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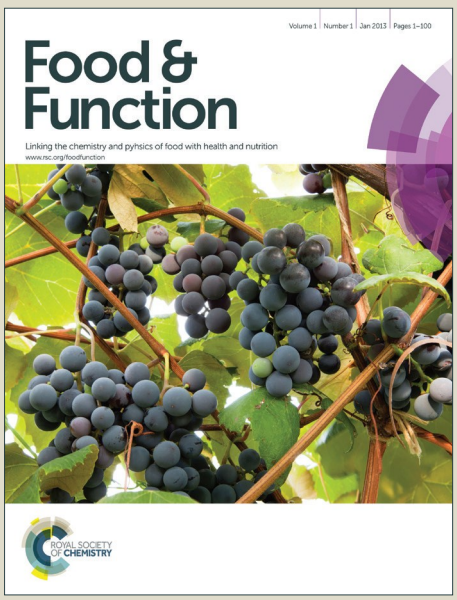

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Naringenin ameliorates renal and platelet purinergic signalling alterations in high-cholesterol fed rats through the suppression of ROS and NFκB Signaling Pathway

Yassine CHTOUROU[1#], Zeineb KAMOUN[1], Wissem Zarrouk[2], Mohammed Kebieche[3]

Choumous Kallel[4], Radhouane Gdoura[1], Hamadi FETOUI[1]

1. Toxicology-Microbiology and Environemental Health Unit (UR11ES70), Faculty of Sciences, University of Sfax, Tunisia.
2. Institute of olive tree. BP1087. 3018. Sfax. Tunisia
3. Molecular Biology Laboratory, Faculty of Nature and Life Sciences, University of Jijel, PB 98, Ouled Aissa, 1800 Jijel, Algeria
4. Hematolology Laboratory, CHU Habib Bourguiba, University of Sfax, 3029 Sfax, Tunisia

[#]**Corresponding Author**:
Dr Yassine Chtourou, Toxicology-Microbiology and Environnemental Health Unit (UR11ES70), Faculty of Sciences, BP1171, 3000 Sfax, University of Sfax, Tunisia.
E-mail address: yacine_chtourou@yahoo.fr

Abbreviation list:

NF-ƙB : nuclear factor-kappa B

TIMPs: tissue inhibitors of metalloproteinases

ROS : reactive oxygen species

NTPDases : triphosphate diphosphohydrolase

iNOS : inducible NO synthase

TNF-α : tumor necrosis factor-α

IL6 : interleukin 6

Abstract

Naringenin (NGEN) is a natural flavonoid aglycone of naringin that has been reported to have a wide range of pharmacological properties, such as antioxidant activity and free radical scavenging capacity. The aim of this study was to investigate the protective effect of NGEN on oxidative and inflammatory parameters, as well as to evaluate the hydrolysis of adenine nucleotides in kidney and platelets membranes of rats exposed to hypercholesterolemic diet (HCD) for 90 days. Kidney oxidative stress and mRNA expression of the ectonucleoside triphosphate diphosphohydrolase (NTPDases), ecto-5'-nucleotidase (CD73), inducible NO synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and the nuclear factor kappa B (NF-κB) genes were evaluated by real time RT-PCR. The co-administration of NGEN (50 mg/kg) for 90 days significantly prevents renal failure in HCD rats indicated by improvment of renal markers. Histopathological observation findings were also consistent with these effects. Moreover, NGEN (50 mg/kg) significantly decreased the lipid profile and inhibited pro-oxidant and inflammation marker levels in kidney of HCD rats. Furthermore, the NTPDases activities were significantly decreased in platelets and kidney membranes of HCD-treated rats and these alterations were improved by NGEN.

In conclusion, this study suggests that naringenin can potentially improve the renal failure and platelets alterations observed in rat fed with hypercholesterolemic diet probably through its antioxidant effects. Naringin could therefore be an effective and affordable dietary therapy for the cardiovascular diseases

Keywords: Naringenin, hypercholesterolemic diet, platelet, kidney, oxidative stress, inflammation

1. Introduction

Hypercholesterolemia-related metabolic syndrome increases cholesterol and triglyceride levels and it remains one of the major risk factors for the development and the progression of Chronic Kidney Disease (CKD)[1]. The incidence and prevalence of CKD are increasing worldwide; however, overweight or obese individuals have an increased risk (40%) of kidney disease compared to individuals of normal weight[2]. Further, obese individuals have a reduced chance of successful kidney transplants and increased tendency of progression to end stage renal failure [3]. Dyslipidaemia in renal disease has been demonstrated in animal studies, however 10- to 12-week diet-induced hypercholesterolemia can be used to generate a valid rodent model accompanied with development of glomerulosclerosis and dysbalance of tissue redox activity partly mediated by increased oxidative stress [4]. In this context, recent studies have linked the relationship of oxidative stress and mitochondrial dysfunction comprise as well as the development of glomerulosclerosis and tubulo-interstitial damage leading to the destruction of the kidney tissue [5]. The mechanisms by which hyperlipidemia contributes to systemic oxidative stress in CKD remain unclear. Inflammatory mediators, including TNFα and IL-1β, are reactive oxygen species (ROS)-activating factors in the kidney and may induce oxygen radical production by mesangial cells (MCs) [6, 7]. Bakker et al.[8] have shown that excess FFAs bound to albumin are filtered through glomeruli and reabsorbed into the renal proximal tubules, which causes interstitial inflammation in the kidney. Moreover, Zhong et al. [9] demonstrated that pro-inflammatory cytokine IL-1β also inhibits ATP-binding cassette A1 (ABCA1)-mediated cholesterol efflux from MCs leading to imbalance of cholesterol uptake, synthesis, and cholesterol efflux, which disrupts cholesterol

homeostasis and causes foam cell formation. The purinergic system consists of cell surface purinergic receptors, which fall into the P1 or adenosine receptor family and the P2 or ATP/ADP receptor family, as well as metabolic enzymes, which convert extracellular purines to each other and nucleoside transporters, which terminate purinergic receptor signaling by purine uptake into the cell [10]. In this regard, control of circulating nucleotide levels is noteworthy in the maintenance of physiological nucleotide-mediated signaling processes and ATP is an important extracellular signal in the regulation of many intracellular processes in normal tubular cells as well as in the progression to chronic renal failure [11]. It is important also to consider that ATP acts as a short range/short timing signaling molecule because the ATP concentration is rapidly declining around the source of the release due to fast degradation by numerous ectonucleotidases [13, 12]. In this context, the specific pattern of ATP hydrolysis in plasma likely has significant implications in the role of ATP in platelet aggregation, ADP promotes platelet aggregation and the nucleoside adenosine is an endogenous inhibitor of platelet aggregation and limits the degree of vascular injury by reducing the release of ROS[14]. Four families of ectonucleotidases exist: ectonucleoside triphosphate diphosphohydrolases (NTPDases), ectonucleotide pyrophosphatase phosphodiesterases (NPPs), ecto-5′-nucleotidase and alkaline phosphatises can modulate platelet function through the hydrolysis of extracellular adenine nucleotides and all are found in the kidney [10]. NTPDase1 (CD39, apyrase) is the major ecto-nucleotidase in the renal vasculature and can be shown to efficiently bind and hydrolyze extracellular ADP (and ATP) to AMP [15]. NTPDase2 and NTPDase3 have been localised in Bowman's capsules and to most nephron segments [10]. Expression of CD39 and CD73 ectonucleotidases on either endothelial or immune cells allows for homeostatic integration and control of vascular inflammatory and immune cell reactions at sites of injury[16]. Nevertheless, these cellular processes and nucleotide-triggered events are further modulated during the development of

atherosclerosis [10]. Recent study has been shown that the protective effects of CD39 in various inflammatory under such episodes of stress conditions, like palmitoylation status and cholesterol content of membrane lipid rafts, can be ameliorated by statins or by incorporation of saturated fatty acids into cell membranes and limitation of lipid peroxidation responses by antioxidants [12]. Naringenin is a flavanon that is naturally produced by several plants, especially the skin of grapefruit [17]. The biological functions of naringenin was widely investigated and include anti-inflammation, anti-oxidation, anti-cancer, cardiovascular protection, anti-diabetes, renal protection, protection against Alzheimer's disease and antihyperuricemic activities [17]. Moreover, Xiao et al. [18] have shown that naringenin inhibits platelet aggregation and release by reducing blood cholesterol levels and the cytosolic free calcium concentration in hyper-lipidemic rabbits. However, the detailed mechanisms are unclear. Hence, the present study was aimed to investigate the protective effects of naringenin on proinflammatory cytokines, pro/antioxidant markers, mitochondria dysfunction as well as the NTPDase, 5′-nucleotidase activities and its expression in renal and platelets in in high-cholesterol fed rats.

2. Materials and methods

2. 1 . Animals and reagents

Forty 2-month-old adult male Wistar rats (220–250 g) were purchased from the Central Pharmacy (SIPHAT, Tunisia). All animal procedures were conducted in strict conformation to the General Guidelines on the Use of living Animals in Scientific Investigations [19] and approved by the Ethical Committee of Sciences Faculty, Sfax University. Animals were kept in an air conditioned room (22 ± 2 °C ; 45% humidity) and housed in individual cages and subjected to-a 12 h light anddark cycle. They were given a regular rodent chow with free access to food and water for twoweeks to acclimatize to the new environment. Unless

otherwise specified, Naringenin and allother chemicals used in this study were obtained from Sigma Chemicals Co. (St. Louis,France).

2. 2 . Experimental protocol

Rats treated in the same manner as in the animal model described and recently published by us [20]. Briefly, rats were randomized into four groups: the first group (C) served as the controls, received *ad libitum* distilled water, standard diet supplied by the Company of Animal Nutrition, Sfax, Tunisia and 0.5 ml vehicle solution of naringenin (NGEN) by gavage. The second group had a hypercholesterolemia diet (HCD) for three months by adding 10 g cholesterol/kg +1 g cholic acid/kg to standard diet. Animals in the third group (HCD+NGEN) were fed a cholesterol-enriched diet supplemented with NGEN (50 mg/kg body weight) administered by oral gavage. The fourth group (NGEN) received normal pellet and received 0.5 ml of naringenin (50 mg/kg body weight) administered by oral gavage. Urinary samples were obtained from each animal housed in a specially designed metabolic cage within 24-h cycles. The volume of each sample was recorded and centrifuged at 3000×g for 5 min. At the end of the experimental period (3 months), the animals of different groups were sacrificed by cervical decapitation to avoid stress conditions. Total blood was collected with citrate (3.8%; 1:9, v/v) as anticoagulant for platelet-rich plasma preparations. The Kidney was quickly excised, rinsed in ice-cold physiological saline, weighed (to calculate Ratio of the Kidney weight to the body weight (%)) and then divided into different parts for homogenization in the appropriate buffer as indicated in the procedures measurement of each parameter.

2.3. Hematological study

White blood cells (WBCs), red blood cells (RBCs), hematocrit (HCT) and platelets were analyzed using standard methods with the fully automated Coulter MAXM (Beckman Coulter, Inc., Fullerton, CA).

2.4. Assessment of renal function and platelet-rich plasma preparations (PRP)

Blood samples were collected into EDTA tubes from the eye vein of rats 24 h before sacrifice and centrifuged for 10 min at 3000×g to obtain clear plasma which were stored at –20°C for subsequent measurement of renal functions. The levels of urea, uric acid and creatinine in plasma and urine were estimated spectrophotometrically using commercial diagnostic kits, respectively, (ref 20151, 20143, 20091) purchased from Biomagreb (Ariana, Tunisia). Creatinine clearance (µl/min), an index of glomerular filtration rate was calculated by UV/P equation, where U is the urinary creatinine level, V the volume of urine sample collected within 24 h and P the plasma creatinine concentration. Platelets were prepared by the method modified by Lunkes et al. [21]. Total blood was collected with citrate (3.8%; 1:9, v/v) as anticoagulant. Platelet rich plasma was obtained by centrifugation at 800×g for 15 min to remove residual blood cells and the remaining blood was further centrifuged at 3,500×g for 10 min and washed twice by centrifugation at 1,400×g with 3.5 mM (HEPES). The washed platelets were resuspended in HEPES isosmolar buffer and adjusted to 0.4-0.6 mg of protein/ml.

2.5.Assessment of lipid contents from Kidney tissues

Total lipids were extracted from 100 mg of tissue by the method of Folch et al. [22].Total cholesterol and triglyceride contents were determined using enzymatic available kits from Biomaghreb (Ariana, Tunisia, Ref. 20111, 20131, respectively). Phospholipids (PL) content was determined by the method of and Davis [23]. Free fatty acids (FFA) were estimated by the method of Falholt et al. [24]. Values were expressed as mg/g tissue.

2.6. Plasma and renal measurement of oxidant stress markers

Hydrogen peroxide (H2O2) was measured using the ferrous ion oxidation xylenol orange version-2 (FOX-2) method of Gay et al.[25]. Concentrations were calculated at 560

nm using a standard curve prepared with H2O2. Nitric oxide production was determined based on the Griess reaction [26]. Absorbance was measured spectrophotometrically at 550 nm using a microplate reader. Nitrite (NO2 -) concentration was determined from a standard nitrite curve generated using NaNO2. The results were expressed μM.

ROS was measured as described in our previous report, based on the oxidation of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) in a 96-well plate assay[20]. Protein samples (25 μg) from kidney or Plasma of all animals were diluted in PBS and incubated with 5.0 μM DCFH-DA in the dark for 15 min at 37 °C. Fluorescence was measured every 15 min for 1 h with excitation and emission wavelengths of 488 and 525 nm, respectively, using a CFX96 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) fluorescence plate reader. Results were expressed as pmoles DCF/mg of protein, using a standard curve with DCF.

The extent of lipid peroxidation by measuring thiobarbituric acid reactive substances (TBARS) in terms of malondialdehyde (MDA) formation was measured according to the method of Draper and Hadley [27]. The MDA values were calculated using 1, 1, 3, 3-tetraethoxypropane as standard and expressed as nmoles of MDA /mg protein.

2.7. Determination of non-enzymatic and enzymatic antioxidants in kidney tissue

Glutathione (GSH) in the heart was determined by the method of Ellman modified by Jollow et al.[28]. Vitamin C (Vit C) determination was performed as described by Jacques-Silva et al. [29]. Vitamin E (Vit E) level was assayed by the extraction method of Katsanidis and Addis [30]. Catalase (CAT) activity was assayed by the method of Aebi [31]. Superoxide dismutase (SOD) activity was estimated according to Marklund [32]. Glutathione peroxidase (GPx) activity was measured according to Flohe and Gunzler [33].

2.8. Assays for mitochondrial complexes activities

Kidney mitochondria were isolated as previously described [34]. NADH dehydrogenase (complex I), succinate-cytochrome c oxidoreductase (complex II-III) and cytochrome c

reductase (complex IV) activities were measured spectrophotometrically using microplate (Jenway 6300) conventional assays as previously described [34].

2.9. kidney membranes and platelet-rich plasma preparations

Kidney membranes were isolated as described previously by Fürstenau et al. [35].

Briefly, about 1.0 g of both right and left kidneys was dissected on ice, washed and were gently homogenized in 10 volumes of an ice-cold medium (0.32 M sucrose, 0.1 mM EDTA, 5 mM HEPES–Tris, pH 7.4) with a Teflon-glass homogenizer, using a discontinuous percoll gradient. The pellet was resuspended in an isosmotic solution and the final protein concentration was adjusted to 0.5–0.8 mg/ml [35].

2.10. Assay of NTPDase and 5-nucleotidase activities

Assays of NTPDases and ecto-5′-nucleotidase activities in the kidney membranes by the method as described previously [21, 35]. The ectonucleoside triphosphate diphosphohydrolase (NTPDase) enzymatic assay were carried out in a reaction medium containing 5 mM KCl, 1.5 mM CaCl2, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 µl. The ecto-5'-nucleotidase (CD73) activity in the Kidney and platelets was determined in a reaction medium containing 10 mM MgSO4 and 100 mM Tris–HCl buffer, pH 7.5, in a final volume of 200 µl. The reaction was initiated by the addition of the substrate (ATP or ADP) at a final concentration of 2.0 mM and stopped with 200 µl of 10 % trichloracetic acid (TCA). The released inorganic phosphate (Pi) molybdate complex was determined by spectrophotometer at 690 nm. All samples were run in triplicate and the specific activity is reported as nmoles Pi released/min/mg of protein.

2.11. Analysis of gene expression by quantitative RT-PCR (qRT-PCR)

Expression of the ectonucleoside triphosphate diphosphohydrolase (NTPDase1-3), ecto-5'-nucleotidase (CD73), inducible NO synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and the nuclear factor kappa B (NF-κB) genes in the kidney tissues of experimental rats (n=4/group) were measured using a reverse transcriptase qRT-PCR technique. Total RNA was extracted using the iScript™ RT-qPCR Sample Preparation Reagent and according to the manufacturer's instructions (170-8898, Bio-Rad). RNA concentrations and purity were determined by measuring the absorbance A260/A280 ratios by using NanoPhotometer™ (Implen GmBH). The cDNA was produced from 2 µg of total mRNA by reverse transcription with superscript reverse transcriptase (Invitrogen, France) using oligo(dT)18 as a primer in a total volume of 20 µl. After incubation for 50 min at 42 °C, the reaction was terminated by denaturating enzyme for 10 min at 70 °C. cDNA (2 µl) was used as a template for PCR according to the recommended protocol using the 2× SYBR Permix Ex Taq™ (TaKaRa) and primers sequences used for the gene amplification are given in Table 1[20,36]. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative changes in gene expression from real-time quantitative PCR experiments. Data were normalized to mRNA levels of the house keeping gene, *β*-actin, and results were expressed relatively to the average values for the control group, which was set to 1.

2.12. Histopathological analysis

Kidney tissues from the normal and experimental rats (n = 3/group) were fixed in 10% buffered formalin and have been processed for paraffin sectioning. Sections of 5 µm thickness were stained with hematoxylin and eosin (H & E), and examined with Leica® microscope fitted with Sony® digital camera to capture images for histological studies.

2.13. Quantitative protein determination

Protein concentration in kidney homogenates was measured by Bradford method using bovine serum albumin as standard.

2.14. Statistical Analysis

Results were expressed as mean ± standard deviation (mean ± SD). All analyses were carried out with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA). Significant differences between treatment effects were determined by one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons with statistical significance of $p < 0.05$.

3. Results

3.1. NGEN ameliorate HCD-induced renal markers and hematological parameters

Results from biochemical plasma and urine study revealed that HCD for a successive 12 weeks significantly ($p < 0.001$) impaired kidney function assessed by an increase in uric acid and urea and a decrease in creatinine clearance in comparison to the control rats (Table 2). Oral co-administration of NGEN significantly improved ($p < 0.001$) these parameters induced by cholesterol treatment.

Upon the examination of hematological parameters (Table 2), a decrease in total red blood cell (RBC) number as well as was accompanied by an increase in platelets concentration and total white blood cell (WBC) number ($p < 0.001$) was observed in rats exposed to HCD as compared to the control group. Hematocrit (HCT) was found to be normal in all the experimental groups. Oral co-administration of NGEN significantly ($p < 0.001$) ameliorated these alterations induced by cholesterol treatment. No changes were observed in the NGEN treated rats alone when compared to the controls (Table 2).

3.2. NGEN restores relative kidney weight and lipid profile changes in high-cholesterol fed rats

As shown in Table 3, the present data recorded that consuming a HCD for 3 months induced a significant increase ($p < 0.001$) in the ratio of the kidney weight to the body weight (%), which was prevented ($p < 0.01$) by NGEN supplementation. Furthermore, a significant

increase ($p < 0.001$) of renal lipid profile such as TC, TG, PL and FFAs was observed in HCD fed rats as compared with the control group. Co-administration of NGEN significantly ($p < 0.001$) reduced the ratio of the kidney weight and attenuated the tissue lipids composition as compared to HCD rats (Table 3). Comparably, no significant changes were observed between control and NGEN-treated rats alone .

3.3. Effects of NGEN treatment on renal oxidative markers and the activities of mitochondrial enzyme markers in high-cholesterol fed rats

As shown in table 4, HCD treatment markedly increased ROS, H2O2, NO2 and TBARS levels as compared with those of the controls in the plasma and kidney tissue ($p < 0.001$). However, the treatment with NGEN significantly ($p < 0.001$) alleviated these changes. Interestingly, NGEN treatment alone does not present any difference with the control groups (table 4).

In order to determine whether impaired mitochondria may play a role in lipid accumulation, we measured mitochondrial activity. The renal mitochondrial enzyme activities such as NADH dehydrogenase (complex I), succinate-cytochrome c oxidoreductase (complex II-III) and cytochrome c reductase (complex IV) (Fig. 1) significantly ($p < 0.001$) decreased considerably in the kidney mitochondria of HCD-treated rats. Co-administration of NGEN along with HCD significantly ($p < 0.001$) increased the activities of these enzymes when compared with HCD-treated rats. Activities of these renal mitochondrial enzymes in NGEN treated rats alone were near to control rats (Figure 1).

3.4. NGEN restores the renal antioxidant status

Vit C, Vit E, GSH, GPx, SOD and catalase levels were significantly ($p < 0.001$) decreased in the HCD group as compared to the control group (Table 5). Co-administration of NGEN along with HCD restored partially ($p < 0.01$) the antioxidant status in the renal tissue

compared with HCD-treated rats. No appreciable change in any of these parameters was observed in the NGEN treated alone group compared to the control group, indicating no adverse effect of NGEN in healthy rats.

3.5. NGEN down-regulated the expression of proinflammatory-mediated genes in kidney

To investigate the role of pro-inflammatory markers on NGEN-mediated renoprotective effect, mRNA expression levels of iNOS, TNF-α and IL-6, the activation of the nuclear factor kappa B (NF-κB) were determined using qRT-PCR (Table 6). The results show concomitant increases ($p < 0.001$) in the mRNA expression levels of TNF-α, IL-6, iNOS and NF-κB in kidneys derived from HCD-treated rats as compared to control. Importantly, gene expression was significantly ($p < 0.001$) down-regulated in HCD+NGEN group compared with HCD group. The administration of NGEN alone did not show a significant effects when compared with control group.

3.6. Effects of NGEN on ectonucleotidase activities and gene expression in the platelets and kidney of HCD-treated rats

Fig. 2 shows the results obtained for ectonucleotidase (NTPDases, 5'-nucleotidase) activities in renal membranes (Fig. 2 a) and platelets (Fig. 2 b) from rats exposed to HCD, NGEN or exposed simultaneously with HCD+NGEN. As can be observed, A significant decrease of ectonucleotidase activities in HCD-treated rats when compared with the control group ($p<0.001$) when ATP, ADP or AMP were used as substrate and those decrease induced by HCD was abolished ($p < 0.001$) by NGEN co-administration. The administration of NGEN alone did not show a significant effects when compared with control group.

To identify related genes that may be regulated over HCD and NGEN exposure, the expression analysis of NTPDase1–3 and 5'-nucleotidase was carried out by qRT-PCR assay (Table 6). Results revealed specific signals accompanied with the down regulation corresponding to mRNA for NTPDase1, NTPDase2 and CD73 in the HCD-treated group when compared to the control group. There were no differences in mRNA transcript levels for NTPDase3. On the other hand, gene expression was significantly ($p < 0.05$) up-regulated in HCD+ NGEN group compared with HCD-treated group (Table 6). The administration of NGEN alone did not show a significant effects when compared with control group.

3.7. Effects of NGEN on the histological injury of kidney in experimental animals

The light microscopy evaluation of kidneys in control and NGEN groups showed normal renal histology with apparent glomerulus with a constant tuft and normal tubular architecture (Fig. 3 a, f). While HCD-treated rats showed distinctive glomerular basement membrane accompagnied by an enlarged Bowman's space and vascular congestion outside fragmented glomeruli and between tubules with mild tubular degeneration (Fig. 3 b-c). NGEN co-treatment significantly induced a marked improvement in histopathological changes in the kidneys of HCD rats (Fig. 3 d-e).

4. Discussion

High-cholesterol diet (HCD) is a well-known model for excess calorie intake, which will contribute to the development of obesity and metabolic syndrome [7]. Feeding for three months by adding 10 g cholesterol/kg +1 g cholic acid/kg to standard diet in this model is characterized by increased renal oxidative/nitrosative damage and is well suited for the study of potential preliminary preventive or therapeutic compounds against hyperlipidemia-induced kidney injury [4]. In this study, we showed that dietary (NGEN) supplementation had a beneficial effect against hypercholesterolemia kidney injury as evidenced by the alleviation of

histopathological changes and the improvement of serum biochemical such as urea, uric acid creatinine clearance and renal lipid profile. NGEN possesses the potential to restore kidney mitochondrial dysfunction and oxidative stress, improve renal antioxidant defense capacity, inhibit NF-κB signaling and is able to modulate the expression of proinflammatory cytokine expressions responsible in the aetiology of renal complications. Such as those linked to inflammation, phosphohydrolysis of extracellular ATP and ADP in the kidney is an essential step in purinergic signaling that regulates key pathophysiological processes. The major new findings of this study are that treatment with NGEN was fitted to modulating the adenine nucleotide hydrolysis alterations induced by HCD diet as indicated by the biochemical and qRT-PCR in the platelets and renal tissue Since hypercholesterolemia-induced oxidative stress plays an essential role in thedevelopment and progression of early nephropathy, as showed in a previous study, elevated serum creatinine, urea and uric acid as a result of both increased generation and decreased excretion is a well known characteristic of renal injury [38, 39]. In the current study, feeding HCD for three months was evidenced by displayed elevated levels of urea and uric acid levels while creatinine clearance was decreased which were taken as direct *in vivo* index of nephropathy in HCD-treated rats (Table 2). One would have expected that NGEN would have ameliorated kidney function parameters in HCD rats. We are tempting to speculate that the prevention of HCD-mitochondrial oxidative stress by NGEN, documented in this study, may be involved, at least in part, in the prevention of renal oxidative stress. Corroborating with these results, several studies have reported that supplementation of NGEN attenuated negative physiological sequels induced by of oxidative stress observed in high fat diet fedstreptozotocin- diabetic due to its direct free radical scavenger activity and its indirect antioxidant properties [40]. Additionally, Tsai et al. [41]demonstrated that the treatment of diabetic mice with NGEN provided antioxidative and antihyperlipidemic activities in the kidney tissue.

Administration of a cholesterol-rich diet results in increased organ lipid profile are well supported by many previous studies [39]. According with this, in our present study, we found that there was a significant elevation in the levels of renal total cholesterol (TC), triglycerides (TG), free fatty acids (FFA) and phospholipids (PL) in HCD fed rats (table 3) as compared with normal, an increase that was prevented by oral administration of NGEN. The approach to lowering lipid nephrotoxicity must be multifaceted and regardless of the specific sources of intracellular ROS production, the close relationship between ROS and hypercholesterolemia has been confirmed by a number of recent studies [7, 20, 37]. Mitochondrial cholesterol accumulation not only sensitizes to oxidative stress but it can contribute to the metabolic reprogramming. The present study endeavored to determine the *in vivo* protective effect of NGEN against the mitochondrial dysfunction and oxidative stress induced by high cholesterol levels in renal tissue. In the present study, our results demonstrated that oral co-administration of NGEN significantly reduced the levels HCD-induced overproduction of ROS, H2O2, NO2- and TBARS in the plasma and renal tissue (table 4). The effect of ROS is balanced by the antioxidant action of non-enzymatic antioxidants as well as by antioxidant enzymes. In this regard, during HCD condition, the activities of renal antioxidant enzymes, the first line of defense against ROS, namely SOD, CAT and GPx and non-enzymic antioxidant namely glutathione, vitamin C and vitamin E, were substantially suppressed (table 5). The ability of NGEN to increase renal reduced glutathione, vitamin C and vitamin E levels and catalase, SOD and GPx activities is in agreement with the finding of Hermenean et al. [43] who found that NGEN corrects oxidative disturbances on carbon tetrachloride-induced acute nephrotoxicity in mouse. It is increasingly evident that elevated levels of ROS can adversely affect cell function by impairing renal mitochondria electron transport chain complex function. However, Koyama et al. detected a reduction in mitochondrial respiratory function using free fatty acid (FFA) bound BSA-overload nephropathy model to induce renal

lipotoxicity/ROS-related inflammation [42]. In agreement with this view, we observed a marked change in mitochondrial complexes such as NADH dehydrogenase, succinatecytochrome c oxidoreductase and cytochrome c reductase activities were significantly decreased in the kidney of HCD-treated rats (Fig. 1). On the other hand, this study reveals that the treatment with NGEN is able to prevent the excessive ROS production and was effective in maintaining the mitochondrial complexes activities. A recent study showed naringenin to be better as compared to hesperedin and hesperitin (its related citrus flavanones) as far as interaction with intracellular enzymes, mitochondrial and cellular membrane is concerned [43]. Moreover, Sahu et al. showed that naringin, the glycosidic form of NGEN, at a dose of 100 mg/kg was found to offer protection and restored the mitochondrial function by maintaining mitochondrial respiratory enzyme activity and thereby preventing the mitochondrial membrane potential ($\Delta\psi m$) [44]. A great number of inflammatory mediators, including the cytokines TNF-α and IL-6 and the reactive nitrogen intermediate NO, may induce mitochondrial damage [7]. TNFα and IL-6 decrease the activity of MRC complex I, ATP production, and mitochondrial membrane potential. These mediators also induce the accumulation of significant amounts of ROS. Activation and nuclear translocation of nuclear factor kappa B (NF-κB), in response to oxidative stress, are thought to be the key factors in the renal inflammatory process by regulating the gene expression of cytokines [20]. In the present study, NGEN co-treatment along with HCD significantly down-regulated HCD induced of inducible nitric oxide synthase (iNOS) and proinflammatory cytokines TNF-α andIL-6 in the kidney by the inhibition of the NF-κB expressions analyzed by qRT-PCR. Our results are consistent with previous ones showing that NGEN reduces cholesterol-induced hepatic inflammation by modulating TNF-α and IL-6 *via* the down-regulation of NF-κB. During inflammation, nitric oxide (NO) and derived oxidants are formed by inflammatory cells. NO plays a number of physiological roles in the regulation of renal function and a

marked increase in iNOS expression along the nephron characterizes experimental renovascular disease, and leads to decreases in tubular filtrate concentration capacity, a measure of intrinsic renal damage [35]. Moreover, NO is a potent inhibitor of LDL oxidation and may be an important antioxidant for LDL *in vivo* but is rapidly inactivated by superoxide anion to form peroxynitrite (ONOO−) as a potent oxidant [45]. Overproduction of NO was also argued to be beneficial in attenuating lipid peroxidation and have been implicated in the regulation of platelet function. Indeed, it has been proposed that the majority (60%) of blood nitrite, in humans, is carried by erythrocytes followed by white blood cells (WBC) andactivated platelets which are frequently used as an indicator of systemic inflammation [45]. In the current study, WBC and platelet counts were all significantly higher in HCD-treated rats than in control rats (Table 2) and were partially alleviated by NGEN. Recently, Annadurai et al. [46] reported that naringenin exhibited lower platelet counts, neutrophils and monocytes mean percentages with a higher mean percentage of lymphocytes in diabetic treated-rats.

Purinergic signaling mechanisms mediated by nucleotides and nucleosides have a complex influence on inflammatory processes and adenosine receptor activation strongly attenuates the production of several proinflammatory cytokines. In the present study, we observed an a decrease of NTPDases and 5′-nucleotidase platelet activities associated with an increase of NO levels in whole blood. An important aspect to be discussed is that the decrease in the NTPDase and 5′-nucleotidase activities can be associated with the increased content of fats in diet, which is related to the deleterious effects attributed to the increase in LDL-c and triglycerides, reduced circulating levels of HDL-c, and a rise of ROS-induced lipid peroxidation in HCD fed animals [47]. Several mechanisms can regulate ectonucleotidase activities, including transcriptional and/or post-translational modifications [35]. Quantitative

RT-PCR analysis of the patterns of ectonucleotidase gene expression after HCD treatment showed a significant decrease in mRNA transcript levels of NTPDase2 and 3, and CD73 (Table 6). Previous studies have already demonstrated that the enzymes able to hydrolyze extracellular nucleotides can be regulated at the transcriptional level following drug treatment [10]. At the time, the mechanism responsible for this effect was unknown. A number of studies have demonstrated that NGEN is a compound that presents anti-aggregant properties related to hydrolysis of nucleotides such as ATP and ADP [19]. In platelets, concentrations of NGEN inhibit platelet activation *in vitro* as well as granular release reaction and platelet Adhesion [19]. Results obtained in this study may represent a potential mechanism of inhibition platelet aggregation by NGEN through ectonucleotidase modulation, which plays an important role in regulating both vascular and thrombotic factors. The increase of ATP, ADP, and AMP hydrolysis caused by NGEN is likely to decrease the ADP levels, the main promoter of platelet aggregation, and increase adenosine, a powerful inhibitor of platelet aggregation [48]. Interestingly, we also observed that NGEN co-treatment prevented the increase in NO, proinflammatory cytokines and the decrease in NTPDase and 5'-nucleotidase activities in kidney tissue of HCD-treated rats. This effect is interesting because the increase in ATP, ADP, and AMP hydrolysis can maintain the levels of extracellular ATP molecule.

5. Conclusion

The results of the present study indicate that naringenin provided a significant protective effect against HCD-induced renal and platelets injurys. The antioxidant, anti-inflammatory and anti-aggregant properties can be considered the main factors responsible for the benificial effect of naringenin. Therefore, naringenin may represent a potential therapeutic option to prevent HCD-induced renal injury and dysfunction.

Conflict of interest

None of the authors have any conflicts of the interest.

Acknowledgments

The present work was supported by the grants of DGRST (Appui a la Recherche Universitairede base, ARUB, UR11ES70) Tunisia.

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Figure captions

Figure 1:

Effect of Naringenin (NGEN) co administration on activities of NADH dehydrogenase (complex I), succinate-cytochrome c oxidoreductase (complex II-III) and cytochrome c reductase (complex IV) mitochondrial enzymes in kidney of high cholesterol diet (HCD)-induced rats.

Values are expressed as mean ± SD of 6 rats per group.

HCD group *vs*. control group: $^{***}p$ <0.001

HCD+NGEN group *vs*. HCD group $^{¥¥¥}p < 0.001$, $^{¥¥}p < 0.01$, $^{¥¥}p < 0.05$

Figure 2:

Effect of high-cholesterol diet (HCD), high-cholesterol diet plus naringenin (HCD+NGEN) and naringenin alone (NGEN) on ectonucleotidase activity (nmoles Pi/mg protein/min) using ATP, ADP and AMP as substrate in Kidney membranes (**A**) and platelets (**B**).

Values are expressed as mean ± SD of 6 rats per group

HCD group *vs*. control group:$^{***}p$ <0.001

HCD+NGEN group *vs.* HCD group $^{¥¥¥}p < 0.001$, $^{¥¥}p < 0.01$, $^{¥¥}p < 0.05$

Figure 3:

Effect of naringenin (NGEN) on kidney histopathological images of hematoxylin and eosin (H&E) staining of high-cholesterol diet (HCD) induced rats (400×). (**A**) and (**F**) Sections from control and NGEN (50 mg/kg)-treated groups show normal histological structure of renal parenchyma. HCD group showed gradually progressing increase in glomerular tuft size and Bowman's space expansion (**B, C**). Kidney tissue from (HCD+NGEN) group showing slight congestion of glomerular tufts (**D, E**).

Figure 1

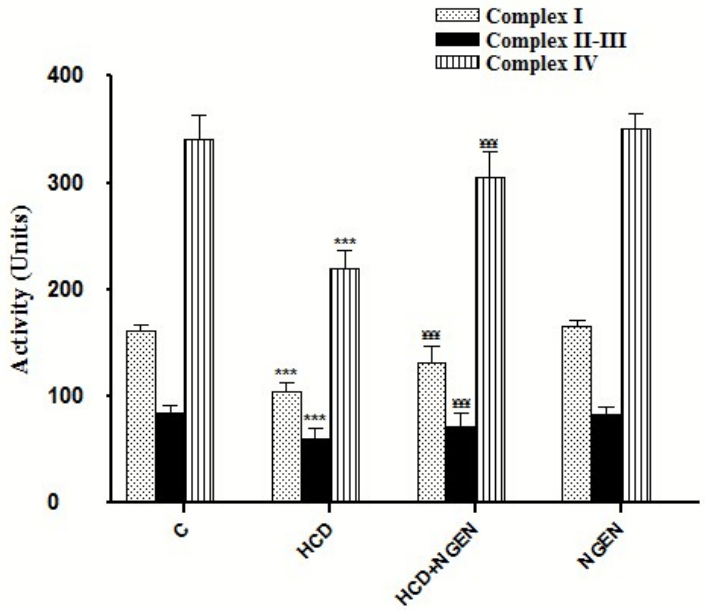

Figure 2

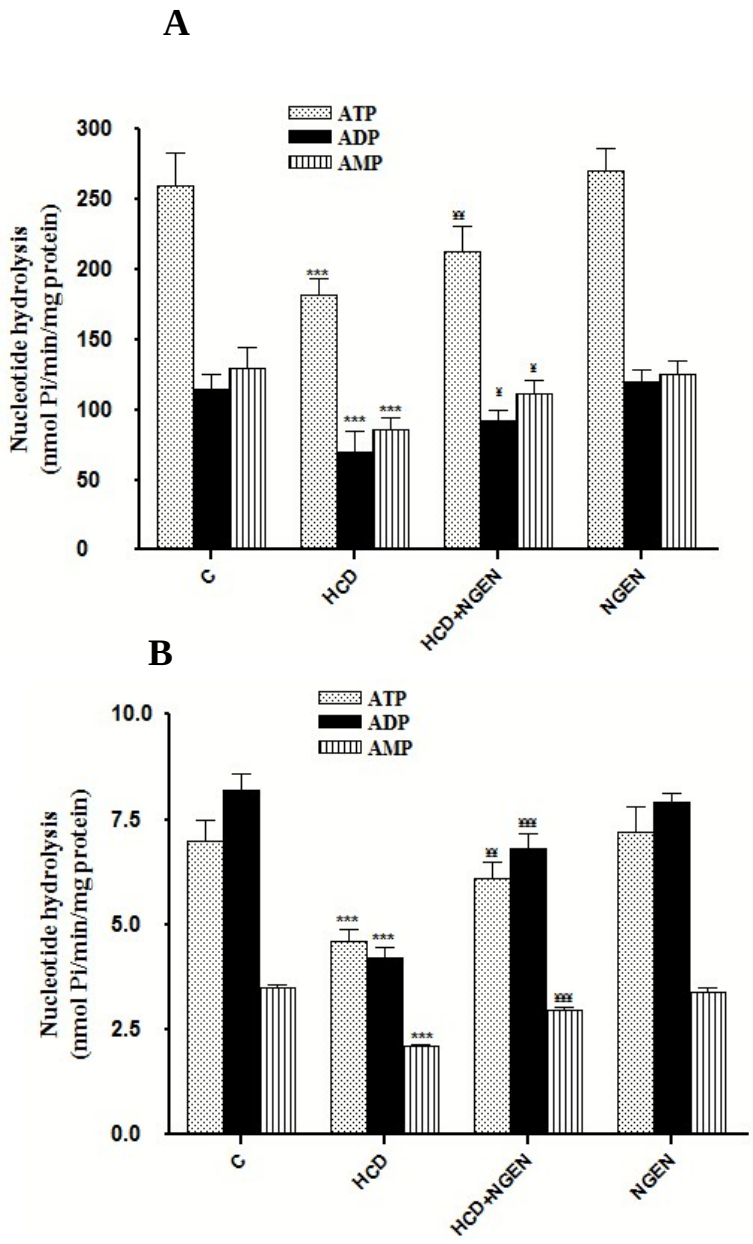

Figure 3

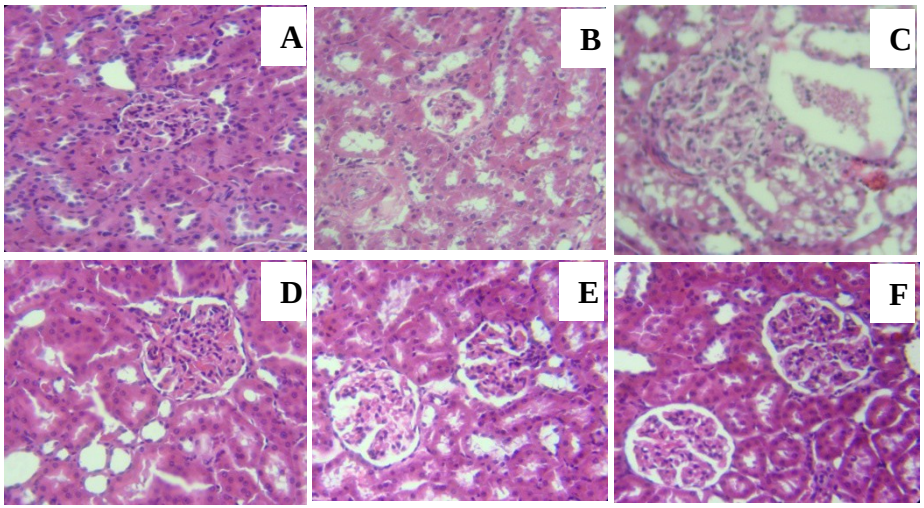

Table 1: Primer sequences used for quantitative RT-PCR reactions

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
β-Actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG
NTPDase1	GATCATCACTGGGCAGGAGGAAGG	AAGACACCGTTGAAGGCACACTGG
NTPDase2	GCTGGGTGGGCCGGTGGATACG	GGGTTCCATGGTGAAGTCAAC
NTPDase 3	CGGGATCCTTGCTGTGCGTGGCATTTCTT	TCTAGAGGTGCTCTGGCAGGAATCAGT
CD73	CCCGGGGGCCACTAGCACCTCA	GCCTGGACCACGGGAACCTT
IL-6	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC
TNF-α	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
iNOS	AGCATCACCCCTGTGTTCCACCC	TGGGGCAGTCTCCATTGCCA
NF-κB	GCCGTGGAGTACGACAACATC	TTTGAGAAGAGCTGCCAGCC

Abbreviations : β-Actin, beta actin ; Ectonucleoside triphosphate diphosphohydrolase, NTPDase; Ecto-5'-nucleotidase, CD73; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha ; NFκB, nuclear factor kappa B

Table 2: Hematological parameters (White blood cells (WBCs), red blood cells (RBCs), hematocrit (HCT)), plasma and urinary levels of urea, uric acid and creatinine clearance of control and rats treated with High cholesterol diet (HCD), naringenin (NGEN) and their combination (HCD+NGEN).

Parameters & treatments	**C**	**HCD**	**HCD+NGEN**	**NGEN**
Urea (mg/dL)				
Plasma	35.44±1.77	52.30±4.80***	41.80±3.0$^{¥¥¥}$	34.70± 2.50
Urine	45.80±1.50	49.40±1.90*	44.28±1.80$^{¥¥}$	45.40± 2.10
Uric acid (mg/dL)				
Plasma	1.7±0.12	3.0±0.28***	2.28±0.14$^{¥¥¥}$	1.76± 0.10
Urine	6.4±0.28	8.54±0.70***	7.14±0.20$^{¥¥¥}$	6.30± 0.25
Creatinine clearance (µl/min)	340±14.50	183± 12.00***	264±18.00$^{¥¥¥}$	348±13.00
Hematological parameters				
WBC (10^3 /µL)	9.55±0.28	15.40± 0.84***	12.55±0.50$^{¥¥¥}$	9.40±0.30
Platelets (10^3/ µL)	680±21.70	1045± 32.80***	848±40.00$^{¥¥¥}$	684±20.80
RBCs (10^6 /µL)	8.34±0.16	7.35±0.23***	7.85±0.13$^{¥¥}$	8.20±0.11
HCT (%)	43.50±0.38	44.74± 0.80	44.52±0.40	44.26±0.50

Values are expressed as mean ± SD of 8 rats per group.

HCD group *vs*. control group: $^{***}p < 0.001$, $^{*}p < 0.05$

HCD+NGEN group *vs*. HCD group $^{¥¥¥}p < 0.001$, $^{¥¥}p < 0.01$

Table 3: Ratio of the kidney weight to the body weight (%) and renal lipid profile (total cholesterol (TC), triglycerides (TG), free fatty acids (FFA) and phospholipids (PL) of control and rats treated with High cholesterol diet (HCD), naringenin (NGEN) and their combination (HCD+NGEN).

Parameters & treatments	**C**	**HCD**	**HCD+NGEN**	**NGEN**
Relative kidney weights (%)	0.69 ±0.026	0.87 ± 0.04***	0.75 ± 0.032$^{¥¥¥}$	0.68 ± 0.02
TC (mg/g tissue)	2.20±0.14	3.26±0.19***	2.43±0.21$^{¥¥¥}$	2.06±0.11
TG (mg/g tissue)	2.54±0.20	5.23±0.16***	3.35±0.22$^{¥¥¥}$	2.45±0.30
FFA (mg/g tissue)	4.10±0.15	7.04±0.3***	4.88±0.31$^{¥¥¥}$	3.90±0.29
PL (mg/g tissue)	13.6±1.5	24.2±1.58***	18.4±1.4$^{¥¥¥}$	13.2±2.3

Values are expressed as mean ± SD of 8 rats per group.

HCD group *vs*. control group: *** $p < 0.001$

HCD+NGEN group *vs*. HCDgroup $^{¥¥¥}$ $p < 0.001$

Table 4: Effect of High cholesterol diet (HCD), naringenin (NGEN) and their combination (HCD+ NGEN) in plasma and tissues hydrogen peroxide (H2O2, nitrite (NO_2^-), reactive oxygen species (ROS) and lipid peroxidation (TBARS).

Parameters & treatments	C	HCD	HCD+NGEN	NGEN
H_2O_2				
Plasma (µmol/L)	22.30 ±2.90	46.00±2.40***	32.50±1.90$^{¥¥¥}$	21.00± 2.00
Kidney(µmol/ mg tissue)	1.48±0.19	4.04±0.30***	2.64±0.28$^{¥¥¥}$	1.30± 0.26
NO_2^-				
Plasma (µM)	0.67±0.03	1.35±0.11***	1.02±0.10$^{¥¥}$	0.60± 0.15
Kidney (µM)	1.56±0.07	2.50±0.16***	1.91±0.14$^{¥¥}$	1.40± 0.16
ROS				
Plasma (pmol DCF/mg protein)	63.1±3.90	94.0± 4.30***	70.00±3.80$^{¥¥¥}$	58.8±5.16
Kidney (pmol DCF/mg protein)	33.50±2.16	79.3± 7.20***	55.20±7.60$^{¥¥¥}$	33.30±3.20
TBARS				
Plasma (µmol/ml)	0.14±0.010	0.52±0.06***	0.35±0.03$^{¥¥¥}$	0.15±0.025
Kidney(nmol/mg protein)	14.17±0.90	31.0±3.40***	22.60±2.07$^{¥¥¥}$	14.05±1.60

Values are expressed as mean ± SD of 8 rats per group.
HCD group *vs*. control group: $^{***}p < 0.001$, $^{*}p < 0.05$
HCD+NGEN group *vs*. HCDgroup $^{¥¥¥}p < 0.001$, $^{¥¥}p < 0.01$

Table 5: Effect of High cholesterol diet (HCD), naringenin (NGEN) and their combination (HCD+ NGEN) on non-enzymatic and enzymatic antioxidants status in kidney tissue: catalase (CAT); glutathione peroxidase (GPx); superoxide dismutase (SOD); Glutathione (GSH); Vitamin C (Vit C) and Vitamin E (Vit E).

Parameters & treatments	**C**	**HCD**	**HCD+NGEN**	**NGEN**
CAT (U/mg protein)	66.25±0.11	43.20 ±1.70***	51.75 ±2.50$^{¥¥}$	62.6 ±2.20
SOD (U/mg protein)	6.54±0.3	4.32 ±0.19***	5.56± 0.18$^{¥¥¥}$	6.3 ±0.25
GPx (U/mg protein)	12.84±1.02	6.06 ±0.50***	8.70±0.4$^{¥¥¥}$	12..3 ± 0.7
GSH (µg/mg protein)	8.67±0.22	5.64±0.56***	6.92±0.44$^{¥¥}$	8.54±0.37
Vit C (µg/mg protein)	2.52±0.13	1.44±0.16***	2.14±0.11$^{¥¥¥}$	2.55±0.12
Vit E (µg/mg protein)	4.20±0.20	2.05 ± 0.13***	3.45±0.35$^{¥¥¥}$	4.40±0.45

Values are expressed as mean ± SD of 8 rats per group.

HCD group *vs*. control group: *** $p < 0.001$

HCD+NGEN group *vs*. HCD group $^{¥¥¥}$ $p < 0.001$, $^{¥¥}$ $p < 0.01$

Table 6: Effect of High cholesterol diet (HCD), naringenin (NGEN) and their combination (HCD+ NGEN) on renal gene expression of proinflammatory markers iNOS, TNF-α, and IL-6, the activation of the nuclear factor kappa B (NF-κB) and ecto-nucleotidases: NTPDase1, NTPDase2, NTPDase3 and 5'-nucleotidase (CD73)

Parameters & treatments	C	HCD	HCD+NGEN	NGEN
Proinflammatory markers				
IL-6	1.00±0.12	1.8.0±0.25***	1.40±0.08$^{¥}$	1.00±0.05
TNF-α	1.00±0.11	2.90.0±0.20***	2.10±0.30$^{¥¥0}$	1.00±0.20
iNOS	1.00±0.13	3.5±0.18***	2.32±0.16$^{¥¥¥}$	1.00±0.10
NF-κB	1.00±0.07	1.94±0.12***	1.4±0.13$^{¥¥}$	1.00±0.15
Ecto-nucleotidase				
NTPDase1	1.00±0.10	0.80±0.04*	0.90±0.03	1.00±0.09
NTPDase2	1.00±0.08	0.6 ±0.12***	0.9±.0.07$^{¥¥¥}$	1.00±0.10
NTPDase3	1.00±0.05	0.85±0.03	0.85±0.13	1.00±0.08
CD73	1.00±0.11	0.74± 0.10***	0.92±0.05$^{¥¥}$	1.00±0.11

Values are expressed as mean ± S.D. of 4 rats per group. ol and HCD group, respectively.

HCD group *vs.* control group: $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05

HCD+NGEN group *vs*. HCD group $^{¥¥¥}p < 0.001$, $^{¥¥¥}p < 0.01$, $^{¥}p < 0.05$