Acute, sub-acute toxicity and antioxidant activities (*in vitro* and *in vivo*) of *Reichardia picroide* crude extract

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ABSTRACT

Ethnopharmacological relevance: *Reichardia picroide* is a species mainly used for alimentary purposes, but it is traditionally known to be used as hypoglycemiant, diuretic, depurative, galactagogue and tonic.
Aim of the study: To date, there are no studies corroborating both its safety and antioxidant activities. The objective of the present study, thus, was to assess the safety profile of *Reichardia picroide* methanolic extract (RPE) and as well as on its antioxidant and antihemolytic activities.
Materials and methods: The acute toxicity of RPE was carried out based on OECD guidelines 425. Signs accompanying toxicity and possible death of animals were monitored for two weeks to ascertain the median lethal dose (LD_{50}) of the RPE. In sub-acute toxicity study, the extract was administered by gavage at the doses of 250, 500 and 1000 mg/kg/day for 21 consecutive days. The antioxidant activity of RPE was investigated through various methods both *in vitro* and *in vivo*.
Results: The admistrated doses did not produce mortality or changes in general behaviors of the tested males and females mice. The LD_{50} was found to be superior to 5000 mg/kg DW. Moreover, daily administration of RPE at doses ranged from 500 to 1000 mg/kg could result in alteration of liver and kidney histology. Significant decrease in liver enzymes (ALT and AST), urea and creatinine levels in female plasma was recorded. The RPE was, *in vitro*, strong in DPPH scavenging and hemolytic inhibition, benificial in lipid peroxidation inhibition and reducing power. In addition, it exhibited, *in vivo*, a strong effect on GSH level increasing and lipid peroxidation inhibition in liver and kidney.
Conclusions: It can be suggested, based on the results of this study, that the crude extract of *Reichardia picroide* was non-toxic in acute administration and the use of this extract is safe at doses ≤ 250 mg/kg. This study supports the application of *Reichardia picroides* in alimentary and traditional medicine purposes. Moreover, antioxidant activity results suggested that *Reichardia picroide* had potent antioxidant activities and could be utilized as new natural antioxidant in food and therapeutics.

1. Introduction

Since antiquity, the use of medicinal plant products has always been present and has increased tremendously over the past three decades up to 80% of the world's population relying on them for some part of primary healthcare (Ekor, 2013). This widespread of plant medicine is attributed to several factors such as various claims on the efficacy or effectiveness of plant medicines, high cost and side effects of most modern drugs, and a movement toward self-medication (Bandaranayake, 2006). Regarding or categorizing medicinal plant products as safe because they are derived from natural sources a misconception. The reality is that "safe" and "natural" are not synon-

ymous. Therefore, there is a need to monitoring their safety (Gori and Firenzuoli, 2015). Unfortunately, many of plant species have not been toxicologically validated (Saad et al., 2006).

Reactive oxygen species are well recognized for their dual role as both deleterious and beneficial species. Beneficial effects of these species occur at low/moderate concentrations and involve physiological roles in cellular signaling pathways (Sorg, 2004). In contrast, the generation of reactive oxygen species beyond the antioxidant capacity of a biological system gives rise to oxidative stress, a deleterious process that can be an important mediator in damaging cell structures, including lipids and membranes, proteins, and DNA (Valko et al., 2006). Oxidative stress facilitates the development of a variety of human diseases (Goetz and Luch, 2008; Libetta et al., 2011).

One solution to this problem is compensating this disequilibrium with antioxidant compounds that are contained in natural plant sources.

Many medicinal plants were frequently used as domestic medicines, which were proved to possess antioxidant and pharmacological properties contributed by multiple compounds in them. These beneficial properties are attributed to various polyphenols present in the plant (Baghiani et al., 2011, 2013).

Reichardia picroide L., locally called "halhal", belongs to Asteraceae family is a Mediterranean endemic species (Quezel and Santa, 1963). Herb leaves are mainly used for alimentary purposes (Cornara et al., 2009; Di Novella et al., 2013; Guarrera and Savo, 2013), but they are also traditionally known to be used as hypoglycemiant (Bonet et al., 1999), diuretic, galactagogue and emollient (Guarrera and Savo, 2013), depurative of intestine (Cornara et al., 2009), and tonic (Loi et al., 2004). Furthermore, herb Roots are used for cough, abdominal pains and kidney problems (Savo et al., 2011). However, to our knowledge, no report is available on both the safety profile and antioxidant activities of *Reichardia picroides*. Based on that, we aim to evaluate the acute and sub-acute toxicity and *in vitro* and *in vivo* antioxidant activities of its methanolic extract.

2. Materials and methods

2.1. Collection of plant

The whole *Rechardia picroides* was collected from Bougaa (36° 19′ 57″ Nord 5° 05′ 19″ Est), Setif (Algeria) in march-april 2013. The voucher specimen was identified by Pr. Oujhih B. Institut of nutrition and agronomy, Batna (Algeria) with the reference number 333/ISVSA/UHL/13. The whole plant was dried in dark and at free air for tow weeks, and powedred using electrical grinder (Retsch SK 100).

2.2. Preparation of crude extraction

Powedred materials were extracted three times at ratio of 1: 10 (w/v) by maceration in methanol for seven days each time at room temperature and with intermittent shaking, according to Sharma et al. (2008) method with slight modifications. The methanol mixture was then filtered and concentrated at 45 °C under reduced pressure using a rotary evaporator (Büchi 461). The RPE was further concentrated by allowing it to stand in an oven at 40 ∘C. The yield of the *Reichardia picroide* extract (RPE) was calculated from the initial weight of the used plant powder.

2.3. Experimental animals

Experiments were performed using young adult male and female (nulliparous and non-pregnant) *Swiss albino* mice and weighing 20 – 30 g. They were obtained from Pasteur institut (Algeria) and housed in plastic cages under normal laboratory conditions (12 h light / dark cycle, 23 ± 2 °C) for an acclimatization period of 7 days prior to the experiments. All the animals were given food and water *ad libitum*. The

experiments were conducted in accordance with the internationally acceptable guidelines for evaluating the safety and efficacy of plant medicines (OECD, 2008).

2.4. In vitro antioxidant activity

2.4.1. Polyphenols content determination

Total phenolics content was estimated by Folin–Ciocalteu method (Li et al., 2007). Such method consists of Folin-ciocalteu's reagent reduction by the phenolic hydroxyl groups, resulting in the formation of a blue product in alkaline solution. Briefly, 200 µl of appropriate dilution of RPE were added to 1 ml of 1:10 diluted Folin-ciocalteu's reagent. After 4 min, the reaction mixture was neutralized with 800 µl of saturated sodium carbonate (75 g/l). Subsequently, the shaken mixture was allowed to stand for 2 h at room temperature, and then measured at 765 nm. Gallic acid (20–140 mg/l) was used for the standard calibration curve. The results were expressed as µg gallic acid equivalent (GAE)/mg of extract.

2.4.2. Flavonoids content determination

The $AlCl_3$ method (Bahorun et al., 1996) was used to determine the total flavonoids content of the RPE, employing the reaction of complex formation between flavonoids and aluminum chloride. Aliquots of 1 ml of extract were added to equal volumes of a solution of 2% $AlCl_3$. The mixture was vigorously shaken, and the absorbance was read at 430 nm after incubation in dark at room temperature for 10 min. Quercetin and rutin (1–40 mg/l) were used as standards for calibration curve. Flavonoids contents were expressed as µg quercetin equivalent (QE)/ mg of extract.

2.4.3. DPPH scavenging assay

DPPH is a purple-colored stable free radical; it becomes reduced to the yellow-colored, diphenyl picrylhydrazine. According to Cuendet et al. (1997) method with slight mod-ification, a 50 µl of various dilutions of extract or standards were mixed with 1250 µl of a 0.004% methanol solution of DPPH. After an incubation period of 30 min in dark at room temperature, the absorbance of the samples was read at 517 nm. Quercetin was used as standards. Lower absorbance indicated higher free radical-scavenging activity.

2.4.4. β-carotene bleaching assay

Antioxidant capacity is determined by measuring the inhibition of RPE compounds and the conjugated diene hydro-peroxides arising from linoleic acid oxidation (Aslan et al., 2006). A stock solution of β-carotene/linoleic acid mixture was prepared as following: 0.5 mg β-carotene was dissolved in 1 ml of chloroform, and then 25 µl linoleic acid and 200 mg Tween 40 were added in round-bottomed flask. Chloroform was evaporated using a rotavapor. 100 ml of distilled water saturated with oxygen (30 min, 100 ml/min) was added with vigorous shaking to form emulsion. A volume 350 µl of RPE, prepared in methanol at concentration of 2 mg/ml, were added to 2500 µl aliquot of reaction mixture, and the emulsion system was incubated up to 48 h in dark at room temperature. Control samples (2 mg/ml) received only the emulsion without any sample, while blank consisted only of corresponding extract or control. After this incubation period, absorbance of the mixtures was measured at 490 nm after 0 h, 1 h, 2 h, 4 h, 6 h, 12 h, 24 h and 48 h of incubation. The rate of bleaching of β-carotene was calculated as antioxidant activity (AA) using the equation: AA% = Ae. 100/Ac, where Ac: control absorbance (BHT), and Ae: absorbance in the presence of RPE.

2.4.5. Reducing power

The extract ability to reduce Fe^{+3} was assessed (Oyaizu, 1986). A volume of 400 µl of extract was mixed with 400 µl phosphate buffer (0.2 M, pH 6.6) and 400 µl of 1% potassium ferricyanide [$K_3Fe(CN)_6$], then the mixture was incubated at 50 °C for 20 min. About 400 µl

(10%) of trichloroacetic acid (TCA) was added to the mixture and centrifuged for 10 min (3000 r/t). Finally, 400 μl of the supernatant solution was mixed with 400 μl of distilled water and 80 μl FeCl$_3$ (0.1%) and the absorbance was recorded at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The results were expressed as EC 50 which means effective concentration at which the absorbance is 0.5. Ascorbic acid was used as standard.

2.4.6. Ferrous iron chealtion assay

Ferrous iron-chelating activity was measured by inhibition of the formation of Fe^{+2}–ferrozine complex after treatment of test extract with Fe^{+2}, following the method of Decker and Welch (1990) modified by Le et al. (2007). The reaction mixture contained 250 μl extract, 50 μl FeCl$_2$ (0.6 mM in water) and 450 μl methanol. The control contained all the reaction reagents except the extract. The mixture was shaken and allowed at room temperature for 5 min 100 μl of ferrozine (5 mM in methanol) were then added, the mixture shaken again, followed by further reaction at room temperature for 10 min to complex the residual Fe^{+2} ion. The absorbance of the Fe+2-ferrozine complex was measured at 562 nm against a blank contained all the reaction reagents except ferrozine. Lower absorbance indicates a higher chelating power. EDTA was used as reference chelator.

2.4.7. Anti-heamolytic assay

The inhibition of mice erythrocytes hemolysis was assessed according to the method described by Girard et al. (2006) with slight modifications. Mice erythrocytes were isolated by centrifugation at 3000 r/t for 10 min and washed three times with phosphate buffer (10 mM, pH 7.4) until the supernatant became colourless. The erythrocytes were then diluted with phosphate buffer to give 2% (v/v) suspension. Briefly, 80 μl of 2% erythrocytes suspension was added to 20 μl of extract (0.1 mg/ml), the mixed was treated then by 136 μl of AAPH (300 mM). We have controlled the whole blood hemolysis with a 96-well microplate reader device. The kinetics of erythrocytes resistance to hemolysis was determined at 37 °C by continuous monitoring by measuring the rate of decrease at 630 nm. Results were expressed as the time corresponding to 50% of maximal hemolysis (half-hemolysis time, HT$_{50}$ in min). Ascorbic acid was used as standard.

2.5. Acute toxicity study

The RPE was administered in a single dose by gavage, at a dose of 2000 and 5000 mg/kg, to one male and one female under fasting for 4 h. Sequentially, at intervals of 48 h, the same dose was administered to four males and four females, five treated animals in total. In parallel, five males and females were treated with vehicle (distilled water) in order to establish a comparative negative control group (OECD, 2008).

2.5.1. Observation

The animals were periodically observed during the first 24 h after administering the RPE and then once a day for 14 days. Observation was carried out focusing attention on animal response and general physical condition. The observation was also carried out the palpation of the abdomen and the thorax searching for palpable masses. We checked the integrity of the mucous membranes and the skin, as well as hair charac-teristics and possible alterations in feces and urine coloration (Malone and Robichaud, 1962). Body weights were recorded at the beginning, then once after every seven days during the study. At the end of the observation period, all animals were sacrificied.

2.5.2. Plasma preparation and biochemical analysis

Biochemical analysis were investigated at the end of the experiments period. Animals were fasting 4 h and then sacrificed. The blood sample was collected in heparine-tubes and then centrifuged at 3000 rpm for 10 min. Plasma biochemical parametres: glycemia, urea,

creatinine, cholesterol, triglycerides, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed using Beckman coulter Synchron CX 9 PRO) and commercial kits (Spinreact, Spain).

2.5.3. Organs weight

Organs (liver, spleen, kidneys, heart, lungs, brain and stomach) were quickly removed and weighed. The relative organ weight of each animal was then calculated relating the absolute organ weight and body weight of the animal on the day of sacrifice.

2.5.4. Histological analysis

Liver and kidneys were fixed in 10% formol and embedded in parrafin. Sections at 5 μm were stained with hematoxylin and eosin and examined under light microscopy (Martey et al., 2010). The analyses of the different organs (liver, kidneys) were performed using an Optika B-500T i-5 microscope (10 and 40×) in order to verify alterations in the tissues. Digital images were obtained using the Optika camera associated to the microscope, and all the images were analyzed using the image processing software, Optika Vision Pro.

2.6. Subacute orale toxicity and in vivo antioxidant activity

2.6.1. Animals treatment

Siwss albinos male mice of 25–30 g were used. The animals were randomly distributed into four groups of nine to ten animals each with similar average body weight.

Group C: served as a neutral control, received distilled water with 4% DMSO.

Group Vit C: received 100 mg/kg of vitamin C with 4% DMSO.

Group D1: received 250 mg/kg of RPE with 4% DMSO.

Group D2: received 500 mg/kg of RPE with 4% DMSO.

Group D3: received 1000 mg/kg of RPE with 4% DMSO.

Distilled water, vitamin C, RPE (250, 500 et 1000 mg/kg) were given orally once day for 21 days. Weight of all mice was noted before treatment (day 0), on day 7, day 14 and day 21 of treatment (Baghiani et al., 2013).

2.6.2. Collection of blood and plasma

On day 21, blood samples were collected in an EDTA centrifuge tube for oxidative hemolysis inhibition assay. Plasma was separated by centrifugation at 3000 rpm for 10 min and then divided in two aliquot. One plasma aliquot was reserved to analyze the antioxidant activity using DPPH scavenging assay, and the last one was reserved to analyze the biochemical parameters for repeated-dose toxicity.

2.6.3. Oxidative hemolysis inhibition assay

The assay is based on the inhibition of free radical-induced membrane damage in erythrocytes by antioxidants. The advantage of this method is that it uses peroxyl radicals as pro-oxidants and erythrocytes as oxidizable targets so that the results obtained reflect biologically relevant radical-scavenging activity and micro-localization of antioxidants.

The anti-hemolysis activity of treated mice whole blood was evaluating using the same *in vitro* protocol with slight modification. Briefly, 136 μl of AAPH (300 mM) was added to 80 μl of mice blood in 96-well microplate. We have controlled the whole blood hemolysis with a 96-well microplate reader device. The kinetics of erythrocytes resistance to hemolysis was determined at 37 °C by continuous monitoring of changes in absorbance at 630 nm. Results were expressed, for the time corresponding to 50% of maximal hemolysis (half-hemolysis time, HT$_{50}$ in min).

2.6.4. DPPH radical-scavenging activity of plasma

Plasma ability to scavenge DPPH radical was measured with the same *in vitro* method. An aliquot of 25 μl of plasma was added to 625 μl of DPPH solution (0.004%), and the reaction mixture was

Table 1
Antioxidant activities of RPE.

Extract/Standard	HT 50 (min)	AA%	EC50 (µg/ml)		
			DPPH scavenging effect	Chelating effect	Reducing power effect
RPE	108.03 ± 2.65[***]	73.88 ± 2.05[***]	38.26 ± 0.00[***]	668.42 ± 0.00[***]	233.05 ± 0.00[***]
Ascorbic acid	111.10 ± 1.51[***]	–	–	–	48.96 ± 1.50
Control	76.92 ± 3.59	–	–	–	–
Quercetin	–	98.79 ± 0.38	2.57 ± 0.00	–	–
EDTA	–	–	–	6.12 ± 0.05	–

[***] $p < 0.001$, the comparison was realized against correspondent standards.

shaken vigorously. After incubation at room temperature for 30 min, the absorbance of this solution was determined at 517 nm.

2.6.5. Histological analysis and preparation of tissue homogenates

Each animal liver and kidneys was divided in two pieces: one piece was fixed in 10% formol for histological analysis and the last one was immediately stored at −20 °C until further antioxidant analyses.

Pieces of liver and kidneys fixed in 10% formol were embedded in parrafin. Sections at 5 µm were stained with hematoxylin and eosin and examined under light microscopy (Martey et al., 2010). The analyses of the different organs (liver, kidneys) were performed using an Optika B-500T i-5 microscope (10 and 40×) in order to verify alterations in the tissues. Digital images were obtained using the Optika camera associated to the microscope, and all the images were analyzed using the image processing software, Optika Vision Pro.

After a swift thawing, pieces of livers and kidneys were weighed and homogenized in ice cold KCl (1.15%) to prepare 10% (w/v) homogenate, using electric homogenizer. Then, the homogenate was centrifuged twice at 4000 rpm/min for 10 min at 4 °C to purge cellular debris and supernatant was collected and stored at −20 °C until quantification of antioxidant enzyme (CAT), GSH and MDA. The protein concentration in the supernatant was measured using commercial Kit.

2.6.6. Estimation of CAT activity

Catalase enzyme catalyzes H_2O_2 into H_2O and O_2. At 240 nm, absorption of hydrogen peroxide can be measured. Absorbance value of H_2O_2 decreases when it is decomposed by CAT. Thus, CAT activity can be calculated by measuring decrease in absorption of H_2O_2. Estimation of CAT activity was carried out according to Claiborne (1986). The activity of CAT was calculated by taking the extinction coefficient of H_2O_2 to be 43.6 M^{-1} cm^{-1} at 240 nm and expressed as µmole/min/mg protein.

2.6.7. Estimation of GSH

The GSH levels were determined in the homogenates of liver and kidneys using Ellman's reagent (5,5 0- dithiobis-(2-nitrobenzoic acid), DTNB) (Ellman, 1959). A volume of 5 ml of sodium phosphate buffer (0.1 M, pH = 8) was added to 25 µl of supernatant homogenate, then 1.5 ml of mixture was added to 10 µl of DTNB (0.01 M). Determination of GSH is based on the reaction of DTNB with GSH and yield a yellow colored chromophore with a maximum absorbance at 412 nm. The amount of GSH present in the tissue was calculated was calculated using its extinction coefficient to be 1.36×10^4 M^{-1} cm^{-1} at 412 nm and expressed as µmoles /g tissue.

2.6.8. Estimation of MDA

The hepatic and renal malondialdehyde (MDA) content was an indicator to determine hepatic and renal lipid peroxidation levels. According to Okhawa et al. (1979), 125 µl of trichloric acid (TCA, 20%) and 250 µl of thiobarbituric acid (TBA, 0.67%) were added to 125 µl of tissue homogenate. The mixture was incubated at 100 C° during 20 min. After incubation, the sample was cooled by cold water, and

MDA-TBA complex was extracted with 1 ml of butanol. The organic phase was separated by centrifugation at 3000 rpm for 15 min and then measured at 530 nm. The concentration of MDA was calculated using its extinction coefficient to be 1.56×10^5 M^{-1} cm^{-1} at 530 nm and expressed as nanomoles MDA/g tissue.

2.7. Statistic analysis

Results were expressed as mean ± standard deviation. The differences between groups were determined by analysis of variance (one-way ANOVA) followed by Dunnett's test. Statistical analysis for *in vitro* results was undertaken using student test. All results were analyzed using GraphPad Prism version 5.00. Differences were considered significant at $p < 0.05$.

3. Results

3.1. In vitro antioxidant activity

The extraction yields calculated showed that crude extract registered a yield of 19.87 ± 6.75%. The content of phenolics compounds and flavonoids was 81.21 ± 0.51 (µg GAE/mg extract) and 12.76 ± 0.10 µg QE/mg extract, respectively. These amounts were equivalent of 1.59 g/100 g of dry weight (DW) and 0.25 g / 100 g DW, respectively.

A significant decrease in the amount of DPPH radical was observed due to the scavenging ability of RPE and standard. The RPE exhibited a higher scavenging effect (IC50 = 38.26 ± 0.00 µg/ml) that remains lower than that of standard (quercetin) with an IC 50 of 2.57 ± 0.00 µg/ml (Table 1). Otherwise, RPE and standard scavenge DPPH radical in dose dependent manner.

As for β-carotene – linoleic acid assay, the addition of the RPE and BHT at 2 mg/ml prevented the bleaching of β-carotene to different degrees. As Table 1 indicates, RPE showed a marked antioxidant activity (73.88 ± 2.05%) when compared to the synthetic antioxidant BHT which had 98.79 ± 0.38% at the same concentration (2 mg/ml).

In reducing power assay, the reduction of ferric cyanide complex to the ferrous form by donating an electron indicates the presence of reductants in the extract. It was observed that the reducing capability of RPE and ascorbic acid increased dependently with increasing concentration. The RPE (EC 50 = 233.05 ± 0.00 µg/ml) found to have a considerable reducing power; however, it remained significantly ($p < 0.05$) lower compared to ascorbic acid (EC 50 = 48.96 ± 1.50 µg/ ml) as shown in Table 1.

Ferrous ion chelating assay aims to determine the capacity of crude extract to bind to ferrous ion catalyzing oxidation. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of color reduction therefore allows estimation of the chelating activity of the coexisting chelator. The obtained data revealed that the ferrous ion chelating effect of RPE and EDTA was concentration dependent; suggesting the chelating ability of ferrous ion was increased when increasing concentration. From the EC 50 (Table 1), the potency of ferrous iron chelating

ability of RPE (EC 50 = 668.42 ± 0.00 µg/ml) was very lower than that of EDTA (EC 50 = 6.12 ± 0.00 µg/ml).

Hemolytic activity of RPE was screened against normal mice erythrocytes. When the erythrocytes were incubated with RPE (without AAPH), hemolysis was maintained at a background level similar to that in the control samples (AAPH untreated samples) (data not shown). However, the incubation of erythrocyte together with AAPH led to remarkable hemolysis. As in Table 1, the RPE increase significantly the half time of erythrocytes hemolysis (HT50 = 108.03 ± 2.65 min) compared to the control (HT50 = 76.92 ± 3.59 min). Otherwise, it was nearly equal to that of ascorbic acid (HT50 = 111.01 ± 1.51 min).

3.2. Acute toxicity

3.2.1. Bihavioral observations and mortality patterns

The present study conducted according to OECD guideline 425 revealed that the RPE did not produce any behavioral changes in both males and females during the study period. Furthermore, any mortality was recorded throughout two weeks and,thus, the LD_{50} is higher than 5000 mg/kg BW for both sexes.

3.2.2. Body weight evolution

Every case of body weight loss was recorded, and the body weight increased gradually throughout the study period. Fig. 1 shows that the weight curve similar to one reported by other animals (males and females) breeding institutions. Statistical analysis of body weight gain did not reveal any significant differences between treated and control groups.

3.2.3. Relative organ weights

The oral administration of RPE caused no significant changes in relative weight of the organs (liver, spleen, kidneys, heart, lungs, brain and stomach) in the treated ones compared to the control mice of both sexes (Table 2).

3.2.4. Biochemical parameters

Statistical analysis of evaluated parameters indicate that there were no significant differences between treated males and the control group. However, a significant ($p < 0.05$) decrease in dose dependent regarding creatinine and triglyceride was recorded in treated females. Furthermore, a significant decrease, but not dose dependently, in ALT and urea was also noted in treated females (Fig. 2).

3.2.5. Liver and kidneys histopathology

The observation of histological sections of the liver and kidneys of treated mice showed a preservation of the cellular architecture (lobular and tubular) of the two organs compared with controls. However, some particularities have been considered. For males, a vascular congestion, inflammatory infiltrated and inflated hepatocytes were recorded in some hepatic tissues of males treated with 2000 mg/kg. At dose of

Table 2

Organs relative weight of mice treated with RPE and control group.

Groups	Control group	2000 mg/kg BW	5000 mg/kg BW
Males			
Liver	68.17 ± 13.20	67.83 ± 7.85[ns]	66.22 ± 9.68[ns]
Kidneys	14.53 ± 1.08	15.28 ± 1.82[ns]	14.61 ± 1.60[ns]
Spleen	8.93 ± 1.52	9.84 ± 2.72[ns]	8.71 ± 4.58[ns]
Heart	5.28 ± 0.75	5.51 ± 1.12[ns]	5.34 ± 0.54[ns]
Lungs	9.51 ± 1.64	13.20 ± 4.77[ns]	10.92 ± 3.71[ns]
Brain	11.79 ± 1.20	10.99 ± 3.22[ns]	12.00 ± 2.75[ns]
Stomach	13.44 ± 2.87	10.98 ± 2.32[ns]	11.34 ± 1.18[ns]
Females			
Liver	62.92 ± 9.58	65.13 ± 4.94[ns]	64.64 ± 10.23[ns]
Kidneys	10.44 ± 1.00	11.98 ± 0.88[ns]	11.17 ± 1.47[ns]
Spleen	6.16 ± 1.64	6.30 ± 1.15[ns]	5.72 ± 1.39[ns]
Heart	4.04 ± 0.60	4.72 ± 0.54[ns]	5.09 ± 0.77[ns]
Lungs	9.73 ± 0.71	9.15 ± 4.03[ns]	10.64 ± 3.54[ns]
Brain	11.98 ± 1.97	11.33 ± 1.12[ns]	11.00 ± 1.90[ns]
Stomach	10.21 ± 1.88	10.86 ± 1.79[ns]	10.78 ± 0.51[ns]

Values expressed as mean ± SD, n=5 animals /group. ns: non significant differences.

5000 mg/kg, some hepatic tissues showed an important congestion and a polymorphe inflammatory infiltrated (Fig. 3). In general, kidneys' histological section did not show any structure modifications compared to control except the presence of glomerular and medullar congestion in some tissues of both 2000 and 5000 mg/kg treated males (Fig. 3).

For females, a moderate congestion was recorded in some liver tissues of males treated with 2000 mg/kg. Furthermore, at dose of 5000 mg/kg, a medullar and cortical congestion with absence of nephrite were showed in some kidneys tissues.

3.3. Subacute orale toxicity and in vivo antioxidant activity

3.3.1. Bihavioral observations and mortality patterns

In the subacute toxicity tests, administration of 250, 500, and 1000 mg/kg doses of RPE to three groups of mice did not show any behavioral changes, visual symptoms of toxicity or mortality in animals during the entire 21-days observation period.

3.3.2. Body weight

During the two first weeks, a non significant weight loss was noted in treated group with RPE at dose 250 mg/kg. Moreover, a weight loss was equally noted during the second week both in the treated groups with RPE (500 and 1000 mg/kg) and control group. However, an improvement was noted in the last week in all groups except control group (Fig. 4).

3.3.3. Relative organ weights

The repeated oral administration of Vit C (100 mg/kg) and RPE (250,500 and 1000 mg/kg) caused no significant changes in relative
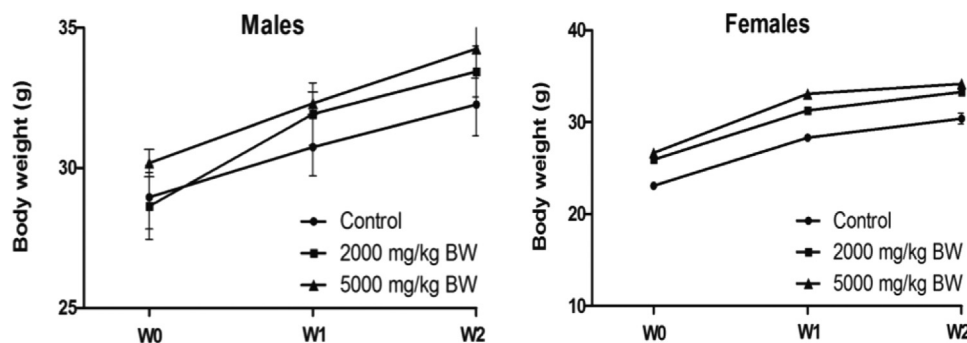

Fig. 1. Body weight of mice treated orally with RPE. Values expressed as mean ± SD, n= 5 animals /group.
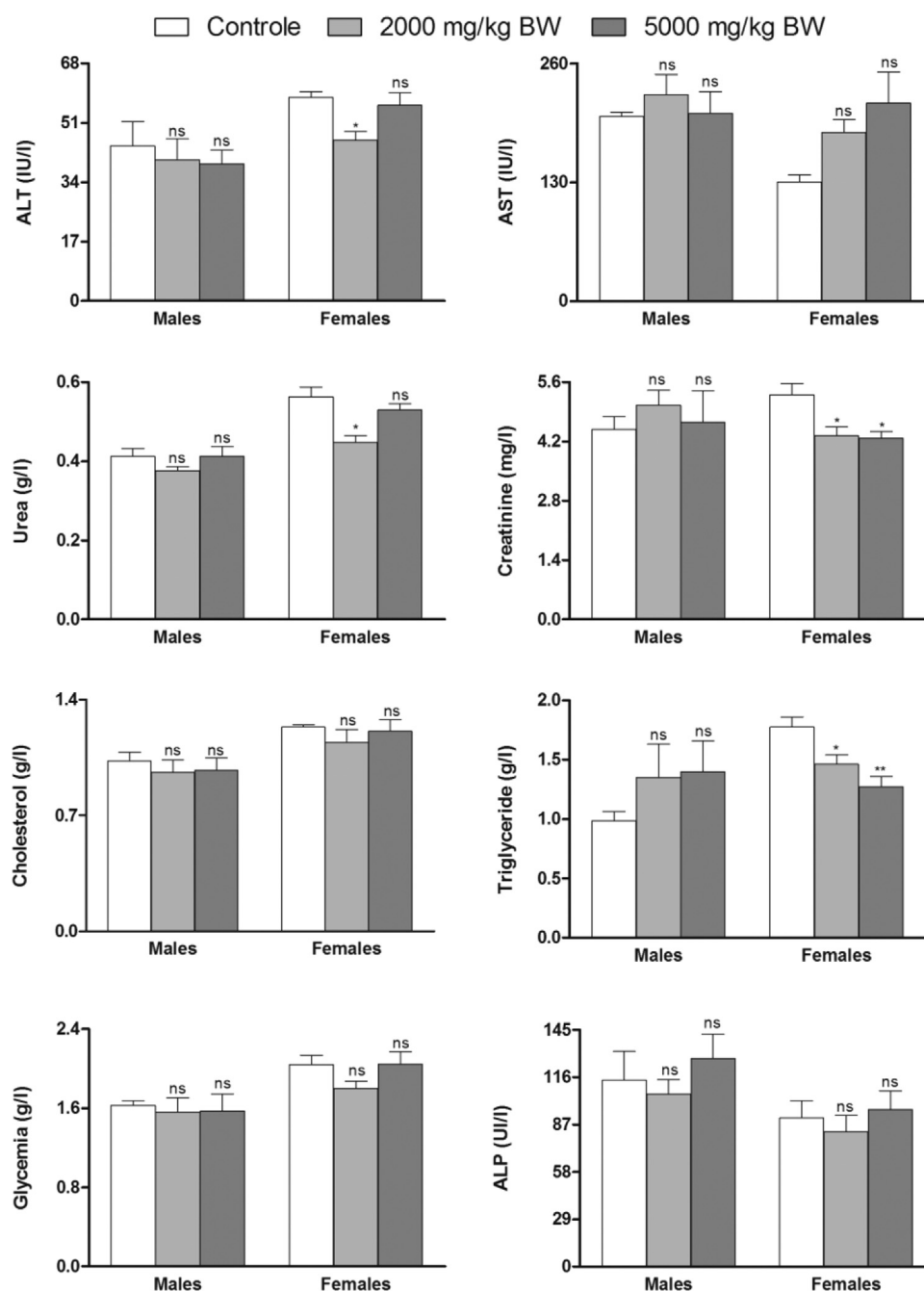
Fig. 2. Biochemical parameters of control and mice treated with RPE measured during the acute toxicity. Values were expressed as mean ± SEM (n = 5). ns: no significant, *: $p < 0.05$, **: $p < 0.01$.

weight of the organs (kidneys, spleen, heart, lungs, brain and stomach) in the treated mice compared to the control mice. However, a significant increase liver relative weight was observed all RPE doses treated mice (Table 3).

3.3.4. Biochemical parameters

Results showed that the statistical analysis evaluated biochemical parameters indicated that there were no significant differences between the treated mice and the control group. However, a significant ($p < 0.05$) decrease in dose non-dependent regarding urea was recorded in treated mice with RPE (250 and 1000 mg/kg) and Vit C (100 mg/ml) (Fig. 5).

3.3.5. Liver and kidneys histopathology

Histopathological evaluation of the vital organs; liver and kidney

(Fig. 6) displayed no relevant macroscopic or histological changes in animals that received Vit C and RPE (250 mg/kg). The liver showed normal architecture and no evidence of lesion; the kidney showed adequate glomeruli and normal tubules. However, histological cutting of liver of mice treated with RPE (500 and 1000 mg/kg) showed in a dose dependent vascular congestions and hepatocyte lysis for the higher dose (1000 mg/kg). Vascular congestion and inflammatory zones with mononuclear cell infiltrates were also observed on kidney sections at 500 and 1000 mg/kg.

3.3.6. Oxidative hemolysis inhibition assay

The effect of RPE and Vit C oral administration on mice erythrocytes was investigated. It appeared from the obtained hemolysis sigmoid curves that the treatment with RPE and Vit C lead to hemolysis delay which is evident as displacement of the curves to the right. Even
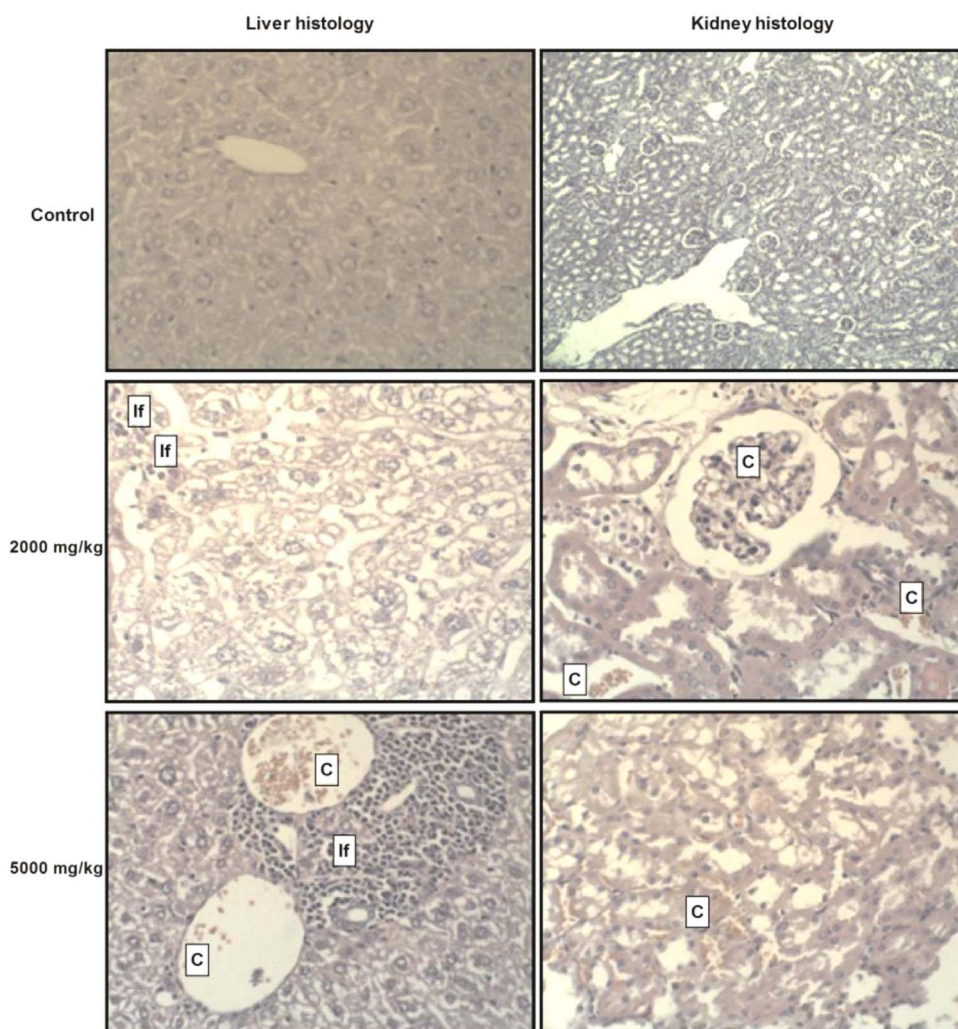
Fig. 3. Histopathological analysis of males' liver and kidney treated with RPE in acute toxicity (x 40). C: congestion, If: inflammatory infiltrate.
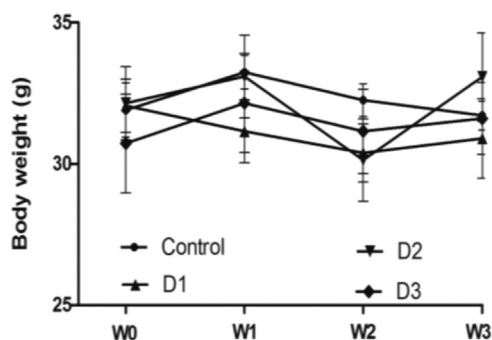

Fig. 4. Body weight of mice treated orally with RPE. Values expressed as mean ± SD, n= 7 animals/group.

though not significant compared to control group (HT 50 = 96.58 ± 7.25 min), the HT50 values revealed an extension of half time of hemolysis in RPE: 250, 500 and 1000 mg/kg (110.91 ± 5.23, 111.64 ± 5.24 and 104. 11 ± 3. 13 min, respectively) and Vit C: 100 mg/kg (HT50 = 113.40 ± 5.90) treated group.

3.3.7. DPPH radical-scavenging activity of plasma

Evaluated by DPPH scavenging assay, it appeared that the plasma scavenging ability was not significantly improved following the administration of RPE and Vit C. The results showed that the group of mice treated with RPE: 250,500 and 1000 mg/kg (scavenging % = 32.71 ±

1.86, 41.30 ± 10.32 and 20.00 ± 1.95%, respectively) and that of Vit C (scavenging % = 30.05 ± 5.96%) were not significantly different compared to the control group (29.24 ± 1.54%).

3.3.8. Estimation of CAT activity, GSH and MDA

Changes in the activity of CAT, content of GSH and MDA in both livers and kidneys of mice were investigated.

The CAT activity in both livers and kidneys homogenates has not undergone any significant changes during treatment with RPE (250, 500 and 1000 mg/kg). Also, no change was observed in positive control group (Table 4).

Following the analysis of this table, we notice that the administration RPE (250, 500 and 1000 mg/kg) caused a significant increase in GSH level in livers (48.59%, 71.48% and 60.03%) and kidneys (26. 02%, 47.81% and 45.51%, respectively). However, Vit C (100 mg/kg) produced a significant increase only in kidneys GSH (46.36%).

According to the results (Table 4), a significant decrease of MDA level was observed in livers of mice treated with Vit C (100 mg/kg) and RPE (250 mg/ml). Even though not significant, the administration of RPE (500 and 1000 mg/kg) decrease the MDA level. In terms of kidneys MDA level, both 250 and 1000 mg/kg administration caused statistical significant decrease.

4. Discussion

The lack of scientific data about the effectiveness and safety of

Table 3
Organs relative weight of mice treated with RPE and control group.

Groups	Control group	Vit C 100 mg/kg BW	RPE 250 mg/kg BW	RPE 500 mg/kg BW	RPE 1000 mg/kg BW
Liver	53.90 ± 3.69	59.40 ± 5.58^{ns}	$68.40 \pm 8.67^{**}$	$62.85 \pm 4.73^{*}$	$65.07 \pm 6.03^{*}$
Kidneys	13.46 ± 1.00	13.67 ± 2.30^{ns}	15.60 ± 1.12^{ns}	12.80 ± 1.70^{ns}	13.92 ± 2.14^{ns}
Spleen	03.70 ± 0.46	05.40 ± 1.04^{ns}	$06.06 \pm 1.62^{*}$	05.19 ± 1.55^{ns}	04.00 ± 0.58^{ns}
Heart	04.80 ± 0.57	04.85 ± 0.33^{ns}	05.43 ± 0.70^{ns}	04.99 ± 0.89^{ns}	04.58 ± 0.46^{ns}
Lungs	06.47 ± 1.00	06.96 ± 1.27^{ns}	07.18 ± 0.43^{ns}	06.65 ± 1.78^{ns}	06.31 ± 1.54^{ns}
Brain	10.96 ± 1.19	12.73 ± 2.70^{ns}	12.35 ± 1.50^{ns}	12.07 ± 2.74^{ns}	11.52 ± 2.37^{ns}
Stomach	07.52 ± 1.75	09.10 ± 1.22^{ns}	09.98 ± 1.42^{ns}	08.59 ± 2.09^{ns}	08.46 ± 1.19^{ns}

Vales expressed as mean $\pm$ SD ($n=6–7$). ns: non significant, $*$: $p < 0.05$, $**$: $p < 0.01$.

medicinal plant is one of the major problems in the use of traditional medicinal preparations. This is probably due to the non-evaluation of mechanism action and toxicological profile of these plants. The present research has provided scientific information on the acute and subacute toxicity of RPE on mice and also on their *in vitro* and *in vivo* antioxidant activity.

The yield of extraction was significantly higher than the one found by Recio et al. (1992). It may be due to the extraction method used. Polyphenols are the most abundant secondary metabolite. They have been associated with color, plant growth, nutritional, and antioxidant properties (Negrào and Faria, 2009).

Since different antioxidant compounds may act through different mechanisms, no single method can fully evaluate the total antioxidant capacity. For this reason, studying the complex antioxidant activities often uses a multiple methods (Huang et al., 2005). Of these, DPPH scavenging, β- carotene bleaching, ferrous ion chelating, reducing power and anti-hemolytic assays are most commonly used for the evaluation of the antioxidant activities of extracts.

Results showed that RPE exhibited a strong DPPH scavenging effect that could be attributed to the presence of polyphenols. Hence, RPE can serve as free radical scavenger.

β-carotene bleaching assay is based on the discoloration of β-carotene owing to its reaction with linoleic acid-generated free radicals in an emulsion system. In the presence of an antioxidant compound,
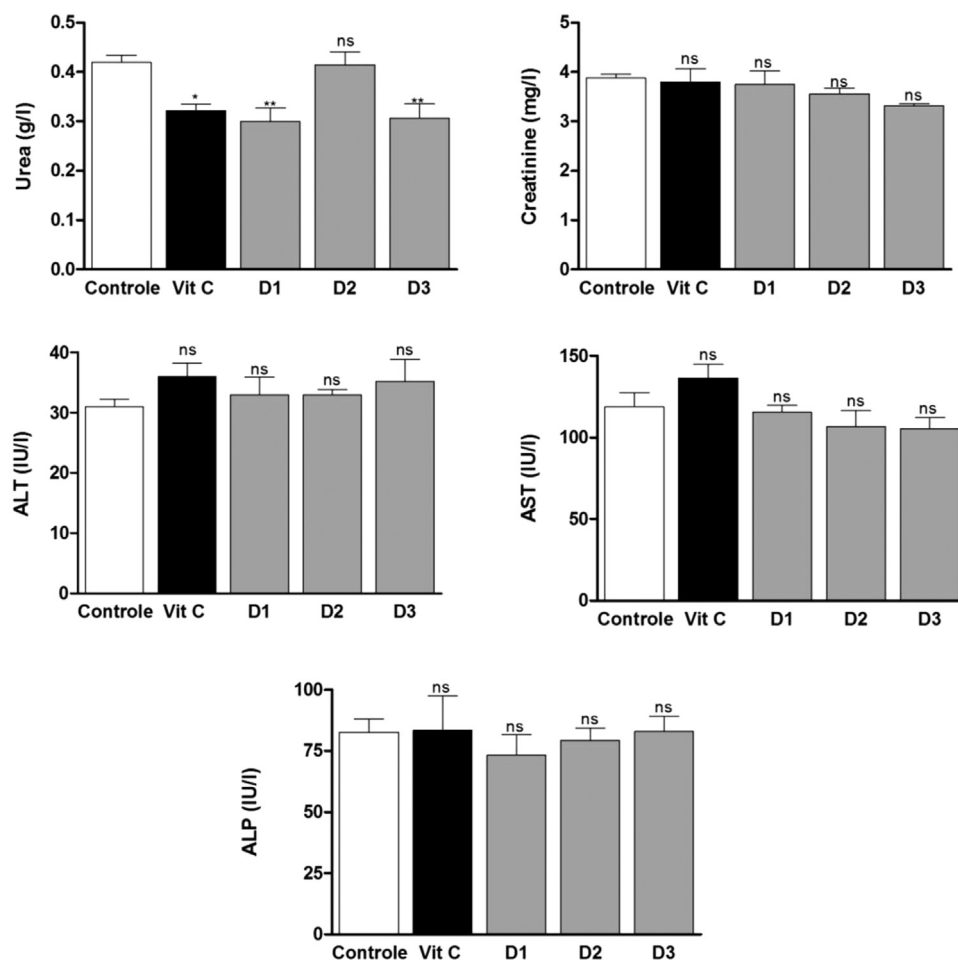

Fig. 5. Biochemical parameters of control and mice treated with RPE and Vit C measured during the acute toxicity. Values were expressed as mean $\pm$ SEM (n = 6–7). ns: no significant, $*$: $p < 0.05$, $**$: $p < 0.01$.
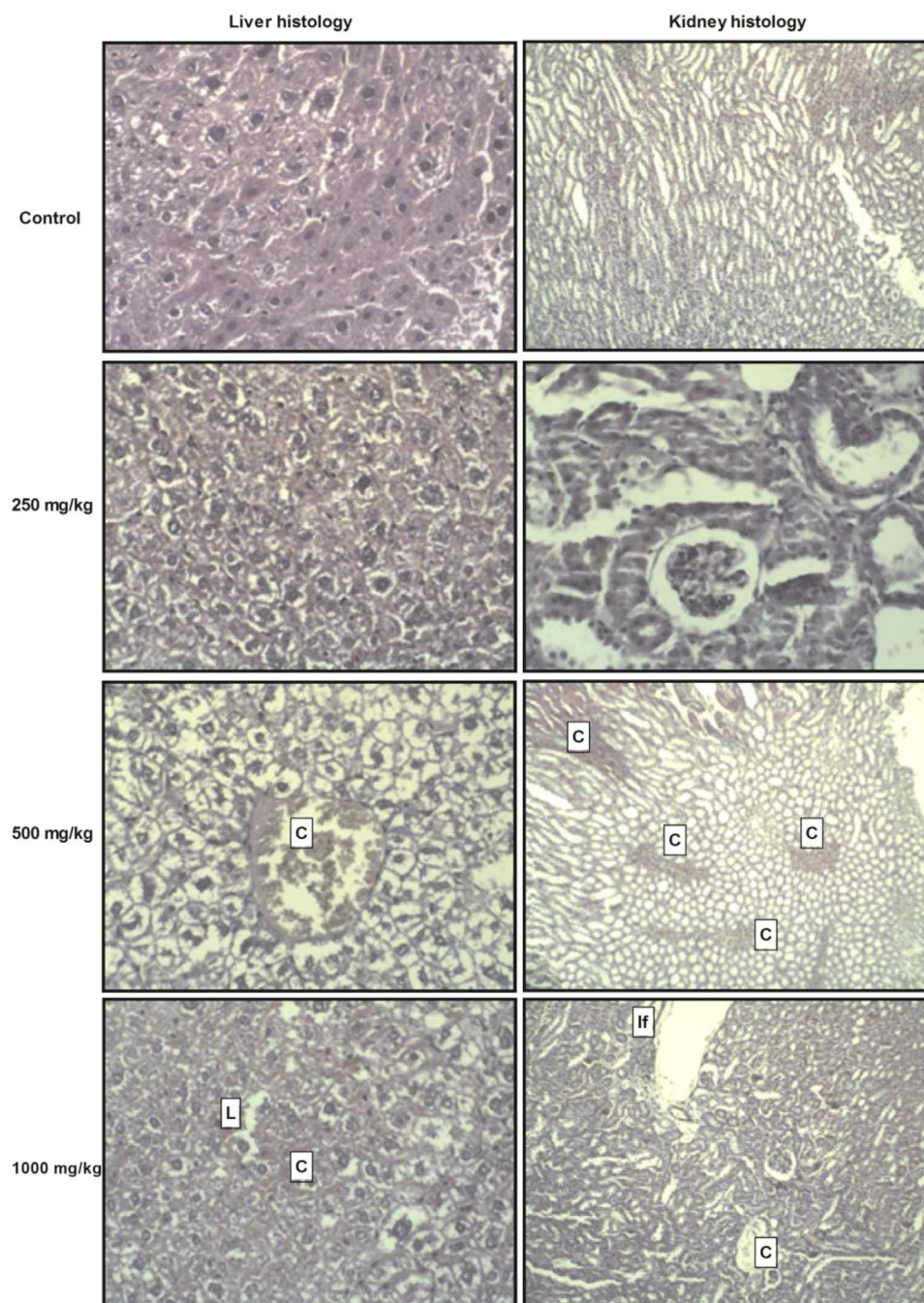
Fig. 6. Histopathological analysis of mice' liver and kidney treated with RPE in sub-acute toxicity (x40). C: congestion, If: inflammatory infiltrate.

Table 4
Estimation of CAT activity (μmole/min/mg of protein), GSH (nmole/g of tissue) and MDA (nmole/g of tissue) in liver and kidney of mice.

Groups		Control group	Vit C 100 mg/kg BW	RPE 250 mg/kg BW	RPE 500 mg/kg BW	RPE 1000 mg/kg BW
Liver	CAT	31.55 ± 4.55	32.84 ± 2.71	32.03 ± 4.28	33.85 ± 6.28	34.26 ± 1.90
	GSH	56.14 ± 2.26	58.06 ± 2.35	83.42 ± 2.48[a,b]	96.27 ± 4.91[a,b]	89.84 ± 1.65[a,b]
	MDA	235.48 ± 5.05	153.55 ± 22.87[c]	178.04 ± 7.66[c]	186.78 ± 14.04	200.23 ± 3.45
Kidney	CAT	29.70 ± 3.14	30.11 ± 3.38	31.90 ± 2.65	32.59 ± 1.90	32.28 ± 3.34
	GSH	55.51 ± 2.61	81.25 ± 2.21[a]	69.96 ± 2.40[a]	82.05 ± 5.61[a]	80.78 ± 2.50[a]
	MDA	117.46 ± 11.44	83.4 ± 2.67[c]	83.38 ± 7.88[c]	95.81 ± 5.94	80.03 ± 4.35[c]

Values were expressed as means ± SEM (n=6) and evaluated by one-way ANOVA.

[a] $p < 0.0001$, compared with control group.
[b] $p < 0.0001$, compared with group treated with Vit C.
[c] $p < 0.01$, compared with control group.

this degradation process is prevented. It also reflects the ability to inhibit the lipid peroxidation *in vitro* (Baghiani et al., 2011). The RPE found to hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system.

The reducing power of the extract may serve as a significant indicator of its potential antioxidant activity. The presence of antioxidant in the extract might cause the reduction of Fe^{3+}/ferric cyanide complex to ferrous form. The RPE showed a considerable reducing power indicating that it can act as electron donors and could react with free radicals to convert them into more stable products and then terminate the free radical chain reactions.

The metal chelating capacity is important since it reduces transition metal acting as catalysts to generate the first few radicals and initiate the radical mediated oxidative chain reactions in biological or food systems. Ion chelating agents also may inhibit the Fenton reaction and lipid peroxidation (Liu et al., 2010). Thus, an antioxidant's ability to chelate metals is an important antioxidant property to measure. The obtained results showed that the RPE had a very weak chelating effect.

Free radicals attack erythrocyte membrane components, and cause the alteration of membrane structure and function, which may result in hemolysis. The AAPH was used to imitate the *in vivo* condition of oxidative stress. It mediates oxidation of lipid in blood erythrocytes membrane induces membrane damage and subsequently leads to hemolysis. The results demonstrated that the peroxyl radicals were initiated by AAPH-induced hemolysis in time-dependent manner. In the present study, the incubation of erythrocyte together with AAPH led to remarkable hemolysis that was agree with previous findings (Phrueksanan et al., 2014). In addition, the results from the AAPH-induced hemolysis of mice erythrocyte are similar to hemolysis of human erythrocytes (Wang et al., 2009). The RPE could inhibit the AAPH-induced red blood cell lysis. The exact mechanisms of RPE for the prevention of erythrocyte hemolysis have yet to be determined. We can suggest that protective effect of RPE against hemolysis may return due to its scavenging activity against free radicals.

Several reports demonstrated that polyphenols including flavonoids have been shown to possess a significant antioxidant activity in various *in vitro* models (Baghiani et al., 2011, 2012; Boumerfeg et al., 2012). Thus also, the pronounced antioxidant activity of the RPE, manifested as inhibition of lipid peroxidation, protection of against erythrocyte hemolysis, scavenging of free radical, was possibly due to its high phenolic content. Although the RPE showed a beneficial effect for the other evaluated activities, this effect remained lower than the one registered by the used standards.

The evidence of RPE *in vitro* antioxidant ability solely were not enough to demonstrate its antioxidant effects in biological systems, on one hand. *In vivo* antioxidant assays, on the other hand, could reflect the related biological implications of dietary consumption, including effect on antioxidant enzymes, oxidation-related metabolic pathways, and activation or repression of gene expression of antioxidant compounds and enzymes.

In acute toxicity experiment, a single oral administration of the RPE at the doses of 2000 and 5000 mg/kg caused neither treatment-related behavior changes nor mortality of mice for both sexes during 14 days of study. Therefore, this suggests that the LD50 is greater than 5000 mg/kg according to the OECD guideline, thus the extract belongs to category 5 of the Global Harmonisation System of Chemical Substances considered as fairly toxic substance.

Some enzymes (such as ALT, AST, ALP) can be used as sensitive indicative of hepatocellular effects (Brandt et al., 2009). In the current study, the enzymes (AST and ALP) did not present any significant variation both in males and females when compared to their respective controls. Moreover, ALT in females treated with 2000 mg/kg BW showed a significant ($p < 0.05$) variation compared to the control group. When compared with control group, a relatively low ALT value for the experiment groups (males and females) was recorded. Consequently, the RPE did not alter ALT, AST and ALP activity of

the treated. This is an indication of non-hepatotoxic potential of RPE. The results of the liver histopathology however indicated some element of the extract's negative effect. The presence of vascular congestion in both males and females treated with 2000 and 5000 mg/kg BW was recorded. Such congestion could be due to the vasoconstriction action of RPE on the wall of blood vessels (Ashley, 2004). The leucocyte infiltration presented in liver of both males and females at dose 2000 and 5000 mg/kg BW could be the result of the liver inflammation. Therefore, further research to consider the possible hepatoprotective or hepatotoxic effect of the RPE is recommended.

Creatinine and urea is considered as biomarkers of renal insufficiency (Lameire et al., 2005). A significant ($p < 0.05$) dose-dependent decrease in creatinine occurred in females, which may indicate muscle wastage (Perrone et al., 1992) but is not consistent with the body weights data since there was weight gain trends in all groups. Otherwise, females treated with 2000 mg/kg recorded a significant decrease in blood urea level. However, this last also depends on extra-renal factors as high proteins food or increased protein catabolism. However, histological analysis revealed the presence of glomular and medullar congestion in males and females for the two doses. From these results, a long term intake of extract should be undertaken to evaluate its toxicity towards kidneys.

We found that the level of triglycerides was significantly decreased in females treated with 2000 mg/kg BW, and this may indicate that the extract stimulates oxidation of triglycerides to fatty acids by lipoprotein lipase (Solera, 2000).

In order to find out any possible effects of RPE on normal metabolic conditions, normal mice were used. The treatment was performed orally for 21 successive days in order to observe the subacute effects and the *in vivo* antioxidant activity of RPE.

Since the weight loss was noted also in control group we cannot conclude that the intake of RPE was responsible for this effect. Therefore, further research to consider the possible weight loss effect of the RPE is recommended. The relative weights of the vital organs like kidneys, spleen, heart, lungs, brain and stomach were found normal indicating no toxic effect in both control and treated groups and there were statistically non-significant differences ($p > 0.05$). Surprisingly, a significant increase in relative weight of liver was reported in RPE treated groups (250, 500, and 1000 mg/kg). Extract is metabolized by the liver and the increase in its weight may be due to response to compensate for increase in demand of its metabolism (Sharma et al., 2014).

Interestingly, none of the studied biochemical parameters showed any evidence of adverse effects in all doses treated groups of mice.

Another important finding of this study is that of the histological assessments of liver and kidneys of mice receiving 500 and 1000 mg/kg dose of RPE for 21 days. The presence of vascular congestions on both liver and kidney sections could be due to the vasoconstriction action of RPE on the walls of blood vessels (Ashley, 2004). Therefore, this finding suggests that administration of the extract at 500 and 1000 mg/kg doses for three weeks duration induces damage to the liver and kidneys of treated animals.

For the purpose of evaluating *in vivo* antioxidant potential, we measured the DPPH scavenging and anti-hemolysis ability as well as the CAT activity, GSH and MDA levels in liver and kidney homogenate.

The results indicate that the RPE did not improve the anti-hemolytic and free radical scavenging activities, in contrast to the importance of these activities of the RPE determined *in vitro*. Or, the anti-hemolytic and free radical scavenging activities have been attributed to their polyphenolic and flavonoid contents. Thus, the main reason behind these results lies on the bioavailability of active compounds, as well as their metabolism in the body. In fact, the absorption of polyphenols in organism is rather low. Another factor is the short half-life of polyphenols in plasma, which are usually in the range of a couple of hours. In addition to poor absorption, polyphenols and especially flavonoids are extensively metabolized in liver and

intestine when ingested (Lotito and Frei, 2006).

In our study, Changes in the activity of CAT were investigated since it was one of the first lines of defense against ROS in the organism by detoxifies hydrogen peroxide into water. No statistically significant changes were observed (p > 0.05) in the activities of this enzyme of both liver and kidney homogenates, comparing to the control group. Also, no changes were observed in the positive control group. This consequence was somehow expected since the consumption of the studied extract did not induce the synthesis of this enzyme in normal mice. Otherwise, this would be an intervention of the body homeostasis. These results are in accordance with the study conducted by Celep et al. (2013) investigating the antioxidant capacity of chery leaves in rats after 21 days of oral administration. They reported that no changes in the activity of CAT were observed in the liver homogenates of normal rats.

Glutathione is the most abundant intracellular thiol-based antioxidant (present in millimolar concentrations), and it serves as a significant first lines of defense against oxidative stress, and it plays an important role in maintaining the integrity of cells (Das and Vasudevan, 2005). Reduced glutathione (GSH) and its elated enzymes play key roles in protecting the cell against the effects of ROS (Nordberg and Arner, 2001). GSH is the most prevalent low-molecular weight antioxidant within cells. The administration of RPE increased significantly the level of GSH, which was possibly due to an improvement in the glutathione status which could be brought about by the constituents of RPE. We have reported that the RPE contains the antioxidant constituent s: total polyphenols amount (81.21 mg/g) and flavonoids (12.76 mg/g). These phytochemicals could have induced the phase antioxidant enzymes and thus increased the biosynthesis of cellular antioxidants, especially glutathione (Dinkova-Kostova et al., 2000).

Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in cell membranes that generates a number of degradation products, resulting in oxidative stress (Najeeb et al., 2012). Hence, the measurement of lipid peroxidation is an important indicator in the assessment of antioxidant potential. MDA, which is an end product of lipid peroxidation, is also one of the most frequently used biomarkers to evaluate the antioxidant activity *in vivo* (Rio et al., 2005). It was stated that decrease in the levels of lipid peroxidation could be related to the ability of RPE to scavenge ROS, thus preventing further damage to membrane lipids (Pradeep et al., 2007). It should be reminded that RPE also showed strong activity against free radical and a remarkable reducing power according to our *in vitro* assay results. Another important point about lipid peroxidation that should be highlighted is the correlation between β-carotene bleaching assay and MDA levels. β-carotene bleaching assay is, as mentioned previously, used as a screening tool for the inhibition of lipid peroxidation in *vitro*. We also noticed that RPE showed good activity in β-carotene bleaching assay, and it induced decrease in the levels of MDA in normal mice.

Notably, RPE exhibited similar or strong GSH level and similar or higher lipid peroxidation inhibition effect than Vit C *in vivo*, probably due to the differences in the affecting factors of antioxidant activities between *in vitro* and *in vivo* assays.

To conclude, to the best of our knowledge, this is the first study investigating the acute and sub-acute oral toxicity and also the *in vitro* and *in vivo* antioxidant activity of methanolic *Reichardia picroide* extract. This study provides valuable data on the acute and sub-acute oral toxicity profiles of *Reichardia picroide* that should be very useful for any future *in vivo* and clinical study of this plant medicine. Results allow us to conclude that the RPE is fairly toxic; with oral LD50 higher than 5000 mg/kg BW on male and female mice. Moreover, daily administration RPE extract at doses ranged from 500 to 1000 mg/kg could result in alteration of the histology of liver and kidney. However, the use of extract of *Reichardia picroide* is safe at doses ≤ 250 mg/kg.

On the basis of obtained results in terms of antioxidant activity, it is concluded that RPE possessed potential antioxidant activity.

Reichardia picroide extract effectively protected AAPH-induced hemolysis and oxidative damage oxidation in erythrocytes and showed a strong DPPH scavenging activity, a benificial lipid peroxidation inhibition effect and reducing power and a weak Fe^{+2} chelating effect. Furthermore, antioxidant assays *in vivo* demonstrated that RPE administration significantly increased GSH level and decreased MDA level in liver and kidney. These results suggested that *Reichardia picroide* had potent antioxidant activities and could be utilized as new natural antioxidant in food and therapeutics.

Acknowledgement

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