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Research Article

SAFRANAL AMELIORATES SODIUM VALPROATE-INDUCED LIVER TOXICITY IN RATS BY TARGETING GENE EXPRESSION, OXIDATIVE STRESS AND APOPTOSIS.

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ABSTRACT

Sodium valproate (VPA) is a potentially hepatotoxic antiepileptic drug. Safranal(SAF), an essential oil derived from saffron, possesses a potent antioxidant, anti-apoptotic and anticancer properties. The current study aimed to investigate the protective effect and underlying mechanisms of SAF on the hepatotoxicity induced by VPA. SAF at the dose of 25 mg/kg was orally administered prior to VPA treatment (500 mg/kg) to rats once daily for 14 consecutive days. SAF treatment attenuated VPA-induced liver dysfunction, structural damage, hepatic ATP depletion and reduction in total antioxidant content. Moreover, SAF attenuated VPA-induced elevation in hepatic oxidant contents and lipid peroxidation. These effects were accompanied by a significant increase in gene expression of carnitine palmitoyltransferase 1A, fibroblast growth factor 21, peroxisome proliferator-activated receptor gamma 1, cytochrome P450 2E1 (CYP2E1) and heme oxygenase 1. SAF combat these effects especially in the group of rats treated VPA plus SAF. Furthermore, VPA provoked apoptotic responses evidenced by increasing protein expression of Bax and caspases-3. SAF pretreatment significantly ameliorated these apoptotic signals indicating its anti-apoptotic actions. SAF attenuates VPA-induced liver injury through decreasing the expression CYP2E1 with consequently, alleviation of oxidative stress and apoptotic signals as well as depletions of ATP.

Keywords: Liver damage, Safranal, Sodium valproate, Oxidative stress, Apoptosis.

Introduction

Valproic acid (2-n-propylpentanoic acid, VPA) is a proven anticonvulsant agent widely used in the treatment of various convulsive disorders and in the management of migraine [1,2]. VPA has been referred as the third most common xenobiotic suspected of causing death due to liver injury after acetaminophen and trogliatzone[3]. VPA induces various liver toxic effects including microvesicular steatosis and necrosis [4-6] as well as irreversible and ultimately fatal liver failure [7]. The exact mechanism of valproate-induced hepatotoxicity is still unclear. However, the abnormalities of mitochondrial functions such as impairment of mitochondrial fatty acid oxidation (FAO),inhibition in mitochondrial membrane potential and ATP depletion are widely thought to be important [7,8]. Being a simple fatty acid, VPA is a substrate for FAO which takes place primary in the mitochondria. Inside the mitochondria, VPA activates to valproyl-CoA by specific acyl-CoA synthases, (ACS). In this system, carnitine palmitoyltransferase 1 (CPT1 A) catalyses the rate limiting step of β-oxidation of VPA. Besides these important enzymes, cytochromes P450 (CYPs) enzymes such as Cytochrome P450 (CYP2E1 & CYP1A1]) can be involved in FAO [9]. CYP2E1 is localized the endoplasmic reticulum and mitochondria and catalyzes VPA metabolism to toxic metabolite with concomitant formation of reactive oxygen species (ROS)[9]. Mitochondrial, peroxisomal, and microsomal FAO is strongly regulated by peroxisome proliferator-activated receptor (PPAR), a nuclear receptor and transcription factor, which can be stimulated by endogenous fatty acids or synthetic drugs such as VPA. VPA appeared to be a pan-activator of PPAR (both PPARα, PPARδ and PPARγ)[10, 11]. PPAR is critically involved in several biochemical regulation processes, including lipid metabolism,

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apoptosis, and cell,survival[2,10,11]. Fibroblast growth factor 21 (FGF21) is a critical mediator of liver lipid metabolism and is under the control of both PPARα andPPARγ[12].

Advances in molecular approaches have clearly demonstrated that the vital mechanism of VPA liver damage is oxidative stress, as evidenced by elevation of total oxidant status (TOS), depletion of total antioxidant status (TAS)[13] as well as elevation of lipid peroxidation [14] and DNA damage [13], which result in necrosis and apoptosis in liver [1]. Since oxidative stress theory plays a corner stone in hepatotoxicity, natural antioxidants have attracted considerable attention for protection against VPA hepatotoxicity in animals [15, 16]. Among these, Safranal is the main component of saffron essential oil exhibits different pharmacological activities [17]. Studies revealed that SAF is able to capture free radicals in different types of tissues, including liver [18]. There is substantial experimental evidence on the ability of safranal at dose levels (25-100 mg /kg) to offer protection against oxidative stress, induced by drugs or toxic agents in different types of tissues, including liver [19-21].

Therefore, this study was designed to evaluate the protective properties of SAF against VPA-induced liver injury and to clarify the underling mechanisms via studying its effect on oxidative stress, gene expression profiling of lipid metabolism and apoptotic markers.

2. Materials and Methods

2.1. Chemicals

Safranal(W338907),O-dianisidine,2,4-dinitophenyl -hydrazine, thiobarbituric acid, Folin's reagent, pyrogallol, SOD enzyme, H2O2, bovine albumin, and sodium carbonate were obtained from Sigma Chemical Co., St. Louis, MO, USA. Radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors [sc-24948] was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Poly vinylidene difluoride (PVDF) membrane and blocking reagent were obtained from Roche Diagnostics GmbH (Mannheim, Germany).The primary antibodies used in the western blotting stage were obtained from BioVision(USA)whereas secondary antibodywas obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sodium Valproate (VPA)[Depakine®] Sanofi-France was purchased from Global Napi Pharmaceuticals, Egypt. All other chemicals were obtained from local commercial suppliers.

2.2. Experimental Animals

Thirty two male Wistar rats [150-200 g] were obtained from the Animal House, National Organization for Drug Control and Research (NODCAR, Cairo, Egypt). Animals were cared in accordance with standard guidelines [22]. They were maintained on standard pellet diet and tap water ad libitum and were kept in polycarbonate cages with wood chip bedding under a 12 hr. light/dark cycle and room temperature 22-24°C. Rats were acclimatized to the environment for two week prior to experimental use. The experimental animal protocol was approved by the ethical committee of NODCAR.

2.3. Experimental design

Rats were randomly divided into four groups (eight rats each). Groups were treated as follows: Control group (CON) was orally,p.o.received vehicle (1% tween 80, 5 mL/kg body weight) for 2 weeks. SAF group was received SAF suspended in 1% tween 80 (25 mg/kg/day,p.o.) for 2 weeks. VPA group was given VPA (500 mg/kg/day,p.o.) for 2 weeks. Rats of the protective group (SAF+VPA group) were received SAF with the same dose and period one hour prior to treatment with VPA (500 mg/kg/day,p.o.). The hepatotoxic dose of VPA (500 mg/kg/day,p.o.)used in the present study is based on the previous hepatotoxic study of VPA in rats [23]. SAF was administrated orally at dose level of 25mg/kg body weight. Doses of SAF were selected based on previously reported pharmacological properties of this plant. SAF at doses varying from 25-100 mg/kg has been reported to suppress toxicity, oxidative damage and inflammations induced by diazinon[24] and isoproterenol [25].

2.4. Sample Preparation

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On the 15th day of experiment, the rats were weighed then blood samples were collected from the retro-orbital venous plexus under diethyl ether anesthesia. Serum was separated using cooling centrifugation at 5000 rpm for 10 min and stored at −20°C until analysis. After the collection of blood samples, all animals were sacrificed under diethyl ether by decapitation. Three liver samples were quickly removed from each animal and washed in an ice-cold isotonic saline. One part was removed and immediately immersed in 10% buffered formalin for histopathological examinations. The other part was homogenized in ice-cold Tris-HCl buffer (150 mMKCl, 50 mMTris, pH 7.4) to give 10% homogenate w/v and stored at -20°C for further biochemical analyses. Aliquots were prepared and used for determination of different biochemical markers. The remaining part of livers was immediately snap frozen in liquid nitrogen and then stored at -80°C for further analysis.

2.5. Assessment of Liver Function

Serum was used for the determination alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and Albumin concentration using bioMerieux reagent kits (bioMerieux, 69280 Marcyl, Etoile, France). Alkaline phosphatase (ALP)activity was determined by using ALP-AMP kit (BioSystems S.A. Costa Brava 30, Barcelona, Spain) and protein concentrations were determined by total protein plus kit (ELITech Clinical System SAS-France) according to the instruction manual.

2.6. Evaluation of Liver Biomarkers of Oxidative Stress

Determination of malondialdehyde(MDA) in liver tissues is based on its reaction with thiobarbituric acid to form a pink complex with absorption maximum at 535 nm [26].Myeloperoxidase (MPO) activity in liver tissues was determined as described in Hillegass et al. [27]. One unit of MPO was defined as the amount of MPO present that degrades one µM peroxide per a minute. The total oxidant content (TOC) of liver samples was determined according as described in Erel method [28]. This method is based on the fact that oxidants present in the sample including hydroperoxides oxidize the ferrous ion–o-dianisidine complex to ferric iron and the resulting ferric ion makes a colored complex with xylenol orange in an acidic medium. TOC of the samples was expressed as nmol H_2O_2 equivalent / mg protein.

2.7. Determination of Liver Antioxidant Enzyme Activity and Total antioxidant.

Catalase activity [CAT] was measured following the method described by Aebi[29] by monitoring H_2O_2 decomposition at 240 nm as a consequence of the catalytic activity of catalase. Superoxide dismutase (SOD) activity in hepatic tissues was determined according to the method described by Nandi and Chatterjee [30]. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogalol auto-oxidation. The total antioxidant capacity in liver was evaluated using ferric reducing antioxidant power (FRAP) assay. The FRAP assay was determined according to the method described by Benzie and Strain[31]. The FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue colored ferrous- tripyridyltriazine complex from colorless oxidized ferric form by the action of electron donating antioxidants. The total protein content in liver was determined according to the Lowry's method modified by Peterson [32]. Absorbance was recorded using a PerkinElmer, Lambda 25 UV/VIS spectrophotometer.

2.8. Histopathological Examination

Pieces of liver from the same lobe were fixed in 10% formalin for about 24 h. TheParaffin sections were prepared and cut into 4 µm thick sections. The sections were passed through xylene, alcohol, water to ensure that the tissue was totally free of wax and alcohol, and then stained with H&E dye. The sections were observed under light microscope (Olympus CX31, Honduras St., London, United Kingdom).

2.9. Isolation of total RNA and*quantitativereal-time polymerase chain reaction [real time-PCR].*

Total RNA was isolated from liver tissues using RNA easy Mini Kit (Qiagen,Valencia, CA, USA) according to manufacturer's instruction and further analyzed for quantity and quality with

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Beckman dual spectrophotometer (USA). Reverse transcription–polymerase chain reaction (RT-PCR) was performed using the extracted RNA for evaluation of PPARγ1, CPT1A, Fgf21, CYP1A1, CYP2E1, ACS-1, HO-1 and GAPDH gene expressions. 1000 ng of the total RNA from each sample were used for cDNA synthesis by reverse transcription using High capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48-well plate using the Step One instrument (Applied Biosystems, Foster City, CA, USA) as follows: samples were first denatured at 95 °C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 95 °C for 15 second, an annealing step at 55-60 °C for 20 second and an extension step at 72 °C for 30 second, followed by a final extension at 72 °C for 10 min. The Primers sequence specific for each gene [Invitrogen] used are demonstrated in Table 1. The value of the cycle threshold (Ct) was used to perform calculations. Ct is defined as the cycle at which PCR amplification reaches a significant value. Expression of the house- keeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as reference gene and values were normalized to the quantity of GAPDH. All signals were expressed relatively to the average values for the control group, which was set to 1.

2.10. Determination of Hepatic Adenine Nucleotides (ATP and ADP) Concentrations.

Hepatic adenine nucleotides (ATP, and ADP) were assayed by high-performance liquid chromatography [Agilent] according to the method of Teerlink*et al.*[33]. The analysis was employed using gradient elution and UV detection at 254 nm. ATP and ADP were quantified by measurement of peak height compared to corresponding standard with each set of experiments. Results were expressed as µmol/g wet tissue. ATP/ADP ratio was then calculated from individual adenine nucleotides values.

2.11. Analysis of Proteins via SDS-PAGE and Western Blotting Method

Liver samples were homogenized in cold in RIPA buffer supplemented with inhibitors for proteases and phosphatases and protein concentrations were determined using the established Bradford dye-binding method (Bio-Rad, Hercules, CA). For direct immunoblotting, aliquots of lysate were mixed with loading buffer containing 2-mercaptoethanol and maintained at 100 ºC for 10 min before loading on 10% SDS-PAGE. Following SDS-PAGE separation, proteins were transferred to PVDF membrane. Membranes were blocked in TBST containing 5% [w/v] non-fat milk and dried for 1 h at room temperature. Membrane strips were incubated with primary antibodies [diluted 1:1000 for bax, caspase-3 and β-actin] overnight at 4 ºC. The primary antibodies used in this study (BioVision) were diluted at a ratio of 1/200, whereas the secondary antibodies were diluted at a ratio of 1/2000 (Santa Cruz Biotechnology). Following extensive washing, membrane strips were incubated with anti-rabbit IgG (1:5000; Cell Signaling Technology Inc., MA, USA) conjugated to horseradish peroxidase for 1 h. Protein bands were detected by a standard enhanced chemiluminescence method and densitometry measurements were made using ImageJ software (Image J; National Institute of Health, Bethesda, USA). The densities of target protein bands were normalized to the corresponding density of β-actin band. All signals were expressed relatively to the average values for the Control group, which was set to 1.

2.12. Statistical Analysis

SPSS (version 18) statistical program (SPSS Inc., Chicago, IL, USA) was used to carry out a one-way analysis of variance (ANOVA) on our data. When significant differences by ANOVA were detected, analysis of differences between the means of the treated and control groups were performed by using Dunnett's test.

3. Results

3.1 SAF Pretreatment Prevented VPA-Induced Liver Damage

Table 2 showedthat, serum AST ($p<0.05$) and ALT ($p<0.001$) activities were significantly increased and ALP tended to increase in the VPA group when compared with control group. In contrast,

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the activities of these enzymes were decreased significantly ($p<0.001$ for ALT and $p<0.05$ for AST) in the SAF + VPA group when compared with VPA-treated rats. Serum albumin was significantly decreased ($p<0.01$) while serum total protein was insignificantly decreased in VPA treated group. SAF pretreatment normalized the levels of these markers in VPA-treated rats when compared with control group. SAF alone did not show any changes in all these markers when compared with control group.

3.2. Effect of SAF on VPA-Induced Histological Changes

As shown in Figure 1, liver of rats in control and SAF groups showed normal histological structure of liver. After VPA treatment, liver architecture was disorganized with focal areas of necrosis. hepatocyt in focal area of necrosis appeared small in size with deeply stained acidophilic cytoplasm and dark nuclei along with congestion of central vein (figure 1, VPA, a).The portal tract was markedly infiltrated with mononuclear cells and showed severe dilated congested portal vessels (figure 1, VPA, b) and some focal cells appeared small in size with deeply stained acidophilic cytoplasm and dark nuclei and other were with pale vacuolated cytoplasm. Liver of rats SAF+VPA group showed normal lobular pattern with small degree of focal necrosis and degeneration of hepatocytes in central area (Figure 1, SAF + VPA,a). The portal tract showed mild congestion of portal vein (Figure 1, SAF + VPA,b). The majority of hepatocytes in the intermediate and peripheral zone were with central dark nuclei and pale vacuolated cytoplasm.

3.3. Effect of SAF on VPA-Induced Hepatic Oxidative Stress and Antioxidant Markers

As presented in Figures 2 and 3, MDA content ($p<0.05$) and TOC ($p<0.001$) contents were increased significantly, whereas TAC was decreased significantly ($p<0.001$) in VPA-treated rats when compared with control group. In addition, MPA activity was slightly increased in the VPA groupcompared with control groupmeanwhile,there was no statistically significant difference in CAT and SOD activities in the liver of VPA-treated rats when compared with control group. However, MDA concentration restored to normal level with significant reduction ($p<0.001$) in the SAF+VPA group compared to those in the VPA group. SAF administration alone did not show any changes in all these markers when compared with control group. Interestedly, SOD activities in the SAF and SAF+VPA groups were significantly higher ($p<0.05$) than those in the control group.

3.4 Effect of VPA and SAF on the Relative mRNA Expression of Genes Involved in FAO and VPA Metabolism in Hepatic Tissues

To better understand the molecular basis for VPA induced fatty liver and disturbance in FAO, we analyzed the expression of genes involved in lipid metabolism and VPA metabolism in liver (Table 3). Compared with control group, the VPA-treated rats showed significant increased expressions of hepatic tissue genes involved in β-oxidation, including PPARγ1, CPT1A and FGF21were significantly higher in VPA groups compared to control group while the mRNA levels of ACS remained unchanged among groups. SAF administration restored values of PPARγ1, CPT1A and FGF21 to the levels of control rats. The increase in PPARγ1, CPT1A and FGF21 expressions in VPA rats was accompanied by a parallel increase in the expression of CYP 2E1 and HO-1.These VPA-induce elevations in CYP 2E1 and HO-1 gene expressions were completely abolished by SAF treatment. There were no statistical differences in CYP 1A1 gene expression among groups.

3.5. Effect of SAF on the VPA-Induced Alteration in Hepatic ATP, ADP and ATP/ADP Ratio

ATP and ADP levels and the ratio of them (Figure 4) were significantly decreased ($P<0.001$) in livers of VPA-treated rats when compared with control group. Most importantly, SAF significantly attenuated the reduction in previous markers and significantly decreased the depletion in each of ATP ($p<0.001$) and the ratio of ATP / ADP ($p<0.05$) in the SAF+VPA group compared with VPA-treated animals.

3.6. Effect of SAF and VPA on Bax and Caspase-3 Protein Expression in Hepatic Tissues.

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Hepatic protein expressions of apoptotic markers are observed in Figure 5. Semi quantitative western blot analysis showed that, the expressions of Bax and caspase- 3 at protein level in VPA group were significantly higher ($p<0.001$) than that in control group. The increased Bax and caspase- 3 levels were significantly reduced ($p<0.001$) by pretreatment with SAF, as compared to VPA-intoxicated rats. Expressions of Bax and caspase- 3 did not significantly alerted in SAF-only treated group as compared to control values.

Table 1: Primers sequence and PCR conditions specific for each gene

Target gene	Primer sequence: 5`- 3`	*Annealing Temperature*	*PCR product base pair [bp]*
PPARγ1	Forward: TTCGGAATCAGCTCTGTGGA Reverse: CCATTGGGTCAGCTCTTGTG	55	160
CPT1A	Forward: TGGTCAACAGCAACTACTACGC Reverse: GAAGACGAATGGGTTTGAGTTC	55	152
Fgf21	Forward: CAAGCATACCCCATCCCTGA Reverse: GGTTTGGGGAGTCCTTCTGA	59	175
CYP1A1	Forward:TCAAAGAGCACT Reverse: GGGTTGGTTACC	60	179
CYP2E1	Forward:CCTTTCCCTCTTCCCATCC Reverse: AACCTCCGCACATCCTTCC	60	150
ACS-1	Forward:GAACGATTGCTCCGGTTT Reverse: TTAGCAGTCTCGGAACCACA	57	148
HO-1	Forward: AAGGAGTTTCACATCCTTGCA Reverse: ATGTTGGAGCAGGAAGGCGGTC	60	251
GAPDH	Forward: CACCCTGTTGCTGTAGCCATATTC Reverse:GACATCAAGAAGGTGGTGAAGCAG	57	134

Table 2: Effect of SAF administration on the activities of ALT, AST and ALP as well as levels of albumin and total proteins in serum of rats treated with VPA

Groups	Control	SAF	VPA	SAF+VPA
ALT (U/ml)	25.53 ± 0.50	25.10 ± 0.61	39.90 ± 2.16***	27.21 ± 1.26 [a]
AST (U/ml)	93.29 ± 2.61	89.29 ± 2.83	115.98 ± 8.76*	90.14 ± 2.96[c]
ALP (U/l)	51.52 ± 1.31	51.52 ± 1.31	62.12 ± 3.64	49.61 ± 3.50
Albumin(g/dl)	3.86 ± 0.05	3.73 ± 0.09	3.41 ± 0.04*	3.63 ± 0.14
Total Proteins (g/dl)	6.79 ± 0.08	6.75 ± 0.10	6.11 ± 0.26	6.87 ± 0.29

Data are presented as mean ± SEM(n = 8). Significance was determined by ANOVA followed by Dunnett's test: ***P<0.001, *P<0.05 vs. control group;[a] P<0.001,[c] P<0.05 vs. VPA group.

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Table 3: Effect of SAF administration on the expression of some genes related to FAO and VPA metabolism in rats treated with VPA. The mRNA of genes was determined by real-time PCR and normalized to the expression of GAPDH.

Groups	Control			SAF			VPA			SAF+VPA		
PPARy1	1.23	±	0.07	1.28	±	0.041	1.86	±	0.19*	1.43	±	0.21
ACS	1.42	±	0.19	1.37	±	0.12	1.58	±	0.12	1.47	±	0.15
CPT1A	1.41	±	0.11	1.36	±	0.12	2.20	±	0.04***	1.47	±	0.13[a]
FGF21	1.85	±	0.05	1.57	±	0.13	2.54	±	0.23*	1.11	±	0.01[b]
CYP1A1	1.67	±	0.04	1.40	±	0.10	1.32	±	0.14	1.52	±	0.18
CYP2E1	1.43	±	0.04	1.50	±	0.15	2.53	±	0.35*	1.86	±	0.21
HO-1	1.68	±	0.09	1.53	±	0.15	2.41	±	0.14**	1.47	±	0.12[a]

Data are presented as mean ± SEM(n = 8). Significance was determined by ANOVA followed by Dunnett's test: ***P<0.001, **P<0.01, *P<0.05 vs. control group; [a]P<0.001, [b] P<0.01 vs. VPA group.

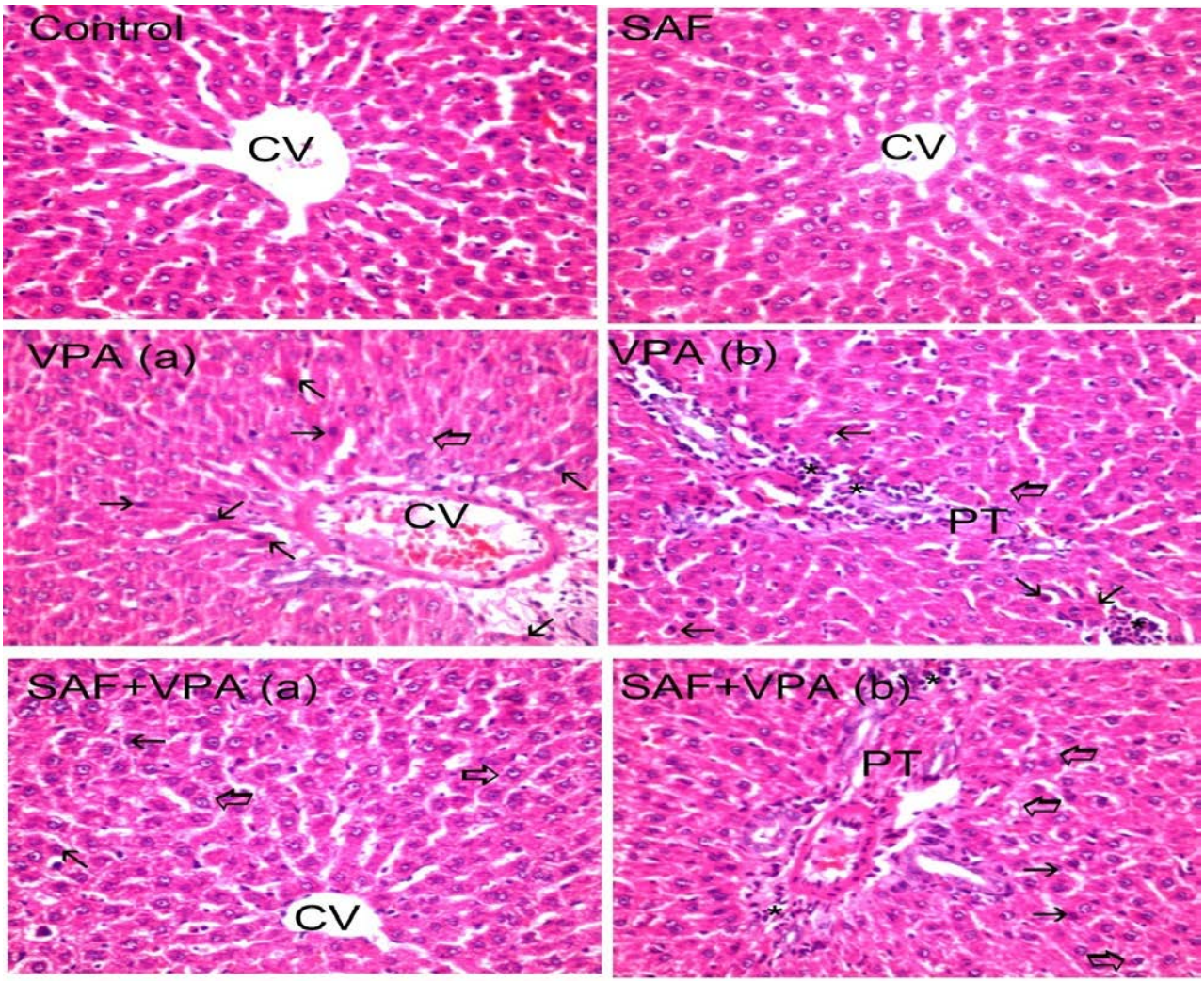

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Figure 1: Representative images of hematoxylin and eosin-stained section in the livers of all groups studied 40x. Liver sections of control and SAF groups showing normal histological structure of central vain (CV) and surrounding hepatocytes.(VPA, a): liver section of VPA group showing disorganized architecture of the liver with focal areas of necrosis, cells stained acidophilic cytoplasm and dark nuclei [thin arrow] and central vein (CV) appears congested. (VPA, b): liver section of VPA group showing mild congestion in the portal tract (PT) area and markedly infiltrated with inflammatory cells (*) with focal eosinophilic cells and pyknotic nuclei (thin arrow). (SAF+VPA, a): liver section of SAF+VPA group showing normal appearance of hepatocytes and the central vein (CV). Some cells are eosinophilic with pyknotic nuclei (thin arrow) and others are with central dark nuclei and pale vacuolated cytoplasm (thick arrows). (SAF+VPA, a): liver section of SAF+VPA group showing mild congestion of portal vein with few inflammatory cell infiltrations (*)Hepatocytes in the intermediate and peripheral zone showing fatty infiltration with pale vacuolated cytoplasm (thick arrow).

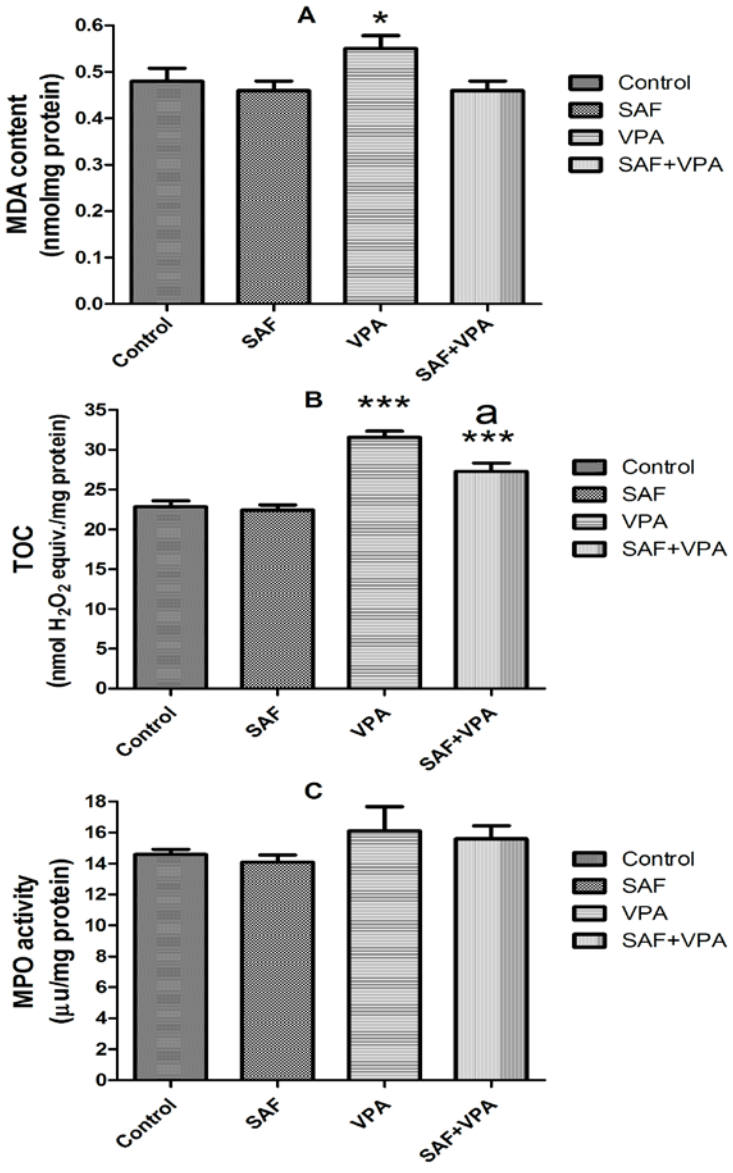

Figure 2: Effect of SAF on VPA-induced alteration in the hepatic oxidative stress markers, (A) MDA, (B) TOC and C (MPO). Values are expressed as mean ± SEM (n=8). Significance was determined by ANOVA followed by Dunnett's test: ***P<0.001, *P<0.05 vs. control group; [a] P<0.001vs.VPA group.

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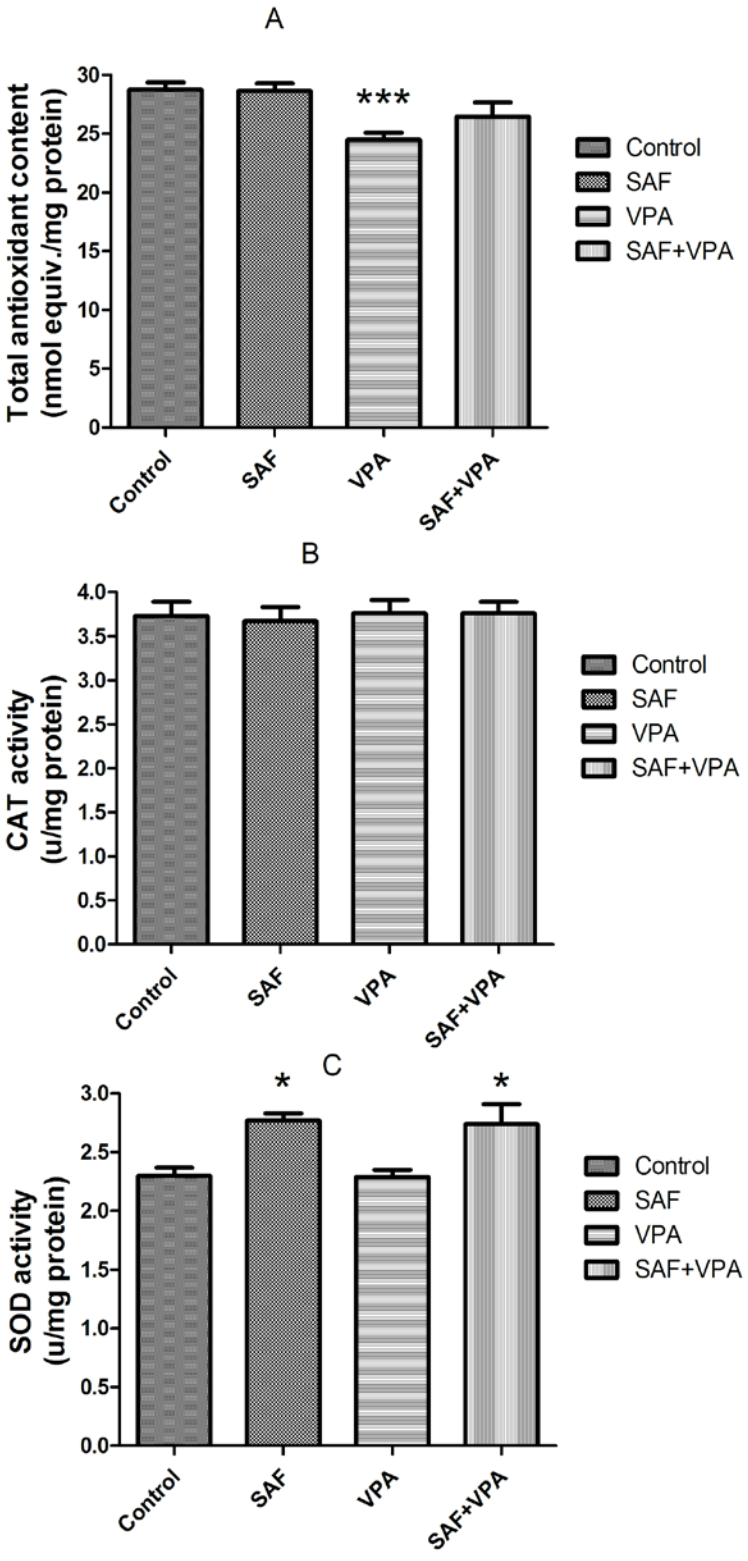

Figure 3: Effect of SAF on VPA-induced alteration in the hepatic antioxidant markers, (A) total antioxidant content, and activities of (B)CAT and (C) SOD. Data are presented as mean ± SME (n = 8). Significance was determined by ANOVA followed by Dunnett's test: ***P<0.001, *P<0.05 vs. control group.

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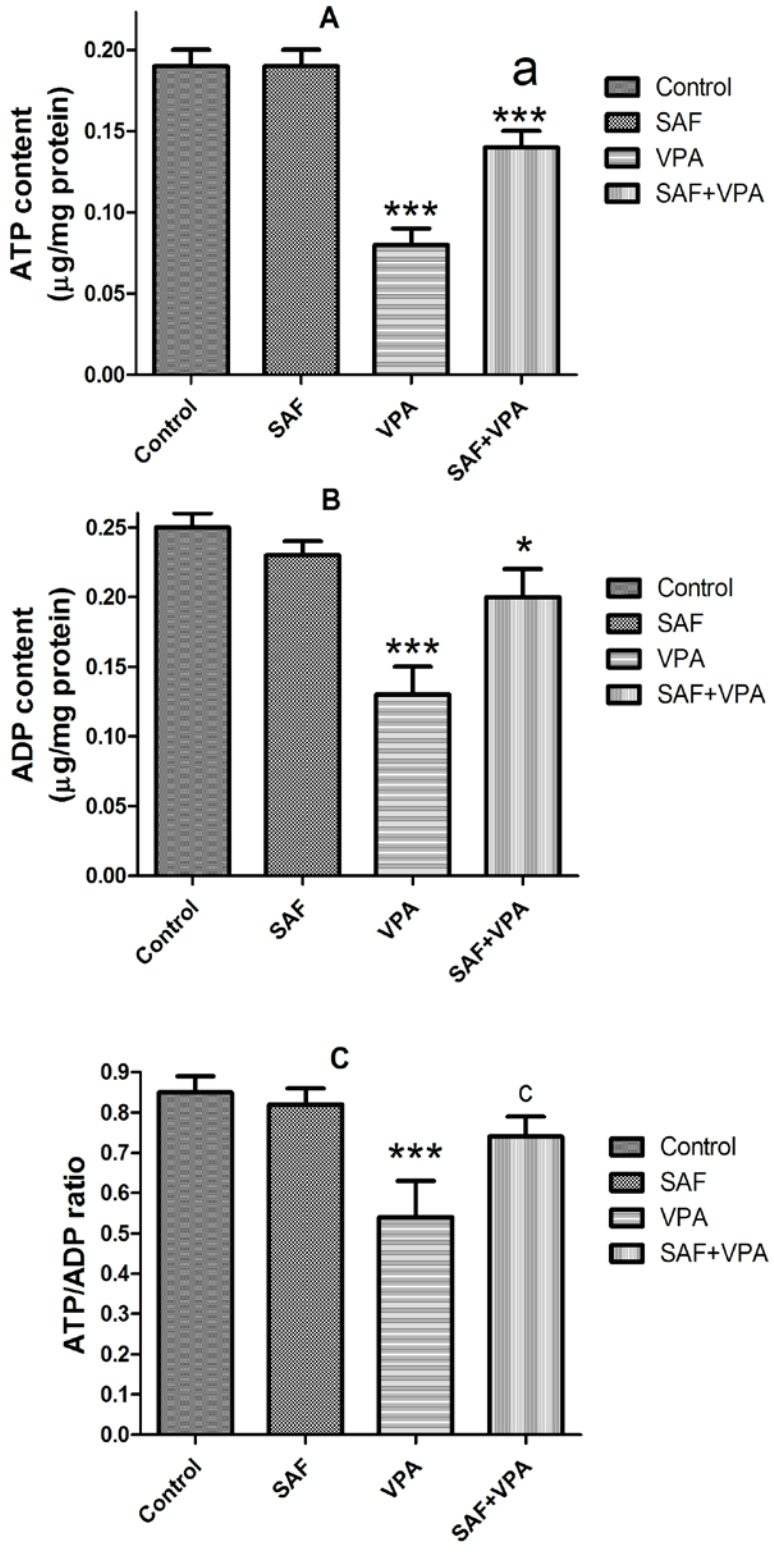

Figure 4: Effect of SAF on VPA-induced alteration in the hepatic ATP, ADP and ATP/ADP ratio. Values are expressed as mean ± SME (n=8). Significance was determined by ANOVA followed by Dunnett's test: ***P<0.001, *P<0.05 vs. control group; [a] P<0.001 [c] P<0.05 vs. VPA group.

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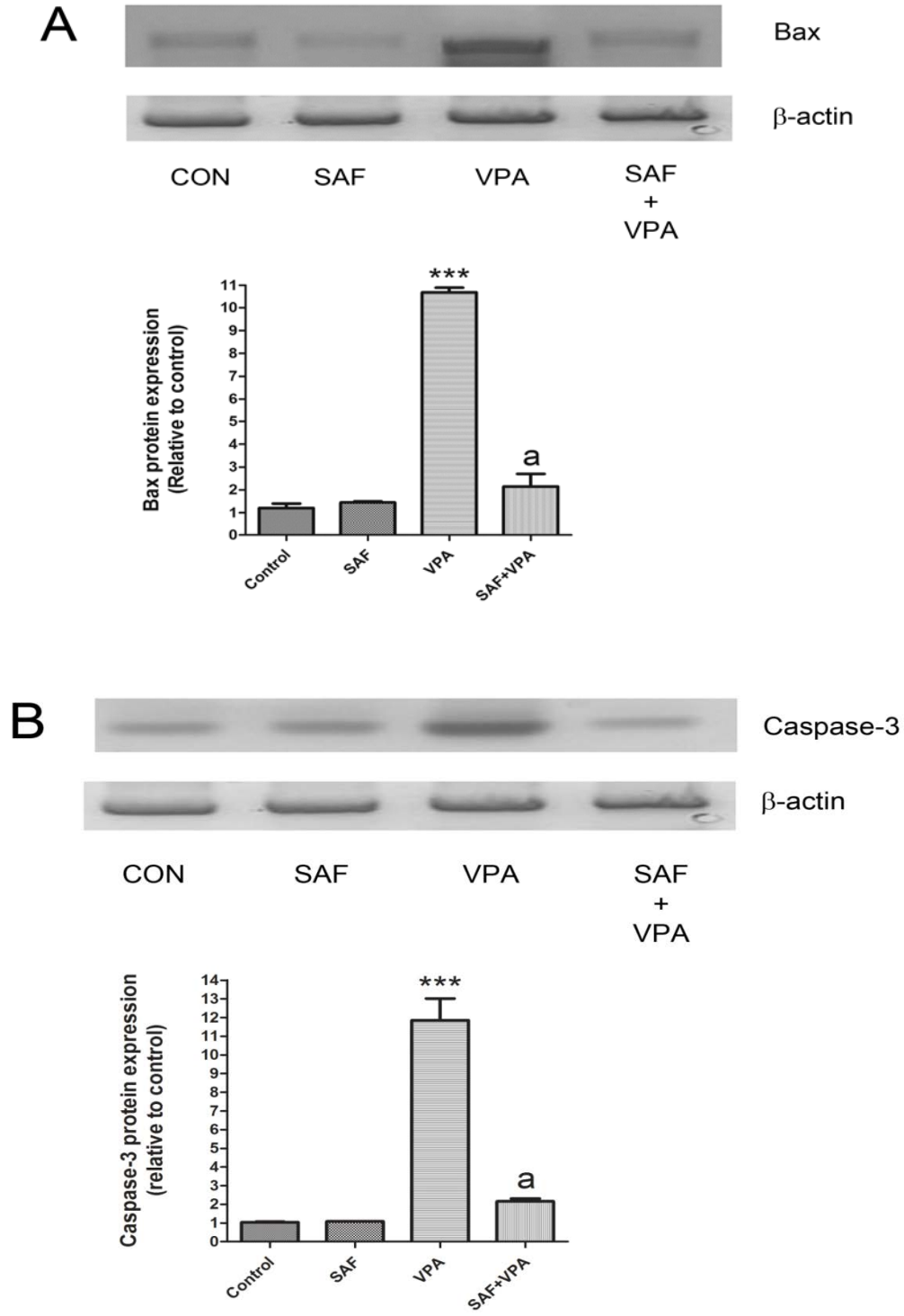

Figure 5: Effect of SAF on hepatic apoptotic effect of VPA-treated rats. The above panels showing the Western blot analysis indicating an increase in protein expression of Bax(Figure 5 A) and caspase-3 (Figure 5 B) in liver of liver of VPA-treated rats. The low graph demonstrated the relative expression of Bax and caspase-3. Data are presented as mean ± SEM (n = 3). Significance was determined by ANOVA followed by Dunnett's test: ***P<0.001, *P<0.05 vs. control group; [a] P<0.001,[c]P<0.05 vs. VPA group.

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4. Discussion

The present study indicates the hepatoprotective effect of SAF against VPA-induced liver toxicity in rats. To explore the hepatoprotective mechanisms of SAF were investigated including its effect on different oxidative stress markers, gene expression of some enzymes related to FAO and VPA metabolism, apoptotic markers as well as ATP contents. Until now, the hepatotoxicity associated with VPA has been fully unfolded [3,6]. VPA-mediated hepatic injury is associated with a dose-dependent rise in serum liver enzymes, decline in plasma albumin, microvesicular steatosis and necrosis [4,14,15,23]. In our study, VPA treatment caused a significant elevation in serum AST, and ALT activities, indicating the liver injury induced by VPA. Serum albumin and protein levels were decreased in rats treated with VPA, which was in consistent with that of previous reports [23,34]. The hepatotoxic effects observed in VPA-treated rats was confirmed histopathologically and characterized by hepatocellular focal necrosis, fatty changes, and inflammatory cell infiltration. However, treatment with SAF effectively improved the VPA-induced elevation in serum AST, and ALT levels, indicating the hepatoprotective effect of SAF against the hepatotoxicity of VPA. In this concern, SAF raised serum albumin and protein levels, suggesting restoration of liver protein synthetic capacity. More such clues were provided from the present histopathology studies, which depicted the capacity of SAF to ameliorate VPA-evoked hepatocellular degeneration, infiltration of inflammatory cells, and induction of focal pericentral necrosis. However the majority of cell in portal area contains pale vacuolated cytoplasm indicating fatty changes.

VPA induces liver damage through various mechanisms such as mitochondrial dysfunction [7], activation of cytochrome P450 [9] and generation of ROS [13].Oxidative stress in a cell is activates when the antioxidant defense system is overwhelmed by the production of ROS [35]. Several studies reported the implication of an increased generation of ROS and oxidative stress in the toxic mechanism of VPA [13-15,36,37]. The presence of hepatic oxidative stress in our VPA-treated rats is supported by the elevation in TOC and MDA as well as the depletion of total antioxidants which were consistent with previous reports [13,23].Therefore, it is confirmed that the cytotoxic mechanism of VPA is mediated by oxidative stress. SAF markedly replenished hepatic TOC and TAC to near baseline and depleted lipid peroxide (MDA) levels, thereby alleviated VPA-induced oxidative stress.

Another mechanism of oxidative stress and liver damage-induced by VPA attributes to cytochrome P450-related activation of reactive VPA metabolites [37]. CYP2E1 is an effective enzyme for ROS production and is one the most powerful inducers of oxidative stress in liver cells [38]. As expected, VPA -induced oxidative stress was accompanied by elevations in CYP2E1 expression in hepatic tissues. Cytochrome P450 desaturation of VPA to form the 4-ene-VPA metabolite is an important step in the bioactivation Pathway [9]. By inhibiting this oxidation, the generation of potentially more toxic metabolites and ROS can be prevented [9,37]. In this research, SAF treatment suppressed CYP2E1 up- regulation in VPA-treated rats. These findings suggest that the hepatoprotective effect of SAF against VPA-induced liver damage may include inhibition of CYP2E1-mediated oxidative stress and toxic metabolites.

Mitochondria are important subcellular organelles involved in the energy production and susceptible to oxidative stress [7]. Hepatocytes are rich in mitochondria, which are the key organelles involved in hepatocellular injury. Mitochondrial dysfunction has been recognized as an important mechanism of VPA-induced liver injury [7]. VPA has been shown to cause impaired FAO in mitochondria. VPA affects oxidation of fatty acids in the mitochondria through different mechanisms that include inhibition of one or several mitochondrial fatty acid oxidation enzymes in the present study we also investigated the effect of VPA gene expression of enzymes contribute in FAO [7]. Unexpectedly, VPA did not decrease the gene expressions of enzymes involved in mitochondrial FAO. The mRNA levels of genes involved in FAO, CPT 1A, FGF21, PPARγ1, and CYP2E1 unregulated in VPA-treated rats, suggesting induction of hepatic fatty acid oxidation. The expression of mitochondrial FGF21 and CPT1A is under the control of PPAR [12]. The obtained result is consistent with previous study which confirmed that VPA treatment to rats resulted in

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upregulations in the genes of fatty acid β-oxidation [4]. Our data suggest that VPA may also aggravate oxidative stress through PPAR and CYP2E1 activations a finding consistent with the previous reports that VPA is a pan-activator of PPAR [PPARα, PPARδ and PPARγ][2,10, 11]. In addition, SAF treatment had reduced the oxidative stress and liver damage in VPA-treated rats but did not affect in the expressions of these 4 genes associated with fatty acid metabolism. This indicates that reduced ROS accumulation rather than increased fatty acid metabolism could be account for VPA-induced liver damage in this study. The up-regulation of heme oxygenase (HO-1) in VPA may be an important adaptive response against the increased oxidative stress produced by elevated CYP2E1. Previous studies showed that HO-1 is upregulated by increased expression of CYP2E1 [39].These results suggest that the inducible form of HO-1 is increased during oxidative injury and believe that HO-1 is an important defense mechanism against CYP2E1-dependent toxicity.

Depletion of ATP is a typical feature of hypoxic and toxic injury which leads to inhibition of two hepatic anabolic processes, namely gluconeogenesis and plasma protein synthesis, which have in common a substantial requirement for ATP [40]. Our data point to decrement in ATP content, ADP content as well as ATP/ADP ratio in hepatic tissues of rats intoxicated with VPA. This indicates that the ATP synthesis decreased as a consequence of ROS formation. In addition, the decreased hepatic ATP content reflects mitochondrial dysfunction since mitochondria produce most ATP utilized by liver. Consistent with previous in vitro study [8], the impairment of mitochondrial function, leading to ATP depletion may indicate that this is an important event in the cytotoxicity triggered by the VPA. ATP depletion could be related to mitochondrial transition permeability (MTP) pore opening which observed after VPA treatment [37]. Any damage to mitochondrial ATP generation results in intracellular acidosis and osmotic injury. The latter is the cause of plasma membrane lysis [37]. VPA-induced ATP deletion can be modulated by disturbance in MTP pore opening as a result of ROS formation due to CYP450 activation. Previous study showed that VPA inhibited mitochondrial membrane potential in HepG2, resulted in decreased ATP level [8]. In the present work, SAF remarkably increased the content of ATP, ADP and ATP/ADP ratio in VPA-treated rats, indicating protective effect of SAF against mitochondrial dysfunction.

Besides liver necrosis and oxidative damage, VPA intoxication induced marked apoptosis of liver as shown by the significant increase of Bax and caspase-3expressions. Hence, the apoptotic effect of VPA could be mainly attributed to triggering the intrinsic mitochondrial dependent apoptotic pathway through the generation of ROS [35] as shown in the VPA-treated group due to oxidative stress observed here. Our results are in accordance with previous studies which reported that VPA causes cellular death in a rat liver cell line via apoptosis and that this was accompanied by activation of caspase-3 [41]. Bax is a pore opening cytoplasmic protein that in response to increased oxidative stress, translocates to the outer mitochondrial membrane, influencing its permeability and inducing cytochrome c loss from the inter membrane space of the mitochondria and subsequent release into the cytosol [35, 37]. Interestingly, VPA-induced alteration in the expressions of Bax and caspase-3 were almost restored to normal levels with SAF supplementation. This indicates that SAF abrogates VPA-induced apoptosis by decreasing ROS and the expression of Bax, then inhibiting caspase-3 expression. The anti-apoptotic effect of SAF can be also attributed to a free radical scavenging capability.

In conclusion, the findings of the present study reinforce the significant role of oxidant/antioxidant status, CYP2E1 and apoptosis in pathogenesis of the VPA-induced hepatotoxicity. The pretreatment of VPA intoxicated rats with SAF protects against VPA-induced liver toxicity as evidenced by reducing hepatic injury markers and improving histopathological changes. Our data further indicate that, SAF not only caused suppression of VPA-induced impaired FAO in mitochondria and up- regulation of CYP2E1 but also abrogation of the resultant apoptotic signals. Accordingly, adjuvant administration of SAF with VPA could provide a new hopeful resolution to the

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deleterious VPA induced liver injury after further confirmatory studies at clinical levels.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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5. References

1. Johannessen SI, Landmark CJ. Antiepileptic drug interactions-principles and clinical implications. Current neuropharmacology. 2010;8(3):254.
2. Sitarz KS, Elliott HR, Karaman BS, Relton C, Chinnery PF, Horvath R. Valproic acid triggers increased mitochondrial biogenesis in POLG-deficient fibroblasts. Molecular genetics and metabolism. 2014;112(1):57-63.
3. Björnsson E, Olsson R. Suspected drug-induced liver fatalities reported to the WHO database. Digestive and liver disease. 2006;38(1):33-8.
4. Lee M-H, Hong I, Kim M, Lee BH, Kim J-H, Kang K-S, et al. Gene expression profiles of murine fatty liver induced by the administration of valproic acid. Toxicology and applied pharmacology. 2007;220 (1):45-59.
5. Sato K, Ueda Y, Ueno K, Okamoto K, Iizuka H, Katsuda S. Hepatocellular carcinoma and nonalcoholic steatohepatitis developing during long-term administration of valproic acid. Virchows Archiv. 2005;447(6):996-9.
6. Verrotti A, Di Marco G, La Torre R, Pelliccia P, Chiarelli F. Nonalcoholic fatty liver disease during valproate therapy. European journal of pediatrics. 2009;168(11):1391-4.
7. Begriche K, Massart J, Robin M-A, Borgne-Sanchez A, Fromenty B. Drug-induced toxicity on mitochondria and lipid metabolism: mechanistic diversity and deleterious consequences for the liver. Journal of hepatology. 2011;54(4):773-94.
8. Komulainen T, Lodge T, Hinttala R, Bolszak M, Pietilä M, Koivunen P, et al. Sodium valproate induces mitochondrial respiration dysfunction in HepG2 in vitro cell model. Toxicology. 2015;331:47-56.
9. Neuman MG, Shear NH, Jacobson-Brown PM, Katz GG, Neilson HK, Malkiewicz IM, et al. CYP2E1-mediated modulation of valproic acid-induced hepatocytotoxicity. Clinical biochemistry. 2001;34(3):211-8.
10. Lampen A, Carlberg C, Nau H. Peroxisome proliferator-activated receptor δ is a specific sensor for teratogenic valproic acid derivatives. European journal of pharmacology.2001;431(1):25-33.
11. Punapart M, Eltermaa M, Oflijan J, Sütt S, Must A, Kõks S, et al. Effect of Chronic Valproic Acid Treatment on Hepatic Gene Expression Profile in Wfs1 Knockout Mouse. PPAR research. 2014;2014.
12. Ge X, Wang Y, Lam KS, Xu A. Metabolic actions of FGF21: Molecular mechanisms and therapeutic implications. Acta Pharmaceutica Sinica B. 2012;2(4):350-7.
13. Schulpis KH, Lazaropoulou C, Regoutas S, Karikas GA, Margeli A, Tsakiris S, et al. Valproic acid monotherapy induces DNA oxidative damage. Toxicology. 2006;217(2):228-32.
14. Tong V, Teng XW, Chang TK, Abbott FS. Valproic acid I: time course of lipid peroxidation biomarkers, liver toxicity, and valproic acid metabolite levels in rats. Toxicological Sciences. 2005;86(2):427-35.
15. Sokmen BB, Tunali S, Yanardag R. Effects of vitamin U [S-methyl methionine sulphonium chloride] on valproic acid induced liver injury in rats. Food and Chemical Toxicology. 2012;50(10):3562-6.
16. Abdella EM, Galaly SR, Mohammed HM, Khadrawy SM. Protective role of vitamin E against valproic acid-induced cytogenotoxicity and hepatotoxicity in mice. The Journal of Basic & Applied Zoology. 2014;67(4):127-39.
17. Rios J, Recio M, Giner R, Manez S. An update review of saffron and its active constituents. Phytotherapy Research. 1998(10):189-93.
18. Assimopoulou A, Sinakos Z, Papageorgiou V. Radical scavenging activity of Crocus sativus L. extract and its bioactive constituents. Phytotherapy Research. 2005;19(11):997-1000.
19. Hariri AT, Moallem SA, Mahmoudi M, Hosseinzadeh H. The effect of crocin and safranal, constituents of saffron, against subacute effect of diazinon on hematological and genotoxicity indices in rats. Phytomedicine.2011;18(6):499-504.

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20. Rezaee R, Hosseinzadeh H. Safranal: from an aromatic natural product to a rewarding pharmacological agent. Iranian journal of basic medical sciences. 2013;16(1):12.

21. Hosseinzadeh H, Tabeshpur J, Mehri S. Effect of Saffron extract on Acrylamide-induced toxicity: In vitro and in vivo assessment. Chin J Integr Med. 2014.

22. Olfert E, Cross B, McWilliam A. Canadian Council on Animal Care, Guide to the care and use of experimental animals. 1993.

23. Abdel-Dayem MA, Elmarakby AA, Abdel-Aziz AA, Pye C, Said SA, El-Mowafy AM. Valproate-Induced liver injury: modulation by the omega-3 fatty Acid DHA proposes a novel anticonvulsant regimen. Drugs in R&d. 2014;14(2):85-94.

24. Hariri AT, Moallem SA, Mahmoudi M, Memar B, Hosseinzadeh H. Sub-acute effects of diazinon on biochemical indices and specific biomarkers in rats: protective effects of crocin and safranal. Food and Chemical Toxicology. 2010;48(10):2803-8.

25. Mehdizadeh R, Parizadeh MR, Khooei A-R, Mehri S, Hosseinzadeh H. Cardioprotective effect of saffron extract and safranal in isoproterenol-induced myocardial infarction in wistar rats. Iranian journal of basic medical sciences. 2013;16(1):56.

26. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Analytical biochemistry. 1978;86(1):271-8.

27. Hillegass L, Griswold D, Brickson B, Albrightson-Winslow C. Assessment of myeloperoxidase activity in whole rat kidney. Journal of pharmacological methods. 1990;24(4):285-95.

28. Erel O. A new automated colorimetric method for measuring total oxidant status. Clinical biochemistry. 2005;38(12):1103-11.

29. Aebi H. Catalase in vitro. Methods in enzymology. 1984;105:121-6.

30. Nandi A, Chatterjee I. Assay of superoxide dismutase activity in animal tissues. Journal of Biosciences. 1988;13(3):305-15.

31. Benzie IF, Strain J. The ferric reducing ability of plasma [FRAP] as a measure of "antioxidant power": the FRAP assay. Analytical biochemistry. 1996;239(1):70-6.

32. Peterson GL. A simplification of the protein assay method of lowry et al which is more generally applicable. Anol Biochem. 1977;83:346 -56.

33. Teerlink T, Hennekes M, Bussemaker J, Groeneveld J. Simultaneous determination of creatine compounds and adenine nucleotides in myocardial tissue by high-performance liquid chromatography. Analytical biochemistry. 1993;214(1):278-83.

34. Rugino TA, Janvier YM, Baunach JM, Bilat CA. Hypoalbuminemia with valproic acid administration. Pediatric neurology. 2003;29(5):440-4.

35. Fruehauf JP, Meyskens FL. Reactive oxygen species: a breath of life or death? Clinical Cancer Research. 2007;13(3):789-94.

36. Yiş U, Seçkin E, Kurul SH, Kuralay F, Dirik E. Effects of epilepsy and valproic acid on oxidant status in children with idiopathic epilepsy. Epilepsy research. 2009;84(2):232-7.

37. Pourahmad J, Eskandari MR, Kaghazi A, Shaki F, Shahraki J, Fard JK. A new approach on valproic acid induced hepatotoxicity: involvement of lysosomal membrane leakiness and cellular proteolysis. Toxicology in Vitro. 2012;26(4):545-51.

38. Boelsterli U. Xenobiotic-induced oxidative stress: cell injury, signaling, and gene regulation, Mechanistic toxicology: the molecular basis of how chemicals disrupt biological targets. Taylor & Francis, London and New York; 2003.

39. Gong P, Cederbaum AI, Nieto N. Heme oxygenase-1 protects HepG2 cells against cytochrome P450 2E1-dependent toxicity. Free Radical Biology and Medicine. 2004;36(3):307-18.

40. Kumar V, Abbas AK, Fausto N, Aster JC. Robbins and cotran pathologic basis of disease, Professional Edition: Expert Consult-Online: Elsevier Health Sciences; 2014.

41. Phillips A, Bullock T, Plant N. Sodium valproate induces apoptosis in the rat hepatoma cell line, FaO. Toxicology. 2003;192(2):219-27.

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