Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 3, 439-444

Silymarin-loaded chitosan nanoparticles alleviate dyslipidemia, oxidative stress, metabolic disturbance and histopathological injury associated with high fat diet-induced non-alcoholic fatty liver disease in rats

Mohamed Fouad Mansour[1*], Zaher Z. Radwan[1], Tarek Khamis[2], Medhat Fawzy[1]

[1]*Department of Biochemistry andMolecular Biology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt.*
[2]*Department of Pharmacology and Laboratory of Biotechnology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt.*

ARTICLE INFO

Recieved: 03 November 2023

Accepted: 06 January 2024

***Correspondence:**

Corresponding author: Mohamed Fouad Mansour
E-mail address: mafouad@zu.edu.eg

Keywords:

NAFLD
Nano-silymarin
Nano-chitosan
Silymarin-loaded chitosan Nanoparticles (SILCSNPs)
High fat diet

ABSTRACT

With an increasing incidence of obesity and metabolic syndrome epidemic, nonalcoholic fatty liver disease (NAFLD) continues to be one of the most prevalent liver illnesses worldwide. One of recommended treatment in NAFLD is silymarin. However, the problem is that silymarin has weak water solubility and limited bioavailability. Therefore, preparation of silymarin in nano-formulation would enhance silymarin's therapeutic effects and bioavailability. This study was designed to evaluate the biochemical and molecular effects of silymarin-loaded chitosan nanoparticles (SILCSNPs) in NAFLD treatment in rats. Fifty rats were divided into five groups include: Group 1: Control group, group 2: HFD-induced NAFLD, group 3: HFD-induced NAFLD that orally received nano-chitosan, group 4: HFD-induced NAFLD that orally received nano-silymarin and group 5: HFD-induced NAFLD that orally received silymarin-loaded chitosan nanoparticles (SILCSNPs). The dose of each treatment was 40 mg/kg/day for 60 days. Lipid parameters (triglycerides, total cholesterol), ALT, AST, hepatic (catalase, SOD and MDA) and mRNA expression of lipogenesis-related genes including ACC (acetyl-CoA carboxylase) and FASN (fatty acid synthase) as well as fatty acids catabolism-related genes including CPT-1 (carnitine palmitoyl-transferase I), PPAR-α (peroxisome proliferator-activated receptor alpha) were measured. Histopathological examination of liver was also conducted. A significant elevation in HDL, catalase, SOD, CPT-1, PPAR-α levels as well as substantial reduction in triglycerides, cholesterol, ALT, AST, MDA, ACC and FASN levels were detected in treated groups in compared to the HFD-induced NAFLD group. Histopathological examination of the liver showed histological amelioration in hepatic tissue in treated groups in compared to the HFD-induced NAFLD group. SILCSNPs revealed a significant potential effect against NAFLD metabolic disturbance and considered an advanced trend in NAFLD treatment.

Introduction

Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are two prominent and developing kinds of chronic liver disease, participate a significant amount of liver fat accumulation (Mokdad *et al*., 2016; Younossi *et al*., 2020). ALD is the most common cause of morbidity, occurs as a part of a larger spectrum of alcohol misuse diseases, and is commonly linked to psychiatric comorbidities (Rehm *et al*., 2009; WHO, 2019). In contrast, NAFLD is an over 5% liver fat accumulation that is not brought on by drugs or alcohol (Chalasani *et al*., 2018). Moreover, NAFLD is a clinically complex collection of illnesses that includes simple steatosis (nonalcoholic fatty liver; NAFL), steatosis with necro-inflammatory alterations (nonalcoholic steatohepatitis; NASH), advanced fibrosis, cirrhosis, and hepatocellular cancer. NAFLD frequency has rapidly increased in recent years as a result of changes in lifestyle (Mashek and Greenberg, 2014). Multiple factors, including nutrition, environment, metabolism, and heredity, interact to cause this condition. It is characterized by an excessive buildup of lipids, which can lead to hepatic illnesses such cirrhosis, fibrosis, and steatohepatitis. NAFLD frequently coexists with metabolic syndromes such obesity, diabetes mellitus, hypertension, and hyperlipidemia because it is a multifactorial condition (Lombardi *et al*., 2017). It has been observed that changes in lifestyle, such dietary adjustments and weight loss, are helpful in treatment of NAFLD (Romero-Gómez *et al*., 2017).

Silymarin is a natural substance that is obtained from the seeds of Silybum marianum. It is used all around the world to treat fatty liver, hepatitis, and liver damage (Abenavoli *et al*., 2012; Salamone *et al*., 2012). Additionally, because of its exceptional hepatoprotective, antioxidant, anti-tumor, hypolipidemic, and anti-inflammatory activities, it is recognized as one of the most promising treatments for NAFLD (Federico *et al*., 2017; Vargas-Mendoza *et al*., 2014). The hepatic hepatoprotective effects of silymarin are caused by a reduction in reactive oxygen species and an increase in cellular glutathione and superoxide dismutase levels (Rašković *et al*., 2011). However, silymarin's weak solubility in water (50–430 micrograms/ml) and low bioavailability (23–47%) limit its absorption abilities (Javed *et al*., 2011; Morazzoni *et al*., 1993). Despite hepatoprotective effects of silymarin, its limited absorption is the fundamental drawback of its oral treatment (Woo *et al*., 2007). Some hypotheses state that the root causes of silymarin's limited bioavailability are: its weak water solubility in stomach pH, poor permeability through gut epithelial cells, and rapid elimination (Clichici *et al*., 2020). In order to boost silymarin's bioavailability, numerous formulations have been created to address this issue. Additionally, the use of nanotechnology may significantly contribute to the enhancement of silymarin's therapeutic effects and bioavailability (Ma *et al*., 2017).

Chitosan, a naturally occurring cationic amino polysaccharide, is regarded as the ultimate biological material because it is biodegradable, nontoxic, and biocompatible (Duceppe and Tabrizian, 2010; Prabaharan and Mano, 2004). Chitosan significantly decreased the increased blood TC, LDL, and LDL/HDL levels brought on by the HFD. Furthermore, in the animals fed the HFD, chitosan slightly increased the serum HDL concentration. To enable HDL to move cholesterol from peripheral organs to the liver for reuse, this action may be related to increased lipid catabolism (Muanprasat and Chatsudthipong, 2017). In addition to the decreased

ISSN: 2090-6277/2090-6269/

liver weight and decreased hepatic TC and TG levels produced by chitosan administration, the histological tests also demonstrate the protective effect of chitosan against hepatic steatosis (Donnelly *et al*., 2005). Moreover, chitosan lowers the high serum levels of AST and ALT caused by HFD raises the possibility that chitosan may protect mice from the liver damage that HFD induces (Tao *et al*., 2019). In recent study, chitosan surpassed the gastrointestinal obstacles of silymarin bioavailability. A polymer based on natural ingredients, chitosan has a variety of functional groups, mucoadhesive properties, regulated drug release, permeating-enhancing effects, and efflux inhibition (Pathomthongtaweechai and Muanprasat, 2021).

To the best of our knowledge, this is the first study evaluates the ameliorative effect of nano-silymarin, nano-chitosan and SILCSNPs in NAFLD treatment. We hypothesized that nano-silymarin, nano-chitosan and SILCSNPs have a beneficial effect on lipid parameters (triglycerides, cholesterol), hepatic antioxidant enzymes (catalase and SOD), hepatic malondialdehyde (MDA), liver function enzymes (ALT and AST). In addition, we postulated that this beneficial effect includes mRNA expression of lipogenesis-related genes including ACC and FASN and fatty acids catabolism-related genes including CPT-1, PPAR-α.

Materials and methods

Preparation of nano-silymarin

According to a prior work, silymarin (Abcam, UK) nanoparticles were created using the desolvation process (Hsu *et al*., 2012) with some modifications. Certain amount of silymarin was dissolved in ethanol and the ethanolic solution added drop-wisely to PVP in water. On a JEOL JEM-2100 high resolution transmission electron microscope, TEM was carried out at a 200 kV accelerating voltage, respectively.

Preparation of nano-chitosan

The ionotropic gelation procedure was used to create chitosan nanoparticles (Hasanin *et al*., 2018). A tripolyphosphate (TPP) aqueous solution was added to a chitosan solution to produce blank nanoparticles. At a respective accelerating voltage of 200 kV, TEM were carried out on a JEOL JEM-2100 high resolution transmission electron microscope.

Preparation of silymarin-loaded chitosan nanoparticles (SILCSNPs)

According to a prior study, silymarin-CS nanoparticles were created using the ionic gelation process (Venugopal *et al*., 2015) with some modifications. Certain amount of silymarin was dissolved in ethanol and the ethanolic solution added drop-wisely to chitosan solution and then TPP added drop-wisely. TEM was carried out using a JEOL JEM-2100 high resolution transmission electron microscope at a 200 kV accelerating voltage, respectively.

Experimental animals

From the farm of laboratory animals at the Faculty of Veterinary Medicine, Zagazig University, Egypt, 50 albino rats (3 months old) weighing 80–100 grams were acquired. The animals spent two weeks getting used to the laboratory environment before the studies began. Animals were kept in stainless steel cages with a 12-hour photoperiod of light and dark, a controlled temperature range of 21–25°C, and a relative humidity range of 50–60%. Throughout the trial, rats had full access to commercial rodent meal pellets (Al wadi Co., Giza, Egypt) and water. All rats were treated in accordance with the general recommendations for the care and use of laboratory animals published by the National Institutes of Health (NIH). The Institutional Animal Care and Use Committee of Zagazig University in Egypt (ZU-IACUC/2/F/302/2022) approved the current study.

HFD-induced NAFLD model

Induction of NAFLD continues for 12 weeks, fifty rats are given the high fat diet (HFD), which is made up of purified high fat diet caused obesity (HFD), It has 23.4 KJ/g of total calories, roughly 58% fat, 25.6% carbohydrate, and 16.4% protein (Shen *et al*., 2022).

Experimental design

The animals were divided into five experimental groups, each of which had ten rats. The first group (control group) was given a typical diet consisting of 12.6 KJ/g of total calories, 11% fat, 62% carbohydrates, and 27.0% protein. The second group (control positive group): Rats induced with NAFLD. The third group (nano-chitosan treated group): Rats induced with NAFLD received 40 mg/kg b. wt. nano-chitosan orally for 60 days. The fourth group (nano-silymarin treated group): Rats induced with NAFLD received 40 mg/kg b. wt. nano-silymarin orally for 60 days. The fifth group (silymarin-loaded chitosan nanoparticles (SILCSNPs) group): Rats induced with NAFLD received 40 mg/kg silymarin-loaded chitosan nanoparticles (SILCSNPs) orally for 60 days.

Sampling

Both blood samples with and without anticoagulants were taken. Biochemical analyses were conducted using serum and plasma. Rats were sacrificed by cervical decapitation at the conclusion of the study period. Immediately after scarification, liver tissues (50 mg) were removed, wrapped in aluminum foil, and stored in a liquid nitrogen container until they were used for gene expression analysis. For measuring antioxidant enzymes (SOD and catalase), another portion of the liver was pulverized and used as a vortex. For histopathology, a portion of the heart, kidney, and liver were taken and preserved in 10% buffered formalin after being rinsed with normal saline (Layton and Suvarna, 2013).

Lipid profile

CHOD/POD Liquid method was used for the determination of cholesterol by using Spinreact kit (Meiattini *et al*., 1978). GPO/POD enzymatic colorimetric technique was used to measure triacylglycerol (TAG) using the Spinreact kit (Young, 1995).

Liver function tests

The levels of serum ALT and AST were measured with NADH Kinetic UV IFCC rec. method according to Spinreact kit (Koller and Kaplan, 1984).

Antioxidant enzymes

Hepatic catalase activity was assessed by enzymatic colorimetric method according to catalase assay kit (Fossati *et al*., 1980). SOD activity was done by enzymatic colorimetric method following the instructions of super oxide dismutase activity assay kit (Arafa *et al*., 2014). Hepatic MDA level was determined by enzymatic colorimetric method according to lipid peroxide (Malondialdehyde) assay kit (Ohkawa *et al*., 1979).

Real – time quantitative PCR (qPCR) Analysis

The real-time RT-PCR was performed in accordance with the manufacturer's instructions using TOP real TM qPCR 2X PreMIX++ (SYBR Green with low ROX) (Cat. # P725 or P750) in a Mx3005P Real-Time PCR System (Agilent Stratagene, USA). (Korea, Enzynomics) Table 1 contains a list of the utilized primers. The expression levels of the target genes were normalized using the mRNA expression of a well-known housekeeping gene, GAPDH. Using the 2-CT approach, the findings are displayed as

Table 1. Primer sequences of real time PCR.

Gene	Forward	Reverse
ACC	AGTTGGGAAGAAGTGCCAGG	AGGCCCAAGGTGTCATAAGC
FASN	TGTACCCTCTAGCTGGACCC	CCAGGCTAAGGGCAATGGAA
CPT-1	TGCAGTCGACTCACCTTTCC	TCAAAGAGCTCCACCTGCTG
PPAR-α	GTCCTCTGGTTGTCCCCTTG	GTCAGTTCACAGGGAAGGCA
GAPDH	GCATCTTCTTGTGCAGTGCC	TACGGCCAAATCCGTTCACA

fold-changes from the control group (Livak and Schmittgen, 2001).

Statistical analysis

SEM (Standard Error of Mean) was used to report the results as mean. The effect of the treatment groups on the various biochemical indicators was assessed using one-way analysis of variance (ANOVA), Duncan multiple testing, and post hoc analysis. A value of P < 0.05 was used to determine statistical significance. The statistical package for Social Sciences version 24.0 (IBM Corp., Armonk, NY) and GraphPad Prism 8.0.2 (GraphPad Software, Inc.) were used for all analyses and charts.

Results

Silymarin was shown molecular weight: 42.44 gm/mol, shape: Spheroidal, size: Less than 50 nm, appearance (form): Powder and appearance (Color): Pale yellow (Fig. 1A). Moreover, chitosan was shown molecular weight: Less than 100 KDa, shape (TEM): Spherical shape, size (TEM): Less than 50 nm, appearance (form): Powder, appearance (Color): White, degree of deacetylation: 85% (Fig. 1B). In SILCSNPs, Chitosan molecular weight: Less than 100KDa, degree of deacetylation: 85% and silymarin molecular weight: 482.44 gm/mol, shape: Spheroidal and size: 25±5nm, appearance: Pale yellow, form: Powder (Fig. 1C).

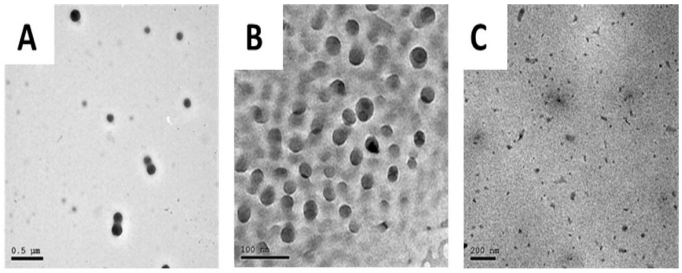

Fig. 1. TEM images of the prepared nanoparticles. TEM images of the prepared silymarine NPS 50 nm (A). TEM images of the prepared chitosan NPS 50 nm (B). TEM images of the prepared silymarin-loaded chitosan NPS (SILCSNPs) and SAED pattern 25±5 nm (C).

A significant increase in the level of cholesterol (P < 0.05) and triglycerides (P < 0.05) was noticed in NAFLD group compared to control group. Such increases in cholesterol and triglycerides levels significantly decreased in nano-silymarin (P < 0.05), nano-chitosan (P < 0.05) and SILCSNPs (P < 0.05) groups compared to NAFLD group (Fig. 2).

ALT (P < 0.05) and AST (P < 0.05) levels increased in NAFLD group compared to control one. These increases in ALT and AST levels significantly decreased in nano-silymarin (P < 0.05), nano-chitosan (P < 0.05) and SILCSNPs (P < 0.05) groups compared to NAFLD group (Fig. 3).

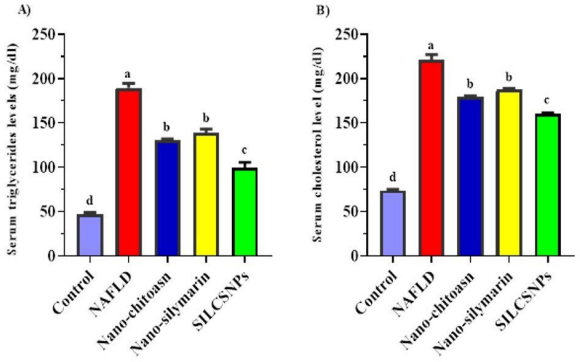

Fig. 2. Effect of nano-silymarin, nano-chitosan and SILCSNPs on lipid parameters in rats induced with NAFLD. (A) Serum triglycerides level (mg/dl). (B) Serum cholesterol level (mg/dl). Means with different superscript were statistically different at p < 0.05.

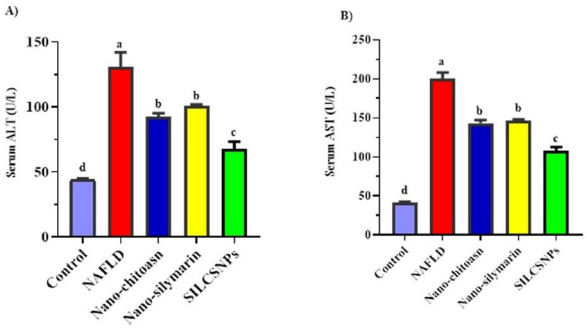

Fig. 3. Effect of nano-silymarin, nano-chitosan and SILCSNPs on liver function tests in rats induced with NAFLD. (A) Serum ALT (U/L). (B) Serum AST (U/L). Means with different superscript were statistically different at p < 0.05.

Levels of SOD (P < 0.05) and catalase (P < 0.05) decreased in NAFLD group compared to control one. These decreases in SOD and catalase levels significantly increased in nano-silymarin (P < 0.05), nano-chitosan (P < 0.05) and SILCSNPs (P < 0.05) groups compared to NAFLD group (Fig. 4).

MDA level significantly increased (P < 0.05) in NAFLD group compared to control one. This increase in MDA level significantly decreased in nano-silymarin (P < 0.05), nano-chitosan (P < 0.05) and SILCSNPs (P < 0.05) groups compared to NAFAOFLD group (Fig. 4).

ACC (acetyl coA carboxylase) and FASN (fatty acid synthase) levels increased (P < 0.05), while CPT-1 (carnitine palmitoyltransferase-1) and PPAR-α (Peroxisome proliferator-activated receptor alpha) levels reduced (P < 0.05) in NAFLD group compared to control one. These increases in ACC and FASN levels significantly decreased in nano-silymarin (P < 0.05),

Table 2. Lesions score of the severity extent in the hepatic tissue.

lesions	Control	NAFLD	Nano-chitosan	Nano-silymarin	SILCSNPs
Steatosis	0	3	1	1	0
Ballooning degenerations	0	3	2	1	0
Necrotic cells	0	1	0	0	0
Lymphocytic infiltrations	0	2	1	1	1

Lesions score system was as follows: 0: no detectable histopathological lesion; 1: Rarely minimal or focal; 2; Multifocal, 3 = patchy or diffuse, as a semiquantitative method.

nano-chitosan (P < 0.05) and SILCSNPs (P < 0.05) groups compared to NAFLD group and such decreases in CPT-1 and PPAR-α levels significantly increased in nano-silymarin (P < 0.05), nano-chitosan (P < 0.05) and SILCSNPs (P < 0.05) groups compared to NAFLD group (Fig. 5).

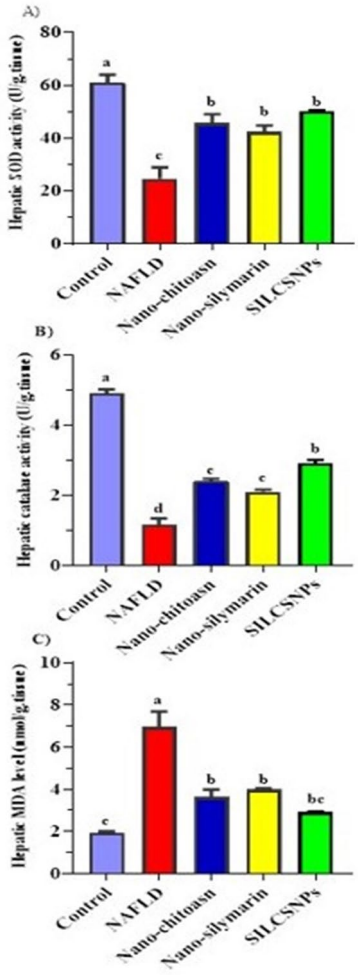

Fig. 4. Effect of nano-silymarin, nano-chitosan and SILCSNPs on anti-oxidant enzymes and malondialdehyde in rats induced with NAFLD. (A) Hepatic SOD activity (U/g.tissue). (B) Hepatic Catalase activity (U/g tissue). (C) Hepatic MDA level (nmol/g.tissue). Means with different superscript were statistically different at p < 0.05.

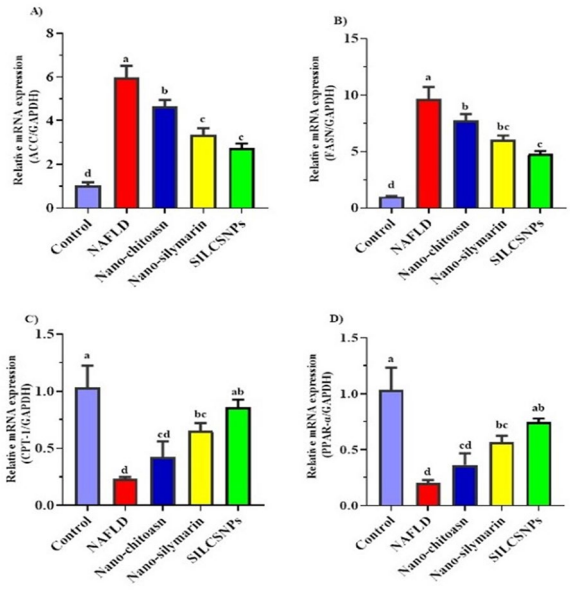

Fig. 5. Effect of nano-silymarin, nano-chitosan and SILCSNPs on hepatic mRNA gene expression in rats induced with NAFLD. (A) Hepatic ACC mRNA expression. (B) Hepatic FASN mRNA expression. (C) Hepatic CPT-1 mRNA expression. (D) Hepatic PPAR-α mRNA expression. Means with different superscript were statistically different at p < 0.05.

Liver showed normal histological structures of hepatic cords and hepatic vasculatures in control group (Fig. 6A). While NAFLD group revealed centrilobular steatosis in 60% of hepatic parenchyma alternated with ballooning degenerated cells. Necrotic some hepatic cells with pyknotic or absent nuclei were seen. Interstitial round cells infiltrations were also detected (Fig. 6B). Nano-chitosan group exhibited presence of ballooned degenerated cells in 20% of examined sections (Fig. 6C). However, Minute clear vacuoles were seen within 15 % of hepatic parenchyma in Nano-silymarin group (Fig. 6D). SILCSNPs showed ameliorative effect of hepatic tissue with presence of minute number of lymphocytes within sinusoids (Fig. 6E). Lesions score of the severity extent in the hepatic tissue was also illustrated (Table 2).

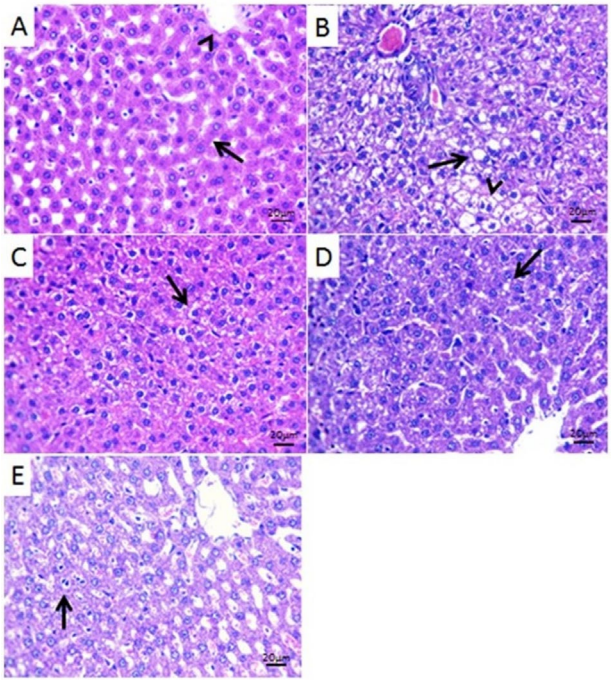

Fig. 6. Histopathological examination of hepatic tissue. (A) Liver of control group. (B) Liver of NAFLD group. (C) Liver of nano-chitosan group. (D) Liver of nano-silymarin group. (E) Liver of SILCSNPs group.

Discussion

The primary goal of this study was to investigate the impacts that nano-silymarin, nano-chitosan and SILCSNPs might have a beneficial effect in NAFLD treatment. The most prevalent hepatic condition in wealthy countries is non-alcoholic fatty liver disease (NAFLD), which can lead to steatohepatitis, cirrhosis, and liver cancer. NAFLD is usually described as the hepatic part of the metabolic syndrome due to its associations with atherogenic dyslipidemia, obesity, and type 2 diabetes (Lim *et al*., 2015). Epidemiology shows that during the past few years, viral hepatitis incidence has dropped while NAFLD prevalence has drastically increased in Western countries. Thus, the most common cause of chronic hepatopathy will soon be NAFLD (Chalasani *et al*., 2012). For NAFLD, silymarin has pharmacological effects on oxidative stress, insulin resistance, and mitochondrial dysfunction (Federico *et al*., 2017). Additionally, silymarin is utilized to treat hepatocellular carcinoma and liver cirrhosis, which are frequent terminal stages of a number of hepatopathies. This is accomplished by changing different molecular patterns (Federico *et al*., 2017). Silymarin exhibits anti-inflammatory effects in NAFLD animal models (Salamone *et al*., 2012). It can also rebuild the liver and bring back normal levels of the hepatic markers alanine transaminase (ALT), aspartate transaminase (AST), and others (Shareef, 2019).

On the other hand, chitosan significantly decreased blood sugar levels in both humans and animals (Lee *et al*., 2021). In addition to significantly lowering dietary fat absorption in the gut, oligosaccharide significantly decreased the amount of total plasma cholesterol (Jo *et al*., 2014). Due to its hypolipidemic properties, chitosan reduced the lipedema caused by a high-fat diet (Wintzingerode *et al*., 1997). Chitosan supplementation also decreased high-fat diet-induced lipedema because of its hypolipidemic activity, which also increased silymarin's oral bioavailability and lipid-lowering efficiency for treating NAFLD (Abd-Elhakeem *et al*., 2016). Additionally, studies on hyperlipidemic animal models have demonstrated that

chitosan considerably lowers blood lipid levels and prevents weight gain in obese animals (Liao *et al*., 2013).

Hepatic lipid accumulation in NAFLD is augmented due to increased fatty acid absorption and de novo lipogenesis. There is no definitive way to inhibit the rising intrahepatic fat accumulation despite potential increases in lipid clearance. At the beginning of the illness, lipid export is increased; however, as the disease worsens and hepatocyte metabolism is progressively compromised, it diminishes or plateaus (Ipsen *et al*., 2018). Attempts to lower lipid levels can potentially accelerate the onset of disease since fatty acid oxidation can cause oxidative stress, which depletes antioxidant capacity and increases harm to cellular DNA. The counter-regulatory systems linked to chronic lipid overload and NAFLD, as well as the molecular mechanisms that regulate hepatic lipid homeostasis, are intricately intertwined and complex (Ipsen *et al*., 2018).

In this study, we detected increased plasma concentration of triglycerides and total cholesterol in NAFLD group compared to control group and this may be attributed to dyslipidemia (Cohen and Fisher). Such increases in triglycerides and cholesterol levels significantly decreased in nano-silymarin, nano-chitosan and SILCSNPs groups compared to NAFLD group. Consistent with these results, prior research showed a drop in the level of total cholesterol and triglycerides levels by administration of silymarin (200 mg/kg for 10 days) in albino mice affected with NAFLD (Sahin *et al*., 2020). In another study, silymarin with dose of 30 mg/kg for four weeks lowered the levels of triglycerides and total cholesterol in rats with NAFLD (Tajmohammadi *et al*., 2018).

Abnormal high levels of serum ALT and AST suggested the presence of hepatotoxicity and liver damage, which are closely linked to hyperlipidemia and hepatic steatosis (Chang *et al*., 2013). Serum AST and ALT are the most sensitive markers used in the detection of liver injury because they are found in the cytoplasm and are consequently released into the blood following cellular damage (Wang *et al*., 2002). A significant increase in AST and ALT activity is directly caused by hepatotoxins' interactions with cellular membranes, mitochondria, or the effects of free radicals. It may therefore be linked to the severe breakdown of liver parenchyma and the subsequent release of enzymes that raises their blood levels. (Sehrawat *et al*., 2006). In our study, ALT and AST activities are increased in NAFLD group in comparison with control group due to ALT and AST release into the bloodstream when liver is damaged or in case of liver fibrosis (Shan *et al*., 2015). Such increases in ALT and AST activities were significantly decreased in nano-silymarin, nano-chitosan and SILCSNPs groups compared to NAFLD group. Similar to this study findings, previous research found that chitosan with a dose 400 mg/kg intragastrically once a day for 7 weeks reduced levels of ALT and AST in mice affected with NAFLD (Tao *et al*., 2019). In another experiment, silymarin with a dose of 200 mg/kg for 10 days reduced levels of ALT and AST in fructose-induced NAFLD mice (Sahin *et al*., 2020).

In this experiment, SOD and catalase activities were decreased in NAFLD group than control group because of increased oxidative stress and liver damage (Dhibi *et al*., 2011). Such decreases in SOD and catalase activities significantly increased in nano-silymarin, nano-chitosan and SILCSNPs groups compared to NAFLD group. In agree with our results, administration of chitosan (400 mg/kg by oral gavage) for seven weeks increased SOD and catalase activities in NAFLD mice (Tao *et al*., 2019). Another experiment reported that silymarin at dose of (100 mg/kg/orally/daily) for 10 weeks increased SOD and catalase activities in rats with nonalcoholic Steatohepatitis (Marzouk *et al*., 2017). Oxidative stress (OS), which happens when reactive species rise and antioxidant systems decline, is the primary cause of the intra- and extrahepatic problems connected to NAFLD (Chen *et al*., 2019). Two important elements that can affect NAFLD's antioxidant system and increase ROS are increased levels of free fatty acids and liver lipid excess. The key mechanisms of OS are endothelial dysfunction, failure of cellular organelles, and stress on the endoplasmic reticulum (ER). These structural and functional abnormalities of liver tissue brought on by ROS have a detrimental effect on extrahepatic tissues and organs (Sies, 2015; Zhang *et al*., 2019).

In our results, MDA level was increased in NAFLD group than control group and this may be attributed to increase oxidative stress-induced liver injury (Liu *et al*., 2016). Such increase in MDA level was significantly decreased in nano-silymarin, nano-chitosan and SILCSNPs groups compared to NAFLD group. Parallel to our study findings, previous experiment reported that chitosan (400 mg/kg/once daily by oral gavage) for seven weeks reduced the level of MDA in NAFLD mice (Tao *et al*., 2019). Another data reported that using of silymarin with two different doses 200 mg/kg and 400 mg/kg for 8 weeks reduced MDA level in male albino wistar rats affected with NAFLD (Mengesha *et al*., 2021).

In these data, genes of lipogenesis including ACC (Imai and Cohen, 2018), and FASN (Su *et al*., 2022), levels increased in NAFLD group than control group, while levels of genes of lipolysis including CPT-1 (Servidio *et al*., 2011), PPAR-α (Souza-Mello, 2015) decreased in NAFLD group than control group and this may be due to increase the synthesis of fatty acids. Such increases in ACC and FASN levels were significantly decreased in nano-silymarin, nano-chitosan and SILCSNPs groups compared to NAFLD group. Moreover, such decreases in CPT-1 and PPAR-α level were significantly increased in nano-silymarin, nano-chitosan and SILCSNPs groups compared to NAFLD group. Our results agreed with the previous findings in which, silymarin (200 mg/kg/12 weeks) dramatically lowered the levels of ACC and FAS in NAFLD mice (Ezhilarasan and Lakshmi, 2022). Moreover, silymarin at a dose of 80 mg/kg administered intraperitoneally for four weeks enhanced the level of PPAR-α gene expression in steatotic model mice (Vaezi *et al*., 2021). In another study, a group of high fat diet with high dose of chitosan (400 mg/kg/7 weeks) significantly increased levels of mRNA expression of CPT-1 and PPAR, while significantly reduced levels of mRNA expression of ACC and FAS in NAFLD mice (Tao *et al*., 2019).

Silymarin stimulates the fatty acid sensor peroxisome proliferator-activated receptor (PPAR), which promotes transcription of the genes required for the hepatocytes' lipid oxidation enzymes. Additionally, silybin stimulates the activity of AMP-activated protein kinase, which blocks the activity of the carbohydrate response element-binding protein and the sterol regulatory element-binding protein to suppress the production of the genes responsible for de novo lipogenesis (Ezhilarasan and Lakshmi, 2022). Chitosan supplementation significantly decreased the protein expression of the lipogenic transcription factors SREBP1c and PPAR- in the liver and adipose tissues brought on by high-fat diets while considerably increasing AMP-activated protein kinase (AMPK) phosphorylation. Furthermore, following supplementing with chitosan, high-fat diet-fed rats displayed noticeably lower expressions of downstream lipogenic genes (FAS, HMGCR, FATP1, and FABP4) in their livers and adipose tissues. These results shown that chitosan decreases high-fat diet-induced lipogenesis in rats via activating AMPK and suppressing genes associated to lipogenesis (Chiu *et al*., 2015).

Conclusion

Finding possibly unique remedies with fewer side effects is advantageous for health problems since nonalcoholic fatty liver disease is a significant health problem and growing worldwide. The present study looked into the rational development and application of nanoparticles (nano-silymarin, nano-chitosan, and SILCSNPs) as successful therapies for NAFLD. We verified that these nanoparticles prevent lipid buildup in rat liver and boost the therapeutic potency of silymarin and chitosan. These results suggest that these nanoparticles especially SILCSNPs would represent a potential therapeutic approach for NAFLD.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abd-Elhakeem, M.A., Farag, N., Maurice, M., 2016. Effects of dietary chitosan nanoparticles on serum lipid concentration in hyperlipidemic rats induced by a high-fat diet. Egyptian Journal of Pure and Applied Science 54, 17-21.

Abenavoli, L., Milic, N., Capasso, F., 2012. Anti-oxidant therapy in non-alcoholic fatty liver disease: the role of silymarin. Endocrine 42, 754-755.

Arafa, M.H., Mohammad, N.S., Atteia, H.H., Abd-Elaziz, H.R., 2014. Protective effect of resveratrol against doxorubicin-induced cardiac toxicity and fibrosis in male experimental rats. Journal of physiology and biochemistry 70, 701-711.

Chalasani, N., Younossi, Z., Lavine, J.E., Charlton, M., Cusi, K., Rinella, M., Harrison, S.A., Brunt, E.M., Sanyal, A.J., 2018. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. Hepatology 67, 328-357.

Chalasani, N., Younossi, Z., Lavine, J.E., Diehl, A.M., Brunt, E.M., Cusi, K., Charlton, M., Sanyal, A.J., 2012. The diagnosis and management of non-alcoholic fatty liver disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. Hepatology 55, 2005-2023.

Chang, H.-C., Huang, C.-N., Yeh, D.-M., Wang, S.-J., Peng, C.-H., Wang, C.-J., 2013. Oat prevents obesity and abdominal fat distribution, and improves liver function in humans. Plant foods for human nutrition 68, 18-23.

Chen, Z., Yu, Y., Cai, J., Li, H., 2019. Emerging molecular targets for treatment of nonalcoholic fatty liver disease. Trends in Endocrinology and Metabolism 30, 903-914.

Chiu, C.-Y., Chan, I.-L., Yang, T.-H., Liu, S.-H., Chiang, M.-T., 2015. Supplementation of chitosan alleviates high-fat diet-enhanced lipogenesis in rats via adenosine monophosphate (AMP)-activated protein kinase activation and inhibition of lipogenesis-associated genes. Journal of agricultural and food chemistry 63, 2979-2988.

Clichici, S., David, L., Moldovan, B., Baldea, I., Olteanu, D., Filip, M., Nagy, A., Luca, V., Crivii, C., Mircea, P., 2020. Hepatoprotective effects of silymarin coated gold nanoparticles in experimental cholestasis. Materials Science and Engineering: C 115, 111117.

Cohen, D.E., Fisher, E.A. 2013. Lipoprotein metabolism, dyslipidemia, and nonalcoholic fatty liver disease. Semin Liver Dis. 33, 380–388.

Dhibi, M., Brahmi, F., Mnari, A., Houas, Z., Chargui, I., Bchir, L., Gazzah, N., Alsaif, M.A., Hammami, M., 2011. The intake of high fat diet with different trans fatty acid levels differentially induces oxidative stress and non alcoholic fatty liver disease (NAFLD) in rats. Nutrition & metabolism 8, 1-12.

Donnelly, K.L., Smith, C.I., Schwarzenberg, S.J., Jessurun, J., Boldt, M.D., Parks, E.J., 2005. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. The Journal of clinical investigation 115, 1343-1351.

Duceppe, N., Tabrizian, M., 2010. Advances in using chitosan-based nanoparticles for in vitro and

in vivo drug and gene delivery. Expert opinion on drug delivery 7, 1191-1207.
Ezhilarasan, D., Lakshmi, T., 2022. A molecular insight into the role of antioxidants in nonalcoholic fatty liver diseases. Oxidative Medicine and Cellular Longevity 2022.
Federico, A., Dallio, M., Loguercio, C., 2017. Silymarin/silybin and chronic liver disease: a marriage of many years. Molecules 22, 191.
Fossati, P., Prencipe, L., Berti, G., 1980. Use of 3, 5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymic assay of uric acid in serum and urine. Clinical chemistry 26, 227-231.
Hasanin, M.T., Elfeky, S.A., Mohamed, M.B., Amin, R.M., 2018. Production of well-dispersed aqueous cross-linked chitosan-based nanomaterials as alternative antimicrobial approach. Journal of Inorganic and Organometallic Polymers and Materials 28, 1502-1510.
Hsu, W.-C., Ng, L.-T., Wu, T.-H., Lin, L.-T., Yen, F.-L., Lin, C.-C., 2012. Characteristics and antioxidant activities of silymarin nanoparticles. Journal of nanoscience and nanotechnology 12, 2022-2027.
Imai, N., Cohen, D.E., 2018. Trimming the fat: acetyl-CoA carboxylase inhibition for the management of NAFLD. Hepatology (Baltimore, Md.) 68, 2062.
Ipsen, D.H., Lykkesfeldt, J., Tveden-Nyborg, P., 2018. Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. Cellular and Molecular Life Sciences 75, 3313-3327.
Javed, S., Kohli, K., Ali, M., 2011. Reassessing bioavailability of silymarin. Alternative medicine review 16, 239.
Jo, S.-H., Ha, K.-S., Lee, J.-W., Kim, Y.-C., Apostolidis, E., Kwon, Y.-I., 2014. The reduction effect of low molecular weight chitosan oligosaccharide (GO2KA1) on postprandial blood glucose levels in healthy individuals. Food Science and Biotechnology 23, 971-973.
Koller, A., Kaplan, A., 1984. The CV Mosby Co St. Louis toronto princeton. Clin Chem 418, 1316-1324.
Layton, C., Suvarna, K., 2013. Bancroft's theory and practise of histological techniques (Co-author).
Lee, J.-Y., Kim, T.Y., Kang, H., Oh, J., Park, J.W., Kim, S.-C., Kim, M., Apostolidis, E., Kim, Y.-C., Kwon, Y.-I., 2021. Anti-obesity and anti-adipogenic effects of chitosan oligosaccharide (GO-2KA1) in SD rats and in 3T3-L1 preadipocytes models. Molecules 26, 331.
Liao, A.-H., Ma, W.-C., Wu, M.-F., 2013. Evaluation of ultrasound combined with chitosan for the control of weight and local fat in mice. Ultrasound in Medicine and Biology 39, 1794-1803.
Lim, S., Oh, T.J., Koh, K.K., 2015. Mechanistic link between nonalcoholic fatty liver disease and cardiometabolic disorders. International Journal of Cardiology 201, 408-414.
Liu, J., Han, L., Zhu, L., Yu, Y., 2016. Free fatty acids, not triglycerides, are associated with non-alcoholic liver injury progression in high fat diet induced obese rats. Lipids in health and disease 15, 1-9.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods 25, 402-408.
Lombardi, R., Onali, S., Thorburn, D., Davidson, B.R., Gurusamy, K.S., Tsochatzis, E., 2017. Pharmacological interventions for non-alcohol related fatty liver disease (NAFLD). Cochrane Database of Systematic Reviews.
Ma, Y., He, H., Xia, F., Li, Y., Lu, Y., Chen, D., Qi, J., Lu, Y., Zhang, W., Wu, W., 2017. In vivo fate of lipid-silybin conjugate nanoparticles: implications on enhanced oral bioavailability. Nanomedicine: Nanotechnology, Biology and Medicine 13, 2643-2654.
Marzouk, M.A., Elsenosy, Y.A., Mahfouz, M.K., ElMageid, A.D.A., Hussein, S.A., 2017. Biochemical Effect of Silymarin Treatment on Blood and Tissue Parameters in Experimental non Alcoholic Steatohepatitis in Rats. Benha Journal of Applied Sciences 2, 65-71.
Mashek, D.G., Greenberg, A.S., 2014. Serum TAG analysis differentiates between genetic and obesity-associated NAFLD. Diabetes 63, 42-44.
Meiattini, F., Prencipe, L., Bardelli, F., Giannini, G., Tarli, P., 1978. The 4-hydroxybenzoate/4-aminophenazone chromogenic system used in the enzymic determination of serum cholesterol. Clinical chemistry 24, 2161-2165.
Mengesha, T., Gnanasekaran, N., Mehare, T., 2021. Hepatoprotective effect of silymarin on fructose induced nonalcoholic fatty liver disease in male albino wistar rats. BMC Complementary Medicine and Therapies 21, 1-13.
Mokdad, A.H., Forouzanfar, M.H., Daoud, F., Mokdad, A.A., El Bcheraoui, C., Moradi-Lakeh, M., Kyu, H.H., Barber, R.M., Wagner, J., Cercy, K., 2016. Global burden of diseases, injuries, and risk factors for young people's health during 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. The Lancet 387, 2383-2401.
Morazzoni, P., Montalbetti, A., Malandrino, S., Pifferi, G., 1993. Comparative pharmacokinetics of silipide and silymarin in rats. European journal of drug metabolism and pharmacokinetics 18, 289-297.
Muanprasat, C., Chatsudthipong, V., 2017. Chitosan oligosaccharide: Biological activities and potential therapeutic applications. Pharmacology and therapeutics 170, 80-97.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical biochemistry 95, 351-358.
Pathomthongtaweechai, N., Muanprasat, C., 2021. Potential applications of chitosan-based nanomaterials to surpass the gastrointestinal physiological obstacles and enhance the intestinal drug absorption. Pharmaceutics 13, 887.
Prabaharan, M., Mano, J.F., 2004. Chitosan-based particles as controlled drug delivery systems. Drug delivery 12, 41-57.
Rašković, A., Stilinović, N., Kolarović, J., Vasović, V., Vukmirović, S., Mikov, M., 2011. The protective effects of silymarin against doxorubicin-induced cardiotoxicity and hepatotoxicity in rats. Molecules 16, 8601-8613.
Rehm, J., Mathers, C., Popova, S., Thavorncharoensap, M., Teerawattananon, Y., Patra, J., 2009. Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. The Lancet 373, 2223-2233.
Romero-Gómez, M., Zelber-Sagi, S., Trenell, M., 2017. Treatment of NAFLD with diet, physical activity and exercise. Journal of hepatology 67, 829-846.
Sahin, E., Bagci, R., Bektur Aykanat, N.E., Kacar, S., Sahinturk, V., 2020. Silymarin attenuated non-alcoholic fatty liver disease through the regulation of endoplasmic reticulum stress proteins GRP78 and XBP-1 in mice. Journal of food biochemistry 44, e13194.
Salamone, F., Galvano, F., Cappello, F., Mangiameli, A., Barbagallo, I., Volti, G.L., 2012. Silibinin modulates lipid homeostasis and inhibits nuclear factor kappa B activation in experimental nonalcoholic steatohepatitis. Translational Research 159, 477-486.
Sehrawat, A., Khan, T.H., Prasad, L., Sultana, S., 2006. Butea monosperma and chemomodulation: protective role against thioacetamide-mediated hepatic alterations in Wistar rats. Phytomedicine 13, 157-163.
Serviddio, G., Giudetti, A.M., Bellanti, F., Priore, P., Rollo, T., Tamborra, R., Siculella, L., Vendemiale, G., Altomare, E., Gnoni, G.V., 2011. Oxidation of hepatic carnitine palmitoyl transferase-I (CPT-I) impairs fatty acid beta-oxidation in rats fed a methionine-choline deficient diet. PloS one 6, e24084.
Shan, W., Gao, L., Zeng, W., Hu, Y., Wang, G., Li, M., Zhou, J., Ma, X., Tian, X., Yao, J., 2015. Activation of the SIRT1/p66shc antiapoptosis pathway via carnosic acid-induced inhibition of miR-34a protects rats against nonalcoholic fatty liver disease. Cell death and disease 6, e1833-e1833.
Shareef, H., 2019. Phytomedicine Silybum marianum (Silymarin) as an Effective Hepato-protective source from nature. Annals of Jinnah Sindh Medical University 5, 39-46.
Shen, S.-H., Singh, S.P., Raffaele, M., Waldman, M., Hochhauser, E., Ospino, J., Arad, M., Peterson, S.J., 2022. Adipocyte-specific expression of PGC1α promotes adipocyte browning and alleviates obesity-induced metabolic dysfunction in an HO-1-Dependent fashion. Antioxidants 11, 1147.
Sies, H., 2015. Oxidative stress: a concept in redox biology and medicine. Redox biology 4, 180-183.
Souza-Mello, V., 2015. Peroxisome proliferator-activated receptors as targets to treat non-alcoholic fatty liver disease. World journal of hepatology 7, 1012.
Su, M., Cao, D., Wang, Z., Duan, Y., Huang, Y., 2022. Fatty Acid Synthase Inhibitor Platensimycin Intervenes the Development of Nonalcoholic Fatty Liver Disease in a Mouse Model. Biomedicines 10, 5.
Tajmohammadi, A., Razavi, B.M., Hosseinzadeh, H., 2018. Silybum marianum (milk thistle) and its main constituent, silymarin, as a potential therapeutic plant in metabolic syndrome: A review. Phytotherapy research 32, 1933-1949.
Tao, W., Sun, W., Liu, L., Wang, G., Xiao, Z., Pei, X., Wang, M., 2019. Chitosan oligosaccharide attenuates nonalcoholic fatty liver disease induced by high fat diet through reducing lipid accumulation, inflammation and oxidative stress in C57BL/6 mice. Marine drugs 17, 645.
Wintzingerode, F.V., Göbel, U.B., Stackebrandt, E., 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS microbiology reviews 21, 213-229.
Vaezi, M., Yaghmaei, P., Ebrahim-Habibi, A., Hayati-Roodbari, N., Irani, S., 2021. The effect of Nitrochalcone on biochemical indicators and PPAR-α gene expression in nonalcoholic male NMRI mice steatosis model. Journal of Basic Research in Medical Sciences 8, 23-31.
Vargas-Mendoza, N., Madrigal-Santillán, E., Morales-González, Á., Esquivel-Soto, J., Esquivel-Chirino, C., y González-Rubio, M. G.-L., Gayosso-de-Lucio, J.A., Morales-González, J.A., 2014. Hepatoprotective effect of silymarin. World journal of hepatology 6, 144.
Venugopal, V., Kumar, J., Muralidharan, S., 2015. Targeted delivery of silymarin to liver cells by galactosylated nanoparticles: *in-vitro* and *in-vivo* evaluation studies. Alb J Pharm Sci 2, 4-8.
Wang, Z., Huang, Y., Zou, J., Cao, K., Xu, Y., Wu, J.M., 2002. Effects of red wine and wine polyphenol resveratrol on platelet aggregation in vivo and in vitro. International journal of molecular medicine 9, 77-79.
Woo, J.S., Kim, T.-S., Park, J.-H., Chi, S.-C., 2007. Formulation and biopharmaceutical evaluation of silymarin using SMEDDS. Archives of pharmacal research 30, 82-89.
WHO, 2019. Global status report on alcohol and health 2018: World Health Organization.
Young, D. S., 1995. Effects of drugs on Clinical Lab. In: Tests, 4th ed., AACC Press.
Younossi, Z. M., Stepanova, M., Younossi, Y., Golabi, P., Mishra, A., Rafiq, N., Henry, L., 2020. Epidemiology of chronic liver diseases in the USA in the past three decades. Gut 69, 564-568.
Zhang, L., Wang, X., Cueto, R., Effi, C., Zhang, Y., Tan, H., Qin, X., Ji, Y., Yang, X., Wang, H., 2019. Biochemical basis and metabolic interplay of redox regulation. Redox biology 26, 101284.