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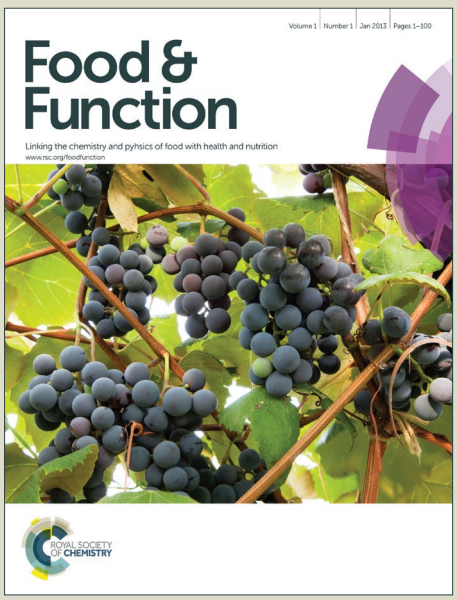

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Citrus flavanones prevent systemic inflammation and ameliorate oxidative stress in C57BL/6J mice fed high-fat diet

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Abbreviation list:

IL-6: interleukin-6

IL-10: interleukin-10

TNF-α: tumor necrosis factor-α

MCP-1: macrophage chemoattractant protein-1

hs-CRP: high-sensitivity C-reactive protein

TBARS: thiobarbituric acid reactive substance

CVD: cardiovascular disease

NF-κB: nuclear factor kappa-B

COX-2: cycloxigenase-2

ALT: alanine transaminase

AST: aspartate transaminase

ABTS^{*+}: 2,2'-azino-di-(3-ethylbenzothiazoline)-6-sulfonic acid

TEAC: Trolox equivalent antioxidant capacity

MDA: malondialdehyde

LDLR: LDL receptor

ABSTRACT

The flavanones hesperidin, eriocitrin and eriodictyol were investigated for their prevention of the oxidative stress and systemic inflammation caused by high-fat diet in C57BL/6J mice. The mice received a standard diet (9.5% kcal from fat), high-fat diet (45% kcal from fat) or high-fat diet supplemented with hesperidin, eriocitrin or eriodictyol for a period of four weeks. Hesperidin, eriocitrin and eriodictyol increased the serum total antioxidant capacity, and restrained the elevation of interleukin-6 (IL-6), macrophage chemoattractant protein-1 (MCP-1), and C-reactive protein (hs-CRP). In addition, the liver TBARS levels and spleen mass (g/kg body weight) were lower for the flavanone-treated mice than in the unsupplemented mice. Eriocitrin and eriodictyol reduced TBARS levels in the blood serum, and hesperidin and eriodictyol also reduced fat accumulation and liver damage. The results showed that hesperidin, eriocitrin and eriodictyol had protective effects against inflammation and oxidative stress caused by high-fat diet in mice, and may therefore prevent metabolic alterations associated with the development of cardiovascular diseases in other animals.

Keywords: Citrus flavonoids; high-fat diet; obesity; inflammation; oxidative stress; C57BL/6J mice

Introduction

Prospective studies show that dietary patterns can modify risks for coronary disease. High-fat diets combined with excess body weight lead to adverse metabolic outcomes, and according to the Framingham Heart Study,[1] the risk for cardiovascular disease (CVD) is particularly increased when abdominal obesity is present. Furthermore, obesity is a major modifiable risk factor that can lead to dyslipidemia, type 2 diabetes and hypertension.[2]In contrast, the consumption of diets rich in fruits, vegetables and unsaturated fatty acids are associated with a lowering of CVD risk, due to their antioxidant nutrients and bioactive compounds.

Adipose tissue accumulation in mice fed high-fat diets increases migration of macrophages, leading to the stimulation of free radical production and secretion of inflammatory cytokines.[3,4]This leads to lipotoxicity in non-fat tissues, causing structural and functional changes in cells, and stimulation of further release of cytokines and chemokines, ultimately resulting in chronic systemic low-grade inflammation.[5]High levels of fat in obese rodents increase systemic levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1), which are related to the development of liver steatosis and insulin resistance.[6,7]

Hesperidin and eriocitrin are flavanone glycosides from oranges and limes, and are deglycosylated by intestinal bacteria to hesperetin and eriodictyol, respectively.[8]Subsequently they are conjugated to glucuronides and sulfates in enterocytes and hepatocytes, both yielding homoeriodictyol and hesperetin conjugated metabolites.[9]Thesemetabolites have antioxidant and anti-inflammatory activities capable of scavenging free radicals and inhibiting *in vitro* inflammation.[10-15]Moreover, in diabetic rats eriodictyol attenuated inflammation and decreased nitric oxide, proinflammatory cytokines and plasma lipid peroxidation.[16]Hesperetin blocked the activation of nuclear factor kappa-B (NF-κB) by TNF-α in mice adipocytes, reduced oxidative

stress, cycloxigenase-2 (COX-2) expression and production of IL-6 in mice with colon carcinogenesis.[17,18]

These studies suggest, therefore, that such citrus flavanones may lessen chronic low-grade systemic inflammation in animals fed high-fat diets, and as a result may reduce the occurrence of diabetes and CVD. To test this, we analyzed the effects of the citrus flavanones hesperidin, eriocitrin and eriodictyol on the antioxidant capacities in liver and blood serum and on the systemic inflammation in C57BL/6J mice fed a high-fat diet.

Materials and methods

Animals and dietary treatment

Sixty nine-week-old male C57BL/6J mice (São Paulo State University, SP, Brazil) were maintained in an isolated macro-environment system, with a 12-h light/12-h dark cycle, and 22 ± 2 ºC, receiving food and water *ad libitum.* The animals was maintained individually in conventional housing inside of the ventilated storage cabinets (Tecniplast, S.A., Buguggiatte, VA, Italy). They were randomly divided into six groups, with similar body weight distributions in each group, ten mice per group, and were fed either a standard diet, a high-fat diet, or a high-fat diet supplemented with ibuprofen, hesperidin, eriocitrin, or eriodictyol. Mice were allocated one per cage, and the body weight was used for the dosage of the supplements, offered in mg/kg body weight. All supplements were mixed into the diet, based on the food intake of the previous day (grams of food ingested/day) with an additional of 10% to ensure the intake of the daily dose. The food intake was monitored at regular intervals of 24 hours, and the supplements were adjusted accordingly. Body weights were recorded weekly and food consumption daily. The high-fat diet contained 21, 34 and 45% energy from protein, carbohydrates and fat, respectively (Rhoster Industry and Trade LTD, SP, Brazil). The control diet contained 15, 76 and 10% energy from protein, carbohydrates, and lipids, respectively (Table 1), based on the AIN93 M diet.[19]After four weeks of treatment, mice were anesthetized with xylazine/ketamine (16/60 mg/g

of body weight) *via* i.p. injection following a 10-h fast, and blood samples were drawn by cardiac puncture. Organs and serum samples were stored at -80°C until analysis. One lobe of each liver sample was fixed in 10% buffered formalin for 48h, rinsed with distilled water and soak in 70% alcohol solution before perform histological analysis by a pathologist. All the animals were handled according to the guidelines of the Brazilian College of Animal Experimentation (COBEA) and the experimental animal protocol was approved by the Animal Use Committee of the Pharmaceutical Sciences Faculty, São Paulo State University, SP, Brazil (n^{o} 18/2013).

Supplementation

Hesperidin supplements were given at a dose of 100 mg/kg body weight, eriocitrin and eriodictyol at 200 mg/kg body weight, and ibuprofen at 20 mg/kg body weight (included as a positive control for anti-inflammatory activity). Doses were selected on the basis of scientific literature that have shown the hesperidin effectiveness to reduce inflammation and oxidative stress in the rodent models.20, 21, 22 Furthermore, supplements were added to the regular diet of the animals to favor the normal physiological pathway of intake and, were offered regularly to maintain a constant level of the compound in the body. Hesperedin purity was > 98 %, and was extracted from the fruit peel of *Citrus sinensis* (*Rutaceae*). Eriocitrin purity was >85% and eriodictyol was > 95%, and both were extracted from the fruit peel of *Citrus limon* (*Rutaceae*).

Blood serum analyses

Serum was obtained by centrifugation and levels of alanine transaminase (ALT), aspartate transaminase (AST), glucose, triglycerides, total cholesterol and HDL-C were evaluated by enzymatic colorimetric assay using commercial kits (Labtest, MG, Brazil). LDL-C was calculated using the Friedewald formula.[23] High-sensitivity C-reactive protein (hs-CRP) was measured by immunoturbidimetric assay with commercial kits (Labtest, MG, Brazil), and

inflammatory cytokines assays (IL-6, IL-10, TNF-α, and MCP-1) were performed by ELISA assays using the Multiplex LuminexMAP detection method (Genese Diagnostics Products Ltd, SP, Brazil).

Organs and liver histology

Organ weights (visceral adipose tissue, liver, spleen and heart) were normalized against body weight of the respective animal (organ weight/body weight) to give a relative percentage. Liver tissues fixed in formalin were embedded in paraffin and sectioned to 4–6 mm of thickness. Deparaffinized liver tissue sections were stained with hematoxylin-eosin and Masson's trichrome, using standardized protocols (Pathology Department of Odontology Faculty from Sao Paulo State University, SP, Brazil). A pathologist analyzed all treated mice hepatic cells by optical microscopy to recognize any morphologic alteration in comparison to control group.

Oxidative stress parameters

Liver and serum oxidative stress were measured by thiobarbituric acid-reactive substances (TBARS).Liver TBARS values were given in μM MDA/mg protein, quantified by Lowry method, [24]and serum TBARS in μM MDA. Total antioxidant activity was measure by the ABTS assay.[25] $ABTS^{*+}$ radical formation was measured at 734 nm, using Trolox (Sigma) as a standard and given as mM of Trolox equivalent antioxidant capacity (TEAC). All analyzes were performed in triplicate.

Statistical analysis

All results are presented as means ± standard deviation. The data distributions were tested for normality, and subsequently, the intergroup variation was measured by one-way ANOVA followed by post hoc analysis (Student Newman Keuls test) to evaluate the effects of diets and/or supplement, with a significance level of $p < 0.05$ (Sigma Stat Software, USA).

Results

Diet- and supplement-induced changes in body weight and organs

The high-fat diet with or without the flavanone supplements showed good acceptance by the animals without harmful effects, and the supplement daily doses reached 97% on average of the calculated dose. Groups fed the high-fat diet with or without supplements showed higher body weight gain, with exception of the hesperidin group, and higher visceral fat compared to the mice fed the standard diet (Table 2). Animals fed the high-fat diet supplemented with hesperidin, eriocitrin or eriodictyol showed lower spleen mass (g/g of body weight) than the unsupplemented high-fat diet group; the spleen mass of the ibuprofen and standard diet groups was intermediate; and the treatment with the unsupplemented high-fat diet increased the heart mass, while eriocitrin showed an intermediate value and hesperidin, eriodictyol and ibuprofen showed the lowest heart masses (Table 2).

Biochemical profile

After four weeks with the high-fat diet, mice blood glucose levels were elevated 20%with the unsupplemented high-fat diet, while the increases were only approximately 8% for the groups fed the high-fat diet supplemented with hesperidin, eriocitrin, eriodictyol and ibuprofen, however this effect was not statistically significant. Furthermore, no effect was observed from the high-fat diet with ibuprofen or citrus flavanones on bloodserum triglycerides compared to the non-supplemented high-fat diet. Total cholesterol, HDL-C and LDL-C were similarly increased by the high-fat diet even with the supplements, where only hesperidin was able to lower the LDL-C levels by28%. Serum hepatic enzymes ALT and AST were not altered by the high-fat diet alone or with flavanones or ibuprofen (Table 3).

Anti-oxidative stress and anti-inflammatory effects

Unsupplemented high-fat diet increased TBARS levels by 75%in the liver and 25%in the blood serum in comparison to standard diet. In contrast, all supplements (ibuprofen, hesperidin, eriocitrin and eriodictyol) were able to maintain liver TBARS at the levels of the standard diet group. Blood serum TBARS, after ibuprofen and hesperidin, were not different to the values observed with the standard diet, but they were lowered 34% by the eriocitrin and eriodictyol supplements. Furthermore, total antioxidant capacity measured by the reduction of $ABTS^{*+}$ radical in the blood serum was significantly lowered by the high-fat diet, and ibuprofen was able to maintain the same level as with the standard diet. Hesperidin, eriocitrin and eriodictyol slightly increased an average of 3.5% the blood serum antioxidant capacity (Table 4).

After four weeks with high-fat diet, IL-6 and MCP-1 increased by7.6 and 3.2foldin the blood serum of mice, showing the effect of the chronic dietary treatment over these inflammatory markers. Ibuprofen and all flavanones were able to decreaseIL-6to the standard diet level, without any significant differences among them.MCP-1production was similarly inhibited by these compounds in comparison to the high-fat diet group. No changes were observed in serum levels of TNF-α and IL-10 for any of the studied groups. Finally, hs-CRP levels were increased by 13% with the high-fat diet, while hesperidin, eriocitrin and eriodictyol supplemented groups were able to lower the hs-CRP by 25%, 25%, and 13%, respectively compared to the standard and unsupplemented high-fat diet groups(Table 4).

Liver histology

Liver sections from mice fed the standard diet showed typical morphology with normal microvesicular depots of fat (Figure 1, A), unlike the mice fed the high-fat diet that showed largely macrovesicular fat depots (Figure 1, B). Hepatocytes from these mice also showed morphologic alterations, as peripheral nuclei (Figure 1, B-1), undefined contours or even fragmented cells (Figure 1, B-2) characteristic of cellular necrosis, and some cells evidenced cellular ballooning (Figure 1, B-3). Although the high-fat diet had high potential to induce

steatosis, the ibuprofen and the citrus flavanone hesperidin did not show cellular alterations and reduced fat depots (Figure 1, C and D). No evidence of cellular ballooning or cell degradation in the liver parenchyma was observed for the mice fed the high-fat diet supplemented with the flavanones and ibuprofen. However, liver sections of mice supplemented with eriocitrin (Figure 1, E) showed histological features similar to the unsupplemented high-fat diet fed mice.

Discussion

The citrus flavanones hesperidin, eriocitrin and eriodictyol were able to prevent key metabolic changes induced by high-fat diet consumption, specifically: (1) increased blood serum levels of IL-6 and MCP-1; (2) elevated TBARS levels in liver and blood serum; (3) liver lipid accumulation and liver damage; (4) decreased antioxidant capacity in the blood serum; and (5) higher mass of heart and spleen. Among the flavanones, only hesperidin showed LDL-C lowering activity, decreasing its level by 28%, an effect that has been reported by others.[26,27] In previous studies this ability was related to the reduction of Apo B secretion and synthesis of VLDL-C, and increased expression of the LDL receptor (LDLR) gene, thereby decreasing the levels of LDL-C circulating.[28, 29] In this present study, all animals that received the high-fat diet with 4.7 times more calories from fat compared with the standard diet, and supplemented with flavanones or not, showed higher body weight and visceral fat, and higher levels of serum lipids and glucose. These deleterious effects of the high-fat diet subsequently led to elevation of key inflammatory markers and increased hepatic lipid deposition.

The presence of hepatic steatosis and a high heart mass, observed in the group fed the unsupplemented high-fat diet, should be a result of the loss of ability to regulate the synthesis and storage of triglycerides in adipose tissue, caused by inflammation.[30,31] Also, the observed increased spleen mass has been previously shown to be related to the inflammatory stimulus of a high-fat diet.[30] However, hesperidin, eriodictyol and ibuprofen efficiently suppressed the heart mass increase, and all of the flavanones prevented spleen mass increase, but ibuprofen did not.

198 The lipotoxicity in heart tissue can induce inflammation and contribute to its remodeling, as
199 shown previously with transgenic mice, where pro-inflammatory cytokine and macrophage
200 infiltration preceded the onset of myocardial dysfunction.[31]The citrus flavanones and ibuprofen
201 were able to prevent the increased mass of heart and spleen possibly by lowering the oxidative
202 stress and inflammation in these organs, as shown by the suppression of systemic levels of IL-6,
203 MCP-1 and TBARS.

204 With an excessive fat supply, the liver is the organ most susceptible to lipotoxicity,
205 because of the large amounts of fat directly received through the portal vein along with
206 circulating lipids and lipoproteins.[32]In the present study, four weeks of the unsupplemented high-
207 fat diet consumption caused severe variations in hepatic structure, showing morphological
208 changes as ballooning, cellular and nuclear degradation characterizing a process of cell death in
209 the presence of hepatic steatosis, presented as macro and microvesicles of fat. A recent study
210 showed that consumption of a high-fat diet by mice could lead to hepatic steatosis in three weeks
211 or less.[33] In comparison, it was previously shown[34]that the consumption of a fast-food diet, rich
212 in both fat and sugar, revealed the presence of hepatic steatosis with pronounced ballooning and
213 progressive fibrosis, while the consumption of a high-fat diet caused hepatic steatosis without
214 other alterations.

215 Supplementation with hesperidin, eriodictyol and ibuprofen decreased lipid droplets in
216 the liver and prevented morphological alterations in hepatocytes of mice fed a high-fat diet,
217 showing the anti-inflammatory action against liver damage. In addition, flavanones significantly
218 reduce the serum levels of C-reactive protein, which is an indicator of liver damage.[35,36]Along
219 with the liver, accumulation of visceral adipose tissue (epididymal, retroperitoneal and peri-
220 renal) has been widely used to measure inflammation in humans and rodents obese.[37-42] In
221 general, those metabolic changes induce the elevation of systemic levels of MCP-1, which has
222 been linked to the infiltration of macrophage and inflammatory cells in adipocytes,
223 proportionally to the increase of the adipose tissue.[4,43,44]

In liver and adipose tissue, high fat levels are associated with increased NF-κB activity, systemic levels of pro-inflammatory cytokines and acute phase proteins.[45] In addition, liver production of C-reactive protein, stimulated by increased levels of IL-6 and TNF-α, lead to a systemic inflammatory response.[46] As observed in this study, mice fed the unsupplemented high-fat diet showed an increase in serum levels of both IL-6 and MCP-1, but no changes in serum levels of IL-10 and TNF-α. In sharp contrast, the supplements hesperidin, eriocitrin, eriodictyol and ibuprofen dramatically suppressed the high-fat diet stimulated elevation of the proinflammatory IL-6 and MCP-1 in the serum, leading ultimately to significantly suppress systemic inflammation in the supplement-treated mice, efficiently defeating pro-inflammatory response.

The metabolites of the flavanones produced in the mice were not analyzed in this study, but other previous studies have shown that mammalian metabolism of these compounds initially produce a series of intact flavanone glucuronides, sulfates, and glucurono-sulfate conjugates mainly at the 3', 4', and 7 positions of the flavanone B and A rings, respectively.[47, 48] Very little, if any, of the unmodified flavanone aglycones are detected as metabolites. Subsequent to the production of the glucuronide and sulfate metabolites, further metabolism produces advanced ring-fission products, including compounds like *m*-coumaric acid, ferulic acid, 3,4-dihydroxyphenylpropionic acid, 3-methoxy-4-hydroxyphenylpropionic acid, *m*-hydroxyhippuric acid, *m*-hydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid, and others.[49]

Research in this field has not advanced to the point of knowing the relative impacts of these diverse metabolites on the sum-total biological effects observed in the test animals following the flavanone doses. Yet, it is known that the effects of glucuronide metabolites of hesperidin are not equal in their effects on cytokine production during inflammation,[50] but rather strong structure/function relationships play roles in biological responses. Also, while assumptions are made that flavonoids, in general are anti-inflammatory, it is also known that in some cases flavonoids promote the production of pro-inflammatory cytokines.[51] Hence, the most

conclusive evidence of anti-inflammatory actions is that obtained by whole animal studies, while in vitro studies point to many possible pathways and outcomes, the selections of which are difficult to predict. However, the benefits of the supplementation observed in this experimental model are consistent with the fact that eriodictyol can exert high antioxidant activity due to its *o*-dyhydroxyl structure in the B ring,[9] and metabolites of eriocitrin and hesperidin increase the concentration of antioxidant enzymes in vivo.[10] Eriodictyol and hesperetin can inhibit cellular oxidative stress and inflammatory damage via regulating antioxidant responses.[52, 53] All these effects are related to lower degree of inflammation and liver damage.

Conclusion

In conclusion, the present study showed that supplementation with hesperidin, eriocitrin and eriodictyol was efficiently able to suppress the systemic state of inflammation induced by a high-fat diet, and to prevent damage to organs such as liver, heart and spleen. Further tests are planned to test whether these flavanones are also useful in preventing metabolic disorders and chronic disease, as CVD and diabetes mellitus.

Conflict of interest

None of the authors have any conflicts of the interest.

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Figure Legend

Figure 1. Histological sections of liver tissue (40 x magnification) of mice fed standard diet (A), high-fat diet (B), high-fat diet supplemented with ibuprofen (C), hesperidin (D), eriocitrin (E), and eriodictyol (F). Numbers in section B: (1) peripheral nuclei, (2) absence of nucleus and (3) cellular ballooning.

Table 1. Composition of high-fat diet and standard diet fed to male C57BL/6J mice for 4 weeks

Composition	G/kg	
Diet	High-fat	Standard
Protein (%kcal)	20.8	14.7
Carbohydrate (%kcal)	33.8	75.8
Fat (% kcal)	45.4	9.5
Energy (kcal/g)	5.4	4.2
Corn starch	78	466
Maltodextrin	117	155
Sucrose	201	100
Casein	240	140
L-cistein	3.5	1.8
Soybean oil	29	40
Lard	207	0
Fiber	58	50
Vitamin mix	12	10
Mineral mix	12	35
Dibasic calcium phosphate	15	0
Calcium carbonate	6.4	0
Potassium citrate	19	0
Choline bitartrate	2.3	2.5
Tert-butylhydroquinone	0.1	8.0

Table 2. Body weight and organ percentage of mice fed high-fat diet supplemented with flavanones and ibuprofen for 4weeks

Diet	Standard	High-fat				
Supplement	None	None	Ibuprofen	Hesperidin	Eriocitrin	Eriodictyol
Weight gain (g)	4.5 ± 0.9a	7.2 ± 3.6b	6.8 ± 3.2b	5.6 ± 0.9ab	7.0 ± 1.4b	7.5 ± 2.5b
Visceralfat(%)	1.0±0.3a	4.5±1.9b	4.6±1.9b	3.8±1.4b	3.8±1.3b	4.5±1.3b
Liver (%)	4.8±0.4b	4.2±0.3a	4.0±0.4a	3.9±0.4a	3.9±0.4a	3.9±0.4a
Spleen(%)	2.6±0.3ab	3.0±0.8b	2.5±0.6ab	2.2±0.5a	2.4±0.4a	2.3±0.4a
Heart(%)	0.47±0.05ab	0.50±0.08b	0.41±0.06a	0.43±0.06a	0.45±0.05ab	0.42±0.07a

Values are mean ± SD. Data analyzed by 1-factor ANOVA, followed by the Student Newman Keuls multiple comparison test. Means in a row followed by different letters differ significantly ($p>0.05$).

Table 3. Biochemical profiles of mice fed high-fat diet supplemented with flavanones and ibuprofen for 4weeks[1]

Diet	Standard	High-fat			
Supplement	None	None	Ibuprofen	Hesperidin	Eriocitrin	Eriodictyol
Glucose(mg/dL)	302 ± 4a	375 ± 59b	321 ± 47ab	337 ± 85ab	332 ± 44ab	316 ± 50ab
Triglycerides (mg/dL)	81 ± 8	71 ± 14	88 ± 17	84 ± 20	90 ± 18	88 ± 20
Total cholesterol (mg/dL)	89 ± 14a	138 ± 2bc	150 ± 27c	122 ± 20b	147 ± 34c	157 ± 22c
HDL-C (mg/dL)	55 ± 9a	83 ± 1bc	88 ± 13c	77 ± 15b	94 ± 22c	99 ± 14c
LDL-C(mg/dL)	18 ± 9a	40 ± 12c	44 ± 13c	29 ± 9b	36 ± 12bc	40 ± 8c
ALT[2](U/L)	52 ± 13	44 ± 23	55 ± 9	41 ± 16	39 ± 14	41 ± 11
AST[3](U/L)	152 ± 36	153 ± 52	161 ± 36	146 ± 45	166 ± 55	133 ± 50

[1]Values are mean ± SD. Data analyzed by 1-factor ANOVA, followed by the Student Newman Keuls multiple comparison test. Means in a row followed by different letters differ significantly ($p>0.05$).

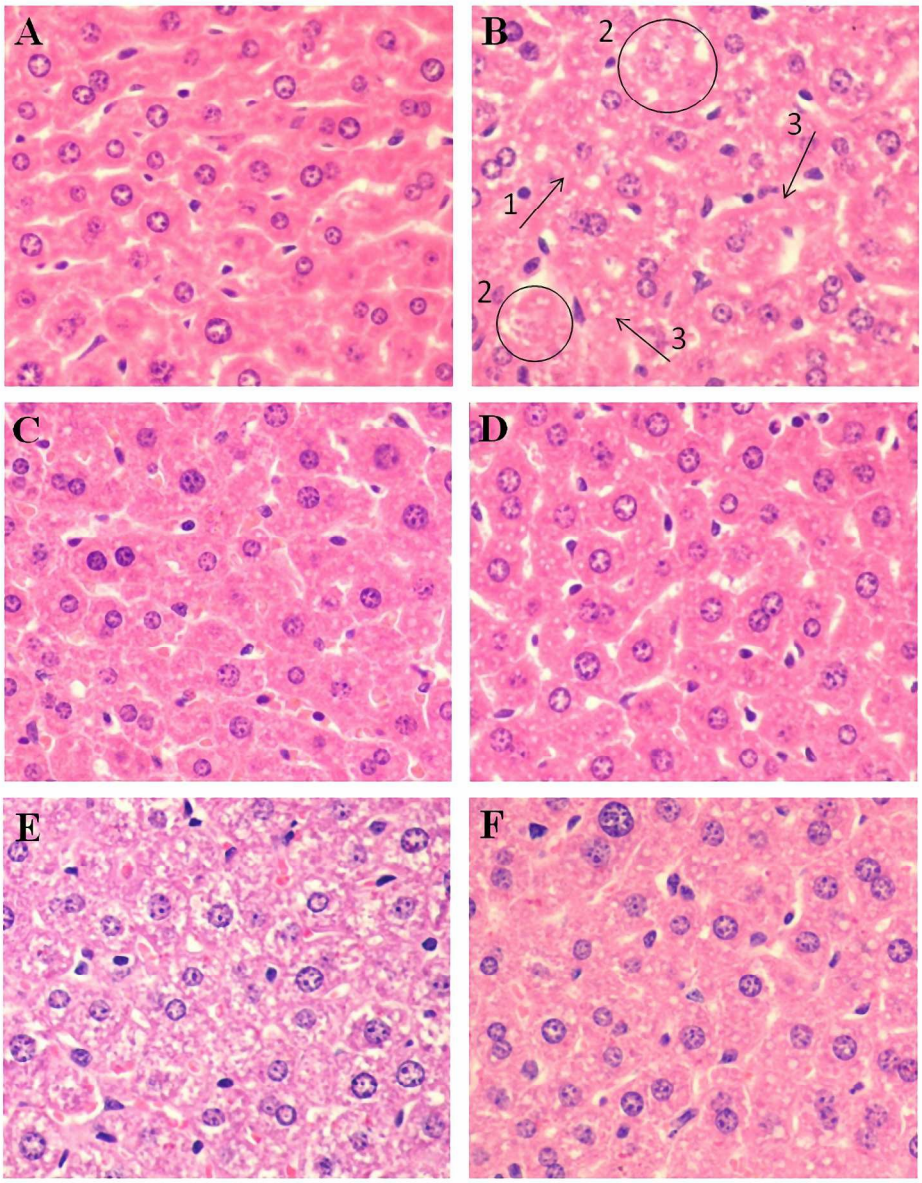
[2]Alanine transaminase.

[3]Aspartate transaminase.

Table 4. Oxidative stress and inflammatory markers of mice fed high-fat diet supplemented with flavanones and ibuprofen for 4weeks

Diet	Standard	High-fat			
Supplement	None	None	Ibuprofen	Hesperidin	Eriocitrin	Eriodictyol
Serum TBARS (μm)	0.95±0.21ab	1.19±0.64b	1.04±0.22ab	0.81±0.17ab	0.62±0.05a	0.63±0.10a
Liver TBARS (μm/mg)	0.08±0.03a	0.14±0.08b	0.03±0.01a	0.07±0.01a	0.06±0.02a	0.05±0.01a
Serum ABTS (mm)	1.50±0.06ab	1.46±0.07a	1.51±0.08abc	1.56±0.08bc	1.59±0.06c	1.55±0.04bc
IL-6 (pg/ml)	8.44±7.2a	64.4±44.3b	21.9±16.7a	5.76±4.32a	3.09±1.54a	5.71±2.53a
IL-10 (pg/ml)	4.97±2.16	3.22±1.77	6.02±4.20	5.27±2.23	3.95±2.03	4.71±1.57
TNF-α (pg/ml)	2.44±0.72	2.54±0.99	2.10±1.69	2.68±0.30	2.05±0.85	2.04±0.18
MCP-1 (pg/ml)	22.8±6.9a	73.3±43.6b	43.4±29.2a	27.6±8.5a	19.3±10.4a	14.1±5.9a
Hs-CRP (mg/L)	0.08±0.0bc	0.09±0.0c	0.08±0.01b	0.06±0.02a	0.06±0.02a	0.07±0.02ab

Values are mean ± SD. Data analyzed by 1-factor ANOVA, followed by the Student Newman Keuls multiple comparison test. Means in a row followed by different letters differ significantly ($p>0.05$).



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In vivo antioxidant and anti-inflammatory effects of citrus flavanones