAAATGGGCTTGTGT-3	5-GTTAACACGAGTGAGGATGTG-3
iNOS	5- CAG CCCTCAGAGTACAACGAT-3	5- CAGCAGGCACACGCAATGAT-3
β-Actin	5-GTGATGCTGGTGCTGA-3	5-GCTAAGCAGTTGGTGG-3

Software, Inc., La Jolla, CA) and all values were presented as means ± SD. Unpaired Student's t-test, one-way ANOVA and Tukey–Kramer test were used for comparison among the groups. Data regarding biochemical studies were analyzed by ANOVA and Student–Neuman–Keuls test was performed for multiple group comparisons. P value ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. Combinational effects of UDCA and CMN on biochemical serum markers

As shown in Table 2. NAFLD-induced animals had higher serum SGOT and SGPT as compared to controls. CMN and UDCA significantly decreased the serum levels of SGOT and SGPT. Whereas, co-administration of these compounds was associated with greater decrease in SGOT and SGPT levels. In NAFLD-induced groups, concentrations of TG, LDL-C, and HDL-C were significantly elevated and combinational therapy of CMN and UDCA partially restored the levels TG and HDL-C while had no effect on LDL-C (Table 2).

3.2. Combinational effects of UDCA and CMN on carbohydrates accumulation in hepatocytes

In NAFLD-induced rats, administration of CMN and UDCA significantly enhanced intra-cytoplasmic carbohydrates storage in hepatocytes, as determined by PAS staining. Also, compared to CMN and UDCA groups, CMN + UDCA-treated group showed significantly higher percentage of cells with positive PAS staining. Histopathological analyses showed that co-administrating CMN and UDCA significantly reduced fatty degeneration, cellular necrosis, edema, and immune cells infiltration as compared to non-treated NAFLD-induced rats (Fig. 1-A and 1-B).

3.3. Combinational effects of UDCA and CMN on protein expression of p53, caspase III, and bcl-2

RT-PCR results showed that compared to the control group, protein expression of p53 and caspase III was significantly higher, while bcl-2 protein expression was significantly lower in untreated NAFLD-induced rats. Administration of CMN and UDCA significantly decreased p53(Fig. 2-A and 2-B) and caspase III (Fig. 3-A and 3-B) and increased

bcl-2 (Fig. 4-A and 4-B) protein expression. Co-administration therapy, however, was associated with superior results, as compared to CMN and UDCA solo therapies.

3.4. Combinational effects of UDCA and CMN on mRNA expression of p53, caspase III, and bcl-2

Compared to controls, untreated NAFLD-induced rats showed greater expression p53 and caspase III bcl-2 levels were decreased (Fig. 3. CMN and UDCA administration significantly decreased p53 and caspase III (Fig. 5-A) and increased bcl-2 mRNA expression (Fig. 5-B). CMN + UDCA group presented significantly greater reductions in p53 and caspase III and increase in bcl-2 mRNA expression than CMN and UDCA groups.

3.5. Combinational effects of UDCA and CMN on TAC, GSH- Px, SOD activities

Compared to the control group, significant reduction in the activities of TAC, GSH- Px, and SOD were observed in the liver of NAFLD-induced (Table 3). Compared to the un-treated NAFLD-induced rats, CMN and UDCA administration significantly increased the levels of TAC, GSH- Px, and SOD.

3.6. Combinational effects of UDCA and CMN on MDA, NO and iNOS levels

As shown in Table 4, in NAFLD-induced rats, serum NO concentration was significantly higher than controls. CMN and UDCA administration significantly decreased serum NO concentration, while there was a significant increase in the levels of MDA (Table 4). Co-administration therapy, however, resulted in greater increase in TAC, GSH- Px, and SOD and decrease in MDA levels than CMN and UDCA groups (Table 4). We next determined the expression of iNOS in liver and found elevation in the levels of iNOS in NAFLD-induced rats. Compared to untreated NAFLD-induced rats, CMN and UDCA led to the reduction of iNOS expression. Similarly, co-administration therapy, was more effective in case as well. (Fig. 6).

4. Discussion

For the first time, our study has shown therapeutic effects of

Table 2
Serum levels of SGOT, SGPT, TG, LDL and HDL in different groups, data presented as Mean ± SD.

	SGOT U/L	SGPT U/L	TG mg/dl	LDL mg/dl	HDL mg/dl
NAFLD-14	261.62 ± 61.78[a]	156.38 ± 3.94[a]	115.66 ± 8.14[a]	91.00 ± 2.64[a]	90.00 ± 4.00[a]
NAFLD-28	604.37 ± 53.24[a]	276.35 ± 4.58[a]	151.33 ± 9.01[a]	116.66 ± 7.02[a]	120.66 ± 4.16[a]
CMN-14	275.70 ± 51.03[a]	76.35 ± 2.33[b]	82.66 ± 2.51[b]	73.3 ± 4.16[b]	72.33 ± 3.21[b]
CMN-28	402.35 ± 62.52[b]	98.61 ± 5.12[b]	93.00 ± 2.64[b]	82.34 ± 3.21[b]	84.33 ± 3.03[b]
UDCA-14	110.28 ± 19.23[b]	55.38 ± 4.01[c]	78.66 ± 8.08[b]	74.66 ± 4.16[b]	72.50 ± 3.04[b]
UDCA-28	351.62 ± 21.33[b]	42.33 ± 3.00[c]	87.66 ± 3.21[b]	85.33 ± 1.15[b]	82.33 ± 2.08[b]
CMN + UDCA-14	86.38 ± 5.34[c]	28.61 ± 4.62[d]	63.66 ± 3.51[c]	53.33 ± 3.05[c]	60.00 ± 4.00[c]
CMN + UDCA-28	52.38 ± 4.29[c]	18.35 ± 3.34[d]	65.33 ± 4.72[c]	69.66 ± 5.50[c]	66.31 ± 60.4[c]

Note; [a,b,c] are representing significant (P < 0.05) differences between marked groups on same days in same columns.
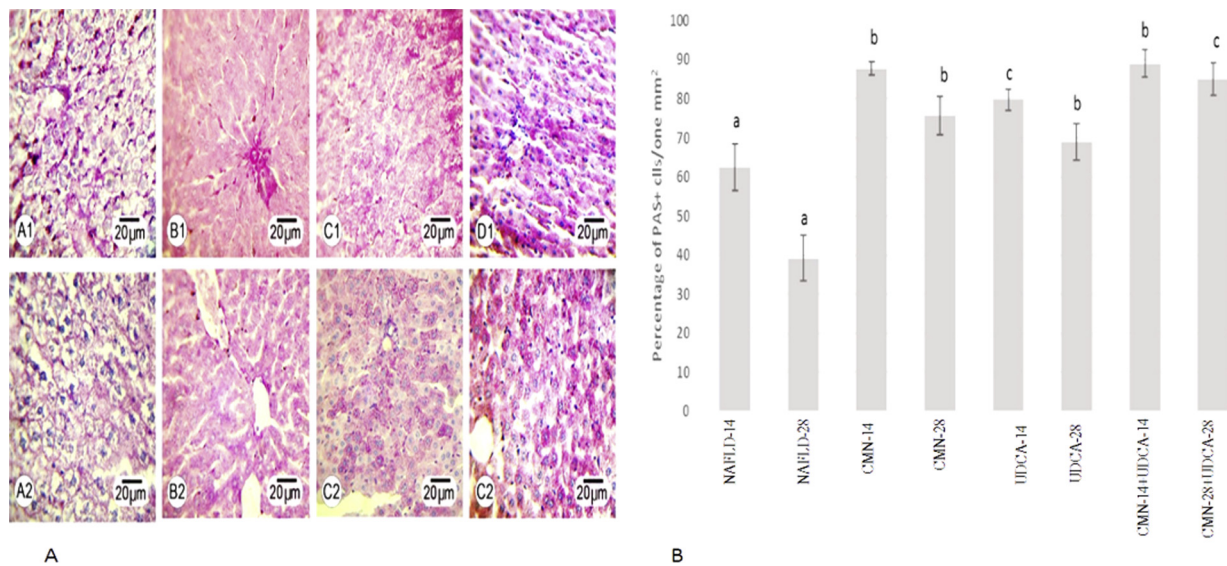
Fig. 1. Cross section from liver tissue; (A) NAFLD-induced (A1: day 14, A2: day 28), (B) CMN-extract-treated (B1: day 14, B2: day28), (C) UDCA-treated (C1: day14, C2: day 28), (D) CMN + UDCA-treated (D1: day14, D2: day28). Note reduced carbohydrate acculturation in hepatocytes of the non-treated NAFLD-induced animals, which is decreased depending on time. However, administrating of CMN-extract and UDCA significantly increased intracytolplasmic carbohydrate ratio. See figures in last column for CMN + UDCA co-administrated groups that are presented with high intracytoplasmic carbohydrate. PAS staining, 600× magnification. **1-B:** Mean percentage of cell with PAS positive reaction in 1 mm² of the liver in different groups, data presented as Mean ± SD. **Note:** [a,b,c] are presenting significant ($P < 0.05$) differences between marked groups on same days.

combinational therapy of CMN with UDCA for the reduction of NAFLD-induced apoptosis, hyperlipidemia, liver injury and inflammation, as well as stabilized mitochondrial membrane integrity.

In this study we showed that NAFLD resulted in over expression of p53 and caspase III expression and reduction in bcl-2 expression/synthesis. Co-administration of CMN with UDCA significantly decreased expression/synthesis of p53 and caspase III and increased bcl-2 expression/synthesis. The importance of p53 and its pro-apoptotic and pro-oxidant downstream target genes in NAFLD are highlighted in the reports, that have shown that the genetic ablation of p53 significantly reduces hepatocellular injury in NAFLD [28,29]. It is seen that p53 regulates lipid accumulation in hepatocytes by directly binding to the glucose-6-phosphatase dehydrogenase (*G6PD*), this complex (p53-

G6PD) results in a significant inhibition of pentose phosphate pathway [30]. However, it should be considered that, p53 not only a tumor suppressor pro-apoptotic protein orchestrates hepatocellular apoptosis, but also negatively affects glucose metabolism by triggering insulin resistance [31,32]. Additionally, enhanced expression of p53 in untreated NAFLD-induced rats positively is correlated with reduced intracytoplasmic carbohydrate storage. In contrast, the CMN and UDCA-treated groups (more significant in CMN + UDCA-received animals) exhibited elevated intra-cytoplasmic carbohydrate ratio associated with diminished steatosis and p53 expression/synthesis. Mitochondrial membrane permeability largely depends on bcl-2-induced integrity. Thus, suppressed expression of bcl-2 leads to caspase-dependent protein release into cytosol, which consequently mediates apoptosis in
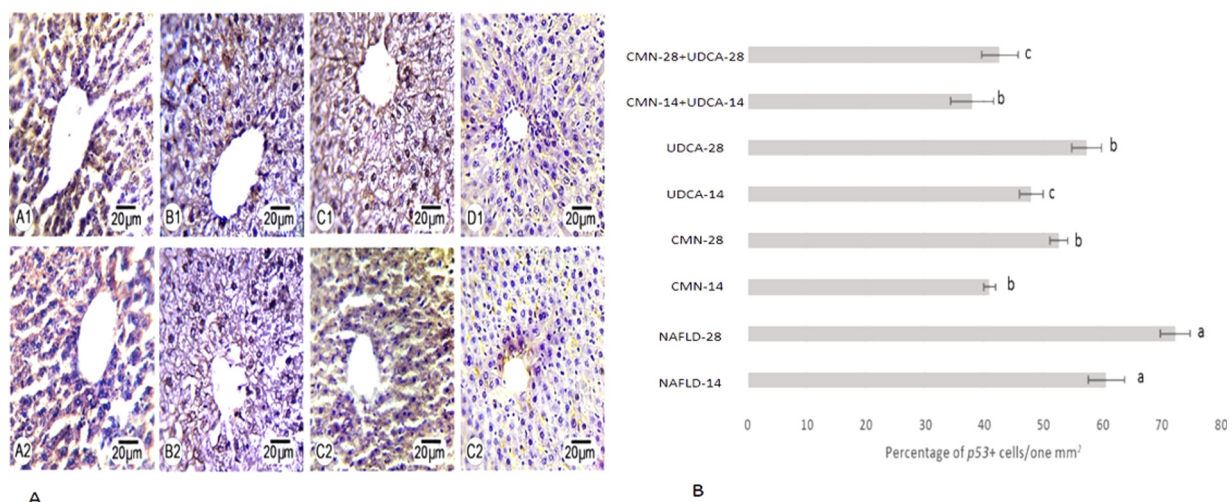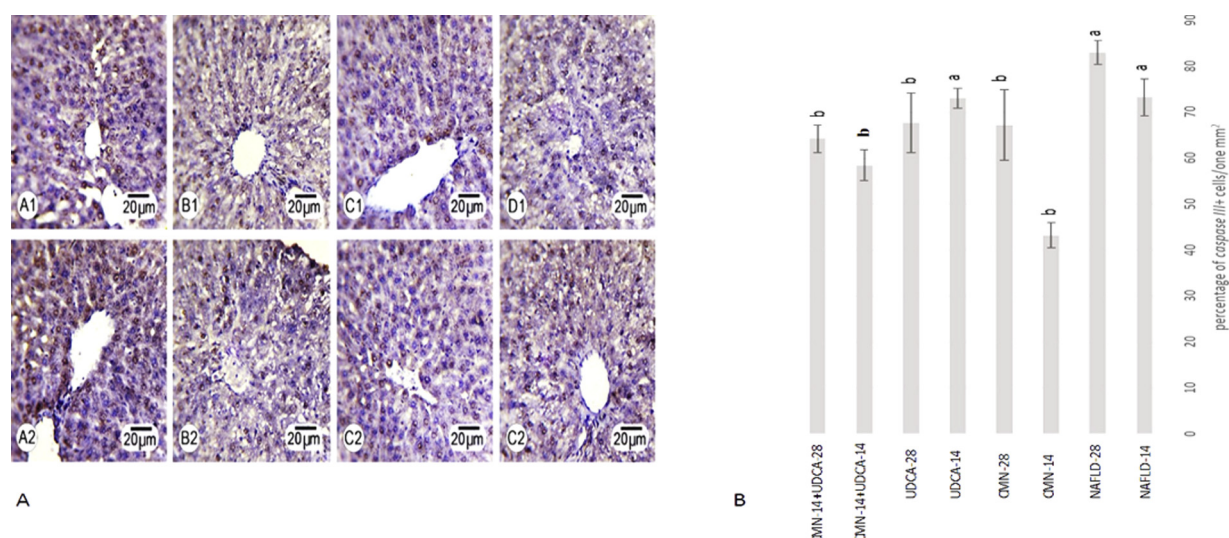

Fig. 2. Cross section from liver tissue; (**A**) NAFLD-induced (A1: day 14, A2: day 28), (**B**) CMN-extract-treated (B1: day 14, B2: day28), (**C**) UDCA-treated (C1: day14, C2: day 28), (**D**) CMN + UDCA-treated (D1: day14, D2: day28). Cross sections from non-treated NAFLD-induced groups are representing time dependently increased *p53* expression. Meanwhile, the animals in CMN-extract and UDCA-treated groups are presented with significantly lower expression of the *p53*. See figures in last column for CMN + UDCA co-administrated groups with remarkably lower *p53* expression versus non-treated animals. Immunohistochemical staining for *p53*, 600× magnification. **2-B:** Mean percentage of cell with *p53* positive reaction in one mm² of the liver in different groups, all data are presented in Mean ± SD. **Note:** [a,b,c] are presenting significant ($P < 0.05$) differences between marked groups on same days.

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Fig. 3. Cross section from liver tissue; (**A**) NAFLD-induced (A1: day 14, A2: day 28), (**B**) CMN-extract-treated (B1: day 14, B2: day28), (**C**) UDCA-treated (C1: day14, C2: day 28), (**D**) CMN + UDCA-treated (D1: day14, D2: day28). Cross sections from non-treated NAFLD-induced groups are representing time dependently increased *caspase III* expression. Meanwhile, the animals in CMN-extract and UDCA-treated groups are presented with significantly lower expression of the *caspase III*. See figures in last column for CMN + UDCA co-administrated groups with remarkably lower *caspase III* expression versus non-treated animals. Immunohistochemical staining for *caspase III*, $600\times$ magnification. **3-B:** Mean percentage of cell with *caspase III* positive reaction in one mm^2 of the liver in different groups, all data are presented in Mean $\pm$ SD. **Note:** [a,b,c,d] are presenting significant ($P < 0.05$) differences between marked groups on same days.
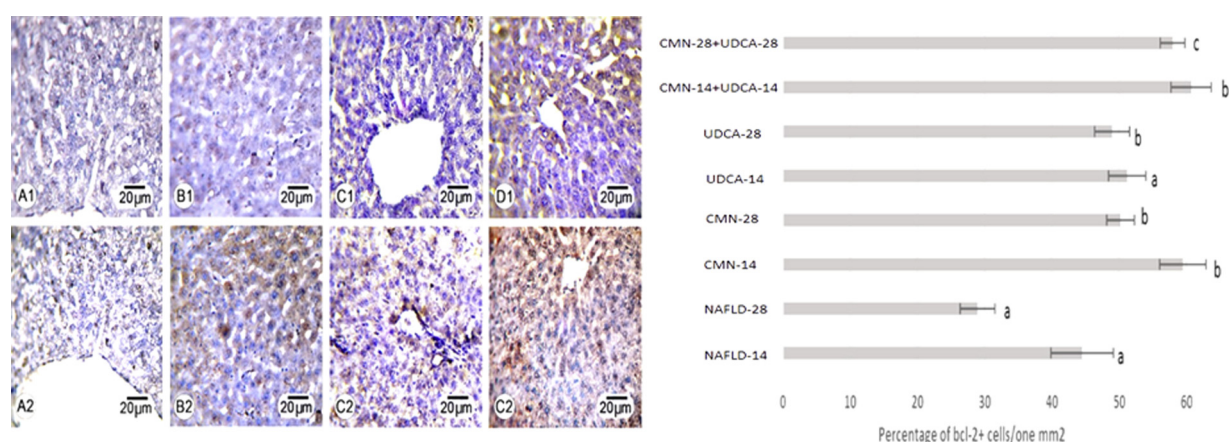

Fig. 4. Cross section from liver tissue; (**A**) NAFLD-induced (A1: day 14, A2: day 28), (**B**) CMN-extract-treated (B1: day 14, B2: day28), (**C**) UDCA-treated (C1: day14, C2: day 28), (**D**) CMN + UDCA-treated (D1: day14, D2: day28). See decreased *bcl-2* in non-treated NAFLD-induced animals, depending on time. However, alone and simultaneous administration of CMN-extract and UDCA resulted in enhanced *bcl-2* expression on both 14 and 28 days after NAFLD-induction. Immunohistochemical staining for *caspase III*, $600\times$ magnification. **4-B:** Mean percentage of cell with *bcl-2* positive reaction in one mm^2 of the liver in different groups, all data are presented in Mean $\pm$ SD. **Note:** [a,b,c] are presenting significant ($P < 0.05$) differences between marked groups on same days.

hepatocytes [33]. However, constitutive expression of bcl-2 is required to avoid spontaneous activation of caspase III and VII [34]. Hence, the bcl-2 is one of the significant proteins that plays an essential role in hepatocellular survival in NAFLD by maintaining the hepatocytes mitochondrial membrane integrity.

Our results showed that NAFLD resulted in severe oxidative stress (as measured by antioxidant status) and inflammation (as measured by SGOT, SGPT, NO, and iNOS levels). Co-administration of CMN with UDCA, remarkably diminished NAFLD-induced pathological effects in the liver of the animals by up-regulating the enzymatic anti-oxidation and reducing the inflammatory response. Previous reports have shown that, inflammation plays an important role in NAFLD patients [35–37], where dysregulation in the production of cytokines plays crtical role [38]. Accordingly, iNOS gene is under the transcriptional control of a several inflammatory mediators such as cytokines and lipopolysaccharide [39–41]. Therefore, decrease in inflammation in liver, white adipose tissue, and intestine has been reported as an effect of UDCA

treatment [42].

In healthy conditions, upregulation anti-oxidation in association with the elevated expression of bcl-2 results in stabilizing mitochondrial membrane, which leads to inhibition of caspase III expression. Moreover, decreased iNOS, the pathologically-induced ROS, triggers lipid peroxidation which in turn initiates release of malindialdehyde (MDA). After binding to hepatocytes or activating hepatic stellate cells, MDA initiates adverse immune response by stimulating collagen synthesis and neutrophil chemotaxis [43].

Collectively, it is evident from our study that single administration CMN and UDCA, respectively and combination of these compounds could partially reduce the NAFLD-induced damage by suppressing the p53 expression at both mRNA and protein level. Therefore, diminished p53 in turn may affect pentose phosphate pathway and/or glucose metabolism and albeit apoptosis.

Thus, we can suggest that, CMN and UDCA up-regulate the hepatocellular survival by protecting the mitochondrial membrane integrity
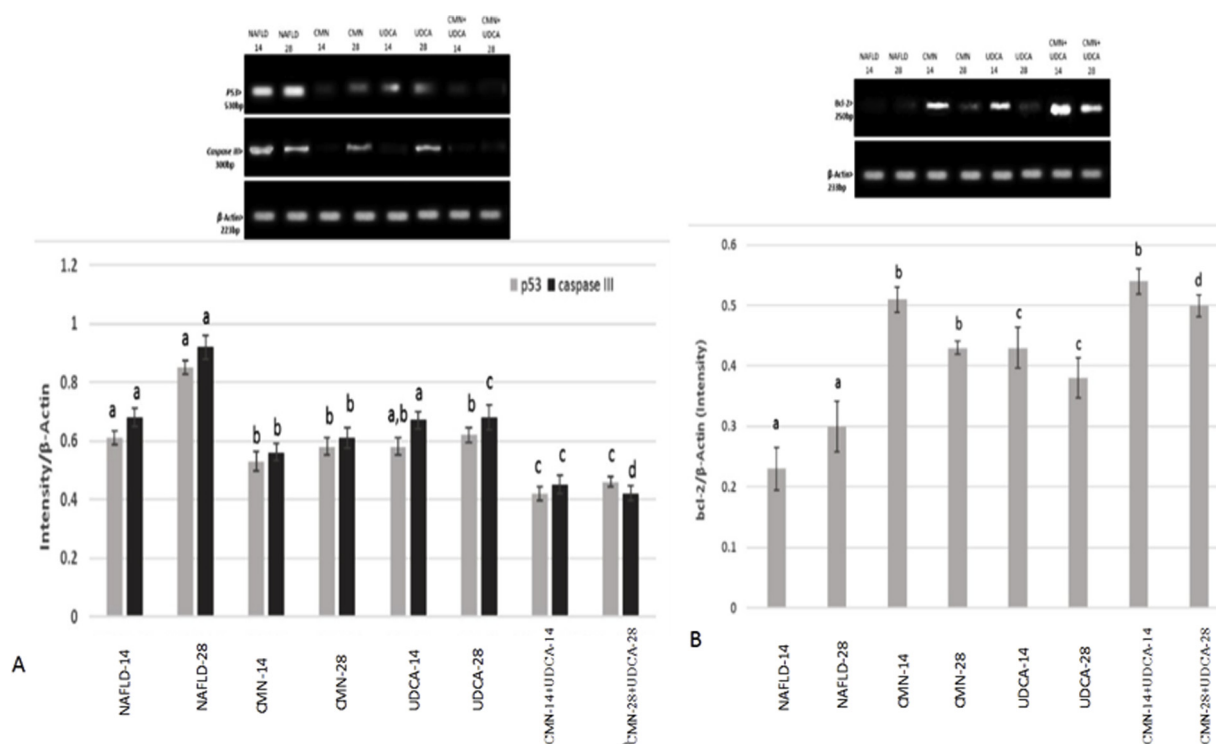
Fig. 5. mRNA expression of p53 and caspase III in different groups on days 14 and 28. CMN-extract and UDCA significantly reduced mRNA contents of p53 and caspase III on both 14 and 28 days after NAFLD-induction. Note semiquantitative data for p53 and caspase III mRNA levels that are represented in Mean ± SD based on β-Actin intensity. 5-B: mRNA expression of bcl-2 in different groups on days 14 and 28. CMN-extract and UDCA significantly enhanced mRNA contents of bcl-2 on both 14 and 28 days after NAFLD-induction. Note semiquantitative data for bcl-2 mRNA levels that are represented in Mean ± SD based on β-Actin intensity. Note: a,b,c,d are presenting significant (P < 0.05) differences between marked groups on same days.

Table 3
Mean alterations of TAC, GPX and SOD in different groups, all data are presented in Mean ± SD.

	TAC (mmol/mg protein)	GPX (U/L)	SOD (U/L)
NAFLD-14	1.55 ± 0.27[a]	41.66 ± 1.52[a]	22.33 ± 2.08[a]
NAFLD-28	0.84 ± 0.15[a]	34.00 ± 4.00[a]	15.33 ± 3.05[a]
CMN-14	2.32 ± 0.09[b]	91.00 ± 5.56[b]	55.00 ± 2.64[b]
CMN-28	1.58 ± 0.25[b]	75.33 ± 5.03[b]	41.66 ± 1.52[b]
UDCA-14	1.95 ± 0.08[c]	89.66 ± 4.50[b]	52.33 ± 2.08[b]
UDCA-28	1.18 ± 0.40[b]	70.33 ± 3.51[b]	40.33 ± 2.51[b]
CMN + UDCA-14	2.83 ± 0.18[d]	97.33 ± 2.51[b]	62.33 ± 2.08[c]
CMN + UDCA-28	1.92 ± 0.39[b]	91.00 ± 4.35[c]	52.00 ± 1.00[c]

Note; a,b,c are representing significant (P < 0.05) differences between marked groups on same days in same columns.

Table 4
Mean alterations of MDA and NO in different groups, all data are presented in Mean ± SD.

	MDA (mmol/mg protein)	NO (mmol/mg protein)
NAFLD-14	13.16 ± 1.21[a]	19.90 ± 1.46[a]
NAFLD-28	21.19 ± 1.16[a]	34.15 ± 1.88[a]
CMN-14	5.55 ± 0.69[b]	10.55 ± 0.78[b]
CMN-28	7.85 ± 0.57[b]	15.32 ± 0.99[b]
UDCA-14	5.84 ± 0.38[c]	11.85 ± 0.57[c]
UDCA-28	9.10 ± 0.84[c]	16.15 ± 0.95[c]
CMN + UDCA-14	3.80 ± 0.45[d]	6.84 ± 0.40[d]
CMN + UDCA-28	6.49 ± 0.57[d]	9.49 ± 0.84[d]

Note; a,b,c,d are representing significant (P < 0.05) differences between marked groups on same days in same columns.

via enhancement of the expression of bcl-2. Whereas, combinational therapy has greater efficacy in this regard.

In conclusion, the CMN in solo and combinational form with UDCA ameliorates NAFLD-induced apoptosis, fatty degeneration and liver injury via up-regulating anti-oxidative responses by the action of GSH-Px and SOD and elevating TAC level, stabilizing mitochondrial membrane integrity by up-regulating bcl-2 and controlling p53 and caspase III expression/synthesis along with anti-inflammatory response by suppressing the production of iNOS, NO, SGPT, SGOT.

Conflict of interest

The authors deny any conflict of interest in any terms or by any means during the study. All charges are provided form research center fund and disbursed accordingly.

Contributions

SG and HEG: Planned the study, wrote the protocol, collected the data and drafted the manuscript and accepted the final draft; BMM and AF: Planned and designed the study, collected the data. LZ, analyzed the data and critically revised the draft and finally approved the manuscript.
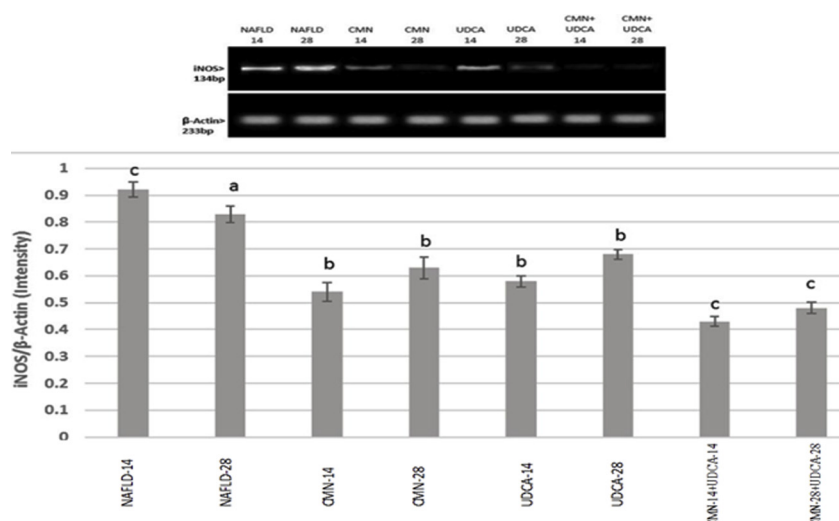
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Fig. 6. mRNA expression of iNOS in different groups on days 14 and 28. CMN-extract and UDCA significantly reduced mRNA contents of iNOS on both 14 and 28 days after NAFLD-induction. Note semiquantitative data for iNOS mRNA levels that are represented in Mean ± SD based on β-Actin intensity. Note: a,b,c are presenting significant (P < 0.05) differences between marked groups on same days.

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