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Peptides from *Liza aurata*: Natural Source Attenuate Paracetamol Induced Nephrotoxicity by Modulation of the Inflammatory Response and DNA Damage

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Abstract

The present study investigates the nephroprotective and the molecular mechanisms effects of *Liza aurata* protein hydrolysates (LAPHs) against paracetamol induced nephrotoxicity and oxidative stress in rats. Oxidative stress as evident by increased malondialdehyde, hydrogen peroxide, advanced oxidation protein product, protein carbonyl, with significant decrease in non protein thiol and glutathione contents, as well as catalase, superoxide dismutase, and glutathione reductase activities. Paracetamol triggered inflammatory response by inducing tumor necrosis factor-α (TNF-α), with the increased expression of cyclooxygenase-2 (Cox-2). Paracetamol also increased alkaline phosphatase (ALP) and lactate deshydrogenase (LDH) activities, along with an increase in blood urea nitrogen, creatinine, low density lipoprotein cholesterol, and total bilirubin levels. LAPHs were found to decrease leakage of LDH and ALP activity and attenuate the increase in biochemical parameters resulted in a subsequent recovery towards normalization. The biochemical parameters and histopathological observation were correlated by regulation of Cox-2, and TNF-α expression. PH-LA, which presented the highest antioxidant and anti-inflammatory activities, was fractionated by G-25 chromatography into five fractions. F2 and F4 which exhibited the highest anti-inflammatory and antioxidant activities, respectively, were further fractionated by reverse phase-high performance liquid chromatography. Our findings claimed that PH-LA can be used in preventing and treating many health problems without any side effects and can be applied in the field of nutraceuticals.

Keywords Nephrotoxicity · Bioactive peptides · Anti-inflammatory · Antioxidant

Introduction

One of the most important health problems that researchers and pharmaceutical industry collaborate globally to tackle is the drug-induced kidney diseases, which are thought to be the result of direct nephrotoxicity and adverse immunological reactions (Dokumacioglu and Iskender 2017). Paracetamol, also known as acetaminophen, is most commonly used in the world as an analgesic and antipyretic drug available without a prescription, both in mono- and multi-component preparations. An acute paracetamol overdose can lead to potentially lethal kidney failure in humans and experimental animals. Paracetamol induced renal toxicity has been increasingly reported in many literature (Adil et al. 2016; Ko et al. 2017).

Paracetamol is metabolized via three metabolic pathways. N-acetyl-p-benzoquinone imine (NAPQI) is a toxic metabolite for the kidneys produced by cytochrome p450 main

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enzyme system. In overdose situation, the conjugation pathways become saturated. The accumulation of NAPQI leads to depletion of reduced glutathione (GSH) and exceeded its capability to synthesize new glutathione. Therefore, NAPQI binds covalently to the cysteine groups of cytosolic and mitochondrial proteins which induce cell death resulting in kidney dysfunction (Hu et al. 1996).

The antidote n-acetylcysteine (NAC) is highly effective at preventing toxicity, provided it is administered within a few hours of the overdose. Subsequently efficacy declines as the interval between the overdose and NAC administration increases (Thomas 2016). However, despite its efficacy in reducing mortality due to acetaminophen poisoning, intravenous NAC can cause anaphylactoid reactions. Due to the complexity of preparation of NAC as well as its side effects, mistakes are possible. However, because of the narrow treatment and limited timing of NAC, new therapeutic interventions are necessary to be developed for the treatment of paracetamol poisoning.

Indeed, biological molecules endowed with antioxidant properties ensure the protection of kidney against the deleterious effects of toxicity induced by paracetamol. In addition, fish protein hydrolysates (FPH) are considered a potential source of amino acids that play a major role in biological processes and have a direct or indirect impact on the maintenance of good health (Nasri et al. 2015). Bioactive peptides are specific peptide motifs encrypted in the primary food proteins with biological effects once they are released from their parent proteins (Ulug et al. 2021). Nowadays, the use of bioactive peptides has gained much interest regarding the increasing demand for efficient and safe renoprotective agents from natural source.

It is known that bioactive peptides have a wide range of physiological effects and positively impact human health. A great diversity of biological activities has been attributed to peptides (Daroit and Brandelli 2021). The biopeptides stay inactive within the parental protein until release by enzymatic hydrolysis. Recently, researchers have focused on generating and characterizing bioactive fish protein hydrolysates and peptides and then studying their potential health benefits (Gao et al. 2021). These bioactive peptides may regulate bodily functions through their myriad peptide sequence and their amino acid composition. In our previous study, RP-HPLC analysis demonstrated that hydrophobicity of *Liza aurata* protein hydrolysates have been proven to be important for the biological activity and the antioxidant activities of LAPHs are related to the high hydrophobic and aromatic amino acid contents (Bkhairia et al. 2016).

Peptides receive great interest over natural proteins due to their nonimmunogenicity and ease of desirable epitope conjugation to direct cell differentiation and other signaling pathways for functional improvement (Zennifer et al. 2020). The bioactive peptides from smooth-hound viscera have recently attracted considerable interest as agents that may subvert many of the problems related to vascular physiology (Abdelhedi et al. 2017). In an attempt to meet the requirements for the treatment of hyperglycemia and hyperlipidemia as much as possible, Ben Slama-Ben Salem et al. (2018) endorsed the anti-diabetic power of peptides from *octopus vulgaris* protein hydrolysates. Experimental data further showed the antioxidative property from marine protein hydrolysates (Chirinos et al. 2018; Ben Slama-Ben Salem et al 2017). However, the protective role of LAPHs against paracetamol induced renal injury has not been investigated yet. Hence, we propose in this study to scrutinize renoprotective effect of LAPHs against paracetamol-induced acute renal injury. In addition, the feasible molecular mechanisms underlying this nephroprotective effect are discussed, involving anti-inflammatory activity.

Materials and Methods

Experimental Animals

Wistar rats were purchased from the Central Pharmacy of Tunis (SIPHAT, Tunisia). All animal procedures were conducted in strict conformation with the local Institute Ethical Committee Guidelines for the Care and Use of laboratory animals of our Institution. Rats were kept in an environmentally controlled breeding room (temperature: 22 ± 2 °C, minimum relative humidity: 60 ± 5%, 12 h dark/light cycle). The rats were randomly divided into seven groups (6 rats in each) out of which five groups were treated with 350 mg LAPHs/kg of body weight. LAPHs obtained following treatment with the endogenous alkaline enzyme extract from the viscera of *L. aurata*, commercial enzymes (Trypsin and Esperase) and proteases from *B. subtilis* A26 and *P. aeruginosa* A2, were named as PH-LA, PH-TR, PH-ES, PH-A26, and PH-A2, respectively. The remaining two groups were normal and paracetamol treated groups. Thus the 6 animal groups were:

Group (Control): rats not treated with paracetamol considered as control.

Group (P): rats treated with 325 mg of paracetamol/Kg body weight for five last days of treatment by intraperitoneal injection.

Groups PH-LA/P, PH-TR/P, PH-ES/P, PH-A2/P and PH-A26/P: treated with LAPHs by gastric gavage at 350 mg/kg body weight, daily for 45 days, and they were injected intraperitoneally for the last 5 days of treatment period by 325 mg paracetamol/kg body weight.

At the end of the experimental period, the rats were refrained overnight and sacrificed by decapitation in order to avoid stress. Blood was collected in EDTA tubes was used to determine hematological parameters while for biochemical

parameters blood was collected in the tubes without any anticoagulant, and the plasma was alienated by centrifugation (1500 rpm, 15 min, 4 °C) and preserved at − 80 °C until further analysis. The hematological parameters were immediately determined. The kidney was quickly excised, weighed and processed for various extractions and stored at − 80 °C until analysis. Ice-cold phosphate buffer (pH 7.4) was used to prepare tissue homogenate followed by centrifugation (5000 rpm, 20 min) to isolate cytosolic supernatant for the determination of all biochemical parameters. A portion of the kidney was stored in RNAlater for RT-PCR and 10% formalin for histopathology study.

Assessment of Serum Biochemical Parameters

Serum levels of cholesterol, low density lipoprotein cholesterol (LDL-c), alkaline phosphatase (ALP), lactate deshydrogenase (LDH), total protein (TP), blood urea nitrogen (BUN), creatinine, total bilirubin (TB), conjugated bilirubin (CB), glucose, and uric acid, were measured in frozen aliquots of serum by standardized enzymatic procedures using commercial kits from Biolabo (Maizy, France) on an automatic biochemistry analyzer (Vitalab Flexor E, USA).

Oxidative Stress Markers

Lipid peroxides were assayed as malondialdehyde (MDA) concentration spectrophotometrically using thiobarbituric acid reactive substances (TBARS) employing the method of Yagi (1976). Measurement of hydrogen peroxide (H_2O_2) was carried out according to the method of Ou and Wolff (1996), by the ferrous ion oxidation xylenol orange (FOX1). The protein carbonyl (PCO) levels were quantified as described by Reznick and Packer (1994). The advanced oxidation protein products (AOPP) levels were performed according to the method of Kayali et al. (2006). Spectrophotometric determination of protein thiol (Np-SH) levels was performed by the method of Ellman (1959).

Antioxidant Enzymes and Glutathione Assays in Kidney

The catalase (CAT) activity was assayed according to the method of Aebi (1984) based on the decomposition of H_2O_2. The degradation of hydrogen peroxide was monitored spectrophotometrically at 240 nm for 1 min and the enzyme activity was expressed as U/mg of protein.

The total superoxide-dismutase (SOD) activity was evaluated by quantitating the inhibition of cytochrome-C reduction in the xanthine–xanthine oxidase system according to Sun et al. (1988). The increase of blue color was measured at the excitation wavelength of 560 nm. One unit of SOD activity as defined as the amount of enzyme required inhibiting the reduction of NBT by 50% and the activity is expressed as units of SOD/mg of protein.

The glutathione peroxidase (GPx) activity was assayed according to Flohé and Günzler (1984), using H_2O_2 and NADPH as substrates. The conversion of NADPH to NADP+ was observed by recording the changes in absorption intensity at 340 nm. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol NADPH per minute. The activity is expressed as U/mg of protein.

The level of glutathione GSH in kidney homogenates was determined by a spectrophotometric method according to Ellman (1959). In this assay, GSH reacts with 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to produce a stable colored product 5-thio-2-nitrobenzoic acid (TNB), which absorbs maximally at 412 nm nm after 10 min. Total GSH content in renal tissue was expressed as µg/mg protein.

DNA Fragmentation Assay

DNA extraction from rat kidney was done by ZR Genomic DNATM-Tissue MidiPrep, Irvine, CA 92614, USA, according to manufacturer's instructions. The quality and quantity of DNA was evaluated. DNA samples were mixed with 10 µl loading solution. Samples were loaded onto a 1% (w/v) agarose gel. DNA fragments were separated by electrophoresis in TBE buffer. Images of the ethidium bromide stained DNA agarose gel was acquired using AlphaImager TM Gel Documentation, USA.

Total RNA Extraction

Total RNA was isolated from 50 mg of kidneys, using TRIzol Reagent (Invitrogen, California, USA). The quality of RNA was evaluated and the A260/A280 ratio is 1.78.

Semi-quantitative RT-PCR of mRNA

MM-LV reverse transcriptase (Invitrogen), was used for reverse transcription (RT) reactions. Oligo (dT_{20}) primer was used to extend all mRNA, and 2 µg of total RNA was used in each reverse transcription reaction in a total volume of 20 µl. PCR was performed using 1.5 µl cDNA, 10 × PCR buffer, 25 mM $MgCl_2$, 250 unit Taq DNA Polymerase (Invitrogen, France), 30 pmol of each gene-specific primer (Table 1), and 20 pmol dNTP in a total volume of 25 µl.

The cycling conditions were as follows: Initial denaturation at 94 °C for 4 min followed by denaturation at 94 °C; annealing at 55 °C (COX-2), 60 °C (TNF-α) and 64 °C (GAPDH) for 40 s and extension at 72° C for 1 min. The number of amplification cycles was determined by using individual primer sets to maintain exponential product amplification (30–35 cycles). DNA bands were visualized

Table 1 Primer sequences for PCR amplification

Genes	Primer sequences	PCR product size (bp)	Accession number
GAPDH F	TGGTCACCAGGGCTGCTTTTAACT	424	M32599
GAPDH R	GCTAAGCAGTTGGTGGTGCAGGA		
COX-2 F	TGTGACTGTACCCGGACAGG	280	NM_017232
COX-2 R	TGCACATTGTAAGTAGGTGGAC		
TNF-α F	AAATGGGCTCCCTCTCATCAGTTC	350	X66539
TNF-α R	TCTGCTTGGTGGTTTGCTACGAC		

with ethidium bromide staining over a UV transilluminator. Quantitation of bands was performed by ImageJ software (http://rsb.info.nih.gov/ij/).

Histopathology

After dissection, kidney tissues were excised and immersed in normal saline and fixed in Bouin solution (picric acid, formaldehyde 40% and glacial acetic acid) for 24 h. Sections were cut at 3–5 µm thicknesses on a rotary microtome, mounted and stained with hematoxylin–eosin. These sections were evaluated for histological changes under light microscopy (Olympus CX41).

PH-LA Fractionation

Size-Exclusion Chromatography (SEC)

A Sephadex G-25 gel filtration column (2.9 cm × 54 cm) was used at a flow rate of 1 mL/min with distilled water. A total of 1 g was suspended in 5 mL of distilled water for injection and fractions of 1.5 mL were collected and monitored at 280 nm. Fractions corresponding to the same peak were pooled together and freeze-dried.

Reverse Phase-High Performance Liquid Chromatography

Sephadex G-25 fractions showing the strongest antioxidants and antiinflammatory activities were filtered through a Phenex-PVDF 17 mm Syringe Filter 0.45 µm (Phenomenex, Torrance, CA, USA) and analysed by RP-HPLC. Analyses were run on a Finnigan HPLC system (Thermo Electron Corporation, San Jose, CA, USA) provided with photodiode array detector (DAD). The column selected was an Aeris PEPTIDE 3.6 m XB-C18 New Column 250 × 4.6 mm (Phenomenex). Elution conditions consisted in 0.1% TFA in distilled water (solvent A) and 0.1% TFA in acetonitrile (solvent B) gradient at a flow rate of 1.0 mL/min. Chromatograms were recorded at 214 nm and assayed for anti-inflammatory and antioxidant activities.

Bioactivity Evaluation of Purified Fractions from PH-LA

Antioxidant Activities

The antioxidant potentiel of purified fractions of PH-LA were determined using three antioxidant tests. 1,1-diphenyl- 2-picrylhydrazy (DPPH) and 2,2-azino-bis (3-éthylbenzothiazoline-6-sulfonique acide (ABTS) radical scavenging activities was determined according to the methods of Bersuder et al. (1998) and Wang et al. (2012), respectively. Ferric reducing antioxidant power (FRAP) assay was performed according to the method of Benzie and Strain (1996).

Anti-inflammatory Activity

The anti-inflammatory activity was assessed using the spectrophotometric measurement of a conjugated diene the result of linoleic acid oxidation by the enzyme 5-LOX (Bekir et al. 2013).

Statistical Analysis

Results were expressed as mean ± standard deviation (SD) of six animals in each group. The Data compared by one-way analysis of variance (ANOVA) with post hoc tests to control group. All statistics were calculated using GraphPad Prism 7 software. p values of < 0.05 or 0.01 was considered significant.

Results and Discussion

Preparation of LAPHs

Protein hydrolysates were prepared from golden grey mullet muscle using different alkaline proteases to obtain bioactive peptides with different amino acid sequences and chain lengths. At the same ration enzyme/substrat, PH-LA showed the highest degree of hydrolysis (DH = 13.05%), followed by PH-TR (DH = 12.67), PH-ES (DH = 12.5), PH-A26 (DH = 9.25) and PH-A2 (DH = 8.05) (Bkhairia et al.

2016). The obtained DH were similar to those previously reported for the hydrolysates from Skipjack Tuna (*Katsuwonus pelamis*) Dark Muscle (Chi et al. 2015) and sole and squid skin gelatins (Giménez et al. 2009). It is of interest to note that the peptides derived from the protein hydrolysates of seafood muscles could serve as potent antioxidants and be used as an efficient and safe additive in food processing. In this context, Bkhairia et al. (2016, 2018) reported that LAPHs are proved to be good sources of antioxidant peptides.

Body Weight and Coefficients of Kidney

Treatment of rats with only a single-dose of paracetamol, during the last 5 days of the experience showed a significant (<0.05) decrease in the average body weight when compared to the control group (data not shown) The administration of paracetamol following pretreatment with LAPHs (350 mg/kg bw) did not significantly (<0.05) cause decrease in the average body weight when compared to the control group. In addition, rats pretreated with PH-ES and PH-A26 showed a moderate weight gain, when compared to control group. At the same time, the rats pretreated with LAPHs showed significant (<0.05) restoration in the kidney index as compared to the paracetamol treated rats. Even in a recent study conducted by Ahn et al. (2021), with single-dose and repeated-dose administration (for 14 days) of the yeast protein hydrolysates, intergroup differences in weight gain, dietary intake, and drinking water intake were not significant. Our findings indicate that the administration of LAPHs had no toxic effect at the experimental dose. Similar results were observed in previous studies showing that fish protein hydrolysates attenuate metabolic disorder (Nasri et al. 2015).

Effects of LAPHs on Biochemical Parameters

The findings presented in Table 2 revealed that, paracetamol group showed significant increase by 71.84%, 55.36%, in ALP and LDH activities, respectively, as well as an increase by 83.3%, 62.49%, 88.58%, 223%, and 74.67%, in BUN, creatinine, TB, CB and glucose levels, respectively, when compared with control group. Contrary, paracetamol lead to a decrease in TP levels by about 55.76%. The decrease in serum total protein (TP) level can be assigned to renal epithelial cell necrosis manifested by a decrease in the number of cells responsible for protein synthesis.

In a study of nephrotoxicity induced by acetaminophen, (Cakir et al. 2010) finds that tubular injury is one of the major findings enclosed to the renal failure induced by paracetamol-overdose and the main evidence of proximal tubular lesion is low-molecular weight proteinuria creatinine and urea are nitrogenous waste produced by the metabolism. They are considered markers of renal cleansing for their exclusive elimination by urine in the kidneys. Kidney dysfunction leads to the increments of creatinine and urea levels in the blood because the rate of creatinine and urea production exceeds the rate of clearance. Recently, Adewuyi et al. (2018) championed hypothesis that high level of uric acid can serve as an antioxidant within extracellular conditions but when it enters the cell, it can cause oxidative stress. Paracetamol induced renal damaging were evidenced by biochemical measurements which are consistent with observation reported by Cakir et al. (2010). Indeed, there is a

Table 2 Influence of LAPHs on clinical biochemical parameters levels from the blood serum in rat kidney in a model of paracetamol-induced nephrotoxicity

	Control (C)	(P)	PH-LA/P	PH-TR/P	PH-ES/P	PH-A2/P	PH-A26/P
Glucose (mmol/L)	5.41 ± 0.39^{c}	9.45 ± 0.41^{a}	6.34 ± 0.47^{bc}	7.21 ± 0.12^{b}	6.89 ± 0.61^{b}	5.21 ± 0.14^{c}	5.91 ± 0.36^{bc}
Cholesterol	2.82 ± 0.11^{c}	1.37 ± 0.34^{a}	2.23 ± 0.34^{b}	2.00 ± 0.17^{b}	2.36 ± 0.21^{c}	2.64 ± 0.42^{bc}	2.52 ± 0.33^{c}
TP (g/L)	77.32 ± 3.21^{a}	34.21 ± 2.12^{c}	49.12 ± 4.13^{b}	50.14 ± 1.16^{b}	57.19 ± 5.13^{b}	49.13 ± 4.78^{b}	60.55 ± 3.16^{b}
Uric acid (µmol/L)	132.06 ± 8.13^{a}	65.32 ± 6.31^{c}	97.32 ± 6.77^{b}	87.12 ± 4.97^{b}	97.12 ± 4.65^{b}	100.87 ± 7.32^{b}	105.63 ± 4.77^{b}
BUN (mmol/L)	5.63 ± 0.26^{c}	10.32 ± 0.52^{a}	6.72 ± 0.34^{bc}	6.38 ± 0.44^{bc}	7.45 ± 0.51^{b}	7.09 ± 0.25^{bc}	6.24 ± 0.48^{bc}
Creatinine (µmol/L)	19.81 ± 0.74^{c}	32.19 ± 1.43^{a}	23.64 ± 0.92^{bc}	25.81 ± 1.16^{b}	24.42 ± 0.77^{bc}	23.08 ± 2.0^{bc}	22.73 ± 1.56^{bc}
LDL-c (mmol/L)	0.88 ± 0.10^{b}	1.95 ± 0.13^{a}	0.94 ± 0.08^{b}	1.10 ± 0.10^{b}	0.96 ± 0.03^{b}	1.06 ± 0.07^{b}	0.91 ± 0.01^{b}
LDH (mmol/L)	1006 ± 24^{a}	1563 ± 0.06^{b}	1241 ± 17^{a}	1205 ± 21^{ab}	1102 ± 33^{a}	1086 ± 27^{a}	1074 ± 35^{a}
TB (µmol/L)	1.84 ± 0.27^{c}	3.47 ± 0.18^{a}	2.56 ± 0.21^{b}	2.05 ± 0.09^{bc}	2.20 ± 0.05^{bc}	1.96 ± 0.25^{bc}	2.31 ± 0.13^{bc}
CB (µmol/L)	0.26 ± 0.05^{b}	0.84 ± 0.10^{a}	0.38 ± 0.03^{b}	0.49 ± 0.10^{b}	0.50 ± 0.02^{b}	0.51 ± 0.07^{b}	0.37 ± 0.09^{b}
ALP (IU/L)	341 ± 27^{e}	586 ± 27^{a}	407 ± 8.0^{c}	413 ± 15^{d}	397 ± 19^{b}	388 ± 10^{b}	361 ± 22^{b}

Glucose; Cholesterol; Total Protein (TP); Uric acid; Blood Urea Nitrogen (BUN); Creatinine; LDL-c, Low Density Lipoprotein cholesterol; LDH, Lactate Dehydrogenase; Total Bilirubin (TB), Conjugated Bilirubin (CB); and ALP, Alkaline Phosphatase; PH-LA, PH-TR, PH-ES, PH-A2 and PH-A26: Protein hydrolysate obtained with crude enzyme from *L. aurata*, trypsin, esperase, *Pseudomonas aeruginosa* A2 and *Bacillus subtilis* A26, respectively. Data expressed as mean ± SD in each group (n= 6). [a,b,c,d,e,f]The means with no common superscripts differ significantly ($p < 0.01$)

significant increase in serum LDL-c level by about 121.59% and a decrease in total cholesterol in group of rats treated with overdose of paracetamol by 51.42%, our results are in agreement with a previous finding of Lebda et al. (2013). Paracetamol seems to cause impairment in lipoprotein metabolism and also alterations in cholesterol metabolism (Lebda et al. 2013).

The pretreatment of rats with LAPHs, recorded significant decrement in creatinine, BUN, TB, CB, ALP, LDH and glucose, while increasing cholesterol and TP content significantly in comparison with paracetamol treated group. LAPHs treated groups, in which and values obtained were similar or slightly higher than those of the control group. In their report, it is worthy to note that PH-LA and PH-A26 effects were almost better than that showed by PH-ES, PH-A2 and PH-TR. Similar findings were observed in previous studies showing the renal protective effect of fish protein hydrolysates (Nasri et al. 2015; Kamoun et al. 2016). In another study, Jemil et al. (2017) have shown the preventive role of fermented sardinelle protein hydrolysates against deterioration of kidney function in wistar rats.

Effect of LAPHs on Renal Oxidative Stress

Effect of LAPHs on Renal MDA and GSH

As shown in Fig. 1A and B, a toxic dose of paracetamol induced an increase in the levels of MDA and a decrease in GSH content by 2.5 fold and 1.3 fold, respectively, in renal tissues as compared to control. Obviously, the treatment with LAPHs significantly ($p < 0.05$) abrogated the elevation of level of MDA by 53.15%, 54.85%, 64%, 58.2% and 62.85% for PH-LA, PH-TR, PH-ES, PH-A2 and PH-A26, respectively.

It has been pointed out that the hydrophobic bioactive peptides, have higher ability to interact with cell membranes and it can cause the blockage of lipid peroxidation and cell damage. Owing to its richness in hydrophobic amino acid that improving cellular uptake and enhancing the antioxidant properties, peptides become more accessible to the hydrophobic polyunsaturated chain of fatty acids within biological membranes to counteract oxidative damage (Borrelli et al. 2018). As response to prophylactic LAPHs treatment, a significant increase of GSH level by 134.3%, 104.8%, 126.83%, 87.99% and 127.68% for PH-LA, PH-TR, PH-ES, PH-A2 and PH-A26, respectively, compared to group of rats exposed to paracetamol intoxication. Consequently, such finding are consistent with the results indicated the potential of the dietary administration of FPH, produced from goby, improving the antioxidant ability and alleviating renal dysfunction in rats (Nasri et al. 2015). The mechanisms of inhibition can attributed either to the interaction of peptides with the radical species or prevention of free radical formation, leading to cytoprotection. In a related study, Kamoun et al. (2016) have noted that some amino acids like aspartic acid regardless of their position in the peptide sequence, facilitate ROS scavenging.

Our finding suggests that the peptides present in the LAPHs efficiently works on the kidney to keep it functioning normally and minimizing cell membrane disturbances.

Effect of LAPHs on Renal Np-SH and H_2O_2, AOPP and PCO Levels

To characterize the effects of LAPHs antioxidant status, the levels of Np-SH, H_2O_2, AOPP and PCO in the kidney samples, were measured respectively and the results are shown in Table 3. The levels of H_2O_2, AOPP and PCO, in

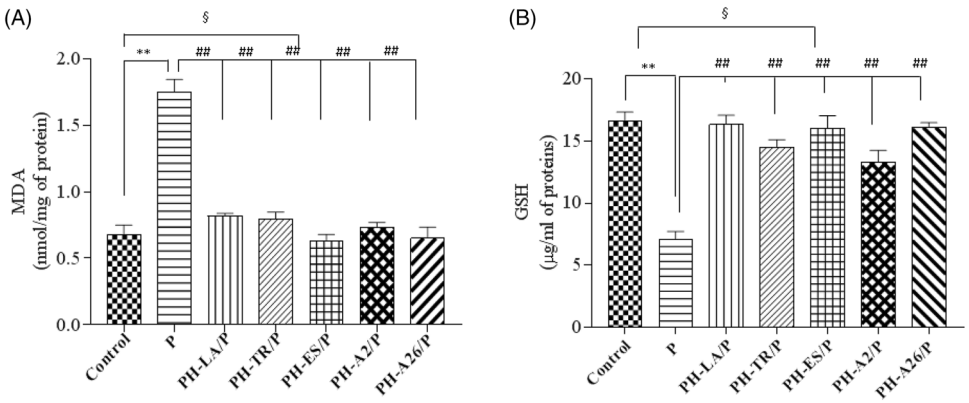

Fig. 1 Effect of LAPHs on the levels of lipids peroxidation (expressed as Malondialdehyde (MDA), nmol/mg protein) (**A**), and reduced glutathione (GSH) content (**B**) in kidney of paracetamol induced nephrotoxicity in rats. Control = control group, P = Paracetamol group, PH-LA, PH-TR, PH-ES, PH-A26 and PH-A2, represent *Liza aurata* protein hydrolysates produced using crude enzyme from *L. aurata*, trypsin, esperase, *Bacillus subtilis* A26 and *Pseudomonas aeruginosa* A2, respectively. Data are expressed as mean ± SEM (n = 6). § $p < 0.05$, ** $p < 0.01$ vs. control group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ vs. paracetamol group

Table 3 AOPP, H_2O_2, PCO and NPSH levels in kidney of control and treated rats with paracetamol (P), or with LAPHs/P

	Control	(P)	PH-LA/P	PH-TR/P	PH-ES/P	PH-A2/P	PH-A26/P
H_2O_2	0.41 ± 0.09^{g}	0.83 ± 0.05^{a}	0.52 ± 0.03^{e}	0.56 ± 0.07^{d}	0.49 ± 0.02^{f}	0.61 ± 0.09^{c}	0.63 ± 0.05^{b}
AOPP	0.62 ± 0.03^{f}	1.08 ± 0.1^{a}	0.73 ± 0.06^{e}	0.81 ± 0.09^{c}	0.61 ± 0.04^{g}	0.85 ± 0.1^{b}	0.77 ± 0.03^{d}
Np-SH	14.73 ± 1.5^{a}	7.92 ± 0.75^{f}	11.12 ± 1.09^{c}	10.23 ± 0.87^{d}	13.69 ± 1.06^{b}	9.44 ± 0.82^{e}	10.5 ± 1.20^{d}
PCO	1.39 ± 0.05^{g}	3.75 ± 0.18^{a}	2.04 ± 0.6^{e}	2.51 ± 0.43^{b}	1.84 ± 0.25^{f}	2.47 ± 0.5^{c}	2.39 ± 0.71^{d}

(Control): control group; (P): Paracetamol treated group; (PH-LA/P), (PH-TR/P), (PH-ES/P), (PH-A2/P), and (PH-A26) are rats treated with PH-LA, PH-TR, PH-ES, PH-A2 and PH-A26, respectively, and injected with overdose of paracetamol during the last five days of treatement
Data expressed as mean ± SD in each group (n = 6). ^{a,b,c,d,e,f} . The means with no common superscripts differ significantly ($p < 0.01$)

the kidney of paracetamol treated group were significantly ($p < 0.05$) higher (by 102.4%, 74.19%, and 169.78%, respectively) compared to that of control animals. Generation of protein carbonyl compounds during paracetamol treatement indicated that proteins were subjected to free radical damage. A significant decrease in the Np-SH level by 46.23% was evident in paracetamol nephrotoxic rats. Data concerning non-enzymatic status in experimental groups revealed that, the level of Np-SH was significantly restored (by 40.4%, 29.16%, 72.85%, 19.19% and 32.57% for PH-LA, PH-TR, PH-ES, PH-A2 and PH-A26, respectively) when compared to the paracetamol group. This effect was associated with a decrease in the levels of H_2O_2, AOPP and PCO, especially the oral administration of PH-LA by about 37.34%, 32.40%, and 46.7%, and PH-ES by about 40.96%, 43.51% and 50.93%, respectively, compared to nephrotoxic rats ($p < 0.05$). Treatment with LAPHs significantly attenuated paracetamol induced carbonyl content and thereby protein oxidation, which are indicative of the antioxidant and free radical scavenging properties of LAPHs. The reduction of H_2O_2, AOPP and PCO, and the increase of Np-SH level in LAPHs treated rats indicated that the bioactive peptides in LAPHs could keep their activity under in vivo conditions.

Effect of LAPHs on Renal Antioxidant Enzymes

As shown in Fig. 2A, B and C, SOD, GPx and CAT activities in the kidney of rats treated with paracetamol decreased significantly as compared to those of control group. The activities of GPx, CAT and SOD, were found to be respectively reduced by 53.47%, 60.94%, 58.35%, as compared to control group.

Our results are in agreement with reports of other workers which suggest that acute over dose of paracetamol to

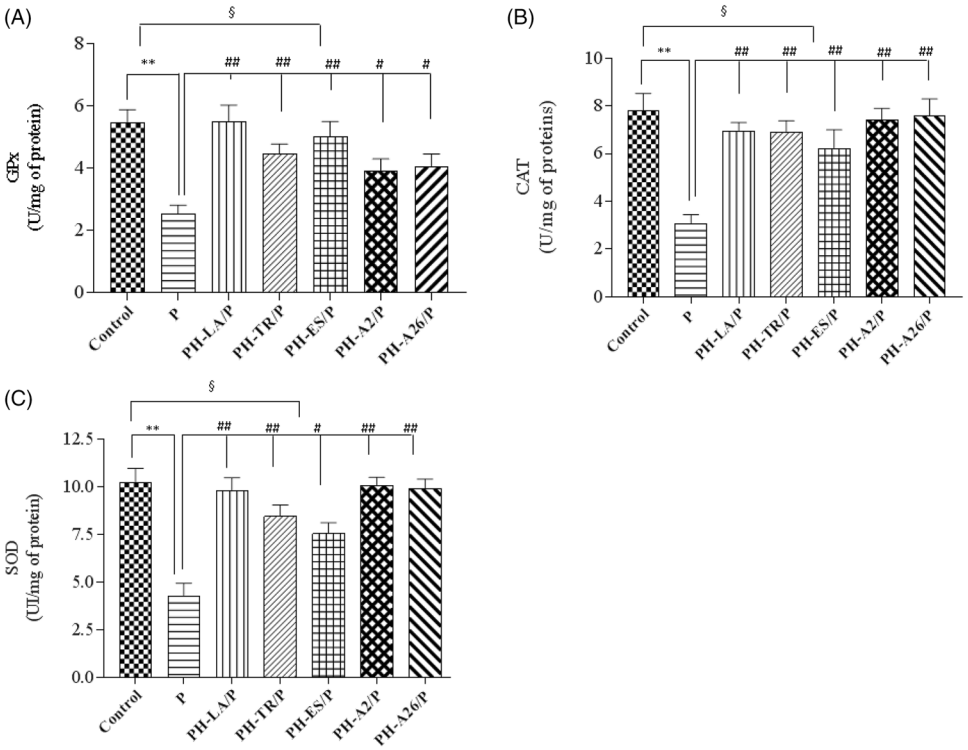

Fig. 2 Effect of LAPHs on the activities of glutathione peroxidase (GPx) (**A**), catalase (CAT) (**B**) and superoxide dismutase (SOD) (**C**), of paracetamol induced nephrotoxicity in rats. Control = control group, P = Paracetamol group, PH-LA, PH-TR, PH-ES, PH-A26 and PH-A2, represent *Liza aurata* protein hydrolysates produced using crude enzyme from L. aurata, trypsin, esperase, Bacillus subtilis A26 and Pseudomonas aeruginosa A2, respectively. Data are expressed as mean ± SEM (n = 6). § $p < 0.05$, ** $p < 0.01$ vs. control group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ vs. paracetamol group

experimental animals depresses their antioxidant system due to increased lipid peroxidation and formation of free radicals (Adil et al. 2016). LAPHs administrated in paracetamol treated rats improved significantly the activities of SOD, CAT and GPx compared with paracetamol values. The administration of LAPHs restored the activities of SOD, CAT and GPx levels to baseline values and even above such values (Figs. 2A, B and C). Furthermore, the significant increase in enzyme activities is considered as an adaptive response against free radical derived from oxidative damage. Our results are in agreement with Ben Hamad Bouhamed et al. (2019) that showed that feather protein hydrolysate reduces the oxidative stress in vivo and increase the antioxidant activities. In an in vivo study, Liu et al. (2015) showed the high capacity of protein hydrolysates from *Mactra veneriformis* in free-radical quenching, effectively by amino acids hydrophobic/donor proton. An excellent radical scavenging properties indicate that FPH exert direct effects on radicals (Liu et al. 2015). Here, it is important to note that hydrophobic amino acids can increase the antioxidant activity by enhancing the solubility in lipid and therefore facilitating donation of protons to lipid-derived radicals. In the present experiment, LAPHs were rich in Arg, Asp,Ala, Gly, Glu, Leu, Lys, and Val (Bkhairia et al. 2016). It has been reported that Asp, Arg, Glu, Gly, and Lys exhibited critial role in the metal chelation or hydrogen/electron donating activity and interrupting the radical chain reactions and/or inhibit their formation. In addition, Liu et al. (2015) confirmed that Ala, Leu, and Val are hydrophobic amino acids which have strong antioxidant abilities. As proved by this experiment, LAPHs exhibited therapeutic potential by stimulating the defense system in rats and showed satisfactory antioxidant efficacy by modulating antioxidant enzymes.

Prevention of Paracetamol-Induced DNA Damage

Figure 3A1, which depicts genomic DNA analysis by agarose gel electrophoresis, confirmed paracetamol mediated apoptosis and displayed marked DNA damage and degradation in kidney cells.

The densitometric analysis revealed a decrease in the percentage of DNA band intensity by 72.27%, compared to control group (Fig. 3A2), corroborates the alterations observed in the genomic DNA isolated from paracetamol treated rats.

Flaks and Flaks (1983) reported that paracetamol can increase the mutation rates through oxidative damage. It is known that ladder pattern of fragmentation is generally observed in apoptosis due to endonuclease mediated internucleosomal fragmentation of DNA. As illustrated in Fig. 3, it was noticed that the genomic DNA isolated from kidney tissues of LAPHs treated groups (lanes 4, 6 and 7), showed partial degradation with mild smearing which may be attributed to the residual damage in the heavily injured cells associated with the ongoing recovery, repair, and renovation processes. Indeed, in the presence of PH-LA and PH-ES, bands intensities were completely restored (Fig. 3A1: lanes 3 and 5, respectively). Besides, quantitative evaluation of DNA protection against paracetamol nephrotoxicity, showed that in presence of PH-LA and PH-ES band intensity was nearly to 90% compared to genomic DNA from control group. Ktari et al. (2017) have reported that the administration of protein hydrolysates from zebra blenny protected heart cells and prevented DNA fragmentation. It has been already reported that LAPHs showed significant protection against paracetamol induced liver injury through a free radical scavenging mechanism of some potent bioactive peptides (Bkhairia et al. 2018).

These results, showed that the protection of the genomic DNA could be caused by the DNA repair enzyme(s) such as OGG1, stimulated by LAPHs, which must be present in the nucleus to reduce the 8-oxo-deoxyguanosine (8-oxodG) incision activity.

Effect of LAPHs of COX-2 and TNF-α Genes Expression

As depicted in Fig. 3B1, B2, results revealed a significant increase in mRNA levels of TNF-α and COX-2 in kidneys of paracetamol nephrotoxic group, compared with control.

The obtained results were similar to those described by Horng et al. (2016) and Ko et al. (2017). El-Agamy et al. (2014) reported that ROS-mediated inflammation plays an important role in the pathogenesis of paracetamol nephrotoxicity. Free radical overproduction activates nuclear transcription factor kappa-B (NF-κB) and, consequently, increases the expression of several pro-inflammatory genes, including cytokines and chemotactic factors such as COX-2 and TNF-α. Ko et al. (2017) and El-Agamy et al. (2014) have noted that paracetamol treatment can induce an inflammatory reaction with increased production of TNF-α in the renal tissue. In contrast, TNF-α and COX-2 genes expression in LAPHs treated groups was decreased significantly compared to paracetamol group. It is of interest to note that the most pronounced decrease was observed in TNF-α and COX-2 mRNA expression for PH-LA and PH-ES groups. Thus, blockade of COX-2 and TNF-α can be an effective in counteracting in paracetamol induced renal damages. Furthermore, it is evident that the anti-inflammatory property of LAPHs can be due to the collective effect from various peptide chains rather than from a single bioactive peptide. Based on our previous findings Bkhairia et al. (2016), showed that LAPHs have high antiradical activity and an ability to chelate iron ions it can be concluded that LAPHs can inhibit expression of COX-2. The mechanism of inhibition of COX-2 and TNF-α expression in LAPHs treated groups might be due

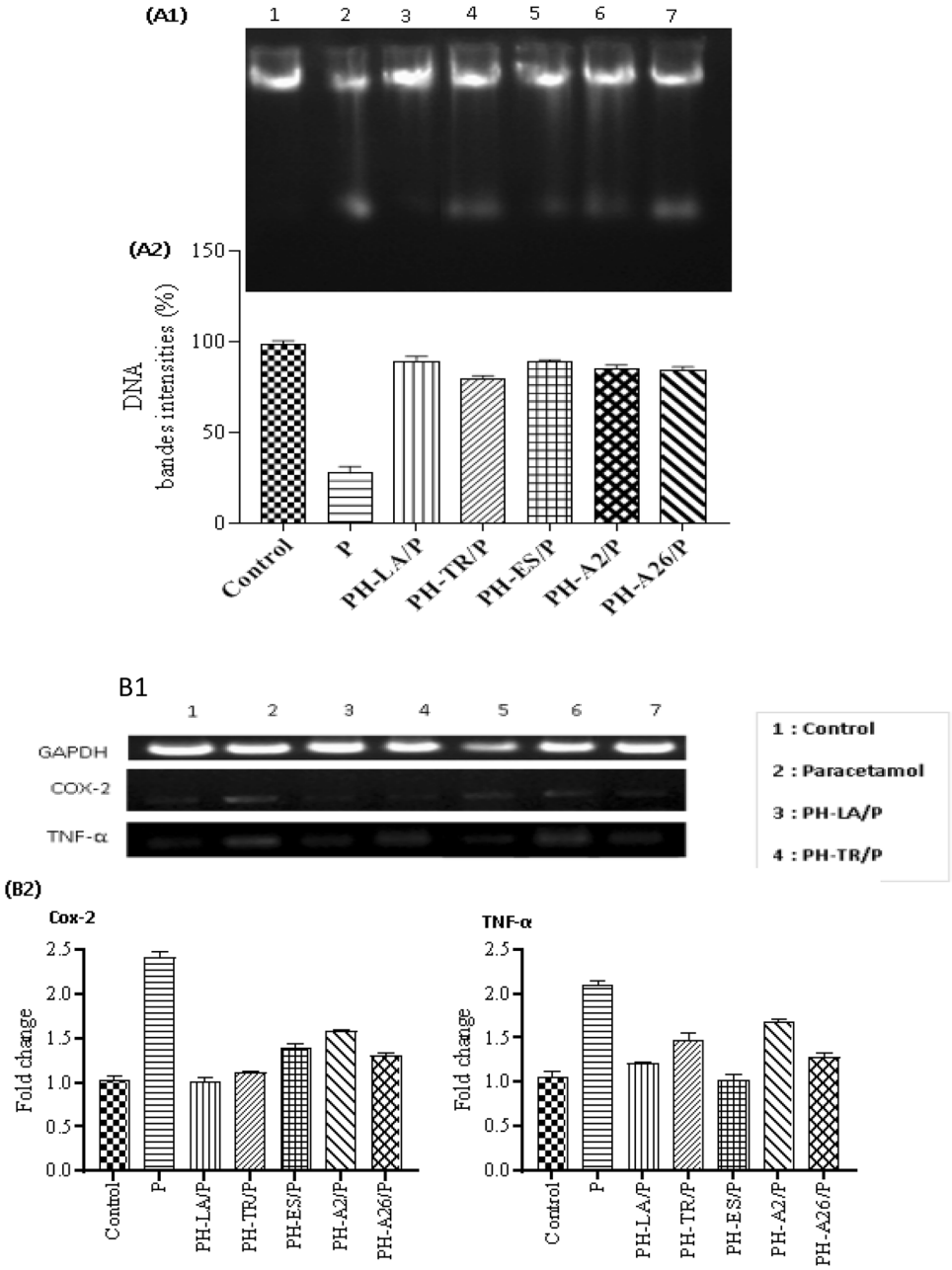

Fig. 3 **A1** Effect of paracetamol/or LAPHs/P on DNA damage. **A1** Agarose (1%) gel electrophoresis of DNA obtained from rat kidney, where Control = normal control group, P = Paracetamol group, and PH-LA/P, PH-TR/P, PH-ES/P, PH-A2/P and PH-A26/P group, represent *Liza aurata* protein hydrolysates produced using crude enzyme from *L. aurata*, trypsin, esperase, *Bacillus subtilis* A26 and *Pseudomonas aeruginosa* A2, respectively and intraperritonialy injected by paracetamol during the last 5 days of treatment. **A2** DNA bandes intensities vs. control group. From the gel image and intensities it is evident that there is intact DNA in the control group. paracetamol treatment introduced DNA damage, marked by the smearing of DNA. Whereas, there is a decrease in DNA smearing with different LAPHs (350 mg/kg bw)/P (325 mg/kg bw). **B1** Pro-inflammatory cytokines TNF-α and cyclooxygenase-2 (COX-2), genes expression in the kidney male rats of treated with paracetamol (P)/or LAPHs/P. GAPDH expression was used as a loading control. **B2** The bar graphs show quantitative relative levels of TNF-α, COX-2, genes expression in paracetamol (P) and PH-LA/P, PH-TR/P, PH-ES/P, PH-A2/P and PH-A26/P treated groups vs. control group

to the interaction of peptides contained in the LAPHs, with the free radicals, leading to antioxidative protection. In agreement with these results, similar conclusions were also reported by Zielińska et al. (2017) who found that there was a relationship between antioxidant potential of protein hydrolysates and anti-inflammatory activity.

Our findings indicated that the protective effects of LAPHs might reflect its function as an antioxidant and antiapoptotic agent. Obviously, this can be regarded as a compensatory mechanism aimed to promote free radical production, induce antioxidant defense and diminish inflammatory damage through pro-oxidant activity.

Histological Assessment

The kidney is a common target for toxic xenobiotics, due to its capacity to extract and concentrate toxic substances, and to its large blood flow share. The morphological changes in the kidneys are shown in the Fig. 4 to support the effect of LAPHs on paracetamol-induced kidney injury.

The kidney tissues in paracetamol rats clearly revealed severe tubular necrosis with dilatation, vacuolar degeneration, epithelial desquamation, together with intraluminal cast formation mainly in the distal convoluted tubules, confirmed

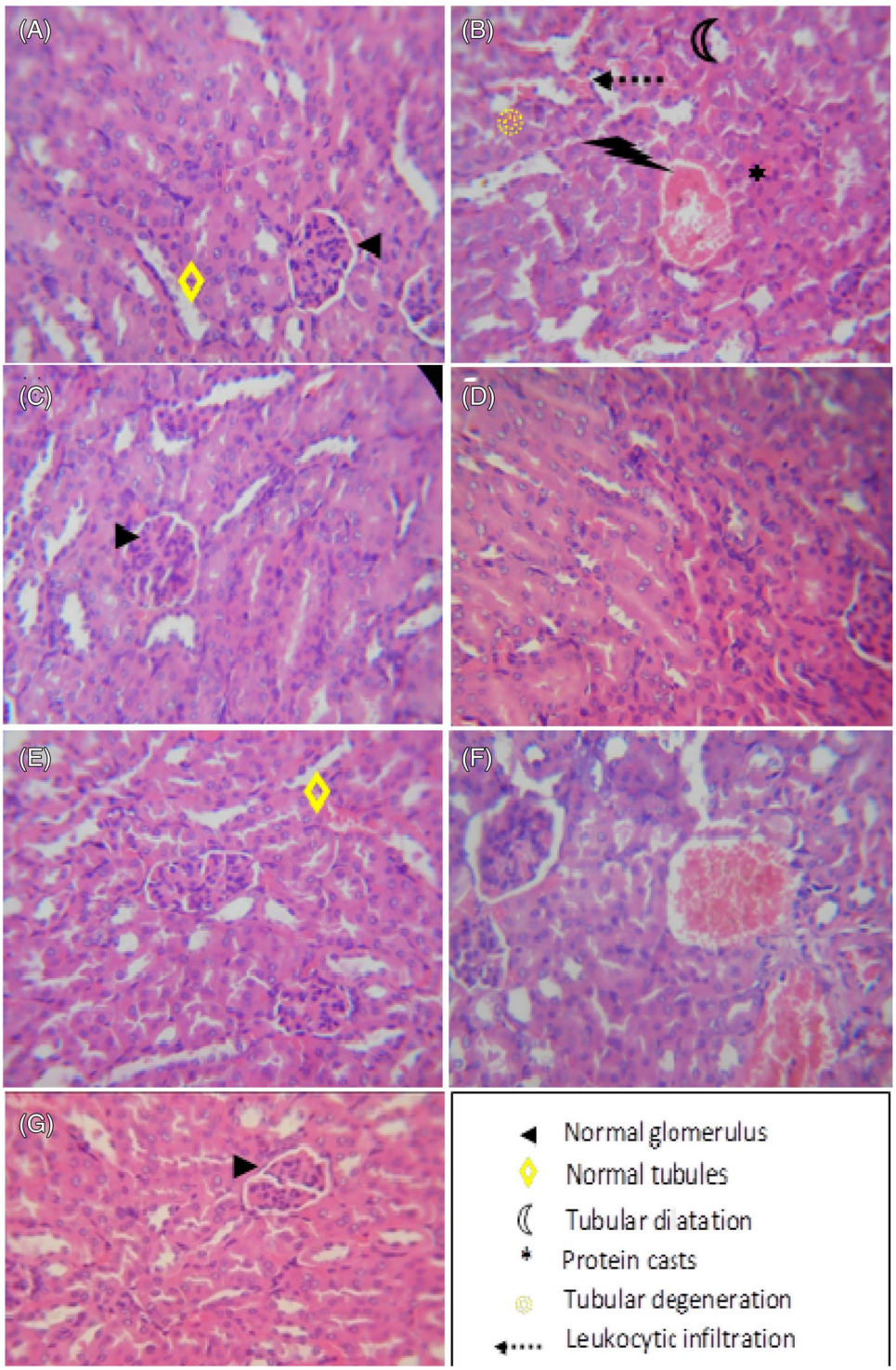

Fig. 4 Representative photographs of kidney sections from control (**a**), paracetamol (**b**) and PH-LA/P, PH-TR/P, PH-ES/P, PH-A2/P and PH-A26/P treated groups. Optical microscopy; hematoxylin–eosin stain (200×)

the biochemical results (Fig. 4b). Our results are in agreement with the previous studies of Reshi et al. (2017).

The kidney architectures of rats treated with LAPHs were noted to undergo marked improvements, indicating the good effect of these hydrolysates against nephrotoxicity. This observation indicated the effectiveness of LAPHs peptides particularly, PH-LA and PH-ES, with which the kidney was completely corrected and renal tissues were apparently normal, (Fig. 4c, e). Rats treated with PH-TR and PH-A26 showed the presence of minimal degree of infiltration and mild tubular degeneration and flattening of proximal tubular cell (Fig. 4d and g). This observation clearly showed a better morphology of kidney tissue in protein hydrolysates treated groups compared with the nephrotoxic groups. The

findings obtained via biochemical assays were further confirmed by histopathological study. Similar results were previously reported by Nasri et al. (2015) showing that protein hydrolysates from the goby fish contain bioactive peptides that possess significant antioxidant properties, and ameliorate renal damage in rats. The histopathological changes are graded and summarized in Table 4.

Fractionation of PH-LA by Size Exclusion Chromatography and RP-HPLC Separation

In order to isolate antioxidant and anti-inflammatory peptides, PH-LA, which exhibited the highest renoprotective potential resulting in antioxidant defense, regulation of Cox-2 and TNF-α expression, was fractionated by size exclusion chromatography on a Sephadex G-25 column followed by reverse phase-high performance liquid chromatography (RP-HPLC).

As illustrated in Fig. 5A, the elution profile showed five separated peaks (F1-F5). The yields of F1, F2, F3, F4 and F5 fractions were 15.11%, 14.23%, 18.47%, 12.08% and 2.69%, respectively.

The fractions associated with each peak were pooled, freeze-dried and evaluated for antioxidant and anti-inflammatory activities. Since natural antioxidants are characterised by complex reactivity and different mechanisms of action, the antioxidant capacity of biopeptides cannot be assessed by using a single method (Tenore et al. 2015). Then, three different spectrophotometric assays, ABTS, DPPH and FRAP tests, were executed.

Thus, fraction F2 and F4 obtained from size-exclusion chromatography that displayed the highest anti-inflammatory (Fig. 5d) and antioxidant activities (Fig. 5a, b and c) was further separated by RP-HPLC (Fig. 6) on a Aeris PEPTIDE 3.6 m XB-C18 New Column 250 × 4.6 mm (Phenomenex). The RP-HPLC profile, of F2 and F4, reported in Fig. 6A and B revealed a very large number of peaks relating to the abundance of peptides generated, and some peaks showed anti-inflammatory and antioxidant activities.

Conclusion

In conclusion this study's, the present findings revealed the protective effects of LAPHs against paracetamol-induced renal toxicity. Its mechanism may be due to the potential of LAPHS in suppressing of the generation of ROS, lipid peroxidation and oxidative stress, generation of TNF-α and COX-2 and DNA damage in kidney tissues. Overall, the present study provides important information about the in vivo antioxidant activity of LAPHs. Peptidic fractions, F2 and F4 obtained from SEC, were found to be the effective anti-inflammatory and antioxidant in different in vitro assays. LAPHs had a considerable protec-tive effect on kidney damage induced by paracetamol. They not only possessed effective protective action but also curative action. They mitigated the nephrotoxicity of paracetamol overdose by different pathways such as increasing the total antioxidant potential of renal cells and regulation of Cox-2, and TNF-α expression.

Finally, our study show the interesting properties and potential medical use of LAPHs that could open new promising opportunities for the production of efficient, safe, and cost-effective bioactive peptides.

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Declarations

Conflict of interest Sofiane Ghorbel declares that he has no conflict of interest. Intidhar Bkhairia declares that she has no conflict of interest. Sabah Dhibi declares that she has no conflict of interest; Maria Maisto declares that she has no conflict of interest; Othman A Alghamdi declares that he has no conflict of interest, Moncef Nasri declares that he has no conflict of interest, and Gian Carlo tenore declares that she has

Table 4 Grading of the histopathological changes on polymorphonuclear (PMN) infiltration in the kidney of control and treated rats with paracetamol (P), or with LAPHs/P

	C	P	PH-LA/P	PH-TR/P	PH-ES/P	PH-A2/P	PH-A26/P
Infiltrated neutrophils	−	+++	+	++	+	++	++
Congestion	−	+++	+	++	+	++	+
Focal necrosis	−	+++	−	+	+	+	++
Glomerular hypertrophy	−	+++	−	++	+	++	+
Inflammatory infiltration	−	+++	−	+	−	++	+
Edema	−	++	−	+	−	++	+
Pyknosis	−	++	−	+	−	+	+

Histological changes were graded based on findings of necrosis and congestion after treatment by toxic dose of paracetamol. Scoring system included: (−) for none (no change), (+) for mild changes, (++) for moderate changes and (+++) for severe changes

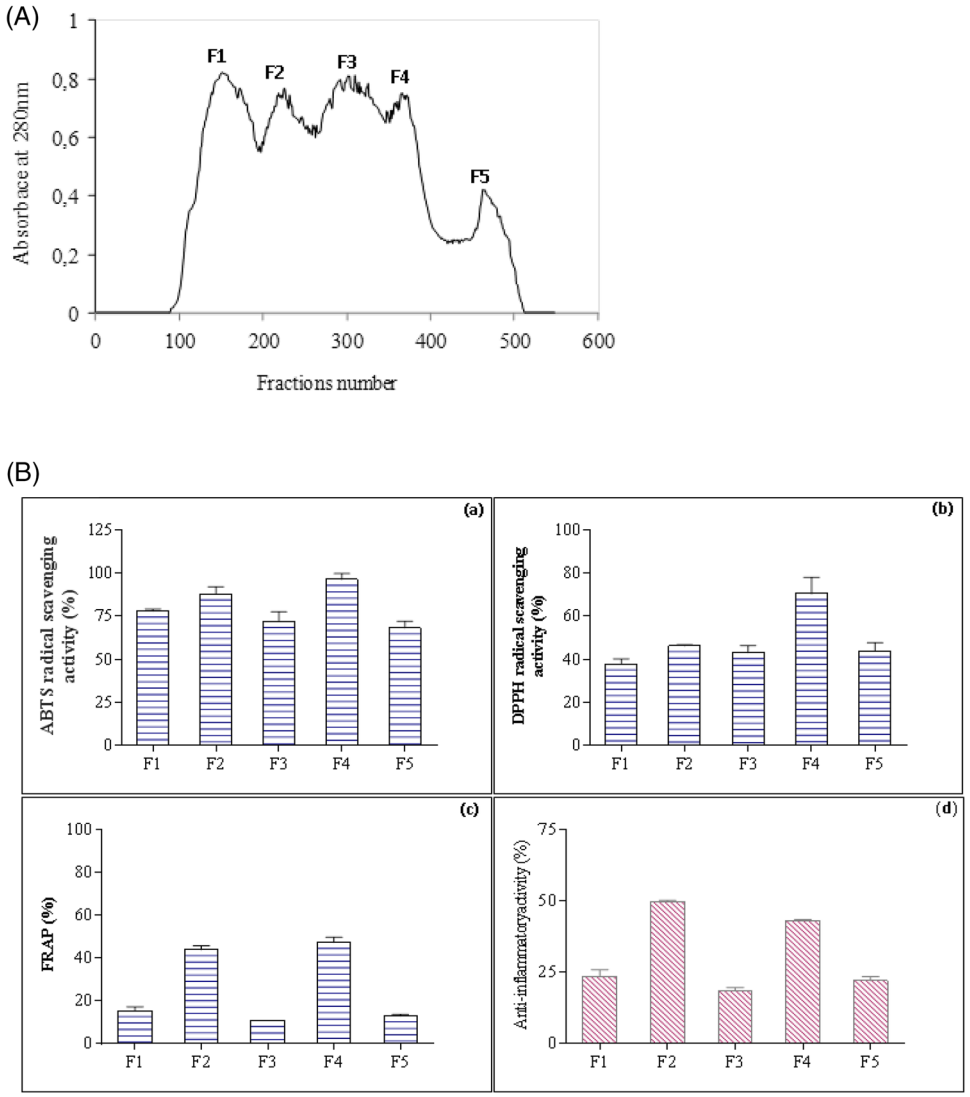

Fig. 5 Fractionation of PH-LA on a Sephadex G-25 gel filtration column (**A**). Fractions were collected and assayed for their antioxidant activities (**B**) (ABTS (a) DPPH radical scavenging activity (b) and FRAP (c)) and anti-inflammatory activity (d)

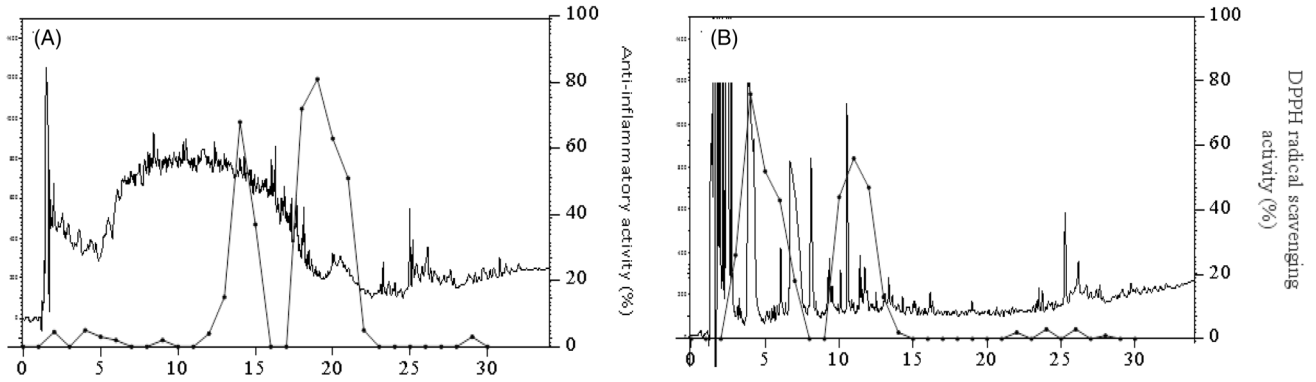

Fig. 6 The selected ractions (F2 and F4) were pooled and separated by reversed-phase chromatographic and automatically collected and assayed for the DPPH radical scavenging activity and ant-inflammatory activity

no conflict of interest. The author declares that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Research Involved in Human or Animal Participants This article does not contain any studies with human participants by any of the authors.

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