International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Apium Graveolens and Rosmarinus Officinalis Protect from Liver Toxicity Induced by Sodium Valproate in Rats and Potentiate its Anticonvulsant Activity on Pentylenetetrazol-Treated Rats

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Abstract

Hepatic injury is a dose-limiting side effect of sodium valproate (VPA), a common used drug in the management of numerous nurological disorders, including epilepsy. The present study was designed to investigate the effect of *Apium Grafionalies* (AG) and *Rosmarinus officinalis* (RO) against VPA-induced liver injury. Hepatic toxicity was induced by the administration of VPA (500 mg/kg/day) once daily for a period of 7 days. The hepatoprotective group received ethanolic extracts of AG and RO (200 mg/kg/day) for 7 days prior to VPA treatment for seven days. VPA liver toxicity was evaluated based on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and, albumin, and total protein levels as well as by histology. Serum ALT and AST activities were elevated and albumin and total protein levels were depleted. Histopathological changes in liver such as fatty degeneration and focal necrosis were associated with significant increase in TUNEL-positive cells and P53 protein in immunohistochemical study indicating the presence of DNA fragmentation.

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This biochemical and histological changes were associated with increased oxidative stress damage as evidenced by increases in protein carbonyl (P.Carbonyl) and decreases in total antioxidant capacity (TAC) in liver. These biochemical and histological disturbances were effectively attenuated on pretreatment with either AG or RO extract, which normalized P.Carbonyl and TAC and attenuated histopathological changes induced by VPA. These observations shed light on the hepatoprotective actions of both AG and RO extracts against experimental VPA toxicity. AG was characterized for its apigenin content while RO was characterized for its rosmarinic acid and carnazol contents. Besides, in a pentylenetetrazole (PTZ) rat clonic convulsion model, pretreatment with AG and RO (200 mg/kg) before VPA protected against PTZ-induced convulsion, where it delayed the onset of clonic convulsion in PTZ treated rats.

Keywords: Sodium valproate; liver toxicity; protection; *Apium gravuonalues; Rosmarinus officinalis*; Liver toxicity;Sodium valproate;Apium Graveolens; Rosmarinus Officinalis; Anticonvulsant activity.

1. Introduction

Sodium valproate (VPA) is a branched-chain saturated fatty acid [2-propyl pentanoic acid] that is widely used for the treatment of epilepsy, and migraine headaches. VPA is being investigated as a novel histone deacetylase inhibitor in the treatment of certain forms of cancer [1]. Despite its effectiveness, VPA has been associated with hepatotoxic effects such as microvesicular steatosis, hepatocellular necrosis and cholestatic liver injury in humans [2-4].

VPA has been referred as the third most common xenobiotic suspected of causing death due to liver injury [5]. Moreover, acute and chronic animal models of VPA toxicity clearly show extensive microvesicular fatty changes, centriolubular degeneration, focal necrosis and infiltration of inflammatory cells in liver [6-8]. At the molecular level, growing body of evidence suggesting that excessive levels of free radicals are generated during VPA exposure, possibly as a consequence of VPA biotransformation by mitochondrial fatty acid â-oxidation and/or alterations in endogenous antioxidants [4, 9]. In rat studies, the depletions of endogenous antioxidant substances [10] and the elevations in oxidative stress markers [6,9-11] have been found to increase after acute and chronic VPA treatments. Conceivably, a big need arises to seek avenues that could alleviate VPA-induced hepatic injury. In fact, several compounds with antioxidant properties such as vitamin C, E, and U, carnitine omega-3, and safranal have been investigated in vitro and in vivo with some degree of success [7-9, 12-13].

In recent years, several natural extractions from plants have been shown to protect against chemical-induced liver toxicity. Conversely, no attempts have been made to reduce VPA liver complications and to boost its therapeutic efficacy by using the natural substances. *Apium graveolens L.* AG [celery, family: Umbelliferae] and *Rosmarinus officinalis* (RO) [Rosmary, family: Lamiaceae] are cultivated in Mediterranean area for their essential oils and are often used as popular aromatic herbs and spices due their characteristic smells. Phytochemical investigations of AG seeds revealed the presence of terpenes like limonene, flavonoids like apigenin and phthalide glycosides [14-16] RO was also found to contain several antioxidant compounds such as phenolic diterpenoids like carnosic acid, carnosol, rosmanol and epirosmanol [17-19] and carotenoids and alpa-tocopherol [20].

AG seeds are traditionally used as a diuretic, tranquilizer, anti-spasmodic and anti-rheumatic [21]. AG seed extracts have been reported to possess a broad spectrum of pharmacological effects including anti-inflammatory [22], anti-cancer [14, 16, 23], anti-hepatotoxic [24-25], anti-hypercholesterolemia [26], anti-ulcer [27] and antioxidant [22,28]. Furthermore, we previously demonstrated that AG seeds can reverse a, VPA-induced reproductive toxicity in rats by ablating oxidative stress [29].

RO is a native Mediterranean plant that has commonly been used against asthma, eczema and rheumatism [30]. RO extracts are found to exhibit different protective effects in rats such anti-hyperglycemic [31], anti-ulcerogenic [32], hepatoprotective [33] antioxidant and anti-genotoxicity [34] and anticancer activity [35] and antidepressant behavior [36]. The extract of RO has been shown to have potent hepatoprotective effects against a variety of hepatotoxic chemical agents including tert-butyl hydroperoxide [37], carbon tetrachloride [30,38-39] and azathioprine [40].

This investigation was set to evaluate whether treatment with either AG or RO ethanol extract could reduce VPA-induced liver toxicity in male rats. To accomplish this, we monitored levels of hepatocellular oxidative stress and markers of hepatic function and histopathologic investigation. On the other hand, the possibility of pharmacologic synergy with VPA was explored in a pentylenetetrazole (PTZ) rat convulsion model.

2. Materials and Methods

2.1. Chemicals

The dried plants, AG seeds, and RO leaves, were purchased from Experimental Plant Station in Faculty of Pharmacy, Cairo University, Giza, Cairo. Plants were collected during the flowering period in March and April 2015. The plants growth environment is typically Mediterranean. The plants were authenticated by plant taxonomist; Dr. Nael M. Fawzi, compared to related specimens available at the herbarium of the Flora and Taxonomy Department, Agricultural Research Center, Giza, Egypt. VPA (Depakin®), it is one of products of Sanofi-Synthelabo Company, Paris, France. 2,4-dinitophenylhydrazine, thiobarbituric acid, reduced glutathione, 5,5-dithiobis (2-nitrobenzoic acid), Folin's reagent, epinephrine, SOD enzyme, H2O2, and bovine albumin were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from common commercial suppliers.

2.2. Experimental animals

Adult male albino rats (150-200g) of the Wistar strain were obtained from the animal house, National Organization for Drug Control and Research (NODCAR). All procedures with animals were performed in accordance with the standard guidelines [41] and approved by local ethical committee of NODCAR. 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 h. light/dark cycle and room temperature 22-24°C. Rats were acclimatized to the environment for two week prior to experimental use.

2.3. Preparation of Plant Extract

To increase the yield of extraction in a shorter time and a lower temperature, the liquid-phase microwave-assisted process was used for extraction of AG and ZO according to the methods described by (Pan and his colleagues 2001). These microwave-assisted extraction applications are based upon the selective heating of the matrix that contains the target extract when the matrix is immersed in a solvent transparent (ethanol and water) the microwaves. This solvent allows for selective heating of particular components within the matrix without using excessive heating. Hundreds grams of dried plants, AG seeds and RO leaves were mixed in 1000 ml of 70% ethanol. Every 10 gm of ground herb mixed with 100 ml of 70% ethanol in 250-ml conical flask. The Mixtures were then irradiated with microwave for two minutes. The output power of the microwave oven is 300 W. The suspensions were irradiated with 25 seconds of power on to give the desired temperature of about 80 °C and 5 second of power-on for heating and then 10 second of power-off for cooling. Then the extracts were finally filtered through gauze and evaporated under vacuum at 40°C using a rotary evaporator. The yields of AG and RO were about 16% and 10% respectively. The dried extracts were dissolved in distilled water before administration.

2.4. Standardizations of AG and RE by high-pressure liquid chromatography

Both of AG and RE were standardized in reference to its major contents exploiting high-pressure liquid chromatography (HPLC) analysis. The separations were carried out on HPLC apparatus (Agilent 1100 series). The dry extract of both AG and RO was dissolved in mobile phase (30% acetonitrile at pH 3.0) and separations were performed on a 250 X 4.6mm i.d., 5 µm reverse-phase Hypersil BDS-C18 analytical column (Thermo Scientific, USA). The mobile phase was acetonitril (A) and acidified water containing 2.5% formic acid (B). The gradient was as follows: 0 min, 5% A; 10min, 15% A; 30 min, 25% A, 35 min, 30% A; 50 min, 55% A; 55 min 90% A; 60 min, 100% A and then hold for 10 min before returning to the initial conditions. The flow rate was 1.2 ml/min for AG and 1.0 ml/ min RO and the absorbance was monitored at 265.5 nm for AG and 280nm for RO according to the method described by [42].

2.5. Rat Liver Toxicity Studies

2.5.1. Experimental Design

VPA was dissolved in a saline solution and was administered to animal in a volume of one ml /100 g body weight. The control animals received an equivalent volume of saline based on body weight. The hepatotoxic dose of VPA (500 mg/kg b.wt.) used in the present study is based on the previous hepatotoxic study of VPA in rats [6]. The two extracts were given orally by gavage at the concentration 200 mg/Kg of body weight, which previously found to prevent liver toxicity induced in rats by other hepatotoxic agents [33]. The rats were randomly divided into six groups (eight rats each) and were treated daily for two weeks as follow: the first control group was received daily an equivalent volume of water for 14 days, the VPA- treated group was treated with water for 14 days and was injected daily an intraperitoneal dose of VPA (500 mg/kg b.wt.) for 7 day after 7 days of water treatment, the AG-treated group was treated orally with AG extract (200 mg/kg b.wt.) for 14 days, the RO-treated group was treated orally with RO extract (200 mg/kg b.wt.) for 14 days, AG plus VPA-treated group was pretreated orally with AG extracts daily for 7 days before VPA treatment for further 7 days, RO plus

VPA-treated group was pretreated orally with RO extracts daily for 7 days before VPA treatment for further 7 days. After 24 hours of last treatment with each of VPA, herbal extracts or vehicle solution administration, blood and organ samples were obtained from the four groups under diethyl ether anesthesia.

2.5.2. Sample Preparation

Blood was collected from the retro-orbital plexus under diethyl ether anesthesia. Serum was separated by centrifugation in a refrigerated centrifuge (4°C) at 3000 rpm for 20 minutes. Fresh serum samples were used for the determination of albumin and total protein levels as well as enzyme activities of ALT and AST. After the collection of blood samples, all animals were sacrificed by decapitations under diethyl ether anesthesia and two liver samples were collected from each animal. The first 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 mM KCl, 50 mM Tris, 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.

2.5.3. Biochemical Assays

2.5.3.1. Assessment of liver damage

ALT and AST activities as well as Albumin and total protein concentrations were determined in serum using Randox reagent kits (Randox Laboratories Ltd., Co. Antrim, United Kingdom) and following their instruction manual

2.5.3.2. Determination of Oxidative Stress Markers

Malondialdehyde (MDA) level, an indirect index of lipid peroxidation (LP) [43] was measured in hepatic tissues. The determination of MDA, as described in [44] is based on its reaction with thiobarbituric acid (TBA) to form a pink complex with absorption maximum at 535 nm. The level of MDA and was expressed was expressed as nmol of MDA/mg protein.

P.carbonyl is vital marker of oxidative stress. It has some advantages in comparison with other markers because of relative early formation and stability of carbonylated proteins [45]. Hepatic P.carbonyl content was determined according to method of [46]. This method is based on spectrophotometric detection of the reaction of 2,4-dinitophenylhydrazine with P.carbonyl to form protein hydrazones at 370nm. The level P.carbonyl was expressed as nmol of carbonyl group per milligram of protein with molar extinction coefficient of 22000 M/cm.

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 [47]. 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 [48]. In all the estimations, absorbance was recorded using a PerkinElmer, Lambda 25 UV/VIS spectrophotometer.

2.5.4. Histopathological examination

Pieces of liver were fixed in 10% neutral phosphate-buffer formalin and the hydrated tissue sections 5μm in thickness were stained with Hematoxylin and Eosin. The sections were examined under a Leica DMRB/E light microscope.

2.5.5. TUNEL assay for DNA fragmentation and immunohistochemical assay for P53

Terminal deoxynuclotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay detects single-strand DNA breaks and double strand DNA breaks, therefore TUNEL positively is seen in both necrosis and apoptosis [49]. Endogenous peroxidase was blocked by incubation in 1.3% H2O2 in PBS before enzymatic labeling. The fragmented DNA was end labeled with horseradish peroxidase (HRP) conjugated-biotinylated nucleotide at 30-OH end using the terminal deoxynucleotidyl transferase recombinant enzyme according to the manufacturer's instructions (Promega, Madison, Wisconsin, USA). Detection was carried out using H2O2 and diaminobenzidine (DAB) substrate. Sections were counterstained with hematoxylin. In the normal section the nuclei stain blue and the positive TUNEL nuclei stained brown.

P53 is a multi-function protein and known as the guardian of the genome [50, 51]. Elevation of P53 protein in response to DNA damage triggers either a transient cell cycle arrest or apoptosis [50, 51]. So that combination of TUNEL and P53 techniques should always be used to detect DNA damage and genotoxic stress.

2.6. Rat Acute, Pentylenetetrazole (PTZ), anticonvulsant Studies

PTZ-induced clonic seizures represent a routine test for screening anticonvulsants [52]. In the present work, the selection of PTZ dose to induce clonic convulsion n our animals and antiepileptic VPA dose was according to Safar and his colleagues [53]. Administration of PTZ (60 mg/kg i.p.) induced: freezing, myclonic twitches, clonic convulsion. The VPA group received the sub effective dose of VPA (100 mg/kg, IP) to study the possibility of pharmacologic synergy of AG and RO with VPA against PTZ-induced clonic convulsion. The animals were divided into six groups of analysis (eight rats each); group 1 received single intraperitoneal PTZ dose (60mg/kg) to induce clonic convulsion [53]. Group 2 is the VPA group injected intraperitoneally with VPA (100mg /kg, ip) 30 min before PTZ administration. AG group received AG (200mg/kg) orally, 1 hour before PTZ treatment (60 mg/kg). RO group received RO (200 mg/kg PO) 1 hour before PTZ administration (60 mg/kg IP). In AG+VPA group animals were received AG (200 mg/kg) 30 min before injection of VPA dose then convulsion was induced by injection PTZ after 30 min of VPA treatment. In RO+VPA group animals were received AG (200 mg/kg) 30 min before injection of VPA dose then convulsion was induced by injection PTZ after 30 min of VPA treatment.

2.6.1. Seizures assessment

After PTZ injection, rats were placed singly in Plexiglas cages and were observed for 30 min. Incidences and latency of clonic convulsive attacks, which lasts over 3 s with an accompanying loss of righting reflex were recorded. Seizure latency for rats showing no convulsive attacks within the observation period was taken as 30

min.

2.7. Statistical Analysis

The data are expressed as group mean ± SEM. The statistical analysis was carried out one-way analysis of variance (ANOVA), with SPSS version 20 statistical program (SPSS Inc., Chicago, IL, USA). ANOVA performed to detect differences between all various groups. When significant differences by ANOVA were detected analysis of a difference between the means of the treated and control groups were performed by using Dennett's t test.

3. Results

3.1. Effect of AG and RO on VPA-induced liver damage

Table 1 revealed that treatment with VPA alone caused a significant increase in serum ALT and AST activities and a significant decrease in serum albumin, and total protein levels compared to the control group. No significant differences were observed in the serum markers of liver damage in the animals treated with AG or RO. However the elevation in serum ALT and AST activity as well as the depletion in albumin and total protein levels was significantly attenuated in groups treated with AG + VPA or RO + VPA in comparison to VPA-treated rats. This improvement was more pronounced in the group of rats pretreated with RO.

Table 1: Effect of AG or RO on serum ALT, AST activity, and albumin and total protein concentrations in control and VPA treated rats

Groups	ALT (U/ml)	AST (U/ml)	Albumin (g/dl)	Total protein (g/dl)
Control	26.13 ± 0.65	118.25 ± 3.18	3.97 ± 0.13	7.04 ± 0.06
AG	27.14 ± 0.81	119.80 ± 2.02	3.99 ± 0.14	7.04 ± 0.05
RO	26.83 ± 0.20	117.62 ± 1.67	3.95 ± 0.15	6.99 ± 0.06
VPA	47.51 ± 1.54[a]	163.50 ± 8.51[a]	3.13 ± 0.20[a]	5.06 ± 0.10[a]
AG+VPA	28.08 ± 1.51[b]	133.12 ± 3.63[b]	3.29 ± 0.15 [a, b]	5.31 ± 0.05 [a, b]
RO+VPA	28.08 ± 1.52[b]	119.02 ± 6.48[b]	3.41 ± 0.17 [a, b]	6.13 ± 0.23 [a, b]

Data are represented as mean ± S.E.M. of eight independent rats of each group. a a<0.05 vs. control, b<0.05 vs. VPA.

3.2. Effect of AG and RO on VPA-induced Histological Changes

Histopathological examination of liver tissue was done to further illustrate VPA-induced liver damage (Figure 1 & 2). Liver of rats in control showed normal histological structure of liver (Figure 1 a & 2 a). Extensive fatty degeneration of the hepatocytes, with focal necrosis, vacuolated cytoplasm was clearly seen in the VPA-treated

group (Figure 1 b & 2 b). In VPA intoxicated rats, the portal tract was

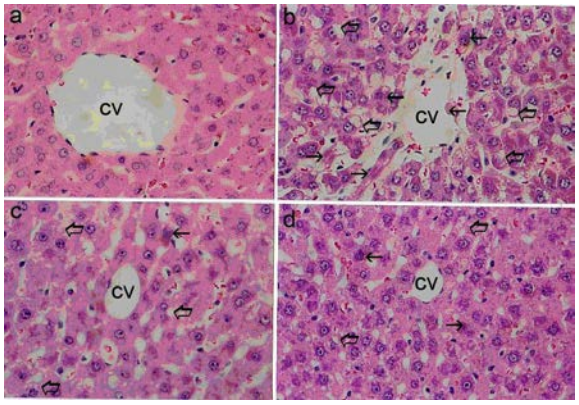

Figure 1: Photomicrographs of liver sections in central area. (a) Liver section from control rats showing the normal arrangement of hepatocytes. Liver section of VPA-treated rats (b) showing extensive degeneration of hepatocytes with focal necrosis (thin arrows), vacuolated cytoplasm (thick arrows) and damaged central vein (CV). Group of rats pretreated with AP (c) or RO (d) before VPA exhibiting relatively normal appearance of hepatocytes with moderate degeneration and focal vacuolated cells (H & E, X 400).

markedly infiltrated with mononuclear cells and showed severe dilated congested portal vessels (Figure 2 b) and some focal cells appeared small in size with deeply stained acidophilic cytoplasm and dark nuclei and other were with pale vacuolated cytoplasm. Animals treated with AG or RO showed normal liver morphology with slight degeneration and vacuolization of hepatocytes (Figure 1 c & 2 c). Rats treated AG + VPA or RO + VPA showed better preserved appearance of hepatocytes (Figure 1 d & 2 d) with a decrease in focal necrosis and inflammations.

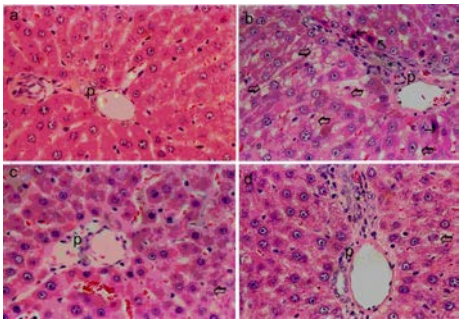

Figure 2: Photomicrographs of liver sections in portal area (p). (a) Liver section from control rats showing normal hepatic architecture. Liver section of VPA-treated rats (b) showing degeneration of hepatocytes with focal eosinohyilic cytoplasm and pyknotic nuclei (thin arrows), vacuolization (thick arrows) and inflammatory cell infiltration (asterisk). (c) Liver section of AG-pretreated rats before VPA showing some degree of swelling and degeneration in hepatocytes. (d) Liver section of rat pretreated with RO before VPA showing better-preserved appearance of hepatocytes with some degree of degeneration and inflammation (H&E, X 400).

3.3. Effect of AG and RO on VPA-induced oxidative stress in liver

The effects of different treatments on the oxidant–antioxidant status of the livers are depicted in Figure 3. VPA treatment alone produced a significant increase in hepatic MDA (Figure 3 A) and P.carbonyl contents (Figure 3 B) with concomitant significant decrease in TAC (Figure 3 C) in liver when compared with the control group. The combined treatment with AG or RO plus VPA significantly decreased hepatic MDA and P.carbonyl contents and significantly increased hepatic TAC when compared with VPA-treated rats (Figure 3). Interestingly, rats received AG or RO alone did not show any changes in these oxidative stress markers.

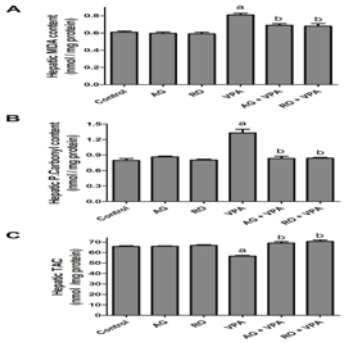

Figure 3: Effect of AG or RO on hepatic MDA (A) and P. Carbonyl (B) contents and TAC (FRAP assay) in control and VPA treated rats. Data are expressed as means ± SEM for eight animals in each group. a $P < 0.05$ vs. control, b $P < 0.05$ vs VPA.

3.4. Effect of AG and RO on VPA-induced DNA fragmentation and up regulation of P53 protein

Immunohistochemical study showed that VPA groups revealed extensive positively brown expression of TUNEL and P53 protein in hepatocytes especially in centriolobular and periportal areas (Figure 4 & 5). AG + VPA treated and RO + VPA treated groups AG and RO attenuated the number of TUNEL and P53 positive cells comparing to VPA-treated group.

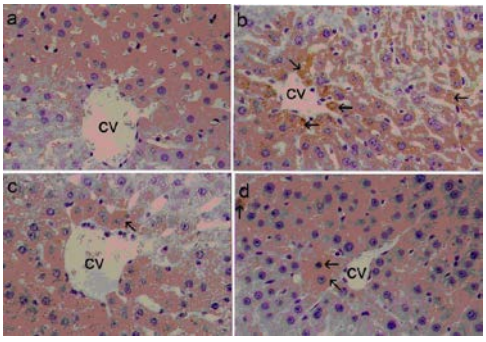

Figure 4: Effect of medicinal herbs on VPA-induced DNA strand breaks were assessed by TUNEL assay. Photomicrographs of liver sections in central area. There are no TUNEL-positive cells in liver section of control rats (a), the nuclei stained blue with hematoxyline. Liver section of VPA-treated rats (b) showing extensive TUNEL- positive hepatocytes (arrows), the hepatocyte nuclei and cytosol stained brown. Group of rats pretreated with AP (c) or RO (d) before VPA exhibiting nearly normal appearance of hepatocytes and there is a marked reduction in TUNEL-positive cells. Sections were counterstained with hematoxyline (X 400).

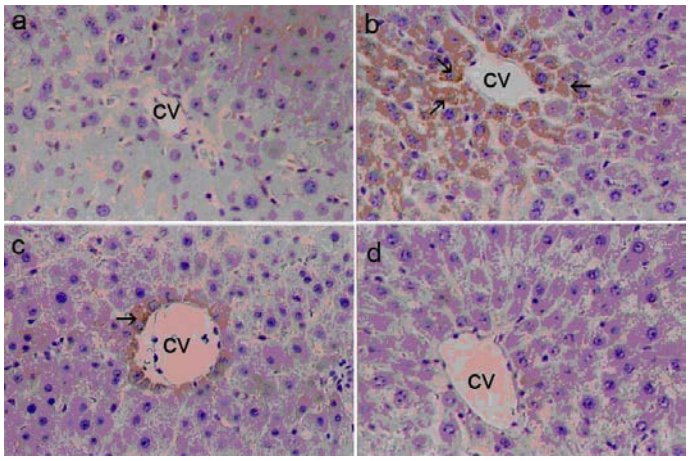

Figure 5: Effect of medicinal herbs on VPA-induced DNA damage was assessed by immunohistochemical localization of P53 protein. Photomicrographs of liver sections in central area. There are no P53-positive cells in liver section of control rats (a), the nuclei stained blue with hematoxyline. Liver section of VPA-treated rats (b) hepatocytes showing frequent P53 immunoreactivity (arrows). Group of rats pretreated with AP (c) or RO (d) before VPA showing a marked reduction in P53-positively-stained cells. (X 400).

3.5. Effect of VPA and AG and RO on clonic convulsion (min) induced by PTZ

Figure 6 depicts that rats subjected to PTZ exhibited clonic convulsions with 4.6 -min average seizure latency. This was manifested as bilateral forelimb myoclonus with rearing, or with loss of postural control. Sub effective dose of VPA showed significant tendency to protect against onset of rat clonic convulsions with 8, 3- min average seizure latency but did not reduce the convulsions' incidence in rats. Each of AG and RO alone did not induce an increase in latency (onset) of rat clonic convulsions with 6 and 5.3- min average seizure latency, when compared with control PTZ value. On the other hand, it was of current interest to seek possible synergy with anticonvulsant effects of VPA. AG and RO successfully delayed the seizure latency to reach 13 and 11.4 min respectively as compared to PTZ treated rats.

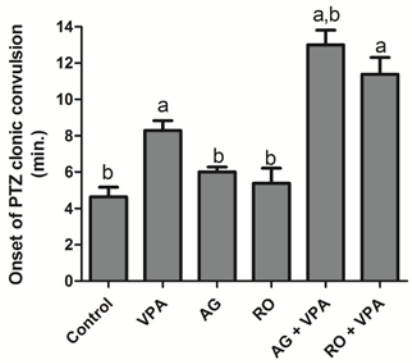

Figure 6: Effect of VPA , AG and RO on PTZ-induced clonic convulsion (min). PTZ was injected 30 min after VPA administration. The combination groups received RO or AG then VPA, respectively; at 30 min intervals, before PTZ was given. Data of clonic convulsion latency are expressed as means ± SEM for eight animals in each group. a $P < 0.05$ vs. PTZ control, b $P < 0.05$ vs VPA.

3.6. Standardizations of AG and RO by HPLC

The HPLC profiles of AG and RO extracts were run in parallel to the corresponding standards under similar analytical conditions (Figure 7 & 8). The standard peak for apigenin was observed at retention time (15.117 min.). While the running of extract resulted in a sharp peak at 15.156 min., which was found to be apigenin present in the extract with other peaks present in HPLC profile of AG. In the present study, apigenin content was about 32 µg/ 200 mg of AG extract.

The HPLC analysis of rosmarinic acid and carnosol of rosemary crud extract are shown in table 2. HPLC analysis of RO extract revealed the presence of some chromatographic peaks among which are rosmarinic acid at 17.654 min and carnosol at 50.246 min (Figure 8). HPLC quantitative analysis showed that rosmarinic acid content was 6.23 mg per 200mg RO crud extract while carnosol was 2.56 mg per 200 mg of RO crud extract.

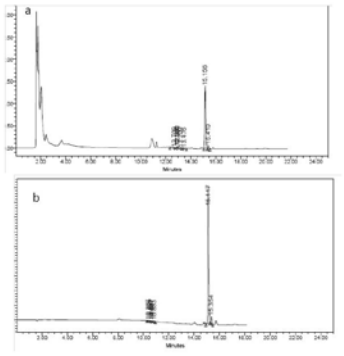

Figure 7: Chromatogram UV obtained at 265.5 nm of AG extract (a) run in parallel to the apigenin standard (b) under similar analytical conditions. The standard peak for apigenin was observed at retention time (15.117 min.). While the running of extract resulted in a sharp peak at 15.156 min., which was found to be apigenin present in the extract with other peaks present in HPLC profile of AG.

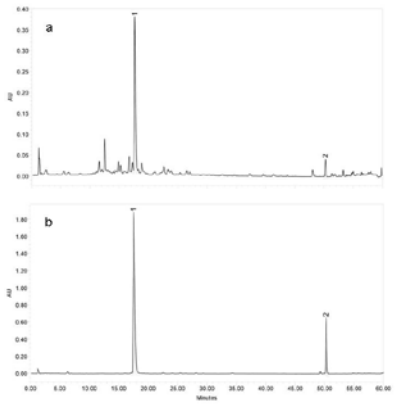

Figure 8: Chromatogram UV obtained at 280 nm of RO extract (a) run in parallel to the rosmarinic acid and carnosol standards (b) under similar analytical conditions. The standard peak for rosmarinic acid (1) was observed at retention time (17.65 min.) and for carnosol was (2) observed at retention time (50.25 min.).

Table 2: Yield, antioxidant capacity and the qualitative–quantitative analyses of the ethanol extract from AG and RO carried out using an HPLC.

Extract	Yield	Antioxidant FRAP assay TAC (mmol/g)	Compound identified	Content / 200 mg extract
AO	16.1%	0.61 ± 0.10	Apigenin	32 µg ± 0.02
RO	10.2%	2.17 ± 0.10	Rosmarinic acid	6.23 mg ± 0.03
			Carnosol	2.56 mg ± 0.03

Values are means ± SME of three experiments.

4. Discussion

The present study demonstrates that treatment with AG and RO extracts significantly reduces the severity of VPA-induced liver dysfunction, cellular steatosis and histopathological changes in male albino rats. Likewise, it reveals that AG and RO enhance the anticonvulsant effects of VPA in a PTZ rat- convulsion model.

In this report, VPA induced liver toxicity in male Wistar rats at dose level 500 mg/kg b.wt. for seven days, which is in agreement with previous experimental animal studies [6, 8, 54] VPA-mediated hepatic injury is characterized by with a dose-dependent rise in serum liver enzymes, decline in plasma albumin, microvesicular steatosis and necrosis [8, 9, 54]. In this 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 [8,55-56]. This hepatotoxicity observed in VPA-treated rats was confirmed histopathological lesions and characterized by hepatocellular focal necrosis, fatty changes, and inflammatory cell infiltration. However, pretreatment of animals by either AG or RO attenuated the liver toxicity induced by VPA as shown by decreased ALT and AST activities and associated decrease of serum albumin and protein levels as well as low histopathological changes in comparison to the VPA-treated group. Similar hepatoprotective effects were obtained from AG and RO treated rats intoxication with paracetamol, thioacetamide [24], CCL4 [25, 33, 38-39], di(2-ethylhexyl) phthalate [57] and creosote [58].

Multiple pathways are implicated in the pathogenesis of VPA-induced liver damage. Overproduction of free radicals and decreased hepatic endogenous antioxidants has been considered as a corner stone in developing VPA-induced liver damage [9-10]. Oxidative stress in a cell is activates when the antioxidant defense system is overwhelmed by the production of ROS and free radicals [59]. In the present work, VPA-induced oxidative stress was confirmed by the elevation of oxidized lipids (MDA) and proteins (P. carbonyl) and as well as the depletion of TAC liver which were consistent with previous studies [8, 10]. Besides liver necrosis and oxidative damage, VPA intoxication significantly increased the number of TUNEL-positive cells, especially in damaged areas of liver. TUNEL assay may detected single-strand DNA breaks as well as double–strand DNA breaks, therefore, TUNEL positively is seen in both necrotic and apoptotic hepatocytes [49]. We have earlier

demonstrated, via increasing of Bax and caspase-3expressions, that apoptosis occurs in the rat liver after similar VPA treatments [9]. These findings concur with those of the previous studies, which reported that VPA induced DNA fragmentation and apoptosis in hepatocyte cell line [60-61], this TUNEL positively effect was accompanied by increased accumulation of P53 protein indicating the presence of DNA fragmentation. Furthermore, excessive amount of free radicals production has been related with an increase in DNA oxidative stress described in series of treatment with VPA epileptic patients [10]. In this study P53 protein accumulation could be a temporary response to DNA fragmentation, which allows the cells to repair its DNA damage or trigger apoptosis. P53 protein acting to induce cell-cycle arrest or apoptosis in response to cell stress or DNA damage, thereby to prevent replication of damaged DNA and to protect the integrity of the genome [51,62]. Therefore, over productions of free radicals and hence oxidative stress may account, at least in part, for hepatic injury associated with VPA treatment. AO and RO pretreatments were effective to decrease VPA -induced oxidative stress and DNA fragmentation (as indicated by TUNEL and P53 protein immunohistochemical techniques). In accordance with our findings, the previous results showed comparable protective effect of RO against oxidative DNA damage induced by several by oxidative stress agents [34, 63- 64].

We used PTZ rat model of convulsion to preliminarily evaluate possible of potential anticonvulsant action of AG and RO. Our results demonstrated an anticonvulsant potential of AG and RO in the PTZ acute convulsion model in rats. These compounds significantly increased the anticonvulsant effect of VPA. The mechanism of their anticonvulsant action is not precisely known and could attribute to their antioxidant effects or might attributed to interaction with GABA.

This protective effect could be the result of direct antioxidant properties found in both herbs, which was reinforced here (FRAP assay) and has been documented previously [17-18, 22, 30, 58]. It is well known that phenolic compounds, which are powerful antioxidants, found in both herbs could responsible for these antioxidant protective effects. The major active phenolic ingredients isolated from celery seeds like limonene, apigenin and phthalide glycosides [14-16] are known to have different pharmacological effects. Limonene and phthalides have been exhibited high activity to induce detoxifying enzyme glutathione S-transferase in the mouse liver [14]. In the same line, Phenolic diterpenes such as carnosic acid, carnosol, rosmanol and rosmanol [17- 18, 65] and carotenoid and alpa-tocopherol [20] have been documented as the principal antioxidant constituents of RO extracts. The efficacy of AG and RO may be attributed to the presence flavonoids apigenin as well as rosmarinic acid and carnosol as active constituents for AG and RO respectively. In the present study, apigenin content was about 32 µg/ 200 mg of AG extract while, rosmarinic acid and carnosol content were about 6.23 mg and 2.56 / 200 mg of RO extract, respectively. Comparable findings have been reported previously [65-67].

Indeed, the protective effect of either AG or RO against VPA toxicity might be mediated not only by their potent antioxidant properties but through their detoxification capacity. Apigenin is an antioxidant and found to be the major component in AG [66]. However, it cannot be excluded that these plant extracts may inhibit several metabolic intermediates and ROS formed during the process of microsomal enzyme activation. Limonene and phthalides have been exhibited high activity to induce detoxifying enzyme glutathione S-transferase in the mouse liver [14]. Celery extract has been caused a significant decrease of cytochrome P450 in the liver of mice

[68]. AG extract also was found to prevent mucosal inflammation through its inhibitory effect on prostaglandin production [27]. Moreover, RO extract was reported to induce xenobiotic detoxification enzymes in rat liver [69]. The preventive action of RO can alternatively be mediated through the induction of detoxification enzymes [33]. However, RO was more effective than AG at preventing liver damage. Histological and AST changes were prevented to greater extent in rats pretreated with RO than in rats pretreated with AG. The high reducing capacity (FRAP assay) of RO extract observed here could contributed in its higher protective effect.

5. Conclusion

In conclusion, the result of the present study suggests an effective strategy to abate VPA-induced hepatic injury. Both of AG and RO act via antioxidant capacity of active phenolic compounds to restore liver function and integrity and to synergize with antiepileptic effects.

6. Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgment

The authors would like to thank Mrs. Hanan Mohamed Mehney, at Department of Hormone Evaluation, NODCAR, Egypt, for her excellent technical help. AG and RO were botanically authenticated by Dr. Nael M. Fawzi, Flora and Taxonomy Department, Agricultural Research Center, Giza, Egypt.

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