Mitochondrial bioenergetics and redox dysfunction in nephrotoxicity induced by pyrethroid permethrin are ameliorated by flavonoid-rich fraction

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Abstract
The present study was designed to evaluate in vitro and in vivo the potential anti-inflammatory and nephroprotective potential of ethyl acetate fraction extracted from Fumaria officinalis (EAF) against permethrin (PER). Male wistar rats were treated daily by gavage during 7 days as follows: group C: negative control rats received 2 mL/kg bw of corn oil, group EAF: positive control rats received EAF at a dose of 200 mg/kg bw dissolved in water, group PER: rats received PER at a dose of 34.05 mg/kg bw and group (PER + EAF): rats received PER (34.05 mg/kg bw) and EAF (200 mg/kg bw). In vitro study showed the ability of EAF to inhibit protein denaturation and heat-induced hemolysis confirming its anti-inflammatory activity. In vivo, PER treatment decreased calcium (Ca) and phosphorus (P) levels and increased lactate dehydrogenase (LDH) activity in plasma. It induced oxidative stress objectified by an increase in the lipid peroxidation and protein oxidation and a perturbation of antioxidant system in kidney and mitochondria. The activities of NADH–ubiquinone reductase, ubiquinol–cytochrome C reductase and cytochrome C oxidase activities were reduced. These alterations were confirmed by histopathological studies. Co-treatment with EAF improved the antioxidant status and mitochondrial bioenergetics. The nephroprotective effects of EAF could be attributed to its modulation of detoxification enzymes and/or free radical scavenging actions.

Keywords Fumaria officinalis · Permethrin · Kidney · Mitochondrial bioenergetics · Oxidative stress

Introduction
At the largest metabolic and excretory organs in mammals, kidney plays an important role to maintain homeostasis (Maschmeyer et al. 2015). Environmental toxicants can cause kidney injury, destroying physical functions leading to health disorders.

Synthetic pyrethroids represent a potent group of pesticides with a strong efficacy and insecticidal activity (Kumar et al. 2016). Permethrin (PER), a type I synthetic pyrethroid, has been widely used in the USA, in agriculture, public health and food preparation and also used extensively in California about 70% of the total usage in urban areas (Saillenfait et al. 2018). Ingestion, inhalational and dermal absorption have been considered as the main pathways of PER exposure (Karmaus et al., 2016). PER is hazardous to animals and humans due to its widespread leading to bioaccumulation in the environment. Wang et al. (2016) have reported that the mechanism of pesticide nephrotoxicity might be related to oxidative stress, apoptosis or an inflammatory response.

In fact, the lipophilic nature of PER facilitates its diffusion into the cell membrane and stimulates excessive synthesis and ROS accumulation leading to oxidative stress (Wang et al. 2017). The latter provokes lipid peroxidation, protein oxidation and DNA fragmentation as well as a pathway dysfunction of several cell’s injury (Georgiadis et al. 2018). Experimental studies have reported that PER cause’s severe damage like hepatotoxicity and nephrotoxicity affecting tubular necrosis and mitochondria alteration (Guvenc et al. 2013; Jellali et al. 2018). This dysfunction is characterized by a loss of the transmembrane electric gradient and therefore of the oxidative phosphorylation process as a consequence of Ca2+...
accumulation (Gunter and Pfeiffer 1990). Given the limited efficacy of drugs treating kidney injuries (Breyer and Susztak 2017), the use of herbal medicine is increased worldwide.

In fact, natural substances are known for their low toxicity and positive biological properties (Fridlender et al. 2015). Several natural constituents such as alkaloids, sesquiterpenes, polyphenols, flavonoids and diterpenoids extracted from medicinal plants, vegetables and fruits are reported to possess promising anticancer activity via different mechanisms such as stimulation of the body immune system, cell proliferation prevention and stimulation of the protective enzyme-like glutathione transferase (Singh et al. 2021). Flavonoids having a low molecular weight among phenolic compounds display significant ROS scavenging activities (Bors et al. 1994; Pannala et al. 1997; Heijnen et al. 2001; Aoiadni et al. 2021). They can be considered as the molecular targets for cellular antioxidants in order to counteract the ubiquinone radicals and a mitochondrial ROS generation (Nichlls and Budd 2000).

Fumaria officinalis (Fumariaceae), called also “fumatory,” has been used as a dietary food and a traditional herbal medicine for the treatment of minor hepatobiliary dysfunction, gastrointestinal diseases, diuretic agents, cancer and skin disorders (Ahmed 2016). It contains polyphenolic compounds such as caffeic acid, rosmarinic acid, p-Coumaric acid, isoquercitrin, rutin, quercitrin, quercetin, kaempferol and apigenin, as well as alkaloids such as chalerythrine, sanquarine, fumaritine and protopine (Paltinean et al. 2017; Stanoevic et al. 2018; Petruczynik et al. 2019).

However, to the best of our knowledge, no investigations have been proceeded to study the nephroprotective effect of ethyl acetate fraction extracted from Fumaria officinalis (EAF). Therefore, the aim of our study was conducted to evaluate the possible protection of the EAF against permethrin (PER)-induced nephrotoxicity in male rats.

Materials and methods

Chemicals and plant materials

Fumaria officinalis aerial part was purchased from Para-chimic Laboratory (ref. HEMF001003. Tunisia). Permethrin (ref.45614; 25/75: cis/trans isomer ratio; purity: 100; Sigma) was purchased from the company Protagri. Sfax, Tunisia. All analytical-grade products were purchased from Sigma Chemical Co. (St. Louis. MO. USA).

Preparation of ethyl acetate fraction

Ethyl acetate fraction from Fumaria officinalis (EAF) was extracted using the method described by Ćujić et al. (2016). Briefly, 100 g of Fumaria officinalis was mixed with 2500 mL of ethanol 60% for 75 min. The filtrate was evaporated to yield a solid residue by rotary evaporator (50 °C). Total flavonoids were fractionated successively with petroleum ether to eliminate the lipophilic compounds and ethyl acetate. Ethyl acetate solution was concentrated to dryness under reduced pressure (50 °C) on a rotary evaporator to give EAF.

Animals

Male albino Wistar rats (180±5.02 g) were purchased from the Society of Pharmaceutical Industries (SIPHAT. Ben Arous, Tunisia). All animals were maintained under controlled conditions (25±2 °C with 12:12 h light/dark periods and a minimum relative humidity (50—60%)). Animals had free access to water and standard pellet diet (SNA, Sfax, Tunisia) (Koubaa et al. 2020). The experimental protocol was conducted according to the international Guidelines for Animal Care (ETS No.123) and approved by the Ethics committee of Sfax Sciences Faculty. After two weeks of acclimatization, rats were divided into 2 lots. The first (n = 3 rats) was used for in vitro study, and the second (n = 24 rats) was served for in vivo study.

Experimental design in vitro

Mitochondrial suspension preparation

Rat kidney mitochondria were isolated by the differential centrifugation procedure according to Hoppel et al. (1979). In brief, 1 g of rat kidney was homogenized in 2 mL of isolation buffer (100 mM saccharose, 50 mM KCl, 50 mM Tris and 5 mM EDTA, pH 7.4) using a Potter–Elvehjem with a loose-fitting Teflon pestle and centrifuged at 600 × g for 10 min at 4 °C to remove nuclei and cell debris. Kidney mitochondria were obtained after supernatant centrifugation at 7000 × g for 10 min at 4 °C, the pellet was resuspended in 1 mL of isolation buffer. Then, the obtained mitochondria were stored at −20 °C until biochemical analysis.

Lipid peroxidation inhibition assay

The principle is based on the reaction of MDA, one of the secondary products of lipid peroxidation, with thiobarbituric acid (TBA) to form a pink MDA-(TBA)2 complex which can be quantified spectrophotometrically at 532 nm (Rajneesh et al. 2008). An aliquot (1 mL) of EAF at graded concentrations (0 to 1 mg. mL−1) was mixed with 100 µL FeSO4 (15 mM) and 50 µL of ascorbic acid (0.1 mmol/L). The mixture was incubated at 37 °C for 1 h. Then, 1 mL of trichloroacetic acid (15%) and 1 mL of thiobarbituric acid (0.67%) were added to the mixture. After boiling at 95 °C and
cooling for 30 min, the mixture was centrifuged at 2,200 x g for 20 min. The absorbance of supernatants was measured at 532 nm. One mL of quercetin at graded concentration (0 to 1 mg. mL\(^{-1}\)) was used also as a positive control at the same condition than EAF. The experiment was carried out in triplicates, and the results were expressed as % of inhibition using the following equation:

\[
\% \text{ Inhibition} = \left( \frac{\text{Absorbance(standard)} - \text{Absorbance(sample)}}{\text{Absorbance(standard)}} \right) \times 100
\]

**Determination of mitochondria swelling**

An increased mitochondrial permeability transition was recognized as a swelling of isolated mitochondria induced by adding Ca\(^{2+}\) and was assayed spectrophotometrically as previously described by Cardoso et al. (2017). Briefly, isolated mitochondria (1 mg. mL\(^{-1}\)) were suspended in 2 mL of buffer D considered as inducer solution (5 µmol/L FeSO\(_4\) and 0.1 mmol. L\(^{-1}\) ascorbic acid) over 60 min at 37 °C and then centrifuged at 1000 x g for 10 min at 4 °C. Control test was determined by suspended in same mixture without FeSO\(_4\). Temperature was maintained at 30 °C and the absorbance was immediately recorded with Shimadzu UV-61 double beam spectrophotometer at 520 nm. The experiment was carried out in triplicates.

**Anti-inflammatory activity determination**

**Protein denaturation inhibition**

The anti-inflammatory activity of EAF was studied using inhibition of albumin denaturation according to methods described by Sakat et al. (2010) and Mizushima and Kobayashi (1968) with slight modifications. For denaturation, a 500 µL aliquot of bovine serum albumin (1%) was added to 100 µL of EAF and kept for 10 min at 37 °C and at 51 °C for 20 min. The mixture was cooled down to room temperature and absorbance was recorded at 660 nm. Diclofenac sodium was taken as a standard (De et al. 2016). The experiment was carried out in triplicates, and percent inhibition of denaturation was calculated from control using Eq. (1).

**Membrane stabilization**

**Red blood cell (RBC) suspension preparation**

Rat’s blood samples (10 mL) were collected by cardiac puncture and centrifuged at 3000 x g for 10 min. The obtained pellets were washed three times with an equal volume of 0.9% NaCl to obtain red blood cell suspension. After centrifugation, RBC layer was diluted with phosphate buffer saline (PBS) (40%, V/V) (Sakat et al. 2010). Red blood cell (RBC) suspension was used for heat-induced hemolysis measurement.

**Heat-induced hemolysis**

The principle of the method is to stabilize red blood cells membrane by heat-induced membrane lysis. A volume of RBC suspension (100 µL) was added to an equal volume of EAF (1000 µg. mL\(^{-1}\)), and the mixture was heated at 56 °C for 30 min. Diclofenac sodium (1000 µg. mL\(^{-1}\)) was used as a standard. Then they were centrifuged at 1000 x g for 10 min at room temperature. The absorbance was recorded at 560 nm according to Sakat et al. method (2010). The experiment was performed in triplicates, and the % inhibition of hemolysis by EAF or by a standard was calculated using Eq. (1).

**Experimental design in vivo**

**Experimental protocols**

Male Wistar rats were divided into 4 groups (n=6 rats per cage for each group) as follows:

- **Group C** represented a negative control: rats received corn oil by gavage at a dose of 2 mL/kg bw for 7 days.
- **Group EAF** represented a positive control: rats received by gavage 2 mL/kg bw of corn oil and EAF 200 mg/kg bw dissolved in 1 mL of distilled water for 7 days. The dose used of EAF was chosen according to Verma (2011).
- **Group PER**: rats received 34.05 mg/kg bw of PER dissolved in corn oil by gavage for 7 days. The chosen dose represented 1/50 of DL\(_{50}\) of PER according to Cantalamessa (1993).
- **Group PER + EAF**: rats received for 7 days PER by gavage at the same dose as PER group and EAF at the same concentration as EAF group.

Food and water consumption and body weights of all groups were monitored daily for 7 days. Animals were sacrificed by cervical decapitation to avoid stress conditions obtained by anesthesia (Liu et al. 1993 and Yang et al. 2020).

**Collection of blood samples and kidney**

After cervical decapitation of rats, blood samples were drawn into heparinized tubes and centrifuged at 1500 x g for 15 min. Plasma samples were kept at –20 °C until analysis of biochemical parameters. The kidneys were collected, washed with ice cold saline solution (0.9% NaCl) and weighed. A kidney portion of 1 g was used for mitochondria isolation. Another portion of kidney (1 g) was homogenized in Tris-buffered saline.
solution (TBS, pH 7.4) using an Ultra-Turrax homogenizer (T25, Germany). The homogenates were centrifuged at 4500 g (4 °C, 20 min) and the resulting supernatants from each group were collected and stored at—20 °C until biochemical analysis. Other kidney portions were used for histological examination.

**Mitochondrial fraction preparation**

Kidney mitochondria were isolated by a differential centrifugation procedure at 4 °C according to Hoppel et al. method (1979).

**Biochemical markers in plasma**

Calcium and phosphorus levels and lactate dehydrogenase (LDH) activity were determined spectrophotometrically at 37 °C using commercial kits according to manufacturer's recommendations (Abbott Park, IL, USA) Architect/Aeroset. Ref 20,051, 20,081 and 2p56-21, respectively).

**Oxidative stress markers determination**

**Protein quantification**

Renal and mitochondrial protein contents were determined in alkaline medium to obtain blue color solutions assayed at 490 nm at 37 °C as described by Lowry et al. (1951). Bovine serum albumin (BSA) was used as a standard.

**Thiobarbituric acid reactive substance (TBARS) determination**

Kidney and mitochondria TBARS levels were estimated at 80° C by the reaction of MDA, one of the secondary products of lipid peroxidation, with thiobarbituric acid (TBA) to form a pink MDA·(TBA)₂ complex measured at 532 nm using the method described by Esterbauer and Cheeseman (1990). Data were expressed as nanomoles/milligram of protein.

**Advanced oxidation protein product (AOPP) determination**

Renal and mitochondrial AOPP levels were determined spectrophotometrically at 37 °C using a plate reader (model Anthos HTIII) and calibrated with chloramine T solution, which, in the presence of potassium iodide, absorbs at 340 nm according to the method described by Witko et al. method (1992). Data were expressed as micromoles/milligram of protein.

**Protein carbonyl (PCO) determination**

Kidney and mitochondria PCO contents were measured by the method based on the reaction of carbonyl groups with 2, 4-dinitrophenylhydrazine (DNPH) forming 2,4-dinitrophenylhydrazones which was quantified spectrophotometrically at 37°C at 370 nm according to Resnik and Packer’s method (1994). Data were expressed as micromoles/milligram of protein.

**Vitamin C content determination**

Vitamin C contents were determined spectrophotometrically at 37 °C using the dinitrophenylhydrazine method described by Jagota and Dani (1982). Briefly, ascorbic acid in the homogenate was oxidized with Cu²⁺ to form dihydro-ascorbic acid reacting with acidic 4-dinitrophenylhydrazine to obtain a red hydrazone assayed spectrophotometrically at 540 nm. Data were expressed as nanogram/milligram of protein.

**Antioxidant enzyme activities in the kidney and mitochondria**

SOD activity was determined based on the photoreduction of the nitroblue tetrazolium (NBT) using the protocol described by Asada et al. (1974) at 37° C. Absorbance was recorded at 580 nm. Data were expressed as units/milligram of protein.

Catalase (CAT) activity was assayed by the decomposition of hydrogen peroxide according to the method described by Aebi (1984) at 37° C. A decrease in absorbance due to H₂O₂ degradation was monitored at 240 nm for 1 min. Results were expressed as micromoles of H₂O₂ consumed/milligram of protein.

GPx activity was determined by measuring the reduced GSH content after incubation of the sample in the presence of H₂O₂. The absorbance was recorded at 412 nm according to Flohé and Günzler’s method (1984) at 37° C. Data were expressed as micromoles of reduced GSH/milligram of protein.

**Renal and mitochondrial glutathione (GSH) content determination**

GSH reacted with 5,5 dithiodis-nitrobenzene (DTNB) forming thionitrobenzene (TNB) of yellow color measured at 412 nm at 37° C according to the method described by Ellman (1959). GSH contents were expressed as micromoles/milligram of protein.

**Renal mitochondrial enzyme activities determination**

Complex I (NADH–ubiquinone reductase), complex III (ubiquinol–cytochrome C reductase) and complex IV (cytochrome C oxidase) activities were evaluated calorimetrically at 37 °C in renal mitochondria according to Malgat et al. method (1999).
Histology

After fixation in 10% of formalin solution for 48 h, kidney tissue was embedded in paraffin, cut into 5 µm section and stained with hematoxylin and eosin (H&E) for light microscopy examination (X 400 magnification). Six slides from each group were examined and the severity of the histological changes along the cross sections of the kidneys (leucocytes infiltration, reduction in Bowman’s space and tubular dilatation) was scored using the blind study methodology according to the following scale: none (-), mild (+), moderate (+++) and severe (++++) damages.

Statistical analysis

In vitro and in vivo data were expressed as means ± standard deviation (SD). Results analysis was performed using SPSS 23.0 analysis software. Statistical analysis was examined by the one-way analyses of variance (ANOVA) followed by the Fisher test (Stat View). The significance was accepted at p < 0.05.

The parametric Pearson correlation coefficient test was determined to assess the bivariate correlation between SOD, CAT and GPx activities with TBARS levels. A probability level of 95% was used.

Results

In vitro study

Assessment of lipid peroxidation

EAF decreased kidney and mitochondrial lipid peroxidation levels in a dose-dependent manner when compared to quercetin (Fig. 1A and B). The percent inhibition at the highest EAF concentration (1 mg.mL⁻¹) was 76.74 ± 4.25% in kidney and 76.02 ± 6.94% in mitochondria. The percent inhibition was similar to that of quercetin at the same concentration (by 77.62 ± 4.02% and 78.03 ± 4.16% in kidney and mitochondria, respectively).

Assessment of mitochondrial swelling

Co-incubation of mitochondria with EAF (1 mg.mL⁻¹) was significantly decreased after 60 min by 89.08 ± 5.23% and 96.41 ± 9.77% (p < 0.001) when compared, respectively, to control and inducer solution assays (Fig. 2). The absorbance values at the lowest EAF concentrations (62.5 µg.mL⁻¹ and 125 µg.mL⁻¹) were 0.26 ± 0.03 and 0.21 ± 0.05. This indicated that EAF had the capacity to improve the impaired mitochondrial function.

Assessment of protein denaturation

As shown in Table 1, EAF (1 mg.mL⁻¹) showed a significant inhibition of protein denaturation compared to control. Inhibition of protein denaturation by EAF (60.06 ± 8.25%) was similar to that of the standard, diclofenac sodium (64.74 ± 9.16%).

Assessment of membrane stabilization

EAF inhibited the heat-induced hemolysis of RBCs when compared to control (Table 1). The percent inhibition of EAF (83.45 ± 4.25%) was similar to that of diclofenac sodium (77.62 ± 9.25%).

In vivo study

Effect of PER on food and water consumption and body and kidney weights

Food intake (p < 0.01) and body weight gain (p < 0.001) were significantly decreased (by 21.06 ± 2.23 and

Mitochondrial swelling and lipid peroxidation showed a significant correlation (R² = 0.966, p < 0.001) (Fig. 3).

Fig. 1 Effect of EAF and quercetin (mg. mL⁻¹) against lipid peroxidation inhibition (%) in the kidney (A) and mitochondria (B). Samples number = 3
179.20 ± 12.36%, respectively) while a significant increase in the water intake and absolute and relative kidney weights (by 8.81 ± 0.65, 6.09 ± 1.23 and 39.47 ± 3.33%, respectively) in the PER-treated rats were recorded when compared to those of control group (Table 2). Co-administration of EAF to PER group restored these variations.

Effect of PER on biochemical markers

The effects of PER on some biochemical parameters (calcium: Ca, phosphorus: P and lactate dehydrogenase activity: LDH) in rats were presented in Fig. 4. PER caused a significant decrease in plasma Ca ($P < 0.001$) and P ($P < 0.01$) levels (by 44.12 ± 7.55% and 42.11 ± 3.69%, respectively) associated with an increase in the plasma LDH (by 296.33 ± 18.69%) when compared to those of control group.

Effect of PER on oxidative markers

PER induced a significant increase ($p < 0.001$) of TBARS, AOPP and PCO levels in the kidney (by 121.76 ± 12.69, 81.65 ± 6.55 and 142.85 ± 15.25%, respectively) and mitochondria (63.15 ± 4.81, 17.48 ± 2.47 and 500 ± 59.25, respectively) and a reduction in vitamin C content ($p < 0.01$) in the kidney (-50.83%) and mitochondria (-51.18%) by when compared to those of controls values (Table 3).

Effect of PER on antioxidant enzyme activities

Permethrin induced a significant increase ($p < 0.001$) of SOD and GPx activities in the kidney (by 5.89 ± 0.69 and 39.13 ± 5.22%, respectively) associated with a rise of CAT and GPx activities in mitochondria (by 100.27 ± 17.99 and 33.33 ± 5.14%, respectively) when compared to those of control group (Fig. 5). A significant reduction ($p < 0.001$) in renal CAT activity (32.22 ± 3.65%) and mitochondrial SOD activity (39.88 ± 1.55%) were also observed. There was no difference in the activities of these enzymes between the control group and the rat treated only with EAF.

Effect of PER on non-enzymatic antioxidant levels

Renal and mitochondrial GSH contents were significantly increased ($p < 0.001$) in the PER-treated rats (by 31.03 and 115.39%, respectively) compared to those of control values (Fig. 6).

Effect of PER on mitochondrial enzyme activities

NADH–ubiquinone reductase (complex I) ($p < 0.05$), ubiquinol–cytochrome C reductase (complex III) ($p < 0.01$) and

| Dose (µg.mL$^{-1}$) | Protein denaturation | Membrane stabilization |
|---------------------|----------------------|------------------------|
|                     | Abs (nm) | % inhibition | Abs (nm) | % inhibition |
| Control             | 0.50 ± 0.01 | - | 0.51 ± 0.02 | - |
| EAF                 | 1000     | 0.20 ± 0.01 | 60.96 ± 8.25 | 0.17 ± 0.01 | 83.45 ± 4.25 |
| Standard (diclofenac) | 1000 | 0.17 ± 0.05 | 64.74 ± 9.16 | 0.23 ± 0.04 | 77.62 ± 9.25 |

Values are mean ± SD, n = 3 determinants.
cytochrome C oxidase (complex IV) \((p < 0.001)\) activities were significantly reduced in permethrin-treated rats (Fig. 7) (by 20.03 \(\pm\) 2.74, 51.62 \(\pm\) 3.33 and 24.69 \(\pm\) 5.14\%, respectively) when compared to those of control groups (C and EAF).

### Table 2: Food and water consumption, weight gain (%) and absolute (g) and relative (%) kidney weights of controls (C, EAF) and treated rats (PER and PER + EAF) after 7 days of treatment

| Treatment       | C          | EAF        | PER          | PER + EAF    |
|-----------------|------------|------------|--------------|--------------|
| Food intake \(a\) | 17.23 \(\pm\) 2.36 | 17.41 \(\pm\) 2.98 | 13.60 \(\pm\) 1.56* | 16.14 \(\pm\) 3.44** |
| Water intake \(b\) | 28.76 \(\pm\) 1.13 | 28.03 \(\pm\) 1.62 | 31.29 \(\pm\) 2.04* | 30.02 \(\pm\) 2.55** |
| % of weight gain \(c\) | 44.04 \(\pm\) 2.03 | 44.29 \(\pm\) 3.08 | -34.88 \(\pm\) 0.38*** | 14.02 \(\pm\) 1.28*** |
| Absolute kidney weight \(d\) | 0.82 \(\pm\) 0.01 | 0.76 \(\pm\) 0.04 | 0.87 \(\pm\) 0.01* | 0.77 \(\pm\) 0.03# |
| Relative kidney weight \(e\) | 0.38 \(\pm\) 0.02 | 0.38 \(\pm\) 0.05 | 0.53 \(\pm\) 0.05** | 0.46 \(\pm\) 0.03# |

\(a\): g/rat/day; \(b\): mL/rat/day; \(c\), \(d\), \(e\): g. Values are mean \(\pm\) SD, \(n = 6\) determinants. PER and PER + EAF-treated groups vs. control group: \(* p < 0.05, ** p < 0.01, *** p < 0.001; \) PER + EAF group vs. PER group: \(# p < 0.05, ## p < 0.001\)

### Pearson correlation between SOD, CAT and GPx activities with TBARS levels

Pearson correlation analysis was performed to clarify the interaction between enzymatic antioxidant systems with lipid peroxidation (TBARS) content in mitochondria (Fig. 8). Our results showed a nonsignificant positive correlation between SOD \((R^2 = 0.5819)\), CAT \((R^2 = 0.6023)\) and GPx \((R^2 = 0.1858)\) activities with TBARS levels.

### Histopathological changes in the kidney

Renal histological sections of controls (C and EAF) showed a normal glomerulus and tubules architecture [Fig. 9(C) and (EAF)]. Kidney of PER-treated rats showed multiple foci of leucocytes infiltration, hypertrophy of glomeruli cells showing reduction in Bowman’s space and cloudy swelling of tubules when compared to those of controls [Fig. 9 (PER)]. However, the supplementation of EAF helps in normalizing the tissue marker levels and protecting from the nephrotoxicities induced by PER [Fig. 9 (PER + EAF) and Table 4].

### Discussion

Earlier reports support the influence of oxidative stress, inflammation, apoptosis and autophagy in the pathophysiology of pesticide-caused nephrotoxicity (Caglayan et al. 2018). One of the major and well-documented causes of inflammation is protein denaturation and cell membrane injury. Hence, alternative approaches are explored using plant-based flavonoids against oxidative stress due to xenobiotic. Our data showed the ability of EAF to inhibit protein denaturation and heat-induced hemolysis compared to the standard drug. This evidence proves that membrane stabilization could be a mechanism of anti-inflammatory action of EAF. Anti-inflammatory properties of plant flavonoids confirmed the previous finding of Bouhlali et al. (2018). They may stabilize the RBC membrane by preventing the discharge of lytic enzymes and other active inflammatory mediators (Yesmin et al. 2020). A strong correlation \((R^2 > 0.760)\) between protein denaturation inhibition action...
acid when compared to PER group. This improvement could be due to the richness of EAF in ferulic acid, which could prevent renal damage in comparison to PER group. This effect can be related to variant chemical structures of flavonoids (Kumar and Pandey 2013).

Permethrin (PER) is one of the toxic chemicals on animals’ health causing several diseases such as neurologic (Omotoso et al., 2020), gastrointestinal (Nasuti et al., 2016) and nephrotoxicity effects (Guvenc et al., 2013) and altered development of animals (Zhu et al., 2020). Our experimental data demonstrated that PER-treated rats caused a significant decrease in the body weight and an increase in absolute and relative kidney weights when compared to those of the control groups. Weight loss could be due to food consumption reduction, inadequate nutrients absorption and/or protein synthesis inhibition. A daily co-administration of EAF to the PER-treated rats improved the body and kidney weights when compared to PER group. This improvement could be due to the richness of EAF in ferulic acid, which reverses almost all the deleterious morphological changes after 15 and 30 days of cadmium-treated rats, as reported by Sanjeev et al. (2019). Quercetin, another compound of EAF found by us (Aoiadni et al. 2021), could increase body weight gain by 70% when compared to imidacloprid group as demonstrated by Hassan et al. (2019).

Moreover, calcium and phosphorus homeostasis are altered in chronic kidney disease (CKD) (Felsenfeld et al. 2015). Since the reabsorption of phosphorus is more accentuated in the proximal tubule and that of calcium is in the proximal and distal tubules (Nogueira et al. 1998). In agreement with the results of George et al. (2017) renal proximal tubules are the targets for toxicity due in part to the expression of transporters that mediate the secretion and reabsorption of xenobiotics. Our experimental findings showed that PER reduced P and Ca levels and increased LDH activity in plasma causing membrane injury as compared to control values. Co-administration of EAF to the PER-treated rats could prevent renal damage in comparison to PER group. This improvement could be explained by the richness of EAF in ferulic acid, found by us (Aoiadni et al. 2021), which increased Ca levels in kidney calculus induced by ethylene glycol in rats (Zhao et al. 2019).

Proximal tubule damage and mitochondrial dysfunction are due to nephrotoxicity induced by permethrin as reported by Bashir et al. (2013). Our in vivo data showed an increase in renal and mitochondrial TBARS, AOPP and PCO levels in PER-treated rats when compared to control values. According to Wang et al. (2016), PER may act as an oxidant or a free radical in kidney tissue inducing oxidative stress. Co-administration of EAF to PER-treated rats improved eventually these parameters to those of control groups. This

| Table 3 | Protein, thiobarbituric acid reactive substances (TBARS), protein carbonyl (PCO), protein oxidation products (AOPP) and vitamin C levels in the kidney and mitochondria of rats after 7 days of treatment |
| --- | --- | --- | --- | --- |
| | Kidney | Groups | EAF | PER | PER + EAF |
| Protein | 12.90 ± 0.04 | 12.78 ± 0.02 | 9.40 ± 0.02*** | 10.40 ± 0.84 4* |
| TBARS | 1.70 ± 0.03 | 1.52 ± 0.40 | 3.77 ± 0.01*** | 2.81 ± 0.61**## |
| AOPP | 5.07 ± 1.27 | 5.80 ± 1.45 | 9.21 ± 2.30*** | 7.06 ± 1.76** |
| PCO | 0.07 ± 0.02 | 0.07 ± 0.01 | 0.17 ± 0.01* | 0.13 ± 0.01* |
| Vitamin C | 2.99 ± 0.13 | 3.06 ± 0.06 | 1.47 ± 0.03** | 2.29 ± 0.01## |
| Mitochondria | 14.12 ± 0.01 | 13.99 ± 3.77 | 12.38 ± 0.03*** | 12.96 ± 0.97## |
| Protein | 0.38 ± 0.02 | 0.40 ± 0.06 | 0.62 ± 0.01*** | 0.57 ± 0.03** |
| TBARS | 1.43 ± 0.36 | 1.41 ± 0.35 | 1.68 ± 0.42* | 1.44 ± 0.36* |
| AOPP | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.06 ± 0.01** | 0.03 ± 0.01## |
| PCO | 1.27 ± 0.42 | 1.22 ± 0.41 | 0.62 ± 0.24** | 0.84 ± 0.28** |

Superscript: a: mg/g of tissue, b: nmol/mg of protein, c: µmol/mg of protein, d: ng/mg of protein, e: mg/mL of mitochondrion suspension. Values are mean ± SD, n = 6 determinants. PER and PER + EAF-treated groups vs. control group: *p < 0.05, **p < 0.01 and ***p < 0.001; PER + EAF group vs. PER group: # p < 0.05 and ### p < 0.001
restoration could be explained by the richness of EAF in ferulic acid thereby minimizing the detrimental action of ROS. The antioxidant action of ferulic acid is attributed to its resonance which stabilizes phenoxy radical structure leading to quench free radicals (Srinivasan et al. 2007). Furthermore, myricetin, a main component of EAF found by us (Aoiadni et al. 2021), can reduce MDA production by quenching the lipid peroxidation chain and shielding the membrane from free radicals causing injuries in kidney of cisplatin-treated mice (Hassan et al. 2017).

Knowing that GSH acts as a non-enzymatic oxygen radical scavenger and as a substrate for various enzymes like GSH-Px, it participates in the ROS reduction (Waheed and Muthu Mohammed 2012). GSH content variation affects renal function in mitochondria and renal ischemia (Baliga et al. 1999; Paller 1988). Our results showed that PER increased significantly renal and mitochondrial GSH content associated with a decrease in renal and mitochondrial vitamin C levels as compared to control groups. Our observation supported the hypothesis that permethrin nephrotoxicity was related to free radicals generation as observed by Hassan et al. (2017). An increased GSH could be due to its oxidation after being conjugated in redox process and converted to GSH reduced form as an adaptive response (Kaur and Kaur 2017). Co-administration of EAF to PER-treated rats restored these parameters when compared to those of PER group. This improvement could be due to the presence of ferulic acid in the extract which ameliorates lipopolysaccharide-induced acute kidney injury by enhancing antioxidant defenses as reported by Mir et al. (2018). As observed by Almaghrabi (2015), quercetin can increase the renal vitamin C levels induced by cisplatin in rats. This can be attributed to its radical scavenging ability (Murakami et al. 2015).

Moreover, antioxidant enzymes, mainly SOD, CAT and GPx, are the first line of defense against ROS. In the present study, PER caused perturbations of antioxidant system in the kidney and mitochondria when compared to control groups. This is in accordance with several studies which have reported the significant changes in SOD and CAT activities in different organs of rodents exposed to insecticide-like methomyl (El-Demerdash...
et al. 2013), cypermethrin (Sankar et al. 2012) and methiocarb (Ozden et al. 2009). Our findings showed that a nonsignificant positive correlation was observed between enzymatic antioxidant system and lipid peroxidation rate ($R^2 = 0.5819$, $p > 0.05$; $R^2 = 0.6023$, $p > 0.05$ and $R^2 = 0.1858$, $p > 0.05$, respectively). This is in agreement with the fact that during the dismutation of $O_2^-$ to $H_2O_2$, the mitochondrial activity of Mn-SOD can be inactivated (Hink et al. 2002). However, supplementation of EAF to PER-treated rats alleviated the oxidative damage reaching control values. Our findings were in agreement with the study of Athira et al. (2016) who have reported the protective effect of flavonoids supplement in nutriments against cisplatin-caused kidney damage. The flavonoids ability to scavenge ROS is determined by the hydroxyl configuration of the flavonoid B-ring, as it donates hydrogen and an electron to superoxide ($O_2^{-\cdot}$) and hydroxyl ($OH^\cdot$) (Sichel et al. 1991). Hence, kaempferol, a bioactive compound in the EAF (Aoiadni et al. 2021), functions as an antioxidant which scavenges oxygen free radicals, hinders lipid peroxidation, prevents the increase in membrane permeability resulting from renal oxidative injury (VijayaPrakasha et al. 2013). Moreover, kaempferol reverses the mitochondrial enzymatic antioxidants damage caused by streptozotocin in diabetic rats (Chandramohan et al. 2015). A observed also by Wang et al (2020) that ferulic acid supplementation restored the antioxidant enzymes activity such as T-SOD, CAT and GSH-Px, following a reduction in MDA level.

The release of $O_2^{-\cdot}$ across the inner mitochondrial membrane causes its depolarization and its dysfunction (Brady et al. 2006; Ye et al. 2016). A loss in the functional mitochondrial number leads to ATP depletion causing interruption of apoptotic signals leading to a secondary necrosis (Sverdlov et al. 2016). Our experimental data revealed that PER significantly decreased complex I (NADH–ubiquinone reductase), complex III (ubiquino-cytochrome C reductase) and complex IV (cytochrome C oxidase) activities in the kidney mitochondria as compared to those of control values. According to Guvenc et al. (2013) caspase-9-dependent and mitochondria-related apoptotic cell death could play a main role in permethrin-induced nephrotoxicity. Co-administration of EAF to the PER-treated rats attenuated these mitochondrial activities: (1) decreased ROS level; (2) mitigated mitochondrial depolarization; (3) reduced mitochondrial swelling which due to an alteration in osmotic pressure; and (4) increased ATP production reaching to control values. As reported by Brown et al. (1998), flavonoids have been found to suppress mitochondrial ROS production by chelating the trace elements involved in its generation. This mitochondrial protective action could be explained by the richness of the extract in naringenin, found by us (Aoiadni et al. 2021), able to rescue kidney mitochondrial function under oxidative stress conditions (Chandran et al. 2019). Another study realized in vitro by Lagoa et al. (2011) reports that quercetin and kaempferol show similar potencies as inhibitors of $H_2O_2$ production by mitochondria in basal and inhibitor-stimulated conditions, which suggest that they inhibit complex I, and possibly also complex III, able to generate $O_2^{-\cdot}$. The out kinetic competition between coenzyme Q1 and flavonoids like kaempferol and quercetin suggests that these flavonoids bind to complex I in the quinone and inhibit the binding pocket as it seems to happen with rotenone, despite their opposed actions in $H_2O_2$ production by mitochondria (Lagoa et al. 2011). According to Qu et al. (2014), quercetin preserves...
Fig. 8 Correlation between (A): SOD activity and TBARS levels ($R^2 = 0.5819$, $p > 0.05$, $n = 16$); (B) CAT activity and TBARS levels ($R^2 = 0.6023$, $p > 0.05$, $n = 16$) and (C): GPx activity and TBARS levels ($R^2 = 0.1858$, $p > 0.05$, $n = 16$) in the kidney mitochondria of rat.

Fig. 9 Light microscopic photographs of the kidney in control (C), permethrin (PER), EAF and permethrin associated with EAF (PER + EAF) groups. Stained with H&E taken at 400 X magnifications. The arrows indicate: G: Glomerulus; T: Tubule; Reduction of Bowman’s space; Tubular dilatation; Leucocyte infiltration.
the structure of mitochondria, implying that it influences mitochondrial structure and function as well as mitochondrial biogenesis.

These alterations were associated with histopathological modifications. In fact, according to Bashir et al. (2013), PER may affect acute tubular necrosis with extensive tubular degenerative variations, cytoplasmic vacuolation and biochemical markers. Our histopathological examination showed that PER caused multiple foci of leucocytes infiltration, hypertrophy of glomeruli cells showing reduction in Bowman’s space and cloudy swelling of tubules in comparison with control groups. Previous findings of Guvenc et al. (2013) have reported that permethrin causes epithelial cells degeneration and necrotic signaling of the renal proximal tubules. Both lysosomal overload and protein-bound toxic moieties may increase sensitivity to nephrotoxicity (Davis and Berndt 2001).

Besides, co-treatment with EAF extract alleviated kidney damage. This improvement may be due to ferulic acid present in EAF (Aoiadni et al. 2021), suggesting its adjuvant therapy in cisplatin-induced nephrotoxicity on apoptosis signaling by improving renal function (Bami et al. 2017; Kelainy et al. 2019). As observed by Erseckin et al. (2020), the ferulic acid able to protect also the kidney injury induced by gentamicin in female rats.

Denoting results of the current study, it is realistically presented for the first time that oral supplementation of EAF ameliorated renal and mitochondrial damage induced by PER administration. EAF showed this effect by reducing inflammation, restoring oxidant/antioxidant status and mitochondrial function (Fig. 10).

**Conclusion**

The present study demonstrated that EAF was able to attenuate in vitro mitochondria swelling via antioxidative mechanisms by reducing lipid peroxidation and inhibiting protein denaturation and heat-induced hemolysis.

Our findings indicated that PER caused kidney damages by inducing oxidative stress and mitochondria dysfunction. Co-administration of EAF in PER-treated rats improved the antioxidant status, the mitochondrial bioenergetics (mitochondrial complex I (NADH–ubiquinone reductase), complex III (ubiquino-cytochrome C reductase) and complex IV (cytochrome C oxidase)) and histopathological changes.

EAF could protect kidney against oxidative stress due to its richness in phenolics components like ferulic acid, kaempferol, quercetine and naringenin.

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**Table 4** Grading of the histopathological changes in the kidney sections of controls (C and EAF) and treated rats for 7 days with PER or with PER associated with EAF

| Kidney histopathological changes  | C     | EAF   | PER  | PER + EAF |
|----------------------------------|-------|-------|------|-----------|
| Hypertrophy of glomeruli cells    | -     | -     | ++   | +         |
| Tubular dilatation               | -     | -     | +++  | -         |
| Leucocyte infiltration           | -     | -     | +++  | -         |

None (-); mild (+); moderate (++); and severe damages (+++)
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Author contributions Nissaf Aoiadni was responsible for investigation, methodology, formal analysis and writing-review. Hajar Jfidji and Hamadi Fetoui were involved in methodology. Abdel fattah El Feki acquired the funding. Fatma Ghorbel Koubaa took part in resources, conceptualization, supervision, and writing, reviewing and editing.

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Data availability All the data are present in the article.

Declarations

Ethical Approval 1204

Consent to Participate and Publish Not applicable.

Conflict of interest The authors declare that they have no conflict of interest.

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