



The extracted sulforaphane from cabbage ameliorates liver functions in rats with diethyl nitrosamine induced hepatotoxicity

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ABSTRACT

Background: Sulforaphane (SFN), a sulfur containing compound naturally occurring in cruciferous vegetables, exhibits several biological activities that make it a potent agent in the treatment of many diseases. **Purpose:** The present study was designed to evaluate the restorative potential of SFN against diethylnitrosamine (DEN)-induced hepatotoxicity in rats. **Materials and methods:** SFN was extracted from cabbage and was characterized by UV, FT-IR and GC spectrophotometry. The antioxidant activity of SFN was evaluated *in vitro* by α , α -diphenyl- β -picrylhydrazyl (DPPH) free radical scavenging method. The hepatoprotective effect of SFN was evaluated in DEN-intoxicated rats comparing to the well-known hepatoprotector, silymarin. Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), albumin and total bilirubin were determined. Histopathological of liver tissues was performed. **Results:** Levels of ALT, AST and total bilirubin were significantly decreased, whereas albumin concentration was increased in rats received SFN either before or after the administration of DEN compared to control. Moreover, Liver histology indicated that SFN repairs hepatic damage induced by DEN in rats. **Conclusion:** Cabbage extracted SFN improves the functions and architecture of liver in DEN-intoxicated rats and maximize the hepatoprotective potential of silymarin.

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1. INTRODUCTION

Hepatotoxicity indicates to liver dysfunction or liver injury that is related to an induction of drugs or xenobiotics which are termed hepatotoxins [1] Liver injury may occur through various pathways including direct toxicity of hepatotoxins

and an immunologically-mediated response affecting hepatocytes, biliary epithelial cells and/or liver vasculature [2]. The response of hepatotoxic triggered by a chemical agent bases on the concentration of the toxicant which may be either toxic metabolite, differential expression of enzymes and concentration

gradient of cofactors in blood across the acinus [3, 4]. Hepatotoxicity related clinical signs or symptoms may include yellowing of the skin, eyes and mucous membranes due to high level of bilirubin in the extracellular fluid that is termed as jaundice, severe abdominal pain, nausea, weakness, severe fatigue, skin rashes, swelling of the feet and/or legs, abnormal and rapid weight gain in a short period of time, dark urine and light colored stool [5, 6].

Diethylnitrosamine (DEN) is classified as a hepatotoxin, carcinogen and mutagen. It has been well characterized to produce reactive oxygen species (ROS) through its metabolism, particularly in the liver. The metabolism of DEN has been shown to stimulate kupfer cells causing high ROS levels which subsequently elevate the rate of mtDNA mutations, which will further impair respiratory chain function [7].

Sulforaphane (SFN, 4-methylsulfinyl-3-butenyl isothiocyanate) is widely found in brassica vegetables, such as broccoli and cabbage. This compound can be synthesized from glucoraphanin by myrosinase enzyme [8]. Various studies have been proposed that SFN has many biological properties as anti-oxidant, anti-inflammatory and anti-tumor. SFN may play an effective role in preventing such damage via inducing phase 2 enzymes, activating the Nrf2/ARE signaling pathway or suppressing nuclear translocation of NF- κ B [9]. The aim of this study is to Dietary sulforaphane repairs diethylnitrosamine-induced hepatic mitochondrial dysfunction in rats.

2. Materials and Methods.

Venom preparation:

Preparation of cabbage sprouts:

Cabbage sprouts were prepared according to the method Azizi and Amiri et al with some modification [10].

Cabbage Seeds were soaked in 20 mL of 5% sodium hypochlorite dissolved in 80 ml distilled water for 20 min and washed three times with distal water. Afterwards, seeds were soaked in 100 mL distilled water overnight. The imbibed seeds were germinated by layering seeds over moist cotton pads in a germination tray. The tray was continuously watered by spray twice per day Germination of cabbage seeds was carried out at 25 °C under photoperiod of 16 hrs light and 8 hrs darkness cycles Sprouted seeds were collected after seven days.

Crucifer aqueous extracts were prepared according to the method of Bertelli et al [11] and [12] with few modifications Briefly, plant material was ground to a fine homogeneous powder then 0.5 gram of powdered material was dissolved in PBS buffer pH 7.0 at 37 C° overnight to allow complete hydrolysis of the sulfur glycosides. The supernatant was filtrated through a piece of gauze then extracted with 5ml dichloromethane three times then the dichloromethane fraction was salted with sodium sulfate anhydrous and dehydrated using a rotary evaporator at 30°C. The residue was dissolved in 2ml of 20% v/v acetonitrile in water and washed two times with equal volume of hexane. After that, the aqueous phase was extracted three times with equal volumes of excess methylene chloride. The methylene chloride layers were pooled, dried over anhydrous sodium sulfate, and filtered through filter paper (Whatman No.1). The filtrate was dried at 33 °C under vacuum in a rotary evaporator. The concentration of sulforaphane was determined using a UV1102 spectrophotometer. The maximum wavelength (λ_{max}) which is unique for sulforaphane is 324 nm. Characteristics of sulforaphane were analyzed by using FT/IR (Model-JASCO FT/IR 4100LE, made in Japan; Range: 4000-400 cm^{-1}). Sulforaphane was detected by GC-MS using Perkin Elmer Clarus 580 Gas

chromatograph according to the method described by Matusheski et al [13], followed by checking the mass spectrum of the extracted sulforaphane in MAINLIB library. More quantities were then extracted, dehydrated by rotary evaporation and dissolved in saline for further animal treatment.

Determination of the antioxidant activity of SFN extract by α -diphenyl- β -picrylhydrazyl (DPPH) free radical scavenging method

The radical scavenging activity of crude extract of Brassica oleracea var. capitata against DPPH radical was based on the method [14, 15]. Different concentrations of the the extract to 3.9 ml of DPPH solution. A blank assay was prepared by addition of 0.1 ml methanol to 3.9 ml of DPPH solution. Both test and blank solutions were stand for 1 hour at room temperature. Then, the absorbance was read at 515 nm. The antioxidant activity against DPPH was calculated according to the following equation:

$$\% \text{ activity} = [(Ac-As)/Ac] \times 100$$

Ac; is the absorbance of the blank, **As**; is the absorbance of sample. The curve of scavenging activity percent versus concentrations was constructed and IC₅₀ of the extract was determined.

Experimental animals

In this study, 70 male rats weighing about 190–250 g were housed in wire mesh cages and were fed standard rat chow and allowed free access to water. They were kept under constant environmental conditions [temperature (23 ± 2°C), relative humidity (80 ± 5%) and light (12 h light/dark cycles)]. All experiments were carried out according to the guidelines of the ethical committee of Faculty of Science, Tanta University.

Study design:

After the rats had acclimatized for a week, they were divided into 7 groups

- Group I (control): Rats received saline for 6 weeks.
- Group II (Silym): Rats received 100mg/kg b.w., silymarin (Silym) orally day after day for six weeks.
- Group III (DEN): Rats received saline for the first 13 days and 100mg/kg b.w., diethylnitrosoamine (DEN) i.p. at the 14th and 28th days.
- Group IV (Silym and DEN): Rats administrated 100mg/kg b.w. silymarin orally day after day for two weeks and 100mg/kg b.w. DEN was administrated i.p. at the 14th and 28th days.
- Group V (SFN and DEN): Rats administrated 10mg/kg b.w. sulforaphane i.p. day after day for two weeks and 100mg/kg b.w. DEN was administrated intraperitoneally at the 14th and 28th days.
- Group VI (SFN & Silym and DEN): Rats administrated 10mg/kg b.w. sulforaphane and 100mg/kg b.w. silymarin orally day after day for two weeks, then 100mg/kg b.w. DEN was administrated i.p. at the 14th and 28th days.
- Group VII (DEN & SFN): Rats injected 100mg/kg b.w. DEN administrated i.p. at the 14th and 28th days, then at the beginning of the 29th day, 10mg/kg b.w. sulforaphane was administrated orally day after day till the 42nd day. At the 43rd day, rats were anesthetized with diethyl ether, blood samples were collected from the orbital venous plexus and serum was separated by centrifugation.

Biochemical assays:

Estimation of activities of aspartate transaminases (AST), alanine transaminases (ALT), albumin and bilirubin concentrations using commercial kits (Spinreact, Spain) were used.

Statistical analysis

One-way analysis of variance (ANOVA) was used to assess significant differences

among groups and the Tuckey test was used to compare all groups with group III "Positive control group" and showed the significant effect of treatment.

Histopathological studies:

After dissecting rats, liver tissues were removed and fixed in neutral buffered formalin 10%, washed in tap water overnight and exposed to ascending concentrations of ethanol (70, 80, 90 and 100%), cleared in Xylene and embedded in paraffin. Sections of the tissues (4-5 μ thick) were prepared and stained with Hematoxylin and Eosin (H&E) for subsequent histopathological examinations (17). They were examined under a complex Olympus light microscope and photographed by a built-in camera.

Statistical analysis

One-way analysis of variance (ANOVA) was used to assess significant differences among groups and the Tuckey test was used to compare all groups with group III "Positive control group" and showed the significant effect of treatment.

3. Results:

3.1. Sulforaphane characterization of FT/IR spectroscopy, UV spectroscopy and GC analysis

Ultraviolet spectrum (UV) of sulforaphane in cabbage seedlings extracted by methylene chloride was registered at 324 nm (Fig.1-a). The functional groups of extracted SFN by FT/IR spectrum (4000-400 cm^{-1}) showed that the peak functional groups are: 3412 (O-H from H₂O absorption), 2926, 2860(CH), 2250, 2104(N=C=S), 1458, 1366(C-H), 1246(C-N), 1011(S=O), 742(C-H), 696(C-S). The functional groups are compatible to the reference standard (Fig.1-b). The GC technique developed by Matusheski et al (Matusheski, et al. 2001) was used to identify SFN in cabbage seedlings by checking the mass spectrum of the dissolved extracts in an HPLC grade methylene chloride followed by checking

this mass spectrum in MAINLIB library. SFN was detected at retention time of 19.184 sec with a peak height of 44,354,976 as (Figure.1-c).

3.2. Antioxidant capacity analysis by DPPH technique

The extract of SFN from cabbage seedlings showed antioxidant possibility by scavenging DPPH radicals through IC₅₀ value which was at 1500 $\mu\text{g/ml}$ (figure. 2)

3.3. The effect of sulforaphane on the levels of liver enzymes and serum albumin

Figure (2) showed The concentration of serum albumin in rats received a mixture of SFN plus silymarin before DEN-induced hepatotoxicity significantly increased compared to DEN group ($p < 0.001$). Moreover, the administration of SFN to rats after DEN induced hepatotoxicity caused a significantly increase in serum albumin compared to DEN group ($p < 0.001$). The concentration of total bilirubin in rats received a mixture of SFN plus silymarin before DEN-induced hepatotoxicity significantly decreased compared to DEN group ($p < 0.001$) and also the administration of SFN to rats after DEN induced hepatotoxicity caused a significantly decrease in total bilirubin compared to DEN group ($p < 0.001$). A significant decrease in ALT level was noted in rats received SFN before DEN-induced hepatotoxicity when compared with DEN group ($p < 0.001$). In addition, ALT level was significantly decreased in rats received a mixture of SFN plus silymarin before DEN-induced hepatotoxicity compared to DEN group ($p < 0.001$). Also, in the SFN therapeutic group, a significant decrease in ALT level was recorded compared to DEN group ($p < 0.001$). Likewise, AST level significantly decreased in preventive SFN group, preventive SFN plus silymarin group and therapeutic SFN group compared to DEN group ($p < 0.001$).

3.4. Histopathological results:

Rats' liver from negative control and silymarin control groups revealed the normal histological structure of normal hepatocytes, centrally located nucleus arranged around central vein and blood sinusoid with kupffer cell (fig. 3a & 3b). Meanwhile, liver sections of DEN only group showed Cytoplasmic vacuolization disrupted pleomorphic with irregular hyperchromatic nuclei (fig. 3c). while pretreated and treated SFN groups showed some areas with mild inflammatory infiltration, vascular congestion in association with vacuolated hepatocytes (fig. 3e & 3g). of rats of Pretreated silymarin group showed dilated central vein with many mono-nuclear lymphocyte infiltrations, swollen hepatocyte with karyolytic and Pyknotic nuclei (fig. 3d). but, rats' liver tissue from pretreated combination of SFN plus silymarin group showed depicted decrease in the hepatotoxic effect of DEN and vascular congestion and focal perivascular cellular infiltration were observed (fig. 3f).

4. Discussion

This study aimed to clarify the importance naturally occurring therapeutic agents obtained from cruciferous vegetables such as cabbage. Sulforaphane (SFN), a sulfur containing organic compound, has several biological activities including antioxidant, cytoprotective, anti-inflammatory, anticancer and antimicrobial [16].

In the present study, the results of SFN characterization agreed with [17] who reported that the spectrum of UV absorption SFN was 324 nm. FT/IR spectrum of SFN (cm^{-1}): 3426 (O-H from H₂O adsorbed), 2923, 2867 (C-H), 2179, 2100 (N=C=S), 1451, 1349 (C-H), 1260 (C-N), 1021 (S=O), 739 (C-H), 688 (C-S) and SFN was detected at retention time at 18.7 min.

Our study showed that, methanolic cabbage sprouts extract was found to have

DPPH radical-scavenging capacity with IC₅₀ value 1500 $\mu\text{g/ml}$ of extract. The results were in agreement with the previous report that indicated methanolic extracts to have the highest DPPH radical scavenging capacity [18]. The differences in sulforaphane content are associated with the variability of plant cultivars and multiple environmental factors, e.g. daily temperatures, soil fertility, pathogen challenge, wounding and plant growth regulators [19].

In our study, liver enzymes (ALT and AST), albumin and total bilirubin concentrations are common markers for cellular damage the results of this study clearly revealed that DEN caused a highly significant increase in total bilirubin concentration, ALT and AST enzymes while decrease in albumin concentration. This increase or decrease due to induction of DEN that indicates that liver is the primary target of DEN. This result agreed with [20]. As a result of the reduction of oxidative stress, cabbage-extracted SFN, individually and in combination with silymarin, ameliorates liver functions of rats compared to DEN control group this result agreed with [21, 22].

Histological investigation of liver tissues indicated marked diffuse vacuolar degeneration fat vacuoles (arrows), necrosis of hepatocytes and markedly focal fibrosis, leading to disintegration of hepatic cords in rats received DEN only this agreed with [23]. While, less injury of central vein and less fat vacuole (arrows) were showed in preventive silymarin and preventive SFN groups and high improvement to become very closed to the negative control group in both of group preventive of mixture of SFN plus silymarin and group therapeutic SFN. our result agreed with [24], proposed that the inactivation of NF- κ B by SFN is likely to be mediated through the interaction with cellular redox regulators like glutathione or thioredoxin. SFN-mediated effects on cellular systems regulating oxidative stress

and activation of several cytoprotective genes, is believed to be mediated through the Nrf2/ARE signaling pathway. In Conclusion, SFN is a naturally ITC that extracted from cabbage seedlings may be effective in improvement mitochondrial dysfunction in liver, has potential anti-inflammatory, antioxidant activities.

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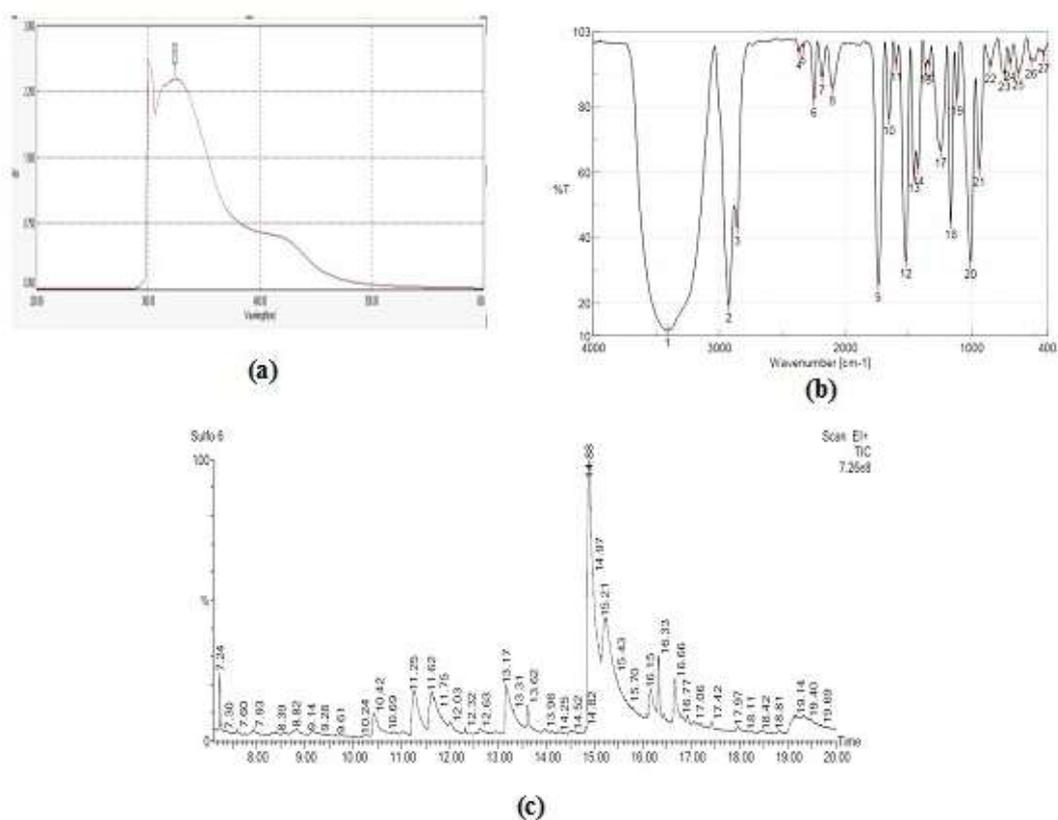


Figure 1: (a) the maximum wavelength of SFN extract by using a UV1102 spectrophotometer (λ_{\max} 324nm). (b) Infrared spectrum of extracted sulforaphane (IR) (cm^{-1}): 3412 (O-H from H_2O adsorbition), 2926, 2860(C-H), 2250, 2104(N=C=S), 1458, 1366(C-H), 1246(C-N), 1011(S=O), 742(C-H), 696(C-S). (c) GC spectrum of sulforaphane which appears with a peak at retention time of 19.184.

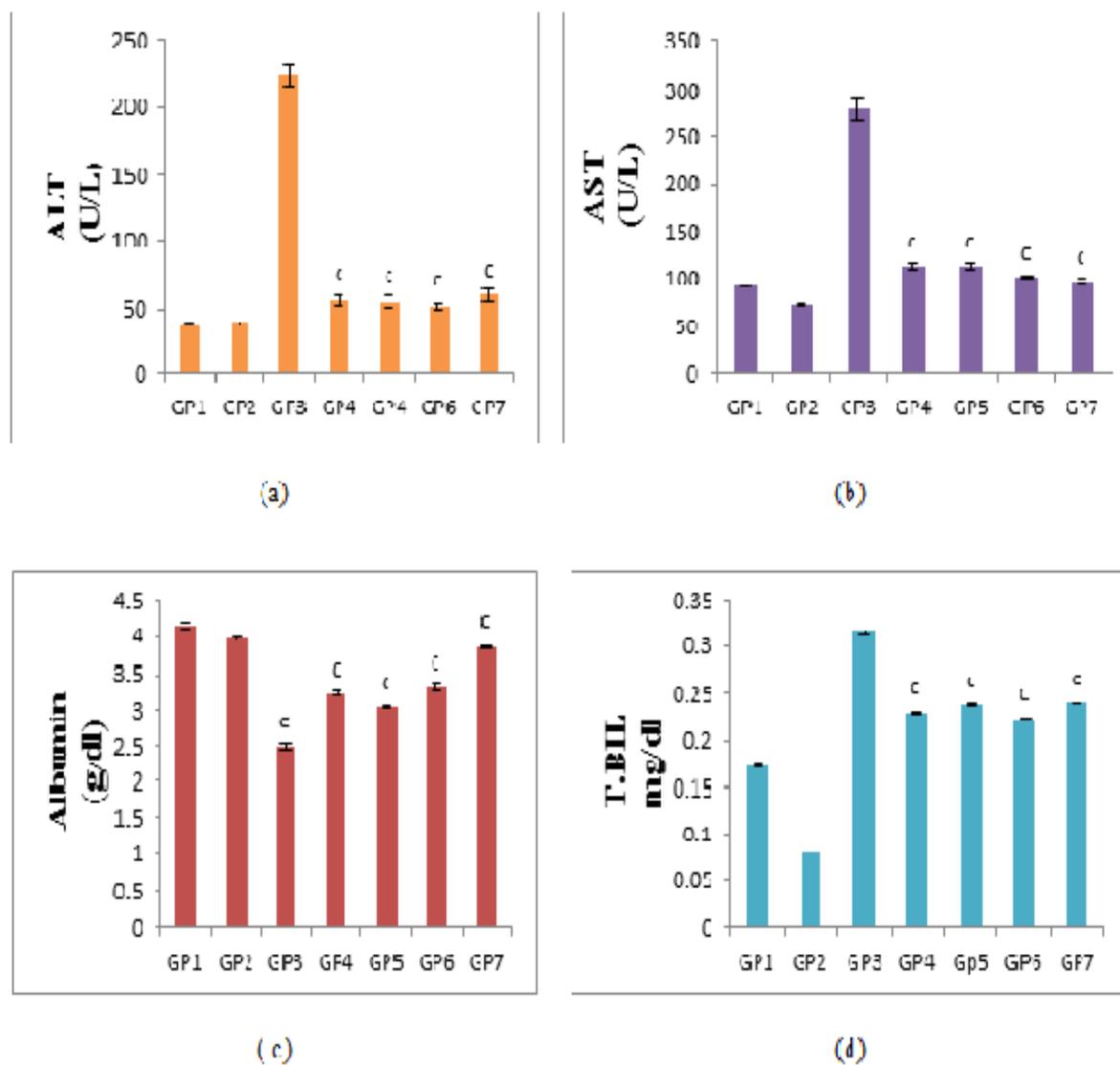


Figure2: Serum ALT & AST activities (U/L) and the concentrations of albumin (g/dl) and total bilirubin (mg/dl) in rats toxicated with DEN and treated with sulforaphane. Results were expressed as Mean±SE, where (c) indicates to $p < 0.001$.

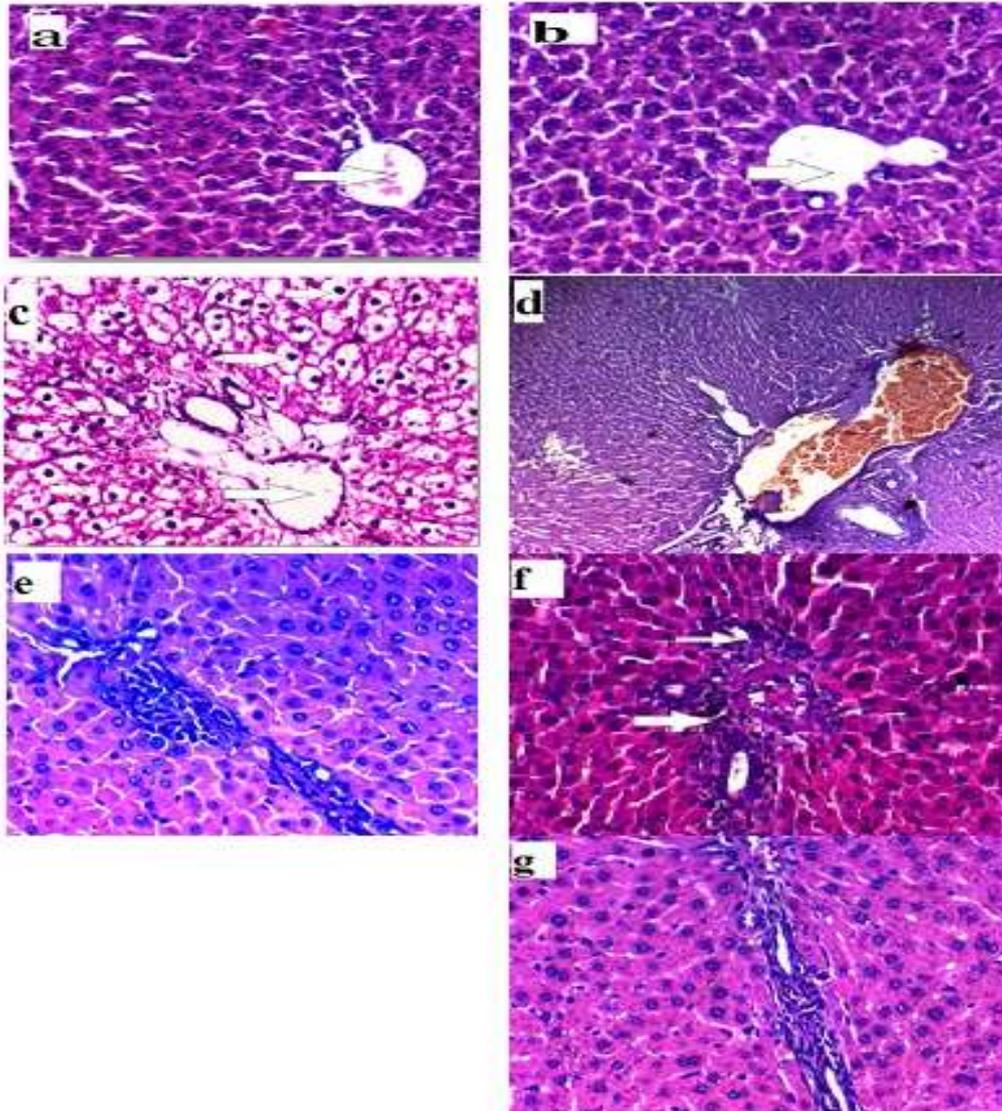


Figure3: The effect of SFN on histological characteristics of the liver (H&E staining,400×)(a)Photomicrograph of a liver in the normal control group(b)Photomicrograph of a liver in rat received silymarin(c) Photomicrograph of a liver in rat received DEN only(d) Photomicrograph of a liver in rat treated silymarin before injection DEN (e) Photomicrograph of a liver in rat treated SFN before injection DEN (f) Photomicrograph of a liver in rat treated SFN and silymarin before injection DEN (g) Photomicrograph of a liver in rat treated SFN after injection DEN.