Protective effects of red wine polyphenols and grape-seed proanthocyanidin extract on acetaminophen-induced liver injury

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Abstract

The present study was designed to examine the potential protective effects of red wine polyphenols (RWPs) and grape seed proanthocyanidin extract (GSPE) against acetaminophen-induced hepatotoxicity. Silymarin was used as a standard reference hepatoprotective agent. A single dose of acetaminophen (800 mg/kg), injected intraperitoneally to male rats, caused a significant increase in serum ALT, AST, alkaline phosphatase (ALP), bilirubin, total cholesterol (TC), triglycerides(TG), tumor necrosis factor alpha (TNF-α), and liver contents of thiobarbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA) and nitric oxide (NO) with significant decrease in serum albumin, HDL cholesterol, reduced glutathione (GSH) and hepatic activities of catalase (CAT), superoxide dismutase (SOD) and caspase-3 in liver tissue as compared with the control group. On the other hand, administration of each of GSPE (100 mg/kg/day, p.o.), RWPs (40 mg/kg/day, p.o.) and silymarin (100 mg/kg/day, p.o.) for 15 consecutive days significantly ameliorated the liver injury which confirmed by the histopathological examination. It was concluded that RWPs and GSPE showed protective effects against acute acetaminophen hepatotoxicity where RWPs were more effective than GSPE; most probably through their antioxidant, anti-inflammatory and anti-apoptotic effects.

Keywords: Acetaminophen; Hepatotoxicity; Antioxidant; Red wine polyphenols; Silymarin; Proanthocyanidins

INTRODUCTION

Acetaminophen, a commonly used analgesic drug is considered safe at therapeutic doses. However, it can cause severe liver damage or even acute liver failure that can be fatal in humans and experimental animals [1,2]. Acetaminophen is metabolized by cytochrome P450 to N-acetyl-p-benzoquinone imine (NAPQI), which reacts rapidly with glutathione (GSH), so acetaminophen overdose may result in a profound depletion of hepatocellular GSH [3]. Once GSH is exhausted, any remaining NAPQI will covalently bind to cellular proteins and induce mitochondrial dysfunction, lipid peroxidation, oxidative stress, peroxynitrite formation and DNA fragmentation and eventually leads to massive hepatocyte necrosis, liver damage or death [4]. Secondary damage may occur due to inflammatory processes that are initiated by the activation of kupffer cells, which are capable of releasing a number of proinflammatory mediators such as TNF-α that can activate other liver cells, inducing the expression of chemokines, which attract and activate circulating inflammatory cells [4]. The role of TNF-α in potentiating the liver damage produced in rats by acetaminophen administration has been demonstrated in several studies [6]. However, conflicting data are also reported, these data concluded that there is a possibility that some inflammatory factors contribute to acetaminophen liver injury at an early injurious phase, but these factors might also facilitate liver regeneration at a late phase [7]. Nitric oxide (NO) has been reported to play an important role in various kinds of liver injury, where the liver produces large quantities of NO by hepatocytes, kupffer cells and endothelial cells in response to tissue damage and inflammation induced by a variety of xenobiotics [4,8]. Caspase-3 is an executioner Caspase which proteolytically cleaves a number of vital proteins [9]. This effect is responsible for the characteristic morphological changes of cells undergoing apoptosis such as cell shrinkage and nuclear condensation [10]. Many conflicting in vitro and animal data have suggested a potential role of apoptosis in acetaminophen-induced hepatotoxicity [11]. Acetaminophen triggers the release of cytochrome c from mitochondria into the cytosol, activation of caspase-3, cleavage of poly (ADP-Ribose) polymerase, and degradation of DNA [12]. Activation of TNF-α has also been implicated in acetaminophen-induced apoptosis [13].

Many plant-derived polyphenols have the potential to be hepatoprotective and therefore can be used to treat acute and chronic liver diseases [14]. Red wines contain a wide variety of polyphenols including two main categories, flavonoids and its derivatives catechins (catechin, epicatechin, dimeric procyanidins and proanthocyanidins), flavonols (mainly quercetin and myricetin) and anthocyanins (malvidin and cyanidin 3-O-gIucosides), the non-flavonoids include stilbenoids (resveratrol), hydroxycinnammic acids (caffeic, caftaric acids) and phenolic acids (gallic acid) [15]. These compounds present in the red wine possess a number of biological effects that may be collectively participating in the antioxidant and free radical scavenging properties

[16]. Regular intake of natural polyphenols which are abundant in grapes and red wine is associated with reduced risk of cardiovascular diseases [17]. RWPs have attracted particular interest due to their in vivo and in vitro antioxidant capabilities. Their beneficial effects on cardiovascular system were described mainly in relation to the French Paradox phenomenon as well as to the Mediterranean diet [18]. RWPs have demonstrated a wide range of biological effects, including vasodilatatory [15], anti-hyperlipedimic [19], anticancer [20] and anti-inflammatory effects [21].

Grape seed proanthocyanidin extract (GSPE), an extract from red grape seeds containing a variety of phenolic compounds, is widely marketed as a dietary supplement with a variety of health benefits [22].

The most abundant phenolic compounds isolated from grape seed are flavan-3-ols (catechins) including catechin, epicatechin, epicatechin gallate, procyanidin dimers (the most common is procyanidin B2), trimers and more highly polymerized procyanidins (often referred to as oligomeric proanthocyanidins or condensed tannins) [23]. GSPEs are powerful free radical scavengers, being more effective than either ascorbic acid, vitamin E or β-carotene [24,25]. Wide variety of biological activities have also been reported, including antithrombotic [26], antitumor [27], anti-inflammatory [28], anti-hyperlipidemic [29], cardioprotective [30] and anti-fibrotic [31] effects.

Therefore, the aim of this study was to evaluate the possible protective utility of GSPE and RWPs against acetaminophen-induced hepatotoxicity in rats and to explain the potential mechanisms whereby GSPE and RWPs mediate their beneficial effects, collecting these data may lead to focusing on the development of novel hepatoprotective agents that are commonly consumed in the human diet.

MATERIALS AND METHODS

Drugs and Chemicals

Acetaminophen was obtained as a gift sample from GlaxoSmithKline (GSK)-Egypt. Red wine polyphenols 95% (Provinols™), prepared from red wine (Cabernet-Sauvignon grape variety) was provided by Seppic inc. (Paris, France).The polyphenols contained in each gram of dry powder by HPLC are 480 mg/g proanthocyanidins, 61 mg/g total anthocyanins, 19 mg/g free anthocyanins, 38 mg/g catechin, 18 mg/g hydroxycinnamic acids, 14 mg/g flavonols and 370 mg/g polymeric tannins.

grape seed proanthocyanidin extract was obtained from Tianjin Jianfeng Natural Product R&D Co., Ltd. (China), the total polyphenolic content >95% (UV) which contains procyanidin B2≥ 1.8% and oligomeric proanthocyanidins ≥ 60% (HPLC).

Silymarin, cremophor EL, bovine serum albumin (BSA), Ellman's reagent, thiobarbituric acid (TBA), reduced glutathione, 1,1,3,3-tetraethoxypropane and pyrogallol were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). All other chemicals used were of the highest grade commercially available.

Acetaminophen and silymarin were prepared in normal saline emulsified with 10% cremophor EL, while GSPE and RWPs were dissolved in warm saline before use.

Animals

Male adult Sprague–Dawley rats weighing 170–200 g were obtained from the animal breeding Laboratory (Helwan, Egypt), and maintained in the animal house of the faculty of pharmacy, Al-Azhar University, Egypt. All animals were fed with standard rat chow and water ad libitum and kept in a temperature-controlled environment (20-22°C) and 40%–60% relative humidity with an alternating 12 h light-dark cycle. The experimental protocol was approved by the local animal care committee and the experimental procedures were carried out in accordance with international guidelines for the care and use of laboratory animals [32].

Experimental protocol

Sixty four animals were divided into 8 main groups (each of 8 rats) and received treatment daily as follows:

Group1: Received daily dose of saline + 10% cremophor EL 2 ml/kg, p.o., for 15 days and served as normal control.

Group 2: Received silymarin in a daily dose of 100 mg/kg, p.o., for 15 consecutive days [33].

Group 3: Received GSPE in a daily dose of 100 mg/kg, p.o. for 15 consecutive days [30].

Group 4: Received RWPs in a daily dose of 40 mg/kg, p.o. for 15 consecutive days [34].

Group 5: Received acetaminophen in a single dose of 800 mg/kg, i.p. [35].

Group 6: Treated with silymarin in a dose of 100 mg/kg/day, p.o. for 15 consecutive days, followed by a single dose of acetaminophen (800 mg/kg, i.p.) on the 15th day.

Group 7: Treated with GSPE in a dose of 100 mg/kg/day, p.o. for 15 consecutive days, followed by a single dose of acetaminophen (800 mg/kg, i.p.) on the 15th day.

Group 8: Treated with RWPs in a dose of 40 mg/kg/day, p.o. for 15 consecutive days, followed by a single dose of acetaminophen (800 mg/kg, i.p.) on the 15th day.

Sample preparation and biochemical assays

The rats were sacrificed 24 h after acetaminophen administration. Blood samples were collected from the retro-orbital venous plexus under light ether anesthesia into non-heparinized capillary tubes, left clot and then centrifuged for 15 min at 5000 rpm to obtain a clear serum which was stored at -20◦C. Subsequently, the activities of serum enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), in addition to the serum levels of total bilirubin, albumin, total cholesterol, triglycerides, and high density lipoprotein cholesterol (HDL-C) were determined colorimetrically using commercial assay kits supplied by Biodiagnostics (Cairo, Egypt).

All animals were sacrificed by cervical dislocation, and then liver was removed, washed with ice-cold saline and stored at -20◦C, subsequently homogenized in ice cold potassium chloride (0.15M, pH 7.4). The homogenates were centrifuged at 5000 rpm for 10 min at 4◦C and the resulting supernatant was obtained and used for determination of total protein content of hepatic tissue according to the method of Lowry et al. [36] using BSA as standard protein. Estimation of the level of thiobarbituric acid reactive substances (TBARSs) was done as malondialdehyde (MDA) in liver tissue, according to the method of Mihara and Uchiyama [37] using 1,1,3,3-tetraethoxypropane as standard. Reduced glutathione was measured according to the method of Ellman [38] using Ellman's reagent [5–5'dithiobis (2-nitrobenzoic acid)] as substrate, while catalase and superoxide dismutase activities were determined according to the methods of Claiborne[39] and Marklund and Marklund [40] using hydrogen peroxide and pyrogallol as substrates respectively.

The nitric oxide level was assayed by indirect measurement of nitrite, a byproduct of NO transformation in living tissues using a colorimetric assay kit as indicated by the manufacturer (Biodiagnostics, Cairo, Egypt).

The level of tumor necrosis factor (TNF-α) in the serum and the activity of caspase-3 in the liver homogenate were determined by enzyme-linked immunosorbent assay technique (ELISA) using rat TNF-α and caspase-3 immunoassay kits according to the recommendations of the manufacturer (Cusabio® Biotech, Wuhan, China).

Histopathological examination of liver tissue

Autopsy samples were taken from the liver of rats in different groups and fixed in 10% neutral buffered formalin for 24 h and decalcification was carried out on formic acid. Washing was done with tap water and then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56◦C in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 μm thicknesses by sledge microtone. The obtained tissue sections were collected on glass slides, deparaffinized, stained with hematoxylin and eosin stain and then examination was done through the light electric microscope [41].

Statistical analysis of data

All values are expressed as means ± SEM. The results were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer method for multiple comparisons using GraphPad Prism version 5 (GraphPad, San Diego, CA) software. Differences were considered significant at $p \leq 0.05$.

RESULTS

Table 1 shows that injection of acetaminophen (i.p.) in a single dose of 800mg/kg caused significant increases in serum ALT (283%), AST (413%), ALP (247%), total bilirubin (570%), total cholesterol (70%), triglycerides (131%) and TNF-α (148%) with significant decreases in HDL cholesterol (49%) and serum albumin levels (31%) as compared with the control group.

Moreover, acetaminophen produced significant increases in hepatic MDA (311%), NO (98%) and caspase-3 (97%) and significant decreases in hepatic GSH content (61%) and the enzymatic antioxidant activities of SOD and CAT (60%&43%, respectively) in comparison with the control group (Table2).

In contrast, administration of GSPE (100 mg/kg/day) for 15 days before acetaminophen significantly reduced the elevated levels of ALT, AST, ALP, bilirubin, TC and TG in serum by 60%, 68%, 52%, 53%, 32% and 30%, respectively, as well as TNF-α (43%) and significantly increased HDL cholesterol (83%) and the serum albumin level (29%), in comparison with acetaminophen-treated group. Furthermore, it decreased MDA, NO and caspase-3 hepatic contents by 54%, 44% & 36%, respectively and increased GSH, SOD and CAT levels (126%, 81% &67%, respectively) in hepatic tissue in comparison with acetaminophen-treated group (Table 1 & 2). Similarly, treatment of animals with RWPs (40 mg/kg/day) for 15 days before acetaminophen significantly reduced the elevated levels of ALT, AST, ALP, bilirubin, TC and TG in serum by 61%, 68%, 55%, 56%, 40% and 27%, respectively, as well as TNF-α level (49%) and significantly increased HDL cholesterol (115%) and the serum albumin level (31%) (in comparison with acetaminophen-treated group), while it decreased MDA, NO and caspase-3 contents by 72%, 47% and 41%, respectively and increased the levels of GSH, SOD and CAT (175%, 100%, and 93%, respectively) in hepatic tissue in comparison with the acetaminophen-treated group.

Histopathological examination of liver sections of the control group showed normal histological structure (Figure 1A). On the other hand, histopathological analysis of acetaminophen-treated rats showed severe focal centrolobular necrosis with central pyknotic nuclei and coagulation necrosis surrounded by ballooning degeneration (Figures 1B, 1C). Pretreatment of the rats with silymarin (Figure 1D) significantly ameliorated the histological parameters induced by acetaminophen. Furthermore, pretreatment of rats with GSPE or RWPs prevented the development of histopathological damage in liver sections (Figures 1E, 1F).

DISCUSSION

Severe liver injury as a result of the overdose or chronic use of acetaminophen remains a significant clinical problem, accounting for 40% of acute liver failure cases [42]. In the present study, we demonstrated the protective effects of GSPE or RWPs on acetaminophen-induced hepatotoxicity and elucidated the mechanisms underlying the effects in rats.

The occurrence of hepatocellular damage and dysfunction induced by a toxic dose of acetaminophen was investigated by measuring the leakage of serum ALT, AST, ALP and bilirubin into the circulation [43, 44].

Furthermore, acetaminophen caused a significant decline in the activity of the antioxidant enzymes (CAT and SOD), significant depletion of GSH and enhancement of MDA and NO production in hepatic tissue. Moreover, it produced marked increases in serum TNF-α and hepatic caspase-3content. These findings are consistent with those of Yan et al. [45], Fouad and Jresat [46] and Kim et al. [47].

It is well documented that acetaminophen hepatotoxicity occurs as a result of excess production of NAPQI metabolite which depletes GSH leading to mitochondrial dysfunction and hepatocellular death. In addition, NAPQI can increase the formation of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide as well as reactive nitrogen species (RNS) such as peroxynitrite. Excessive levels of ROS and RNS can attack biological molecules such as DNA, protein and phospholipids leading to lipid peroxidation and depletion of the antioxidant enzymes that further results in oxidative stress [4, 46]. Increase in ROS Production can also activate transcription factors including nuclear factor kappa B which regulates the production of inflammatory mediators implicated in hepatotoxicity such as TNF-α [48] and sensitizes to TNF-α induced apoptosis [13]. The role of TNF-α in acetaminophen-induced liver injury has been demonstrated by Blazka et al. [6] who reported that up-regulation of TNF-α or IL-1α with specific antibodies showed protection against acetaminophen-induced liver injury in mice.

Apoptosis plays a critical role in acetaminophen-induced hepatic injury, since inhibiting apoptosis prevents the development of acute liver failure [49]. Moreover, a recent study showed that hepatic caspase-3 is activated in both wild type and CXCR2 knockout mice within one hour of acetaminophen treatment [50].

Our results revealed that pretreatment with GSPE or RWPs ameliorated serum biochemical alterations. In addition, GSPE and RWPs normalized lipid profile, restored normal serum level of albumin, which indicates preservation of cellular integrity and hepatic synthetic capacity, suggesting anti-hyperlipidemic effects which are consistent with previous results of Vinson et al. [51] and Hassan and Al-Rawi [52].

The present study demonstrated that pretreatment with GSPE or RWPs significantly ameliorated the lipid peroxidation induced by acetaminophen as manifested by the decreased MDA level, accompanied by the increased GSH content and enhanced activities of CAT and SOD. These results could be attributed to the potential antioxidant effects of GSPE [24] and RWPs [53]. In addition, RWPs normalized the elevation of TNF-α production after acetaminophen toxicity, while GSPE significantly ameliorated this effect. GSPE or RWPs have also remarkably reduced production of NO approaching the control levels. These findings might be useful for attributing the anti-inflammatory effect and are in agreement with those obtained by Li et al. [28], Marfella et al. [54], Nunes et al. [55] and Zhang et al. [56].

Moreover, our data revealed that GSPE and RWPs prevented the elevated level of caspase-3, a marker of apoptosis in hepatocytes. These results are in agreement with previous studies of Rezzani et al. [34], Boghdady [57] and Ulusoy et al. [58] who found similar anti-apoptotic effects in kidney and heart tissues.

The histopathological findings demonstrated that administration of acetaminophen induced various degenerative changes in hepatic cells, which confirmed the biochemical evidence of the oxidative stress and inflammatory response. In contrast, pretreatment with GSPE or RWPs obviously prevented the histopathological changes induced by acetaminophen.

Our data showed that RWPs was more effective as a hepatoprotective agent than the standard silymarin and GSPE. This could be explained by the fact that the majority of wine polyphenols derived from whole grape components, which include the grape seed, grape skin, and grape juice when present in combination exhibit a synergistic effect than when present individually [59-61].

CONCLUSION

GSPE or RWPs protected the hepatic tissue against acetaminophen-induced hepatotoxicity in rats, where RWPs was more effective than GSPE. The antioxidant activities may be the main principal responsible for such hepatoprotective effects. The anti-inflammatory and anti-apoptotic mechanisms can be considered Therefore, GSPE or RWPs represent a potential candidate to prevent hepatic injury caused by acetaminophen overdose. It is suggested to analyze composition of various polyphenols in RWPs and GSPE to delineate their mode of action.

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TABLE 1. Effect of pretreatment with silymarin, GSPE and RWPs on serum ALT, AST, ALP, bilirubin, albumin and lipid profile levels of acetaminophen-treated animals

Group / Parameter	Control	Silymarin	GSPE	RWPs	Acetaminophen	Silymarin + Acetaminophen	GSPE + Acetaminophen	RWPs + Acetaminophen
ALT (U/L)	25.3 ± 2.5	22.9 ± 1.9	24.2 ± 1.3	21.7 ± 2.0	96.8 ± 4.6[a]	40.7 ± 1.7[a,b]	39.2 ± 2.0[a,b]	38.0 ± 2.1[a,b]
AST (U/L)	38.6 ± 2.8	32.9 ± 2.4	29.7 ± 2.6	28.0 ± 2.4	198.1 ± 5.5[a]	73.3 ± 4.7[a,b]	63.4 ± 3.1[a,b]	62.5 ± 3.9[a,b]
ALP (U/L)	80.1 ± 6.2	79.8 ± 4.2	80.9 ± 3.9	76.6 ± 4.9	278.0 ± 11.6[a]	148.0 ± 7.4[a,b]	134.0 ± 10.8[a,b]	125.0 ± 7.6[a,b]
Total bilirubin (mg/dl)	0.88 ± 0.07	0.73 ± 0.07	0.64 ± 0.06	0.62 ± 0.05	5.9 ± 0.47[a]	2.5 ± 0.22[a,b]	2.8 ± 0.17[a,b]	2.6 ± 0.18[a,b]
Albumin (g/dl)	5.1 ± 0.15	5.5 ± 0.16	5.3 ± 0.35	5.5 ± 0.34	3.5 ± 0.16[a]	4.7 ± 0.11[b]	4.5 ± 0.15[b]	4.6 ± 0.12[b]
Cholesterol (mg/dl)	107.0 ± 8.8	95.1 ± 8.7	81.6 ± 6.8	72.7 ± 7.0	182.0 ± 11.0[a]	122.0 ± 8.9[b]	125.0 ± 6.9[b]	110.0 ± 9.5[b]
Triglycerides (mg/dl)	101.0 ± 8.1	108.0 ± 3.4	93.8 ± 8.9	98.4 ± 7.5	233.0 ± 8.7[a]	174.0 ± 5.3[a,b]	164.0 ± 9.2[a,b]	170.0 ± 8.8[a,b]
HDL-C (mg/dl)	45.0 ± 2.7	50.0 ± 4.4	54.5 ± 4.4	67.9 ± 5.7[a]	22.9 ± 1.3[a]	38.5 ± 2.6[b]	41.9 ± 3.5[b]	49.2 ± 3.1[b]

Data are expressed as means ± SEM of eight rats per group.

[a]Significantly different from the control group.

[b]Significantly different from the acetaminophen-treated group using one-way ANOVA followed by Tukey–Kramer test for multiple comparison at $p \leq 0.05$.

TABLE 2. Effect of pretreatment with silymarin, GSPE and RWPs on liver contents of MDA and GSH and activities of liver enzymes, nitric oxide and caspase-3 as well as serum TNF-α level in acetaminophen-treated animals

Group / Parameter	Control	Silymarin	GSPE	RWPs	Acetaminophen	Silymarin + Acetaminophen	GSPE + Acetaminophen	RWPs + Acetaminophen
MDA (nmol/g tissue)	25.8 ± 1.6	21.6 ± 1.9	25.3 ± 2.4	13.7 ± 0.09[a]	106.0 ± 5.8[a]	44.4 ± 3.9[a,b]	49.1 ± 3.7[a,b]	29.5 ± 2.3[b,c,d]
GSH (µmol/g tissue)	17.2 ± 1.0	21.1 ± 1.4	17.7 ± 0.90	25.2 ± 2.3[a]	6.8 ± 0.60[a]	14.4 ± 1.0[b]	15.4 ± 0.80[b]	18.7 ± 0.80[b]
CAT (U/mg protein)	1.0 ± 0.11	1.1 ± 0.08	1.2 ± 0.07	1.4 ± 0.08[a]	0.57 ± 0.07[a]	0.99 ± 0.06[b]	0.95 ± 0.07[b]	1.1 ± 0.06[b]
SOD (U/mg protein)	5.2 ± 0.35	4.6 ± .032	5.4 ± 0.45	5.2 ± 0.41	2.1 ± 0.15[a]	3.9 ± 0.23[b]	3.8 ± 0.31[b]	4.2 ± 0.33[b]
NO (µmol/L)	53.1 ± 2.1	52.0 ± 2.2	48.9 ± 3.4	44.5 ± 2.4	105.0 ± 3.7[a]	65.4 ± 3.2[b]	59.2 ± 2.4[b]	55.4 ± 3.1[b]
TNF-α (pg/ml)	43.5 ± 3.9	42.6 ± 3.5	43.1 ± 3.5	36.1 ± 2.9	108.0 ± 4.9[a]	64.2 ± 2.8[a,b]	61.8 ± 4.5[a,b]	55.5 ± 2.9[b]
Caspase-3 (ng/g tissue)	0.35 ± 0.04	0.38 ± 0.03	0.29 ± 0.04	0.34 ± 0.02	0.69 ± 0.01[a]	0.50 ± 0.02[a,b]	0.44 ± 0.05[b]	0.41 ± 0.03[b]

Data are expressed as means ± SEM of eight rats per group.

[a]Significantly different from the control group.

[b]Significantly different from the acetaminophen-treated group.

[c]Significantly different from silymarin + acetaminophen-treated group.

[d]Significantly different from GSPE + acetaminophen-treated group using one-way ANOVA followed by Tukey–Kramer test for multiple comparison at $p \leq 0.05$.

TABLE3. Effect of pretreatment with silymarin, GSPE and RWPs on histopathological findings of liver tissues of acetaminophen-treated rats

Histopathological Findings	Control	Acetaminophen	Silymarin + Acetaminophen	GSPE + Acetaminophen	RWPs + Acetaminophen
Centrolobular necrosis	(-) none	(++++) very severe	(+) mild	(-) none	(-) none
Ballooning degeneration	(-) none	(++++) very severe	(+) mild	(-) none	(-) none
Inflammatory cell infiltration	(-) none	(+++) severe	(+) mild	(-) none	(-) none
Dilatation and congestion of portal vein	(-) none	(-) none	(++) moderate	(-) none	(-) none
Dilation of central vein	(-) none	(+) mild	(++) moderate	(-) none	(++) moderate
Kupffer cell proliferation	(-) none	(+) mild	(+++) severe	(+++) severe	(++) moderate

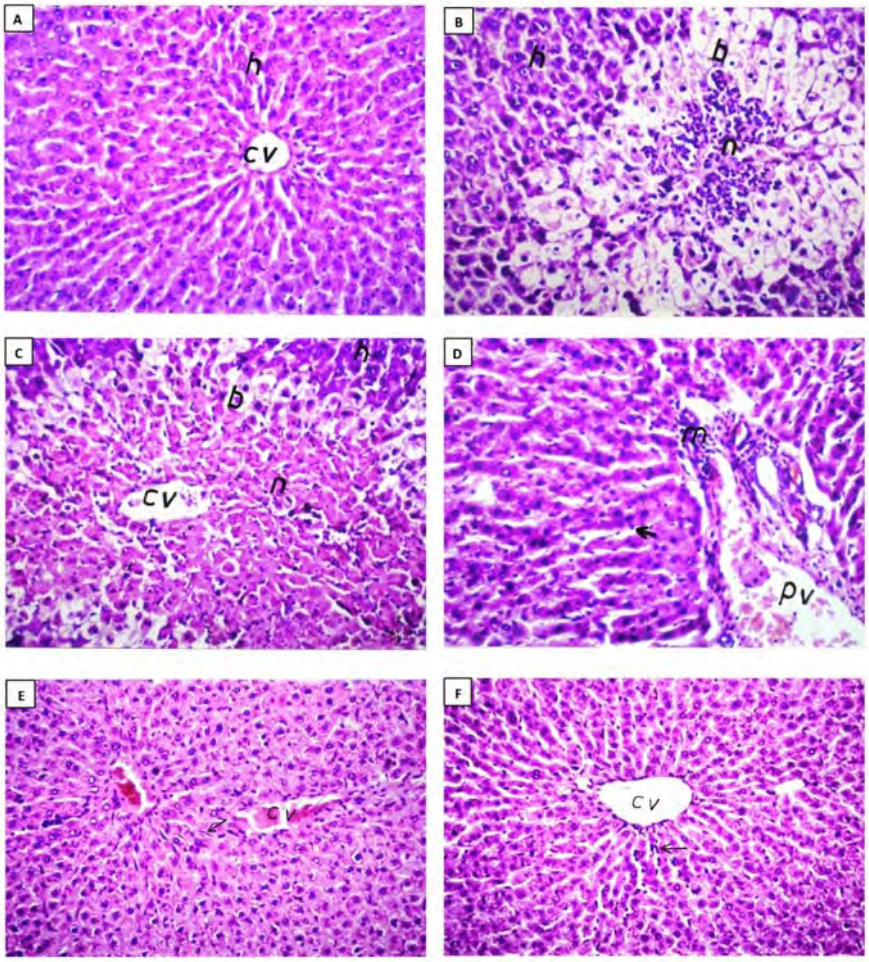

Figure1. Histology of liver samples of the control, Acetaminophen-treated group, Silymarin + Acetaminophen group, GSPE + Acetaminophen group and RWPs + Acetaminophen group. (A) Control group: normal histological structure of the central vein (CV) and surrounding hepatocytes (h); (B,C) Acetaminophen-treated group: focal centrolobular necrosis (n) surrounded by ballooning degeneration (b) in most of hepatic parenchyma; (D) Silymarin + Acetaminophen -treated group: dilatation and congestion in portal vein (PV) with few inflammatory cells infiltration (m) and diffuse kuppfer cells proliferation (arrow) between hepatocytes; (E) GSPE + Acetaminophen -treated group: diffuse kuppfer cells proliferation between hepatocytes; (F) RWPs + Acetaminophen -treated group: dilatation in central vein with few kuppfer cells proliferation (arrow) between hepatocytes . Hematoxylin–Eosin staining, magnifications: ×40.