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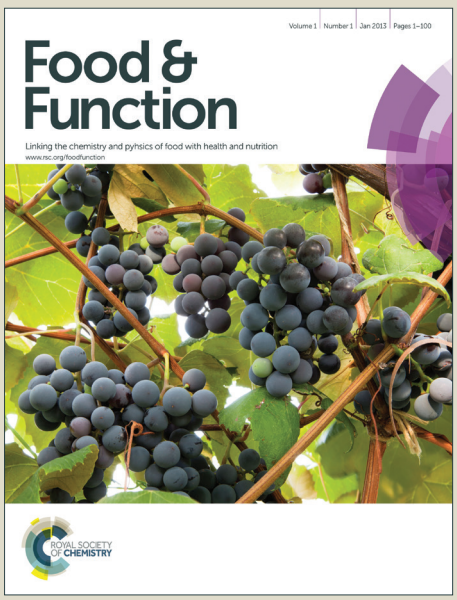

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Cholesterol regulatory effects and antioxidant activities of protein hydrolysates from zebra blenny (*Salaria basilisca*) in cholesterol-fed rats

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Abstract

This study aims to explore the hypocholesterolemic effects and antioxidative activities of zebra blenny protein hydrolysates (ZBPHs) in rats fed a hypercholesterolemic diet. The rats were fed during eight weeks a standard laboratory diet (normal rats), a high-cholesterol diet (HCD) (1%) or a HCD and orally treated with ZBPHs or undigested zebra blenny proteins (UZBP) (400 mg/kg/day). Results showed that hypercholesterolemic diet induced the elevation of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C). Treatment with ZBPHs increased the level of high-density lipoprotein cholesterol (HDL-C) and decreased significantly the levels of TC, TG, and LDL-C. In addition, ZBPHs treatment showed a significant normalization of thiobarbituric acid-reactive substances (TBARS) levels as well as catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities in renal and hepatic tissues. Furthermore, ZBPHs may also exert significant protective effects on liver and kidney functions, evidenced by a marked decrease in the level of serum urea, uric acid, creatinine, alkaline phosphatase (ALP), and alanine aminotransferase (ALAT). Histological studies confirmed that ZBPHs effectively protected the livers and kidneys against hypercholesterolemia-mediated oxidative damage. Therefore, the study strengthens the hypothesis that ZBPHs can be used as a novel antioxidant and hypocholesterolemic compounds against hyperlipidemia induced atherosclerosis.

Keywords: Zebra blenny (*S. basilisca*), protein hydrolysate, antioxidant, hypocholesterolemic.

1. Introduction

Hypercholesterolemia is one of the primary risk factors for coronary arteries damage. It has been linked to the development of various cardiovascular diseases (CVD)[1], leading to death in the developed countries. Various studies have demonstrated that the increased formation of free reactive oxygen species (ROS) contributes to CVD progression.[2, 3] In fact, large amounts of ROS production decrease the intracellular antioxidant defense, causing lipid peroxidation, protein oxidation, and DNA breaks.[4]

Dietary proteins and protein hydrolysates have often been reported to regulate human functions. Previous findings demonstrate that protein hydrolysates from several origins (e.g., fish, soybean, milk and sunflower)[5,6,7,8] may promote a significant decrease in blood cholesterol concentration. Wergedahl et al.[9] have reported a decrease of hepatic cholesterol concentration in obese rats by fish protein hydrolysates. In addition Ben Khaled et al.[5] have found an increase of HDL-C and a decrease of TC, TG, and LDL-C levels in rats fed with cholesterol enriched diet with sardinelle protein hydrolysates or whole sardinelle protein. Thus could be explained according to previous reports[6,7] by a reduction of cholesterol biosynthesis, suppression of cholesterol micellar solubility, changes in bile acid synthesis and a reduced absorption of lipid cholesterol and bile acid.

Zebra blenny (*Salaria basilisca*), belonging to Blenniidae family, is a very common benthic fish known to generate bioactive peptides under enzymatic hydrolysis. Our earlier studies demonstrated that treatment with zebra blenny protein hydrolysates (ZBPHs) obtained by treatment with crude enzymes from the same species (ZBPH-Z), sardinelle (ZBPH-S) and smooth hound (ZBPH-SH) significantly attenuated hyperglycemia and hyperlipidemia and could attenuate diabetic renal damage and hypertension and the levels of oxidative stress in alloxan-induced diabetic rats.[10,11] Accordingly, the present work was undertaken to investigate the potential effect of ZBPHs to lower serum cholesterol and oxidative stress levels in rats fed cholesterol-supplemented diets.

2. Materials and Methods

2.1. Fish samples

Zebra blenny (*S. basilisca*), sardinelle (*Sardinella aurita*) and smooth hound (*Mustelus mustelus*) were freshly purchased from the fish market of Sfax City, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1 : 3 (w/w) and transported to the research laboratory within 30 min. After washing the fish, their viscera and the muscle were separated and rinsed with cold distilled water. The muscle of zebra blenny was stored in the sealed plastic bags at -20 °C until use for protein hydrolysate production. The viscera were used immediately for the extraction of digestive enzymes.

2.2. Preparation of endogenous enzyme extracts and ZBPHs

Viscera (150 g) from zebra blenny, sardinelle, and smooth hound were homogenized for 1 min with 300 ml of extraction buffer (10 mM Tris–HCl, pH 8.0). The homogenates were centrifuged at 8500 rpm for 30 min at 4 °C. The pellets were discarded, and the supernatants were then collected and used as the crude alkaline protease extracts for the production of zebra blenny protein hydrolysates (ZBPHs) as previously reported by Ktari et al.[12] The obtained hydrolysates with the crude alkaline protease extracts from zebra blenny, sardinelle, and smooth hound were referred to ZBPH-Z, ZBPH-S, and ZBPH-SH, respectively. UZBP was used as control.

2.3. Characterization of ZBPHs by size exclusion chromatography

Peptide fractions of the hydrolysates were separated using column chromatography as described by Chen et al.[13] The lyophilized hydrolysate (200 mg/mL was fractioned by gel filtration on Sephadex G-25 column (3 cm × 53 cm), and eluted with deionized water. Each fraction (4 mL) was collected at a flow rate of 30 mL/h, and monitored at 280 nm the

absorbance to separate peptide fractions. cytochrome C (13400 Da), B12 vitamin (1355.5 Da), insulin (5800 Da) and glutathion (307.3 Da) were used as the comparable standards of molecular weight. Determinations were performed in triplicate. Data were expressed as means followed by standard deviations which were in all cases lower than 5%.

2.4. Animals and treatment

In the present study adult male Wistar rats, weighing 200±10 g, purchased from the Central Pharmacy (SIPHAT, Tunis, Tunisia), were used. All rats were kept in a controlled breeding room (temperature: 22±2 °C, humidity: 60±5%, 12 h dark/light cycle) and they had free access to tap water and alimentation. The standard diet was supplied by Society of Animals Nutrition (Sfax, Tunisia), and composed of corn, soya and vitamins minerals compound.[11] The animals were maintained in accordance with the international guidelines for Care and use of laboratory animals and approved by the Local Animal Care Committee at Sfax University.

A total of 42 rats were used in the experimental assays of the present study. They were randomly divided into 7 groups of six each.

Group 1: rats were fed with the standard diet (Normal rats (NR)).

Group 2: rats were fed daily with a HCD prepared by adding 10 g cholesterol/kg diet + 1 g cholic acid/kg diet to standard diet for eight weeks (hypercholesterolemic rats (HCR)).

Group 3: rats were fed daily with a HCD and treated with a cholesterol-lowering drug (Crestor) by gastric gavage at 10 mg per kg of body weight for eight weeks (HCR+Crestor).

Groups 4, 5, 6, and 7: rats were fed daily with a HCD and treated with ZBPH-Z (HCR+ZBPH-Z), ZBPH-S (HCR+ZBPH-S), ZBPH-SH (HCR+ZBPH-SH), and UZBP (HCR+UZBP), respectively, by gastric gavage at 400 mg per kg of body weight for eight weeks.

2.5. Collection of blood and tissue samples

At the end of the treatment period, the rats were killed by cervical decapitation to avoid animal stress. The blood was collected without anticoagulant for serum separation. Livers and kidneys from control and experimental groups of rats were excised and cleaned. Some livers and kidneys were rinsed, homogenized in Tris Buffered Saline (0.05 M, pH 7.4) and centrifuged at 10.000 × g for 10 min at 4°C. The supernatants were collected and stored at -80°C until analysis. The amount of protein in the supernatant was measured, according to the method of Lowry et al.[14] using bovine serum albumin as standard. Other samples were immediately fixed in 10% buffered formalin solution for histological studies.

2.6. Serum and liver homogenate analysis

Serum urea, uric acid and creatinine levels were estimated using commercially available diagnostic kits (Biomagreb, Tunisia). Serum alanine aminotransferase (ALAT) and alkaline phosphatase (ALP) activities, and serum and hepatic lipid levels of TG, TC, HDL-C, LDL-C were measured in frozen aliquots of serum or liver homogenate by standardized enzymatic procedures using commercial kits from Biolabo (Maizy, France) on an automatic biochemistry analyzer (Vitalab Flexor E, USA).

2.7. Antioxidant defense system assays

2.7.1. Catalase activity

Catalase activity was assayed by the decomposition of hydrogen peroxide according to the method of Aebi.[15] Twenty microliters of the supernatant were added to a cuvette containing 780 µl of a 100 mM potassium phosphate buffer (pH 7.4), and then the reaction was initiated by adding 200 µl of 500 mM H_2O_2 to make a final volume of 1.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was expressed as µmol H_2O_2 consumed/min/mg of protein.

166 *2.7.2. Superoxide dismutase (SOD) activity*

167 SOD activity was measured according to the method of Asada et al.[16] An aliquot (50
168 µl) of liver or kidney extract supernatant (diluted 20 times) was mixed with 1 ml of
169 ethylene-diaminetetraacetic acid (EDTA) methionine, tampon PO_4, NBT and riboflavin and
170 then incubated under the light at 25 °C for 25 min. The activity was measured at 580 nm.
171 One unit was determined as the amount of enzyme that inhibited the oxidation of NBT by
172 50%. The activity was expressed as units of SOD/mg protein.

173

174 *2.7.3. Glutathione peroxidase (GPx) activity*

175 GPx was measured according to Flohe and Gunzler.[17] Briefly, the reaction mixture
176 contained 0.1 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml of tissue homogenized in
177 phosphate buffer (0.1 M, pH 7.4), 0.2 ml glutathione (4 mM) and 0.5 ml H_2O_2 (5 mM). The
178 mixture was incubated for 10 min at 37 °C, 1 ml 5% trichloroacetic acid (TCA) was then
179 added to precipitate proteins and centrifuged at 3200 × *g* for 20 min. The supernatant was
180 assayed for glutathione content using DTNB (5,52 -dithiobisnitrobenzoic acid) reagent (10
181 mM). The activity was expressed as µmol of GSH consumed/min/mg of protein.

182

183 *2.8. Thiobarbituric acid-reactive substances (TBARS) assay*

184 Lipid peroxidation was estimated colorimetrically by measuring TBARS in liver and
185 kidney tissues by the method of Buege and Aust.[18] 125 µl of each liver or kidney extract
186 supernatant was mixed with 50 µl of TBS and 125 µl of TCA-BHT, vortexed and then
187 centrifuged at 1000 rpm for 10 min. An aliquot (200 µl) of the supernatant was mixed with
188 40 µl of HCl and 160 µl Tris-TBA, vortexed and then incubated at 80 °C for 10 min. The
189 resulting absorbance was measured at 530 nm.

190

191

2.9. Assays for hepatic and renal histology

Some livers and kidneys, collected from control and tested rats, were fixed in 10% buffered formalin solution for 48 h. They were subjected to standard routine tissue processing technique and embedded in paraffin. Sections of 3-µm thickness were then stained with hematoxylin-eosin and examined under an Olympus CX41 light microscopy.

Six slides were prepared from each liver and kidney. All sections were evaluated for the degree of liver and kidney injuries. Each liver and kidney slide was examined and assigned for severity of changes using scores on a scale of none (-), mild (+), moderate (++) and severe (+++) damages.

2.10. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student's test for comparison between groups. All values were expressed as means ± SD. A P value of <0.05 was considered significant.

3. Results and discussion

In our experimental study, cholesterol treated rats did not show any sign of death.

3.1. Determination of molecular weight distributions of ZBPHs

In a previous paper, we have shown that at the same enzyme/substrate ratio (E/S=3 U/mg) and after incubation for 7 hours, ZBPH-Z showed the highest degree of hydrolysis (DH = 10%), followed by ZBPH-SH (DH = 9.1%) and ZBPH-S (DH = 6.65%).[12]

Size-exclusion chromatography on Sephadex G-25 was performed to analyze the overall molecular weight distribution of the three hydrolysates. The profiles reported in Fig. 1 revealed the differences in the degree of protein hydrolysis which depending on the crude

enzyme used. The molecular weight distribution of the different hydrolysates divided into seven fractions was shown in Table 1.

In the present study, the results showed that ZBPH-Z, which had the highest DH contained more small-sized peptides with molecular weight below 7000 Da, whereas ZBPH-S, which showed the lowest DH, peptides with molecular weight above 10000 Da were predominant. The obtained results were in accordance with DH observed for ZBPHs.

3.2. Effect of ZBPHs on serum and liver lipid levels in HCD-induced hyperlipidemic rats

Serum and liver levels of TC, TG, LDL-C and HDL-C of control and experimental groups were shown in Table 2. Consumption of the HCD leads to an increase of TC levels (94.87% and 150%) and TG levels (55.44% and 53.59%) in serum and liver of HCR, respectively, as compared to those of control group. Numerous studies have been done to seek the effect of dietary cholesterol on hepatic lipid homeostasis. They suggested that cholesterol stimulates acyl-CoA cholesterol acyltransferase (ACAT) and other enzymes involved in fatty acid biosynthesis, which increase cholesterol esters formation and the synthesis of TG and fatty acids.[19,20,21] There were also a significant increase in serum and liver LDL-C levels by 253.84% and 339.53%, respectively, as well as a decrease in HDL-C levels by 18.31% and 27.78%, respectively, in HCR when compared with those of standard diet-fed rats, indicating that the hypercholesterolemic model was successfully established. The high levels of LDL-C found in HCR may be attributed to a down-regulation in LDL receptors by cholesterol included in the diet.[22]

It is interesting to note that the administration of ZBPHs and UZBP for eight weeks to HCD-fed rats, at a dose of 400 mg/kg, promoted a significant reduction ($p<0.05$) in serum and hepatic TC, TG and LDL-C levels and increase in serum and hepatic HDL-C levels. Indeed, the TG levels decreased by 33.13%, 21.02%, 38.22%, and 21.66% in serum and by 23.62%, 15.96%, 27.03%, and 12.98% in liver, whereas HDL-C levels increased by 29.31%,

31.03%, 43.1%, and 27.58% in serum and by 53.85%, 38.46%, 30.77%, 30.77% in liver after administration of ZBPH-S, ZBPH-Z, ZBPH-SH and UZBP, respectively. We might conclude that Crestor as well as UZBP were found to be less efficient than ZBPHs in the protection against hyperlipidemia. In fact, the strong antihyperlipidemic effects of the ZBPHs might be caused by some potent bioactive peptides obtained by the enzymatic hydrolysis of zebra blenny proteins. This is an important advantage in the prevention and treatment of hypercholesterolemia, particularly among the Tunisians, whose hypercholesterolaemia prevalently presents itself as lipoprotein abnormality.[23]

UZBP given to rats by oral administration are subjected to protease digestion in the gastrointestinal tract, releasing the bioactive peptides. Peptides generated *in vivo* or by fish crude alkaline protease extracts hydrolysis may inhibit cholesterol absorption, possibly due to the suppression of the micellar solubility of cholesterol. Indeed, it is hypothesized that a peptide with high bile acid-binding capacity can inhibit the reabsorption of bile acid in the ileum or decrease the micellar solubility of cholesterol in small intestinal epithelial cells and therefore decrease the blood cholesterol concentration.[24,25] Another hypothesis on cholesterol-lowering mechanism is that some peptides can upregulate LDL receptors, which are chronically suppressed by hypercholesterolemia or dietary cholesterol administration.[26]

In consistence with the present data, a number of other protein hydrolysates have been observed to have similar pattern of hypolipidemic effects. Ben Khaled et al.[5] have reported that administration to hypercholesterolemic rats of protein hydrolysates obtained from sardinelle (*Sardinella aurita*) by treatment with crude enzyme preparations from *Bacillus pumilus* A1, *Bacillus mojavensis* A21 and crude enzyme extract from sardinelle viscera, significantly decreased the levels of TC, TG, LDL-C and the atherogenic index (defined as the ratio of LDL-C and HDL-C) and increased the HDL-C concentrations. Liu et al.[27] also reported that oral administration of protein hydrolysates from *Rhopilema esculentum*

obtained by sequentially treatment with pepsin and papain decreased TC and TG and increased HDL-C in rats fed with high-fat diet.

Lin et al.[28] have reported that freshwater clam protein hydrolysate, obtained by treatment with Protamex, reveals a significant *in vitro* bile acid-binding capacities indicating a strong cholesterol-lowering effect. Likewise, Lin et al.[29] have also reported that cholesterol-enriched diet supplemented with this protein hydrolysate displayed a very pronounced hypocholesterolemic and hypolipidemic effect when administrated to Sprague–Dawley rats. In another study, Iwami et al.[30] have suggested that protein hydrolysates or peptides with high bile acid-binding capacity could inhibit the re-absorption of bile acids in the ileum and reduce blood cholesterol levels. Additionally, Soares et al.[31] have recently shown that major peptides from Amaranth (*Amaranthus cruentus*) protein exhibited an *in vitro* inhibition of the HMG-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis and commonly targeted for drugs capable of a cholesterol-lowering effect[32], suggesting its hypocholesterolemic effect.

Further studies are necessary to clarify the mechanism of action of bioactive peptides from zebra blenny proteins on hypocholesterolemic effect.

3.3. Liver and kidney oxidative damage

Oxidative stress is currently suggested as a mechanism underlying hypercholesterolemia.[33] Lipid peroxidation is initiated by free radical attack on membrane polyunsaturated fatty acids leading to their transformation and fragmentation to alkanes and reactive aldehyde compounds.[34] The measurement of TBARS, the end-product of lipid peroxidation, is commonly used to monitor this process *in vitro* and *in vivo*. Figure 2 showed the levels of TBARS in the liver and kidney tissues of normal and experimental animals. The TBARS levels were significantly increased ($p<0.05$) in both tissues by 247.79% and 58.77%, respectively, when the rats were fed with HCD compared with those of normal rats.

The increase in TBARS levels suggested an enhanced lipid peroxidation leading to tissue damage and the failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals. However, the administration of ZBPH-SH, ZBPH-Z, ZBPH-S, and UZBP to rats fed a HCD yielded a 3.05-fold, 3.17-fold, 2.9-fold and 2.39-fold reduction in liver TBARS levels and 1.77-fold, 1.59-fold, 1.66-fold and 1.41-fold reduction in kidney TBARS levels, respectively, compared to those of HCR group. This normalization might be accomplished by the presence of bioactive peptides in ZBPHs. According to Ktari et al.[12], these peptides have antioxidant activities by preventing the generation of free radicals. The radical scavenging activity of some peptides could be ascribed to the presence of determined amino acids within their sequence which could donate protons to electron-deficient radicals.[35] Furthermore the antioxidant capacity could be affected by the peptide conformation, abundance and also the position of certain amino acids within the peptide sequence.[36]

It was interesting to note that ZBPHs were found to be more efficient than UZBP and Crestor in protection against peroxidative damage under the challenge of oxidative stress associated with hypercholesterolemia. In fact, UZBP and Crestor treated rats showed only 2.39-fold and 1.43-fold reduction in liver TBARS levels and only 1.41-fold and 1.4-fold reduction in kidney TBARS levels, respectively, compared to those of HCR. The results presented above were in agreement with several other findings previously reported in the literature with regards to the hypocholesterolemic effects of protein hydrolysates.[5,37]

3.4. Liver and kidney antioxidant enzyme activities

Key antioxidant enzymes SOD, GPx and catalase limited the effects of oxidant molecules in tissues and acted as free radical scavengers against oxidative cell injury.[38] These enzymes worked together to eliminate reactive oxygen species (ROS). Small deviations in physiological concentrations might have dramatic effects on the resistance of

cellular lipids, proteins and DNA to oxidative damage.[39] As reported in Fig 3A and 3B, the activities of the antioxidant enzymes SOD, GPX and catalase in liver and kidney of rats fed a HCD, respectively, significantly increased as compared to those of NR. The obtained results were in agreement with reports of other workers.[40,41,42] Besides, the increase in antioxidant enzyme activities is seen as an adaptation process against free radicals production. The administration of ZBPHs and UZBP to HCR reverted the SOD, GPx and catalase activities. Further, the results suggested that ZBPHs were more efficient than UZBP in neutralizing oxidative stress. These findings could be attributed to particularly low molecular weight as well as to amino acid sequence and composition of generated peptides which prevent the generation of free radicals. Indeed, according to the literature, it has also been shown that the antioxidant activity of the protein hydrolysates increases with the increase of degree of hydrolysis.[43,44] Further, several works reported that antioxidant peptides are usually constituted by 2 to 10 amino acidic residues.[45]

3.5. Hepatic function evaluation

The development of oxidative stress causes cell damages or tissue injuries. Furthermore, the latters caused functional impairment as evidenced with hepatic function tests, like elevated serum of ALAT and ALP activities and inflammatory mediators which demonstrated the severity of cholesterol induced hepatic damage.[46,47] ALAT and ALP are normally found in circulation in small amounts because of hepatic growth and repair. In the clinical examination, the increased levels of these enzyme activities in serum served as biomarkers for liver injury.[48] The impact of oral administration of Crestor, ZBPHs and UZBP on the hepatic status of experimental rats was studied by estimating liver-function specific enzyme markers like ALAT and ALP (Table 3). The findings revealed that the activities of ALAT and ALP in serum of rats were significantly elevated by 25.5% and 38.1%, respectively, compared to those of controls, following cholesterol-rich diet

346 administration to rats. Consequently, elevated activities of ALAT and ALP observed in the
347 current study could be a common sign of impaired liver function. A significant decrease in
348 these enzyme activities was observed in all the treated groups. When compared to HCR, the
349 ZBPH-SH treated rats showed the highest decrease in ALAT activity (38.12%), followed by
350 ZBPH-S treated rats (37.59%), UZBP treated rats (32.28%) and ZBPH-Z treated rats
351 (21.65%). ZBPH-SH treated rats showed also the highest decrease in ALP activity (37.7%)
352 followed by ZBPH-S, ZBPH-Z, and UZBP treated rats (36.27%, 35.72%, and 34.55%,
353 respectively) ($P<0.05$). It was interesting to note that ZBPHs and UZBP were found to be
354 more efficacious than Crestor in terms of hepatoprotective activity in cholesterol induced
355 liver damage in rats. The present data corroborated with previous studies of our laboratory
356 showing an improvement of hepatic function after administration of fish protein
357 hydrolysates to HCD-fed rats.[5,31] Cholesterol induced hepatic damage appears to confer a
358 second "hit" that results in a distinct hepatic phenotype characterized by increased
359 inflammation. In agreement with these views, Chtourou et al.[47] have reported that the
360 inflammatory response stimulated by addition of cholesterol increased TNF-α, IL-1β and IL-
361 6 expression and levels in liver.
362
363 *3.6. Nephropathy function evaluation*
364 Many reports present the hypothesis that hyperlipidemia is involved in the progression
365 and exacerbation of chronic progressive glomerular and tubulo-interstitial diseases.[49,50,51]
366 Kidney indices of toxicity of our experimental groups were investigated. As shown in Table
367 4, HCD treatment produced a significant ($p<0.05$) increase in serum levels of urea, uric acid,
368 and creatinine by 19.14%, 23.91%, and 5.67%, respectively, in comparison with control
369 group. These results are in accordance with several studies that have showed that uric acid is
370 a powerful scavenger of free radicals and provides 60% of free-radical scavenging capacity
371 in plasma.[52] Interestingly, eight weeks treatment with ZBPHs, Crestor or UZBP significantly

372 reversed these changes to near normal values. Nevertheless, ZBPHs seemed to be more
373 effective than UZBP in attenuating lipid nephrotoxicity. In fact, administration of ZBPH-Z,
374 ZBPH-S, ZBPH-SH and UZBP to HCR decreased significantly the urea levels by 10.8%,
375 19.43%, 23.98% and 7.31%; and creatinine levels by 7.05%, 7.97%, 5.68% and 4.77%,
376 respectively ($p<0.05$) as compared to those of HCR group indicating that ZBPHs could
377 avoid renal damage induced by hypercholesterolemia. Uric acid levels in HCR treated with
378 ZBPHs were significantly lower than that of NR. These results could be explained by an
379 adaptation response of the cell against oxidative damage.
380
381 *3.7. Histopathological studies in liver and kidney*
382 The hepatic tissue defensive effects of ZBPHs in experimental groups of rats were
383 ascertained by histological studies (Figs. 4A). In control group, normal liver histological
384 aspect with distinct hepatic cells and a central vein were observed (Fig. 4A, a). HCD
385 treatment exhibited several histopathological changes, such as a marked leucocytes
386 infiltration, vacuoles formation and steatosis (Figs. 4A, b_1, b_{11}, b_2, and b_{21}). The latter
387 anomaly was known as fatty liver disease and marked by a reversible condition wherein
388 large vacuoles of triglyceride fat accumulate in liver cells. In rats treated with crestor,
389 ZBPHs, and UZBPs, liver histological examination showed a partial alleviation.
390 Interestingly, in ZBPH-Z treated rats, the histopathological changes were completely
391 alleviated indicating the effectiveness of peptides in mediating cholesterol induced
392 hepatotoxicity (Fig. 4A, d).
393 Upon kidney histological examination of NR, renal tissue architecture showed normal
394 glomeruli and tubules (Fig 4B, a).While, kidney in HCR exhibited a narrowed Bowman's
395 space, tubular obstruction, vacuoles formation and an infiltration of leucocytes (Fig 4B, b).
396 According to Ben amara et al.[53], the narrowed Bowman's space could be the result of
397 reduction in the glomerular filtration rate objectified by a reduced creatinine clearance and a

decline of a 24-h urinary excretion of creatinine. Furthermore, the leucocytic infiltrations considered, according to Abdel-Rahman and Zaki[54], as a prominent response of the body tissue facing any injurious impacts. Moreover, Sutton et al.[55] have reported that tubular obstruction was proposed to extend the initial injury to the renal tubules. These modifications could be due to the accumulation of free radicals resulting from an increased lipid peroxidation in the renal tissues.[53]

The severe kidney damages, shown in HCR significantly decreased when Crestor, ZBPHs and UZBPs were administered to HCD-induced hyperlipidemic rats for eight weeks. In fact, in these groups, the kidney histological aspect was similar to that of controls (Figs. 4B, c, d, e, f, and g). The histopathological changes are graded and summarized in Table 5.

Conclusion

The present study showed that zebra blenny protein hydrolysates were efficient in the protection against hyperlipidemia by decreasing serum TC, TG, LDL-C and lipid peroxidation in animals fed a high-cholesterol diet. Furthermore, the hypolipidemic effect of zebra blenny protein hydrolysates could be attributed to some potent hypocholesterolemic peptides. Therefore, they may be considered as a promising functional agent to prevent atherosclerotic disease. However, the accurate mechanism is not yet clear. Further studies are needed in order to check the potential therapeutic use of these hydrolysates.

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Figure captions

Fig. 1. Effects of different treatments on oxidative stress parameters: lipid peroxidation (TBARS) in liver **(a)** and kidney **(b)** of control and experimental groups of rats.

NR are normal rats; HCR are hypercholesterolemic rats; HCR+Crestor, HCR+ZBPH-Z, HCR+ZBPH-S, HCR+ZBPH-SH, and HCR+UZBP are groups of hypercholesterolemic rats treated with Crestor, ZBPH-Z, ZBPH-S, ZBPH-SH, and UZBP, respectively.

Values are given as mean ± S.D. for 8 rats in each group. Values are statistically presented as follows: *P<0.05 significant differences compared to controls, #P<0.05 significant differences compared to hypercholesterolemic rats, @P<0.05 significant differences to diabetic rats treated with Crestor.

Fig. 2. Antioxidant enzyme activities (SOD, GPx and catalase) in liver **(A)** and kidney **(B)** of control and experimental groups of rats. **(a)** Superoxide dismutase (SOD), **(b)** Glutathione peroxidase (GPx) and **(c)** catalase.

NR are normal rats; HCR are hypercholesterolemic rats; HCR+Crestor, HCR+ZBPH-Z, HCR+ZBPH-S, HCR+ZBPH-SH, and HCR+UZBP are groups of hypercholesterolemic rats treated with Crestor, ZBPH-Z, ZBPH-S, ZBPH-SH, and UZBP, respectively.

Values are given as mean ± S.D. for 8 rats in each group. Values differ significantly at p< 0.05. Statistical analysis as in the legend of Fig. 1.

Fig. 3. Histological sections of livers **(A)** and kidneys **(B)** of experimental rats. **(a)** normal rats; **(b, b_1, b_{11}, b_2, b_{21})** hypercholesterolemic rats; **(c)**, **(d)**, **(e)**, **(f)**, **(g)** hypercholesterolemic rats treated with Crestor, ZBPH-Z, ZBPH-S, ZBPH-SH, and UZBP, respectively.

Optic microscopy; hematoxylin–eosin stain; magnification b_1 and b_2: (200×) and a, b, b_{11}, b_{21}, c, d, e, f, and g (400×).

Arrows indicate: (➔) Hepatocyte; (⟶) Central vein; (⟶●) Vacuolization; (- - ➔) Steatosis; (····➔) Leucocyte infiltration; (····●) Bowman's space; (⟶◗) tubular lumen.

Fig. 1.

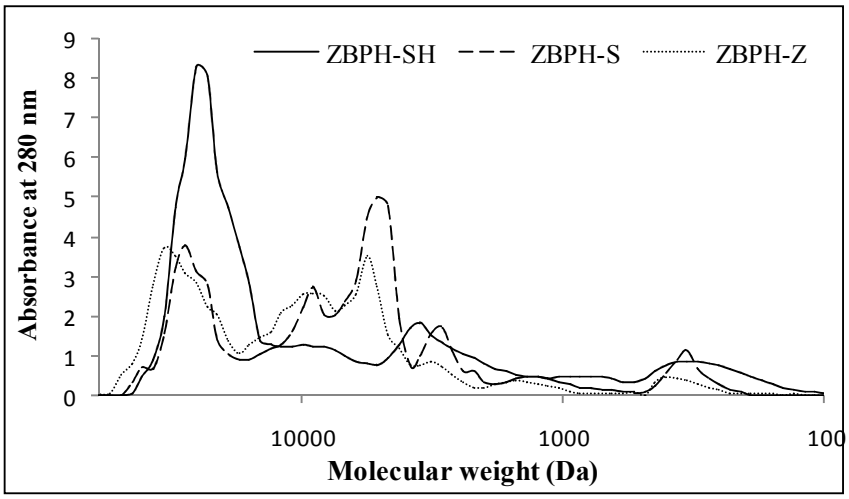


Fig. 2.

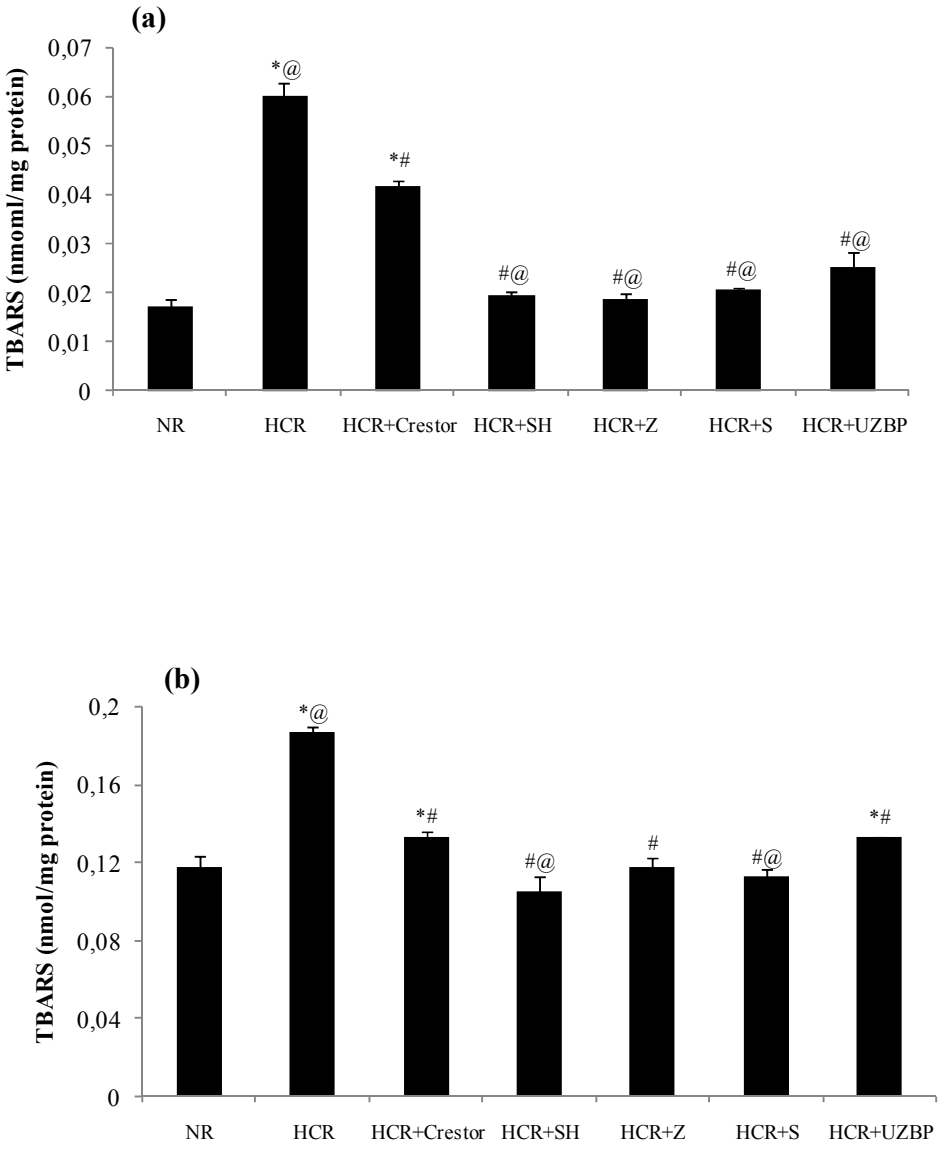
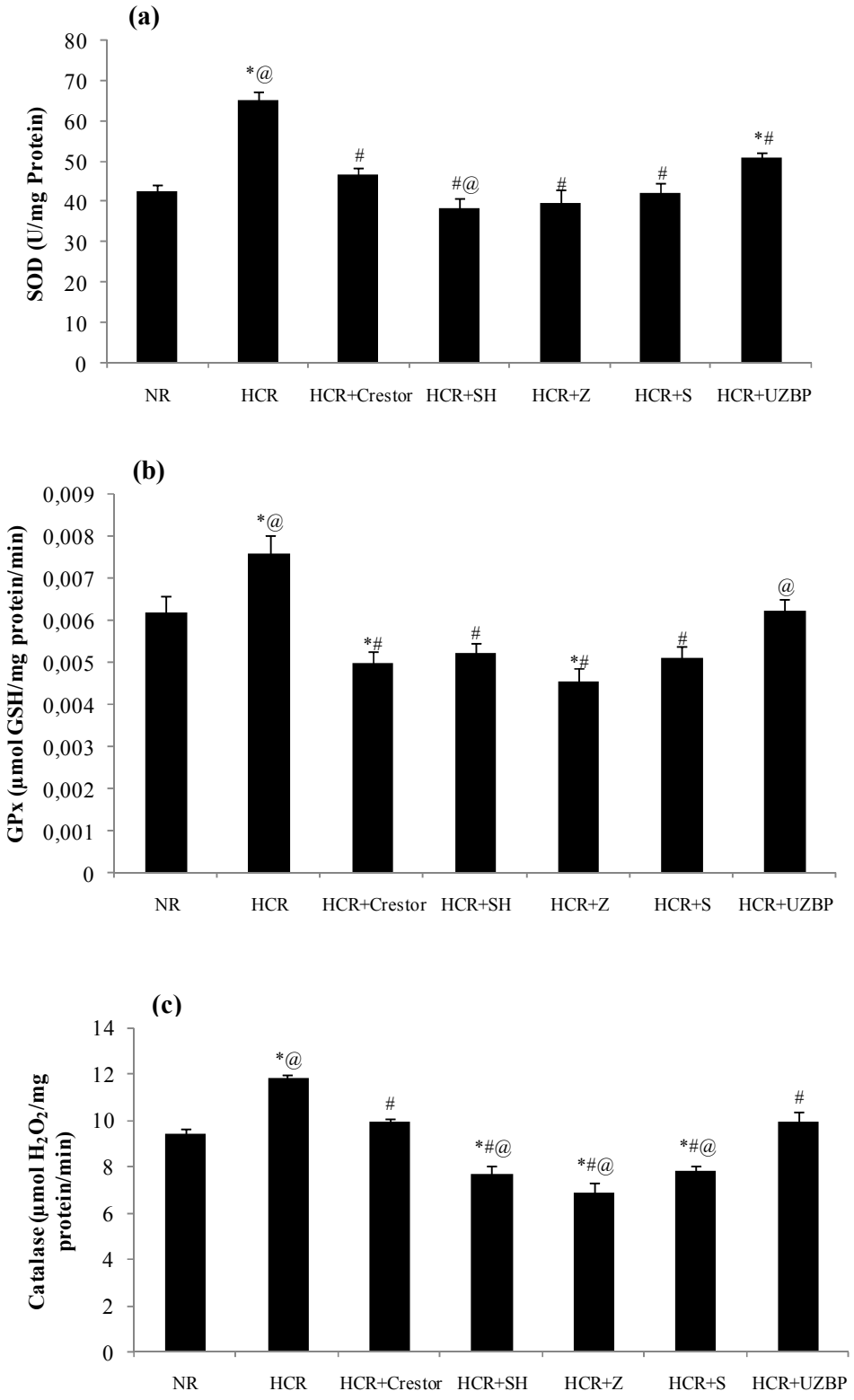


Fig. 3.

(A)



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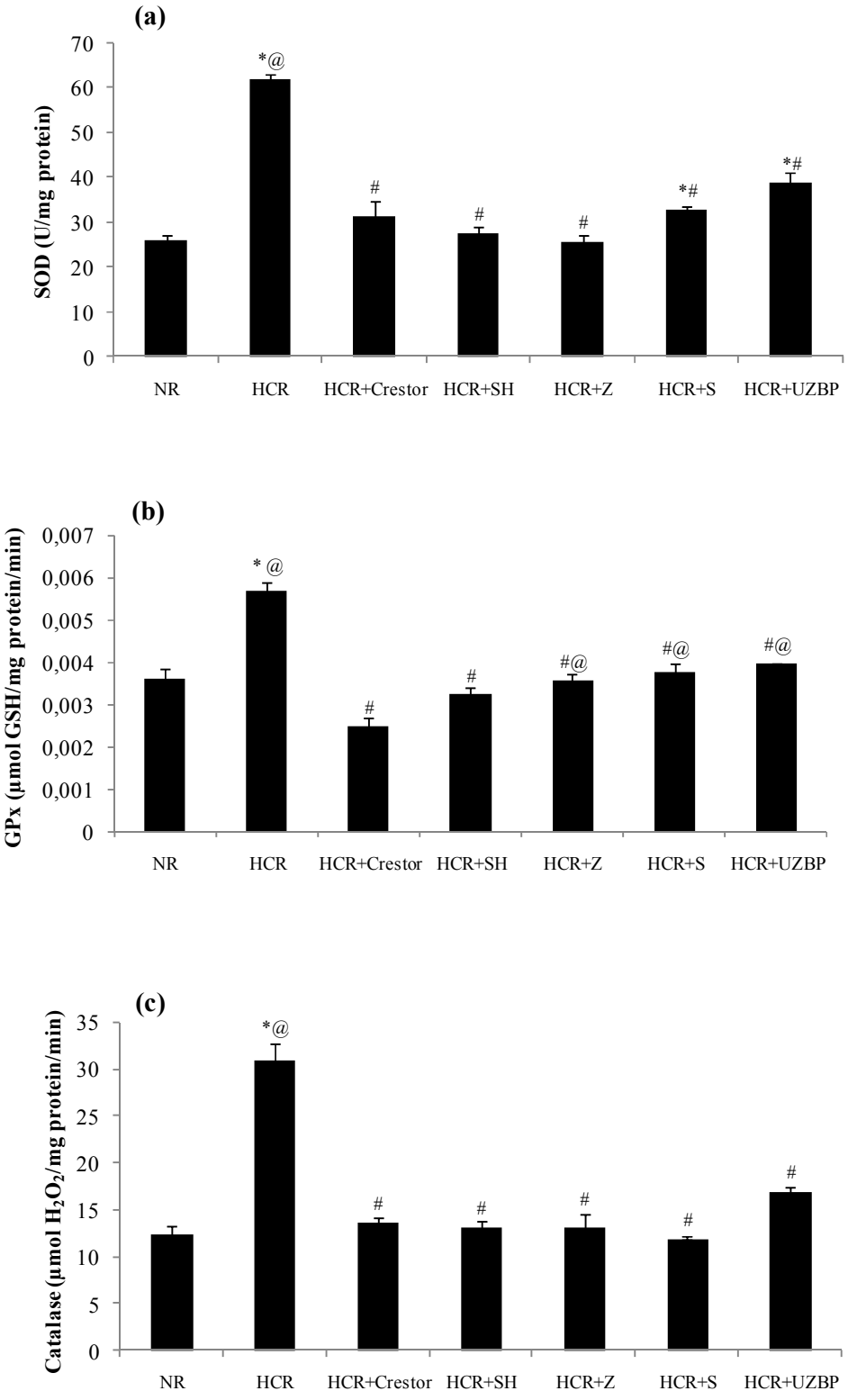
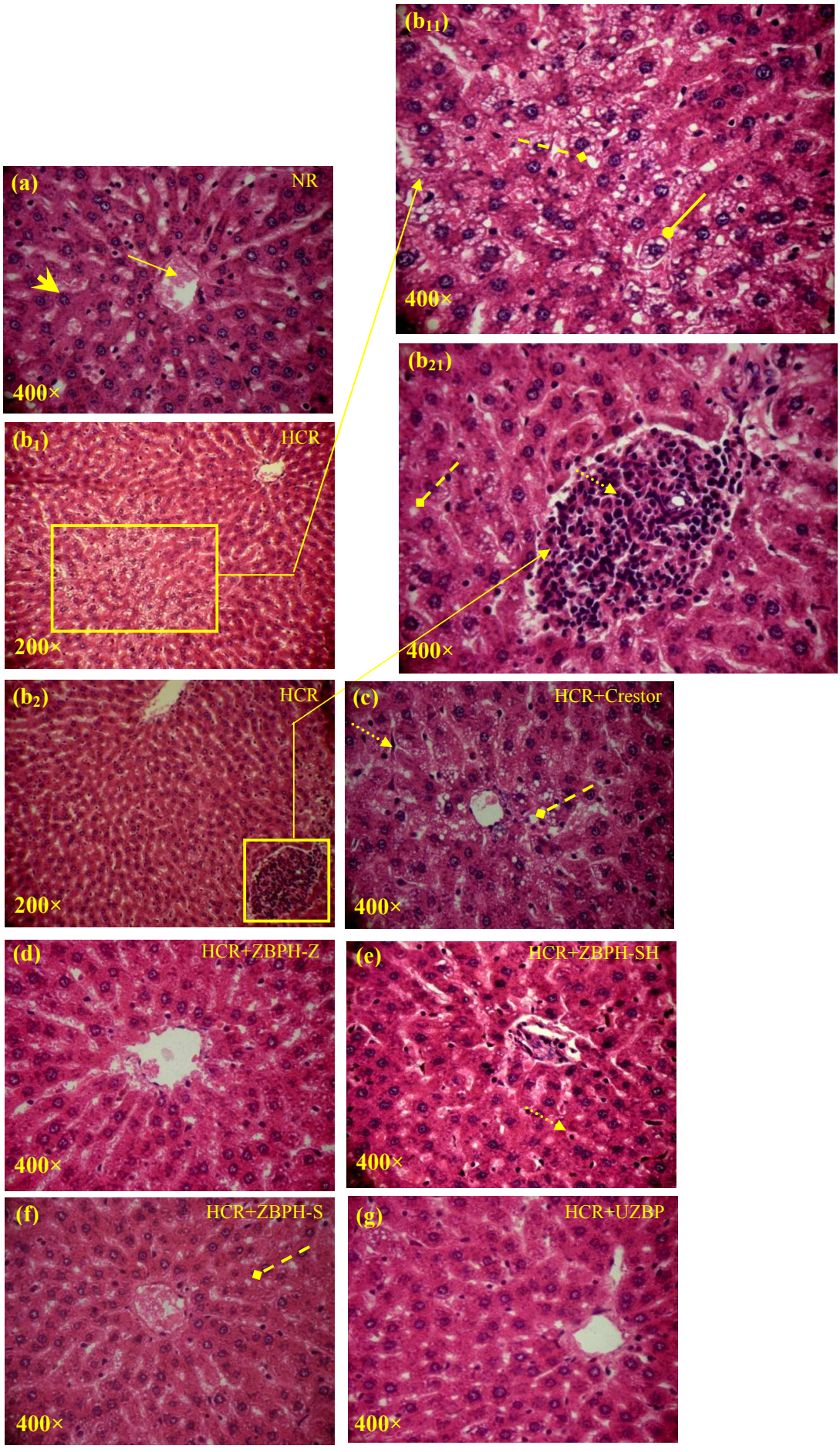


Fig. 4.

(A)



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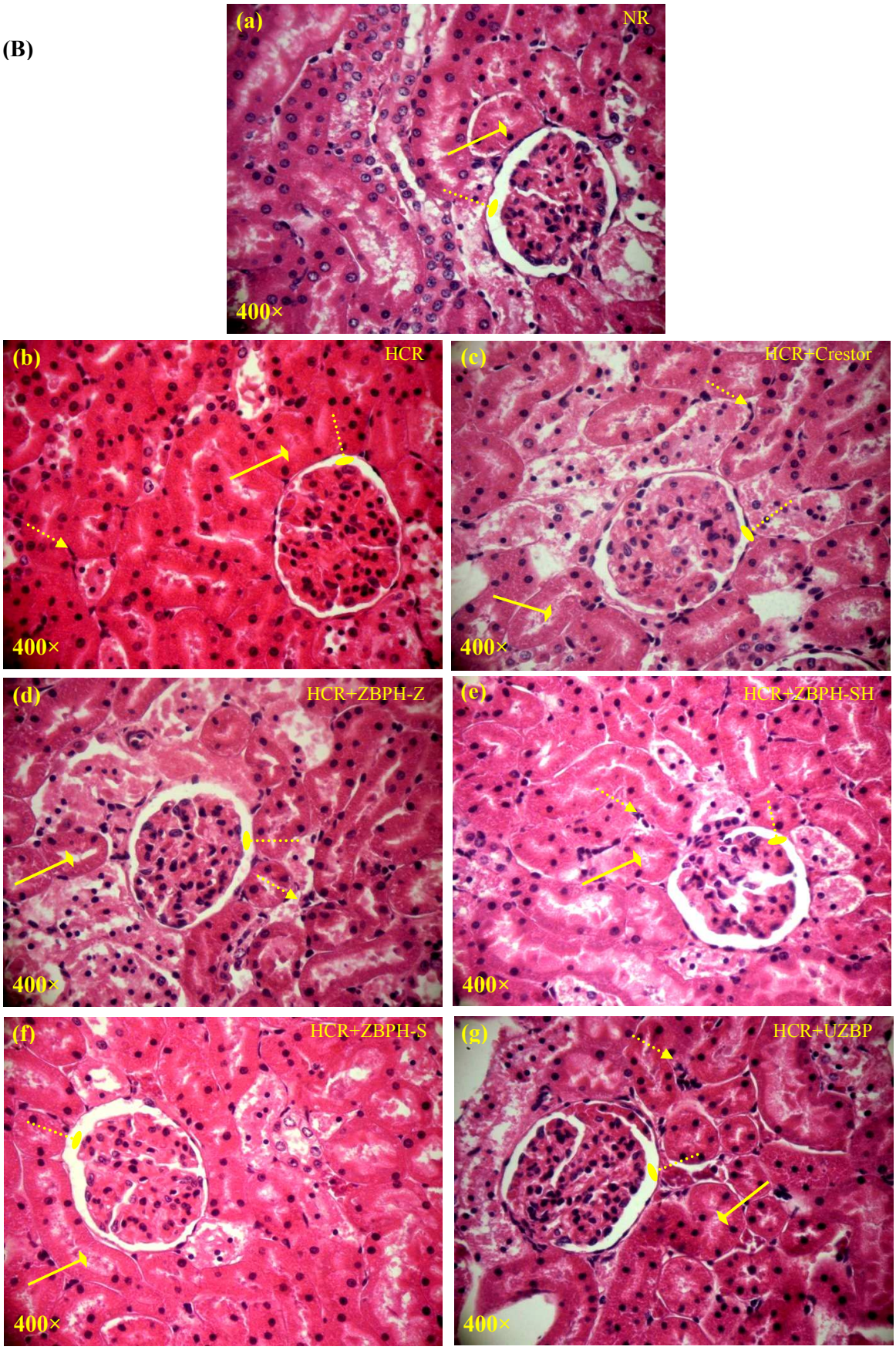

Table 1. Molecular weight distribution of ZBPHs

	ZBPH-Z	ZBPH-S	ZBPH-SH
>10000 Da	29	40.8	54
10000 to 7000 Da	20.5	26.21	11
7000 to 3000 Da	33.8	23	12.1
3000 to 1000 Da	8.87	5.45	9.54
1000 to 500 Da	1.42	0.67	3.33
500 to 100 Da	6.32	3.85	9.72
<100 Da	0	0	0.26

ZBPH-Z, ZBPH-S and ZBPH-SH are zebra blenny protein hydrolysates produced using zebra blenny proteases, sardinelle proteases and smooth hound proteases, respectively.

Table 2. Total cholesterol, triglycerides, LDL-cholesterol, and HDL-cholesterol in serum and liver of hypercholesterolemic rats treated with ZBPHs and UZBP.

Groups	NR	HCR	HCR+Crestor	HCR+ZBPH-Z	HCR+ZBPH-S	HCR+ZBPH-SH	HCR+UZBP
Serum (mmol/l)							
TC	1.56±0.08	3.04±0.17$^{*@}$	2.38±0.15$^{\#*}$	2.02±0.03$^{*\#}$	1.9±0.12$^{*\#}$	1.92±0.11$^{*\#}$	2.06±0.1$^{*\#}$
TG	1.01±0.07	1.57±0.09$^{*@}$	1.14±0.08$^{\#}$	1.24±0.03$^{\#}$	1.05±0.09$^{\#}$	0.97±0.11$^{\#}$	1.23±0.08
LDL-c	0.39±0.03	1.38±0.18*	1.04±0.16*	0.55±0.05$^{\#@}$	0.53±0.05$^{\#@}$	0.66±0.03$^{*\#@}$	0.7±0.04$^{*\#@}$
HDL-c	0.71±0.04	0.58±0.01$^{*@}$	0.79±0.03$^{\#}$	0.76±0.01$^{\#}$	0.75±0.02$^{\#}$	0.83±0.03$^{*\#}$	0.74±0.00$^{\#}$
Liver (mg/g WT)							
TC	1.18±0.07	2.95±0.11$^{*@}$	1.44±0.12$^{\#}$	0.88±0.03$^{\#@}$	1.17±0.02$^{\#}$	1.18±0.09$^{\#}$	1.3±0.13$^{\#}$
TG	3.06±0.17	4.7±0.19$^{*@}$	3.34±0.11$^{\#}$	3.95±0.19*	3.59±0.08$^{\#}$	3.43±0.13$^{\#}$	4.09±0.04$^{*@}$
LDL-c	0.43±0.09	1.89±0.09$^{*@}$	0.65±0.13$^{\#}$	0.55±0.09$^{\#}$	0.49±0.2$^{\#}$	0.36±0.05$^{\#}$	0.52±0.04$^{\#}$
HDL-c	0.18±0.01	0.13±0.00$^{*@}$	0.16±0.00$^{*\#}$	0.18±0.01$^{\#}$	0.2±0.01$^{\#}$	0.17±0.01$^{\#}$	0.17±0.00$^{\#}$

NR are normal rats; HCR are hypercholesterolemic rats; HCR+Crestor, HCR+ZBPH-Z, HCR+ZBPH-S, HCR+ZBPH-SH and HCR+UZBP are groups of hypercholesterolemic rats treated with Crestor, ZBPH-Z, ZBPH-S, ZBPH-SH, and UZBP, respectively.
Values are given as mean ± S.D. for 8 rats in each group. Values differ significantly at $p<0.05$. Statistical analysis as in the legend of Fig. 2.

Table 3. Serum ALT and ALP enzyme activities in different rat groups.

Groups	NR	HCR	HCR+Crestor	HCR+ZBPH-Z	HCR+ZBPH-S	HCR+ZBPH-SH	HCR+UZBP
ALAT (UI/L)	60.0±2.3	75.3±5.4*	62.6±4.3	59.0±2.4$^{\#}$	47.0±2.7$^{*\#@}$	46.6±5.5$^{*\#@}$	51.0±2.9$^{*\#@}$
ALP (UI/L)	264. 7±27.8	365.6±32.9*	311.2±27.9	235.0±1.3$^{\#@}$	233.0±23.5$^{\#@}$	227.8±33.1$^{\#@}$	239.3±6.2$^{\#@}$

NR are normal rats; HCR are hypercholesterolemic rats; HCR+Crestor, HCR+ZBPH-Z, HCR+ZBPH-S, HCR+ZBPH-SH and HCR+UZBP are groups of hypercholesterolemic rats treated with Crestor, ZBPH-Z, ZBPH-S, ZBPH-SH, and UZBP, respectively.
Values are given as mean ± S.D. for 8 rats in each group. Values differ significantly at $p<0.05$. Statistical analysis as in the legend of Fig. 2.

Table 4. Kidney indices of toxicity (Urea, uric acid, and creatinine) of control and experimental groups of rats.

Groups	NR	HCR	HCR+Crestor	HCR+ZBPH-Z	HCR+ZBPH-S	HCR+ZBPH-SH	HCR+UZBP
Urea (mmol/l)	7.00±0.06	8.34±0.10*	7.94±0.38*	7.44±0.27$^{\#}$	6.72±0.39$^{\#@}$	6.34±0.49$^{\#@}$	7.73±0.22
Uric acid (µmol/l)	76.67±5.75	95±6.72*	75±2.84$^{\#}$	<60$^{*\#@}$	<60$^{*\#@}$	<60$^{*\#@}$	63±0.00$^{*\#@}$
Creatinine (µmol/l)	51.67±0.76	54.60±1.2*	51.20±1.11$^{\#}$	50.75±1.18$^{\#}$	50.25±0.69$^{\#}$	51.50±1.03$^{\#}$	52.00±1.08

NR are normal rats; HCR are hypercholesterolemic rats; HCR+Crestor, HCR+ZBPH-Z, HCR+ZBPH-S, HCR+ZBPH-SH and HCR+UZBP are groups of hypercholesterolemic rats treated with Crestor, ZBPH-Z, ZBPH-S, ZBPH-SH, and UZBP, respectively.

Values are given as mean ± S.D. for 8 rats in each group. Values differ significantly at $p<0.05$. Statistical analysis as in the legend of Fig. 2.

Table 5. Grading of the histopathological changes in the liver and kidney sections.

	Histopathological changes in the liver sections			**Histopathological changes in the kidney sections**		
Groups	Steatosis	leukocytes infiltration	Vacuolization	Reduced Bowman's space	Tubular obstruction	leukocytes infiltration
NR	-	-	-	-	-	-
HCR	+++	+++	+++	++	++	++
HCR+Crestor	++	+++	++	-	-	+
HCR+ZBPH-Z	+	+	+	-	-	+
HCR+ZBPH-S	-	-	-	-	-	+
HCR+ZBPH-SH	-	-	-	-	-	+
HCR+UZBP	+	+	+	-	-	+

Scoring was categorized as none (-), mild (+), moderate (++) and severe (+++).