

Original Research Article

Immunoreactivity of desmin antibody against perisinusoidal cell activation using *Citrus limon* aqueous extract

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Abstract

Purpose: To examine the effect of *Citrus limon* aqueous extracts (CLE) on high-fat diet (HFD)-induced steatosis using desmin immunoreactivity (perisinusoidal cell activation) and cholesterol levels.

Methods: A total of 30 male Swiss albino mice were randomly assigned to 3 groups ($n = 10$). Group 1 was the control, group 2 received HFD (1 %w/w cholesterol), and group 3 received HFD in combination with CLE (2.0 mL/kg body weight, 1:5 m/v lemon juice daily by gavage) for 16 weeks. Hepatic steatosis was assessed by H & E staining and quantified by ImageJ. Assessment of perisinusoidal cell activation was done by anti-desmin immunohistochemistry (Clone D33, Dako), while density of positive cells was quantified by ImageJ.

Results: Administration of HFD resulted in significantly more desmin-immunopositive cells compared to control ($p < 0.05$). However, treatment with CLE reduced desmin-positive cells, comparable to control ($p > 0.05$). Serum total cholesterol was significantly higher in group 2 compared to group 1 ($p < 0.05$). Treatment with CLE reduced serum total cholesterol to levels comparable to control ($p > 0.05$).

Conclusion: Treatment with CLE reduces desmin-immunopositive cells and serum total cholesterol, demonstrating its potential hepatoprotective and lipid-lowering effects.

Keywords: *Citrus limon*, Desmin, Hepatic stellate cells, Immunohistochemistry, Steatosis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a complex and serious metabolic disorder characterized by excessive fat build-up in the liver without significant alcohol use. The spectrum ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis, which are closely related to obesity, insulin resistance, dyslipidemia, and systemic inflammation [1,2]. Hepatic stellate cells (HSCs) are found

in the space of Disse and are central to NAFLD progression. At rest, HSCs retain retinoids and help maintain sinusoidal integrity. HSCs undergo myofibroblastic activation and drive fibrosis in response to hepatocyte injury and lipid overload, characterized by constitutive expression of desmin.

Desmin immunohistochemistry (IHC) is a validated marker to monitor HSC activation. [3]. Citrus Limon is a good source of polyphenols and flavonoids (such as

eriocitrin, hesperidin, naringin, and neohesperidin) with antimicrobial, anti-tumorigenic, lipid-lowering and cardioprotective properties [4,5]. In herbal medicine, lemon has long been used for hepatic disorders, and the extract has been found to ameliorate diet-induced NAFLD by modulating lipid metabolism and inhibiting inflammatory signaling [6,7]. No study has yet assessed the effect of Citrus limon aqueous extract (CLE) on hepatic steatosis (hematoxylin and eosin (H & E) histology), perisinusoidal cell activation (desmin IHC with digital quantification), and serum lipids in a single high-fat diet (HFD) murine model. Therefore, this study investigated the effect of CLE on HFD-induced steatosis using the H & E staining procedure.

EXPERIMENTAL

Animals and experimental design

Thirty male Swiss albino mice (8 – 12 weeks old) were sourced from the animal house, Faculty of Science. The animals were allowed to acclimatize under standard conditions (12 h light / dark cycle, 22 ± 0.5 °C, humidity of 40 – 60 %) with unrestricted access to feed and water ad libitum. The mice were randomly assigned to 3 groups (n = 10). Group 1 was control (received standard feed), group 2 received HFD (standard feed supplemented with 1 %w/w cholesterol (Sigma-Aldrich, USA)), and group 3 received HFD in addition to oral gavage of CLE (2.0 mL/kg body weight (1:5 m/v diluted fresh lemon juice)) for 16 weeks. The HFD was prepared on alternate days and stored at 4 °C before being equilibrated to room temperature [8]. Ethical approval was obtained from the Institutional Board of Animal Health, Faculty of Science, Babylon University and the study was conducted in compliance with the guidelines for the care and use of laboratory animals [9].

Preparation of *Citrus limon* extract (CLE)

Fresh lemon fruits were harvested from a farm in Hilla, Babylon, Iraq. They were identified by matching them with specimens in the herbarium of the College of Science, University of Babylon. They were washed with deionized water. The pulp was homogenized with deionized water 1:1 (m/v) and centrifuged at 5,000 rpm for 10 min. The supernatant (100 % CLE) was diluted 1:5 (m/v) with deionized water. The CLE was freshly prepared daily before gavage and administered at a dose of 2.0 mL/kg body weight of the 1:5

CLE (equivalent to ~ 0.4 mL/kg undiluted juice) as used in murine hypolipidaemic studies [10].

Evaluation of parameters/indices

Histomorphology

At the end of the 16 weeks, the mice were sacrificed under chloroform anaesthesia. Liver tissues were excised and fixed in 10 % neutral buffered formalin for 24 h. The tissues were processed, embedded in paraffin, and sectioned at 5 µm. Sections were stained with haematoxylin and eosin (H & E). Steatosis was assessed on a scale. Grades of 0, 1, 2, and 3 were defined as < 5, 5 – 33, 34 – 66, and > 66 %, respectively of hepatocytes with lipid vacuolation, respectively. ImageJ software (v1.54, NIH, USA) was used to measure lipid vacuolation area in each section, done on five random fields at 200× magnification.

Immunohistology

Tissue sections (5 µm) were deparaffinized and rehydrated. Thereafter, they were heated in citrate buffer (pH 6.0) to retrieve the epitope. A solution of 3 % H₂O₂ was added to prevent endogenous peroxidase activity. The sections were incubated overnight at 4 °C with the use of a monoclonal mouse anti-human desmin antibody. Detection was performed using the LSAB+HRP kit (K0697, Dako) and EnVision™ system (Dako), and the sections were counterstained with haematoxylin. The IHC Toolbox plugin of ImageJ was used to quantify desmin-positive cells (brown cytoplasmic staining) over five non-overlapping fields per section at a magnification of 200×. All groups were imaged on the same microscope with the same camera settings. The number of desmin-positive cells per high-power field (HPF) was recorded as mean ± standard deviation (SD).

Biochemical analysis

Blood samples (5 mL) were obtained by cardiac puncture and centrifuged at 3000 rpm for 15 min. Serum total cholesterol was measured using enzymatic colorimetric kits (Biomaghreb, Tunisia) following the manufacturer's instructions.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS, version 25.0; SPSS Inc., IBM Corp., Armonk, NY, USA). Data were presented as mean ± SD and compared using one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test for

multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Histomorphology

Group 1 (control) displayed normal hepatic architecture without lipid vacuolation (steatosis grade 0). Group 2 was found to have extensive macrovesicular steatosis, with large lipid vacuoles that displaced the nucleus peripherally. There was also focal inflammatory infiltration and widening of perisinusoidal spaces (grade 2 – 3, $p < 0.05$ compared to group 1). Group 3 showed greatly reduced lipid vacuolation and preserved cord architecture (grade 0 – 1), which was not significantly different from group 1 ($p > 0.05$), indicating strong hepatoprotection (Figure 1).

Desmin-positive perisinusoidal cell density

Immunostaining for anti-desmin showed that perisinusoidal cells were recognized by brown cytoplasmic reactivity. Photomicrographs were taken at 200× magnification using the same microscope settings (Figure 1).

Serum lipid profile

Treatment with HFD significantly elevated total cholesterol levels compared to control and group 3 ($p < 0.05$). However, there was no significant increase in total cholesterol level following treatment with CLE compared to control ($p > 0.05$; Figure 2).

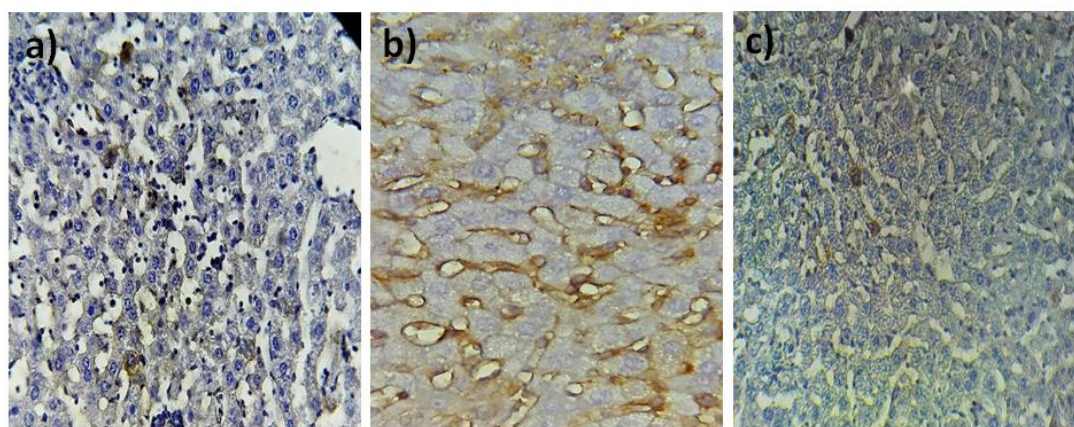


Figure 1: Desmin immunohistochemical staining of mouse liver sections (200×). (A) Control group showing normal desmin-positive perisinusoidal stellate cells (4.2 ± 0.9 cells/HPF), reflecting baseline quiescent stellate cell population. (B) Group 2 showed significantly higher desmin-positive cells (9.8 ± 1.4 cells/HPF; $p < 0.05$ vs control), indicating significant perisinusoidal stellate cell activation driven by HFD-induced lipid overload. (C) Group 3 showed reduced desmin-positive cells vs group 2 (4.7 ± 1.1 cells/HPF, $p > 0.05$), confirming that CLE effectively suppressed perisinusoidal stellate cell activation

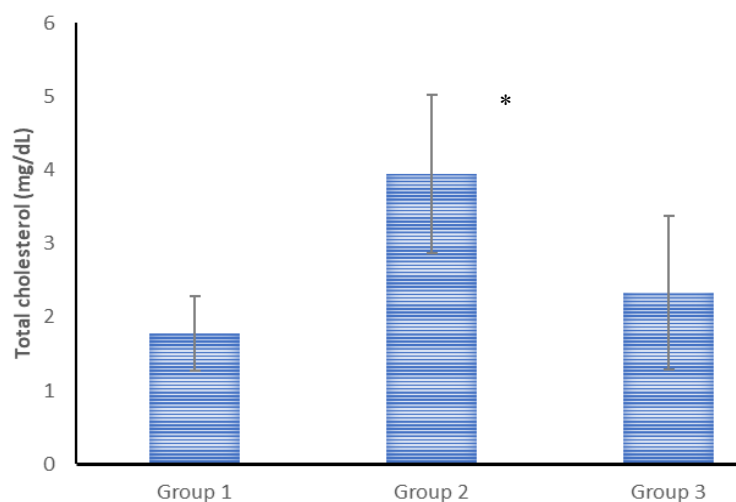


Figure 2: Serum total cholesterol (mg/dL) among the three groups. * $P < 0.05$ vs group 1 (control)

DISCUSSION

The integrated histological, immunohistochemical, and biochemical evidence showed that CLE has a significant hepatoprotective effect on HFD-induced NAFLD in mice. Furthermore, CLE demonstrated the presence of attenuated macrovesicular steatosis, suppressed perisinusoidal stellate cell activation evidenced by reduced desmin immunoreactivity, and normalized serum total cholesterol. The HFD containing 1 % cholesterol induced macrovesicular steatosis. In group 3, supplementation with CLE successfully reduced histological steatosis and serum cholesterol, which is consistent with previous studies with lemon [11,12].

Previous studies have also shown that eriocitrin and hesperidin were key active constituents. Eriocitrin lowers oxidative stress and regulates glucose and lipid metabolism in HFD-fed obese mice [12,13]. Furthermore, a clinical trial showed that hesperidin improves hepatic steatosis and normalizes hepatic enzymes in patients with NAFLD [14]. Citrus peel extracts work through activation of AMP-activated protein kinase (AMPK) signaling, inhibiting new fat creation via sterol regulatory element-binding protein-1 (SREBP-1) and fatty acid synthase (FAS) downregulation. They also modulate mechanistic target of rapamycin complex 1 (mTORC1) and endoplasmic reticulum redox homeostasis [7,15]. Desmin, a type III intermediate filament protein constitutively expressed in HSCs (Ito cells), serves as a marker of these cells in both their quiescent and activated states. When exposed to HFD conditions, lipid metabolites produce reactive oxygen species (ROS) and pro-inflammatory cytokines (especially transforming growth factor- β 1 [TGF- β 1] and tumor necrosis factor- α [TNF- α]) that paracrinally activate quiescent HSCs. Following activation, HSCs proliferate and upregulate desmin and α -SMA, and at the same time secrete collagens I and III to initiate perisinusoidal fibrosis [16,17]. This showed that the animals in group 2 had significant HSC activation due to lipid overload in the liver. The fact that the response is suppressed in group 3 may be attributed to the presence of antioxidant and anti-inflammatory flavonoids that CLE contains. Accumulation of ROS, which may trigger the activation of stellate cells, is reduced and TGF- β 1 signaling is suppressed. By diminishing oxidative stress-mediated injury on hepatocytes, CLE attenuates the paracrine signaling that drives Ito cell activation in the liver. As a result, it was able to maintain the density of desmin-positive cells at the level seen in the control, a finding consistent with other studies [7,18]. This pathway

established a link that is both direct and coherent in relating biochemical findings (reduced steatosis and cholesterol) to immunohistochemical observation (attenuated desmin immunoreactivity) in this experimental design.

Limitations of the study

This study has some limitations. The phytochemical composition of CLE was not characterized using high-performance liquid chromatography (HPLC), and as a result, it was not possible to identify any metabolite responsible for the activity. Co-staining with α -SMA, which would have provided a more specific assessment of the activated myofibroblastic HSC phenotype, was not done. Masson's trichrome staining for hepatic collagen deposition, and molecular analyses (SREBP-1, FAS, TGF- β 1, AMPK mRNA/protein), which would have identified more molecular and cellular pathways, were not done. Future investigations should address these concerns.

CONCLUSION

Aqueous extract of *Citrus limon* effectively reduces HFD-induced hepatic steatosis, inhibits perisinusoidal hepatic stellate cell activation, and normalizes serum cholesterol levels in a NAFLD mouse model.

DECLARATIONS

Acknowledgements

None.

Conflict of interest

The authors declare no conflict of interest.

Contribution of authors

All authors contributed to the conception, design, execution, and reporting, and take full responsibility for the integrity of the data.

Data Availability

De-identified data supporting the findings of this study are available from the corresponding author upon reasonable request.

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