Universidade Estadual Paulista
"Júlio de Mesquita Filho"

Faculdade de Ciências Farmacêuticas

Capacidade antioxidante de polimetoxiflavonas e identificação de seus metabólitos em ratos

Marilia Caroline Martini Rodrigues

Dissertação apresentada ao Programa de Pós-Graduação em Alimentos e Nutrição para obtenção do título de Mestre em Alimentos e Nutrição.

Área de concentração: Ciências Nutricionais.
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MARILIA CAROLINE MARTINI RODRIGUES

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Dissertação de Mestrado apresentada à Faculdade de Ciências Farmacêuticas da Universidade Estadual Paulista – UNESP, Campus de Araraquara como requisito para obtenção do título de Mestra em Alimentos e Nutrição.

Araraquara, 28 de novembro de 2016.

BANCA EXAMINADORA

THAIS BORGES CESAR

ALCEU AFONSO JORDÃO JÚNIOR

AMANDA MARTINS BAVIERA

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RESUMO

Objetivo: Verificar a capacidade antioxidante das polimetoxiflavonas tangeritina (TAN), nobelitina (NOB) no soro sanguíneo e fígado. **Métodos**: Grupos de ratos foram tratados com 200 mg/kg pc/dia de TAN ou NOB, ou placebo, por 15 dias. Compostos parentais e seus metabólitos foram avaliados no fígado por análise cromatográfica, além de α-tocoferol e retinol no soro sanguíneo. Resultados: Ambos os suplementos TAN e NOB foram capazes de reduzir o malonaldeído (MDA) no sangue do rato em 22% e 18%, respectivamente, mas apenas o NOB aumentou a reação redox em 3%. os níveis sanguíneos de retinol e α-tocoferol aumentaram sob TAN em 59% e 20%, respectivamente, mas não foram afetados por NOB. Oito metabólitos NOB foram detectados no fígado, mas apenas dois metabólitos TAN foram identificados em baixa concentração. **Conclusão**: o grupo que recebeu NOB melhorou a capacidade antioxidante e reduziu a peroxidação lipídica, enquanto o aumento dos níveis de retinol e α-tocoferol após a suplementação de TAN pode ter contribuído para a diminuição da peroxidação lipídica no sangue.

Palavras-chave: nobelitina, tangeretina, metabólitos, atividade antioxidante, retinol, alfa-tocoferol

ABSTRACT

Objective: The effects of tangeretin (TAN) and nobiletin (NOB) on antioxidant activity in the blood and liver of rats were evaluated. **Methods**: Groups of rats were treated with 200 mg/kg bw/day of TAN or NOB, or placebo, for 15 days. Parental compounds and their metabolites were assessed in the liver by chromatographic analysis, in addition to α-tocopherol and retinol in the blood serum. **Results**: Both TAN and NOB supplements were able to reduce malonaldehyde (MDA) in the rat's blood by 22% and 18%, respectively, but only NOB increased redox reaction by 3%. Blood levels of retinol and α-tocopherol increased under TAN by 59% and 20%, respectively, but were not affected by NOB. Eight NOB metabolites were detected in the liver, but only two TAN metabolites were identified in low concentration. **Conclusion**: NOB improved antioxidant capacity and reduced lipid peroxidation, while increased levels of retinol and α-tocopherol after TAN supplement may have contributed to decreased blood lipid peroxidation.

Keywords: nobiletin, tangeretin, metabolites, antioxidant activity, retinol, alpha-tocopherol

Sumário

INTRODUÇÃO

As frutas cítricas são fontes ricas de fitoquímicos, tais como flavonoides, carotenoides, vitaminas A, C e E, elementos minerais, entre outros compostos (ZHOU, 2012), os quais possibilitam a essas frutas atividades biológicas importantes como antioxidantes, anti-inflamatórias, hipolipidêmicas, antineoplásicas, neuroprotetoras. Essas atividades estão intimamente ligadas à produção de radicais livres no organismo.

Radicais Livres e Antioxidantes

Os radicais livres (RL) são espécies cujas reatividades resultam da presença de um ou mais elétrons desemparelhados na estrutura atômica (HALLIWELL, 1997) e que são geradas continuamente durante os processos metabólicos. A alta reatividade desses compostos os possibilita reagir com biomoléculas tais como carboidratos, lipídios, proteínas e ácidos nucleicos (ALVES *et al.,* 2010; FERREIRA; MATSUBARA, 1997). Existem espécies radicalares e não radicalares. Os radicais livres nos quais os elétrons desemparelhados encontram-se centrados em átomos de oxigênio ou de nitrogênio são denominados espécies reativas de oxigênio (ERO) ou espécies reativas de nitrogênio (ERN). Entre as principais ERO radicalares, destacam-se o ânion superóxido (O_2•-), radical hidroxila (HO•), peroxila (ROO•), alcoxila (RO•); e para as não radicalares destacam-se o peróxido de hidrogênio (H_2O_2), ácido hipocloroso/hipoclorito (HOCl/OCl-) e o oxigênio

singlete ($^1O_2$). O óxido nítrico (NO•) e o peroxinitrito (ONOO-) constituem as principais ERN.

A geração de radicais livres constitui um processo contínuo e fisiológico, cumprindo funções biológicas relevantes, tais como produção de energia, fagocitose, regulação do crescimento celular e síntese de biomoléculas importantes (BARREIROS; DAVID; DAVID, 2006).

Durante os processos metabólicos, esses radicais atuam como mediadores para a transferência de elétrons nas várias reações bioquímicas, porém em condições nas quais ocorre produção excessiva de RL, o equilíbrio redox é alterado, ocasionando desequilíbrio entre os sistemas pró e antioxidantes, e por consequência, danos oxidativos, que culminam em estresse oxidativo (VASCONSELOS *et al.*,2007; BARREIROS; DAVID; DAVID, 2006; FINKEL; HOLBROOK, 2000). Esse desequilíbrio redox apresenta efeitos prejudiciais à saúde, principalmente eventos intracelulares importantes para o desenvolvimento e complicação de várias doenças, como por exemplo, aterosclerose, diabetes mellitus, inflamações, cardiopatias, doenças neurodegenerativas (doença de Alzheimer, esclerose múltipla, mal de Parkinson), câncer, entre outras (PADURAIU *et al.,* 2013; HALLIWELL; GUTTERIDGE, 1995; NIKI, 2010; DURACKOVÁ, 2010; RODRIGUES, 2007; VASCONCELOS *et al.*, 2007; VALKO *et al.*, 2006; FINKEL; HOLBROOK, 2000).

O excesso de radicais livres no organismo é neutralizado por antioxidantes. Eles são capazes de interceptar os radicais livres gerados pelo metabolismo celular ou por fontes exógenas, impedindo o ataque sobre

os lipídeos, os aminoácidos das proteínas, a dupla ligação dos ácidos graxos poliinsaturados e as bases do DNA, evitando a formação de lesões e perda da integridade celular (BIACHI; ANTUNES, 1999).

Os antioxidantes são divididos em endógenos (produzidos pelo corpo), como a glutationa peroxidase (GPx), a superóxido dismutase (SOD) e a catalase (CAT), ou exógenos (absorvidos através de dieta), como o *α*-tocoferol (vitamina-E), β-caroteno (pro-vitamina-A), retinol (vitamina A), ácido ascórbico (vitamina C), e compostos fenólicos, nos quais se destacam os flavonoides (HALLIWELL *et al*., 1995; PIETTA, 2000; BARREIROS; DAVID; DAVID, 2006; BARREIROS *et al*., 2006; OLIVEIRA *et al.*, 2009; KAJARIA *et al.*, 2012; PADURAIU *et al.,* 2013).

A atividade antioxidante se refere à capacidade de um composto bioativo em manter a estrutura e a função celular eliminando eficazmente os radicais livres, inibindo reações de peroxidação lipídica e impedindo outros danos oxidativos (BRAVO*,* 1998). Essa ação inclui a regulação de enzimas antioxidantes e detoxificantes, a modulação da sinalização celular redox e a expressão de genes (LÓPEZ-ALARCÓNA; DENICOLA, 2013).

Antioxidantes Endógenos e Exógenos

Vitamina E

Um potente antioxidante exógeno é a vitamina E, representada pelo alfa-tocoferol, protege especialmente os ácidos graxos poli-insaturados (PUFAs) de membranas biológicas e as lipoproteínas da oxidação em hidroperóxidos (BRAMLEY *et al*., 2000). Em humanos as concentrações mais elevadas de

α-tocoferol encontram-se principalmente nas lipoproteínas HDL e LDL (DEBIER; LARONDELLE, 2005).

A vitamina E restringe os radicais livres, extingue o oxigênio singleto ($^1O_2$), reduzi o ferro ferroso (Fe^{2+}) ao ferro férrico (Fe^{3+}) para minimizar a catálise, efeito sinérgico com selênio para proteger as mitocôndrias contra danos causados por radicais livres e suas membranas contra danos à peroxidação, impedem a oxidação do carotenoide, aumentando a sua capacidade antioxidante (LEVANDER; AGER; BECK, 1995; AMITAVA; KIMBERLY, 2014).

Por impedir danos aos tecidos mediados por radicais livres, acredita-se que a vitamina E desempenhe um papel fundamental em retardar a patogênese de várias doenças degenerativas, como câncer, aterosclerose, doenças cardiovasculares, doenças das vias aéreas, Alzheimer, entre outras doenças neurológicas (DUTTA; DUTTA, 2003; TRABER, 2007; FARINA; ISAAC; LI *et al.,* 2015; MILLER; TURNER; CORNISH, 2015). No fígado, o alfa-tocoferol se liga à proteína de transporte do alfa-tocoferol (α-TTP), que o libera para ser transportada pela VLDL na corrente sanguínea, podendo ir para outros tecidos ou retornar ao fígado (AZZI; STOCKER, 2000; TRABER, 2007; BORTOLI; COZZOLINO, 2012).

Vitamina A e precursores

Outros antioxidantes exógenos importantes são os carotenoides que agem como desativadores do oxigênio singleto ($^1O_2$) ou como sequestradores dos radicais peroxila (LOO•), reduzindo a oxidação do DNA e lipídios, que está associada a doenças degenerativas, como câncer e

doenças cardíacas. carotenoides apolares, como o β-caroteno, são regeneradores, combatendo os radicais formados com mais eficiência no interior da membrana (WOODALL, *et al.,* 1997). Os retinóides (vitamina A) possuem grupos polares que os localizam na membrana celular na região próxima à fase aquosa, entretanto, apresentam atividade antioxidante cerca de cinco vezes menor que a do β-caroteno.

Glutationa Peroxidase

Há ainda as enzimas antioxidantes endógenas, nas quais uma de suas principais representantes é a glutationa peroxidase (GPx), uma família de múltiplas isoenzimas, encontrada no citosol e nas mitocôndrias, que catalisa a redução de H_2O_2 ou hidroperóxidos orgânicos em água ou álcoois correspondentes usando a glutationa reduzida como doador de elétron. Ela é responsável pela detoxificação de peróxidos orgânicos e inorgânicos, sendo sua ação dependente da glutationa reduzida (GSH), que é oxidada em glutationa oxidada (GSSG). Os níveis de GSH são mantidos por meio da oxidação do NADPH resultante do ciclo das pentoses (BALLATORI, 2009).

Além dos antioxidantes endógenos, como já ditos anteriormente, existem, os antioxidantes exógenos, adquiridos através da alimentação, como os flavonoides.

Flavonoides

Os flavonoides são o grupo mais comum e amplamente distribuído de polifenois de plantas e mais de 5000 diferentes já foram descritos. (HARBONE, 1993; YANG *et al.,* 2001; MARCHAND, 2002; MANACH *et*

*al.,*2004; GONÇALVES, 2008). Eles são formados nas plantas a partir dos aminoácidos aromáticos fenilalanina e tirosina, e malonato de dietila (HARBORNE, 1986). Eles possuem uma estrutura comum consistindo em 2 anéis aromáticos (A e B) ligados por 3 átomos de carbono, formando heterociclo oxigenado (anel C) (MANACH *et al.,*2004). Segundo Lima (2008), os flavonoides estão envolvidos em várias funções na planta: propriedades sensoriais (cor, aroma, sabor e adstringência), crescimento, processo germinativo da semente, defesa contra pragas, entre outros. Em animais e humanos, observa-se que são capazes de reagir com radicais livres, neutralizando-os, o que os caracteriza como potentes agentes antioxidantes.

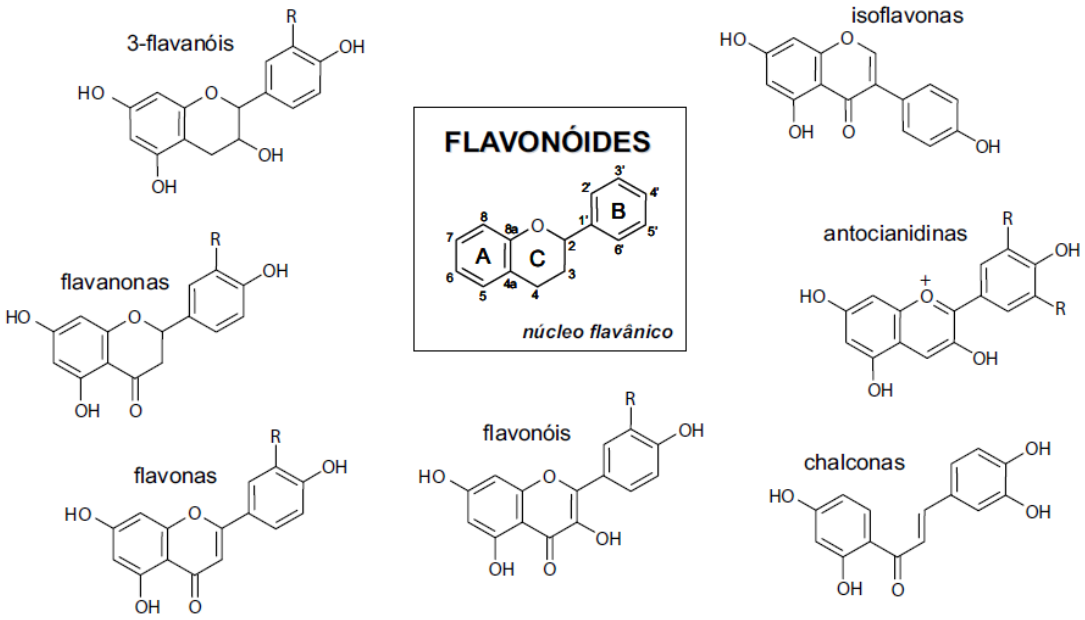

Figura 1. Estrutura molecular dos flavonoides (Fonte Mateus, 2009)

Os flavonoides têm um papel direto na remoção de espécies reativas de oxigênio (ROS), o que pode neutralizar a oxidação lipídica *in vitro* e melhorar a atividade enzimática dos antioxidantes endógenos e diminuir a formação de peróxidos *in vivo* (NAKAO *et al.*, 2011).

A atividade antioxidante dos ácidos fenólicos está vinculada, principalmente, às propriedades redutoras de sua estrutura, as quais atuam tanto na captura de espécies reativas, quanto na inativação de metais de transição, participando assim, das etapas de iniciação e propagação do processo oxidativo (SOUZA *et al.*, 2007). A ressonância do anel aromático que compõe a estrutura dos compostos fenólicos é responsável pela estabilidade dos produtos intermediários formados durante a interação destes compostos com as espécies reativas (RAMALHO; JORGE, 2006). A atividade de eliminação de RL parece diretamente relacionada com o número de grupos hidroxilos substituídos no anel B, especialmente em C-3'. Quando o número de grupos hidroxilo diminui, a capacidade de extinção de OH cai rapidamente (HUSAIN *et al.,* 1987).

Existem, na literatura, controvérsias a respeito do mecanismo de ação dos flavonoides. Eles atuam como antioxidantes na inativação dos RL tanto nos compartimentos celulares lipofílicos quanto hidrofílicos, doando átomos de hidrogênio e inibindo reações em cadeia provocadas pelos RL (HARTMAN; SHANKEL, 1990; ARORA *et al.,* 1998). Assim, a ação antioxidante eficaz dos flavonoides depende principalmente de três fatores: o potencial quelante de metal, que é fortemente dependente de grupos hidroxilas e carbonilas em torno da molécula; a presença de substituintes doadores de hidrogênio/elétrons, capazes de reduzir os radicais livres; e a capacidade do flavonoide de deslocar o elétron não emparelhado, para a formação de um radical fenoxil estável (SHAHIDI; AMBIGAIPALAN, 2015).

Os flavonoides que possuem um grupamento hidroxila livre são mais fisiologicamente ativos do que os compostos metilados em sua atividade de eliminação de RL. Portanto, flavonoides que possuem um radical hidroxila têm uma maior capacidade de varrimento de espécies de RL, ao passo que os flavonoides totalmente metoxilados, como as polimetoxiflavonas, podem efetivamente inibir as enzimas como a sintase induzível do óxido nítrico (iNOS) e NADPH oxidase, que geram radicais livres como NO e ânion superóxido, obtendo também um potencial antioxidante (MURAKAMI *et al.*, 2000; CHOI *et al.*, 2007; LI *et al.*, 2007).

Polimetoxiflavonas

Existe uma subclasse de flavonoides denominada flavonas polimetoxiladas ou polimetoxiflavonas (PMF) localizadas principalmente na casca de cítricos; possuem sucessivas metilações em seus grupos hidroxílicos e alta solubilidade em lipídios (ROBBARDS; ANTALOVICH, 1997; GORINSTEIN *et al.*, 2001). Elas possuem um derivado benzogama-pirano na posição C3 (Figura 2), e também exercem função de defesa contra patógenos causadores de doenças nas plantas (DEL RIO *et al.*, 1998).

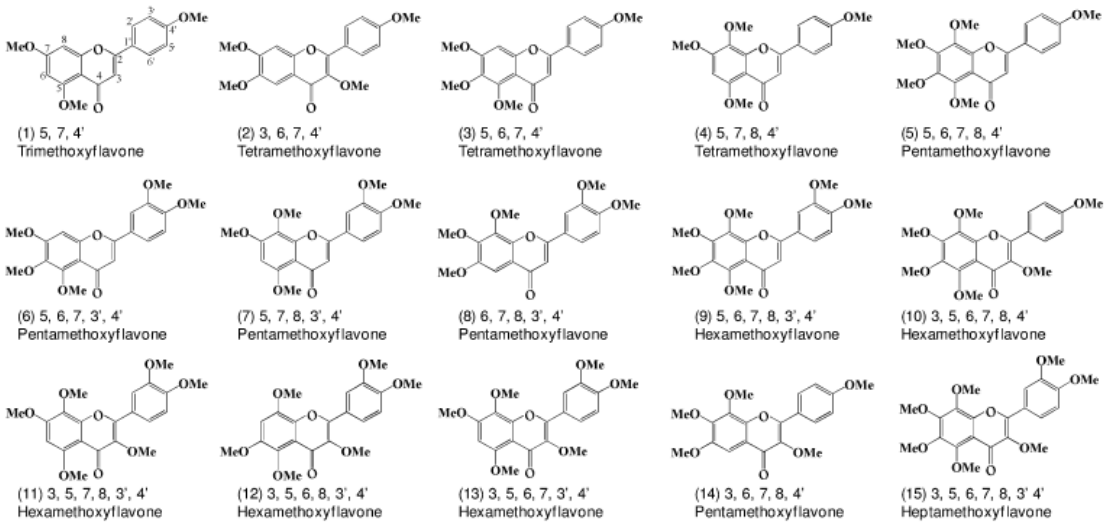

Figura 2. Estrutura molecular de polimetoxiflavonas isoladas a partir de frutas cítricas (Fonte: UCKOO, 2012)

Cerca de vinte PMF têm sido isoladas e identificadas a partir de diferentes espécies cítricas. Os tipos e concentração destes compostos variam entre os diferentes alimentos de origem vegetal (DUGO *et al.,*1995; LI *et al.,*2006), ocorrendo em maior abundância na casca da laranja (*Citrus sinensis*) e tangerina (*Citrus reticulata)* (MANTHEY *et al.,* 2001). Mouly e col. (1999) isolaram do suco de laranjas comerciais várias PMF, como a sinensetina, a hexametoxiflavona, a nobelitina, a heptametoxiflavona, a tetra-O-metil escutelareína e a tangeritina. Foi confirmado que esses flavonoides se encontravam no pericarpo do fruto e que, provavelmente, sua quantidade no suco depende do processo de extração utilizado.

A tangeritina (TAN) e a nobelitina (NOB) constituem as principais PMF encontradas nas espécies cítricas; já a isosakuranetina (ISR), a sinensetina (SIN), a tetrametilescutelareina (TMS) e a heptametoxiflavona (HMF) estão

presentes em menores quantidades nas frutas cítricas (MANTHEY *et al.,* 2011).

A **TAN** tem cinco grupos metoxi (-COH_3) sobre o núcleo flavona distribuídos nas posições 5, 6, 7, 8 - do anel A e 4' - do anel B (Figura 3). Sua denominação IUPAC é: 5,6,7,8,4'-pentametoxiflavona.

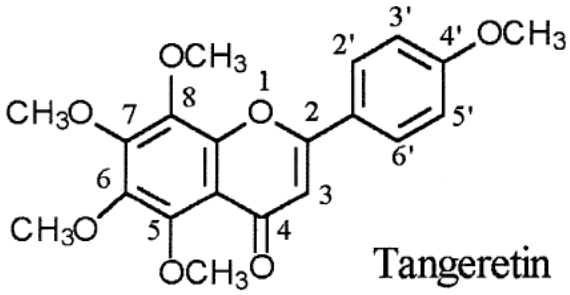

Figura 3. Estrutura molecular da TAN (Fonte: Nielsen *et al.*, 2000)

A **NOB** tem seis grupos metoxi (-COH_3) sobre o núcleo flavona distribuídos nas posições 5, 6, 7, 8 - do anel A e 3', 4' do anel B (Figura 4). Alguns de seus nomes IUPAC são: 2-(3,4-dimetoxifenil)-5,6,7,8-tetrametoxi-4H-1-benzopiran-4-o, ou 5,6,7,8, 3′,4′-hexametoxiflavona (LI *et al*., 2014). É uma das mais estudadas PMF, por ser a mais abundante em cascas de cítricos (LI *et al.,* 2006).

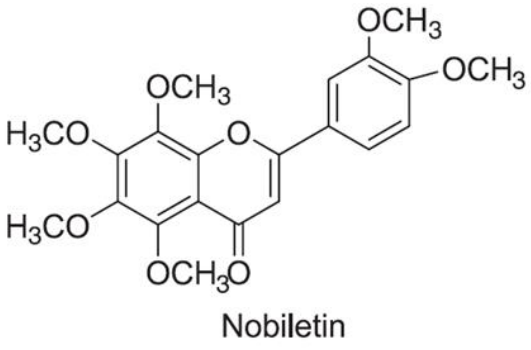

Figura 4. Estrutura molecular da NOB (Fonte: Singh *et al.,* 2011)

Atividades das PMF

Pesquisadores têm voltado sua atenção para o estudo da farmacocinética das PMF, por possuírem importante ação farmacológica (MANTHEY *et al.,* 2011; ASAMI *et al.,* 2010) como anti-inflamatórios (MANTHEY *et al.,* 2001; LI *et al.,* 2006; SHIN, *et al.,* 2012), anticarcinogênicos (MANTHEY *et al.,* 2001; LAI *et al.,* 2011; QIU *et al.,* 2010), hipolipidêmicos (KUROWSKA; MANTHEY, 2004; WHITMAN *et al.,* 2005; LI *et al.,* 2006) e prevenção e tratamento da obesidade (SERGEEV *et al.,* 2009).

A tangeritina e a nobelitina têm propriedades citotóxicas sobre as células cancerígenas e função de ajudar na circulação sanguínea de pacientes com doenças coronarianas (DEPYPERE *et al.*, 2000). Esses compostos atuam na prevenção do câncer, uma vez que interagem com radicais livres, os quais contribuem para a carcinogênese (IWASE *et al.*, 2001).

Estudos epidemiológicos têm mostrado a importância dos flavonoides cítricos na saúde. Kean e colaboradores (2015) apontaram que a ingestão regular de alimentos ricos em flavonoides cítricos está associada a um risco reduzido de doenças crônicas. Kurowska e Manthey (2004) demonstraram que estes compostos também podem reduzir a produção hepática de lipoproteínas contendo colesterol, reduzindo, assim, a concentração de colesterol total no plasma e, consequentemente, o risco de doenças cardiovasculares.

Dados apontam que a TAN e a NOB têm potente atividade inibidora contra o fator de necrose tumoral-α (TNFα) em monócitos humanos

estimulados com lipopolissacarídeos (LPS) (MANTHEY *et al.,*2001). A TAN e a NOB inibiram o crescimento de carcinoma de célula escamosa humana (HTB43), ao passo que outros flavonoides como a quercetina e o tamoxifeno (diidroquercetina) não tiveram efeito algum (KANDASWAMI *et al.,* 1991). Essas duas PMF também são capazes de inibir a proteína iNOS induzida por LPS (IHARA *et al.,* 2012), e também a expressão do RNA mensageiro responsável pela supressão da ativação de fator de transcrição nuclear Kappa-B (NFκ-B) e a fosforilação de p38-MAPK (proteína quinase ativada por mitógeno) (MANTHEY; BENDELE, 2008; HAGENLOCHER *et al*, 2016). A TAN e a NOB exercem papel primordial na resposta inflamatória, acarretando em decréscimo nas concentrações de NO (óxido nítrico) e citocinas, que por sua vez ocasionam menor adesão e influxo de leucócitos, controlando assim, a resposta inflamatória (MAIA, 2015).

Choi *e col*. (2007) comprovaram efeitos anti-inflamatórios da NOB sobre as células RAW 264.7 (macrófagos) ativadas por LPS com a diminuição da produção de RO, e para a NOB e TAN com atividade inibidora da produção de NO.

Jang *e col.* (2013) mostraram que a NOB e a TAN inibiram a expressão de IL-4, TNF assim como a ativação de NF-kB, AP-1 e p38 no tecido da pele de ratos, e que essas PMF reduziram a expressão de IL-4 e TNF em células RBL-2H3 (células de leucemia com receptores de IgE de elevada afinidade).

Tangeritina

TAN também desempenha um papel múltiplo na proliferação de células cancerosas e na fase metastática por inibir a adesão e invasão celular

(KAWAII *et al.,*1999), modular o sistema enzimático de metabolização de drogas (CANIVENC-LAVIER *et al.,*1996; SIESS *et al.,*1996), induzir os leucócitos à inibição parcial do desenvolvimento de HL-60 (originadas de paciente com leucemia promielocítica aguda, não têm marcadores específicos para células linfoides, mas expressam receptores de superfície para fragmentos FC e proteínas do sistema de complemento) (HIRANO, ABE, OKA, 1995). A Tan promove, ainda, um mecanismo de inibição das células cancerígenas mamárias humanas e a citólise na qual ela inibe a fosforilação da quinase de sinalização extracelular regulada (ERK) (SLAMBROUCK *et al.*, 2005). Apesar de não ser uma boa captadora de radicais livres, ela inibe a atividade da 15-lipoxigenase (15-LOX, com ação pró-inflamatória), sugerindo que ele pode exercer um efeito modulador sobre a oxidação lipídica enzimática (MALTERUD; RYDLAND, 2000). Ela é capaz de aumentar a comunicação intercelular por junções de hiato *(gap junction)* entre células normais e células mutantes e, assim, inibe a proliferação de células cancerígenas (CHAUMONTET *et al.,* 1994). TAN pode prender o ciclo celular na fase G1 por inibir cinases dependentes de ciclina (CDK) e pelo aumento de proteínas inibidoras de CDK (PAN *et al*., 2002;. MORLEY *et al.*, 2007).

TAN tem o potencial de reduzir colesterol e TG e modular o metabolismo de lipoproteínas contendo ApoB pela ativação de receptores ativados por proliferadores de peroxissomas (PPARs), que são fatores de transcrição pertencentes à família de receptores nucleares que regulam a homeostase da glicose, metabolismo de lipídeos e inflamação (TAVARES; HIRATA,

HIRATA, 2007), reduzir o potencial de apoB e modular o metabolismo de lipoproteína contendo através da ativação do receptor ativado por proliferador de peroxissoma (PPAr) (KUROWSKA; MANTHEY, 2004; KUROWSKA *et al.*, 2004).

Lee *e col.* (2016) observaram que a TAN inibiu significativamente a produção de NO, TNF-α, IL-6 e IL-1β em células BV2 (células imunes da micróglia-SNC) estimuladas com LPS, alem de inibir expressões de mRNA induzidas por LPS de sintase de óxido nítrico indutível (iNOS) e citocinas, uma vez que a iNOS medeia a síntese de NO, indicando que a TAN suprimiu os mediadores pró-inflamatórios.

HO-1 é uma enzima anti-inflamatória e antioxidante bem conhecida regulada através da via de sinalização Nrf2/ARE (KEUM, 2012). Lee *e col.* (2016) mostraram que a TAN aumentou os níveis de mRNA e de proteína da HO-1 aumentando a atividade de ligação de DNA de Nrf2 ao local de ARE, revelando que a TAN desempenha não apenas papéis anti-inflamatórios, mas também papéis antioxidantes em células BV2 estimuladas por LPS.

Nobelitina (NOB)

Yoshimizu *e col.* (2004) demonstraram que a NOB, em comparação com 42 flavonoides, foi a que teve atividade anti-proliferativa mais forte contra seis linhagens de células cancerosas humanas. Além de inúmeras atividades farmacológicas a ela associadas, tais como antidemência, antiapoptótica, antioxidante e efeitos anti-inflamatórios (ISHIWA *et al.,*2000; NAGASE *et al.,* 2005; WALLE, 2007).

NOB foi capaz de atenuar o fator de crescimento de hepatócitos (HGF) em células HepG2, inibindo a adesão, invasão e migração das mesmas (SHI; LIAO; SHIH; TSAI, 2013). NOB também possui caráter anti-inflamatório, uma vez que ela suprimiu a produção de prostaglandina E2 (PGE2) e a expressão da proteína COX-2 in vitro, após estímulos associados à inflamação, e envolvidos na carcinogênese do cólon de humanos e roedores (KOHNO *et al.*, 2001).

Administração oral de 200mg/kg de peso de NOB em camundongos ob/ob (modelo para obesidade) reduziu nível de glicose sanguíneo em animais jejuados ou não (LEE *et al.*, 2010). Em um modelo com ratos obesos, alimentados com dieta rica em gordura, o tratamento com NOB diminuiu o ganho de peso corporal, o peso do tecido adiposo branco e TG plasmático, melhorou níveis de adiponectina plasmática e tolerância à glicose, devido à regulação da expressão de genes relacionados com o metabolismo de lipídeos e de adipocinas, e também pela regulação da expressão de marcadores inflamatórios e a atividade da via de sinalização de insulina (LEE *et al.,* 2013).

Whitman *e col.* (2005) demonstraram que a NOB pode reduzir as concentrações circulantes de lipoproteínas de densidade muito baixa (VLDL) e lipoproteínas de baixa densidade (LDL) no sangue, e pode inibir diretamente a formação de células espumosas derivadas de macrófagos no local da lesão. Ainda nesse estudo, foi verificado que a NOB reduziu as concentrações plasmáticas de triglicerídeos (TG) e colesterol, e reduziu TG hepático.

Li *e col.* (2007) observaram os efeitos anti-inflamatórios da NOB através da inibição significativa da produção de ROS intracelular induzida por LPS ou juntamente com os seus metabolitos (3'- e 4'-desmetilnobiletina) e a desdemetilação (3', 4'-didemetilNOB) diminuindo a produção de NO induzida por LPS e expressão de proteína iNOS e COX-2 em RAW264.7, no qual os metabolitos tiveram uma melhor atividade anti-inflamatória do que a própria NOB.

Yoshigai *e col.* (2013) mostraram que a NOB exerceu atividade anti-inflamatória em hepatócitos suprimindo tanto a produção de óxido nítrico (NO) induzida pela IL-1β como a expressão de iNOS.

Metabolismo dos flavonoides/PMF

O metabolismo dos flavonoides (Figura 6) depende da forma na qual eles se encontram no alimento, podendo estar na forma aglicona ou glicosilada (conjugados à molécula de carboidrato), a qual apresenta menor reatividade e maior solubilidade em água (SANTOS, 2009). A naringina e hesperidina são formas glicosídicas de flavanonas presentes em frutas cítricas, nas quais a deglicosilação é necessária para que o composto seja absorvido pela borda em escova das vilosidades intestinais, uma vez que somente a forma aglicona e os conjugados à glicose podem ser absorvidos no intestino delgado (KUMAR; PANDEY, 2013).

As flavonas metoxiladas têm uma grande vantagem sobre as outras em relação à biodisponibilidade oral. Aparentemente os compostos que contêm apenas um ou dois grupamentos metoxis podem ser metabolicamente mais

estáveis do que as flavonas polimetoxiladas, pois se sugere que a estrutura molecular é mais estável, embora sejam necessários mais estudos nesta área (WALLE, 2007)

As PMF se apresentam na forma aglicona, os grupos metoxis da molécula possuem uma natureza hidrofóbica quando comparados com os grupos hidroxis, o que torna as PMF (NOB, TAN E HMF) mais lipofílicas do que flavonoides poli-hidroxilados, tais como a quercetina, a luteolina e naringina (MURAKAMI *et al*., 2001). Em decorrência disso, as PMF têm maior permeabilidade através do intestino delgado e são mais prontamente absorvidas na circulação sanguínea (MURAKAMI *et al.*, 2001; WALLE, 2007; ASSINI *et al.,* 2013).

Uma vez na corrente sanguínea as PMF chegam ao fígado (hepatócitos) no qual elas podem sofrer o processo de conjugação, por meio de metilação, sulfatação e/ou glucuronidação (conjugação ao ácido glucurônico), a fim de aumentar sua afinidade pela água (hidrofilicidade) e facilitar a excreção, esse mecanismo pode ocorrer também no intestino, nos enterócitos (CROZIER *et al.,*2009; MAIA, 2015).

A excreção dos flavonoides pode ser realizada por meio da bile ou pelo sistema urinário. No rim, os flavonoides também podem sofrer conjugação e serem excretados na urina. Já a excreção biliar pode contribuir para reabsorção intestinal devido à ação enzimática bacteriana, alterando a meia vida dos flavonoides (BOHN, 2014; MAIA, 2015). No cólon, os flavonoides glicosilados podem sofrer a ação de enzimas provenientes da microbiota local, como a β-glucuronidase, sendo degradados e absorvidos na forma de

seus metabólitos (aglicona/ácidos fenólicos), via sistema porta-hepática (THILAKARATHNA; RUPASINGHE, 2013).

Citocromo P450 (CYP) é o sistema enzimático chave envolvido no metabolismo das PMF, e é capaz de catalisar reações de hidroxilação e de desmetilação. A via metabólica da PMF é considerada idêntica entre as espécies. O número e a posição dos grupos hidroxila e metoxila no anel B das PMF têm uma grande influência sobre o metabolismo das mesmas (BREINHOLT *et al.*, 2003; NIELSEN *et al.*, 1998; LI, 2009). As posições 3' e 4' no anel B das PMF são os sítios primários de biotransformação. A partir de estudos *in vitro* em células Caco-2, foi observado que a NOB fica acumulada na monocamada das células (MURAKAMI *et al.,* 2001) e após 4h de incubação mais de 48% dela permeia para o lado basolateral (como a veia porta), mostrando que a NOB tem elevada permeabilidade e uma tendência a acumular-se no compartimento intracelular (LI, 2009). A NOB possui uma propriedade distinta de acumular-se em uma grande variedade de órgãos, incluindo o estômago, intestino delgado e grosso, fígado e rim durante o período de 1-4h após uma dose única (MURAKAMI *et al.*, 2001, 2002).

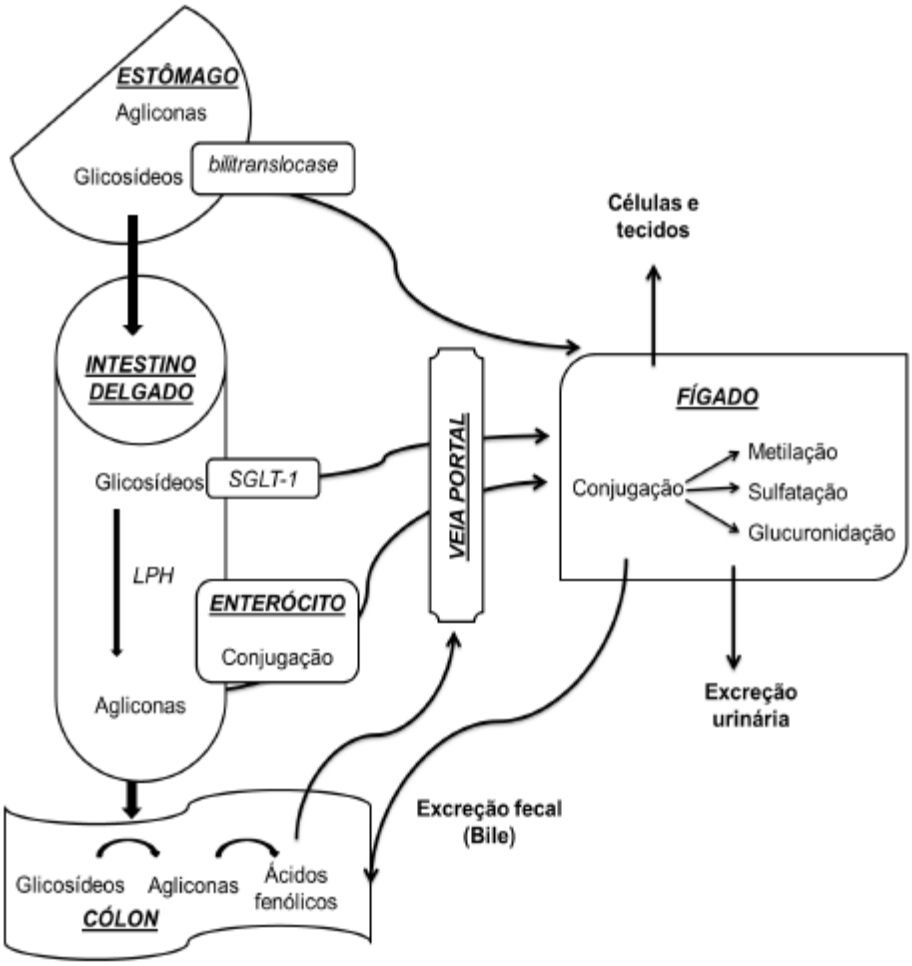

Figura 6. Metabolismo dos flavonoides. SGLT-1: transporte de glicose sódio dependente-1. LPH: enzima lactase florizina hidrolase (MAIA, 2015).

Existem poucos estudos sobre a biodisponibilidade oral de metoxiflavonas ou polimetoxiflavonas. A NOB foi administrada em ratos, em conjunto com a luteolina não metilada (5,7,3',4'-tetrahidroxiflavona) em doses de 25 mg/kg, e seu conteúdo no fígado e nos rins foi maior que os níveis de luteolina (MURAKAMI *et al.,* 2002). A TAN foi administrada a hamsters como 1% da sua dieta durante 35 dias, e foi encontrada considerável absorção intestinal, com base na excreção urinária de vários

metabolitos destes animais, e também detectados no soro e órgãos desses animais (KUROWSKA; MANTHEY, 2004). Manthey *e col.* (2011) em uma análise comparativa entre a absorção de NOB e TAN em ratos, observaram que a administração oral em óleo vegetal apresentou níveis séricos de NOB maiores que os de TAN. Esta descoberta sugeriu uma diferença potencialmente significativa na biodisponibilidade de NOB e TAN e, como consequência, possíveis diferenças na eficácia destes dois compostos, quando administrados em animais.

A solubilidade da TAN/NOB foi observada por Li *e col.* (2009), por meio do ensaio LYSA (lyophilisation solubility assay), no qual é considerada uma alta solubilidade, valores maiores que 500µg/mL, média entre 100 e 200µg/mL, e baixa solubilidade valores menores que 100µg/mL. Em geral, a solubilidade das PMF é muito baixa, sendo que a das PMF hidroxiladas é maior do que as totalmente metoxiladas, como por exemplo, a solubilidade da 5-demetilNOB (32µg/mL), 3'-demetilNOB (29µg/mL) e 4'-demetilNOB (22µg/mL) é mais alta do que a da NOB (12µg/mL). Outro exemplo é a solubilidade de 3-hidroxi-5,6,7,8,3',4'-hexametoxiflavona (ou 3-hidroxiNOB) (37µg/mL), que é muito mais alta do que a da HMF (8µg/mL). Assim, quanto mais grupos hidroxila as PMF tiverem, melhor será a sua solubilidade, e o grupo hidroxila na posição 3 do anel C das PMF tende a aumentar muito a solubilidade das PMF: a solubilidade da 3-hidroxiNOB (37µg/mL) é muito mais alta do que a da 3'-metoxiNOB (ou HMF) (8µg/mL) (LI *et al.*, 2009).

A solubilidade mínima aceitável que é necessário para um fármaco ativo por via oral ou nutriente também depende da permeabilidade. Li *e col.* (2009)

concluíram que a permeabilidade das PMF é de média a elevada, o que significa que elas podem atravessar facilmente a membrana fosfolipídica, devido à presença de vários grupos metoxis. TAN possui a maior permeabilidade ($1,62x10^{-6}$cm/s), seguido da NOB ($1,38x10^{-6}$cm/s).

Os efeitos benéficos de polifenóis dependem tanto do seu consumo quanto de sua absorção e biodisponibilidade, a qual pode variar de acordo com a estrutura molecular do composto (MANACH *et al.,* 2004). Vários estudos indicam que os metabolitos formados a partir da metabolização sofrida pelos compostos durante a passagem pelo intestino e fígado, têm ação superior ao composto original, e são, portanto, os responsáveis pelos efeitos benéficos observados (LI *et al*., 2009; MANTHEY *et al.*, 2011).

Juntamente com o rápido desenvolvimento em tecnologias de purificação e identificação de compostos bioativos de plantas, estudos sobre compostos cítricos bioativos e atividades antioxidantes irão atrair mais e mais atenção no futuro (ZOU, 2015).

Assim, este trabalho teve como objetivo, avaliar a capacidade antioxidante e a peroxidação lipídica no soro sanguíneo e fígado de ratos tratados, por via oral, com as polimetoxiflavonas, TAN e NOB, por meio dos métodos ABTS e TBARS, além de α-tocoferol, β-caroteno e retinol no soro sanguíneo e glutationa peroxidase no fígado desses animais; e identificar os metabólitos formados na urina desses ratos.

Capitulo 1.

Differential effect of nobiletin and tangeretin on the antioxidant activity and levels of α-tocopherol and retinol in rats

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ABSTRACT

Polymethoxylated flavones (PMFs) have been associated with increased antioxidant activity in animal models. The effects of tangeretin (TAN) and nobiletin (NOB) on antioxidant activity in the blood and liver of rats were evaluated. Groups of rats were treated with 200 mg/kg bw/day of TAN or NOB, or placebo, for 15 days. Parental compounds and their metabolites were assessed in the liver by chromatographic analysis, in addition to α-tocopherol and retinol in the blood serum. Both TAN and NOB supplements were able to reduce malonaldehyde (MDA) in the rat's blood by 22% and 18%, respectively, but only NOB increased redox reaction by 3%. blood levels of retinol and α-tocopherol increased under TAN by 59% and 20%, respectively, but were not affected by NOB. Eight NOB metabolites were detected in the liver, but only two TAN metabolites were identified in low concentration. In Conclusion, NOB improved antioxidant capacity and reduced lipid peroxidation, while increased levels of retinol and α-tocopherol after TAN supplement may have contributed to decreased blood lipid peroxidation.

Keywords: nobiletin, tangeretin, metabolites, antioxidant activity, retinol, alpha-tocopherol

1. INTRODUCTION

Blood levels of antioxidants can vary in response to normal oxidative metabolism, controlling the production of reactive oxygen species (ROS) that damage cell molecules. However, in the course of the infection or inflammation that follows most human diseases, there is a greater demand for antioxidants to neutralize oxidative damage [1]. Diets containing plenty of fruits and vegetables may meet those demands, but dietary supplements can be designed to offer a variety of antioxidants in ample amounts. Antioxidants extracted from citrus fruits, such as vitamin C, carotenoids, flavanones (hesperidin and naringenin), and polymethoxyflavones (PMFs), such as nobiletin (NOB) and tangeretin (TAN), can be used as nutraceuticals to benefit health [2]. Currently, the use of supplements by the general population has been encouraged by broad segments of society, such as health professionals, the pharmaceutical industry and social networks, as an alternative and faster way to improve well-being by combating conditions associated with unhealthy and stressful lifestyles. Despite apparent benefits, the extensive use of supplemental antioxidants can unbalance the redox state, causing negative effects, depending on the dose and the physiological state [1]. The benefits of NOB and TAN supplementation are associated with their ability to block oxidative stress and modulate enzymes that cause the accumulation of free radicals and ROS [3-5]. This leads to inhibition of early stages of inflammation and overproduction of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1b (IL-1b), interleukin 6 (IL-6), and nitric oxides (NO) [6-7]. These combined effects improve overall redox balance and decrease systemic and local inflammation [8], mitigating damage caused by disease-or drug-induced oxidative stress [9]. Although the protective activity of PMFs has been demonstrated in animals [3, 9-10] and humans [11], the effect of regular ingestion of higher doses under physiological conditions is still unknown. It was suggested that the indiscriminate use of antioxidants, not clinically prescribed, could disturb the oxidative balance, limit the extent of its effect, or even act as a pro-

oxidant agent [12]. Therefore, it is relevant to know whether supplementation of a given dose of NOB and TAN, under physiological conditions, will contribute to the stability of the antioxidant system or lead to undesirable effects [13]. More research is warranted for recommendation of these supplements, aimed at maintaining health, preventing diseases, or treating a specific pathology [11]. The main objective of this study was to evaluate biological parameters related to the antioxidant activity of NOB and TAN, after systematic supplementation of 200 mg/kg of body weight (bw)/day for 15 days in apparently healthy animals. This translated dose for an average 70 kg person is about 2.3 g/day [14], and it is considered a high dose since the general recommendation of citrus bioflavonoids for humans is around 500 mg/d to provide protection against chronic diseases [15]. The hypothesis of this study is that systematic doses of NOB or TAN (200 mg/kg/d) maintain the redox balance in the blood and liver, due to the increased availability of endogenous antioxidant compounds, which are spared with the supplementation of PMFs. For this purpose, blood concentrations of α-tocopherol (vitamin E) and retinol (vitamin A derivative) were measured, as well as the levels of hepatic glutathione peroxidase (GPx), and the antioxidant effect of NOB and TAN on lipid peroxidation and total antioxidant capacity in the blood. The second objective was to quantify the hepatic production of NOB and TAN metabolites, which are eventually associated with health benefits.

2. MATERIAL AND METHODS

2.1 Purification of Tangeritin and Nobiletin

Cold pressed orange oil residues containing around 10% PMFs, obtained from a local flavoring company, were initially purified by silica gel column chromatography (Biotage), using linear gradients of hexane and ethyl acetate for partial separation of tangeretin [(TAN) 5,6,7,8,4’-pentamethoxy-flavone] and nobiletin [(NOB) 5,6,7,8,3’,4’-hexamethoxyflavone]. Purification of TAN and NOB compounds were obtained by reverse phase liquid

chromatography (RediSep C18 Reverse Phase) with linear gradients of methanol/aqueous formic acid, followed by crystallization with acetone and hexane. The purity of TAN and NOB was >95% compared to authentic standards using methods previously reported [16]. The diagram in Figure 1 briefly describes the purification process.

2.2 Animals and treatment

Thirty male rats (Wistar) weighing 150-200 g from the Animal Center of São Paulo State University (UNESP) were housed in individual metabolic cages and were adapted for 7 days to the environmentally controlled temperature (23 ± 1 °C), humidity (55 ± 5%) and a 12 h light/dark cycle. Rats had free access to water and a commercial diet (Presence Nutricao Animal®) throughout the 15 days of the experiment under the same conditions. The basic composition of the rat chow contained: bran of soybean, wheat, and rice; ground whole corn; refined soybean oil; meat and fish meal; dextrin; sodium chloride; calcium iodate; and trace minerals and vitamins. High-Performance Liquid Chromatography (HPLC) analysis of the commercial rat chow showed retinol and α-tocopherol occurred at concentrations of 0.04 and 1.10 mg per 20 g of commercial chow, respectively. This protocol was conducted in compliance with recommendations of the Brazilian College of Animal Experimentation (COBEA) and the experimental procedures received prior institutional approval by the Ethical Board for Animal Experimentation, School of Pharmaceutical Sciences, UNESP (Protocol # 68/2015).

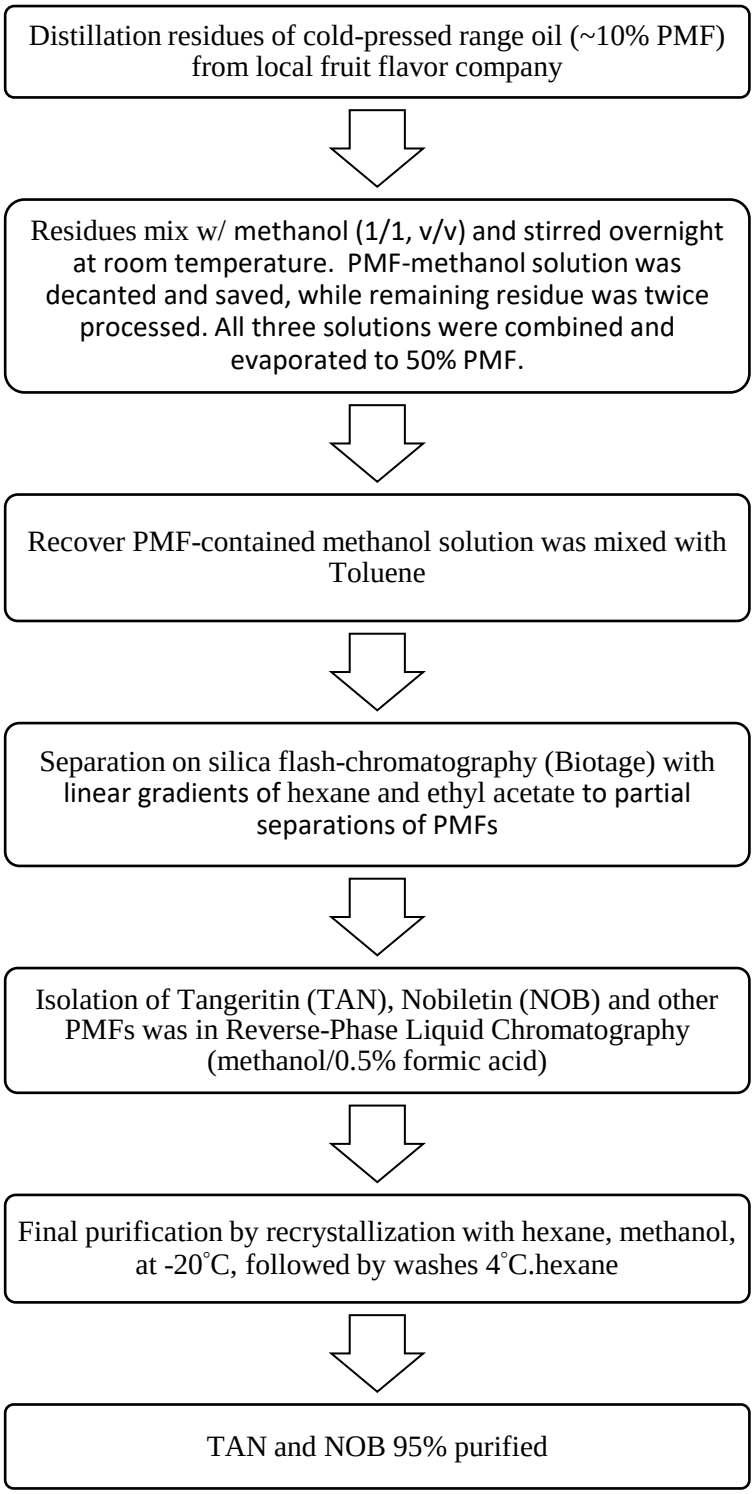

Figure 1. Flowchart of Nobiletin (NOB) and Tangeritin (TAN) purification by reverse phase liquid chromatography (adapted from Gonçalves et al, 2019)

2.3 Experimental design

The purified TAN and NOB were mixed separately with commercial plain yogurt (Nestle®) with a homogenizer operating at 820 × g for 10

minutes at a controlled ambient temperature (25 °C) (Figure 2). After the adaptation period, rats were assigned into three groups according to similar mean values of body weight: control group (n = 10), NOB group (n = 10), and TAN group (n = 10). Each experimental group was treated with 200 mg/kg/day of NOB or TAN mixed in 1 mL plain yogurt by gavage once a day at 4:30 PM. The selected dose of 200 mg/kg bw/day in the current study was based on the protective effects of NOB and TAN against induced brain and liver damage in rats without toxic events [6-7, 9]. The control group received 1 mL of plain yogurt (Nestlé) as a placebo. Body weight was checked every three days for the PMF dose adjustment. After 15 days of treatment, the rats were euthanized by decapitation and blood samples were collected in heparinized tubes (Hemofol®, 5000 UI/mL, Brazil). Blood serum was obtained by centrifugation (10,000 × g for 15 min at 25 °C), and livers were removed and frozen (-80 °C) until further analysis.

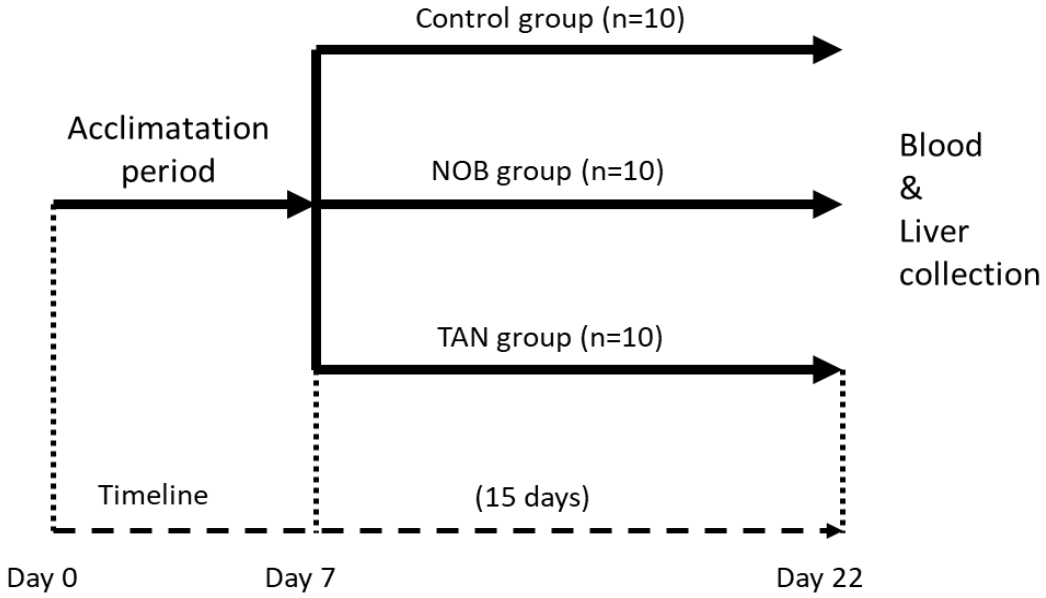

Figure 2. Experimental design. After the acclimation period (7 days), Nobiletin (NOB) and Tangeretin (TAN) groups received, by gavage once a day a solution of NOB or TAN (200mg/kg) in 1ml of vehicle (plain yogurt). Control group received 1mL of vehicle for 15 days.

2.4 Antioxidant capacity in the blood serum and liver

Blood serum oxidative stress was measured by comparing levels of serum lipid peroxidation using thiobarbituric acid-reactive substances (TBARS) and

quantified as μM malondialdehyde (MDA) [17]. Malondialdehyde (MDA) is the end product of lipid peroxidation and reacts with thiobarbituric acid (TBA) to form the MDA-TBA adduct in a 1:2 ratio with TBA. The absorbance of each test was obtained in a 96-well microplate reader at 540 nm. Total antioxidant capacity in blood serum was measured as the Trolox equivalent antioxidant capacity (TEAC) using the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay [18]. The absorbance was measured at 734 nm to verify the formation of ABTS+ and, to prepare the calibration curve, Trolox (Sigma) was used as a standard. All analyzes were performed in triplicate. To assess liver oxidative stress, the organs were thawed and one gram of each was homogenized in 4 mL of 1.15% potassium chloride at 4 °C, centrifuged (10,000 × g for 10 min at 4 °C), and the supernatants collected. Levels of proteins in liver supernatants were determined using bovine serum albumin as standard for the assay [19]. Liver MDA values were given in μM MDA per mg protein. Liver glutathione peroxidase (GPx) activity was determined and the results were expressed as mmol of NADPH consumed/min/mg protein (liver) [20]. Bovine serum albumin was also used as the protein standard in the glutathione peroxidase (GPx) activity.

2.5 Analysis of retinol, α-tocopherol, and β-carotene in the blood serum

Retinol (≥ 95% pure), α-tocopherol (≥ 95% pure), and β-carotene (≥ 95% pure) were purchased from Sigma® . This analysis and preparation of the standards and blood samples were according to [21]. Sample preparation was in glass tubes, cover with aluminum foil to minimize light-induced degradation of vitamins. Blood serum samples (200 μL) were combined with methanol (600 μL), thoroughly mixed, and centrifuged at 10,000 × g for 4 min at room temperature. Supernatants were collected and reduced to dryness under nitrogen flow, and the residues were dissolved into the mobile phase. Chromatographic analysis was performed with Ultra-Performance Liquid Chromatograph (UPLC) (Shimadzu Nexera X2), equipped with a photodiode array UV-Vis detector (PDA-SPD-M20A), auto sampler (Shimadzu Nexera X2 SIL-30 AC), and column compartment/heater, both enabling the control of

the temperature. An Acquity UPLC C18 column (2.0 mm × 200 mm, 2.2 µm particles) was used for separation. Isocratic acetonitrile-methanol-dichloromethane (75:5:20, v/v/v) was used as mobile phase; the flow rate was 0.3 mL/min. UV detection was accomplished at 325 nm for retinol, 450 nm for β-carotene, and 292 nm for α-tocopherol [21]. Injection volume was 7 µL and the compounds were separated within 10 min. Data were collected and processed by LabSolutions Shimadzu Corporation software.

2.6 Detection of liver PMF metabolites

To analyze the metabolites in rat liver, 2.0 mL of methanol was added to each tube containing the complete freeze-dried supernatant of 1.0 g of homogenized tissue. The methanol-added samples were dispersed in a vortex at high speed for 2 min. The samples were centrifuged for 10 min 10,000 × g at room temperature under vacuum in a Savant concentrator. The clear supernatants were placed into individual 4 mL vials. The remaining sample precipitates were reextracted using the above procedure. The first and second supernatants were combined and evaporated at 60 °C under vacuum to near dryness then mixed with methanol to 2.0 mL and clarified through a 0.45-micron PTFE filter. Detection and analysis of the metabolites in the liver samples were performed by HPLC (Waters 2695 Alliance®), connected in parallel with a Photodiode Array Detector (PDA), and a Micromass ZQ quadrupole Mass Spectrometer with an electrospray ionization source (ESI), using a Waters C8 XBridge (4.6 × 150 mm) column, using linear gradients of aqueous 0.5% formic acid and acetonitrile, (90/10; v/v), as described previously [22]. Detection of the NOB and TAN metabolites were by positive ion ESI-MS using single ion monitoring and by UV absorbance with a Waters 996 diode-array detector. Rat liver metabolites were quantified by comparisons with previously purified 4’-hydroxy-NOB, NOB4’-glucuronide, 4’-desmethyl TAN and Tan-4’-O-glucuronide standards [23].

2.7 Statistical analysis

The results of metabolites in rat livers are described in table and chromatogram charts. The variables to assess lipid peroxidation and antioxidant capacity in rat blood serum and liver were expressed as mean ± SD, and differences were considered statistically significant when $p \leq 0.05$. Data were statistically compared using one-way analysis of variance (ANOVA) and SIDAK as post-hoc. Analyses were performed in SPSS Statistics software (v.21, SPSS: An IBM Company, Chicago, IL).

3. RESULTS

3.1 Body weight and food intake

At the beginning of the supplementation period, there were no differences among body weights of the groups: control, TAN, and NOB (Figure 3), but on the 7th day, the rats supplemented with TAN had a higher body weight than the controls, while the weight of NOB rats was intermediate between the TAN and controls. At the end of the experiment (15th day), the rats treated with TAN gained 26% more weight in comparison to NOB and controls (Figure 3 and Table 1). At the end of the experiment, the animals with the highest body weight were also those that ingested the highest daily and total amount of food (Table 1 and Figure 3). Consequently, they also had higher energy consumption (Table 1), as all rats were fed ad libitum with the same diet. Therefore, the discrepancy in weight gain between TAN and the other groups may be based on the greater appetite of these individuals, or on other unmeasured variables. Although the sample size was calculated taking into account differences within and between groups (ANOVA one-way), there are limitations in interpreting unexpected results, as the estimate is based on the main hypothesis previously determined.

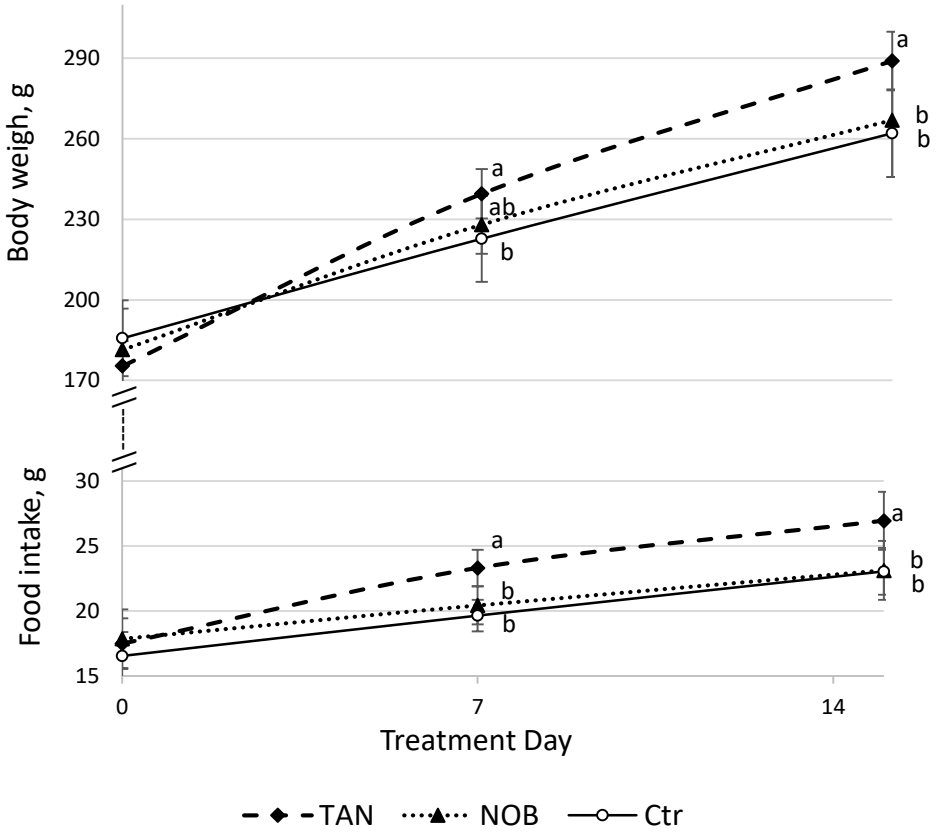

Figure 3. Body weight and food intake of rats treated with standard diet (control group), and diet supplemented with 200mg/kg.bw of tangeretin (TAN group) or nobiletin (NOB group) during the experimental period of 15 days.

Table 1. Effect of NOB and TAN on body weight, food and energy intake in rats fed with normal caloric diet (AIN-93)

Group	**Control** (n=10)	**NOB** (n=10)	**TAN** (n=10)
Initial Weight (g)	186 ± 14^{a}	181 ± 15^{a}	175 ± 9^{a}
Final Weight (g)	262 ± 16^{b}	267 ± 21^{b}	289 ± 11^{a}
Weight Gain (15 d)	76 ± 13^{a}	86 ± 23^{a}	114 ± 6^{b}
Intake (g/d)	21.4 ± 2.4^{b}	21.8 ± 1.9^{b}	25.1± 2.6^{a}
Energy Intake (kcal/d)	81 ± 9^{b}	83 ± 7^{b}	96 ± 10^{a}

Data are presented as mean ± SD.
One-way analysis of variance (ANOVA) and SIDAK post-hoc. Different letters show that there is statistical difference between groups ($p \leq 0.05$)

3.2 Redox status and antioxidant capacity

Both TAN and NOB administered to healthy rats reduced the blood serum MDA in comparison to the control group. MDA values decreased by an average of 22% with TAN, while NOB reduced the blood serum MDA by 18% ($p \leq 0.05$) (Table 2). The serum antioxidant activity tested by the ABTS+ assay increased 3% in the NOB group ($p \leq 0.05$), while there was no change in the TAN group. Yet, α-tocopherol increased 20%, and retinol increased 59% in the group treated with TAN ($p \leq 0.05$), but NOB and control showed no changes (Table 2). Chromatographic analysis of blood serum did not detect β-carotene in samples from any group. Analysis of liver peroxidation showed no significant differences among the three groups. Interventions with either PMF had no effects on the GPx levels in the liver.

Table 2. Antioxidant capacity in the blood serum and liver of rats supplemented with daily dose (200 mg/kg bw) of tangeretin (TAN), nobiletin (NOB) or vehicle (Control).

Tissue	Antioxidants Parameters		Control (n=10)	NOB (n=10)	TAN (n=10)
Blood Serum	**MDA**	(μM/L)	1.48 ± 0.40[a] (Δ%)	1.21 ± 0.26[b] (-18%)	1.15 ± 0.39[b] (-22%)
	ABTS	(mM/L)	1.68 ± 0.06[b] (Δ%)	1.73 ± 0.05[a] (3.0%)	1.70 ± 0.02[ab] (1.2%)
	α-tocopherol	(μM)	6.63 ± 0.89[b] (Δ%)	6.18 ± 1.72[b] (-6.8%)	7.97 ± 1.04[a] (20%)
	retinol	(μM)	1.35 ± 0.13[b] (Δ%)	1.27 ± 0.49[b] (-5.9%)	2.14 ± 0.4 7[a] (59%)
Liver	**MDA**	(μM/g)	0.40 ± 0.18[a] (Δ%)	0.48 ± 0.14[a] (20%)	0.54 ± 0.11[a] (35%)
	GPx	(mmol/min/mg)	0.08 ± 0.02[a] (Δ%)	0.06 ± 0.01[a] (-25%)	0.07 ± 0.03[a] (-13%)

Data are presented as mean ± SD.
Antioxidant variables: MDA (malondialdehyde), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline - 6-sulfonic acid, α-tocopherol), α-tocopherol (vitamin E) and retinol (derivative of vitamin A) were measured in the blood serum, while the activity of MDA and GPx (glutathione peroxidase) were measure in the liver.
Δ% is percent difference between experimental group (NOB or TAN) versus Control.
One-way analysis of variance (ANOVA) and SIDAK post-hoc. Different letters show that there is statistical difference between groups ($p \leq 0.05$).

3.3 PMF metabolites in rat liver

Profiles of glucuronide and sulfate conjugates, as well as hydroxylated aglycone metabolites in the livers of TAN and NOB-fed rats were analyzed by HPLC-ESI-MS and PDA (UV) detection [22]. Protonated molecular masses ([M + H]+) and retention times of these compounds are shown in Table 3, and the MS and UV chromatograms are shown in Figure 4. The ESI-MS of the conjugated compounds exhibited neutral losses of 176 atomic mass units (amu), suggesting losses of glucuronic acid substituents, or 80 amu for the loss of a sulfate group. HPLC-MS analysis of the individual liver extracts showed two TAN metabolites: T1 (TAN-4'-O-glucuronide) at an average liver concentration of 0.011 ± 0.0046 µg/g FW and T2 (4'-desmethyl TAN, i.e., 4'-hydroxy-5,6,7,8- tetramethoxyflavone) at an average liver concentration of 0.031 ± 0.0054 µg/g FW. Trace levels of other metabolites exhibiting 359 m/z fragment ions were also detected in a pooled and concentrated liver extract (data not shown), but were not quantifiable in the individual liver extracts. HPLC-MS analyses of the individual liver extracts additionally showed eight NOB metabolites, N1-N8 (Table 3), and most of them were similar to those previously reported in rat blood serum [24]. The ESI-MS, elution time, and concentration of each of these compounds, as well as their suggested molecular compositions, are listed in Table 3. From these measured concentrations, total amounts of the cumulative TAN metabolites (0.042 µg/g FW), occurred at 170 times lower than the cumulative levels of the NOB metabolites (7.18 µg/g FW). A substantial portion of the NOB metabolites occurred as a NOB-O-sulfate (N5) at 2.74 µg/g FW. Also, unlike TAN, NOB metabolism in the rat livers produced measurable levels of di-desmethyl metabolites [(M+H)+ (389)-2(CH2)] = 375 m/z, including metabolites N4A and N4B. Of the NOB metabolites, most occurred as conjugates of glucuronic acid and sulfate, and a smaller portion of the metabolites occurred as free aglycones (a cumulative average of 1.75 µg/g FW). Only trace levels of the original NOB and TAN were detected in the individual liver extracts.

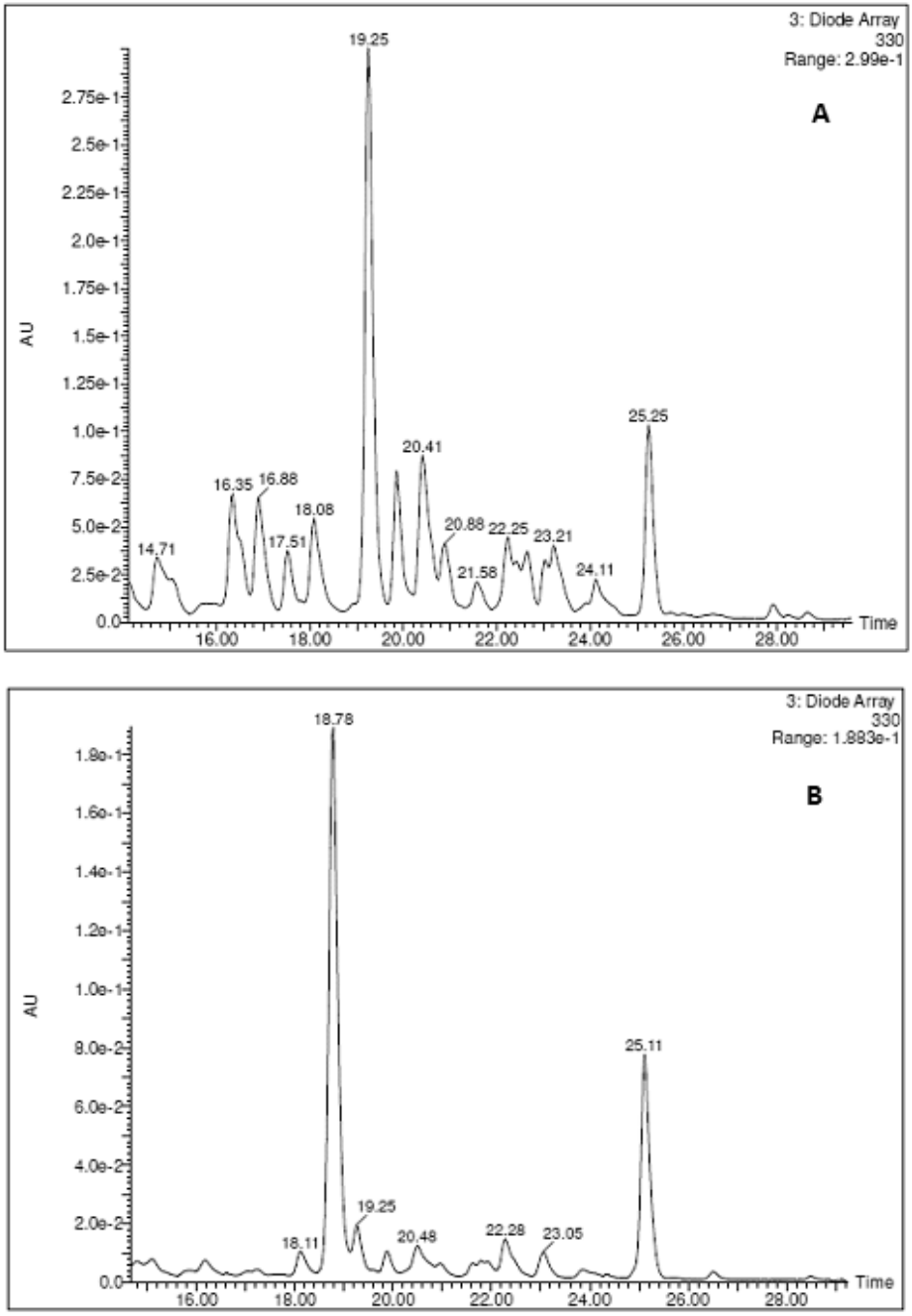

Figure 4. Profile of UV chromatogram (330 nm) of NOB (A) and TAN (B) metabolites in the rat's liver. The molecular masses of the ions $(M + H)^+$ hydroxylated, glucuronide and sulfated are listed in Table 2.

Table 3. Proposed structure, elution time and liver concentration of metabolites of rat supplemented with tangeritin and nobiletin

Metabolites	Proposed Structure	$[M + H]^{+1}$ ESI-MS^+	Elution time (min)	Liver (µg/g)
NOB 1	NOB-*O*-glucuronide	565/389	20.3	1.18 ± 0.64

NOB 2	NOB-*O*-glucuronide	565/389	20.7	1.39 ± 0.58
NOB 3	NOB-*O*-glucuronide	565/389	17.6	0.12 ± 0.09
NOB 4	di-desmethyl NOB	375	21.8	0.10 ± 0.05
NOB 5	NOB-*O*-sulfate	469/389	22.9	2.74 ± 1.22
NOB 6	mono-desmethyl NOB	469	23.3	0.64 ± 0.29
NOB 7	mono-desmethyl NOB	469	24.8	0.15 ± 0.11
NOB 8	mono-desmethyl NOB	455	25.8	0.86 ± 0.44
			Σ_{NOB}	**7.18**
TAN 1	TAN-4-*O′*-glucuronide	359	19.7	0.011 ± 0.005
TAN 2	4′-*O*-hyroxy-5,6,7,8-pentamethoxy flavone	535	25.8	0.031 ± 0.005
			Σ_{TAN}	**0.042**

NOB (1,2,3,4,5,6,7 and 8): Eight metabolites detected in the liver of NOB-treated rats. Two metabolites are hydroxylated aglycones with protonated molecular masses of 389 *amu* (NOB 1) and 375 *amu* (NOB 4), two conjugated with glucuronic acid, 565 *amu* (NOB 3) and 551 *amu* (NOB 4), and four conjugated with sulfate groups, 469 *amu* (NOB 5, NOB 6, NOB 7) and 455 *amu* (NOB 8).
TAN (1 and 2): Two metabolites detected in the liver of animals supplemented with TAN identified as hydroxylated aglycone with protonated molecular masses of 359 *amu* (TAN 1) and one glucuronic acid conjugate, 535 *amu* (TAN 2).

4. DISCUSSION

This study showed that systematic doses of NOB or TAN (200 mg/kg/d) as dietary supplements for rats during a short time interval (15 days) reduced lipid peroxidation by 20% in circulating blood. In addition, a significant increase in the blood antioxidant capacity was observed after NOB treatment (3%, $p \leq 0.05$). At the end of the supplementation period, significant levels of NOB and TAN metabolites were detected in the liver, but not the parental compounds, suggesting that they were bio transformed before, or soon after, reaching the organ. The antioxidant activity measured in the hepatic tissue did not change with either supplement, but higher levels of retinol and α-tocopherol were detected in the blood after TAN oral administration. Animals treated with NOB had weight gain similar to the control group fed a standard diet, but the TAN group had higher intake and consequently higher body weight at the end of the experiment.

Supplementation of NOB and TAN, or placebo, was performed by gavage and therefore had no direct effect on the flavor of the food. This is important because NOB and TAN are very bitter compounds [25]. Feng et al. [26] suggested an anti-obesity effect of TAN due to lower weight gain and body fat in rats that consumed 0.04% and 0.08% TAN mixed with a high-fat diet. Compared to the current experiment, these doses were 4.6 to 9.1 times higher, the initial body weight was 50% higher and, the intervention was longer (6 wk). In contrast, Nery et al. [27] found similar results to the current study in mice fed a highfat diet plus 100 mg/kg bw TAN for 4 weeks. The authors showed an increase in weight gain compared to the standard diet, but no change in animals fed a high-fat diet without TAN. It has been recognized that the biological performance of flavonoids is dependent on their bioavailability, which can be very low due to the hydrophobic nature of these compounds. Glycosylated flavonoids, such as hesperidin, naringin, and eriocitrin, are mainly absorbed in the distal portion of the large intestine (colon), after deglycosylation by bacteria from the intestinal microbiota [23]. In contrast, polymethoxylated aglycones, such as NOB and TAN, show higher oral bioavailability than flavanones due to the lipophilic nature of multiple methoxy groups that decrease their hydrophilicity [28]. Such compounds are absorbed in the small intestine by passive diffusion, undergoing oxidative demethylation by cytochrome P450 in the intestinal wall (phase I metabolism), and can be conjugated by UGTs and SULTs enzymes (phase II metabolism). In the liver, phase II enzymes can produce glucuronate, sulfate, or methylate metabolites, which are much more hydrophilic. Part of them is secreted into the bile, performing the enterohepatic cycle, which increases the residence time and the effect of these compounds. Hepatic metabolites of NOB and TAN reach the bloodstream and peripheral tissues, and finally, they are eliminated by the urinary system or feces [2]. NOB and TAN are the main fraction of PMFs in the citrus peel, representing about 95% of the total PMF in balanced quantities [29]. However, they differ in bioaccessibility and absorption, with NOB being more bioavailable than TAN [5, 30]. Previously, we have shown

that oral administration of 50 mg/kg bw of NOB, solubilized in corn oil, produced a higher content of metabolites in the blood of rats than the same dose of TAN, suggesting greater bioavailability of NOB [31]. We also showed that NOB produced eight metabolites, four glucuronides, and four aglycones, but TAN produced only two, an aglycone and a glucuronic acid conjugate [31]. In the present study, we detected TAN and NOB metabolites in the livers of each supplemented group, demonstrating that oral administration of 200 mg/kg bw of TAN or NOB was bioaccessible. Chromatographic analysis of the liver, obtained from all groups, revealed two metabolites of TAN, a hydroxylated aglycone, and a glucuronide metabolite, while eight metabolites of NOB were detected, four hydroxylated aglycones, three glucuronides and one sulfate metabolite, but no parental compounds. Other authors have described similar quantities of TAN and NOB metabolites [5, 31-33]. In our study, a 170 times higher concentration of total NOB metabolites in the liver was found in comparison to TAN metabolites. These results suggest that NOB metabolites presumably reached the bloodstream improving the antioxidant capacity in the blood, while very low levels of TAN metabolites were transported out of liver cells. Some recent work has focused on the activity of the main metabolites of NOB and TAN, such as 5-dimethyl NOB [5-hydroxy-6,7,8,3’,4’-pentamethoxyflavone) and 5-dimethyl TAN (5-hydroxy-6,7,8,4’-tetramethoxyflavone). These compounds decreased lipid peroxidation and ROS production in Saccharomyces cerevisiae, a eukaryote model under oxidative stress [5]. Another study showed the anti-inflammatory effect of the most abundant colonic metabolite of NOB in mice, 3’,4’-didemethyl NOB (3’,4’-dihydroxy-5,6,7,8-tetramethoxyflavone), which was able to inhibit NO production and iNOS and COX-2 gene expression, by suppression of nuclear translocation of NF-κB and AP-1 transcription factors in RAW 264.7 macrophages [32]. Assessing the activity of antioxidant compounds in control animals helps to understand how to avoid oxidative stress and consequent biochemical and physiological damage. This is one of the main benefits of dietary supplements that attract consumers who seek to prevent the degenerative diseases due to aging. In this sense, our results

showed that healthy rats supplemented orally with NOB, increased the antioxidant capacity of the blood by 3%, assessed by ABTS, while reducing lipid peroxidation by 18%, assessed by blood MDA levels. In turn, the TAN did not show a significant change in the antioxidant capacity in the blood, but its intake was associated with a 22% reduction in serum MDA levels, suggesting a protective effect against lipid peroxidation. It is interesting to note that previously we had found similar protective effects against oxidative stress and inflammation from citrus flavanones to C57BL/6J mice fed a highfat diet [34]. The efficiency of oral intake of PMFs, with regard to the mechanism of action and safety, is another relevant topic to consider for their preventive use. Previous results indicate the benefits of NOB and TAN at a dose similar to this study, 200 mg/kg bw/day, without mentioning any toxic effects [6-7, 9]. However, these studies used experimental models of oxidative stress induced by chemical agents or diseases. For example, the neuroprotective role of TAN was investigated in an experimental model of pilocarpine-induced epilepsy in rats previously supplemented with 50, 100, or 200 mg/kg bw/day. It was observed that the TAN inhibited the lesions in a dose-dependent manner, and 200 mg/kg being the most effective dose to reduce neuronal loss, with the number of viable cells in the injured animals similar to the control group [9]. In another study, doses of 100 or 200 mg/kg bw/day of NOB administered intragastrically to rats for 9 days, before the induced ischemic brain injury, inhibited overproduction of pro-inflammatory cytokines, such as TNF-α, IL-1b, IL-6 and NO, suggesting anti-inflammatory and neuroprotective effects [6]. Intraperitoneal injection with 200 mg/kg bw of alcoholic extract containing NOB and TAN significantly attenuated brain damage in a rat ischemiareperfusion model [7]. It was previously mentioned that in the absence of oxidative damage induction, TAN did not influence any redox balance marker in the liver after four weeks of intervention [35]. These results are in accordance with the present study, which found no changes in the MDA or GPx in the liver of TAN-treated animals. In addition, experimental models of injury induction by drugs or pathologies showed that supplementation with TAN or NOB provided protection to the liver and

extrahepatic tissues, assessed by tissue and inflammatory markers. For example, restoration of superoxide dismutase (SOD), GPx, and catalase levels have been observed in the liver and other organs, and a reduction in systemic lipid peroxidation, with decreased levels of MDA and NO in the bloodstream, in mice previously treated with high doses of ethanol [3]. Pre-treatment with NOB in acute liver damage induced by concanavalin A in mice reduced the levels of liver enzymes alanine aminotransferase and aspartate transaminase, decreased the intracellular generation of ROS, and suppressed the release of inflammatory cytokines TNF-α and interferon gamma (IFN-γ) [24]. In another study with cisplatin-induced acute liver damage in rodents, TAN decreased TNF-α and interleukin 10 (IL-10) inflammatory markers, lipid peroxidation (MDA), inflammatory systemic NO responses and increased glutathione (GSH), and GPx activities, improving liver function [36]. It has been suggested that the mechanisms underlying TAN protection against hepatocyte-induced oxidative damage occur by the positive regulation of molecular signaling pathways, such as heme oxygenase 1 (HO-1), neurofibromatosis type 2 (Nrf2), NAD(P)H quinone dehydrogenase 1 (NQO-1) and mitogenactivated protein kinase (MAPK) [37]. NOB and TAN express greater anti-inflammatory potential than glycoside flavonoids, such as hesperidin and narirutin, with NOB having the greatest anti-inflammatory effect among all, and the apparent anti-inflammatory capacity of TAN is, very likely, due to its cytotoxicity [4]. NOB metabolites show antioxidant activity by inhibiting the production of intracellular ROS [38] and blocking the expression of the nitric oxide synthases (iNOS) and cyclooxygenase 2 (COX-2) protein in murine macrophages [32-33, 38]. In an inflammatory skin model, 3’,4’-dihydroxy-5,6,7,8-NOB inhibited MAPK and improved intracellular signaling pathway important in regulating the cell cycle (PI3K/Akt), leading to negative regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and decreasing the accumulation of ROS [39]. TAN metabolites were able to enhance antioxidant capacity and cell survival, as well as inhibit inflammatory responses through downregulation of NF-kB, TNF-α, INOS signaling pathways [39], and up-

regulation of Nrf2 expression that mediates these protective impacts [38]. Numerous studies confirm that exogenous antioxidants from dietary fruits and vegetables work together with endogenous antioxidants against oxidative stress [40]. Thus, it is expected that the supplements of TAN and NOB jointly with endogenous vitamins, such as α-tocopherol and retinol, will help to balance the antioxidant status in animals. However, in the present study, increased levels of retinol and α-tocopherol under TAN treatment, suggested that TAN may interfere in the balance of these vitamins. Previously it was shown that female rats exposed to dimethylbenz(a)-anthracene (DMBA) induced breast cancer, underwent a chemotherapeutic effect with TAN, protecting tissue damage, reducing lipid peroxidation, increasing antioxidant enzymes, and preserving antioxidant compounds, such as vitamin C and E [41]. On the other hand, the higher levels of antioxidant vitamins can be associated with an unbalance of the circulating antioxidants, which suggested some degree of toxicity for TAN. In a very interesting study, a U-shaped curve for the toxicity of TAN was detected, meaning that repetitive lower doses of TAN (50 to 100 mg/kg bw) can be as toxic to cells and tissues as acute and larger doses (1000 to 3000 mg/kg bw). According to these authors, supplementation of potential consumers with TAN will require much more extensive evaluations to establish its safety profile [13].

5. CONCLUSIONS

This study showed that systematic doses of NOB and TAN reduced lipid peroxidation, but only NOB increased the antioxidant capacity in the blood. Significant levels of NOB and TAN metabolites were found in the liver, suggesting that the parent compounds were absorbed and bio transformed after oral ingestion. High blood levels of retinol and α-tocopherol after ingestion of TAN showed a metabolic pathway distinct for this PMF. Therefore, we conclude that under physiological conditions, regular doses of TAN and NOB can improve antioxidant activity, but prolonged use of TAN

should be treated with caution, and broader studies of liver toxicity at different doses should be further explored, including clinical studies.

6. ACKNOWLEDGMENTS

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Considerações Finais

As PMF são amplamente metabolizadas após seu consumo, por via oral, possuem capacidade de reduzir os níveis dos biomarcadores de peroxidação lipídica sanguínea e a nobelitina foi capaz de ter uma atividade antioxidante sanguínea expressiva. Por meio deste estudo foi possível, também, identificar uma variedade de metabólitos gerados a partir da administração de TAN ou NOB, tanto em sua forma hidroxilada, quanto conjugadas a grupos sulfatos ou ao ácido glucurônico. Os tratamentos não alteraram os níveis de glutationa-peroxidase (GPx) porém, no grupo tratado com TAN, houve um aumento nos níveis de α-tocoferol e o retinol, sendo observada uma prevenção do gasto de antioxidantes já presentes no organismo dos ratos.

Para uma próxima etapa sugere-se o isolamento dos metabolitos encontrados neste estudo, além de testes *in vitro* e *in vivo* com estes, para a verificação de seus efeitos benefícios e de suas doses necessárias para essa obtenção.

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Anexo Comitê de Ética

Protocolo CEUA/FCF/CAr nº 42/2015
Interessada: MARILIA CAROLINE MARTINI RODRIGUES
Orientadora: Profa. Dra. Thais Borges César
Projeto: Biodisponibilidade e capacidade antioxidante da suplementação crônica da nobelitina, tangeritina e heptametoxiflavona em ratos

Parecer nº 68/2015 – Comissão de Ética no Uso de Animais

A Comissão de Ética no Uso de Animais da Faculdade de Ciências Farmacêuticas – UNESP (campus Araraquara), reunida em 12 de agosto de 2015, considerou que o protocolo para uso de animais na pesquisa: "Biodisponibilidade e capacidade antioxidante da suplementação crônica da nobelitina, tangeritina e heptametoxiflavona em ratos", apresentado pela pós-graduanda MARILIA CAROLINE MARTINI RODRIGUES, sob orientação da Professora Doutora Thais Borges César, do Departamento de Alimentos e Nutrição desta Faculdade, está estruturado dentro dos princípios éticos na experimentação animal do Conselho Nacional de Controle de Experimentação Animal - CONCEA, manifestando-se FAVORÁVEL à sua execução.

O relatório final do protocolo de pesquisa deverá ser entregue em JANEIRO de 2016 em formulário para este fim.

Araraquara, 17 de agosto de 2015.

Prof. Dr. CARLOS CESAR CRESTANI
Coordenador da CEUA