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Research Article

*Sodium Valproate-Induced Hepatic Dysfunction in Albino Rats and Protective Role of *n*-Butanol Extract of *Centaurea sphaerocephala* L.*

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ABSTRACT

The objective of the present study was to evaluate the protective effect of *n*-butanol extract of *Centaurea sphaerocephala* (*C.sphaerocephala*) and Vitamin E against sodium valproate-induced hepatotoxicity and oxidative stress in male rats. Male rats were divided into eight equal groups treated with plant extract (50mg/kg, 100mg /kg), Vit. E (100mg/kg) and VPA (300mg/kg). At the end of the experiment, animal were scarified and samples (blood and liver's tissue) were removed isolated for biochemical and histological study. VPA-treated rats showed hepatic injury characterized by a significant increase in biochemical parameters (serum transaminase, cholesterol and triglycerides). Also, VPA induced oxidative stress exhibited a significant increase in MDA level and significant decrease in GSH levels, CAT and GPx activities. These effects were accompanied by histopathological changes in liver. While the pretreatment by *n*-butanol extract of *C. sphaerocephala* reversed the alteration induced by VPA and reduced its toxic effects. The results showed a significant decrease in serum markers and liver's lipid peroxidation whereas GSH level and the activities of GPx, CAT enzymes were significantly increased. Histopathological observations correlated with the biochemical parameters. VPA-induced hepatotoxicity involved free radical production, the antioxidant and free radical scavenging property of *Centaurea sphaerocephala* would have provided the protection against hepatic damage.

Keywords: Valproic acid; *Centaurea sphaerocephala* L; Hepatotoxicity; Oxidative stress; Lipid peroxidation; protective effect.

INTRODUCTION

Valproic acid (VPA) is a well-established anticonvulsant drug used in the treatment of many forms of generalized epilepsy and psychiatric disorders to control epileptic seizures and regulate the mania associated with bipolar disorder[1,2]. VPA is well tolerated at therapeutic doses and it has inherent toxicity[3]. Two types of serious side-effects limit the use of this drug: hepatotoxicity and teratogenicity[4] However, Administration of VPA produced many metabolic and morphological aberrations in the liver[5]. Also, histopathological and biochemical studies indicated that VPA evoked hepatic necrosis, apoptosis and steatosis[6].

Furthermore, VPA increased intracellular reactive oxygen species (ROS) levels in several tissues, including liver, brain and small intestine[7]. But the mechanism by which VPA induces liver injury remains unknown[8]. A possible VPA biotransformation and/or alterations in natural antioxidants might contribute to the VPA associated complications.

However, the main cause of VPA hepatotoxicity was shown to be due to generate the free radical scavenger[9]. Oxidative stress, as a result of compromised antioxidant capacity and/or increased production of reactive oxygen species (ROS) has been also proposed as one mechanism for VPA-induced hepatotoxicity[10].

Lipid peroxidation may be involved as an additional mechanism of VPA-induced liver damage in rats[11]. Injection a single dose of VPA ~~in~~ to rats resulted in a dose-dependent elevation levels of lipid peroxidation in plasma and liver[12]. However, antioxidants were the primary candidates to counteract such toxic effect. Glutathione (GSH) as a major antioxidant and redox regulator play an important role in the defense against oxidants and electrophiles[13]. Consequently, any mechanism which removes ROS or prevents hepatic GSH depletion or induce activation and production of GSH dependent enzymes may provide protection for hepatotoxicity in VPA-treated patient[14]. Also Cells can be protected from oxygen-derived radical injury by naturally occurring free- radical scavengers and antioxidant pathways, including vitamins A, C, E, SOD, catalase and glutathione peroxidase[15]. Moreover, many therapeutic studies are offered to plants

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since plants are a natural source of antioxidants and hence reduce oxidative stress[16].

The genus *Centaurea* (Asteraceae) contained more than 500 species. 45 species growing in Algeria, including 7 in the Sahara[17,18]. Many species of the genus *Centaurea* have been used in traditional medicine to cure various ailments (diabetes, diarrhea, rheumatism, malaria, hypertension)[19].

To our knowledge, no traditional uses or pharmacological studies are reported so far for this species. So, as a part of our ongoing research program on beneficial health effects of plants and herbs[20,21] , we investigate in the present study, the ability of the protective effect of *n*-butanol extract of *Centaurea sphaerocephala* an Algerian endemic plants and vitamin E on VPA-induced liver damage in male rats.

MATERIALS AND METHODS

Plant material and extraction procedure

Aerial parts of *C. sphaerocephala* were collected from the area of El Kala, Algeria (21 m, 36° 53′ 44″ N, 8° 26′ 35″ E) in May 2012 and authenticated on the basis of Quezel and Santa (1963)[18] by Professor M. Kaabache, specialist in the identification of Algerian *Centaurea species* (Ferhat Abbas University, Setif 1, Algeria). A voucher specimen (CSA0512-EK-ALG-65) was deposited in the Herbarium of the VARENBIOMOL research unit, Frères Mentouri University Constantine 1.

The leaves and flowers (2000 g) of this plant were macerated for 24 h, three times with methanol-water (70:30, v/v) at room temperature. After filtration, the filtrate was concentrated under vacuum (up to 35 °C), the remaining solution (400 mL) was dissolved in distilled H_2O (800 mL) under magnetic stirring and maintained at 4 °C overnight to precipitate a maximum amount of chlorophylls. After filtration, the resulting solution was extracted successively with chloroform (CHCl3), ethyl acetate (EtOAc) and n-butanol (n-BuOH). The organic solutions were dried with sodium sulfate (Na_2SO_4), filtered using common filter paper and concentrated in vacuum (up to 35 °C) to obtain the following extracts: $CHCl_3$(5 g), EtOAc (4.94 g) and n-BuOH (34 g).

Animals and Treatment

Male Wistar albino rats weighing (150-200 g) were obtained from Pasteur institute (Algiers, Algeria). Animals were housed in plastic cages, with controlled laboratory conditions of light/dark cycle (12 h/12 h), temperature (22±2°C) and relative humidity, with food and tap water. Rats were adapted for 2 weeks before the indicated treatments. All experimental procedures were performed between 8–10 a.m. and care was taken to avoid stress full conditions. Also, all experimental assays were carried out in conformity with international guidelines for the care and use of laboratory animals. Animals were left for 10 days before being randomized into experimental groups of 8 animals and four animals per cage. The study protocol was approved by the Institutional Animal Ethical Committee. Rats were housed four per cage and were randomly divided into 8 groups (8 animals in each group):

Group1, non-treated served as control; Group2 and Group 3, received plant extract (50 mg/kg) and (100mg/kg) respectively; Group 4 treated with 300 mg/kg per day sodium valproate; Group 5, rats received Vitamin E (100mg/kg); Groups 6, 7, 8 received respectively, plants extract (50 and 100mg/kg), vitamin E (100 mg/kg) 1 hour before treatment with VPA (300mg/kg).Treatments were given for 14 days by gavage.

After treatment, blood samples were drawn from the caudal vena cava, collected in test tubes containing EDTA, and centrifuged to obtain serum for analysis of biochemical parameters. The rats were sacrificed by decapitation after deep ether anesthesia; livers were isolated to measure the levels of antioxidant enzymes, MDA and histopathological studies.

Preparation of tissues samples

Livers were perfused with ice NaCl 0.9% solution to remove blood cells, removed quickly and placed in the same solution. After blotted on filter paper, weighed, and homogenized in ice-cold KCl 1.015% with the addition of 6 µl of 250 µM butylated hydroxytoluene to prevent the formation of new peroxides during the assay. The homogenization procedure was performed under standardized condition. Homogenates (20%) were centrifuged and the supernatant was kept on ice until assayed or conserved in freezer – 80°.

Lipid peroxidation determination

Lipid peroxidation (LPO) was determined by measuring the formation of TBRAs using the colorimetric method of Uchiyama[22]. 3ml of phosphoric acid (1%) and 1ml of thiobarbituric acid (TBA, 0.67%), aqueous solution were added to 0.5 ml of liver homogenate (20%) pipetted into centrifuge tube. The mixture was heated for 45 min in a boiling water bath. Then the mixture was cooled at room temperature, and 4 ml of *n*-butanol was added and mixed vigorously. After centrifugation, the absorbance was measured at 532 nm. MDA was used as the standard.

Measurement of reduced glutathione

Reduced glutathione (GSH) content in the liver was measured chemically according to the method described by Elman[23] using Elman's reagent. This method is based on the reactive cleavage of 5, 5′-dithiobis-(2-nitrobenzoic acid) by sulfhydryl group to yield a yellow color with maximum absorbance at 412 nm against reagent blank.

Evaluation of GPx activity

GPx activity in the liver was measured chemically according to the method described by Flohe[24]. This method is based on the reduction of H_2O_2 in the medium by GPx in the presence of GSH. Briefly 0.2ml supernatant obtained from tissues, 0.4ml GSH (0.1 mM), 0.2ml TBS solution (Tris 50mM, NaCl 150mM PH 7.4) were added to the tubes and mixed. After 5 min incubation at 25 °C, 0.2 ml of H_2O_2 (1.3mM) was added in the mixture. The reaction was stopped after 10 min by addition of 1 ml trichloroacetic acid (TCA 1%, w/v), and then the tubes maintained at 0-5°C in an ice bath for 30min. After centrifugation, 0.48ml supernatant was taken and added to each tube, and then 2.2 ml TBS solution and 0.32 ml DTNB (1mM) were added. The optical density was measured at 412 nm in the spectrophotometer after 5 min.

Evaluation of the catalase activity

The enzymatic activity of catalase was measured as described by (Claiborne, 1985)[25]. The homogenate was

centrifuged at 10000 rpm for 45 min at 4° C; the final supernatant is the source used for the evaluation of the activity of catalase. The disappearance of H_2O_2 was determined spectrophotometrically at 240 nm. Catalase activity was expressed as U/mg of protein. In order to express the antioxidant enzyme (GPx, catalase) activities per gram of protein, total protein concentration was determined calorimetrically by using the method of (Lowry, 1951)[26].

Plasma biochemical analysis

The liver marker enzymes, aspartate transaminase (AST) and alanine transaminase (ALT) also, total cholesterol and triglycerides were estimated using commercial kits (Spinreact, SPAIN).

Histopathological examination

For histopathological analysis, hepatic tissue fragments were taken and fixed in neutral formalin 10 % solution. The fixed specimens were then trimmed, washed and dehydrated in ascending grades of alcohol. These specimens were then embedded in paraffin, cut into 5µm thick sections and stained with Harris hematoxylin and eosin for microscopically examination[27].

Statistical analysis

Data are expressed as mean ± SD and statistical interferences were based on student's test for mean values comparing control and treated animals using Graph Pad Prism 5.01 Retail+5.02 Update, Version 5. The statistical significance was accepted at a level of $P<0.05$.

RESULTS

Impact of VPA, vitamin E and n-butanol extract of Centaurea sphaerocephala on serum transaminases levels.

As shown in Figure 1, the administration of toxic dose of VPA (300mg/kg) caused a significant increase in liver enzymes (AST and ALT) with the values 130.32±2.11U/l, 95.72±4.14U/l respectively. This increase was statistically significant ($P<0.001$) compared to control group 75.14±2.42U/l, 66.63±1.01U/l respectively. Animals pretreated with *n*-butanol extract (100mg/kg) and Vit. E (100mg/kg) showed a significant decrease ($P<0.01$, *$P<0.001$*) in these liver enzymes compared to VPA-treated animals. While, plasma levels of these enzymes in rats pretreated group with extract (50mg/kg) were significantly decrease ($P<0.05$) and ($P<0.01$) respectively.

The protective effect of n-butanol extract of C. sphaerocephala and vitamin E on cholesterol and triglycerides levels.

The VPA treated rats exhibited a significant increase ($p<0.001$) the cholesterol and triglyceride serum levels compared to control group. The pretreatment with both doses of plant extract and Vit. E (100mg/kg) decreased significantly ($p<0.01$) the total cholesterol compared to VPA-group. A significant reduction in triglycerides was observed in rats pretreated with *n*-butanol extract (50mg/kg, 100mg/kg) ($p<0.01$, $p<0,001$) and Vit E ($p<0,001$) compared to VPA-treated rats (Figure 2).

The protective effect of n-butanol extract of C. sphaerocephala and vitamin E on VPA- induced lipid peroxidation in liver

The administration of VPA induced a significant increase ($P<0.01$) in lipid peroxidation in liver tissue compared to control. While the pretreatment with *n*-butanol extract (100mg/kg) and Vit E (100 mg/kg) produced a significant decrease ($P<0.01$) in lipid peroxidation in liver compared to VPA group (Fig 3).

Effect of VPA, n-butanol extract of C. sphaerocephala and Vitamin E on liver GSH levels

As showed in Figure 4, a significant decrease in GSH levels of liver's tissue was observed in VPA group ($P<0.001$) compared to control or untreated rats. While co-administration of plant extract (100mg/kg) and vit E (100mg/kg) with VPA increased significantly ($P<0.01$) the level of GSH compared to VPA group, in the other side, group pretreated with 50mg/kg has significant decrease with ($P<0.05$) in GSH level.

Effect of VPA, n-butanol extract of C. sphaerocephala and Vitamin E on GPx activity in liver.

As illustrated in Figure 5, VPA induced significant decrease ($P<0.001$) in GPx activity compared to control or normal group. Furthermore, there was found a marked significant increase ($P<0.001$) in GPx activity after co-treatment with plant extract (100mg/kg, 50mg/kg) and VitE (100mg/kg) compared to VPA group (Figure5).

Effect of VPA, n-butanol extract of C. sphaerocephala and Vitamin E on Catalase activity in rats' liver

CAT activity was significantly decreased ($P<0.01$) in liver rat's tissue after administration of VPA (300mg/kg) compared to control. Furthermore, *n*-butanol extract of *C. sphaerocephala* (100mg/kg) and Vit E (100mg/kg) showed a significant increase ($P<0.05$; $P<0.01$) respectively in catalase activity compared to its activity in VPA group (Figure 6).

Histological examination

Effect of VPA and n-butanol extract of C. sphaerocephala on liver histology

As shown in Figure 7 (A) the liver of control or untreated rats showed normal histological architecture. Liver's VPA treated-rats (300mg/kg), showed dilatation and vascular congestion (D, a); steatosis (D, b) and hepatic necrosis (D, c). While the liver's section of plant extract treated rats showed a normal histological picture that closely approximate of the control group (Figure7 B, C).

Also, sections belonging to groups pretreated or co-administrated by VPA and Vit. E or VPA and *n*-butanol extracts showed relatively normal ultrastructure compared to VPA group (Figures 7, G, F and H).

DISCUSSION

The use of VPA as an anticonvulsant has been supported by clinicians, which was subsequently challenged due to its side-effects and induced toxicity[28]. The most serious of those being hepatotoxicity[29], teratogenicity[30] and neurotoxicity[31] which are associated with increased reactive oxygen species (ROS) formation[32].

The mechanism of hepatic injury has been studied extensively but is still unclear. Some authors hypothesized that VPA aberrant metabolism with the formation of toxic metabolites or mediation of lipid peroxidation might be the underlying mechanism of serious hepatic reactions[33,34].

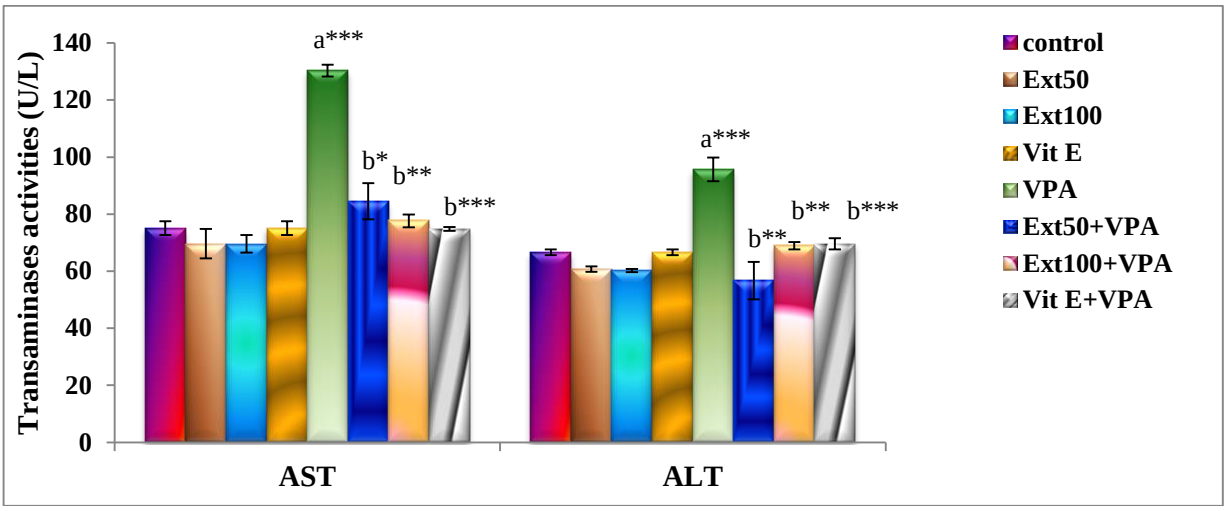

Figure 1: Effect of VPA (300mg/kg), Vit. E (100mg/kg) and *n*-butanol extract (50mg/kg, 100mg/kg) of *C. sphaerocephala* L. on serum aspartate transaminase (AST) and alanine transaminase (ALT) levels in experimental rats. Data are reported as means ± SD. (*P<0.05; **P<0.01; ***P<001). a: compared to control group, b: compared to VPA group.

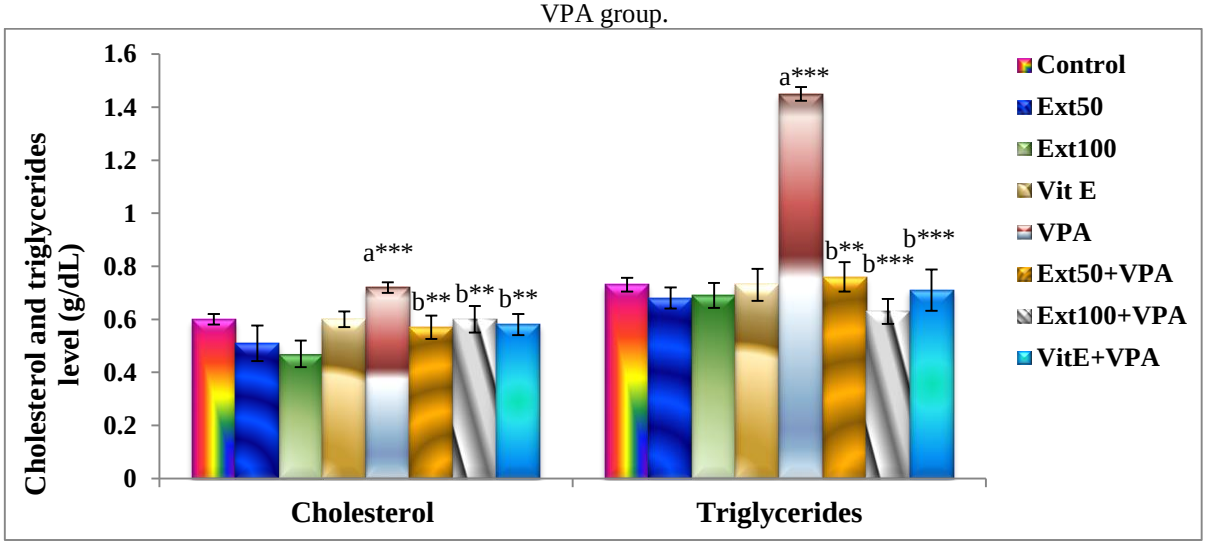

Figure 2: The protective effect of *n*-butanol extract of *C. sphaerocephala* (50mg/kg and 100mg/kg) and Vit E (100mg/kg) against VPA-induced toxicity. Effect on serum cholesterol and triglycerides levels in rat's liver. Data are reported as means ± SD. a: group compared to control group, b: group compared to VPA group. (*P<0.05; **P<0.01; ***P<0.001).

Lipid peroxidation is one of the excessive ROS consequences while causing cell damage. It was shown that VPA induced lipid peroxidation in rat hepatocyte cultures[35,36].

In the present study, administration of VPA to rats caused a significant increase of lipid peroxidation as indicated by the significant increase in MDA level compared to the control group; suggesting that VPA activated the formation of free radicals in hepatic tissue. These results confirmed by others findings which demonstrated that VPA exposure stimulated the generation of ROS[37,38]. Also, study reported elevated serum LPO levels in epileptic children who had VPA therapy when compared to pretreatment group[39]. Another study reported increased in plasma LPO levels in epileptic adults who were treated with VPA[40].

It is well known that reduced glutathione (GSH) is a major antioxidant and redox regulator, which is present in all cell types. Is the most abundant cellular thiol, and plays an important role in the defense against oxidants and electrophiles [41]. Also it is a substrate for glutathione peroxidase (GPx) and detoxifies foreign compounds and biotransformation drugs[42]. In our investigation, GSH level, CAT and GPx activities decreased in rats' liver of VPA-treated group compared to control animals. The increased production of ROS caused inactivation of antioxidant enzymes which reflects their consumption through the oxidative stress. In agreement with this finding, the significant decrease of GSH content in VPA-treated rats suggested that it might be due to exhaustion of GSH stores and increase in the oxidative stress. These results are in agreement with others studies[43,44].

Also, the activity of erythrocytes GPx decreased in patients

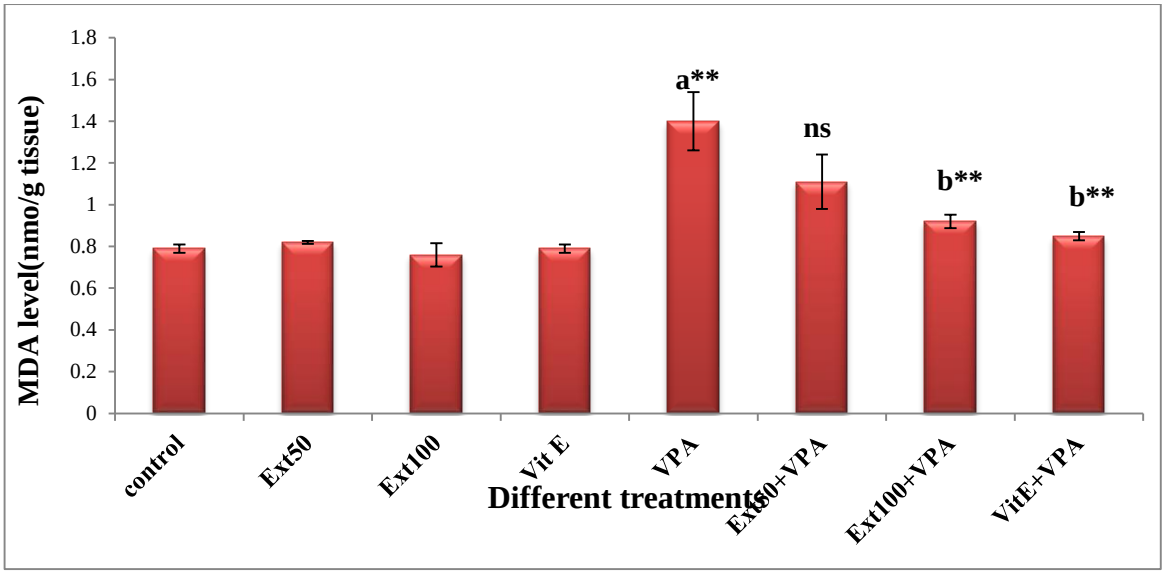

Figure 3: Effect of VPA (300mg/kg), *n*-butanol extract of a *C. sphaerocephala* (50mg/kg, 100mg/kg) and vitamin E (100mg/kg) on lipid peroxidation (TBARs content) in rat's liver. Data are reported as means ± *SD. *P<0.05; **P<0.01; ***P<001).) ns: non-significant.* a: compared to control group, b: compared to VPA group.

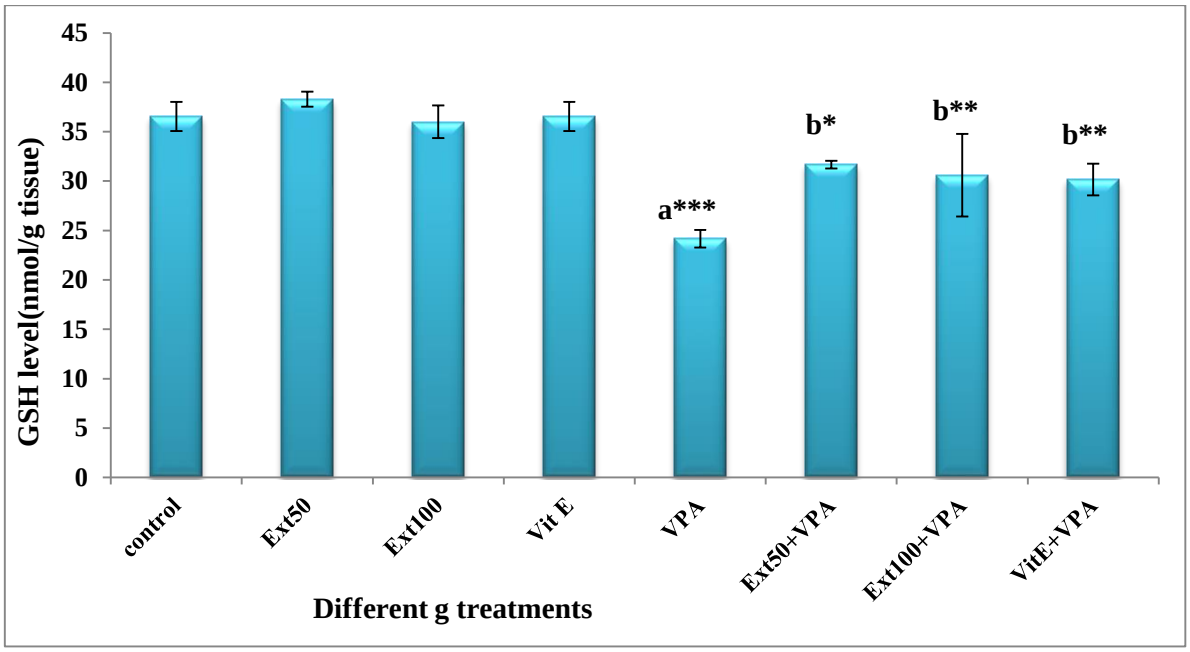

Figure 4: The effect of *n*-butanol extract of *C. Sphaerocephala* (50mg/kg, 100mg/kg), Vitamin E (100mg/kg) and VPA (300mg/kg) on GSH level in rat's liver. Data are reported as means ± SD. a: group compared to control group, b: group compared to VPA group. (*P<0.05; **P<0.01; ***P<0.001).

treated with VPA[45] and in rats administered VPA intraperitonally[46].

One of the most sensitive and dramatic indicators of hepatocyte injury is the release of intracellular enzymes such as AST, ALT after VPA administration. The elevated activities of these enzymes indicated a hepatocellular damage[47]. Our results showed that VPA administration caused severe acute liver damage in rats, demonstrated by the significant elevation of plasma AST and ALT levels, suggesting that excessive VPA might cause critical injury to the organ. These findings concurred with the results of other studies[48,49] . Also, in the current study; the VPA-treated rats exhibited significantly higher Cholesterol and triglycerides levels than the control rats. This increase consistent with the finding of other study which reported that administration of VPA caused significant increase in the levels of lipid profile (cholesterol, triglycerides, phospholipids and free fatty acids)[11]. Moreover, histological studies of VPA-induced toxicity have shown extensive factor lead to sever distortion of liver architecture, vascular congestion, microvesicular steatosis with and hepatic necrosis which is in agreement with other studies[50,51].

Plants produced significant amount of antioxidants such as polyphenols, phenols and flavonoids. These compounds scavenge a wide range of free radicals, including the most

active hydroxyl radical, which may initiate lipid peroxidation and prevent the loss of the lipophilic (α-tocopherol) and hydrophilic (ascorbate) antioxidants, by repairing tocopheryl and ascorbate radicals[52].

In our study, administration of *n*-butanol extract of *C. sphaerocephala* (50mg/kg,100mg/kg) or vitamin E (100 mg/kg) simultaneously with VPA to male rats resulted in normalization of lipid peroxidation process as well as glutathione content, glutathione peroxidase and catalase activity in rats' livers . Permitting the prevention of hepatic dysfunction and maintaining the normal level of serum transaminases, cholesterol and triglycerides following inhibition of their hepatic leakage by preventing lipid peroxidation. So, the protective efficacy of *C. sphaerocephala* may be due to the presence of several active components. These results are in agreement with other studies which demonstrated that the antioxidant and free radical scavenging property of medicinal plants extract would have provided the protection against hepatic damage caused by valproic acid[53,54]. Also, in this study we showed that, treatment with *C. sphaerocephala* improved histological changes in the liver caused by VPA.

CONCLUSION

Results of this study showed that VPA administration reduced antioxidants and increased lipid peroxidation which leads to organ damage. Also, it was observed that *C. sphaerocephala* exerted significant protection against VPA-induced toxicity by its ability to ameliorate the lipid

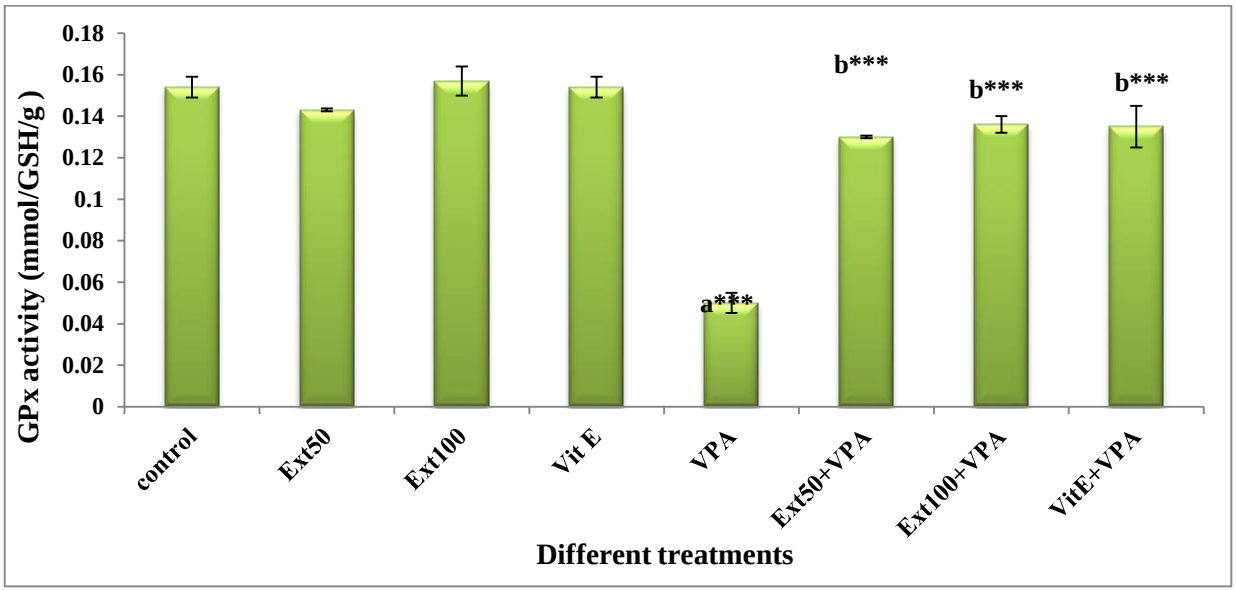

Figure 5: Effect of VPA (300mg/kg), *n*-butanol extract of *C. sphaerocephala* (50mg/kg, 100mg/kg) and vitamin E (100mg/kg) on GPx activity in liver's rats. Data are reported as means ± *SD. (*P<0.05; **P<0.01).* a: compared to control group, b: compared to VPA group.

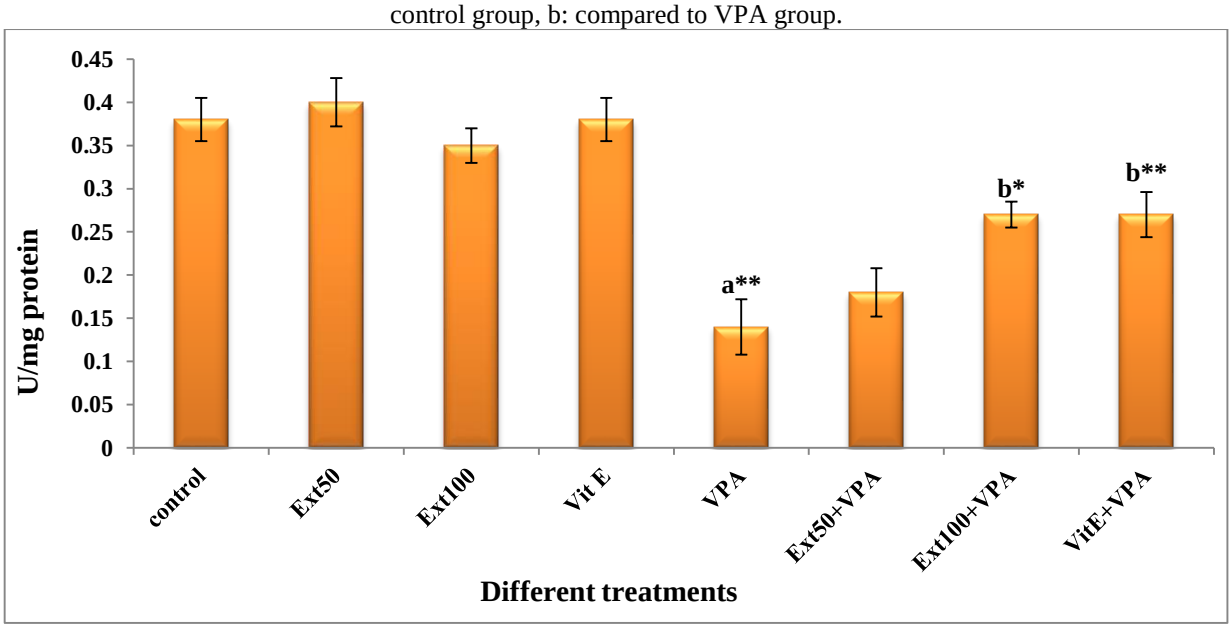

Figure 6: Effect of VPA (300mg/kg), *n*-butanol extract of *C.sphaerocephala* (50mg/kg, 100mg/kg) and Vit. E (100mg/kg) on catalase enzyme activity in rat's liver. Data are reported as means ± SD. a: group compared to control group, b: group compared to VPA group. *P<0.05; **P<0.01; ***P<0.001.

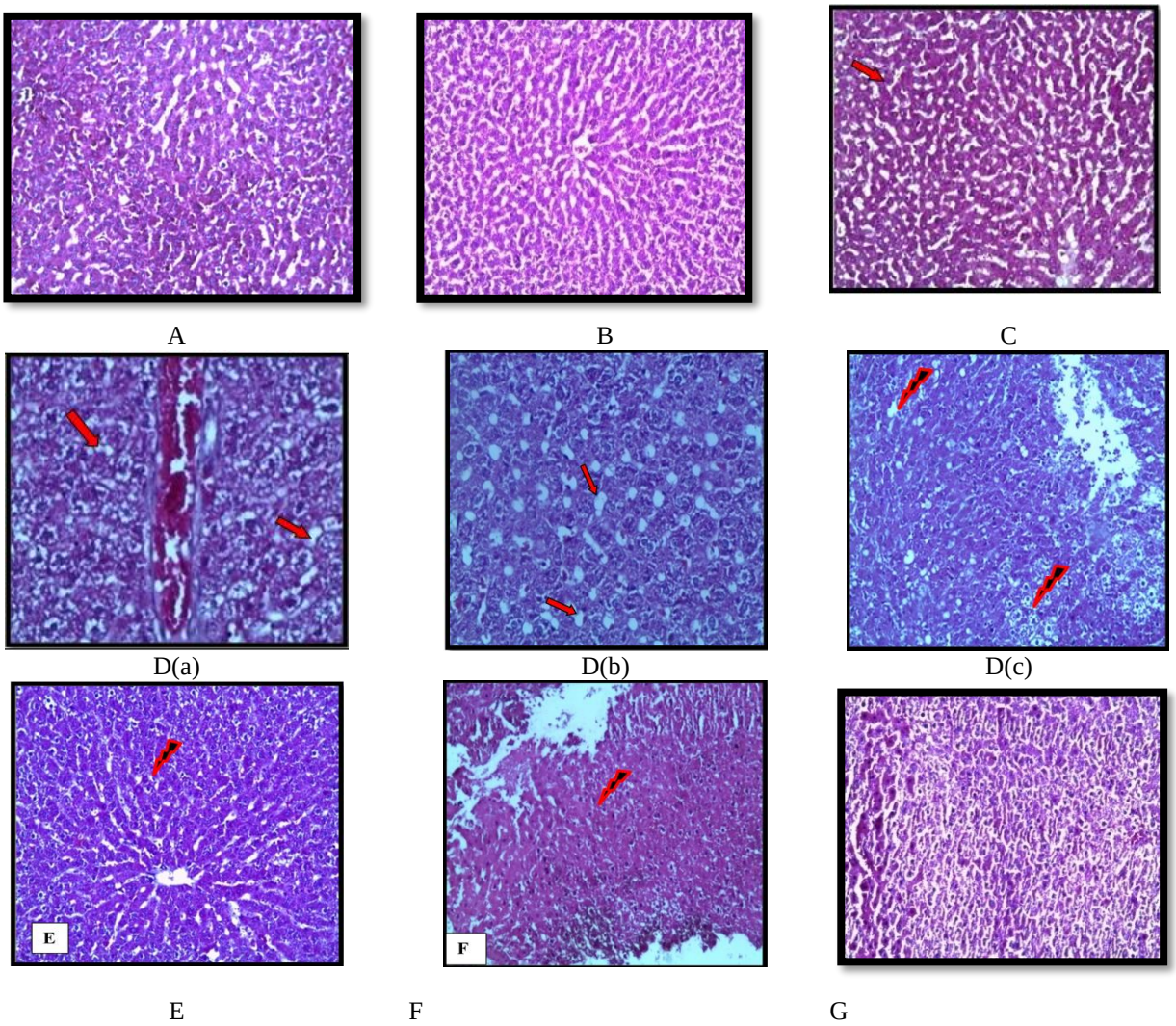

Figure 7: Photomicrographs of rat's liver section (H&E, ×100, ×400). (A): Control group showing normal hepatic architecture, (× 100). (B & C): Rats' livers treated with *n*-butanol extract of *C. sphaerocephala* alone (50 mg/kg and 100 mg/kg) respectively. Treated group showed normal histology almost similar to the control group (×100). (D): VPA (300mg/kg) treated group showing distortion of normal architecture and irregularly-shaped hepatocytes as; vascular congestion (D, a), (×400); Steatosis (D, b), (×400) and necrosis, dilated and congested sinusoids veins (D, c), (×400). (E), (F): Livers 'section of rats treated with VPA (300mg/kg) and *C. sphaerocephala* extract (50mg/kg or 100mg/kg) respectively showed conserved hepatocytes (×400). (G): Livers 'section of rats treated with VPA (300mg/kg) and vitamin E (100mg/kg) showing a histological picture comparable to that of the control group with minimal damage of hepatocytes (×400).

peroxidation through the free radical scavenging activity, which enhanced the levels of antioxidant defense system. This effect could be attributed to its antioxidant properties.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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