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Original Article

Anti-carcinogenic effects and mechanisms of actions of *Citrus limon* fruit peel hydroethanolic extract and limonene in diethylnitrosmine/2-acetylaminofluorene-induced hepatocellular carcinoma in Wistar rats

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Abstract: Hepatocellular carcinoma (HCC) is the third most common cause of cancer death and disability in the world. Citrus species and their constituents have many biological activities including antioxidant, anti-inflammatory and anti-carcinogenic properties. This study aimed to assess the anti-carcinogenic effects and postulate the possible mechanisms of action for Citrus limon fruit peel hydroethanolic extract (CLFPHE) and limonene in diethylnitrosamine (DEN)/2-acetylaminofluorene (2AAF)-induced HCC in male Wistar rats. For analysis and characterization of CLFPHE, gas chromatography-mass spectrum (GC-MS) and high performance liquid chromatography (HPLC) methods were applied. A HCC was elaborated by DEN intraperitoneal injection (150 mg/kg/week) for two weeks followed by oral delivery of 2AAF (20 mg/kg) four times a week for three weeks. The DEN/2AAF-administered rats were treated with CLFPHE (50 mg/kg) and limonene (20 mg/kg) by oral gavage every other day for 24 weeks. CLFPHE and limonene significantly attenuated the harmful effects of DEN on liver function. Histopathological analysis confirmed that both treatments inhibited DEN/2AAF-induced tumorigenesis in association with the suppression of serum tumor markers including AFP, CEA, and CA19.9 and liver proliferator indicator (Ki-67). Moreover, CLFPHE and limonene prevented the oxidative stress and enhanced the antioxidant defenses in DEN/2AAF-administered rats. These ameliorations were manifested by decreases in liver lipid peroxidation, increases in GSH, SOD and GPx levels and upregulation of Nrf2. The treatments also abated inflammation by suppressing TNF-α and IL-1β levels and IL-8 and NF-κB expression. CLFPHE and limonene substantially decreased hepatic BCL-2, IQGAP1, IQGAP3, HRAS, KRAS and Ki-67 while they elevated BAX, P53, PDCD5 and IQGAP2 expressions. Our findings suggest that CLFPHE and limonene may abate HCC development via enhancement of apoptotic, antioxidant, cell anti-proliferatory and anti-inflammatory effects.

Keywords: Hepatocellular carcinoma, Citrus limon peel extract, limonene, diethylnitrosamine, acetylaminofluorene

Introduction

Hepatocellular carcinoma (HCC) ranks third in the world among cancer-related deaths and may be associated with cirrhosis or liver fibrosis that resulted from repeated liver damage. The immune system is involved in the liver fibrosis development, the degree of damage to necrotic-inflammatory tissue, and an increase in the incidence of HCC [1, 2]. Liver fibrosis is characterized by altered liver vascularization, extracellular matrix composition, and medication metabolism, making animal models with fibrosis highly relevant to evaluate HCC medicines for their efficacy against tumour initiation and/or progression [3].

Diethylnitrosamine (DEN) is hepatocarcinogenic and has toxic effects in model of animal [4]. Pharmaceutical substances, cured meat, cos-

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metics, and tobacco smoke and cooked foods all contain DEN [5]. Enzymes of the cytochrome P450 family activated DEN, ultimately producing reactive electrophile species that are cytotoxic, carcinogenic, and oxidative [6]. As a result, DEN has been shown to cause oxidative stress, cell damage, free radical production, and DNA structural changes [7]. A persistently produced DEN rat model mimics the pathophysiology of HCC. This involves damage to the liver, chronic inflammatory response, proliferation of hepatocytes, cirrhosis and liver fibrosis, disorganized vasculature, and alters in the immune microenvironment of the liver [8].

Inflammation is a natural immune response to hazards signals. Disruption of tissue and/or infection is both possible outcomes. While transient and properly terminated inflammation is beneficial, chronic inflammation raises the risk of cancer [9]. Cancer-related inflammation, which involves complex interactions between stromal and epithelial cells, can occasionally result in epigenetic changes that fuel the progression of cancer and even start tumor development. However, chronic inflammation more generally leads to the production of growth factors that encourage the emergence of new tumors [10]. Oncogenic and proinflammatory signaling pathways are also closely related, also recognized for activating genes governing inflammatory cell signaling processes and carcinogenesis-related procedures are numerous oncogenic chemicals [11].

Apoptosis is one kind of controlled cell death which might take on by mitochondria-mediated pathways (intrinsic apoptosis) or surface death receptors (extrinsic apoptosis) [12]. During pathophysiological stimuli, as a method of cell death as a mechanism for cell death, programed cell death is essential for maintaining the homeostasis of cells [13]. Cancerous cells typically disrupt the ratio of proteins that prevent and promote apoptosis to avoid apoptosis. The cancer cells are also able to avoid apoptosis due to decreased caspase action and compromised death receptor (DR) signaling [12, 14].

Citrus species, plants belonging to the rutaceae family, are widely distributed worldwide [15]. Citrus fruit peels can provide functional compounds and preservatives for the more recent food products [16]. Due to their antioxidant properties, polyphenolic compounds (such as phenolic acids and flavonoids) are significant fruit phytochemicals [17]. Phenolic compounds' capacity to eliminate free radicals, inhibit radical cycles, as well as chelate metals related to their antioxidant property [18].

Fruits that naturally contain limonene, a monoterpene, include grapefruit (95%), tangerine (94%), orange (91%), mandarin (72%), lemon (65%), and elemi (50%) [19]. Plant-based nutrients are widely used because of their powerful effects on the human body and high antioxidant content, which enable them to be used as a resource for dynamic and highly natural additives that reduce oxidative stress and lipid peroxidation [20]. D-limonene has been demonstrated in numerous studies to possess antioxidant qualities, reduce lipid peroxidation, and guard against cell damage caused by free radicals.

Consequently, this study aims to assess the anti-carcinogenic efficacy and to hypothesize the action's mechanisms of *Citrus limon* fruit peel hydroethanolic extract and limonene in DEN/2AAF-induced HCC in male Wistar rats *via* investigating the effects on cell proliferation, apoptosis, inflammation, oxidative stress and antioxidant defense system.

Materials and methods

Chemicals

DEN, 2AAF and limonene were purchased from Sigma Chemicals Co., St. Louis, MO, USA, and were kept at 2-4°C. The additional substances utilized in the procedures and experiments were all of the analytical variety.

Collection of Citrus limon fruit peel and preparation of extracts

Citrus limon fruit peel was obtained from Local market of Minya (Egypt). Following several rounds of washing in tap water and then distilled water, it was dehydrated by air in the shade. Citrus limon fruit peel verified by staff members of the Taxonomy Division, Botany Department, Faculty of Science, Beni-Suef

University, Egypt. Citrus limon fruit peel crushed using an electric grinder, then extracted at room temperature by maceration in 70% aqueous ethanol until exhaustion. The filtrate was then concentrated when vacuumed in a rotary evaporator after filtering. The result of residue was kept at -20°C until use in biological assessment.

Gas chromatography-mass spectrometry analysis

Gas chromatography-mass spectrometry (GC-MS) is an analytical method that combines the capabilities of GC and mass MS. This approach is used to detect various elements inside a test sample. Furthermore, it may detect trace levels of components in material that were previously thought too degraded for detection. GC-MS allows for the evaluation and identification of minute amounts of a substance, making it a useful tool in analytical chemistry and forensic investigations [21]. The chemical components of Citrus limon fruit peel hydroethanolic extract were determined using Agilent Technologies' 7890A/5975C Inert MS-GC system with a triple-axis detector. The analysis was carried out at the Central Laboratory for Postgraduate Studies in Advanced Sciences at Beni-Suef University. A 1 µl splitless mode injection of a hydroethanolic extract from fruits and seeds was used for the analysis. The temperature of the injection port was maintained at 250°C. The oven temperature programme began at 120°C and increased by 5°C per minute to 220°C. After that, it increased by 8°C per minute to 280°C for five minutes. The study took 32.5 minutes to complete, using helium gas as the carrier gas at a flow rate of 1.0 ml/min. By comparing the mass spectra of the constituent parts with the derivative spectra found in the Library Search Report (C:\Database\NIST11.L; C:\Database\demo.l), the components were identified. The system took use of the triple-axis detector's capability to conduct accurate chemical analysis.

HPLC analysis of phenolic constituents

HPLC analysis was performed using an Agilent 1260 series equipment at Central Laboratories Network, National Research Centre, and Cairo, Egypt. A Zorbax Eclipse Plus C8 column (4.6 mm × 250 mm i.d., 5 µm) was used to perform the separation. At a flow rate of 0.9 ml/min, the mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B). A linear gradient was used to program the mobile phase, as seen below: 0 min (82% A); 0-1 min (82% A); 1-11 min (75% A); 11-18 min (60% A); 18-22 min (82% A); and 22-24 min. A multi-wavelength detector was used to detect at 280 nm. Each sample solution had an injection volume of 5 µl, and the temperature of the column was kept constant at 40°C.

Study of cytotoxicity in vitro

Cell culture and cell lines: HCC cell lines of HepG2 were obtained from the Egyptian tissue culture department at the Holding Company for Biological Products and Vaccines (VACSERA). Due to the possibility of cross-contamination or infection, we have verified the authenticity of HepG2 cells that were employed in this investigation. Three steps made up our standard authentication process. 1) To verify the general health of these cells before experimentation, we evaluated the morphology of these cells (cell size, shape, and performance). 2) To ensure that the HepG2 cells were healthy, their proliferation was measured during their log growth phase. Population doubling times were determined by analyzing growth curves. 3) A PCR-based method was employed to identify multiple mycoplasma strains through detection and identification of mycoplasma contamination.

Culture of cells: The cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI1640; Lonza Verviers SPRL, Belgium) in 75 cm^2 cell culture flasks at a concentration of 5×10^4 with 10% FBS and 50 µg/mL gentamycin. The cultures were kept at 37°C and incubated in 5% CO_2. The cells were trypsinized and passaged once they had attained a confluence of 70%-80%. MTT was used in the test, which involved the metabolization of MTT and the creation of a purple hue using a method developed by Serva Electrophoresis in Germany. An appropriate quantity of cells was established onto 96-well plates and exposed for 24 hours (hrs) to various concentrations of CLFPHE and limonene (10,000, 5000, 2500, 1250, 625, 312.5, 165.25, 78.12, 39.06, 19.53, 9.76, and 4.88

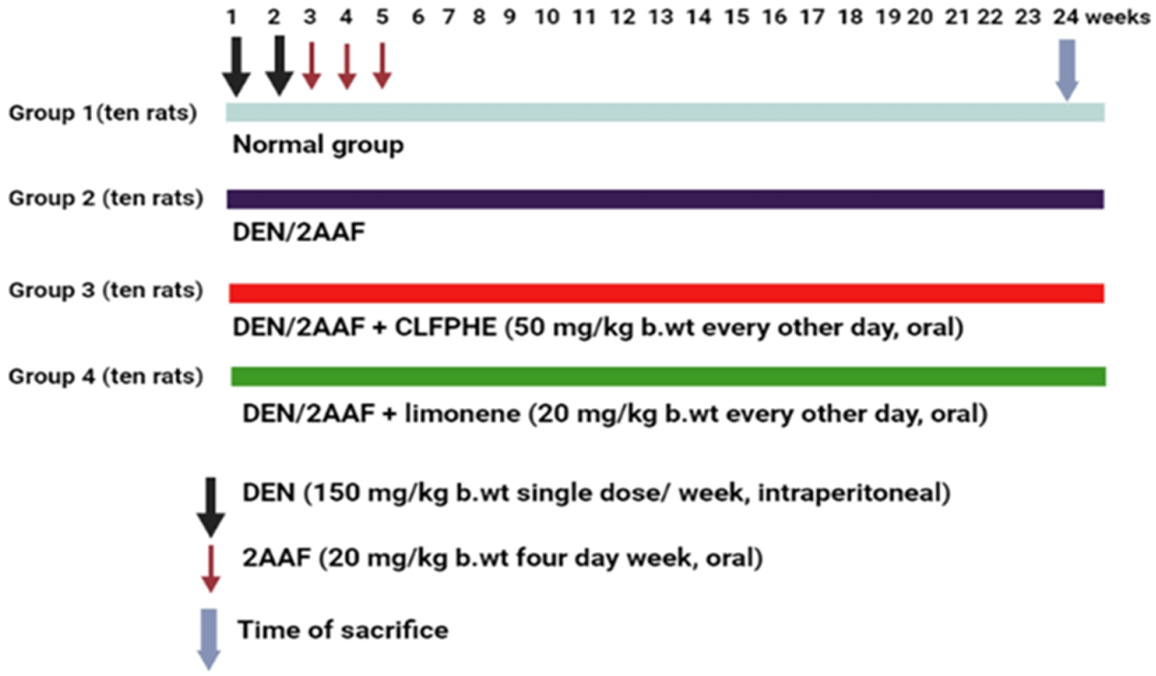

Figure 1. Schematic of the experimental design and animal grouping.

μg/mL) for 24 hrs. Both CLFPHE and limonene were dissolved in dimethyl sulfoxide (DMSO). The cells were treated with 50 L of MTT (0.5 mg/mL) for 4 hrs in an incubator with 5% CO_2 after being cleaned in sterile phosphate-buffered saline (PBS). DMSO was used to dissolve formazan crystals. Using a microplate reader (Sunrise, TECAN, Inc., USA), the optical density at 590 nm was detected. [(ODt/ODc)] × 100% was used to determine the viability percentage, where ODt is the mean OD of wells treated with the substance of test and ODc is the OD of the control. The concentration of inhibitors of 50% is described as the amount that prevents the viability of HepG2 cells by 50% (IC_{50}). By utilizing dose-response graphs, IC_{50} can be determined [22].

In vivo investigation

Animals of experiments: Adult male Wistar rats weighing between 120-140 g were employed in the present investigation. The animals were purchased from the Egyptian Organization for Biological Products and Vaccines (VACSERA), located in Cairo's Helwan Station. Two weeks of animal observation were performed prior to the experiment to ensure that there were no recurrent infections. Rats were maintained in good-aerated polypropylene cages in Faculty of Science Animal House (Beni-Suef University, Egypt) at average cycle of daylight (10-12 hrs/day), a temperature range of 20-25°C, and sufficient food and water at all times. The Beni-Suef University's Experimental Animal Ethics Committee gave its seal of approval to all experimental methods and the study protocol (Ethical Approval Number: FS/2018/18). This committee oversees the Faculty's use and care of animals in research. Precautions were taken to minimize animal pain and distress.

Design of experiment: Four groups of adult male Wistar rats were established, each with twelve rats. Group I was the control group. The remaining three groups received for two weeks DEN intraperitoneally (i.p.) twice weekly at a dose of 150 mg/kg body weight (b.w.) and during the subsequent 3 weeks 2AAF (20 mg/kg b.w.) via oral gavage four times per week [23]. Group II was considered as positive control group and received DEN/2AAF; groups III and IV received DEN/2AAF and were given oral treatments with CLFPHE (50 mg/kg b.w.) [24] and limonene of (20 mg/kg b.w.) [25] respectively for 24 weeks, every other day during the duration of the experiment (**Figure 1**).

Blood and liver tissue sampling: Twenty-four weeks, diethyl ether inhalation was used to give animals a mild anesthetic before they were sacrificed. Samples of blood were collected, followed by clotting, were centrifuged at 3000 r.p.m. for 15 minutes and serum were gathered, maintained at a temperature of -30°C for measuring tumor markers and liver function parameters. After the sacrifice, decapitation by head dislocation and dissection were performed. Each animal's liver was removed, washed by sterile saline perfusion (0.9% NaCl) and blotted with filter paper to remove saline. From each, four pieces of liver were removed. One liver sample (1 g) was homogenized in 10 mL of 0.9% sodium chloride to produce 1% homogenate (weight/volume) after being washed with ice-cooled sterile saline solution. After centrifuging liver homogenates at 3000 r.p.m. for 15 minutes, the supernatants were used to study oxidative stress, antioxidant activities, and the concentrations of inflammation-related biomarkers like TNF-α, IL-1β, and Nrf2. The second piece of liver was kept at -70°C in sterilized tubes for RNA isolation and real-time (RT)-polymerase chain reaction (PCR) assays. Additionally, a third liver tissue sample was also kept at -70°C pending Western blot analysis of Ki-67. After being fixed for a day in 10% neutral buffered formalin, the fourth liver portion was prepared for cutting into sections and histopathological analysis using the H&E stain.

Detection of liver function markers in serum: Kits provided by HUMAN (Gesellschaft für Biochemica und Diagnostica mbH, Max-Planck-Ring 21, 65205 Wiesbaden, Germany) were used to assess ALT, AST, ALP activities and total bilirubin and albumin levels.

Detection of tumor markers levels in serum: ELISA kits purchased from R&D systems (Minneapolis, MN, USA) were used to measure AFP, CEA, and CA19.9 serum levels.

Detection of antioxidant status and oxidative stress detection: Liver LPO, GSH, GPx and SOD were examined with kits from Bio-diagnostic company (Dokki, Giza, Egypt).

Detection of hepatic TNF-α, IL1β and Nrf2: Levels of TNF-α, IL-1β, and Nrf2 in the tissue of liver rat measurements were made in accordance with the product's instructions (R&D Systems) (Minneapolis, MN, USA).

RNA isolation and qRT PCR

Total RNA was extracted from lung tissue homogenates using the manufacturer's instructions and the TRIzol reagent (Gibco, Invitrogen; Eugene, OR, USA). At 260 nm, RNA absorbance was measured using the approach of Sthoeger et al. [26]. The quality of the RNA was determined by calculating the ratio of absorbances recorded at 260 and 280 nm. The first strand of cDNA was generated using Applied Biosystems®' high-capacity cDNA reverse transcription kit (Foster City, California, USA). For quantitative PCR studies of target gene mRNA expression, the ABI Prism 7500 System and 96-well optical reaction plates were used.

To summarize, the cDNA was amplified via PCR (Applied Biosystems) utilizing a 25-μL reaction mixture that contained 1.25 μL of cDNA sample, 11.05 μL of nuclease-free water, 12.5 μL of SYBR Green Universal Master mix, and 0.1 μL of 10 μM forward and reverse primers (final concentration of each primer was 40 nM). **Table 1** shows the primers used in these experiments. The real-time PCR data was analyzed using the relative gene expression technique (ΔΔCT) provided in Applied Biosystem User Bulletin No. 2. Normalization of each sample and gene was done using the β-actin gene.

Western blots analysis

Protein was extracted from liver samples using a ReadyPrepTM protein extraction kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. Protein was then determined using a Bradford assay kit (SK3041; Bio Basic Inc., Markham, Ontario, Canada L3R 8T4), separated using 10% SDS-PAGE (Cat. #161-0181; Bio-Rad Laboratories, Inc.), and transferred to polyvinylidene difluoride (PDVF) membranes. Next, skim milk (5%) mixed in Tris-buffered saline with Tween 20 (TBST) was used to block the membranes. Following that, the membranes were incubated for a whole night at 4°C with the appropriate primary antibodies against Ki-67 (Cat. #AB9260; EMD Millipore Corporation, Teme-

Table 1. Primer sequences for rats

Genes	Sequence	GenBank Accession Number
IQGAP1	F: 5'GCGGCTTCCAACAAGATGTTT3' R: 5'CAGCAGTTCATGGATGGGGT3'	NM_001108489.1
IQGAP2	F: 5'GGAATCTACGGACGCTGGAG3' R: CACAGTACTGGGTGTGTCCC3'	XM_039103456.1
IQGAP3	F: 5'AGCCTATGATCGTCTCACAGC3' R: 5'CACAGGTACTGGTAGGCGAC3'	NM_001191709.1
NF-κB	F: 5'TTCAACATGGCAGACGACGA3' R: 5'TGCTCTAGTATTTGAAGGTATGGG3'	NM_001276711.1
IL-8	F: 5'CAGAGACTTGGGAGCCACTC3' R: 5'CAGAGTAAAGGGCGGGTCAG3'	X77797.1
BCL-2	F: 5'TAAGCTGTCACAGAGGGGCT3' R: 5'TGAAGAGTTCCTCCACCACC3'	NM_016993.1
BAX	F: 5'CTGGATCCAAGACCAGGGTG3' R: 5'CCTTTCCCCTTCCCCCATTC3'	NM_007527.3
P53	F: 5'GTTTTTGTTCCTGAGCCCCG3' R: 5'GAGCAAGGGGTGACTTTGGG3'	NM_030989.3
PDCD5	F: TGAAGCGATTCCAACCGAGT3' R: 5'GCTCCGTGGGTCTGTCTAAG3'	NM_001106247.1
HRAS	F: 5'CTGTCCTGACACCAGGCTC3' R: 5'ATGGACCCTCCTGTAGCCAT3'	NM_001130441.1
KRAS	F: 5'AGACACGAAACAGGCTCAGG3' R: 5'GCATCGTCAACACCCTGTCT3'	NM_031515.3
β-actin	F: 5'TCACTATCGGCAATGTGCGG3' R: 5'GCTCAGGAGGAGCAATGATG3'	NM_031144.3

cula, CA, USA) and β-actin (Sc-8432; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The chemiluminescent substrate (ClarityTM Western ECL substrate, Bio-Rad Laboratories, Inc.) was then used to develop the membranes, and the corresponding HRP-conjugated secondary antibodies (goat anti-rabbit IgG-HRP-1 mg goat monoclonal antibody; Novus Biologicals, Littleton, CO, USA) were used to probe them. Charge-coupled device (CCD) camera-based imager was employed to capture the chemiluminescent signals. Applications of the ChemiDoc MP imager were used for analyses.

Histopathological investigation

Once the animals were dissected, the livers were taken out. Before being studied histopathologically, the liver was placed into 70% alcohol and fixed in neutral buffered formalin for 24 hrs. For slide microtome sectioning, paraffin wax tissue blocks with a 4 micron thickness were created. Haematoxylin and eosin (H&E) stain was applied to the deparaffinized tissue samples before being attached to glass slides [27]. The investigation was done utilizing an electric light microscope.

Analytical statistics

The final outcomes were displayed as mean ± standard error. The one-way analysis of variance (ANOVA) was employed to identify statistical contrasts between groups (SPSS version 20 software, Chicago, IL, USA), proceeded by Duncan's test comparing various groups with a significance level of $P < 0.05$.

Results

GC-MS analysis

GC-MS analysis of the *Citrus limon* fruit peel hydroethanolic extract demonstrated phytocomponents. The described phytocomponents are shown in **Figure 2** and summarized in **Table 2** with their molecular weight (MW), molecular formula, retention time (RT), and relative abundance represented as peak area % and activity.

HPLC analysis

Using HPLC analysis, the majority of 19 phenolic compounds were found in *Citrus limon* fruit peel hydroethanolic extract. High concentrations of catechin, chlorogenic acid, syringic acid, gallic acid, daidzein, rutin and naringenin were among the chemicals discovered in **Figure 3** and **Table 3**.

Cytotoxicity and the MTT assay

The MTT assay was employed to assess the viability of HepG2 cells after treatment with CLFPHE and limonene at different dosages (0-10,000 μg/mL) for 24 hours (**Figure 4**). CLFPHE and limonene significantly retarded HepG2 viability, with IC_{50} values of 316.0 and 26.35 μg/mL for CLFPHE and limonene, accordingly.

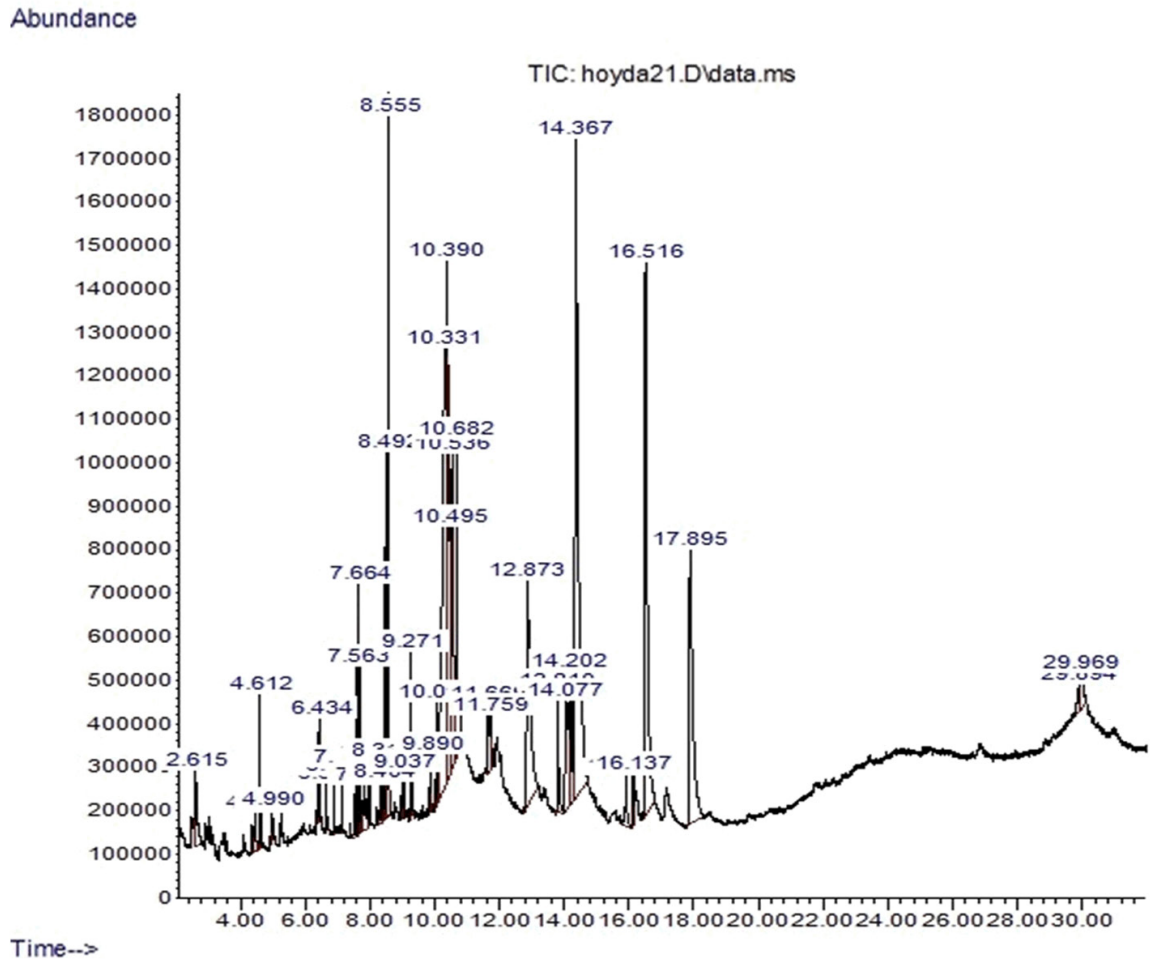

Figure 2. GC-MS analysis of the *Citrus limon* fruit peel hydroethanolic extract.

In vivo investigation

The influence of CLFPHE and limonene on serum liver function: Compared to rats under normal management, administration of the DEN/2AAF resulted in a significant increase in serum ALT, AST, and ALP activities and total bilirubin level, as well as a significant decrease in albumin level. When compared to the DEN/2AAF-administered control, CLFPHE and limonene significantly decreased ALT, AST, and ALP activities, as well as total bilirubin level, and increased albumin level (**Table 4**).

The influence of CLFPHE and limonene on serum levels of AFP, CEA, and CA19.9: The administration of DEN/2AAF resulted in a significant increase ($P < 0.05$) in serum AFP, CEA and CA19.9 levels in comparison to rats of normal control group. Orally treatment of DEN/2AAF-administered rats with CLFPHE and limonene produced a significant amelioration ($P < 0.05$) of tumor marker levels in comparison to DEN/2AAF-administered control (**Figure 5**).

The influence of CLFPHE and limonene on the hepatic oxidative stress and anti-oxidant defense system: DEN/2AAF administration significantly increased LPO and decreased GSH content in liver tissue in comparison to rats under normal control, whereas the therapy with CLFPHE and limonene resulting in a substantial diminishment in LPO and a significant an improvement in GSH content in comparison with DEN/2AAF control group. Additionally, administration of DEN/2AAF notably decreased SOD and GPx activities in comparison to rats under normal conditions, treatment with CLFPHE and limonene repaired SOD and GPx activities. All together, these findings suggest

Table 2. The chemical profile of the *Citrus limon* fruit peel hydroethanolic extract as identified by GC-MS analysis

RT	Name of the Compound	Molecular Formula	MW	Peak Area %
2.613	beta.-Pinene	$C_{10}H_{16}$	136.23	1.33%
4.467	3-Cyclohexen-1-ol,4-methyl-1-(1-methylethyl)-, (R)-	$C_{10}H_{18}O$	154.24	0.39%
4.610	3-Cyclohexene-1-methanol, alpha,alpha,4-trimethyl-, 1-acetate, (1R)-	$C_{12}H_{20}O_2$	196.29	0.93%
4.988	Hexanoic acid, 3,7-dimethyl-2,6-octadienyl ester, (E)-	$C_{16}H_{28}O_2$	252.39	0.23%
6.436	Cyclohexene,4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)-	$C_{15}H_{24}$	204.35	0.52%
6.659	2,6-Octadien-1-ol, 3,7-dimethyl-,(Z)-	$C_{10}H_{18}O$	154.25	0.25%
6.905	3-Carene	$C_{10}H_{16}$	136.23	0.33%
7.147	Cyclohexane,1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-,[1S (1.alpha.,2. beta.,4.beta.)]-	$C_{15}H_{24}$	204.35	0.34%
7.563	Caryophyllene	$C_{15}H_{24}$	204.35	1.02%
7.666	1,3,6,10-Dodecatetraene,3,7,11-trimethyl-, (Z,E)-	$C_{15}H_{24}$	204.35	1.45%
7.849	1,6,10-Dodecatriene,7,11-dimethyl-3-methylene-, (Z)-	$C_{15}H_{24}$	204.35	0.62%
7.992	alpha-Santalol	$C_{15}H_{24}O$	220.35	0.40%
8.318	1,6-Cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl)-	$C_{15}H_{24}$	204.35	0.42%
8.404	Eudesma-4(14),11-diene	$C_{15}H_{24}$	204.35	0.27%
8.490	alpha.-Farnesene	$C_{15}H_{24}$	204.35	2.49%
8.553	Cyclohexene,1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-, (S)-	$C_{15}H_{24}$	204.35	4.16%
9.039	No match found			
9.274	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-,[1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]-	$C_{15}H_{24}O$	220.35	0.98%
9.892	1,2,4-Methenoazulene,decahydro-1,5,5,8a-tetramethyl-, [1S-(1α,2α,3aβ,4α,8aβ,9R*)]-	$C_{15}H_{24}$	204.35	0.35%
10.332	1-(p-Toluidino)-1-deoxy-.beta.-d-idopyranose	$C_{13}H_{19}NO_5$	269.3	11.93%
10.390	Undecanoic acid	$C_{11}H_{22}O_2$	186.29	10.30%
10.492	No match found			1.75
10.538	2(1H)-Naphthalenone,4a,5,6,7,8,8a hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-	$C_{15}H_{22}O$	218.35	4.11
10.681	Cyclohexene,1-methyl-4-(5-methyl-1-methylenehexyl)-	$C_{15}H_{26}$	206.37	3.88
11.671	Lactose	$C_{12}H_{22}O_{11}$	342.30	1.19
11.757	9-Ethyl-9-borabicyclo[3.3.1]nonane	$C_{10}H_{19}B$	150.07	0.47
12.873	N-(4-Hydroxy-2-benzothiazolyl)acetamide	$C_9H_8N_2O_2S$	208.24	6.04
13.811	Palmitic acid	$C_{16}H_{32}O_2$	256.42	1.22
14.074	No match found			
14.200	Hexadecenoic acid ethyl ester	$C_{18}H_{36}O_2$	284.5	1.66
14.366	2H-1-Benzopyran-2-one, 5,7-dimethoxy-	$C_{11}H_{10}O_4$	206.19	16.14
15.923	No match found			
16.134	6-Dodecanol	$C_{12}H_{26}O$	186.33	0.52
16.518	Anthracene, 9-ethyl-	$C_{16}H_{14}$	206.28	10.46
17.897	7H-Furo[3,2-g][1]benzopyran-7-one,4,9-dimethoxy-	$C_{13}H_{10}O_5$	246.21	7.51
29.896	Silane,trimethyl[5-methyl-2-(1-methylethyl)phenoxy]-	$C_{13}H_{22}OSi$	222.40	0.39
29.970	2,4-Dimethylbenzo[h]quinoline	$C_{15}H_{13}N$	207.27	0.93

that both CLFPHE and limonene can help to reduce DEN/2AAF-induced oxidative stress by keeping the antioxidant defense system of the cell (**Table 5**).

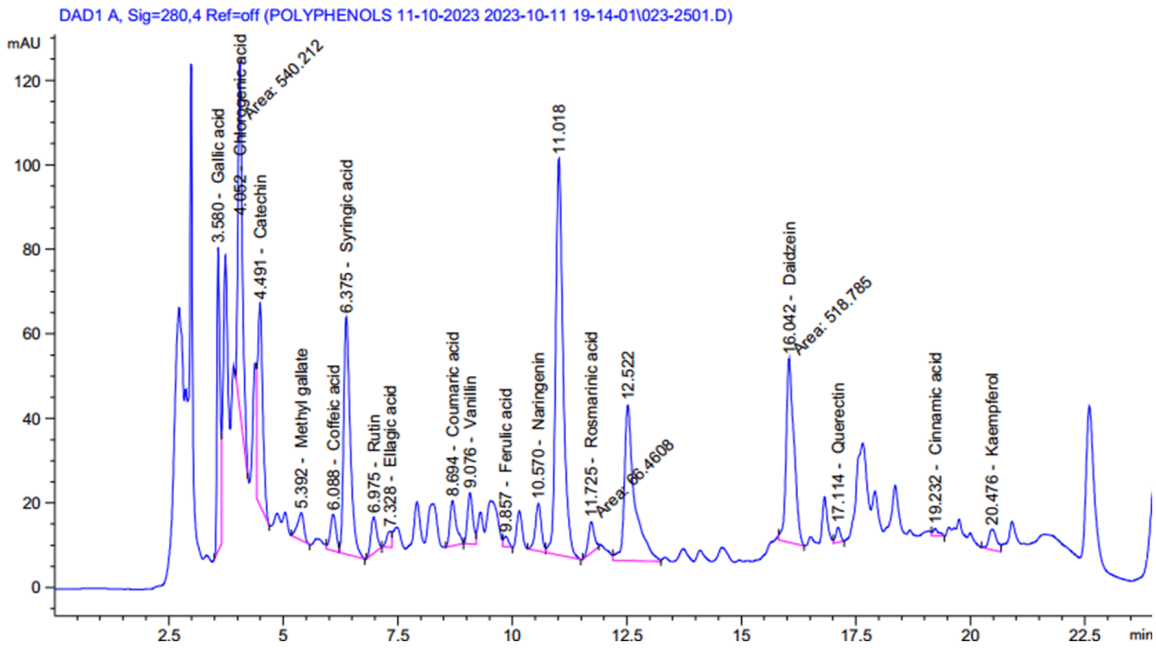

Figure 3. HPLC analysis of phenolic constituents of *Citrus limon* fruit peel hydroethanolic extract.

Table 3. Phenolic constituents of the ethanolic extract of *Citrus limon* fruit peel hydroethanolic extract using HPLC analysis

Name of the Compound	Area	Conc. (μg/ml)	Conc. (μg/g)
Gallic acid	322.35	28.69	1434.61
Chlorogenic acid	540.21	73.15	3657.71
Catechin	376.39	85.63	4281.43
Methyl gallate	63.42	3.29	164.51
Coffeic acid	75.79	6.08	303.90
Syringic acid	561.40	42.83	2141.26
Pyro catechol	0.00	0.00	0.00
Rutin	75.04	12.58	628.82
Ellagic acid	28.53	2.61	130.30
Coumaric acid	101.32	3.73	186.30
Vanillin	96.90	3.65	182.61
Ferulic acid	18.31	1.10	55.06
Naringenin	102.89	9.73	486.43
Rosmarinic acid	66.46	7.32	366.07
Daidzein	518.78	30.13	1506.39
Querectin	25.36	3.37	168.46
Cinnamic acid	15.60	0.29	14.51
Kaempferol	59.63	4.08	203.79

The influence of CLFPHE and limonene on the hepatic TNF-α, IL-1β and Nrf2 levels: Rats given DEN/2AAF revealed a substantial rise in hepatic TNF-α and IL-1β levels while a substantial decrease in Nrf2 level compared to the normal control rats. The other hand, supplementing with CLFPHE and limonene to DEN/2AAF-administered rats led to a substantial improvement from the elevated TNF-α and IL-1β in addition the decrease in Nrf2 in comparison to DEN/2AAF-administration lonely (**Figure 6**).

The influence of CLFPHE and limonene on the hepatic NF-κB and IL-8 expressions: The administration of DEN/2AAF resulted in significant increases ($P < 0.05$) in hepatic NF-κB and IL-8 expressions in comparison to normal rats, and these alterations were significantly ($P < 0.05$) improved by CLFPHE and limonene treatments. The efficacies of CLFPHE and limonene were more or less similar (**Figure 7**).

The influence of CLFPHE and limonene on the hepatic BCL2, BAX, P53 and PDCD5 mRNA expressions: The giving DEN/2AAF resulted in a significant increase ($P < 0.05$) in BCL2 expression in comparison to normal rats; this response was significantly ($P < 0.05$) improved by treatments with CLFPHE and limonene, which had more or less similar effects.

On the other hand, DEN/2AAF administration led to a significant reduction ($P < 0.05$) in the

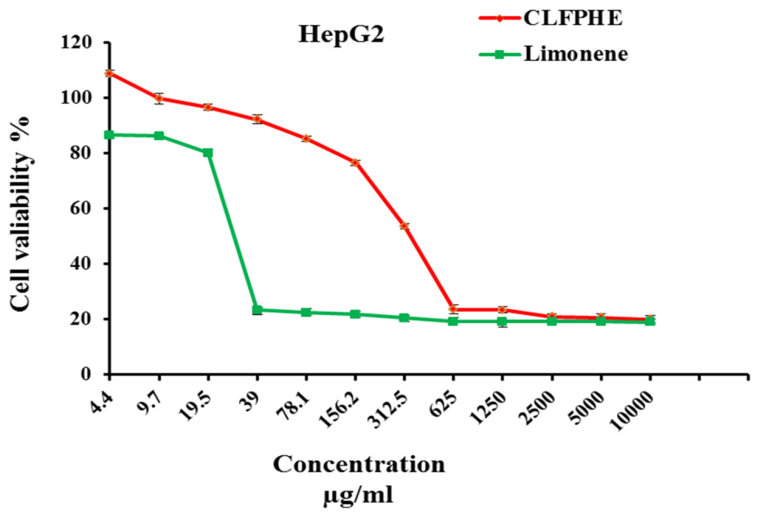

Figure 4. Effects of CLFPHE and limonene with different doses (0-10,000 µg/mL) for 24 h, on HepG2 cells' viability percent.

mRNA expression of BAX, P53 and PDCD5 in comparison to normal rats. The treatment of DEN/2AAF-administered rats with CLFPHE and limonene resulted in a significant increase ($P < 0.05$) of the lowered BAX, P53 and PDCD5 expression (**Figure 8**). While liver P53 expression was normalized by CLFPHE and limonene, BAX expression increased significantly ($P < 0.05$) above normal values and PDCD5 expression was still significantly lower ($P < 0.05$) than its normal levels.

The influence of CLFPHE and limonene on the hepatic IQGAP1, IQGAP2, IQGAP3, HRAS and KRAS expressions: Wistar rats given DEN/2AAF exhibited an increased expression of IQGAP1, IQGAP3, HRAS and KRAS and a lower level of expression of IQGAP2. The treatments of DEN/2AAF with CLFPHE and limonene successfully improved ($P < 0.05$) these alteration (**Figure 9**). While the treatments produced a significant downregulation ($P < 0.05$) of the elevated IQGAP1, IQGAP3, HRAS and KRAS expression, they induced a significant upregulation ($P < 0.05$) of IQGAP2.

The influence of CLFPHE and limonene on the hepatic Ki-67 protein expression: The DEN/2AAF group showed a significant overexpression ($P < 0.05$) of the liver of Ki-67 indicating cellular hyperproliferation along with the elevations of tumor markers. Oral supplementation of CLFPHE and limonene to DEN/2AAF-administered rats produced a significant amelioration ($P < 0.05$) of the elevated Ki-67 protein expression (**Figure 10**).

Histopathological results

In **Figure 11**, histopathological examination of liver section of normal rat liver displaying the typical liver lobule. The hepatocytes exhibited incredibly eosinophilic and cytoplasm with granules and unique nuclei. In among the strands of the hepatocytes, hepatic sinusoids are displayed. A photomicrograph from a part of the liver of rat administered DEN showing congested portal tract in association with moderate infiltration of inflammation in the portal tracts and compressed hepatocytes that surrounding it leading to interruption of the hepatic lobule. The tumor cells exhibited well-differentiated forms and resemble hepatocytes form and present as trabeculae, cords, and nests. The primary developing of septa between the hepatic lobules was noticed. A photomicrograph from section of rat's liver given administered DEN and CLFPHE exhibiting the portal tract appeared nearly as normal form. A single tumor cells with large nuclei and hepatic lobule appeared nearly as normal form. A section of liver of rat given DEN and limonene showing the hepatic lobule displayed like normal structure and the portal tract appeared nearly as normal form.

Discussion

HCC is the fifth most typical malignancy and the second-most common reason of death from cancer worldwide [28]. DEN, either alone or in combination with cancer promoters like 2AAF, has been used to cause HCC [29]. In the current research, we employed a combination of DEN and 2AAF to enhance liver cancer development in Wistar rats. Hence, the design of hepatocarcinogenesis caused by DEN as a triggering factor and 2AAF as promoter was selected at this time to assess the safety and effectiveness of CLFPHE and limonene.

Within this investigation, DEN induced hepatocellular injury is amply demonstrated by the

Table 4. The influence of CLFPHE and limonene on serum activities of liver function

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	Total Bilirubin (mg/dL)	Albumin (g/dL)
Normal	39.48±3.74[a]	98.92±1.92[a]	212.50±5.51[a]	0.20±0.02[a]	3.76±0.12[c]
DEN/2AAF	68.05±4.79[b]	164.33±7.06[c]	498.66±31.71[c]	0.85±0.07[c]	2.97±0.06[a]
DEN/2AAF+CLFPHE	48.10±3.94[a]	122.33±8.44[b]	341.50±43.72[b]	0.48±0.04[b]	3.29±0.09[b]
DEN/2AAF+limonene	45.20±4.21[a]	139.00±5.93[b]	324.33±21.44[b]	0.44±0.05[b]	3.23±0.04[b]

Data are presented as mean ± standard error. There are six identified samples in each category. Means, which have different symbols (a, b and c) in the same column, are significantly different at $P < 0.05$.

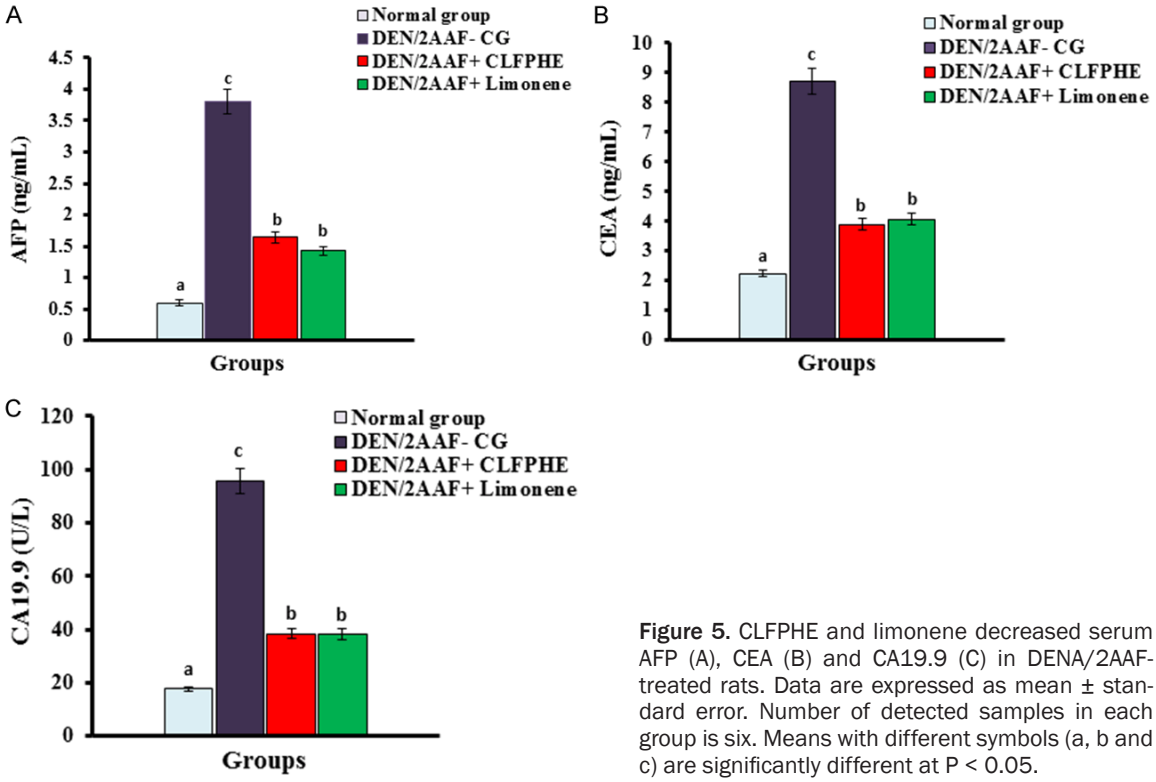

Figure 5. CLFPHE and limonene decreased serum AFP (A), CEA (B) and CA19.9 (C) in DENA/2AAF-treated rats. Data are expressed as mean ± standard error. Number of detected samples in each group is six. Means with different symbols (a, b and c) are significantly different at $P < 0.05$.

Table 5. The influence of CLFPHE and limonene on the hepatic oxidative stress and anti-oxidant defense system

Groups	LPO (nmolMDA/100 mg tissue)	GSH (nmol/100 mg tissue)	SOD (U/g tissue)	GPx (mU/100 mg tissue)
Normal	22.47±0.58[a]	69.90±3.41[c]	33.6±0.49[c]	123.13±1.3[c]
DEN/2AAF	63.80±0.56[c]	33.47±0.55[a]	9.30±0.32[a]	65.47±1.02[a]
DEN/2AAF+CLFPHE	34.43±6.20[b]	40.47±1.81[b]	19.90±0.81[b]	102.23±2.0[b]
DEN/2AAF+limonene	36.67±4.77[b]	42.60±1.08[b]	21.03±1.15[b]	98.27±1.08[b]

Data are presented as mean ± standard error. There are six identified samples in each category. Means, which have different superscript symbols (a, b and c) in the same column, are significantly different at $P < 0.05$. CLFPHE, *Citrus limon* fruit peel hydroethanolic extract; LPO, lipid peroxidation; GSH, glutathione; SOD, superoxide dismutase; GPx, glutathione peroxidase.

major rise in the activities of serum ALT, AST, ALP, and total bilirubin in addition to decreased serum albumin, in line based on earlier investigations [30]. The increase in LPO and ROS play an important role in the damaging effects of DEN (**Figure 12**). Increases in serum ALT and

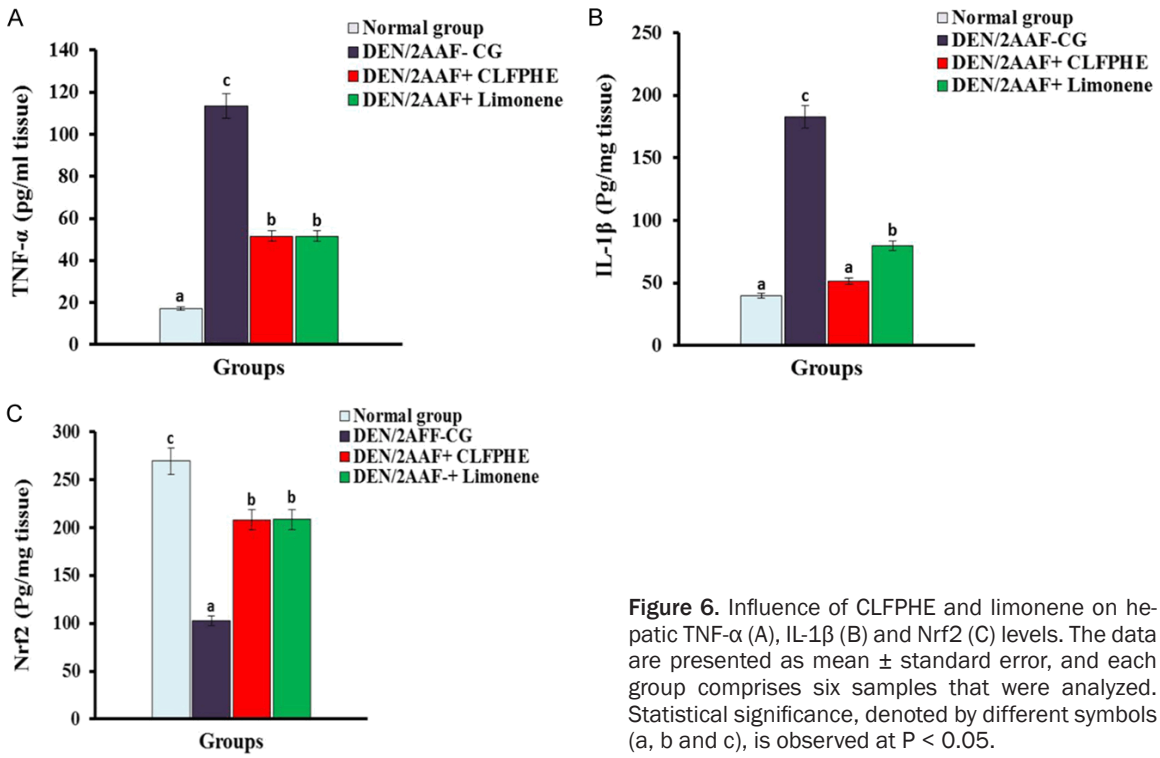

Figure 6. Influence of CLFPHE and limonene on hepatic TNF-α (A), IL-1β (B) and Nrf2 (C) levels. The data are presented as mean ± standard error, and each group comprises six samples that were analyzed. Statistical significance, denoted by different symbols (a, b and c), is observed at $P < 0.05$.

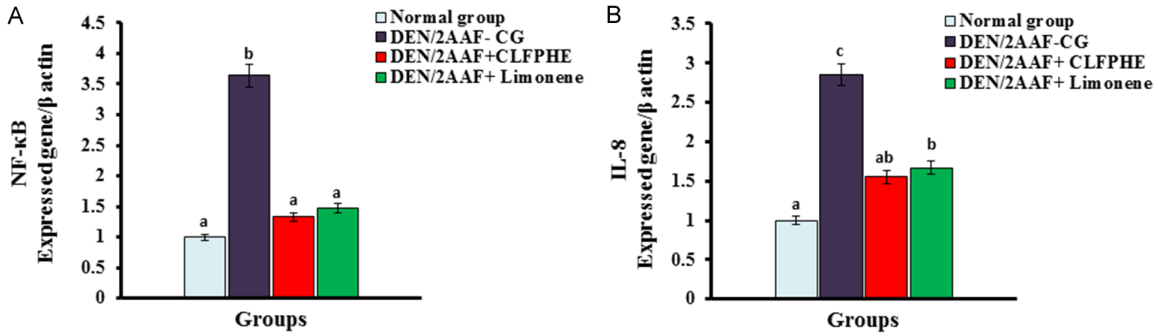

Figure 7. Influences of CLFPHE and limonene on (A) NF-κB and (B) IL-8 expressions in DENA/2AAF-supplied rats. The data are presented as mean ± standard error, and each group comprises six samples that were analyzed. Statistical significance, denoted by different symbols (a, b and c), is observed at $P < 0.05$.

AST are resulting from a leak from injured hepatic cells [31]. The increase in serum bilirubin level and ALP activity indicate liver inflammation and hepatobiliary illnesses with pathological changes to biliary flow [32, 33]. Changes in the metabolism of proteins and free amino acids as well as their production in the liver can be attributed for the decrease in serum albumin [34]. The improvement in the membrane-bound enzyme, ALP, by CLFPHE and limonene reflected that these treatments are able to stop membrane integrity loss [35-37]. These results are in accordance with those provided by Zhou et al. [38] who found that lemon ethanol extract is hepatoprotective for liver damage and Bacanlı et al. [39] who showed that treatment with limonene prevented changes in liver enzymes.

DEN/2AAF-administration significantly raised levels in the serum of tumor markers including AFP, CEA, and CA19.9 and that rise might be due to DEN/2AAF-caused liver damage and displays the elevated risk of liver cancer. Those tumor marker findings are also corresponding with the earlier research of Ahmed et al. [30]

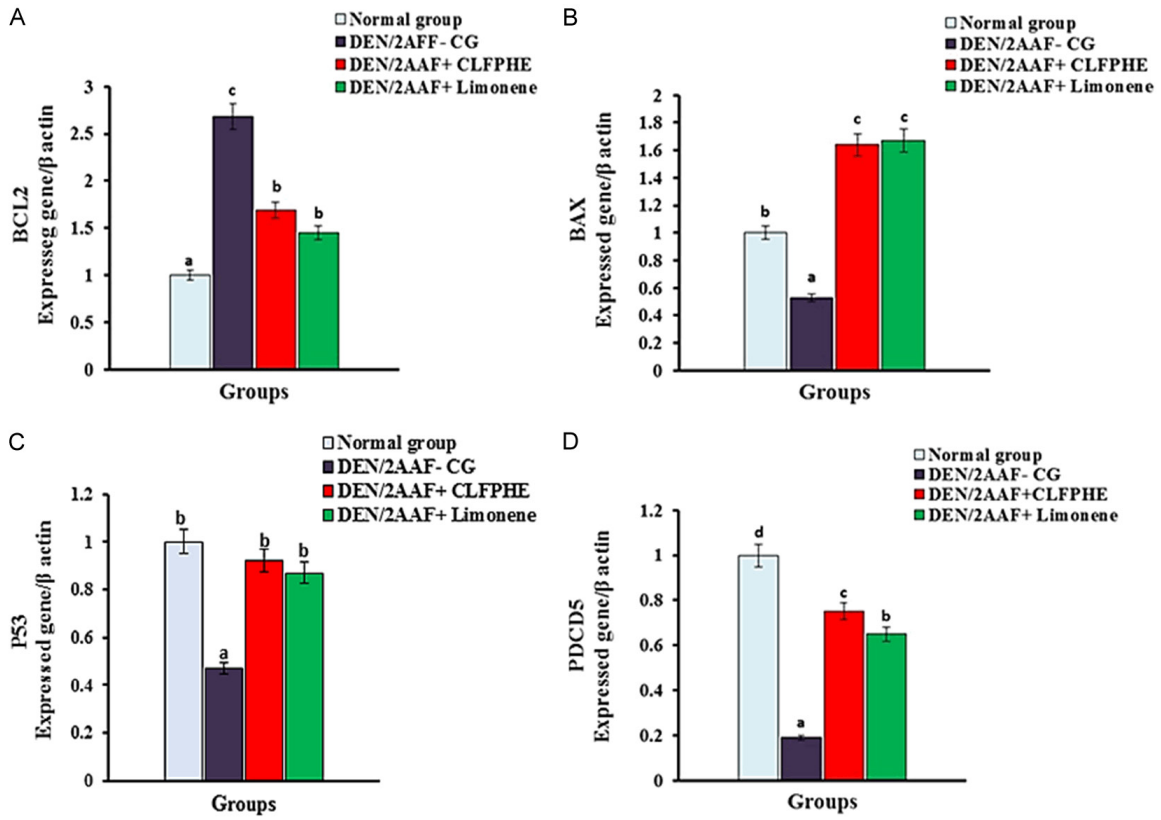

Figure 8. Influences of CLFPHE and limonene on hepatic BCL2 (A), BAX (B), P53 (C), and PDCD5 (D) expressions in DEN/2AAF-supplied rats. The data are presented as mean ± standard error, and each group comprises six samples that were analyzed. Statistical significance, denoted by different symbols (a, b and c), is observed at $P < 0.05$.

and Yassin et al. [40] according to which, the administration DEN to rats prompted a rise in the circulating AFP, CEA and CA19.9 levels. Newly regenerated and modified cells when liver damage occurs result in the newly expressed and production of AFP and CEA from these cells [41]. A raised level of CA19.9 in serum was additionally realized in a small proportion of HCC patients [42]. The current evidence supports these discovering changes in the liver's histology which showed the existence of lesions that are malignant or precancerous. The supplementation of CLFPHE and limonene reduced the high levels of serum AFP, CEA and CA19.9, so giving evidence of a decline risk for HCC and liver damage.

As part of the ongoing investigation, DEN-triggered HCC caused liver oxidative stress and changed the endogenous antioxidant enzymes. DEN decreased the endogenous antioxidant defense mechanism by increasing LPO and decreasing the activity of GPx and SOD as well as GSH content this outcome corresponds to Ahmed et al. [30] and Ahmed et al. [43]. Previous research indicates that DEN was processed in hepatic tissue by the way one acts of cytochrome p450 enzymes and lastly, elevated oxidative stress [44, 45]. DEN administration raised the LPO reaction together with the suppression of endogenous antioxidant enzymes [46]. ROS stimulate oxidative stress and cytotoxicity by destroying biomolecules such as DNA, lipids, and proteins. The decreased levels of GSH, GPx, and SOD frequently observed because reduction in their biosynthesis during damage of liver [47, 48]. The levels of these non-enzymatic and enzymatic antioxidant are decreased as a result of excessive use in the elimination free radicals which are generated throughout the metabolism of DEN in the liver [49].

The treatment with CLFPHE and limonene reduced the higher level of LPO in the liver; consequently, they decreased the free radicals'

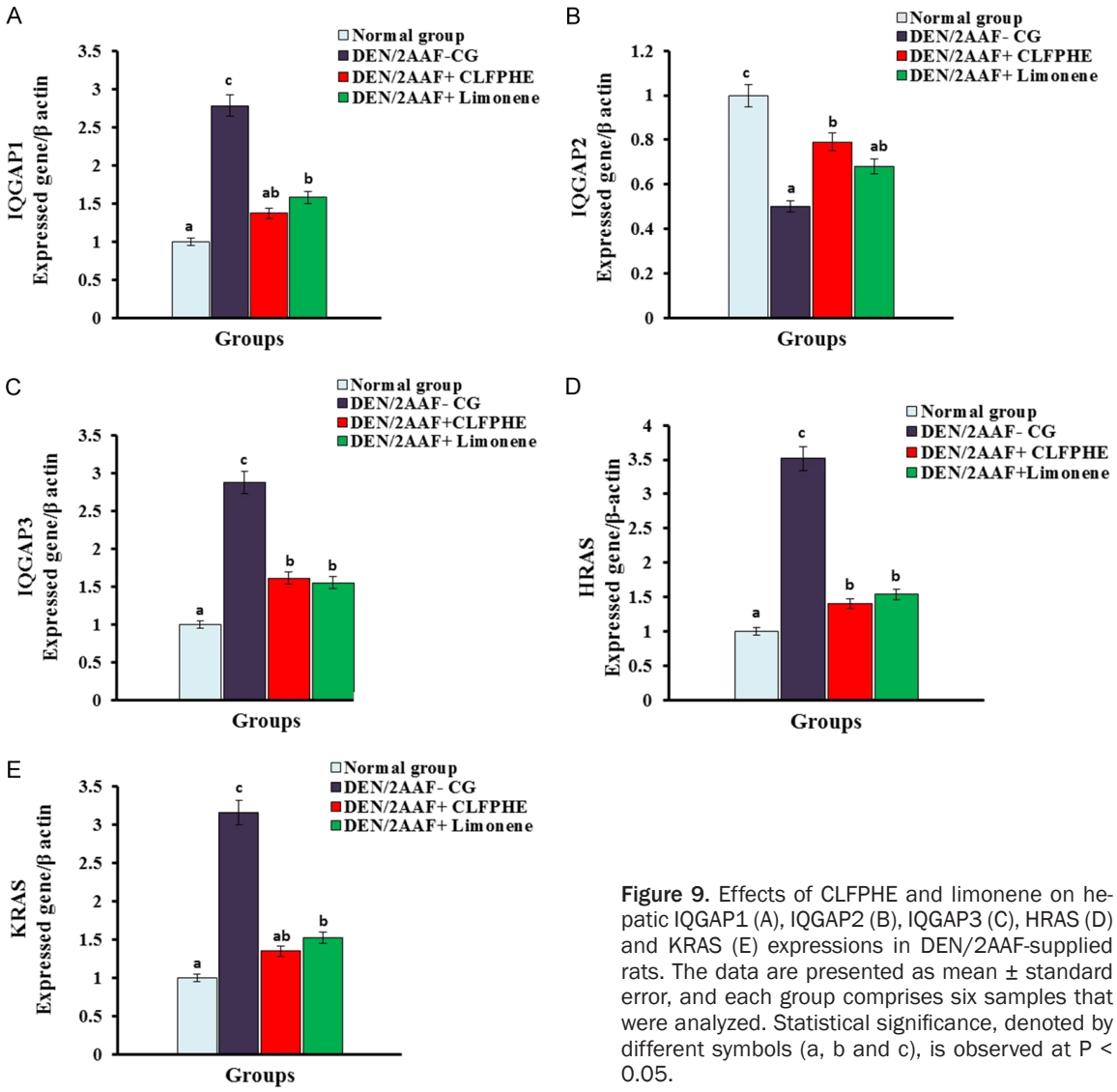

Figure 9. Effects of CLFPHE and limonene on hepatic IQGAP1 (A), IQGAP2 (B), IQGAP3 (C), HRAS (D) and KRAS (E) expressions in DEN/2AAF-supplied rats. The data are presented as mean ± standard error, and each group comprises six samples that were analyzed. Statistical significance, denoted by different symbols (a, b and c), is observed at $P < 0.05$.

formation prompted by DEN/2AAF. Additionally, they resulted in an improvement in the decreased SOD and GPx activities additionally to GSH content in liver of DEN/2AAF-administered rats. Several additional studies also reveal the effective antioxidant efficacy of limonene [39, 50]. Antioxidants are essential for cleaning up the tissue and cells from ROS, preventing sickness from causing cellular and tissue damage [44]. Natural compounds' potential, as a case study phenols and flavonoids, to a cure injury to the liver according to reports, related to their antioxidant activity [51-53]. It is well known that limonene has antioxidant properties and can prevent the formation of hazardous free radicals [54]. The modification of ROS and antioxidant status is among the key mechanisms by which CLFPHE produces its anticancer effects (**Figure 12**). The bioactive compounds in citrus that have antioxidant properties, especially flavonoids, carotenoids, terpenes, and limonoids, can reduce disorders associated with oxidative stress [55, 56].

Additionally, DEN treatment increased the overall levels of TNF-α, IL-1β, NF-κB, and IL-8, even though decreasing Nrf2 levels, these result agreed with Tawfik et al. [31]; Ali et al. [57] and Zoheir et al. [58] who suggest that DEN-induced liver damage may be mediated through the increased regulation of pro-inflammatory cytokines and transcription factors, resulting in chronic inflammation and oxidative stress. The release of TNF-α, IL-1β, and IL-8, among other

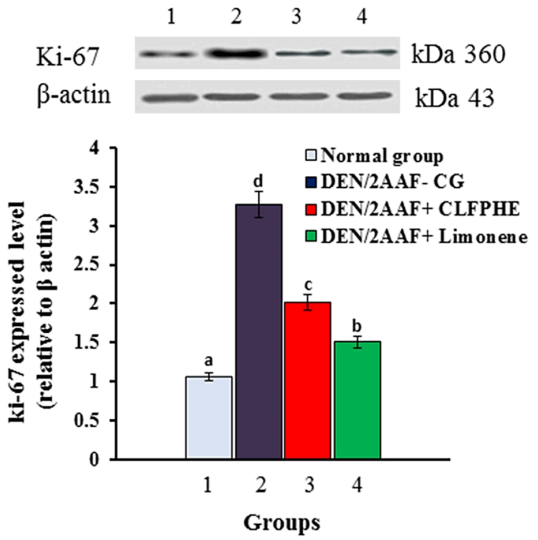

Figure 10. Influence of CLEPHE and limonene on hepatic Ki-67 level in DEN/2AAF-supplied rats. The data are presented as mean ± standard error, and each group comprises six samples that were analyzed. Statistical significance, denoted by different symbols (a, b c and d), is observed at $P < 0.05$.

proinflammatory factors, leads to the cellular and molecular processes that leading to fibrosis; in addition, chronic inflammation can also promote the development of HCC by inducing genetic mutations and alterations in signaling pathways [59]. NF-κB controls the level of TNF-α and IL-1β which enhance cell proliferation and inflammation [60]. IL-1β is a cytokine that promotes inflammation and is regarded as a biological marker of inflammation [61]. Furthermore, the elevated IL-1β level may also be associated with chronic inflammation that resulted from ROS produced as result of DEN and 2AAF administration [62]. IL-8 has been linked to angiogenesis, cancer cell invasion, proliferation, and survival [63]. In addition to its implication to regulate the production of antioxidant enzymes and phase II detoxifying enzymes (**Figure 12**), Nrf2 also influences the course of diseases by protecting cells from diverse insults through its anti-inflammatory properties [64, 65].

The treatment with CLFPHE and limonene could shield rats from DEN-induced inflammation by inhibiting the expression of TNF-α, IL-1β, IL-8 and NF-κB and increasing Nrf2 level reflecting the anti-inflammatory effects of these treatments. The use of medicinal plants as well as their active ingredients has received widespread acclaim for the treatment of diseases linked to inflammation [66]. The high concentration of limonene in *Citrus limon* is most likely the cause of its anti-inflammatory properties [67]. D-limonene lowers a wide range of inflammatory mediators. Numerous reports referred to limonene anti-inflammatory actions by reducing several inflammatory mediator levels like TNF-α, NF-κB, and IL-1β [68]. The anti-inflammatory properties of D-limonene have been demonstrated in earlier times due to the suppression of redox-dependent NF-κB and further inflammatory cytokines following which are significant figures in exhibition of inflammation in line with prior reports [69, 70]. As a lipophilic molecule with a low molecular weight, limonene as well said to it can demonstrate its anti-inflammatory properties via saturating the cell membrane, protecting the cell from inflammation [71].

Apoptosis is a unique type of cell death that is initiated and essential for maintaining cellular homeostasis. It is often inhibited in both normal tissues and tumorigenic processes [72]. Deregulatory actions of apoptosis and increased hepatocyte proliferation there have identified as critical factors in the development of HCC [73]. During cancer progression, apoptosis is absent from cancer cells. The families of pro- and anti-apoptotic factors are in charge of controlling it [74]. Rats given DEN/2AAF exhibited elevated expression of antiapoptotic BCL2 and decreased expression of proapoptotic BAX in comparsion to controls, accordance with earlier reports Subramaniam et al. [75], Rückert et al. [76]. Hepatic BCL2 upregulation in the DEN/2AAF group indicates that malignant cells were refusing to apoptosis, which may have been caused by increased BCL2 gene copy numbers, transcription, and/or translation, as was seen for other cancer-related genes. Tumors typically express higher levels of BCL2 [77, 78]. In addition, compared to controls, rats given DEN had lower levels of p53 and PDCD5 expression in the liver, which may be required for resistance to apoptosis and DEN-induced carcinogenesis, this result with the same line of Mohamed et al. [24] and Zoheir et al. [58]. P53 reduced levels may be required for apoptosis obstruction and may influence DEN/2AAF-triggered cancer development. P21 protein activation, which prevents cell division, and the beginning of the apoptosis process, which kills

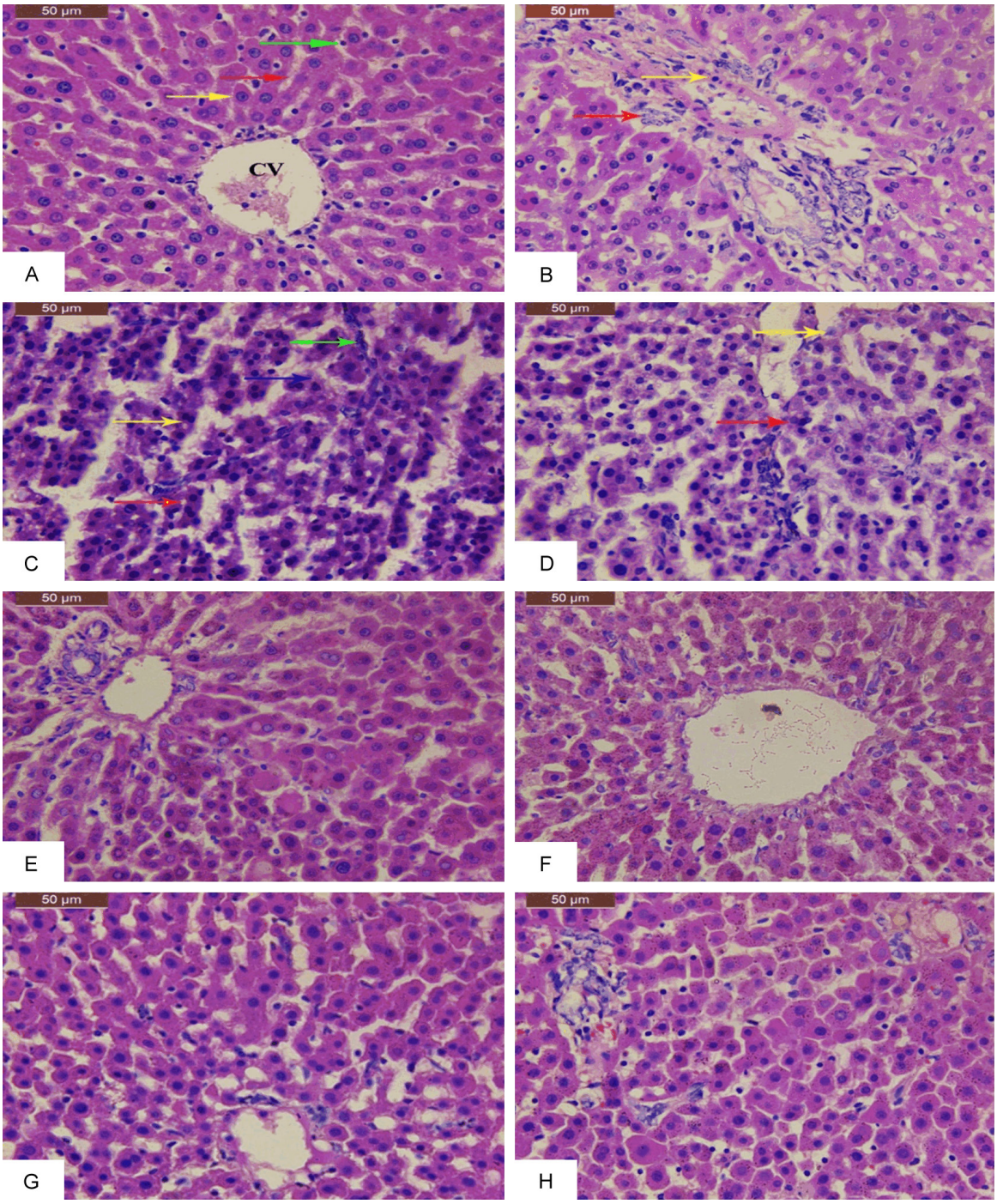

Figure 11. Histological characteristics of normal control rats' liver sections. (A) Section showing intact lobules with portal triads among them. Each lobule consists of cords formed from regularly arranged hepatocytes (yellow arrow) enclosing sinusoids (red arrow) lined with Kupffer cells (green arrow), and a central vein (CV) located in the center (H&E stain, Scale bar: 50 µm). Liver sections from DEN/2AAF-administered rats from (B-D) showing (B) congested portal tract that was associated with moderate inflammatory infiltration (red arrow) in the portal tracts and compressed hepatocytes that surrounding it (yellow arrow) (H&E stain, Scale bar: 50 µm). (C) Showing interruption of the hepatic lobule. The tumor cells exhibited with well-differentiated forms and resemble hepatocytes form and present as trabeculae (blue arrow), cords (yellow arrow), and nests (red arrow). Note the primary growing of septa between the hepatic lobules (green arrow) (H&E stain, Scale bar: 50 µm). (D) Showing interruption of the hepatic lobule. The tumor cells exhibited with well-differentiated forms and resemble hepatocytes form and present as cords (red arrow), and nests (yellow arrow) (H&E stain, Scale bar: 50 µm). Liver sections from DEN/2AAF-administered

rats treated with CLFPHE (E and F) showing the portal tract appeared nearly as normal form. Note a single tumor cells with large nuclei. The hepatic lobule appeared nearly as normal form (H&E stain, Scale bar: 50 μm). Limonene treatment (G and H) showing the hepatic lobule displayed like normal structure, the portal tract appeared nearly as normal form (H&E stain, Scale bar: 50 μm).

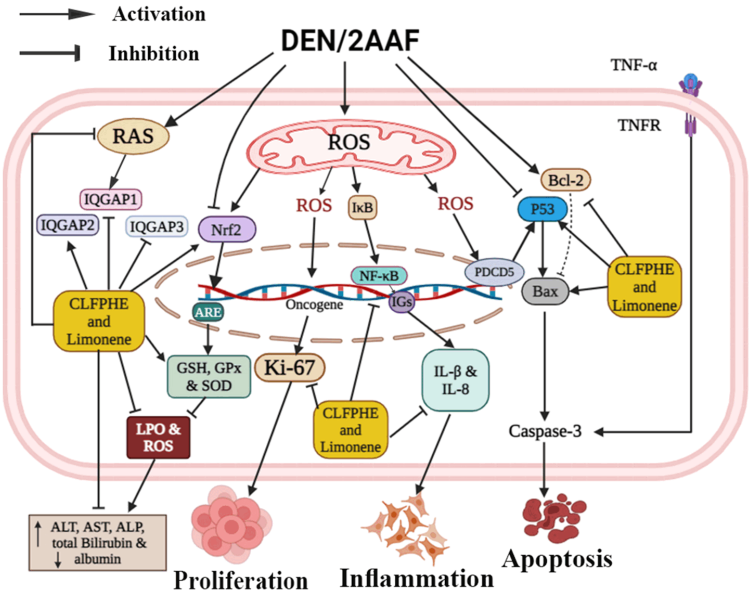

Figure 12. Schematic diagram showing the mechanism of actions of CLFPHE and limonene against DEN/2AAF, which suppress oxidative stress, inflammation, proliferation and induced apoptosis in carcinogens lung cells. ROS, reactive oxygen species; IGs, inflammatory genes; ARE, antioxidant response element; NF-κB, Nuclear factor-kappa-B; BCL2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; p53, Tumor suppressor protein 53; iNOS, Inducible nitric oxide synthase; IFN-γ, Interferon-gamma; IL-6, Interleukin-6; IL-10, Interleukin-10.

cancerous cells, are two of the molecular mechanisms with which P53 reduces the formation of tumors [79]. PDCD5 is participating in apoptosis, which is thought to act as a tumor suppressor in a variety of tumor types, including breast and lung cancer [80]. In the presence of DNA damage, PDCD5 can interact with and stabilize P53 to encourage apoptosis [81, 82].

As an alternative, CLFPHE and limonene enhanced apoptosis in liver of DEN/2AAF-administered rats by decreasing BCL2 expression and increasing BAX, p53, and PDCD5 expressions; thus, the induction of apoptosis by these treatments may be contributed to their ability to prevent HCC (**Figure 12**). Limonene is the principal portion of lemon and is referred to as safe in the Federal Aromatic Substances [83, 84]. Also, various reports have indicated that limonene has anticancer properties against various cancer types due to its ability to cause apoptosis [85, 86].

IQGAPs are a group of conserved protein types found in eukaryotic cells, starting with yeast to humans, and include three homologs that are related; regulating a wide range of processes that is cytoskeleton remodeling, cytokinesis, trafficking in proteins, adhesion of cells, proliferation, migration, and the growth of tumors [87-89]. IQGAP1 and IQGAP3 expression was raised in control rats given DEN and 2AAF compared to normal, while IQGAP2 expression was decreased. These results are parallel with these obtained by Mohamed et al. [24]. The accumulated evidence implies that IQGAP1 is an oncogene while IQGAP2 may be a tumor suppressor, according to Colin et al. [90]. IQGAP3 serves a significant part in controlling mitosis and keeping genomic reliability and stability, and the physiological level of IQGAP3 is necessary for cell proliferation and migration [91, 92]. IQGAP3 expression was noticeably elevated levels of tissue cancer in contrast to that in healthy tissues and was closely related to large tumor size, developed tumor stage, poor differentiation, and intra and extra-hepatic metastasis in HCC [93]. IQGAP3, like IQGAP1, is also regarded as oncogene in HCC. In HCC tissues, IQGAP3 is overexpressed. Its presence is linked to increased tumor size, advanced tumor stage, and poor tumor differentiation [93, 94] and IQGAP3 promotes intrahepatic and extrahepatic metastasis, resulting in a significantly shorter patient survival time [95].

The RAS family of proto-oncogenes, involving KRAS, NRAS and HRAS, encoding a group of small GTPases, which are triggered in reaction for development factors and other extracellu-

lar stimuli and cause subsequent signalling flows, for example the mitogen-activated protein kinase (MAPK) pathway [96]. The manifestations of HRAS and KRAS increased in DEN/2AAF-treated rats in comparsion to rats under normal condition, according to Zoheir et al. [97], who reported that HRAS and KRAS mRNA expressions are elevated in a model of animals using DEN to cause HCC. The prognostic importance of KRAS and HRAS expression has undergone evaluation in a number of cancers [98].

CLFPHE and limonene showed a noticeable decline in IQGAP1, IQGAP3, HRAS and KRAS and a rise in IQGAP2 expressions; thereby offering proof that reduced probability of HCC. In accordance with this evidence, D-Limonene is also effective in primary chemotherapy for hepatocellular carcinoma [99].

Proliferation of cells is well known to be essential in the different stages of carcinogenesis. However, Ki-67 is the only protein, which is only based on cell proliferation; accordingly, its expression is frequently used as an indicator for the multiplication of cells [100]. Ki-67 is one of noticeable marker proteins used by histologists to recognize proliferating cells [101]. Rats treated with DEN dramatically raised the expression of Ki-67, consistent with earlier research of Ahmed et al. [30] and Yassin et al. [40], who demonstrated that Ki-67 is overexpressed in hepatocarcinogenesis caused by DEN. Treatments with CLFPHE and limonene reduced Ki-67 gene expression reflecting the anti-proliferatory effects of these treatments (**Figure 12**). *Citrus limon* has been shown a time-dependent inhibitory effect on tumor cell proliferation in various tumor cell lines by activating apoptotic cell processes [102]. D-Limonene has the anti-proliferative role on multiple chemical carcinogenesis animal models [103]. Numerous studies revealed that flavonoids could be effective in the prevention of cancerous growth by preventing the metastasis process, the reduction of cancer cell accessibility in circulatory systems, proapoptosis, preventing cell cycle progression and antiangiogenesis [104].

Conclusions

The potential for prevention of CLFPHE and limonene on DEN/2AAF-induced HCC can be explained using of oxidant-antioxidant system control and management of the inflammatory state as well as cell proliferation and apoptosis. Therefore, these therapies might serve as preventive antioxidant and anti-inflammatory agents (**Figure 12**). However, to evaluate the extract and limonene effectiveness and safety in humans, additional clinical studies and pharmacological investigations are required.

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Disclosure of conflict of interest

None.

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