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Biodistribuição e farmacocinética da eriocitrina em ratos e seus efeitos regulatórios em camundongos induzidos à obesidade por dieta hiperlipídica

Paula Souza Ferreira

Araraquara

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Paula Souza Ferreira

Tese apresentada ao Programa de Pós-graduação em Alimentos e Nutrição para obtenção do título de Doutor em Alimentos e Nutrição.

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Tese de Doutorado apresentada à Faculdade de Ciências Farmacêuticas da Universidade Estadual Paulista – UNESP, Campus de Araraquara como requisito para a obtenção do título de Doutora em Alimentos e Nutrição.

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Dedicatória

Dedico este trabalho:

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Sumário

Resumo

Objetivos: Avaliar os efeitos da eriocitrina nas alterações do metabolismo, inflamação e estresse oxidativo causados pela dieta hiperlipídica em camundongos e seu metabolismo, farmacocinética e biodistribuição em ratos. **Métodos:** Sessenta camundongos C57BL/6J foram divididos aleatoriamente em seis grupos (n=10 cada), quatro grupos foram alimentados com dieta hiperlipídica por quatro semanas, e então suplementados com 10, 25, 50 ou 100 mg/kg de eriocitrina por mais quatro semanas. Outros dois grupos incluíram um grupo alimentado com dieta padrão, e outro com dieta hiperlipídica sem suplemento por oito semanas. Foram avaliados o perfil lipídico, glicêmico, lipídios e gordura hepática, inflamação e estresse oxidativo sistêmicos. Para o estudo farmacocinético, e de biodistribuição, 36 ratos Wistar machos receberam 100 mg/kg de eriocitrina e foram divididos em 12 grupos de 3 ratos cada, de acordo com os tempos de coleta: 0, 0,5, 1, 3, 4, 5, 6, 8, 10, 12, 15 e 24 h. Foram coletados o sangue, órgãos e urina para análises de HPLC-MS e espectroscopia de infravermelho dos metabólitos de eriocitrina. **Resultados:** No primeiro estudo, o grupo alimentado com dieta hiperlipídica apresentou aumento do peso corporal, gordura abdominal e dos níveis séricos de glicose, insulina, triglicerídeos, colesterol total, resistina, leptina e peroxidação lipídica ($p < 0,05$). No entanto, reduções desses marcadores foram observadas com todas as doses de eriocitrina, entre as quais a dose de 25 mg/kg foi a mais efetiva, diminuindo significativamente os níveis séricos de triglicerídeos (-33%), e melhorando os níveis séricos de glicose (-25%), insulina (-35%), resistina (-18 %) e peroxidação lipídica (-21%). No segundo estudo, nove metabólitos de eriocitrina foram identificados. A meia-vida daqueles detectados no plasma foi de 3 e 3,2 horas. Os metabólitos da eriocitrina foram amplamente distribuídos nos órgãos dos ratos (pâncreas>rins>fígado>coração>músculo>baço>tecido adiposo) e exibiram uma biodisponibilidade total > 1%. **Conclusão:** Estes resultados indicam que a eriocitrina pode ajudar a reduzir distúrbios metabólicos relacionados ao desenvolvimento de diabetes mellitus tipo 2 em obesos, devido aos seus efeitos sobre a glicemia e insulinemia, e pela considerável deposição de seus metabólitos no pâncreas, o que sugere um efeito direto da eriocitrina na proteção da função pancreática.

Palavras-chave: Eriocitrina; obesidade; resistência insulínica, diabetes *mellitus* tipo 2; metabolismo; farmacocinética; biodistribuição.

Abstract

Aim: To assess the effect of eriocitrin on metabolic, inflammatory and oxidative stress changes caused by high-fat diet induced obesity in mice; and its metabolism, pharmacokinetics and biodistribution in rats. **Methods:** Sixty C57BL/6J mice were randomly divided into six groups (n = 10 each), four of them were fed a high-fat diet for four weeks, and then supplemented with 10, 25, 50 or 100 mg/kg eriocitrin for another four weeks. Two other groups included one group fed a standard diet, and one group fed high-fat diet for eight weeks, both without supplementation. The lipidemia, glycemia, hepatic fats, systemic inflammation and oxidative stress were evaluated. For the pharmacokinetic and biodistribution study, 36 male Wistar rats were orally administered 100 mg/kg eriocitrin and divided into 12 groups of 3 rats each, according to the following time points: 0, 0.5, 1, 3, 4, 5, 6, 8, 10, 12, 15 and 24 h. Blood, organs, and urine were collected at each time point, and the eriocitrin metabolites were extracted and analyzed by HPLC-MS and infrared spectroscopy. **Results:** In the first study, the group fed high-diet presented increased body weight, abdominal fat, and glucose, insulin, triglycerides, total-cholesterol, resistin, leptin and lipid peroxidation in the serum ($p < 0.05$). However, positive effects of eriocitrin were observed with all of the doses tested, whereas the dose 25 mg/kg bw was the most effective. It decreased significantly the triglycerides (-33%), and improved glucose (- 25%), insulin (-35%), resistin (-18%) and lipid peroxidation (-21%) in blood serum. In the second study, nine metabolites of eriocitrin were identified, and the half-life of metabolites detected in plasma was 3 and 3.2 hours. Eriocitrin metabolites were widely distributed in the rat body (pancreas> kidneys>liver>heart>muscle>spleen>adipose tissue) and exhibited a total bioavailability of 0.5%. **Conclusion:** These results indicate that oral supplementation with eriocitrin may help to reduce metabolic disorders related to the development of diabetes mellitus type 2 in obese individuals, whereas the high concentrations of eriocitrin metabolites found in pancreas suggest a direct role of eriocitrin in the protection of pancreatic function.

Keywords: Eriocitrin; obesity; insulin resistance, diabetes mellitus type 2; metabolism; pharmacokinetics; biodistribution.

Introdução

O papel da obesidade no desencadeamento de doenças crônico-degenerativas está fortemente ligado ao consumo de dietas hipercalóricas, especificamente as dietas com alto teor de gordura saturada, levando a um estado de inflamação crônica e subaguda, acompanhada do acúmulo excessivo de lipídios no tecido adiposo, fígado, coração, artérias e músculos (1). Este estado representa um padrão alimentar não saudável observado em grande parte da população ocidental, onde a ingestão continuada de dietas hiperlipídicas tem sido preditiva de vários distúrbios metabólicos. Em todo o mundo, pelo menos 2,8 milhões de pessoas morrem a cada ano como resultado de excesso de peso ou obesidade. Os riscos para doença cardíaca coronariana, acidente vascular cerebral isquêmico e diabetes mellitus tipo 2 aumentam com o aumento do índice de massa corporal (IMC), que também aumenta o risco de vários tipos de câncer (2). A deposição anormal de lipídios nos tecidos (colesterol, ceramidas, triglicerídeos e ácidos graxos livres) é uma linha característica comum dos distúrbios metabólicos observados na obesidade. A dislipidemia da síndrome metabólica, caracterizada por uma superprodução hepática de lipoproteínas de muito baixa densidade (VLDL) e baixa produção de lipoproteínas de alta densidade (HDL) contribui para a deposição de lipídios nos tecidos. Essa desregulação do metabolismo lipídico contribui para a patogênese das doenças cardiovasculares e diabetes mellitus tipo 2 (3).

Vários são os mecanismos que podem estar envolvidos no efeito da obesidade e consumo de dietas ricas em gorduras, principalmente as saturadas, sobre a inflamação e o estresse oxidativo, como por exemplo, a produção de espécies lipídicas tóxicas, remodelação das membranas lipídicas, receptores inatos e nucleares, apoptose e, possivelmente a quimiotaxia da microbiota intestinal (4).

Foi mostrado que a alta ingestão de glicose e gordura saturada podem induzir inflamação através do aumento do estresse oxidativo e das atividades de fatores de transcrição, como o fator nuclear kappa B (NFkB) (5). Uma única infusão intravenosa de triglicerídeos em indivíduos eutróficos aumentou os níveis de ácidos graxos livres no sangue e levou a uma resposta inflamatória como a observada em indivíduos obesos. Os ácidos graxos saturados podem ativar diretamente as respostas pró-inflamatórias em células vasculares endoteliais, adipócitos e células mielóides. A sobrecarga de gordura nos tecidos resulta tanto na ativação do estresse oxidativo, quanto das vias inflamatórias (6).

Os ácidos graxos, láurico, mirístico e palmítico estão presentes na molécula de lipídio A do lipopolissacarídeo (LPS) da membrana exterior de bactérias gram-negativas, onde desempenham um papel importante na ativação do receptor do tipo Toll 4 (TLR-4) (7). Alternativamente, os ácidos graxos podem modular vias inflamatórias através da ligação a G-receptores acoplados a proteínas e a receptores X do fígado (LXR), que são expressos em células do sistema imunológico e de tecidos metabolicamente ativos, afetando a inflamação e o metabolismo da glicose. Outro mecanismo pode envolver o aumento da translocação de LPS do intestino para a circulação, causado pela ingestão de dietas ricas em gordura, que tornam a mucosa intestinal mais suscetível a entrada de substâncias tóxicas produzidas por bactérias (8,9).

A elevação de ácidos graxos livres no sangue e acúmulo ectópico de lipídios nos tecidos provoca alterações funcionais e estruturais nas células (10). Essa lipotoxicidade pode causar lesões teciduais e apoptose por meio do estresse oxidativo do retículo endoplasmático, levando à liberação de citocinas ou quimiocinas pelas células lesadas (10). Além disso, o aumento da produção de espécies reativas de oxigênio e a diminuição de sustâncias antioxidantes estão

associados à diminuição da capacidade de diferenciação e proliferação dos adipócitos (11,12). Tal falha no processo adipogênico fundamenta o paradigma de expansão do tecido adiposo, no qual a sua incapacidade para armazenar o excesso de triglicerídeos da dieta provoca um ambiente diabetogênico, promovendo o aumento do fluxo e a deposição de ácidos graxos em órgãos insulino dependentes, levando à secreção de adipocinas que aumentam o estresse oxidativo, a inflamação local e sistêmica, além de promover um ambiente hipóxico (13). Estudos têm mostrado que o consumo de dietas hiperlipídicas pode levar à hipertrofia do coração, e que a obesidade induzida por dieta está associada ao aumento da utilização de ácidos graxos e redução das taxas de utilização de glicose pelo miocárdio, que ocorre pela redução do conteúdo e translocação do GLUT-4, um transportador de glicose regulado por insulina, encontrado principalmente no tecido adiposo e músculo estriado. Este processo representa um sério risco cardiovascular e pode levar à disfunção do miocárdio (14).

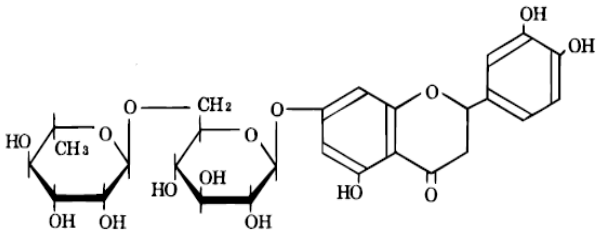
Em camundongos, o consumo de dieta hiperlipídica aumenta as vias para produção de radicais livres no fígado e no tecido adiposo, sugerindo que o estresse oxidativo seja um dos passos iniciais da desregulação metabólica da obesidade, estimulando a produção de moléculas pró-inflamatórias e proteínas de fase aguda, como a proteína C-reativa (15,16). Em humanos obesos, não diabéticos, a peroxidação lipídica, representada por espécies reativas ao ácido tiobarbitúrico (TBARS), foi positivamente correlacionada com o índice de massa corporal (IMC) e a circunferência da cintura (17). De forma geral, o acúmulo de gordura em indivíduos obesos aumenta o estresse oxidativo e a inflamação sistêmica devido ao aumento da produção de citocinas pró-inflamatórias, como a interleucina-6 (IL-6), fator de necrose tumoral-α (TNF-α) e a proteína quimiotática de monócitos-1 (MCP-1) (17).

O fator de necrose tumoral α (TNF-α), que é uma das citocinas mais comumente elevadas no plasma de obesos, pode inibir a fosforilação do substrato receptor de insulina-1 (IRS-1) nos músculos e tecido adiposo, diminuindo a transdução do sinal para a translocação de GLUT-4, levando à resistência insulínica. Ele também aumenta a liberação de ácidos graxos livres e a deposição de gordura no fígado, e pode acelerar o processo de aterosclerose através da indução da expressão de moléculas de adesão em células endoteliais e vasculares (13). A exposição crônica a interleucina-6 (IL-6) pode estimular a hipertrigliceridemia pelo estímulo a secreção hepática de VLDL, e impedir a estimulação da insulina pela ativação da tirosina fosfatase ou pela interação com proteínas supressoras da sinalização de citocinas e o receptor de insulina, além de estimular a produção hepática de proteína C-reativa (15). O excesso de IL-6 e o acúmulo de triglicerídeos no fígado estão associados ao aumento do baço em indivíduos com esteatose hepática, e o MCP-1 pode induzir a infiltração e acúmulo de macrófagos ativados no baço, contribuindo para a produção de TNF-α (18,19).

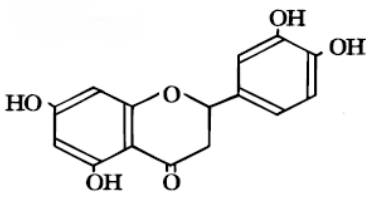
Por conseguinte, os flavonoides cítricos têm sido descritos por ajudar a regular o metabolismo lipídico, limitando a lipotoxicidade e estimulando a utilização de glicose e ácidos graxos nos tecidos (20). Existe uma relação inversa entre o consumo de frutas cítricas e fatores de risco para doenças cardiovasculares, incluindo melhora da pressão arterial, do peso corporal, e da dislipidemia (21). O papel antioxidante dos flavonoides cítricos se caracteriza por sua ação de sequestrar e neutralizar radicais livres, contribuindo com as defesas endógenas contra o estresse oxidativo. Os flavonoides cítricos abrangem vários subgrupos, incluindo flavanonas (naringina, hesperidina e eriocitrina), flavonas (diosmina), e polimetoxiflavonas (nobiletina e tangeretina). Eles têm sido reconhecidos como agentes com ação anti-inflamatória e antioxidante na obesidade e síndrome

metabólica. A eriocitrina (eriodictiol-7-*O*-rutinosideo ou 3', 4', 5,7-tetrahidroxiflavanona-7-β-D-glucopiranosídeo), um flavonóide abundante em limões e limas, foi relatado como o antioxidante mais potente destas frutas em estudos *in vitro.*2,3 Ela é encontrada principalmente na casca (albedo + flavedo) e vesículas de suco, sendo 7,5 vezes mais abundantes na casca do limão do que no suco, enquanto sua concentração no suco representa metade da do ácido ascórbico (22). As propriedades biológicas da eriocitrina têm sido relacionadas à redução de alterações metabólicas observadas em indivíduos obesos (23-25).

Foi mostrado que a eriocitrina e sua aglicona, eriodictiol (**Figura 1**), apresentaram potente atividade antioxidante *in vitro*, e foram relatadas com atividades anti-inflamatórias e antioxidantes, sendo capazes de suprimir o estresse oxidativo em ratos induzidos ao diabetes com estreptozotocina e em ratos submetidos ao exercício agudo (24-27). Além disso, a administração oral de eriocitrina melhorou a dislipidemia e diminuiu a deposição de gordura no fígado em modelo experimental de obesidade. Processo que foi atribuído à ativação da biogênese mitocondrial, aumento do tamanho da mitocôndria e produção de ATP em células HepG2 (28). O efeito anti-inflamatório do eriodictiol foi mostrado na redução dos níveis de oxido nítrico, citocinas pró-inflamatórias e moléculas de adesão em ratos diabéticos (24). Em células HepG2 o eriodictiol aumentou a absorção de glicose e o acúmulo de ácidos graxos em adipócitos 3T3-L1, pela estimulação da insulina, indicando melhora da resistência à insulina devido ao aumento da expressão do PPARγ2 (expresso no tecido adiposo) e da proteína de ligação de ácidos graxos específica de adipócitos (aP2) (29). Foi mostrado que o eriodictiol regula moléculas de adesão e a migração trans-endotelial de monócitos, que são eventos imunes envolvidos na patogênese de doenças inflamatórias, como a aterosclerose (30).



Eriocitrina (eriodictiol-7-*O*-rutinosideo)



Eriodictiol

Figura 1. Estrutura química da eriocitrina e eriodictiol (31).

O eriodictiol tem sido também identificado como ativador do fator de transcrição relacionado ao NF-E2 (Nrf2), induzindo enzimas de desintoxicação de fase 2 em queranócitos, células da retina humana, e em modelo de isquemia cerebral (32,34). O Nrf2 regula a transcrição de enzimas de fase 2 que protegem contra danos causados ao DNA pelo estresse oxidativo e evita a morte celular (33). Recentemente, foi revelado que a via Nrf2 pode ser ativada de maneira dependente do PPAR, protegendo hepatócitos humanos normais do elevado teor de glicose, sendo este um dos mecanismos de ação do roziglitazone (35). Ambas hesperidina e eriocitrina aumentaram a atividade de enzimas antioxidantes, como a catalase e glutationa (GSH) no fígado, e diminuíram o nível de 8-OHdG (indicador de dano oxidativo ao DNA) na urina de ratos diabéticos, suprimindo a peroxidação lipídica no soro, fígado e rins, onde a ação antioxidante da eriocitrina mostrou-se mais potente que da hesperidina (26).

A ação antioxidante da eriocitrina e do eriodictiol se deve a capacidade de eliminar cadeias de radicais livres e quelar íons metálicos que catalisam a peroxidação lipídica. A presença do grupo catecol no anel B (anel B dihidroxilado), e da dupla ligação juntamente com função carbonila no anel heterocíclico, ou a

polimerização da estrutura nuclear os tornam capazes de doar elétrons, proporcionando estabilidade à molécula através da conjugação e ressonância de elétrons que confere propriedade anti-radical direcionada a íons OH e ânion superóxido. Além disso, a presença de grupos funcionais envolvendo ambos os grupos hidroxila do anel B e o grupo 5-hidroxi do anel A são importantes na capacidade das flavanonas em quelar íons metálicos ativos e evitar a quebra catalítica de peróxido de hidrogênio (reação de Fenton). Flavonoides com mais grupos OH apresentam maior atividade antioxidante e efeito anti-inflamatório, como é o caso da eriocitrina (36). Essas propriedades defendem contra o estresse oxidativo e evitam danos teciduais pela oxidação das membranas celulares.

No entanto, a bioatividade de tais moléculas depende da sua estrutura e metabolismo, que depende da sua biodisponibilidade. Essa, por sua vez, é baseada na matriz alimentar ingerida, e afetada pela abundância no alimento, quantidade ingerida, e metabolismo interindividual (23). Quando ingerida, a eriocitrina é deglicosilada por ação de bactérias intestinais e convertida a eriodictiol, sendo este conjugado com glucuronídeos e sulfatos nos enterócitos, para posterior absorção. As formas conjugadas são então transportadas ao fígado, onde sofrem novas reações de sulfatação, glucuronidação e metilação, formando uma variedade de metabólitos glicuronídeos e sulfatos conjugados de eriodictiol, homoeriodictiol e hesperitina. Em seguida eles são liberados para a circulação aptos a exercerem sua atividade biológica (37,38).

Como apresentado, a eriocitrina possui efeitos que podem reduzir a inflamação e distúrbios metabólicos devido à sua capacidade de eliminar o excesso de radicais reativos, impedindo o desencadeamento de processos inflamatórios. Estudos prévios mostraram um rápido desaparecimento dos metabólitos de eriocitrina do compartimento sanguíneo, sugerindo uma maior afinidade deste

pelos tecidos (39). Entretanto, pouco se conhece sobre seu metabolismo e possíveis metabólitos responsáveis pela sua ação nos diferentes órgãos. Desta forma, o emprego da eriocitrina em modelo experimental se faz importante como forma de avaliar sua atividade biológica, doses de efeito relacionadas à redução da inflamação e metabólitos ativos no sangue e demais tecidos. Para isso, foram realizados dois estudos que serão apresentados nos dois capítulos subseqüentes desta tese. No primeiro estudo, o metabolismo da eriocitrina foi estudado a partir da sua administração oral e subsequente identificação e estudo farmacocinético e de biodistribuição de seus metabólitos no sangue, urina e órgãos de ratos Wistar. No segundo, o efeito de diferentes doses de eriocitrina foi testado contra a inflamação, o estresse oxidativo e distúrbios do metabolismo lipídico e glicêmico associados à obesidade induzida por dieta hiperlipídica em camundongos C57BL/6J.

Capítulo 1.

Pharmacokinetics and Biodistribution of Eriocitrin in Wistar Rats

Artigo a ser enviado para publicação na revista "Journal of Agricultural and Food Chemestry".

Pharmacokinetics and Biodistribution of Eriocitrin in Wistar Rats

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Abstract

Eriocitrin has been shown to play a role in the improvement of oxidative stress conditions that lead to the development of diabetes mellitus and atherosclerosis. We investigated the pharmacokinetics and distribution of eriocitrin metabolites in Wistar rats orally administered with eriocitrin. Plasma, urine and organs were collected in 12 different time-points from 0 to 24 hours for HPLC-MS analysis. For the first time, the metabolism and distribution of orally administered eriocitrin were shown. Nine metabolites of eriocitrin were identified in rat tissues and urine (eriodictyol, homoeriodictyol, hesperetin and six glucuronidated metabolites). Overall, eriocitrin metabolites were widely distributed in the rat tissues, and homoeriodictyol-7-*O*-glucuronide and homoeriodictyol aglycone were the major metabolites. The half-life of the metabolites detected in plasma was between 3 and 3.2 hours and the total bioavailability of eriocitrin was less than 1%.

Keywords: eriocitrin, metabolites, biodistribution, pharmacokinetics, identification

INTRODUCTION

Recent studies have focused on the beneficial effects of bioactive compounds from diets based on fruits and vegetables, due to the activity of phenolic compounds and flavonoids in the chemoprevention of oxidative stress and inflammatory processes involved in the genesis of degenerative diseases.[1] Eriocitrin (eriodictyol-7-*O*-rutinoside or 3', 4', 5,7-tetrahydroxyflavanone-7-β-D-glucopyranoside), an abundant flavonoid in lemon and lime fruits, was reported as the most potent antioxidant in these fruits in vitro.[2,3] It is found mainly in the fruit peel (albedo + flavedo) and juice vesicles, being 7.5 times more abundant in lemon peel than in the juice, whereas its concentration in the juice represents half that of ascorbic acid.[2]

Eriocitrin metabolites were detected in human and rat blood plasma following oral administration of lemon peel extract and/or pure eriocitrin, and conjugated forms of eriodictyol, homoeriodictyol and hesperetin were identified as the main metabolites.[4,5] Pharmacokinetic studies show a rapid disappearance of eriocitrin metabolites from the blood compartment, suggesting affinity for tissues, other than blood.[6] Moreover, dietary eriocitrin was shown to decrease the hypertrophy of kidneys and lipid peroxidation in liver, kidneys and blood serum of streptozotocin-induced diabetic rats (Miyaki et al., 1998).[7] Eriocitrin metabolites are associated with prevention of diabetes mellitus, cardiovascular disease and some cancers.[7-13] Nevertheless, the free radical scavenging effects of flavonoids are indicated to prevent the formation of advanced glycated end products (AGE) and other diabetic complications associated with high oxidative stress conditions such as atherosclerosis, nephropathy, neuropathy, retinopathy and erectile dysfunction. These properties may also protect pancreatic cells from oxidative damage and help β-cells regeneration.[14] In our previous study, eriocitrin increased the blood serum

antioxidant capacity and had protective effects against inflammation in high-fat diet-fed mice.[15]

Therefore, the identification and distribution of eriocitrin metabolites *in vivo* may provide significant pharmacological information for understanding its biological activity, as well as assist in the development of potential drugs from eriocitrin and its metabolites. Despite the in vitro and in vivo effects of eriocitrin reported thus far, information on its metabolism and tissue distribution is still scarce, and the disposition and concentration range of eriocitrin metabolites in the tissues have not yet been reported. Based on this, the present study focused on the identification and distribution of eriocitrin metabolites in blood plasma, organs and urine of Wistar rats.

MATERIALS AND METHODS

Materials and Chemicals

Eriocitrin > 85% purity was extracted from the peel of Citrus limon (L.) (Rutaceae). Pure eriodictyol, hesperetin, homoeriodictyol, hesperetin-7-O-glucuronide, hesperetin-3'-O-glucuronide, mangiferin (mangiferin dica bark) and Sulfatase type H1from Helix pomatia were purchased from Sigma Aldrich Chemical Co. (Merck KGaA, Darmstadt, Germany). Other reagents were of commercial grade, and all the solvents were of HPLC grade.

Experimental design

Thirty six male Wistar rats (234±22 g) from the Animal Center of São Paulo State University (UNESP) at Botucatu Campus, SP, Brazil, were maintained in standard conditions (22 ± 2°C; 12-h light\dark cycles) with free access to food and

drinking water. The experimental protocols were approved by the Animal Use Board of UNESP, School of Pharmaceutical Sciences, Araraquara, SP, Brazil (16/2015). For the biodistribution and pharmacokinetic study, rats were individually kept in metabolic cages and randomly divided into twelve groups (n=3) according to the determined time-points: 0 (base), 0.5, 1, 3, 4, 5, 6, 8, 10, 12, 15, 24 hours. After 10 h fasting, eriocitrin was administered by gavage in the dose of 100 mg/kg body weight in a 0.9% saline, and urine samples were collected in each time point until euthanasia. The rats were anaesthetized with Ketamine/Xylazine (100 mg/kg/10mg/kg) and the blood samples were collected from the left ventricle by cardiac puncture into heparinized tubes, and centrifuged at 2000 rpm/10 minutes to obtain plasma. Liver, kidneys, heart, spleen, adipose tissue, pancreas, brain and gastrocnemius muscle were collected subsequently by frontal excision. The whole organs were immediately excised, rinsed with ice-cold saline (0.9%), wiped on filter paper, weighed and stored. Urine was collected individually into dark bottles, centrifuged and the volume of clean supernatant removed, measured and stored. The urine of 24 h was collected in two fractions of 12 h each to avoid spoilage and loss of metabolites. All samples were stored at -20 °C until analysis. The experimental procedures and sample pre-preparation were carried out at the Laboratory of Nutrition, from the Food and Nutrition Department of UNESP, School of Pharmaceutical Sciences, Araraquara, SP, Brazil. The sample extractions and analysis were carried out at the Quality in Citrus and Subtropical Products Laboratory, from the United States Department de Agriculture (USDA), Fort Pierce, Florida, USA.

Metabolite Extractions

Urine metabolite extraction. The pH of each rat urine was adjusted to 3 with 5% formic acid and cleared through C18 Sep Pak cartridges (Waters Sep-Pak, Milford, MA, USA) preconditioned with 10 mL of methanol and 20 mL of de-ionized water. The metabolites were eluted from the cartridge with 20 mL of methanol, and methanolic extracts were transferred to a separatory funnel with160 mL of 0.5% formic acid and 100 mL of ethyl acetate. The mixture was vigorously shaken for 1 minute and left to rest for phase separation. This procedure was repeated twice and the organic phases were combined and re-extracted two more times with 100 mL of ethyl acetate. The organic phases were combined, retransferred to the funnel and mixed for 1 minute with 60 mL of water for back-wash. The organic phase containing the metabolites was partially evaporated using a rotary evaporator (Rotavapor R-114, Buchi, New Castle, USA) to remove the ethyl acetate, and dried under vacuum using a centrifugal evaporator at 40 ºC (SpeedVac SVC 200H, Savant, Holbrook, NY, USA). The resulting extracts were re-dissolved in 400 µL of DMSO containing an internal standard (IS), mangiferin, for HPLC-MS analysis.

Blood plasma metabolites extraction. Two milliliters of plasma, for each rat were combined with 6 mL of methanol, vortexed and centrifuged at 10,000 rpm/4 min at room temperature. The supernatants were collected and dried at the centrifugal vacuum concentrator. The extracts were then re-suspended in 700 µL methanol, vortexed and centrifuged at 10,000 rpm for 2 minutes. The supernatants, 400 µL of plasma extracts, were filtered through a syringe filter (0.2 µm) into a new vial, dried, and re-dissolved in 100 µL of methanol for HPLC-MS analysis.

Liver metabolite extraction. Three grams of the two lobes of liver were removed, washed with 0.9% saline and homogenized with 10 mL of methanol using a tissue homogenizer (Omni THQ, Omni International, Inc, Kennesaw, GA, USA) at 18,000

rpm/1 min. For lipid removal, the volume was completed to 25 mL with methanol and 25 mL of hexane were added. The solution was vigorously mixed for 30 seconds and centrifuged at 8,000 rpm/8 min. The supernatant (hexane) was removed and the pellet (methanolic extract) was reserved. Two other extractions were made by adding 50 mL of methanol to the pellet and re-centrifuging. The three methanolic fractions were combined and dried under vacuum. The dried extracts were reconstituted with 500 µL of methanol, sonicated for 10 minutes, centrifuged three times at 10,000 rpm/1 min and the supernatants combined and dried. For the HPLC-PDA-MS analysis, the liver extracts were re-dissolved in 4 mL of DMSO, homogenized and centrifuged at 2000 rpm/2 min. The supernatants were collected, injected into two combined C-18 Sep Pak cartridges pre-conditioned with 10 mL of methanol and 15 mL of water. The metabolites were eluted from the Sep Pak with 15 mL of methanol, dried under vacuum and re-suspended in 1 mL of DMSO containing an IS (mangiferin) for HPLC-MS analysis.

Other organs metabolites extraction. Whole organs were homogenized with 4 mL of methanol by using the tissue homogenizer at 18,000 rpm/1 min. After homogenization in methanol, 4 mL of water were added to the samples and homogenized for another minute. The homogenates were centrifuged at 7,000 rpm/5 min at 20 ºC. The supernatants were collected, filtered and dried under vacuum. For the HPLC-MS analysis**,** the extracts were re-suspended with 1mL of methanol, sonicated for 15 minutes, vortexed and centrifuged at 10,000 rpm/2 min. Clear supernatants were dried, re-suspended with 400 µL DMSO (containing IS) and filtered through a Whatman Anotop syringe filter 0.2 µm (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) for analysis.

HPLC-PDA-MS Analysis

The metabolites of eriocitrin in urine and tissues were analyzed by an optimized HPLC method for maximum flavanone sensitivity[16] using a Waters 2695 Alliance system connected in parallel with a 996 photo diode array (PDA)detector and a Micromass ZQ single-quadrupole mass spectrometer equipped with ESI source. The post-column split to the PDA and mass (ZQ) detector was 10:1. Compound separations were achieved with a XBridgeC8 column (6 x 150 mm) using linear gradients of acetonitrile:0.5% formic acid, initially composed of 10:90 (v/v), and increased in acetonitrile content to 20:80, 25:75, 40:60, 70:30, and then decreased to 10:90 (v/v) at 10, 15, 23, 40 and 53 min, respectively, at a flow rate of 0.75 mL/min. Data handling was done by Mass Lynx software version 4.1 (Micromass, Division of Waters Corp., Beverly, MA). Mass spectrometer (MS) parameters were as follows: negative ESI ionization mode; capillary voltage, 3.0 kV; extractor voltage, -4 V; source temperature, 100 °C; desolvation temperature, 250 °C; desolvation N_2 flow, 550 L/h; cone N_2 flow, 100 L/h; cone voltage, 20 and 40 V. Detection and identification of the metabolites were based on their characteristic UV spectra (230 – 400 nm), molecular masses and fragmentation patterns as described previously.[16-18] Eriocitrin aglycons and glucuronides were identified by ions at *m/z* 287 and 463, respectively; and both homoeriodictyol and hesperetin aglycones and glucuronides by ions at *m/z* 301 and 477, respectively. Quantifications of metabolites were achieved by using peak area (PA)/µg conversion factors of authentic metabolite standards with integrated mass-extracted peak areas, obtained either in the scan mode for urine samples (50-1000 amu) or in the single ion response (SIR) mode for the organs and blood plasma extracts. Different amounts of external standards (eriodictyol, homoeriodictyol, hesperetin, hesperetin-7-*O*-glucoronide and hesperetin-3'-*O*-glucuronide), and IS mangiferin were dissolved in

DMSO to build external calibration curves. The conversion factors (PA μg^{-1}) of metabolites standards were linear over the concentrations observed in the rat samples and the limit of quantification was below 2.0 ng/g.

Eriocitrin Metabolite Purification

To obtain standards of eriocitrin metabolites from urine, previously extracted urine samples from another batch of eriocitrin-fed male Wistar rats (n=10) were re-suspended in 20% acetonitrile for purification of the flavanone metabolites. The solution was injected into a preparative HPLC system coupled to a PDA detector Varian ProStar model 210 (Agilent Technologies, Santa Clara, CA, USA) and an automated collector Foxy 200 (Teledyne Isco, Lincoln, NE, USA). Separations were achieved with an Atlantis C18 column (19 x 100 mm, 5 µm particle size) at a flow-rate of 5 mL/min. A linear gradient composed of solvents A (0.5% formic acid) and B (acetonitrile) was as follows: 20% of B for 34 minutes, then 23% from 35 to 49 min, 40% from 50 to 59 min, and 20% at 60 min. The effluent from the column outlet was monitored at the range of 240 to 500 nm and the data was acquired on the Varian MS Workstation Software (Agilent Technologies, Santa Clara, CA, USA). Each peak fraction was collected according to the elution profile, dried, weighed and re-suspended in DMSO to be analyzed by HPLC-MS. Eriocitrin metabolites isolated from this technique were used for metabolites identification in rat tissues.

An aliquot of each conjugated metabolite isolated from urine was enzymatically hydrolyzed and submitted to the HPLC-MS analysis for confirmation of their respective aglycons. For this, 1/10 of each flavanone metabolite isolated from rat urine was combined to 1 mL of 250 mM acetate buffer at pH of 4.9 containing 50 units of Sulfatase type H1 from Helix pomatia, which has both sulfatase and β-glucuronidase activity. The samples were incubated for 2 hours at 37º C. Then, 100 µL of HCL 3.7% were added to stop the enzyme activity and

extractions were twice made with 10 mL of ethyl acetate.[19] The organic phases were combined, dried under vacuum and re-dissolved in 1 mL of methanol. One hundred microliters of each sample were combined to 50 µL of mangiferin as IS, and 20 µL were injected into the HPLC-MS system for analysis.

Fourier-transform spectroscopy (FTIR)

Dried eriocitrin metabolites were reconstituted with methanol and applied as thin films on PTFE infrared cards (International Crystal Laboratories, Garfield, NJ). Infrared spectra of eriocitrin metabolites were recorded at room temperature with a PerkinElmer Spectrum One FTIR spectrometer, at a scanning range of 4000–450 cm-1.

Pharmacokinetic Analysis

A non-compartmental pharmacokinetic model was chosen to fit the concentration–time data from individual rats for each group using the software PKSolver,[20] which provided the estimates of T_{max}, C_{max} and area under the curve (AUC). Additionally to the pharmacokinetic analysis, a percentage of absorption was calculated for total eriocitrin metabolite (glucuronides and aglycones), as following: $absorption\,\% = \frac{AUC_{0-24h}\,(urine)}{mean\ eriocitrinoraldose\ per\ rat\ (19.9\text{x}10^{6}\ \text{ng})} \times 100.$

Statistical Analysis

Results are presented as mean ± standard error of the mean (S.E.M.). Where possible, the data were analyzed for statistical significance using GraphPad Prism version 6 (GraphPad Software, Inc. La Jolla, CA, USA). When three or more data were available for statistical analysis, the ANOVA one-way followed by Tukey multiple-comparison test was performed. When only data pairs were available, the

Student's t-test was employed. Paired tests were considered when the data to be compared were at the same time point (same rats). A P-value <0.05 was considered statistically significant for all of the analyzes.

RESULTS

Eriocitrin Metabolites

The HPLC-MS parameters of eriocitrin metabolites and suggested structures are presented in Table 1. Six glucuronides and three aglycones were isolated from urine of rats treated with eriocitrin as previously described. The three aglycons derived from eriocitrin metabolism were identified as eriodictyol, homoeriodictyol and hesperetin by comparison with original compounds previously isolated from rat urine (data not shown). The conjugated metabolites were identified by comparison with pure compounds of eriodictyol, homoeriodictyol and hesperetin and original conjugated standards of hesperetin (hesperetin-7-*O*-glucuronic acid and hesperetin-3´-*O*-glucuronic acid).

Each flavanone derived from eriocitrin metabolism presented two conjugated isomers in the MS spectrum. The first two conjugated metabolites eluted were eriodictyol glucuronides. Both exhibited a deprotonated molecular ion of m/z 463 and a fragment ion of m/z 287, corresponding to eriodictyol with the neutral loss of m/z 176, which is characteristic of glucuronic acid. The next two conjugated metabolites eluted were homoeriodictyol glucuronides, both with a deprotonated ion of m/z 477 and a fragment ion of m/z 301, corresponding to homoeriodictyol with the loss of glucuronic acid. The last two conjugated metabolites also exhibited the m/z 477 and m/z 301 ions and were identified as hesperetin-7-*O*-glucuronic acid

and hesperetin-3´-*O*-glucuronic acid by comparison with hesperetin conjugated standards.

In the present study, only monoglucuronidated metabolites of eriocitrin were found, but the molecular position of glucuronic acid in eriodictyol and homoeriodictyol conjugates was not definitely confirmed. However, according to their elution order in reversed phase HPLC, they were suggested to be eriodictyol-7- and 3´-*O*-glucuronic acid; and homoeriodictyol-7- and -4´-*O*-glucuronic acid, respectively (Table 1).

Table 1. HPLC and Mass Spectrometry Parameters of Compounds Isolated from Rat Urine After Oral Administration of Eriocitrin.

Metabolite	Retention time (min)	$[M - H]^-$ m/z	Suggested structure
ERD-glu1	14.4	463	eriodictyol-7-*O*-glucuronic acid
Eriodictyol	20.94; 21.00	287	aglycon
ERD-glu2	17.24	463	eriodictyol-3'-*O*-glucuronic acid
hERD-glu1	17.95	477	homoeriodictyol-7-*O*-glucuronic acid
hERD	24.21; 24.25	301	aglycon
hERD-glu2	18.5	477	homoeriodictyol-4'-*O*-glucuronic acid
HSPTN-glu1	18.9	477	hesperetin-7-O-glucuronic acid
HSPTN	24.74; 24.87	301	aglycon
HSPTN-glu2	19.9	477	hesperetin-3'-*O*-glucuronic acid

The infra-red (IR) spectra of eriocitrin metabolites are shown in Table 2. All of the metabolites presented a broad absorption peak after 3000 cm-1, indicating the presence of hydroxy groups (OH); and broad, intense, and asymmetric peaks between 1609 and 1642 cm-1, corresponding to aromatic ketonic carbonyl (C=O) stretchings. Absorption peaks from 1500 to 1580 cm-1 indicated the presence of C=C aromatic stretching vibrations, whereas stretching vibrations from 1060 to 1291 cm-1 indicated the presence of aliphatic and conjugated ethers (C-O). All

metabolites showed weak vibrations near the values from 2956 to 2847 cm-1 for C-H stretches of alkanes. Weak vibrations at 2956 represent CH3 asymmetric stretch, and 2920/2913/2905 represent asymmetric CH2, and 2847 represents CH2 symmetric stretch.

Table 2. FTIR Spectrum (cm-1) of Metabolites Detected in Rat Urine After Oral Administration of Eriocitrin.

Metabolite	Ethers (C-O)	Aromatic Carbons C=C	Carbonyls (C=O)	Alkanes C-H	Hydroxyls (OH)
ERD-glu1	1291, 1194, 1172, 1060	1609, 1530, 1580	1642/1609	2956, 2920, 2847	3420
ERD-glu2	1259, 1187, 1161, 1067	1508	1617	2956, 2913, 2847	3434
hERD-glu1	1277, 1190, 1168, 1082	1580, 1515	1642, 1629/1613	2913, 2847	3347
hERD-glu2	1266, 1158, 1083, 1125, 1062	1583, 1512,	1638,1615	2913, 2847	3230
HSPTN-glu1	1266, 1179, 1082	1577, 1534	1627, 1577	2927, 2847	3391
HSPTN-glu2	1266, 1183, 1161, 1082	1515	1641, 1617/1594	2905, 2840	3304

Pharmacokinetic Profile of Eriocitrin Metabolites

A pharmacokinetic study was performed to observe the behavior and distribution of free and conjugated metabolites of eriocitrin in rats (Table 3). The identity of eriocitrin metabolites in rat tissues were confirmed by comparison to authentic compounds (i.e. the compounds previously identified in rat urine and original standards of hesperetin-7-*O*- and hesperetin-3´-*O*-glucuronic acid) in the

HPLC-MS system. Both free and glucuronidated metabolites were observed in rat tissues and urine. The concentration-time curves of the free and conjugated forms of eriocitrin metabolites in urine and in all tissues evaluated (liver, kidneys, pancreas, spleen, heart and muscle) are shown in Figures 1 and 2. Based on these findings, seven of the nine eriocitrin metabolites identified in rat urine were detected in the various tissues analyzed (Table 3). Three of them were the aglycons eriodictyol, homoeriodictyol and hesperetin; and the other four were: two eriodictyol glucuronides (ERD-glu1 and ERD-glu2); and two homoeriodictyol glucuronides (hERD-glu1 and hERD-glu2).

Overall, the results demonstrated that eriocitrin metabolites were widely distributed, with some difference in the tissue uptake of glucuronides and aglycons (Table 3). According to AUC_{0-24}, the more abundant metabolite of eriocitrin is hERD-glu1 and its respective aglycon, homoeriodictyol, either comprising 33% of the total eriocitrin metabolites in rat body. hERD-glu1 was the most widely distributed metabolite, being detected in 7 of the 9 analyzed tissues (blood plasma, liver, heart, pancreas, spleen, muscle and kidneys), whereas homoeriodictyol aglycon was found in 5 (liver, pancreas, spleen, muscle and kidneys). Others metabolites are ranked as hERD-glu2 (15%), ERD-glu1 (7.6%), ERD-glu2 (4.4%), hesperetin aglycon (4.3%) and lastly, eriodictyol aglycon (2.6%). Only very low concentrations of the two homoeriodictyol glucuronides were detected in blood plasma, while in urine they were found in much higher levels.

In urine, AUC_{0-24} percentages of metabolites reflected those found in the body tissues. hERD-glu1 exhibited 22% of the total excreted metabolites, followed by hERD (20%), ERD-glu2 (15%), ERD-glu1 (14%), ERD (13%), hERD-glu2 (10 %) and lastly HSPTN (5.6%). Most eriocitrin metabolites presented the Cmax between six and ten hours after ingestion; except ERD-glu1, which reached its peak

concentration at 5 hours after ingestion. The plasma half-life of metabolites detected in blood plasma, estimated by PKSolver[18] was 3 and 3.2 hours and no metabolites were detected after 24 hours (Figure 1, A).

Table 3. Pharmacokinetic Parameters[a] of Eriocitrin Metabolites in Rat Plasma, Tissues and Urine, After Oral Administration of 100 mg/kg of Eriocitrin (n = 3).

Organ	Kinetic	Glucuronidated Metabolites				Aglycon Metabolites		
		ERD-glu1	ERD-glu2	hERD-glu1	hERD-glu2	ERD	hERD	HSPTN
Urine[e]	*Cmax*	1237 ± 465	1309 ± 492	2089 ± 785	1112 ± 390	807 ± 242	1332 ± 872	463 ± 264
	Tmax	24	24	24	24	24	24	24
	AUC_{0-24}	13835	14266	21512	9587	12701	18742	5392
Plasma	*Cmax*[b]	-	-	7.7 ± 0.8 b	2.5 ± 1.1 b	-	-	-
	Tmax[c]	-	-	10	6	-	-	-
	AUC_{0-24}[d]	-	-	54	20	-	-	-
Kidneys	*Cmax*	-	-	134 ± 26 b	-	81 ± 8	628 ± 384 a	97 ± 56 ab
	Tmax	-	-	6	-	6	6	6
	AUC_{0-24}	-	-	1082	-	440	3535	659
Liver	*Cmax*	-	-	119 ± 71 b	42 ± 21 ab	-	130 ± 97 B	23 ± 4 a
	Tmax	-	-	6	6	-	6	6
	AUC_{0-24}	-	-	684	236	-	849	41
Pancreas	*Cmax*	-	-	325 ± 90 a,A	89 ± 17 a,B	-	156 ± 83 ab,AB	-
	Tmax	-	-	10	6	-	6	-
	AUC_{0-24}	-	-	2262	584	-	822	-
Spleen	*Cmax*	210 ± 109 A	-	-	-	-	34 ± 15 b,B	3.9 ± 0.8 b,B
	Tmax	5	-	-	-	-	6	6
	AUC_{0-24}	1268	-	-	-	-	147	20
Muscle	*Cmax*	-	54 ± 0	83 ± 19 b	-	-	26 ± 11 b	-
	Tmax	-	6	10	-	-	10	-
	AUC_{0-24}	-	266	464	-	-	121	-
Heart	*Cmax*	-	53 ± 5	117 ± 33 b	-	-	-	-
	Tmax	-	10	10	-	-	-	-
	$AUC0_{-24}$	-	459	886	-	-	-	-
Adipose Tissue	*Cmax*	-	-	18 ± 7 b	-	-	-	-
	Tmax	-	-	6	-	-	-	-
	AUC_{0-24}	-	-	134	-	-	-	-

[a]Data are presented as mean ± standard error. ND = not detected. [b]Maximum concentration in ng/g or ng/mL (plasma and urine); [c]Time to reach the maximum concentration in hours; [d]Area under the curve values in ng/g*h or ng/mL*h (plasma and urine). [e]Urine data were not compared to body tissues data. Uppercase and lowercase letters show significant differences between column and row data, respectively.

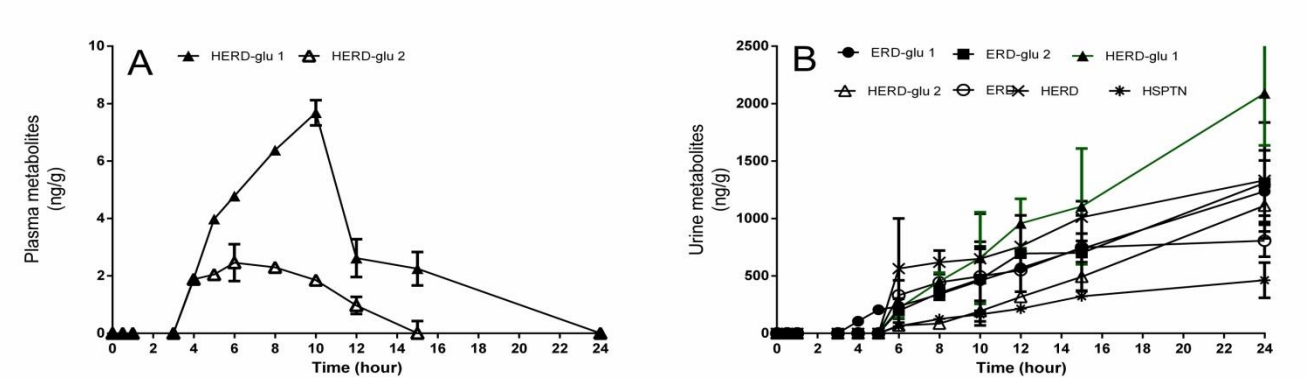

Figure 1. Concentration-Time Curves of Eriocitrin Metabolites in Rat Blood Plasma (A) and Urine (B) after the Oral Administration Of 100 Mg/Kg of Eriocitrin.

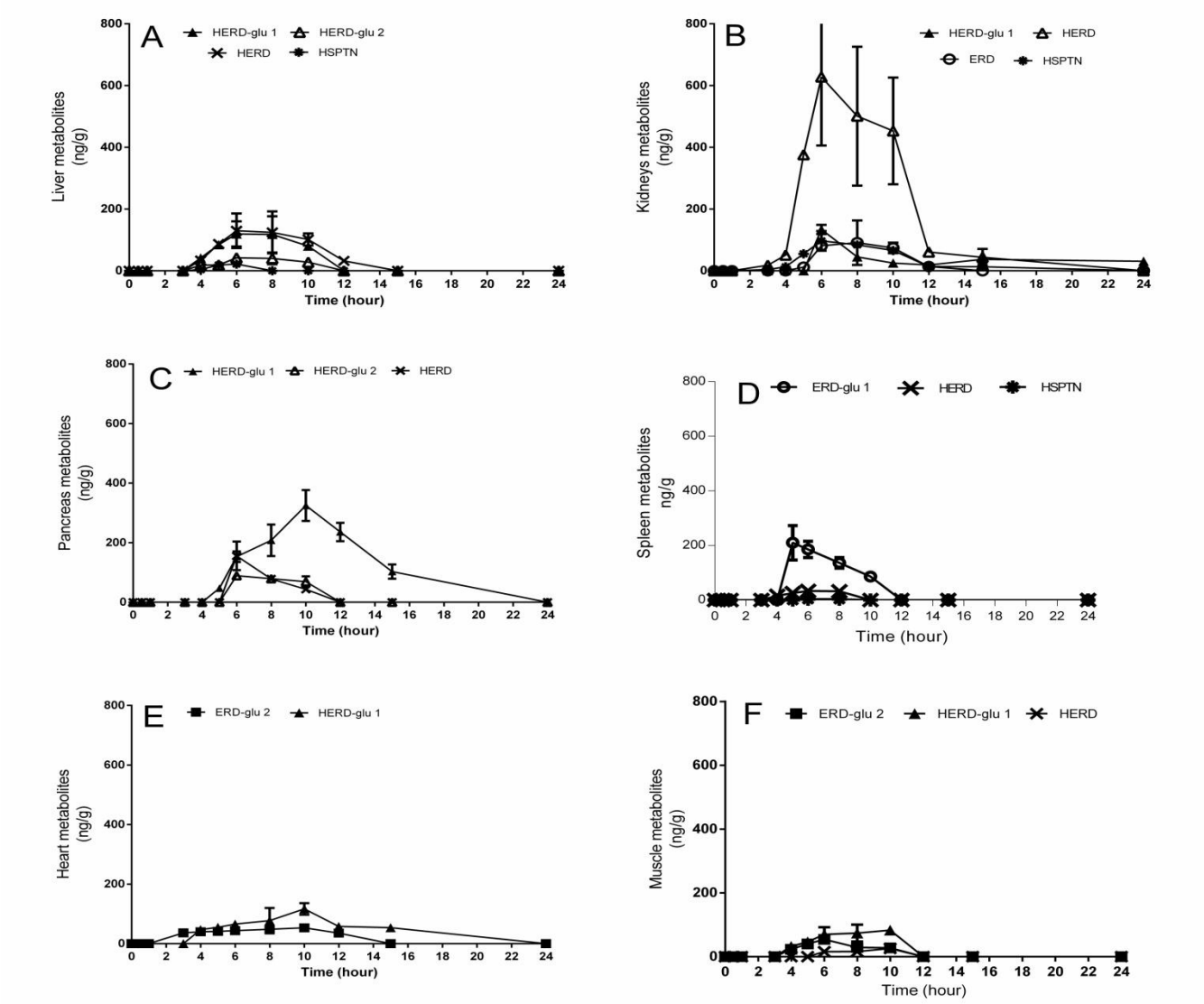

Figure 2. Concentration-Time Curves of Eriocitrin Metabolites in Rat Liver (A), Kidneys (B), Pancreas (C), Spleen (D), Heart (E), and Muscle (F) after the Oral Administration of 100 Mg/Kg of Eriocitrin.

Also, some statistically significant differences were found in metabolite concentrations (Table 3). When comparing eriocitrin metabolites in tissues, hERD-glu1 concentration in pancreas was significantly higher than in liver, kidneys, heart, muscle, adipose tissue and plasma. hERD-glu2 concentration in pancreas was higher than in plasma, whereas in liver it was intermediary. The higher concentration of homoeriodictyol aglycon was found in the kidneys, with a statistically similar concentration in the pancreas. When analyzing eriocitrin metabolite concentrations in each particular tissue, only pancreas metabolites showed statistically significant differences. In this organ, hERD-glu1 concentration was significantly higher than that of hERD-glu2, and similar to homoeriodictyol aglycon.

Analyzing the tissue distribution (Table 4), blood plasma and adipose tissue presented the lower AUC values of total eriocitrin metabolites (glucuronides and aglycons), while kidneys, pancreas and liver presented the higher levels. Spleen, heart and muscle presented considerable levels, ranging from 5.7 to 9.5%. When comparing glucuronides and aglycons percentages, 56% of total metabolites were glucuronides and 44% were aglycons. The percentage of glucuronides ranged from 19% to 100% in the analyzed tissues. Glucuronides comprised 65% of the total metabolites excreted in urine and were the major forms in the spleen (88%), muscle (86%), pancreas (78%) and liver (51%); and the only form detected in plasma, heart and adipose tissue, whereas aglycons were the major form in the kidneys (81%). Total eriocitrin absorption was less than 1%, as estimated by the ratio between total metabolites excreted in the urine divided by the eriocitrin oral dose.

Table 4. Distribution and Bioavailability of Glucuronides and Aglycons Derived from the Metabolism of Orally Administered Eriocitrin[a] (n = 3).

Tissue	Gluc	Aglyc	Total		Gluc	Aglyc	% Absorption[c]
	$AUC_{0\text{-}24}$[b]	$AUC_{0\text{-}24}$	$AUC_{0\text{-}24}$	%	%/organ	%/organ	
Plasma	74	0	74	0.5	100	0	0.00
Kidneys	1082	4634	5716	38.0	19	81	0.03
Liver	920	890	1810	12.0	51	49	0.01
Pancreas	2846	822	3668	24.4	78	22	0.02
Spleen	1268	167	1435	9.5	88	12	0.01
Muscle	730	121	851	5.7	86	14	0.00
Heart	1345	0	1345	9.0	100	0	0.01
Adipose	134	0	134	0.9	100	0	0.00
Tissues' total	8399	6634	15033	100	56	44	0.08
Urine	59201	36834	96035	100	62	38	0.48

[a]*Dose 100 mg/kg of body weight ;* [b]*ng/mL/h;* [c] $absorption\ \% = \frac{AUC_{0-24h}\ (urine)}{eriocitrinoraldose\ (19.9\text{x}10^6\ \text{ng})} \times 100$.

DISCUSSION

In the present study, three flavanone aglycons and six glucuronides were isolated from rat urine after the oral administration of eriocitrin. The three aglycons were eriodictyol, homoeriodictyol and hesperetin, which produced two glucuronidated metabolites each. The pharmacokinetic study showed that eriocitrin metabolites were widely distributed in rat body, where both free and glucuronidated metabolites were found. To our knowledge, this is the first study to report the in vivo identification, distribution and pharmacokinetics of eriocitrin metabolites.

The identification of the flavonoid skeleton of each conjugated metabolite was crucial for the elucidation of the multiple glucuronidated products derived from eriocitrin metabolism. Two monoglucuronidated isomers of each flavanone were found: two metabolites of eriodictyol with peaks at m/z 463, and two monoglucuronidated metabolites of both homoeriodictyol and hesperetin, with peaks at m/z 477. These results are in agreement with a previous study that

showed that the enzymatic glucuronidation of eriodictyol or hesperetin produced two monoglucuronidated isomers each, with almost identical MS profiles.[21]

Similarly, a previous HPLC-MS/MS analysis revealed two monoglucuronidated metabolites of eriodictyol in liver and intestine microsomes of mice after intragastric administration.[22] It is known that the metabolism of flavanone glycosides, as eriocitrin, occur in the distal parts of the gut, because the microbial hydrolysis is necessary to release the aglycon before absorption. For instance, the conversion of hesperetin in a tissue fraction of the colon resulted in the formation of only mono-conjugates.[23]

It is known that the presence of a sugar moiety, number, nature of the glycosides, and the position of the flavonoid structure influence the mobility and retention in the RP-HPLC.[21] Therefore, in the present study the two isomers of each glucuronidated flavonoid (eriodictyol, homoeriodictyol and hesperetin) derived from eriocitrin were determined. According to Davis and coworkers,[21] for flavonoid glycosides and glucuronides the evidence points towards a general elution order by position of conjugation of 5<7< 3< 4´<3´. It can be easily determined when only monoglucuronidated products are formed and the flavonoid B-ring has only one hydroxyl group. In their study, the flavonoids constituted two distinct groups distinguished by the presence or the absence of a 3´ hydroxyl group on the B-ring. The sole reaction product for compounds that lacked this structural feature was the 7-*O*-glucuronide, indicating it as a primary metabolite. Hence, the isomer of hesperetin was identified as hesperetin 3'-*O*-glucuronide as it presented the free 3´ hydroxyl group on the B-ring.

A similar generalization can be made for the homoeriodictyol and hesperetin glucuronides in the present study. As isomers, they present a free hydroxyl group on the 4´ and 3´ position, respectively, and can be sequentially assigned as

homoeriodictyol-7-*O*-glucuronide, homoeriodictyol-4´-*O*-glucuronide, hesperetin-7-*O*-glucuronide and hesperetin-3'-*O*-glucuronide. Although we included eriodictyol glucuronides in this prediction, only its first derivative was assigned as eriodictyol-7-*O*-glucuronide. However, the position of the glucuronic acid in its second derivative could not be conclusively assigned, because both the 3´ and 4´ positions in the B-ring are hydroxylated, but only one B-ring glucuronide was formed. Although no rules are known for differentiating the 3´- and 4´-*O*-glucuronides of flavonoids, we suggest that it is eriodictyol-3´-*O*-glucuronide because of the important activating properties of the 3´ hydroxyl group.[21]

The FTIR spectra of eriocitrin metabolites give further evidence of their glucuronic acid substitutions. The presence of glucuronic acid substitutions on all of the metabolites of eriocitrin were confirmed by the presence of hydroxyl groups (OH), exhibited by broad peaks after 3000 cm-1. Also, vibrations from 2956 to 2847 cm-1 indicated CH2 groups in the middle ring of the flavanone and the presence of glucuronic acid substitutions primarily due to the presence of alkanes (C-H) of glucuronic acid. The aromatic ketonic carbonyl groups (C=O) from flavanone skeleton were represented by the presence of peaks between 1609 and 1642 cm-1. Furthermore, the presence of aromatic vibrations (C=C) and of aliphatic and conjugated ethers (C-O) were confirmed by peaks from 1500 to 1580 cm-1, and peaks from 1060 to 1291 cm-1, respectively.[24-27]

Several reports on animals and humans have noted that the major metabolites of orally administered flavanones were glucuronidated, although a small portion of sulfated or sulfo-glucuronidated were reported.[28-30] In human blood, the glucuro and/or sulfo conjugated metabolites of eriodictyol, homoeriodictyol and hesperetin were observed after the oral ingestion of lemon peel extracts containing flavanone glycosides and flavanone aglycons. However, the free forms were not

detected in the period analyzed (up to four hours after intake).[5] Thus, a great variety of flavonoid metabolites can exist in vivo, including the free forms.

The activity of the β-glucuronidase enzyme in a variety of tissues was not correlated with the proportions of conjugated metabolites after the ingestion of luteolin; some of its free forms were suggested to escape the intestinal conjugation and hepatic sulfation/methylation.[31,32] Our study showed a considerably high amount of deconjugated metabolites in the kidneys and urine. In agreement, a preliminary study indicated the conjugated forms of eriodictyol, homoeriodictyol and hesperetin as the main metabolites of eriocitrin in rat plasma after oral administration, whereas both free and conjugated forms were detected in the urine. It was suggested that these metabolites were deconjugated in the kidneys, releasing the glucuronic acid and/or sulfate moiety before urinary excretion.[4]

There is little information on the biodistribution and pharmacokinetics of eriocitrin in a 24-hour period, which is necessary to observe the complete process of absorption and metabolism of this flavonoid. Free aglycons are directly captured in the enterocyte of the small intestine by passive diffusion,[33] while the glycosylated forms are absorbed in the colon, hydrolyzed into their respective aglycons by the colonic microbiota and then absorbed into the enterocytes. On the other hand, in the liver contribute to delay the appearance of these metabolites in the blood.[34] The maximum peak of an extract prepared from lemon peel aglycones was observed at only one hour after ingestion, while the absorption of the extract prepared from the glycosides was slower.[5] As observed in previous studies, the delayed absorption of eriocitrin in our study may have been caused by the first pass intestinal metabolism and conversion of eriodictyol to homoeriodictyol and hesperetin in the liver.

The free eriodictyol was detected only in the kidneys and urine, while free homoeriodictyol was widely distributed in liver, pancreas, spleen, muscle, kidneys

and urine; and free hesperetin was detected in liver, spleen, kidneys and urine. Matsumoto and coworkers[35] monitored eriodictyol and its conjugates in rat plasma and indicated that eriodictyol was immediately re-methylated in rat liver since the corresponding peaks of eriodictyol and its conjugates could not be detected. They also indicated that eriodictyol undergoes heavy phase II metabolism due to the significantly higher excretion of the conjugated metabolites. Therefore, the low detection of eriodictyol glucuronides in the present study may be due the direct metabolism of eriodictyol in the intestine. Nevertheless, this is a dynamic system in which eriodictyol is rapidly metabolized to homoeriodictyol, hesperetin and their glucuronides.[6]

Eriodictyol in the liver is de-glucuronidated and methylated to a greater extent in homoeriodictyol (3'-*O*-methylated compound) and, to a lesser extent in hesperetin (4'-*O*-methylated compound), followed by re-glucuronidation to form homoeriodictyol and hesperetin glucuronides.[4] This agrees with previous observation that, after oral ingestion of hesperidin, hesperetin glucuronides reach peak plasma concentration two hours earlier than homoeriodictyol-glucuronides, which are formed from the demethylation and re-methylation of hesperetin.[35]

Pharmacokinetic analysis revealed homoeriodictyol-7-*O*-glucuronide (hERD-glu1) as the major metabolite derived from oral ingestion of eriocitrin. In addition, it was the most widely distributed and in higher concentration in the analyzed tissues than its homoeriodictyol-4'-*O*-glucuronide isomer (hERD-glu 2). As in the present study, the predominant plasma metabolites of hesperidin were shown to be the 7- and 3´-*O*-glucuronides.[36] Similarly, a physiologically based kinetic model predicted the -7- and -3´-*O*-glucuronides as the major circulating metabolites of hesperidin, whereas the detection of hesperetin aglycon was limited.[23]

The predominance of this metabolite stems from the lower efficiency of glucuronidation at the 4' position of homoeriodictyol caused by a steric hindrance between the 3'-*O*-methoxy group and the 4'-*O*-hydroxyl group of homoeriodictyol. A previous study showed that, after oral ingestion of hesperidin, steric hindrance in the hesperetin 3'-hydroxyl group led to higher concentration of hesperetin 7-*O*-glucuronide.[35]

It is known that flavonoids are highly accumulated in organs related to their metabolism, like the liver, kidneys and small intestine, whereas in tissues like brain, white adipose tissue and skeletal muscle the accumulation is low.[37,33,32] A previous study showed that metabolites of oral hesperetin were widely distributed in rat tissues, with high accumulation in the liver, aorta, kidneys, skin, pancreas and spleen. However, in heart, brain and muscle, the concentration was very low.[33]

In our study, the higher accumulation of eriocitrin metabolites was found in the kidneys, pancreas, liver and spleen, while minor concentrations were found in the heart, muscle and adipose tissue. Likewise, after five minutes of intravenous administration of a naturally occurring homoeriodictyol (homoeriodcityol-7-*O*-β-glucopyranoside), it was majorly found in rat liver, kidneys and small intestine. However, after one hour, it was mostly found in the pancreas, whereas its concentration almost did not change in the spleen and increased in the heart. Contrarily, it was not detect in brain and very little was found in adipose tissue, muscle and heart.[38] In the present study, the eriocitrin metabolites found in liver and kidneys exhibited peak concentrations at the same time point, reinforcing the synergistic action of these two organs in the flavonoid metabolism.

Curiously, homoeriodictyol and hesperetin were the first metabolites to be detected in the kidneys, while eriodictyol and hERD-glu1 appeared later, indicating that eriodictyol remained in the liver and was continuously converted to

homoeriodictyol, hesperetin and their conjugates. Previously, it was suggested that eriodictyol, homoeriodictyol and hesperetin are cleared from the blood, and incorporated into body tissues, owing to the lipophilic nature of the compounds.[6] The distribution, duration and intensity of physiological actions of flavonoids, as well as their metabolism and elimination, are correlated with their affinities towards serum albumin. The presence of the free 7-OH on flavanones' structure enhances their binding capacities towards bovine serum albumin through the formation of hydrogen bonds between the biding sites of albumin and the free hydroxyl group of flavanones. On the other hand, polar glycosidic groups affect the flavonoid orientation in relation to the hydrophobic environment of serum albumin, increase their molecular size and cause steric hindrances in the binding pocket.[39] Thus, we suggest that the lower levels of aglycons derived from eriocitrin metabolism in rat tissues was in part due to their strong affinity to serum albumin, that may have limited their distribution and contributed to higher accumulation in the kidneys, while being eliminated. Differently, the glucuronidated forms were more easily dissociated and therefore, more able to be distributed to other tissues.

A previous study reported that plasma concentrations of eriocitrin metabolites could be detected up to four hour after the i.v. injection of eriodictyol in rats, whereas the urine concentrations could be detected for up to 24 hours.[6] In the present study, the only metabolite detected after 24 hours was hERD-glu1 in the kidneys. This may be due to the low bioavailability of eriocitrin. In our study only 0.5% of the ingested dose of eriocitrin was recovered in urine in the form of glucuronides and aglycons. A previous study showed that 80% of a 25 mg dose of hesperetin-7-*O*-rutinoside was recovered intact after the perfusion into the jejunum of healthy volunteers. Also, no hesperetin metabolites were detected in blood and only traces were excreted in urine.[40]

Considering only the major urinary metabolites of eriocitrin, Miyaki and coworkers[4] estimated an absorption rate of 5.68%, being 11 times greater than the absorption estimated in our study. Our results may have been limited by the choice to study only the flavanone metabolites, since other products of eriocitrin metabolism, such as ring fission products, may eventually occur. In addition, the low fraction excreted in the urine indicates that eriocitrin and its metabolites were eliminated via non-renal routes, such as the intestinal and entero-hepatic routes. Although the concentrations obtained for most orally administered flavonoids are low, it is suggested that these low concentrations do not necessarily indicate a lack of biological activity, since several studies in humans and animals have shown that oral consumption of flavonoids exerts a clear biological effect.[41]

Studies in vitro and in vivo showed that conjugated flavonoids have less bioactivity than its aglycon counterparts, and that in situ de-conjugation is necessary for compound activation.[41] For example, i.v. and i.p. administration of eriodictyol protected against APAP-induced hepatotoxicity, while when it was orally administered, the same protection was not observed, which was reported as a result of higher bioavailability of eriodictyol aglycon.[22] Similarly, in a previous study by our group, oral ingestion of eriodictyol reduced hepatic injury of mice fed a high-fat diet, whereas eriocitrin did not have the same effect.[15] However, recent data from in vitro study showed that the 7- and 3´-*O*-glucuronides of hesperetin were equally active as the hesperetin aglycon in the inhibition of protein kinase A (a regulator of glycogen, sugar, and lipid metabolism). It was pointed that conjugation does not necessarily result in loss of biological activity, and that in vivo effects of hesperidin are at least in part due to its conjugated metabolites.[23] In the present study, glucuronidated forms of eriocitrin were widely distributed to rat tissues, and the 7-*O*-glucuronide of homoeriodictyol was the major form in most of the tissues

analyzed. Also, only conjugated forms of homoeriodictyol (hERD-glu1 and hERD-glu2) were detected in plasma. Interestingly, the 7-*O*-glucuronide of hesperetin showed more potent hypotensive, vasodilatory and anti-inflammatory activities in rat aortic endothelial cells when compared to its 3´-*O*-glucuronide.[6]

Previous findings on intravenously administered eriodictyol and hesperetin showed plasma half-life values of 3-7 hours and urine excretion values of 3–4%, which are consistent with the plasma half-lives found for homoeriodictyol glucuronides in the present study (3 and 3.2 hours). However, the plasma half-life was suggested to underestimate the overall half-life of flavanones because serum concentrations are commonly at the limit of sensitivity.[6] The effect of glucuronosyl transferases on the glucuronidation of flavanones is indicated to limit their appearance in plasma. Moreover, results of a physiologically based kinetic model predicted similar circulating plasma patterns of hesperetin metabolites at dose levels ranging from 0.01 to 50 mg/kg of body weight of hesperidin, showing that the dose may not be the limiting factor for the flavanones appearance in plasma.[23] The pharmacokinetic results of the present study were able to show the *in vivo* metabolism and distribution of eriocitrin. For instance, the high levels of hERD-glu1 found in the pancreas is very insightful, since dietary eriocitrin have been shown to improve blood glucose levels as well as diabetic disorders.[8,9]

Furthermore, eriocitrin and eriodictyol were reported to be potent antioxidants in vivo, capable of neutralizing reactive oxygen species through its flavanone structure design as well as through preservation and activation of the glutathione antioxidant system, which plays a fundamental role in cellular defense against reactive free radicals and other oxidant species.[42,43] Of note, oxidative processes play a pivotal role in the pathogenesis of diabetic complications. The enhancement of glucose autoxidation, protein glycation, and subsequent oxidative degradation of

glycated protein induced by high blood glucose levels can induce the degradation of pancreatic β-cell function. In this sense, the free radical scavenging effects of eriocitrin may help to prevent the formation of advanced glycated end products (AGE) and protect pancreatic islet cells from oxidative stress as well as help in the regeneration of β-cells as shown for epicatechin and quercetin.[7,14]

Thus, although some effects might be attributed to the conjugated metabolites themselves, it remains to be demonstrated whether the de-conjugation is necessary for the effect of eriocitrin metabolites in different tissues. For this reason, it is crucial to understand the metabolism of eriocitrin in order to gain more insights about its possible in vivo effects, while knowing the in vivo distribution of eriocitrin metabolites can maximize its desired effects in specific tissues.

ABBREVIATIONS USED

AUC_{0-24}: area under the curve from zero to 24 hours

Cmax: maximum concentration

Tmax:time to reach the maximum concentration

HPLC: high performance liquid chromatography

PDA: photo diode array

MS: Mass spectrometry

ESI: electrospray ionization

SIR: single ion response

DMSO: dimethyl sulfoxide

FTIR: Fourier transform infrared

APAP: acetaminophen

AGE: advanced glycated end products

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REFERENCES

1. Rodrigo R, Libuy M, Feliu F, Hasson D. Polyphenols in disease: from diet to supplements. *Curr Pharm Biotechnol.* 2014; 15:304-317.

2. Miyake, Y.; Yamamoto, K.; Morimitsu, Y. and Osawa, T. Isolation of C-glucosyl flavone from lemon peel and antioxidative activity of flavonoid compounds in lemon fruit. *J. Agric. Food Chem*.1997a, 45, 4619-4623.

3. Miyake, Y.; Yamamoto, K.; Osawa, T. Isolation of eriocitrin (eriodictyol 7-rutinoside) from lemon fruit (Citrus limon BURM. f.) and its antioxidative activity. *Food Sci. Technol. Int. Tokyo.* 1997b, 3, 84-89.

4. Miyake, Y.; Shimoi, K.; Kumazawa, S.; Yamamoto, K.; Kinae, N.; Osawa, T. Identification and antioxidant activity of flavonoid metabolites in plasma and urine of eriocitrin-treated rats. *J Agric Food Chem.* 2000, 48, 3217-3224.

5. Miyake, Y.; Sakurai, C.; Usuda, M.; Fukumoto, S.; Hiramitsu, M.; Sakaida, K.; Osawa, T.; Kondo, K. Difference in plasma metabolite concentration after ingestion of lemon flavonoids and their aglycones in humans. *J Nutr Sci Vitaminol (Tokyo).* 2006, 52, 54-60.

6. Yáñez, J.A.; Remsberg, C.M.; Miranda, N.D.; Vega-Villa, K.R.; Andrews, P.K.; Davies, N.M. Pharmacokinetics of selected chiral flavonoids: hesperetin, naringenin and eriodictyol in rats and their content in fruit juices. *Biopharm Drug Dispos.* 2008, 29, 63-82.

7. Miyake, Y.;Yamamoto, K.; Tsujihara, N.; Osawa, T. Protective effects of lemon flavonoids on oxidative stress in diabetic rats. *Lipids.*1998, 33, 689–695.

8. Zhang, W.Y.; Lee, J.J.; Kim, Y.; Kim, I.S.; Han, J.H.; Lee, S.G.; Ahn, M.J.; Jung, S.H.; Myung, C.S. Effect of eriodictyol on glucose uptake and insulin resistance in vitro. *J Agric Food Chem.* 2012, 60, 7652-8.

9. Bucolo, C.; Leggio, G.M.; Drago, F.; Salomone, S. Eriodictyol prevents early retinal and plasma abnormalities in streptozotocin-induced diabetic rats. *Biochem Pharmacol.* 2012,84, 88-92.

10. Minato, K.; Miyake, Y.; Fukumoto, S.; Yamamoto, K.; Kato, Y.; Shimomura, Y.; Osawa, T. Lemon flavonoid, eriocitrin, suppresses exercise-induced oxidative damage in rat liver. *Life Sci*. 2003, 72, 1609-1616.

11. Lee, S.E.; Yang, H.; Son, G.W.; Park, H.R.; Park, C.S.; Jin, Y.H.; Park, Y.S. EriodictyolProtects Endothelial Cells against Oxidative Stress-Induced Cell Death throughModulating ERK/Nrf2/ARE-Dependent Heme Oxygenase-1 Expression. *Int J Mol Sci.* **2015a**, 16, 14526-14539. doi: 10.3390/ijms160714526.

12. Ogata, S.; Miyake, Y.; Yamamoto, K.; Okumura, K.; Okumura, K.; Taguchi, H.Apoptosis induced by the flavonoid from lemon fruit (Citrus limon Burm. f.) and its metabolites in HL-60 cells. *Biosci. Biotechnol. Biochem.*2000,64, 1075 - 1078.

13. Lee, J.; Noh, A.L.; Zheng, T.; Kang, J.H.; Yim, M. Eriodicyol inhibits osteoclast differentiation and ovariectomy-induced bone loss in vivo. *Exp Cell Res.* **2015b**, 339, 380-388. doi: 10.1016/j.yexcr.2015.10.001.

14. Ezuruike, U.F.; Prieto, J.M. The use of plants in the traditional management of diabetes in Nigeria: pharmacological and toxicological considerations. *J Ethnopharmacol*. 2014, 155, 857-924.

15. Ferreira, P.S.; Spolidorio, L.C.; Manthey, J.A.; Cesar, T.B. Citrus flavanones prevent systemic inflammation and ameliorate oxidative stress in C57BL/6J mice fed high-fat diet. *Food Funct*. 2016, 7, 2675-2681. doi: 10.1039/c5fo01541c.

16. Silveira, J.Q.; Cesar, T.B.; Manthey, J.A.; Baldwin, E.A.; Bai, J.; Raithore, S. Pharmacokinetics of flavanone glycosides after ingestion of single doses of fresh-squeezed orange juice versus commercially processed orange juice in healthy

humans. *J Agric Food Chem*. 2014 Dec 31;62(52):12576-84. doi: 10.1021/jf5038163.

17. Yáñez JA, Remsberg CM, Miranda ND, Vega-Villa KR, Andrews PK, Davies NM. Pharmacokinetics of selected chiral flavonoids: hesperetin, naringenin and eriodictyol in rats and their content in fruit juices. Biopharm Drug Dispos. 2008 Mar;29(2):63-82.

18. Amaral S, Mira L, Nogueira JM, da Silva AP, Helena Florêncio M. Plant extracts with anti-inflammatory properties--a new approach for characterization of their bioactive compounds and establishment of structure-antioxidant activity relationships. Bioorg Med Chem. 2009 Mar 1;17(5):1876-83.

19. Rondini, L.; Peyrat-Maillard, M.N.; Marsset-Baglieri, A.; Berset, C. Sulfated ferulicacid is the main in vivo metabolite found after short-term ingestion of free ferulic acid in rats. *J Agric Food Chem*. 2002 May 8;50(10):3037-41.

20. Zhang, Y.; Huo, M.; Zhou, J.; Xie, S. PKSolver: An add-in program for pharmacokineticand pharmacodynamic data analysis in Microsoft Excel. *Comput Methods Programs Biomed.* 2010, 99, 306-314.

21. Davis, B.D.; Brodbelt, J.S. Regioselectivity of human UDP-glucuronosyl-transferase 1A1 in the synthesis of flavonoid glucuronides determined by metal complexation and tandem mass spectrometry. *J Am Soc Mass Spectrom*. 2008, 19, 246-256.

22. Wang, Z.; Lan, Y.; Chen, M.; Wen, C.; Hu, Y.; Liu, Z.; Ye, L. Eriodictyol, Not Its Glucuronide Metabolites, Attenuates Acetaminophen-Induced Hepatotoxicity. *Mol Pharm*. 2017, 14, 2937-2951.

23. Boonpawa, R.; Spenkelink, A.; Punt, A.; Rietjens, I.M.C.M. Physiologically based kinetic modeling of hesperidin metabolism and its use to predict in vivo

effective doses in humans. *Mol Nutr Food Res*. 2017; 61. doi: 10.1002/mnfr.201600894.

24. Berezin, K.V.; Likhter A. M.; Shagautdinova, I.T.; Chernavina, M.L.; Novoselova, A.V. FT-IR and DFT study of lemon peel. *Proc. of SPIE*, 2006, 10336,103360I-2.

25. Prima, E.C.; Hidayat, N.N.; Yuliarto, B.; Suyatman; Dipojono, H.K. A combined spectroscopic and TDDFT study of natural dyes extracted from fruit peels of Citrus reticulata and Musa acuminata for dye-sensitized solar cells. Spectrochim Acta A Mol Biomol Spectrosc. 2017,171, 112-125.

26. Erenler, R.; Sen, O.; Aksit, H.; Demirtas, I.; Yaglioglu, A.S.; Elmastas, M.; Telci, İ. Isolation and identification of chemical constituents from Origanum majorana and investigation of antiproliferative and antioxidant activities. J *Sci Food Agric*. 2016, 96:822-836.

27. Vijay, M.M.J.; Eswarappa, B.; Bodke, Y.D.; Jayadevaiah, K.V.; Basavaraja, H.S. Isolation of Phytoconstituents from the Stem Bark of Bauhinia Variegata Linn. *PharmaTutor.* 2014, 2, 150-156.

28. Booth, A.N.; Jones, F.T.; DeEds, F. Metabolic fate of hesperidin, eriodictyol, homoeriodictyol, and diosmin. *J. Biol. Chem.* 1958, 230, 661-668.

29. Manach, C.; Morand, C.; Gil-Izquierdo, A.; Bouteloup-Demange, C.; Remesy, C. Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *Eur. J. Clin. Nutr*. 2003, 57, 235-242.

30. Shen, C.; Qian, Z.; Chen, R.; Meng, X.; Hu, T.; Chen, Z.; Li, Y.; Huang, C.; Hu, C.; Li J. Single Dose Oral and Intravenous Pharmacokinetics and Tissue Distribution of a Novel Hesperetin Derivative MTBH in Rats. *Eur J Drug Metab Pharmacokinet*. 2016, 41, 675-688.

31. Shimoi, K.; Okada, H.; Furugori, M.; Goda, T.; Takase, S.; Suzuki,M.; Hara Y.; Yamamoto, H.; Kinae, N. Intestinal absorption of luteolin and luteolin 7-*O*-beta-glucoside in rats and humans. *FEBS Lett.* 1998, 438, 220-224.

32. Bieger. J.; Cermak, R.; Blank, R.; de Boer, V.C.; Hollman, P.C.; Kamphues, J.; Wolffram, S. Tissue distribution of quercetin in pigs after long-term dietary supplementation. *J Nutr*. 2008, 138, 1417-1420.

33. Takumi, H.; Mukai, R.; Ishiduka, S.; Kometani, T.; Terao, J. Tissue distribution of hesperetin in rats after a dietary intake. *Biosci Biotechnol Biochem*. 2011, 75, 1608-1610.

34. Thilakarathna, S.H.; Rupasinghe, H.P. Flavonoid bioavailability and attempts for bioavailability enhancement. *Nutrients.* 2013, 5, 3367-3387. doi: 10.3390/nu5093367.

35. Matsumoto, H.; Ikoma, Y.; Sugiura, M.; Yano, M.; Hasegawa, Y. Identification and quantification of the conjugated metabolites derived from orally administered hesperidin in rat plasma. *J Agric Food Chem*. 2004, 52, 6653-6659.

36. Yamamoto, M.; Jokura, H.; Hashizume, K.; Ominami,H.; Shibuya, Y.; Suzuki, A.; Hase, T.;Shimotoyodome, A. Hesperidin metabolite hesperetin-7-O-glucuronide, but not hesperetin-3'-O-glucuronide, exerts hypotensive, vasodilatory, and anti-inflammatory activities. *Food Funct*. 2013, 4, 1346-1351.

37. Barros, P.P.; Silva, G.H.; Gonçalves, G.M.S.; Oliveira, J.C, Pagnan, L.G.; Arco-e-Flexa, L. Hepatoprotective Effect of Quercetin Pretreatment Against Paracetamol-Induced Liver Damage and Partial Hepatectomy in Rats. *Braz. Arch. Biol. Technol.* 2017, 60.

38. Zhao, Y.; Wang, X.; Zhao, Y.; Gao, X.; Bi, K.; Yu, Z.; HPLC determination and pharmacokinetic study of homoeriodictyol-7-O-beta-D-glucopyranoside in rat plasma

and tissues. *Biol Pharm Bull.* 2007, 30, 617-620.

39. Liu, S.; Guo, C.; Guo, Y.; Yu, H.; Greenaway, F.; Sun, M.Z. Comparative binding affinities of flavonoid phytochemicals with bovine serum albumin. *Iran J Pharm Res.* 2014, 13, 1019-1028.

40. Actis-Goretta, L.; Dew, T.P.; Lévèques, A.; Pereira-Caro, G.; Rein, M.; Teml, A.; Schäfer, C.; Hofmann, U.; Schwab, M.; Eichelbaum, M.; Crozier, A.; Williamson, G. Gastrointestinal absorption and metabolism of hesperetin-7-O-rutinoside and hesperetin-7-O-glucoside in healthy humans. *Mol Nutr Food Res*. 2015, 59, 1651-1662.

41. Perez-Vizcaino, F.; Duarte, J.; Santos-Buelga, C. The flavonoid paradox: conjugation and deconjugation as key steps for the biological activity of flavonoids. *J Sci Food Agric.* 2012, 92, 1822-1825.

42. Li, C.Z.; Jin, H.H.; Sun, H.X.; Zhang, Z.Z.; Zheng, J.X.; Li, S.H.; Han, S.H. Eriodictyol attenuates cisplatin-induced kidney injury by inhibiting oxidative stress and

inflammation. *Eur J Pharmacol*. 2016;772, 124-130.

43. Hanneken, A.; Lin, F.F.; Johnson, J.; Maher, P. Flavonoids protect human retinal pigment epithelial cells from oxidative-stress-induced death. Invest *Ophthalmol Vis Sci.* 2006, 47, 3164-3177.

Capítulo 2.

Eriocitrin regulatory effects in high-fat diet induced obesity in C57BL/6 mice

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Abstract

Eriocitrin has been shown to play a role in decreasing oxidative stress conditions, which is related to the development of metabolic disturbances and chronic diseases. We assessed the effect of eriocitrin supplementation on high-fat diet fed mice. Sixty male C57BL/6J mice were randomly assigned into six different groups of 10 mice each. Four of the groups were fed high-fat diet for four weeks, and subsequently, fed high-fat diet supplemented with eriocitrin at the doses of 10, 25, 50 or 100 mg/kg bw for four additional weeks. Control groups consisted of mice fed standard diet (10% kcal from fat) and non-supplemented high-fat diet (60% Kcal from fat). Mice from groups fed non-supplemented high-fat diet exhibited increased body weight, abdominal fat, and blood serum levels of glucose, insulin, triglycerides, total-cholesterol, resistin, leptin and lipid peroxidation ($p< 0.05$). However, eriocitrin supplementation was effective in ameliorating metabolic disturbances induced by high-fat diet consumption; mainly those related to hyperglycemia. The best collective results of eriocitrin were found with a dose 25 mg/kg bw, which decreased serum levels of triglycerides ($p<0.05$), and improved serum levels of glucose, insulin, resistin and lipid peroxidation, being the most effective dose.

Key-words: Citrus flavonoids; eriocitrin; obesity; insulin resistance; antioxidants.

Introduction

Obesity is characterized as a chronic, low-grade systemic inflammation, with increased production of inflammatory cytokines and acute phase proteins. It is suggested that diet-induced inflammation is caused by nutrient overload from the diet, however, the sequence of events responsible for its initiation is not yet known. In particular, the intake of diets rich in saturated fatty acids is accompanied by inflammatory processes in adipose, hepatic and vascular tissues (1,2). Excess fat deposition in liver increases the activity of nuclear factor-κB (NFκB) and the expression of pro-inflammatory cytokines (2). Macrophage infiltration into adipose tissue is considered the main source of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and interleukin 6 (IL-6), and others. Adipose tissue *per se* produces the inflammatory cytokines leptin, adiponectin, and resistin, which affect physiological functions of white adipose tissue and other organs (3,4).

The development of metabolic syndrome associated with obesity is mediated by exacerbated production of inflammatory cytokines and oxidative stress. In mice, the intake of high-fat diet increases pathways for the production of free radicals in liver and adipose tissue, and stimulates the production of pro-inflammatory molecules and acute phase proteins, suggesting that oxidative stress is one of the initial steps of obesity-related metabolic dysregulation (5,6). In obese humans, lipid peroxidation, represented by thiobarbituric acid reactive species (TBARS), is positively associated with body mass index (BMI) and waist circumference.[7] The lipotoxicity caused by high-fat diet dysregulates cardiac, hepatic and insulin functions, due to lipid accumulation in heart, liver and muscles, increasing the release of free-fat acids and cytokines, which contribute to the perpetuation of

systemic inflammation (8,9). On the other hand, the intake of food rich in antioxidant compounds is associated to the reduction of risk factors for chronic diseases, especially for cardiovascular disease and diabetes *mellitus* type 2, being known as protective foods.[10] In this sense, the flavonoids from citrus fruits are reported as potent antioxidants, with anti-inflammatory, hypolidaemic and hypoglycemic effects, and have been shown to contribute to the reduction of chronic disease risk factors (11,12).

The flavanone eriocitrin (eriodictyol-7-*O*-rutinoside), which is found in different parts of lemon fruit (*Citrus limon*) (ranked as: peels>seed>whole fruit) (13) after ingestion is converted to eriodictyol by intestinal bacteria. Eriodictyol is then absorbed into enterocytes and glucuronidated, sulfated and delivered to liver through the portal circulation. In the liver, conjugated eriodictyol is hydrolyzed and re-glucuronidated/sulfated and methylated, forming a variety of conjugated metabolites of eriodictyol, homoeriodictyol and hesperetin, which return to blood circulation and distribute to body tissues to exert their biological activities (14-16) Previous studies reported that eriocitrin and eriodictyol are potent antioxidants *in vitro* and *in vivo* (17), capable of suppressing oxidative stress in rats induced to diabetes by streptozotocin (18) and in rats with exercise-induced oxidative stress (19). Eriodictyol was shown to protect against cisplatin-induced kidney injury by inhibiting oxidative stress and inflammation through activation of nuclear factor erythroid 2–related factor 2 (Nrf2), a redox-sensitive transcription factor that positively regulates the expression of genes encoding antioxidants; and through inhibition of NF-κB (20). It also significantly inhibited lipid peroxidation in diabetic rats, and reduced the retinal levels of TNF-α, intercellular adhesion molecule 1 (ICAM-1), vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (12). In addition, a recent study from our laboratory showed that eriocitrin

and eriodictyol inhibited systemic inflammation and reduced oxidative stress markers in liver and blood of mice fed high-fat diet (21).

Eriocitrin and eriodictyol antioxidant activity is mainly provided by the flavonoid B-ring 3' and 4' orto-dihydroxy structure, which stabilizes the molecule to the neutralization of free radicals.[17] Therefore, citrus flavanones, as eriocitrin, can act as metal chelating and radical scavenging substances, capable of neutralizing reactive oxygen species (ROS) and improving the endogenous antioxidant system. They are indicated as inducers and preservatives of antioxidant enzymes, such as glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GSR) and superoxide dismutase (SOD) (19,22-24).

In this sense, as inflammation and oxidative stress play critical roles in the pathogenesis of obesity-related diseases, and eriocitrin exhibit both anti-inflammatory and antioxidant effects, the present study aimed to explore the effect of different doses of orally administered eriocitrin in C57BL/6 mice.

Materials and methods

Experimental design

Sixty male C57BL/6J mice, six weeks old (20±1 g), from University of São Paulo, Ribeirão Preto, SP, Brazil, were kept in a HEPA-filtered ventilated cabinet with constant temperature of 22-25 ºC and 12-hour light/dark cycles. After one week of adaptation in these conditions they were randomly divided in six groups of ten animals each, being: (1) control group, fed a standard diet containing 10% of calories from fat (AIN-93M); (2) High-fat diet group, group fed a 45% calories high-fat diet; (3) group fed high-fat diet supplemented with 10 mg/kg of eriocitrin; (4) group fed high-fat diet supplemented with 25 mg/kg of eriocitrin; (5) high-fat diet

supplemented with 50 mg/kg of eriocitrin, and; (6) high-fat diet supplemented with 100 mg/kg of eriocitrin. The experimental period was divided into two periods of four weeks, being the first four weeks destined to weight gain and obesity development by the high-fat diet feeding. From the fifth to eighth week, each group was supplemented with the determined dose of eriocitrin combined with the high-fat diet. All mice had free access to food and water during the experimental period. The weight gain was measured weekly and the food intake daily. This experimental protocol was approved by the Animal Use Committee of the São Paulo State University (UNESP), School of Pharmaceutical Sciences, Araraquara, SP, Brazil (16/2015).

Supplementation

Eriocitrin was offered in 10, 25, 50 and 100 mg/kg of body weight (bw).[25-28] Each dose was homogenized in the high-fat diet to mimic a natural intake by the food. The diet was offered at 110% of the last day food intake in grams/day, while eriocitrin doses were offered at 120% in milligrams/body weight/day of the determined dose to each group to avoid losses by any lower food intake and to assure enough food to mice.

Diets

The high-fat diet (Rhoster, Indústria e Comercio LTDA, Araçoiaba da Serra, SP, Brazil) was prepared with purified ingredients to contain 45% kcal from fat (lard), 21% kcal from protein (casein) and 34% kcal from carbohydrates (28% corn starch and 72% sucrose), with a caloric density of 5.35 kcal/g. The standard diet was based on AIN 93M and contained 9.5% kcal from fat (soybean oil), 14.7% kcal from protein (casein) and 75.8% kcal from carbohydrates (82% corn starch and

18% sucrose), with caloric density of 4.22 kcal/g (29). Eriocitrin and energy intake was calculated based on daily food intake.

Sample collection

The mice were fasted for 10 hours before euthanasia and were anesthetized *via* intraperitoneal (*i.p.)* with xilazin (16 mg/kg): ketamin (60 mg/kg). The euthanasia was performed by cardiac puncture and blood was collected into tubes with anticoagulant gel and centrifuged at 2800 rpm for 15 min for blood serum obtainment. Abdominal adipose tissue (retro-abdominal, epididymal and peri-renal), liver, spleen, heart and kidneys were collected after frontal excision, rinsed with 0.9% saline, blotted in filter paper, weighed and immediately frozen in liquid nitrogen. Blood serum and organs were stored at - 80 ºC until analysis. Livers left lobes and epididymal fat pads were reserved for histological kept in 10% buffered formalin for 48 h, then rinsed with deionized water and stored in 70% ethanol until analysis.

Histological analysis

Hepatic fixed tissues were paraffin-embedded, sectioned at 4-6 μm and stained with hematoxylin-eosin. Liver morphology, fat deposition and damage were analyzed at an optical microscope with magnifying power of x200 by using standard protocols from The Pathology Laboratory from The São Paulo State University (UNESP), School of Dentistry, Araraquara, SP, Brazil.

Blood and liver metabolic parameters

Blood serum glucose, total cholesterol, HDL-cholesterol, triglyceride, alanine and aspartate aminotransferases (ALT and AST) were determined using

commercial enzymatic assay kits (Labtest, Lagoa Santa, MG, Brazil). LDL-cholesterol concentration was calculated by Friedewald's formula: LDL = (CT - HDL) - (TG/5). Free fatty acids (non-esterified fatty acid) in blood serum were also measured using an enzymatic assay kit (Cayman Chemical, Ann Arbor, MI, USA). The same tests used for blood lipids were used to determine hepatic lipids (triglycerides and total cholesterol). Hepatic lipids were extracted with chloroform:methanol (2:1), dried under N_2 at - 60 °C and solubilized with Triton X-100 according to Folch's method (30). Fasting blood glucose and insulin concentrations were used to estimate insulin resistance using the Homeostatic model assessment (HOMA) index, as following: HOMA−IR = Insulin (μU/mL) × glucose (mmol/ L)/22.5 (31).

Inflammatory markers

Inflammatory cytokines TNF-α, IL-1β, IL-6, IL-10, MCP-1, adiponectin, resistin and leptin were assayed by ELISA Multiplex kits using Luminex xMAP detection method (Merck KGaA, Darmstadt, Germany) and us-CRP was determined by immunoturbidimetry using a commercial kit (Labtest, Lagoa Santa, MG, Brazil).

Oxidative stress markers

The thiobarbituric acid-reactive substances method (TBARS) was used to determine blood serum lipid peroxidation as previously described (32). TBARS levels were spectrophotometrically measured at 535 nm and expressed as μM MDA. Total antioxidant activity was determined by the ABTS assay as previously described (33). Formation of ABTS•+ radical was measured at 734 nm, using Trolox (Sigma-Aldrich® St, Louis, MO, USA) as standard. Values were expressed as mM

of Trolox equivalent antioxidant capacity (mMeq TEAC). All analyzes were performed in triplicate.

Statistical analysis

Results are presented as mean ± standard deviation. The effects of eriocitrin and/or obesity on the studied groups were analyzed by one-way ANOVA, followed by Tukey test at GraphPad Prism software. Statistically significant differences were considered when $P < 0.05$.

Results

Food intake, energy intake, body and organ weight parameters

Mice body weight and food intake parameters are shown in **Table 1**. At the beginning of the study all mice presented similar body weight, however, mice fed high-fat diet, with or without eriocitrin supplementation, presented a statistically significant increase of 27% in final body weight compared to the control group. Accordingly, all of the groups fed high-fat diet presented 127% higher weight gain than the control group ($p<0.05$). Of the groups fed high-fat diet, the lowest weight gain was of the group supplemented with 10 mg/kg body weight (bw) of eriocitrin, while the highest weight gain was of the group supplemented with 100 mg/kg bw of eriocitrin.

The groups fed high-fat diet, supplemented or not, exhibited lower food and energy intake than the control group, whereas the higher food intake was of the group supplemented with 100 mg/kg bw, which had energy intake similar to that of the control group. Despite lower food intake, the groups fed high-fat diet presented an averaged abdominal fat accumulation > 4.6 times higher than that of mice fed

standard diet ($p < 0.05$). Yet, the analysis of organ weights did not show any significant difference between the groups studied.

Nevertheless, eriocitrin intake, calculated by the groups mean daily food intake, showed that its intake was within the expected range in all groups: 99 ± 24% at group supplemented with 10 mg/kg bw; 93 ± 24% at group supplemented 25 mg/kg bw; 94 ± 23% at group supplemented with 50 mg/kg bw and; 95 ± 27% at group supplemented with 100 mg/kg bw.

Table 1. Food and energy intake, and body and organ weights of mice fed standard diet, high-fat diet and high-fat diet supplemented with different doses of eriocitrin.

Parameters	Standard diet	High-fat diet				
Supplementation	Not supplemented	Not supplemented	Eriocitrin 10 mg/kg	Eriocitrin 25 mg/kg	Eriocitrin 50 mg/kg	Eriocitrin 100 mg/kg
Food intake (g/d)	3.95 ± 0.60^{c}	2.72 ± 0.49^{a}	2.69 ± 0.39^{a}	2.70 ± 0.26^{a}	2.77 ± 0.30^{ab}	2.99 ± 0.33^{bc}
Energy intake (kcal/d)	16.6 ± 2.5^{b}	14.6 ± 2.6^{a}	14.4 ± 2.1^{a}	14.4± 1.4^{a}	14.8 ± 1.6^{a}	16.0 ± 1.76^{b}
Initial body weight (g)	20.70 ± 1.36	19.78 ± 1.19	19.45 ± 1.48	19.27 ± 0.89	20.45 ± 0.77	19.66 ± 0.86
Final body weight (g)	27.21 ± 2.44^{a}	34.47 ± 3.75^{bc}	32.34 ± 4.07^{b}	33.23 ± 2.50^{bc}	36.51 ± 2.95^{c}	36.11 ± 2.53^{bc}
Weight gain (g)	6.51 ± 1.59^{a}	14.69 ± 3.28^{bc}	12.90 ± 3.40^{b}	13.96 ± 2.30^{bc}	16.06 ± 2.83^{bc}	16.45 ± 1.93^{c}
Abdominal fat (g)	0.55 ± 0.14^{a}	2.31 ±0.76^{b}	2.33 ± 0.85^{b}	2.55 ± 0.47^{b}	2.96 ± 0.50^{b}	2.59 ± 0.48^{b}
Liver (g)	1.27 ± 0.18	1.15 ± 0.12	1.27 ± 0.17	1.13 ± 0.13	1.22 ± 0.08	1.13± 0.10
Heart (g)	0.13 ± 0.01	0.14 ± 0.02	0.14 ± 0.01	0.12 ± 0.01	0.14 ± 0.02	0.14 ± 0.01
Spleen (g)	0.08 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
Kidneys (g)	0.32 ± 0.06	0.37 ± 0.04	0.38 ± 0.04	0.35 ± 0.05	0.38 ± 0.03	0.36 ± 0.01

Results are presented as mean ± standard deviation. Statistically significant differences were detected by one way ANOVA followed by Tukey test, considering $p<0.05$. Different letters indicate significant differences between groups.

Metabolic parameters

Blood and liver metabolic parameters are shown in **Table 2**. After eight weeks, blood serum glucose and insulin levels significantly increased for all groups fed high-fat diet, except at the group supplemented with 25 mg/kg bw of eriocitrin, which presented intermediate levels of both. However, no significant difference was observed at HOMA-IR index values of the studied groups.

Blood serum triglyceride concentration was significantly increased for the non-supplemented high-fat diet group, however, the groups supplemented with 10, 25 and 100 mg/kg bw of eriocitrin presented a significant reduction in this lipid, with values even lower than that of the control group. Similarly, liver triglyceride concentration was elevated by the non-supplemented high-fat diet, but presented intermediate concentrations at the groups supplemented with 10, 25 and 100 mg/kg bw of eriocitrin.

In blood serum, total cholesterol concentration was significantly higher at the group fed high-fat diet compared to control group. Although a statistically significant difference was observed for groups supplemented with eriocitrin compared to non-supplemented groups, their values were very similar, and would not be clinically considerable. Similarly, the LDL-cholesterol level increased significantly for all groups fed high-fat diet and was not affected by any of the eriocitrin doses used in this study. No statistically significant difference was observed for blood serum HDL-cholesterol, free fatty acids, or liver enzymes neither in liver total cholesterol

of mice fed high-fat diet, supplemented or not with eriocitrin during the analyzed period (**Table 2**).

Table 2. Blood and liver metabolic parameters of mice fed standard diet, high-fat diet and high-fat diet supplemented with different doses of eriocitrin.

Parameters	Standard diet	High-fat diet				
Supplementation	Not supplemented	Not supplemented	Eriocitrin 10 mg/kg	Eriocitrin 25 mg/kg	Eriocitrin 50 mg/kg	Eriocitrin 100 mg/kg
Glucose (mg/dL)	191.7 ± 25.9^{a}	360.7 ± 56.6^{b}	354.5 ± 50.7^{b}	269.2 ± 45.3^{ab}	330.1 ± 52.2^{b}	310.1 ± 54.9^{b}
Insulin (mU/L)	1.75 ± 1.01^{a}	4.29 ± 0.70^{b}	4.52 ± 1.90^{b}	2.78 ± 0.75^{ab}	4.37 ± 1.26^{b}	4.28 ± 1.26^{b}
HOMA-IR	0.83 ± 0.53	3.88 ± 1.11	2.56 ± 2.19	1.89 ± 0.75	3.60 ± 1.32	3.39 ± 1.44
Triglycerides (mg/dL)	72.4 ± 11.8^{ab}	85.0 ± 12.9^{b}	63.1 ± 13.5^{a}	56.8 ± 9.7^{a}	86.1 ± 10.9^{b}	57.0 ± 7.0^{a}
Total-cholesterol (mg/dL)	88.9 ± 14.6^{a}	138.8 ± 29.2^{b}	128.4 ± 33.2^{ab}	133.1 ± 34.4^{ab}	132.0 ± 21.4^{ab}	129.9 ± 18.1^{ab}
LDL- cholesterol (mg/dL)	18.9 ± 2.5^{a}	49.8 ± 16.2^{b}	44.6 ± 11.7^{b}	50.7 ± 9.7^{b}	51.5 ± 15.8^{b}	54.7 ± 15.4^{b}
HDL- cholesterol (mg/dL)	53.2 ± 11.9	69.2 ± 14.4	61.9 ± 9.7	58.7 ± 4.2	63.3 ± 6.7	63.8 ± 5.1
Free fat acids (µM)	13908±1861	13584±1197	10693±1649	12403±949	12943±1236	13854±2418
ALT (U/L)	38.1 ± 19.0	36.0 ± 14.1	38.4 ± 18.4	37.7 ± 6.1	31.0 ± 10.2	45.0 ± 20.1
AST (U/L)	204.9 ± 111.6	223.4 ± 107.4	223.8 ± 84.1	227.4 ± 63.1	232.6 ± 60.8	256.0 ± 91.9
Liver Triglycerides (mg/dL)	71.5 ± 15.6^{a}	122.6 ± 30.8^{bc}	89.8 ± 15.4^{ab}	95.5 ± 16.6^{abc}	145.4 ± 40.5^{c}	93.8 ± 26.7^{abc}
Liver Total Cholesterol (mg/dL)	10.2 ± 0.4	10.4 ± 1.1	11.2 ± 1.1	10.5 ± 0.6	10.4 ± 1.1	9.7 ± 1.4

Results are presented as mean ± standard deviation. Statistically significant differences were detected by one way ANOVA followed by Tukey test, considering a p value <0.05. Different letters indicate significant differences between groups.

Inflammatory and oxidative stress parameters

Of the inflammatory markers analyzed in the present study, three of them were the adipokines adiponectin, resistin and leptin (**Table 3**). Leptin and resistin have both inflammatory and hormonal activities involved in appetite regulation and insulin resistance, respectively. The systemic levels of both them were significantly increased in mice induced to obesity by the high-fat diet, whereas no change was observed for adiponectin levels. No influence of eriocitrin supplementation was observed for leptin levels. However, resistin values were moderately reduced in groups supplemented with the eriocitrin doses of 10, 25 and 100 mg/kg bw. Systemic levels of the other inflammatory markers analyzed, including IL1β, IL-6, TNF-α, MCP-1 and us-CRP were not affected by high-fat diet or by eriocitrin for the all doses tested.

The effect of eriocitrin on oxidative stress is shown in Table 3. No significant changes were detected in total blood serum antioxidant capacity with high-fat diet feeding, supplemented or not, at all of the doses tested. On the other hand, serum TBARS levels were significantly increased in mice fed the non-supplemented high-fat diet, while intermediate levels of TBARS were observed for groups supplemented with 25, 50 and 100 mg / kg bw of eriocitrin.

Table 3. Inflammatory and oxidative stress parameters of mice fed standard diet, high-fat diet and high-fat diet supplemented with different doses of eriocitrin.

Parameters	Standard diet	High-fat diet				
Supplementation	Not supplemented	Not supplemented	Eriocitrin 10 mg/kg	Eriocitrin 25 mg/kg	Eriocitrin 50 mg/kg	Eriocitrin 100 mg/kg
Adiponectin (pg/mL)	$3.47x10^6 \pm 6.44x10^5$	$3.86x10^6 \pm 8.11x10^5$	$3.60x10^6 \pm 2.37x10^5$	$3.58x10^6 \pm 7.38x10^5$	$4.21x10^6 \pm 6.61x10^5$	$4.04x10^6 \pm 6.17x10^5$
Resistin (pg/mL)	1055 ± 164^a	1887 ± 391^b	1594 ± 487^{ab}	1554 ± 616^{ab}	1932 ± 420^b	1675 ± 451^{ab}
Leptin (pg/mL)	546 ± 340^a	9791 ± 4123^b	10857 ± 4698^b	9187 ± 3233^b	10875 ± 4040^b	11849 ± 3199^b
IL-1β (pg/mL)	1.98 ± 0.68	1.79 ± 0.60	1.50 ± 0.66	1.20 ± 0.51	2.43 ± 1.49	1.41 ± 0.50
IL-6 (pg/mL)	5.44 ± 4.15	4.29 ± 3.50	2.26 ± 1.97	3.13 ± 1.70	3.46 ± 2.90	1.51 ± 1.05
IL-10 (pg/mL)	2.95 ± 0.73	2.57 ± 1.32	2.32 ± 1.09	1.92 ± 1.10	4.75 ± 3.17	1.78 ± 0.65
TNF-α (pg/mL)	3.16 ± 0.61	3.01 ± 0.46	2.79 ± 0.26	2.67 ± 0.13	3.04 ± 0.68	2.62 ± 0.35
MCP-1 (pg/mL)	34.5 ± 18.5	29.9 ± 4.03	36.1 ± 13.4	23.2 ± 7.0	32.7 ± 14.9	25.7 ± 11.0
us-CRP (mg/L)	0.012 ± 0.001	0.016 ± 0.008	0.014 ± 0.005	0.015 ± 0.005	0.0120 ± 0.004	0.014 ± 0.004
TEAC (mMeq)	1.40 ± 0,02	1.43 ± 0.02	1.43± 0.02	1.43 ± 0.06	1.41 ± 0.08	1.38 ± 0.06
TBARS (μM)	7.3 ± 0.9^a	11 ± 0.9^b	12 ± 2.2^b	8.9 ± 1.4^{ab}	10 ± 2.2^{ab}	8.6 ± 1.9^{ab}

Results are presented as mean ± standard deviation. Statistically significant differences were detected by one way ANOVA followed by Tukey test, considering a p value <0.05. Different letters indicate significant differences between groups.

Histological analysis

The morphological findings of liver sections of mice fed standard diet were histologically normal, exhibiting globular hepatocytes with one or two centralized nuclei and apparent basophilic nucleoli. The cytoplasm was eosinophilic and did not present substantial macro or microvesicular fat vesicles (Figure 1, a). On the other hand, livers from mice fed the high-fat diet exhibited the classical hepatic steatosis, with varying sizes of fat vesicles at distinct areas of the tissue (Figure 1, b). At some regions, numerous small fat vesicles (microvesicular steatosis) were observed in cytoplasm, and in others, large fat vesicles were observed (macrovesicular steatosis). In those cases, hepatocytes were larger compared to those of the control group, and many of them lacked well defined contours, and/or were fragmented with undefined nuclei and nucleoli. However, no leukocyte infiltrates were observed in hepatic parenchyma and the portal space region was within normal expectations. Livers from mice from groups supplemented with eriocitrin, regardless of the dose, exhibited hepatic steatosis similar to that observed in mice fed the non-supplemented high-fat diet, with no leukocyte infiltrates in the parenchyma and portal space within normal expectations (Figure 1, c, d, e and f).

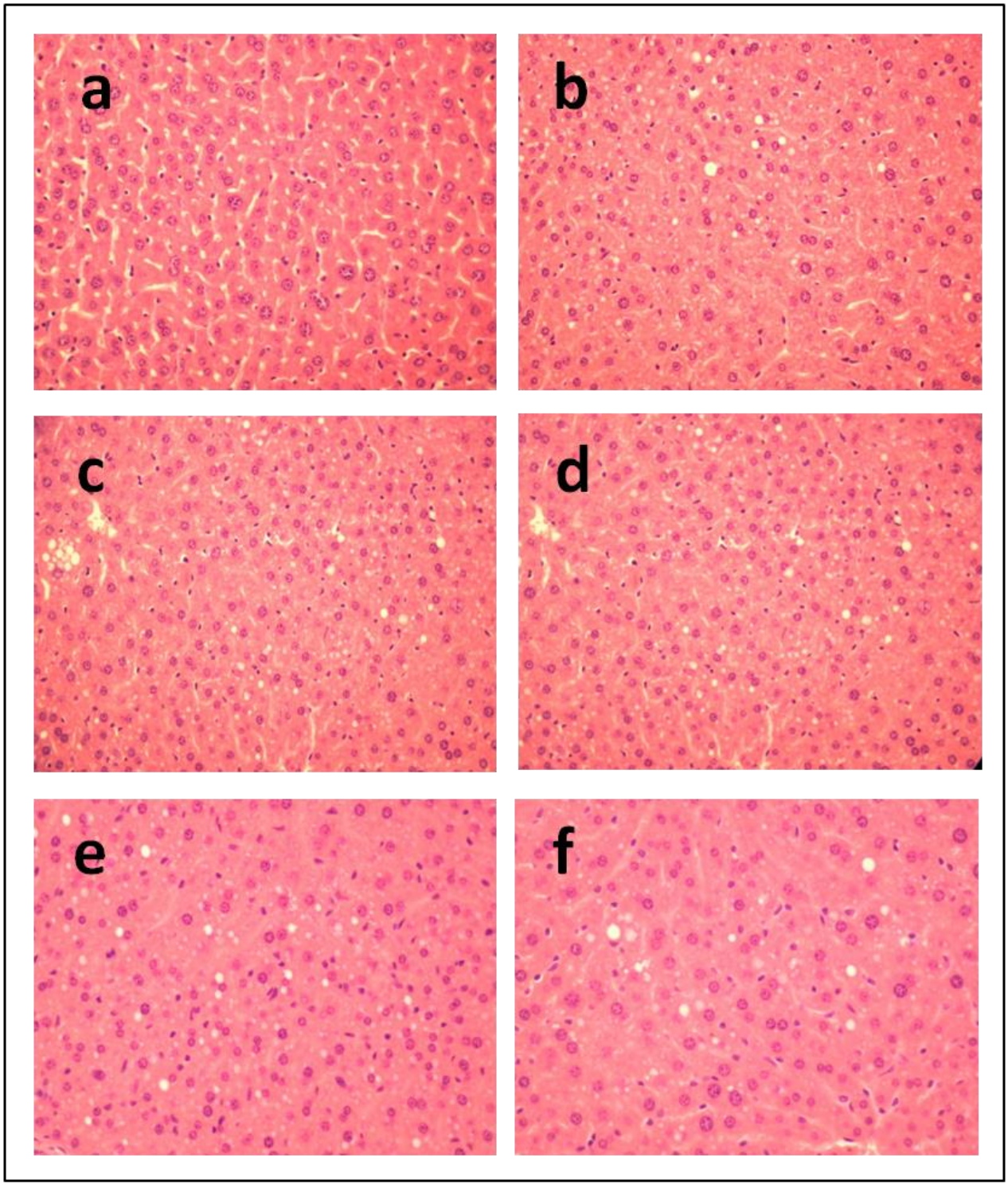

Figure 1. Hepatic histological sections of mice fed standard diet (a), high-fat diet (b), high-fat diet supplemented 10 mg/kg bw of eriocitrin (c); high-fat diet supplemented 25 mg/kg bw of eriocitrin (d); high-fat diet supplemented 50 mg/kg bw of eriocitrin (e) and; high-fat diet supplemented 100 mg/kg bw of eriocitrin (f). Please note well-defined contours, globular cells, and micro, almost not visible, lipid droplets in "a".

Note apparent lipid droplets, undefined contours and bigger cells in "b"(the same pattern is seen in "c, d, e and f").

Discussion

The results of the present study re-enforce the deleterious potential of high-fat diet induced obesity. Compared to the control group, mice in the high-fat diet group exhibited higher accumulation of abdominal adipose tissue, liver triglycerides and liver fat deposition (demonstrated by liver histology). These changes were accompanied by elevated levels of glucose, insulin, resistin, leptin, triglycerides, total-cholesterol and LDL-C in blood serum. On the other hand, eriocitrin supplementation showed metabolic improvements, anti-inflammatory and antioxidant responses of obese mice. The eriocitrin dose of 25 mg/kg bw improved blood serum glucose and insulin, while doses of 10, 25 and 100 mg/kg bw significantly reduced serum triglycerides. Similarly, blood serum resistin was moderately improved at doses of 10, 25 and 100 mg/kg bw, whereas TBARS levels were moderately improved at doses of 25, 50 and 100 mg/kg bw. The mice fed high-fat diet, with or without eriocitrin at any dose, exhibited higher body weight gain and abdominal fat accumulation than did mice on standard diet, even with a lower food (g/day) and energy intake (kcal/day). This decline in food intake is seen in rodents fed high-fat diets and is considered a compensatory mechanism related to an attempt to maintain energy balance (34,35).

Organ damage and hypertrophy are typical symptoms of diabetes mellitus type 2 and sometimes they occur before the clinical appearance of the disease (36). However, in our study we did not observe any change in organ weight of high-fat diet fed mice, with or without eriocitrin supplementation. Also, no change was observed in total antioxidant capacity

or cytokine levels in blood serum. On the other hand, blood serum lipids and liver fat deposition were increased in all mice fed high-fat diet, with no effect of eriocitrin at the different doses tested, except in triglycerides. Previously, a strong suppressing effect of eriocitrin on oxidative stress in liver and kidneys of diabetic rats was shown by decreased levels of TBARS and 8-OHdG, which are makers of lipid peroxidation and DNA damage, respectively (18).

Similar to our results, the plasma of high-fat diet fed mice was evaluated during sixteen weeks and did not exhibit changes in levels of IL-1β, TNFα and non esterified fatty acids at any time (NEFA). It was indicated that inflammatory markers are highly variable in mice fed high-fat diet, particularly at the beginning of high-fat feeding, which is typical of acute inflammatory responses. Moreover, mice liver triglycerides were significantly elevated after 16 weeks on the high-fat diet and the Kupffer cell area was increased at all times tested and appeared to peak at 4 weeks, but did not increase further. The plasma LDL- and HDL-cholesterol were elevated at three days and again at 16 weeks, but were unchanged between these times. Therefore, the elevated levels of inflammatory cytokines and changes in a number of acute phase proteins in mice fed high-fat diet is indicated as a rapid and transient inflammatory response. It was shown that the inflammatory response to high-fat diets is developed in distinct stages; the first resulting from an acute activation of Kupffer cells in the liver, and the second, between 12 and 16 weeks when inflammation in white adipose tissue and muscle becomes apparent (35). In accordance with our previous study elevated levels of IL-6, TNF-α, MCP-1 and us-CRP, as well as a

decrease of blood serum total antioxidant capacity, were observed in mice after four weeks on the high-fat diet, and were significantly improved by eriocitrin treatment (21).

Oxidative stress coupled with hepatocyte apoptosis is believed to play a key role in the pathogenesis of non-alcoholic fat liver disease (NAFLD). Emerging data suggest that hepatocyte apoptosis may be involved in the progression of simple steatosis to non-alcoholic steatohepatitis (NASH). Several studies have reported that antioxidants can attenuate oxidative stress and hepatic steatosis; however, any improvement of liver fat deposition and damage caused by high-fat diet was observed after eriocitrin supplementation at the different doses used, a result which is similar to that found in our previous study (21). In the current study, we could not detect any change in blood serum total antioxidant capacity of mice fed high-fat diet, with or without eriocitrin supplementation, during the period of assessment. In contrast, lipid peroxidation in blood serum was significantly increased by the high-fat diet, and improved by eriocitrin supplementation at doses from 25 mg/kg to 100 mg/kg. The fact that the high-fat diet and eriocitrin did not change serum total antioxidant capacity, but influenced serum lipid peroxidation may indicate the action of endogenous response to oxidative stress caused by the high-fat diet. It is known that endogenous antioxidant molecules are stronger than exogenous antioxidants. Thus, we suggest that the oxidative stress caused by high-fat diet stimulated the endogenous antioxidant system and activated cellular antioxidant enzymes and molecules, which in turn counteracted the oxidative damage caused by high-

fat diet as well as overlapped eriocitrin antioxidant action, making its small improvements unnoticed during the period of assessment, even with positive effects at lipid peroxidation (37). In accordance with this hypothesis, it was shown that both treatment with eriodictyol or cisplatin (a nephrotoxic chemotherapeutic antitumor drug) alone significantly upregulated the expression of Nrf2 and heme oxygenase 1 (HO-1) enzyme in injured kidneys, while treatment with eriodictyol dose-dependently increased the cisplatin induced expression of both them. The protective effect of eriodictyol against cisplatin-induced kidney injury was demonstrated as being result of activation of Nrf2 and inhibition of NF-κB, which upregulated the activities of SOD, CAT and GSH-PX in kidneys and inhibited the production of oxidative and inflammatory molecules (20). In addition, the administration of eriocitrin to exercise-trained rats suppressed the formation of HEL, a lipid hydroperoxide modified lysine residue, suggesting that eriocitrin directly trapped the oxygen radicals generated by exercise and/or promoted the activation of endogenous defense mechanisms such as the induction of antioxidative enzymes (38).

Therefore, flavonoids may not be protective under particular conditions, but are suggested to provide protection from oxidative stress toxicity with long-term treatment through induction of Nrf2 and HO-1. The activation of Nrf2 induces phase-2 genes to protect cells from oxidative damage through binding to the antioxidant response element (ARE) and inducing phase-2 proteins, which allows the cell to mount a more prolonged and sustained defense that continue to function after the antioxidants are consumed (39,40). Eriocitrin and its aglycon eriodictyol can act direct as

antioxidants, neutralizing reactive oxygen species by donating hydrogen ions or by modulating cell-signaling pathways (17, 20). Curiously, the expression of HO-1 in adult retinal pigment epithelial (RPE) cells was markedly more robust after exposure to eriodictyol compared with quercetin and fisetin, while Nrf2 expression was similar among all three (40). The increase of the Nrf2 protein level in response to an eriodictyol glucoside was attributed mainly to the stabilization of Nrf2. One of the primary roles of intracellular redox-balancing proteins is to maintain cellular glutathione and, therefore, to reduce levels of reactive oxidant species. Treatment of human renal mesangial (HRMC) cells with eriodictyol glucoside showed a dose-dependent increase in glutathione levels, augmenting the cellular redox capacity, which is the major mechanism in counteracting oxidative stress induced injury (22). Eriocitrin oral administration was suggested to play an important role in the suppression of glutathione oxidation and maintenance of glutathione antioxidative system by suppressing the increase of GSSG and depletion of GSH in rat livers caused by acute exercise-induced oxidative stress (19). Therefore, exogenous compounds are essential for intact functioning of endogenous antioxidant defense system, and there is continuous demand for exogenous antioxidants to prevent oxidative stress in abnormal conditions (41).

In contrast to our findings, Hiramitsu and Cols. (42) observed amelioration of hepatic steatosis after oral administration of eriocitrin (32 mg/kg/day for 28 days) in zebrafish induced to obesity by high-fat diet. Eriocitrin suppressed the increase of serum triglycerides and decreased lipid

droplets in zebrafish liver. However, its effect was attributed to the elevation of mitochondrial size and mtDNA content, demonstrated by increased production of ATP. Eriocitrin was predicted to increase *ppara* and other β-oxidation enzyme genes, being indicated as the main anti-dyslipidaemic component of lemon polyphenols. Moreover, eriocitrin was shown to downregulate two lipid metabolism pathways, retinoid X receptor (RXR) and sterol regulatory element-binding transcription factor 1 (SREBF1) via PPARα activity (42). However, the lipid-lowering effect of eriocitrin was suggested to be limited to hyperlipidaemic conditions. Others citrus flavonoids also have been shown to improve lipid profiles in high-fat diet-induced obesity, with no difference in the treatment upon normal feeding (43). Therefore, the changes observed after high-fat diet feeding in the present study may be limited to the treatment time as well as to the period of results assessment, not allowing observation of the expected changes caused by high-fat diet induced obesity as well as a more evident protection of eriocitrin in the altered parameters.

Although substantial changes were not observed, the decreases of glucose, insulin, resistin and lipid peroxidation values reflect an effect of eriocitrin on improvement of glucose metabolism in obese mice. A previous study demonstrated that dietary eriodictyol attenuated the degree of retinal inflammation of diabetic rats with plasma lipid peroxidation by decreasing the retinal levels of inflammatory cytokines, adhesion molecule and nitric oxide synthase. However, a hypoglycemic effect was not observed, showing rather a protective effect mechanism of the molecule. In the referred study, effects

of eriocitrin were observed at doses of 10 and 100 mg/kg, however the data of dose 100 mg/kg was superimposable with 10 mg/kg (12).

It was suggested that lemon polyphenols may prevent or improve obesity and insulin resistance. Supplementation with lemon polyphenols modulated lipid metabolism and prevented metabolic syndrome in mice fed high-fat diet for 12 weeks. The mice body weight gain and body fat accumulation were suppressed through increased peroxisomal β-oxidation induced by upregulation of the mRNA level of acyl-CoA oxidase in liver and white adipose tissue, which was suggested to be mediated via upregulation of the mRNA level of PPARα (44). Furthermore, prevention of PPARγ expression in adipocytes has been reported to be associated with adipogenesis and activation of insulin-induced glucose transport. Eriodictyol, in particular, increased insulin-stimulated glucose uptake in human hepatocellular liver carcinoma cells (HepG2) and in differentiated 3T3-L1 adipocytes under high-glucose conditions. The ability of eriodictyol to stimulate insulin-induced glucose uptake was indicated as a result of increased Akt phosphorylation by activation of the PI3K/Akt pathway in HepG2 cells, and of adipogenesis promotion by increased expression of the mRNA of PPARγ and aP2, and of PPARγ2 protein levels in 3T3-L1 adipocytes, with pharmacological efficacy comparable to that of rosiglitazone (45). In addition, it was reported that eriocitrin inhibited dipeptidyl peptidase-4 (DPP-4) activity and prevented the metabolism of incretins (GLP-1 and GIP), being a natural gliptin-like alternative to improve beta cell health and suppress glucagon, preventing post-prandial and fasting hyperglycemia (46).

In the present study, the best eriocitrin collective effects were found with supplementation at the dose of 25 mg/kg bw, while different doses had effects on different parameters. It is known that isolated compounds can act as pro-oxidants at high doses, either by presenting direct pro-oxidant behavior or by interfering with the normal doses of reactive oxygen species that are required for normal cell functioning (47). The oral supplementation of hesperidin at two different concentrations (50 mg/kg and 200 mg/kg bw) for six weeks to irradiated mice has been reported. The lower dose of hesperidin (50 mg/kg bw) enhanced splenocyte proliferation on day 10 after irradiation, while lipid peroxidation levels on days 10 and 30 tended to decrease with increased hesperidin dose. In the *in vitro* experiment, low concentrations of hesperidin (5, 10, 25, or 50 lM) enhanced the proliferation of cultured splenocytes, but high concentrations (150 or 200 lM) suppressed this effect.[48] Based on a physiologically based kinetic (PBK) model, *in vivo* dose-response curves derived from *in vitro* concentration-response curves were predicted for hesperidin. It was concluded that the plasma maximum concentration of total hesperetin (aglycon and metabolites) at a Western dietary and a supplementary intake amounting to 2.8-8 and 7-29 mg/kg bw of hesperidin would be high enough to induce effects on endothelial cell migration and pro-inflammatory molecules *in vivo*. However, the plasma maximum concentration of total hesperetin upon these dietary or supplementary intakes would generally not be high enough to induce a similar effect on the inhibition of protein kinase A activity *in vivo (*49). Thus, different protective effects of natural compounds may be influenced by the

dose, and this may be why different responses were observed for the different doses of eriocitrin used in this study.

Additionally, the form of administration of natural compounds is as important as the dose administered. Protective effects were observed with intravenously and intraperitoneally administered eriodictyol, but not with intragastric administration. It was shown that intragastric administration of eriodictyol displayed lowering of parent (aglycon) and increasing metabolite plasma concentrations, and did not elicit protective effects against acetaminophen hepatotoxicity when compared with intraperitoneal injection.[50] Thus, the bioavailability of parent compound (eriodictyol) also needs to be considered for improvement of eriocitrin biological actions, and to its development as a nutraceutical compound. The present findings suggest that eriocitrin has a potential effect on the amelioration of obesity-induced insulin resistance, demonstrated by improved levels of glucose, insulin, resistin, lipid peroxidation (TBARS) and triglycerides in blood serum. Although the potency of eriocitrin effects was, likely, limited by the duration of the experiment and period of data assessment, these findings are good enough to pursue the development of eriocitrin as new anti-diabetic nutraceutical.

References

1. Eder K, Baffy N, Falus A, Fulop AK. The major inflammatory mediator interleukin-6 and obesity. Inflamm Res. 2009 Nov;58(11):727-36.

2. van Diepen JA, Berbée JF, Havekes LM, Rensen PC. Interactions between inflammation and lipid metabolism: relevance for efficacy of anti-inflammatory drugs in the treatment of atherosclerosis. Atherosclerosis. 2013 Jun;228(2):306-15.

3. Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB. Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders. Front Endocrinol (Lausanne). 2016 Apr 13;7:30.

4. Hsieh PS, Lu KC, Chiang CF, Chen CH. Suppressive effect of COX2 inhibitor on the progression of adipose inflammation in high-fat-induced obese rats. Eur J Clin Invest. 2010 Feb;40(2):164-71.

5. Matsuzawa-Nagata N, Takamura T, Ando H, Nakamura S, Kurita S, Misu H, Ota T, Yokoyama M, Honda M, Miyamoto K, Kaneko S. Increased oxidative stress precedes the onset of high-fat diet-induced insulin resistance and obesity. Metabolism. 2008 Aug;57(8):1071-7.

6. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003 Dec;112(12):1796-808.

7. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest. 2004 Dec;114(12):1752-61.

8. Mei M, Zhao L, Li Q, Chen Y, Huang A, Varghese Z, Moorhead JF, Zhang S, Powis SH, Li Q, Ruan XZ. Inflammatory stress exacerbates ectopic lipid deposition in C57BL/6J mice. Lipids Health Dis. 2011 Jun 30;10:110.

9. Lira FS, Rosa JC, Cunha CA, Ribeiro EB, do Nascimento CO, Oyama LM, Mota JF. Supplementing alpha-tocopherol (vitamin E) and vitamin D3 in high fat diet decrease IL-6 production in murine epididymal adipose tissue and 3T3-L1 adipocytes following LPS stimulation. Lipids Health Dis. 2011 Feb 27;10:37.

10. Bendinelli B, Masala G, Saieva C, Salvini S, Calonico C, Sacerdote C, Agnoli C, Grioni S, Frasca G, Mattiello A, Chiodini P, Tumino R, Vineis P, Palli D, Panico S. Fruit, vegetables, and olive oil and risk of coronary heart disease in Italian women: the EPICOR Study. Am J Clin Nutr. 2011 Feb;93(2):275-83.

11. Assini JM, Mulvihill EE, Huff MW. Citrus flavonoids and lipid metabolism. Curr Opin Lipidol. 2013 Feb;24(1):34-40.

12. Bucolo C, Leggio GM, Drago F, Salomone S. Eriodictyol prevents early retinal and plasma abnormalities in streptozotocin-induced diabetic rats. Biochem Pharmacol. 2012 Jul 1;84(1):88-92.

13. Xi W, Lu J, Qun J, Jiao B. Characterization of phenolic profile and antioxidant capacity of different fruit part from lemon (Citrus limon Burm.) cultivars. J Food Sci Technol. 2017 Apr; 54(5):1108-1118.

14. Tripoli E, Guardia M, Giammanco S, Majo DD, Giammanco M. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review, Food Chemistry, 2007,104:466-479.

15. Miyake Y, Shimoi K, Kumazawa S, Yamamoto K, Kinae N, Osawa T. Identification and antioxidant activity of flavonoid metabolites in plasma and urine of eriocitrin-treated rats. J Agric Food Chem. 2000 Aug;48(8):3217-24.
16. Nielsen IL, Chee WS, Poulsen L, Offord-Cavin E, Rasmussen SE, Frederiksen H, Enslen M, Barron D, Horcajada MN, Williamson G. Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. J Nutr. 2006 Feb;136(2):404-8.
17. Miyake Y, Mochizuki M, Okada M, Hiramitsu M, Morimitsu Y, Osawa T. Isolation of antioxidative phenolic glucosides from lemon juice and their suppressive effect on the expression of blood adhesion molecules. Biosci Biotechnol Biochem. 2007 Aug;71(8):1911-9.
18. Miyake Y, Yamamoto K, Morimitsu Y, Osawa T. Characteristics of antioxidative flavonoid glycosides in lemon fruit. Food Sci Tech Intern, 1998,4:48–53.
19. Minato K, Miyake Y, Fukumoto S, Yamamoto K, Kato Y, Shimomura Y, Osawa T. Lemon flavonoid, eriocitrin, suppresses exercise-induced oxidative damage in rat liver. Life Sci. 2003 Feb 21;72(14):1609-16.
20. Li CZ, Jin HH, Sun HX, Zhang ZZ, Zheng JX, Li SH, Han SH. Eriodictyol attenuates cisplatin-induced kidney injury by inhibiting oxidative stress and inflammation. Eur J Pharmacol. 2016 Feb 5;772:124-30.
21. Ferreira PS, Spolidorio LC, Manthey JA, Cesar TB. Citrus flavanones prevent systemic inflammation and ameliorate oxidative stress in C57BL/6J mice fed high-fat diet. Food Funct. 2016 Jun 15;7(6):2675-81.

22. Hu Q, Zhang DD, Wang L, Lou H, Ren D. Eriodictyol-7-O-glucoside, a novel Nrf2 activator, confers protection against cisplatin-induced toxicity. Food Chem Toxicol. 2012 Jun;50(6):1927-32.
23. Jing X, Ren D, Wei X, Shi H, Zhang X, Perez RG, Lou H, Lou H. Eriodictyol-7-O-glucoside activates Nrf2 and protects against cerebral ischemic injury. Toxicol Appl Pharmacol. 2013 Oct 26. pii: S0041-008X(13)00466-3.
24. Lee ER, Kim JH, Kang YJ, Cho SG. The anti-apoptotic and anti-oxidant effect of eriodictyol on UV-induced apoptosis in keratinocytes. Biol Pharm Bull. 2007 Jan;30(1):32-7.
25. de Oliveira DM, Dourado GK, Cesar TB. Hesperidin associated with continuous and interval swimming improved biochemical and oxidative biomarkers in rats. J Int Soc Sports Nutr. 2013 May 24;10:27.
26. Kumar B, Gupta SK, Srinivasan BP, Nag TC, Srivastava S, Saxena R, Jha KA. Hesperetin rescues retinal oxidative stress, neuroinflammation and apoptosis in diabetic rats. Microvasc Res. 2013 May;87:65-74.
27. Mahmoud AM, Ashour MB, Abdel-Moneim A, Ahmed OM. Hesperidin and naringin attenuate hyperglycemia-mediated oxidative stress and proinflammatory cytokine production in high fat fed/streptozotocin-induced type 2 diabetic rats. J Diabetes Complications. 2012 Nov-Dec;26(6):483-90.
28. Lee YR, Jung JH, Kim HS. Hesperidin partially restores impaired immune and nutritional function in irradiated mice. J Med Food. 2011 May;14(5):475-82.

29. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr. 1997;5:838-41.

30. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957 May;226(1):497-509.

31. Haffner SM, Greenberg AS, Weston WM, Chen H, Williams K, Freed MI. Effect of rosiglitazone treatment on nontraditional markers of cardiovascular disease in patients with type 2 diabetes mellitus. Circulation. 2002 Aug 6;106(6):679-84.

32. Kohn HI, Liversedge M. On a new aerobic metabolite whose production by brain is inhibited by apomorphine, emetine, ergotamine, epinephrine, and menadione, Journal of Pharmacology and Experimental Therapeutics, 1944,82:292-300.

33. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999 May;26(9-10):1231-7.

34. Chin-Chance C, Polonsky KS, Schoeller DA (2000) Twenty-four-hour leptin levels respond to cumulative short-term energy imbalance and predict subsequent intake. J Clin Endocrinol Metab 85: 2685–2691.

35. Williams LM, Campbell FM, Drew JE, Koch C, Hoggard N, Rees WD, Kamolrat T, Thi Ngo H, Steffensen IL, Gray SR, Tups A. The development of diet-induced obesity and glucose intolerance in C57BL/6 mice on a high-fat diet consists of distinct phases. PLoS One. 2014 Aug 29;9(8):e106159.

36. de Simone G, Wang W, Best LG, Yeh F, Izzo R, Mancusi C, Roman MJ, Lee ET, Howard BV, Devereux RB. Target organ damage and incident type 2 diabetes mellitus: the Strong Heart Study. Cardiovasc Diabetol. 2017 May 12;16(1):64.

37. Taha R, Blaise G. Nrf2 activation as a future target of therapy for chronic diseases, Functional Foods in Health and Disease, 2014,4(11):510-523.

38. Kato Y, Miyake Y, Yamamoto K, Shimomura Y, Ochi H, Mori Y, Osawa T. Preparation of a monoclonal antibody to N(epsilon)-(Hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against
exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun. 2000 Aug 2;274(2):389-93.

39. Prestera T, Holtzclaw WD, Zhang Y, Talalay P. Chemical and molecular regulation of enzymes that detoxify carcinogens. Proc Natl Acad Sci USA. 1993;90:2965–2069.

40. Hanneken A, Lin FF, Johnson J, Maher P. Flavonoids protect human retinal pigment epithelial cells from oxidative-stress-induced death. Invest Ophthalmol Vis Sci. 2006 Jul;47(7):3164-77.

41. Bouayed J, Bohn T. Exogenous antioxidants--Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses. Oxid Med Cell Longev. 2010 Jul-Aug;3(4):228-37.

42. Hiramitsu M, Shimada Y, Kuroyanagi J, Inoue T, Katagiri T, Zang L, Nishimura Y, Nishimura N, Tanaka T. Eriocitrin ameliorates diet-induced

hepatic steatosis with activation of mitochondrial biogenesis. Sci Rep. 2014 Jan 15;4:3708.

43. Alam, M. A. et al. Naringin improves diet-induced cardiovascular dysfunction and obesity in high carbohydrate, high fat diet-fed rats. Nutrients, 2013,5, 637–650.

44. Fukuchi Y, Hiramitsu M, Okada M, Hayashi S, Nabeno Y, Osawa, T, Naito M. Lemon Polyphenols Suppress Diet-induced Obesity by Up-Regulation of mRNA Levels of the Enzymes Involved in β-Oxidation in Mouse White Adipose Tissue. J Clin Biochem Nutr. 2008 Nov; 43(3): 201–209.

45. Zhang WY, Lee JJ, Kim Y, Kim IS, Han JH, Lee SG, Ahn MJ, Jung SH, Myung CS. Effect of eriodictyol on glucose uptake and insulin resistance in vitro. J Agric Food Chem. 2012 Aug 8;60(31):7652-8.

46. Gupta A, Jacobson GA, Burgess JR, Jelinek HF, Nichols DS, Narkowicz CK, Al-Aubaidy HA. Citrus bioflavonoids possess dipeptidyl peptidase-4 inhibition activity similar to gliptin antidiabetic medication. Biochem Biophys Res Commun. 2018 p: S0006-291X(18)30937-9.

47. Koçyiğit A, Selek S. Exogenous Antioxidants are Double-edged Swords. Bezmialem Science 2016;2:70-5.

48. Lee YR, Jung JH, Kim HS. Hesperidin partially restores impaired immune and nutritional function in irradiated mice. J Med Food. 2011 May;14(5):475-82.

49. Boonpawa R, Spenkelink A, Punt A, Rietjens IMCM. Physiologically based kinetic modeling of hesperidin metabolism and its use to predict in vivo effective doses in humans. Mol Nutr Food Res. 2017 Aug;61(8).
50. Wang Z, Lan Y, Chen M, Wen C, Hu Y, Liu Z, Ye L. Eriodictyol, Not Its Glucuronide Metabolites, Attenuates Acetaminophen-Induced Hepatotoxicity. Mol Pharm. 2017 Sep 5;14(9):2937-2951.

Considerações Finais

Os resultados do presente estudo reforçam o potencial deletério do consumo de uma dieta rica em gordura e da obesidade. Evidenciado pela desregulação do metabolismo lipídico (aumento significativo dos níveis séricos de triglicerídeos, colesterol total e LDL-C) e da glicose (aumento dos níveis de glicose, insulina, resistina e leptina no soro), além do acúmulo de gordura hepática, como mostrado pelas análises histológicas e bioquímicas de colesterol e triglicerídeos no fígado. Por sua vez, de forma dose-dependente, a suplementação com eriocitrina reduziu significativamente a trigliceridemia e melhorou os níveis de glicemia, insulinemia, resistina e TBARS no soro sanguíneo dos camundongos.

Embora tenham sido observados efeitos regulatórios da eriocitrina em todas as doses testadas, os resultados deste estudo mostram uma melhor resposta metabólica, anti-inflamatória e antioxidante com a dose de 25 mg/kg de eriocitrina, sugerindo um potencial uso da eriocitrina contra a resistência à insulina e prevenção de danos metabólicos causados pela obesidade e consumo de dietas ricas em gorduras. É necessário, portanto, avaliar a atividade da eriocitrina na referida dose em um maior período de tempo a fim de se observar efeitos mais significativos, já que no período estudado não foi possível observar todas as mudanças esperadas pela suplementação de eriocitrina.

O tempo escolhido no presente estudo, assim como o momento da suplementação (em camundongos já obesos) podem ter prejudicado a observação dos efeitos esperados. Em estudo anterior, onde foi avaliado o

efeito da eriocitrina na prevenção de danos causados pelo consumo da dieta hiperlipídica durante o curso de indução da obesidade, a eriocitrina inibiu a inflamação sistêmica e reduziu a dislipidemia e esteatose hepática de camundongos alimentados com dieta hiperlipídica. Além disso, a utilização do composto na forma não glicosilada (eriodictiol) deve ser considerada, uma vez que a baixa biodisponibilidade da eriocitrina, observada no estudo farmacocinético, pode ter reduzido seus os efeitos biológicos da eriocitrina no presente estudo.

É importante ressaltar que a alta concentração dos metabólitos de eriocitrina encontrados no pâncreas indica uma ação não só sistêmica, mas local nesse órgão, sugerindo um efeito protetor direto na função pancreática, que deve ser considerado em estudos futuros. Desta forma, os resultados deste estudo apontam para a necessidade de investigação das atividades biológicas dos metabólitos de eriocitrina em órgãos diretamente relacionados ao metabolismo de nutrientes, como o fígado e pâncreas, a fim de otimizar seu uso como composto coadjuvante no tratamento de distúrbios metabólicos relacionados à obesidade.

Referências

1. Lira FS, Rosa JC, Cunha CA, Ribeiro EB, do Nascimento CO, Oyama LM, Mota JF.Supplementing alpha-tocopherol (vitamin E) and vitamin D3 in high fat diet decrease IL-6 production in murine epididymal adipose tissue and 3T3-L1 adipocytes following LPS stimulation. Lipids Health Dis. 2011 Feb 27;10:37.

2. WHO. Global Health Observatory (GHO) – Obesity: Situation and trends. [cited 2018 may 01] Available from: http://www.who.int/gho/ncd/risk_factors/obesity_text/en/

3. Unger RH, Scherer PE. Gluttony, sloth and the metabolic syndrome: a roadmap to lipotoxicity. Trends Endocrinol Metab. 2010 Jun;21(6):345-52.

4. Schilling JD, Machkovech HM, Kim AH, Schwendener R, Schaffer JE. Macrophages modulate cardiac function in lipotoxic cardiomyopathy. Am J Physiol Heart Circ Physiol. 2012 Dec 1;303(11):H1366-73.

5. Dhindsa S, Tripathy D, Mohanty P, Ghanim H, Syed T, Aljada A, Dandona P. Differential effects of glucose and alcohol on reactive oxygen species generation and intranuclear nuclear factor-kappaB in mononuclear cells. Metabolism. 2004 Mar;53(3):330-4.

6. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. Gastroenterology. 2007 May;132(6):2169-80.

7. Lee JY, Sohn KH, Rhee SH, Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J Biol Chem. 2001 May 18;276(20):16683-9.

8. van Diepen JA, Berbée JF, Havekes LM, Rensen PC. Interactions between inflammation and lipid metabolism: relevance for efficacy of anti-inflammatory drugs in the treatment of atherosclerosis. Atherosclerosis. 2013 Jun;228(2):306-15.

9. Oh DY, Lagakos WS. The role of G-protein-coupled receptors in mediating the effect of fatty acids on inflammation and insulin sensitivity. Curr Opin Clin Nutr Metab Care. 2011 Jul;14(4):322-7.

10. Mei M, Zhao L, Li Q, Chen Y, Huang A, Varghese Z, Moorhead JF, Zhang S, Powis SH, Li Q, Ruan XZ. Inflammatory stress exacerbates ectopic lipid deposition in C57BL/6J mice. Lipids Health Dis. 2011 Jun 30;10:110.

11. Lee H, Lee YJ, Choi H, Ko EH, Kim JW. Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. J Biol Chem. 2009 Apr 17;284(16):10601-9.

12. Sparks JD, Cianci J, Jokinen J, Chen LS, Sparks CE. Interleukin-6 mediates hepatic hypersecretion of apolipoprotein B. Am J Physiol Gastrointest Liver Physiol. 2010;299:980–9.

13. Murdolo G, Piroddi M, Luchetti F, Tortoioli C, Canonico B, Zerbinati C, Galli F, Iuliano L. Oxidative stress and lipid peroxidation by-products at the crossroad between adipose organ dysregulation and obesity-linked insulin resistance. Biochimie. 2013 Mar;95(3):585-94.

14. Mali VR, Ning R, Chen J, Yang XP, Xu J, Palaniyandi SS. Impairment of aldehyde dehydrogenase-2 by 4-hydroxy-2-nonenal adduct formation and

cardiomyocyte hypertrophy in mice fed a high-fat diet and injected with low-dose streptozotocin. Exp Biol Med (Maywood). 2014 May;239(5):610-8.
15. Matsuzawa-Nagata N, Takamura T, Ando H, Nakamura S, Kurita S, Misu H, Ota T, Yokoyama M, Honda M, Miyamoto K, Kaneko S. Increased oxidative stress precedes the onset of high-fat diet-induced insulin resistance and obesity. Metabolism. 2008 Aug;57(8):1071-7.
16. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003 Dec;112(12):1796-808.
17. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest. 2004 Dec;114(12):1752-61.
18. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest. 2006 Jun;116(6):1494-505.
19. Tarantino G, Pizza G, Colao A, Pasanisi F, Conca P, Colicchio P, Finelli C, Contaldo F, Di Somma C, Savastano S. Hepatic steatosis in overweight/obese females: new screening method for those at risk. World J Gastroenterol. 2009 Dec 7;15(45):5693-9.
20. Mulvihill EE, Huff MW. Protection from Metabolic Dysregulation, Obesity, and Atherosclerosis by Citrus Flavonoids: Activation of Hepatic PGC1α-Mediated Fatty Acid Oxidation. PPAR Res. 2012;2012:857142.

21. O'Neil CE, Nicklas TA, Rampersaud GC, Fulgoni VL 3rd. 100% orange juice consumption is associated with better diet quality, improved nutrient adequacy, decreased risk for obesity, and improved biomarkers of health in adults: National Health and Nutrition Examination Survey, 2003-2006. Nutr J. 2012 Dec 12;11:107.

22. Miyake Y, Yamamoto K, Morimitsu Y, Osawa T. Isolation of C-glucosyl flavone from lemon peel and antioxidative activity of flavonoid compounds in lemon fruit. J. Agric. Food Chem. 1997;45(12),4619-4623.

23. Assini JM, Mulvihill EE, Huff MW. Citrus flavonoids and lipid metabolism. Curr Opin Lipidol. 2013 Feb;24(1):34-40.

24. Bucolo C, Leggio GM, Drago F, Salomone S. Eriodictyol prevents early retinal and plasma abnormalities in streptozotocin induced diabetic rats. Biochemical Pharmacol. 2012; 84:88–92.

25. Miyake Y, Mochizuki M, Okada M, Hiramitsu M, Morimitsu Y, Osawa T. Isolation of antioxidative phenolic glucosides from lemon juice and their suppressive effect on the expression of blood adhesion molecules. Biosci Biotechnol Biochem. 2007 Aug;71(8):1911-9.

26. Miyake Y, Yamamoto K, Morimitsu Y, Osawa T. Characteristics of antioxidative flavonoid glycosides in lemon fruit. Food Sci Technol Int. 1998;4:48–53.

27. Minato K, Miyake Y, Fukumoto S, Yamamoto K, Kato Y, Shimomura Y, Osawa T. Lemon flavonoid, eriocitrin, suppresses exercise-induced oxidative damage in rat liver. Life Sci. 2003 Feb 21;72(14):1609-16.

28. Hiramitsu M, Shimada Y, Kuroyanagi J, Inoue T, Katagiri T, Zang L, Nishimura Y, Nishimura N, Tanaka T. Eriocitrin ameliorates diet-induced hepatic steatosis with activation of mitochondrial biogenesis. Sci Rep. 2014 Jan 15;4:3708.

29. Zhang WY, Lee JJ, Kim Y, Kim IS, Han JH, Lee SG, Ahn MJ, Jung SH, Myung CS. Effect of eriodictyol on glucose uptake and insulin resistance in vitro. J Agric Food Chem. 2012 Aug 8;60(31):7652-8.

30. Huang T, Tseng KY, Tsai S, Liu H, Ho C; Lin HY, Cheng LT, Chuang KP. Eriodictyol decreases very late antigen-4 (VLA-4) expression, cellular adhesion, and migration through an NFκB-dependent pathway in monocytes. J Funct Foods 2010 Oct;2(4):263-70.

31. Miyake Y, Shimoi K, Kumazawa S, Yamamoto K, Kinae N, Osawa T. Identification and antioxidant activity of flavonoid metabolites in plasma and urine of eriocitrin-treated rats. J Agric Food Chem. 2000 Aug;48(8):3217-24.

32. Hu Q, Zhang DD, Wang L, Lou H, Ren D. Eriodictyol-7-O-glucoside, a novel Nrf2 activator, confers protection against cisplatin-induced toxicity. Food Chem Toxicol. 2012 Jun;50(6):1927-32.

33. Johnson J, Maher P, Hanneken A. The flavonoid, eriodictyol, induces long-term protection in ARPE-19 cells through its effects on Nrf2 activation and phase 2 gene expression. Invest Ophthalmol Vis Sci. 2009 May;50(5):2398-406.

34. Jing X, Ren D, Wei X, Shi H, Zhang X, Perez RG, Lou H, Lou H. Eriodictyol-7-O-glucoside activates Nrf2 and protects against cerebral ischemic injury. Toxicol Appl Pharmacol. 2013 Dec 15;273(3):672-9.

35. Wang X, Wang Z, Liu JZ, Hu JX, Chen HL, Li WL, Hai CX. Double antioxidant activities of rosiglitazone against high glucose-induced oxidative stress in hepatocyte. Toxicol In Vitro. 2011 Jun;25(4):839-47.
36. Lee ER, Kim JH, Kang YJ, Cho SG. The anti-apoptotic and anti-oxidant effect of eriodictyol on UV-induced apoptosis in keratinocytes. Biol Pharm Bull. 2007 Jan;30(1):32-7.
37. Miyake Y, Sakurai C, Usuda M, Fukumoto S, Hiramitsu M, Sakaida K, Osawa T, Kondo K. Difference in plasma metabolite concentration after ingestion of lemon flavonoids and their aglycones in humans. J Nutr Sci Vitaminol (Tokyo). 2006 Feb;52(1):54-60.
38. Nielsen IL, Chee WS, Poulsen L, Offord-Cavin E, Rasmussen SE, Frederiksen H, Enslen M, Barron D, Horcajada MN, Williamson G. Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. J Nutr. 2006 Feb;136(2):404-8.
39. Yáñez JA, Remsberg CM, Miranda ND, Vega-Villa KR, Andrews PK, Davies NM. Pharmacokinetics of selected chiral flavonoids: hesperetin, naringenin and eriodictyol in rats and their content in fruit juices. Biopharm Drug Dispos. 2008 Mar;29(2):63-82.