UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Engenharia de Alimentos

AMANDA ROGGIA RUVIARO

EFFECTS OF CITRUS RESIDUE BIOTRANSFORMATION IN VASCULAR PROTECTION

EFEITO DA BIOTRANSFORMAÇÃO DO RESÍDUO DE CITRUS NA PROTEÇÃO VASCULAR

CAMPINAS
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Thesis presented to the Faculty of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Food and Nutrition, in the area of Experimental Nutrition and Applied to Food Technology.

Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Alimentos e Nutrição, na área de Nutrição Experimental e Aplicada à Tecnologia de Alimentos.

Supervisor/ Orientador: Profª. Drª. Gabriela Alves Macedo

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA AMANDA ROGGIA RUVIARO, ORIENTADA PELA PROFa. DRa. GABRIELA ALVES MACEDO.

CAMPINAS
2018

Agência(s) de fomento e nº(s) de processo(s): CNPq, 140537/2015-5; FAPESP, 2015/04555-2

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Claudia Aparecida Romano - CRB 8/5816

R942e

Ruviaro, Amanda Roggia, 1990-
Effects of citrus residue biotransformation in vascular protection / Amanda Roggia Ruviaro. – Campinas, SP : [s.n.], 2018.

Orientador: Gabriela Alves Macedo.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. Antioxidantes naturais. 2. Hidrólise enzimática. 3. Hipertensão. 4. Polifenóis. 5. Tanase. I. Macedo, Grabriela Alves. II. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Efeito da biotransformação do resíduo de citrus na proteção vascular
Palavras-chave em inglês:
Antioxidants
Enzymatic hydrolysis
Hypertension
Polyphenols
Tannase
Área de concentração: Nutrição Experimental e Aplicada à Tecnologia de Alimentos
Titulação: Doutora em Alimentos e Nutrição
Banca examinadora:
Grabriela Alves Macedo
Thais Borges Cesar
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Data de defesa: 08-11-2018
Programa de Pós-Graduação: Alimentos e Nutrição

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A ata da defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

AGRADECIMENTOS

À Universidade Estadual de Campinas e ao Departamento de Alimentos e Nutrição, por permitirem a realização deste trabalho.

À minha orientadora Gabriela Alves Macedo, por nos incentivar a sermos melhor. Agradeço pela recepção, pela confiança, pelos ensinamentos e conselhos. Meu respeito e admiração.

À professora Dr.ª Juliana Alves Macedo, pelas contribuições, ensinamentos e carinho.

Ao professor Dr. Edson Antunes, pela colaboração, ensinamentos e valiosas sugestões para o desenvolvimento deste trabalho. Agradeço a toda a sua equipe do laboratório de Farmacologia da FCM/Unicamp, em especial ao Eduardo C. Alexandre, ao Alberto F. O. Justo e à Gláucia Mello, pela paciência, pela parceria e por toda a ajuda para a realização dos experimentos.

A todas as pessoas que tive o prazer de conviver na Faculdade de Engenharia de Alimentos, incluindo professores, funcionários e alunos pela atenção e auxílio prestados durante essa etapa.

Aos colegas e amigos Laboratório de Bioprocessos e do Laboratório de Compostos Bioativos: Karina Leão, Lívia Reis, Amanda Ávila, Paula Barbosa, Isabela Martins, Vânia Nakajima, Annayara Fernandes, Renata Gandra, Lívia Dias, Débora Kono, Bruninha Sampaio, Naice Monteiro, Camila Rubia, Júlia Zunin, Vânize Genova, Jeane Melo, Dani Branta, Paula Speranza, Mônica Carmo, Tais Moala, Tauan Gomes, Aline Rodrigues, Andressa Vianna, pelos ensinamentos, pela troca de experiências, pela paciência e disposição em ajudar sempre. Agradeço pelos finais de semana de laboratório e os de lazer, pelos cafés na praça, pelos bolos da tarde, pela cia de bandejão. Foi um prazer conviver e aprender com vocês. Um agradecimento especial à Paulinha, minha companheira de bancada. Obrigada pela paciência comigo e pela parceria nesses anos todos.

Ao Chico, por toda a ajuda, colaboração e momentos de descontração. A Cidinha, por ser tão atenciosa e solícita.

Aos meus amados pais, Maria Tereza e Rosmeri, e irmão, Heitor: a saudade é diária. Agradeço por compreende minha ausência e me receber sempre de braços abertos. Agradeço por incentivar minhas escolhas e por não medirem esforções para estar comigo em todos os momentos.

Às minhas queridas avós, Dona Edy (*in memorian*) e Vó Dóris, pelo carinho, consolo, paciência e amor sem medidas.

A todos os meus familiares, especialmente à Lísia, Maurício, Miguelito, tia Rozi e tio Erlon por me abraçarem em Campinas.

Ao meu namorado Igor, agradeço pela paciência, amor, carinho, compreensão e parceria, tornando meus dias mais leves e felizes. Obrigada pelo apoio e pelas palavras de incentivo constantes. Agradeço a sua família por me receber de braços abertos e com muito carinho.

Aos amigos Lívia, Karinex, Geina, Aline, Géssika, Andressita, Tassia, Lalá, Natássia, Felipe, Elson, Erick. Vocês tornaram minha estadia em Campinas muito mais feliz. Só posso agradecer pela amizade e carinho.

Às minhas *homemates*, Ana Laura Tibério, Fernanda Ortolan, Kemilla Rebelo e Gláucia Quinete, pela paciência, tolerância, companhia e compreensão.

Aos meus amigos de longa data, agradeço pela cumplicidade. Mesmo de longe sempre se fizerem presentes.

Aos membros da banca examinadora, pela disponibilidade e colaboração com o trabalho.

À Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP, pelo apoio financeiro através do projeto de auxílio à pesquisa [2015/04555-2].

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, pela concessão da bolsa de estudo no Brasil e da taxa de bancada [140537/2015-5].

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

Certa que não se chega ao sucesso sozinha, registro aqui meu agradecimento sincero a todos aqueles que não foram mencionados, mas que tiveram, de alguma forma, participação nesse trabalho.

"Optimism is the best way to see life"

ABTRACT

Brazil is the world's largest orange juice producer, resulting in the production of tons of industrial citrus waste. These residues are considered an important source of phenolic compounds, especially flavanones. Phenolic compounds are known due to their bioactive effects, which involve their antioxidant properties and cardiovascular protection. Recent studies have shown that phenolics in the aglycone form exhibited greater bioactivity than in the glycosidic form. However, these studies use high cost analytical standards, since there are no commercial sources of these aglycones. One of the strategies to produce extracts rich in aglycones is the enzymatic biotransformation. Based on this, this study aimed to evaluate the effects of enzymatic biotransformation on the antioxidant and antihypertensive potential of citrus extracts from industrial citrus wastes. Two citrus residues were studied, one from the extraction of orange juice and another from pectin extraction. Different enzymatic processes were applied using the enzymes cellulase, pectinase, tannase and β-glucosidase alone or in combination. The phenolic profile of the citrus extracts was analyzed by HPLC-DAD. The antioxidant potential of the extracts was evaluated by Folin-Cicalteou, ORAC, DPPH and FRAP assays. The in vitro antihypertensive potential of the extracts was evaluated by the inhibition of the angiotensin converting enzyme (ACE) and by the vasorelaxant effect in rat iliac artery rings. Parallel tests were conducted using analytical standards and commercial drugs to elucidate the pathways involved in the antihypertensive effect of citrus extracts. The results showed that extracts of pectin extraction residues showed higher concentration of hesperidin in relation to extracts of juice residues. HPLC analysis clearly showed the changes in the phenolic profile of citrus extracts after the enzymatic treatment with tannase and β-glycosidase, leading to the production of 3044 and 5338 μg/ g hesperetin from extracts of juice and pectin residues, respectively. Among the extracts studied, the extract of pectin residues biotransformed presented higher antioxidant activity and was the most effective in inhibiting ACE activity (Emax = 100% and IC_{50} = 0.21 mg/ ml). This extract was also the most efficient in inducing vasorelaxation (Emax = 100% and EC_{50} = 0.47 mg/ ml). Our results confirmed the higher anti-ACE (100% and 76%) and vasorelaxant (EC_{50} = 20 μM and 1.3 mM) effects of the hesperetin aglycone in relation to the hesperidin glycoside, justifying the best results of biotransformed extracts rich in flavanones aglycones. The vasorelaxation induced by extracts from pectin extraction residues was independent of the endothelium. Furthermore, relaxation was potentiated when the GCs enzyme was oxidized due to oxidative stress, being comparable to BAY58-2667. Thus, this work showed that enzymatic biotransformation can be used to reduce and reuse residues from the world's largest citrus processing industry, since they could be used in the pharmaceutical and food industry as ingredients for the development of products for the prevention and in the treatment of vascular diseases.

Keywords: antioxidant; enzymatic hydrolysis; hypertension; polyphenols, tannase.

RESUMO

O Brasil é considerado o maior produtor mundial de suco de laranja, resultando na produção de toneladas de resíduos industriais de citrus. Esses resíduos são considerados uma fonte importante de compostos fenólicos, especialmente de flavononas. Os compostos fenólicos são conhecidos devido aos seus efeitos bioativos, que envolvem suas propriedades antioxidantes e de proteção cardiovascular. Estudos recentes têm mostrado que os fenólicos na forma aglicona apresentam maior bioatividade de que na forma glicosídica. Entretanto, esses estudos utilizam padrões analíticos de alto custo, já que não existem fontes comerciais dessas agliconas. Uma das estratégias empregada para produzir extratos ricos em agliconas é a biotransformação enzimática. Baseado nisso, esse estudo teve como objetivo avaliar os efeitos da biotransformação enzimática no potencial antioxidante e anti-hipertensivo de extratos cítricos preparados a partir de resíduos industriais de citrus. Dois resíduos cítricos foram estudados, um proveniente da extração de suco de laranja e outro resíduo proveniente da extração de pectina. Diferentes processos enzimáticos foram aplicados empregando as enzimas celulase, pectinase, tanase e β-glicosidase de forma isolada ou combinada. O perfil fenólico dos extratos cítricos foi analisado por HPLC-DAD. O potencial antioxidante dos extratos foi avaliado pelos métodos de *Folin-Cicalteou*, ORAC, DPPH e FRAP. O potencial anti-hipertensivo *in vitro* dos extratos foi avaliado pela inibição da enzima conversora de angiotensina (ECA) e pelo efeito vasorelaxante em anéis de artéria ilíaca de rato. Testes paralelos foram conduzidos utilizando padrões analíticos e drogas comerciais para elucidar os mecanismos envolvidos no efeito anti-hipertensivo dos extratos de cítricos. Os resultados mostraram que os extratos dos resíduos da extração de pectina apresentaram maior concentração de hesperidina em relação aos extratos dos resíduos de suco. A análise por HPLC mostrou claramente a mudança do perfil fenólico dos extratos de cítricos após o tratamento enzimático com tanase e β-glicosidase, levando a produção de 3,044 e 5,338 μg/ g de hesperetina a partir dos resíduos da extração de suco e de pectina, respectivamente. Entre os extratos estudados, o extrato dos resíduos de pectina biotransformado apresentou maior atividade antioxidante e foi o mais eficaz em inibir a atividade da ECA ($E_{máx}$ = 100% e IC_{50} =0,21 mg/ ml). Esse extrato também foi o mais eficiente em induzir o relaxamento de vasos ($E_{máx}$ = 100% e EC_{50} =0,47 mg/ ml). Nossos resultados confirmaram o maior potencial anti-ACE (100% e 76%) e vasorelaxante (EC_{50} = 20 μM e 1,3 mM) da aglicona hesperetina em relação ao glicosídeo hesperidina, justificando os melhores resultados dos extratos biotransformados ricos em flavononas agliconas. O vasorelaxamento induzido pelos extratos dos resíduos da extração de pectina foi independente do endotélio. Ainda, o relaxamento foi potencializado quando a enzima GCs estava oxidada devido ao estresse oxidativo, sendo comparável ao BAY58-2667. Assim, esse trabalho mostrou que a biotransformação enzimática pode ser utilizada para a redução e reutilização de resíduos da maior indústria processadora de citrus do mundo, podendo ser utilizados na indústria farmacêutica e alimentícia como ingredientes para o desenvolvimento de produtos para a prevenção e para auxiliar no tratamento de doenças vasculares.

Palavras chave: antioxidante; hidrólise enzimática; hipertensão; polifenóis, tanase.

LISTA DE ABREVIATURAS E SIGLAS – CAPÍTULO I

Ach: acetilcolina

AT-1: receptores de tipo I de angiotensina II

AV**C**: acidente vascular cerebral

Ca^{2+}: cálcio

CaM: calmodulina

DCVs: doenças cardiovasculares

DNT(s): doenças não transmissíveis

NCD(s): non communicable diseases

ECA: enzima conversora de angiotensina

EDCFs: fatores de constrição derivados do endotélio

EDRFs: fatores de relaxamento dependente do endotélio

EROs: espécies reativas de oxigênio

GCs: guanilato ciclase solúvel

GMPc: guanosina monofosfatada cíclica

GTP: guanosina trifosfatada

H_2O_2 : peróxido de hidrogênio

HA: hipertensão arterial

mm Hg: milímetros de mercúrio

NO: óxido nítrico

NOS: oxido nítrico-sintase

$O2^{\bullet -}$: ânion superóxido

$OH^{\bullet}$: radical hidroxila

$ONOO^{\bullet -}$: peroxinitrito

PA: pressão arterial

SRAA: sistema renina-angiotensina-aldosterona

VSMC: vascular smooth muscle cells

LISTA DE ABREVIATURAS E SIGLAS – CAPÍTULO II

CJB: citrus juice by-products

CPB: citrus pectin by-products

DM: dry matter

DPPH: DPPH radical scavenging capacity

GAE: gallic acid equivalent

HPLC: High performance liquid chromatography

ORAC: oxygen radical absorption capacity

TE: trolox equivalent

TPC: Total polyphenols content

LISTA DE ABREVIATURAS E SIGLAS – CAPÍTULO III

B: β-glucosidase

BCP: β-glucosidase + cellulase + pectinase

C: cellulase

CJB or CJBs: citrus juice by-products

CP: celulase + pectinase

DM: dry matter

DMSO: dimethyl sulfoxide

DPPH: DPPH radical scavenging capacity

GAE: gallic acid equivalent

h: hour

HPLC: High performance liquid chromatography

n.d.: not detected

ORAC: oxygen radical absorption capacity

P: pectinase

RT: reaction time

T: tannase

TCP: tanase + cellulase + pectinase

TE: trolox equivalent

TPC: Total polyphenols content

tr: traces

LISTA DE ABREVIATURAS E SIGLAS – CAPÍTULO IV

ACE: angiotensin-converting enzyme

CJR: citrus juice residue extract

CJRTB: citrus juice residue extract treated with tannase and β-glucosidase

CPR: citrus pectin residue extract

CPRTB: citrus pectin residue extract treated with tannase and β-glucosidase

DPPH: DPPH radical scavenging capacity

$E_{max:}$ maximum effect

FRAP: ferric reducing antioxidant power

GAE: gallic acid equivalent

HA: hippuric acid

HPLC: High performance liquid chromatography

$IC_{50:}$ the half maximal inhibitory concentration

LE: lyophilized extract

ORAC: oxygen radical absorbance capacity

TB: tannase + β-glucosidase

TE: trolox equivalent

TPC: Total polyphenols content

LISTA DE ABREVIATURAS E SIGLAS – CAPÍTULO V

Ach: acetylcholine chloride

cGMP: cyclic guanosine monophosphate

DMSO: dimethyl sulfoxide

DPPH: DPPH radical scavenging capacity

E−: endothelium-denuded

E+: endothelium-intact

$EC_{50:}$ negative logarithm of the half maximal effective concentration

$E_{max:}$ maximal response effect

eNOS: enzyme endothelial NO synthase

FRAP: ferric reducing antioxidant power

Hd: hesperidin

HPLC: High performance liquid chromatography

Ht: hesperetin.

$IC_{50:}$ half maximal effective concentration in mg per ml

JUI: crude extract of citrus juice by-products

JUIE: biotransformed extract of citrus juice by-products

KCl: potassium chloride

KH: Krebs-Henseleit solution

LE: lyophilized extract

NO: nitric oxide

O_2•: superoxide anion

ODQ: 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one

PE: (R)-(-)phenylephrine hydrochloride

PEC: crude extract of citrus pectin by-products

$pEC_{50:}$

PECE: biotransformed extract of citrus pectin by-products

ROS: reactive oxygen species

sGC: soluble guanylyl cyclase

SNP: sodium nitroprusside

TE: trolox equivalents

VSMCs: vascular smooth muscle cells

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INTRODUÇÃO GERAL

De acordo com os dados da Organização Mundial da Saúde (2011;2017) e com a Organização Pan-Americana da Saúde (2017), estima-se que as Doenças Cardiovasculares (DCVs) causam aproximadamente 7.5 milhões de mortes no mundo todo.

As DCVs englobam várias doenças, como a hipertensão e as doenças vasculares, as quais estão associadas a uma disfunção endotelial e ao aumento do estresse oxidativo vascular. Esses eventos levam a perda da integridade do endotélio vascular, que é responsável pela secreção de inúmeras substâncias vasoativas que regulam a pressão arterial, como o óxido nítrico. Além disso, o aumento na produção de radicais livres no meio vascular inibe a ação de substâncias vasorelaxantes e favorecem a ação de agentes vasoconstritores. Como consequência, a homeostase vascular é comprometida, favorecendo o desenvolvimento de doenças que afetam o sistema vascular. (BLEAKLEY et al., 2015; GONZÁLEZ, 2014; VASCONCELOS et al., 2007).

Devido as diversas complicações associadas à hipertensão e às doenças que envolvem o sistema vascular, além da elevada prevalência dessas doenças, estas são consideradas problemas de saúde pública. Desta forma, estratégias e produtos têm sido estudados como o objetivo de prevenir e auxiliar no tratamento de doenças vasculares e suas complicações. Entre os compostos estudados, os fenólicos presentes nos alimentos de origem vegetal tem recebido grande atenção nos últimos anos.

As frutas cítricas são reconhecidas como uma importante fonte de compostos fenólicos. A laranja ganha destaque nesse trabalho devido à sua importância comercial no Brasil, especialmente no estado de São Paulo. O Brasil ainda é considerado o maior produtor de laranja do mundo, atingindo uma produção de aproximadamente 17 mil toneladas na última safra (FAOSTAT, 2018). A produção de laranja destina-se principalmente para a produção do suco de laranja, sendo que o Brasil é responsável por mais de 80% do total de exportações de suco de laranja (NEVES et al., 2010). Os resíduos sólidos gerados após a extração do suco correspondem a aproximadamente 50% da massa da fruta e são compostos principalmente por casca e bagaço. Esses resíduos são destinados à produção de diferentes subprodutos, sendo utilizados principalmente como componentes de ração animal.

Os resíduos de cítricos são uma fonte promissora de compostos bioativos, uma vez que os compostos fenólicos são encontrados principalmente na casca e nas paredes vegetais (ABEYSINGHE et al., 2007; ESCOBEDO-AVELLANEDA et al., 2014; SUN et al., 2010). Assim, estudos recentes tem dado destaque para os resíduos gerados pela indústria

processadora, especialmente pela alta concentração das flavanonas hesperidina e naringenina, além de outros fenólicos minoritários, como ácido elágico, diosmetina e tangeritina (BARBOSA; RUVIARO; MACEDO, 2018; PEREIRA et al., 2017; SUN et al., 2010).

Os efeitos bioativos dos glicosídeos hesperidina e naringina encontrados naturalmente em frutos cítricos e seus subprodutos vem sendo relatado por diversos estudos (BENAVENTE-GARCÍA; CASTILLO, 2008; DI MAJO et al., 2005; KIM; KIM, 2016; LÓPEZ-CARRERAS et al., 2015; SUN et al., 2010; TRIPOLI et al., 2007; ZOU et al., 2016). Entretanto, estudos *in vitro* têm demonstrado que na forma aglicona essas flavanonas apresentam maior efeito biológico. Esse efeito tem sido associado a maior biodisponibilidade (LONDOÑO-LONDOÑO et al., 2010), maior potencial antioxidante (FERREIRA et al., 2013; MADEIRA; MACEDO, 2015), maior efeito vasorelaxante e de proteção cardiovascular (CALDERONE et al., 2004; ORALLO; ÁLVAREZ; BASARAN, 2004; ORALLO; CAMIÆA, 2005) das agliconas em relação aos seus glicosídeos. De Souza et al. (2016) compararam os efeitos *in vivo* da hesperetina e suas formas glicosiladas em ratos. Os autores notaram que a suplementação com a aglicona hesperetina aumentou a defesa antioxidante do organismo, sugerindo uma associação entre a biodisponibilidade da hesperetina com o efeito antioxidante potencializado no organismo.

As agliconas raramente são encontradas em frutas cítricas e seus derivados. Além de serem fenólicos de difícil obtenção, não existem fontes comerciais desses compostos. Assim, os estudos que avaliam os efeitos dessas agliconas costumam utilizar padrões analíticos de alto custo. Diante disso, se faz necessário à busca por fontes naturais de agliconas, como a hesperetina e a naringenina, e a realização de testes biológicos que demonstrem seu efeitos bioativos.

Levando isso em consideração, nosso grupo de pesquisa tem estudado processos biotecnológicos com o objetivo de aumentar a extração das flavanonas agliconas a partir de resíduos da indústria cítrica (MADEIRA et al., 2014; MADEIRA; MACEDO, 2015; NAKAJIMA et al., 2016; RUVIARO; BARBOSA; MACEDO, 2018). Esses estudos destacam a importância de tratamentos enzimáticos para obtenção de hesperetina e naringenina a partir de fontes naturais de fácil obtenção e de baixo custo.

Quando se fala em processo biotecnológico, a biotransformação enzimática tem se mostrado um método viável para extração de fenólicos de diferentes resíduos agroindustriais, além de ser considerado seguro para o manipulador e *eco-friendly*, já que minimiza o uso de compostos tóxicos na extração, como os solventes orgânicos (MADEIRA; TEIXEIRA; MACEDO, 2015; RADENKOVS et al., 2018). Uma enzima que aparece como alternativa

para aumentar o potencial bioativo de diferentes resíduos agroindustriais é a tanase. Sabe-se que essa enzima é capaz de agir em fenólicos complexos e atuar em ligações glicosídicas entre sacarídeos e fenólicos glicosilados (FERREIRA et al., 2013).

Levando tudo isso em consideração, dois resíduos industriais do processamento de citrus foram avaliados no presente estudo. Os resíduos da indústria processadora de suco de laranja, que são provenientes do que sobrou da extração de suco, e contém casca, resíduos da polpa (membranas e gomos) e sementes não recuperadas, além de pectina. E os resíduos da indústria de extração de pectina, que são provenientes da extração de pectina a partir dos resíduos anteriores.

Avaliou-se o efeito da biotransformação enzimática, empregando diferentes enzimas, no potencial antioxidante e anti-hipertensivo *in vitro* de extratos ricos em compostos fenólicos elaborados a partir desses resíduos. Além disso, foram realizados testes paralelos com padrões analíticos e drogas comerciais, a fim de elucidar os mecanismos de ação envolvidos no potencial bioativo dos extratos produzidos.

Esse trabalho teve como objetivo encontrar alternativas para a redução e reutilização de resíduos da maior indústria processadora de citrus do mundo, contribuindo para questões ambientais e econômicas. Além disso, tivemos como interesse produzir extratos ricos em agliconas e com alto potencial bioativo, que podem ser utilizados na indústria farmacêutica e alimentícia como ingredientes para o desenvolvimento de produtos para a prevenção e para auxiliar no tratamento de doenças vasculares, como a hipertensão.

Essa tese está organizada em cinco capítulos. No primeiro capítulo é apresentada uma revisão bibliográfica, que envolve os temas de Doenças Cardiovasculares, fenólicos cítricos e o processo de biotransformação enzimática.

O segundo capítulo é baseado no artigo “Comparison of different Brazilian citrus by-products as source of natural antioxidants” publicado na revista Food Science and Biotechnology, o qual descreve a caracterização fenólica dos dois tipos de resíduos cítricos industriais, um resíduo proveniente do processo de extração do suco de laranja e um resíduo proveniente do processo de extração de pectina.

O terceiro capítulo, baseado no artigo “Enzyme-assisted biotransformation increases hesperetin content in citrus juice by-products” publicado na revista Food Research International, descreve a aplicação de diferentes processos enzimáticos em resíduos da indústria processadora de citrus para obtenção de compostos fenólicos com maior bioatividade.

O quarto capítulo, baseado no artigo “Flavanones biotransformation of citrus by-products improves antioxidant and ACE inhibitory activities in vitro” submetido para a revista “Journal of Functional Foods”, apresenta os resultados da atividade antioxidante e anti-hipertensiva de extratos cítricos biotransformados.

O quinto capítulo, baseado no artigo “Aglycone-rich extracts from citrus by-products induced Endothelium-Independent relaxation in isolated arteries” que será submetido à revista Food Chemistry, avalia o potencial antioxidante e vasorelaxante de extratos de cítricos biotransformados.

CAPÍTULO I. REVISÃO BIBLIOGRÁFICA

1. INTRODUÇÃO

As doenças cardiovasculares (DCVs) são classificadas como doenças não transmissíveis (DNT) e incluem doenças do coração, doenças cerebrovasculares e doenças dos vasos sanguíneos. As DCVs ainda são consideradas a principal causa de morte do mundo, sendo que aproximadamente 17,7 milhões de pessoas morreram devido as DCVs em 2015, o que representa 31% de todas as mortes em nível global (WHO, 2011; IFPMA, 2016; Opas, 2017).

Sabe-se que inúmeros fatores de risco estão associados ao desenvolvimento dessas doenças (Figura 1). Entre eles, os fatores de risco comportamentais mais significantes são a dieta inadequada, o sedentarismo, o tabagismo e o uso abusivo de álcool, os quais podem se causar nos indivíduos hiperglicemia, sobrepeso, obesidade, hipercolesterolemia e, principalmente, hipertensão arterial (HA). Estima-se que, do total de mortes provocadas pelas DCVs, mais da metade (aproximadamente 9,4 milhões) são causadas por complicações devido à hipertensão, considerada um dos principais fatores envolvidos no acidente vascular cerebral (AVC), nas cardiopatias isquêmicas e no infarto agudo do miocárdio (AHA; 2017; WHO, 2011, 2013).

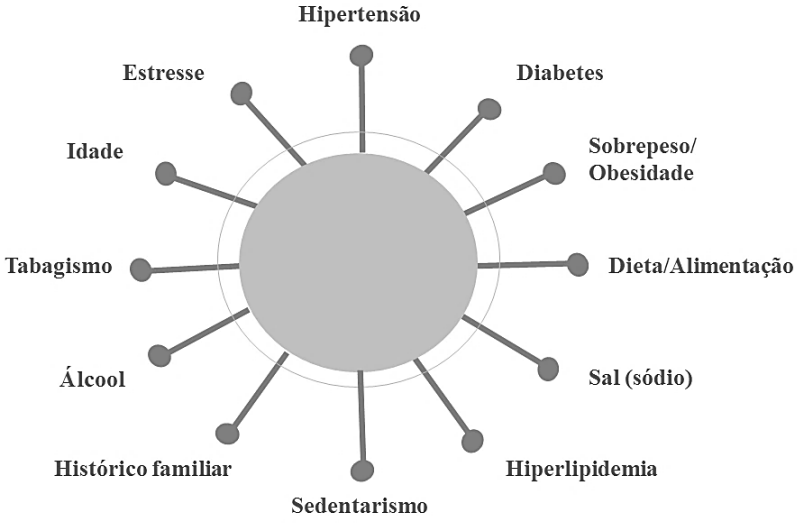


Figura 1. Fatores de risco associados ao desenvolvimento de Doenças Cardiovasculares. Fonte: Adaptado de (AHA, 2017; WHO, 2013)

Em 2013, a Organização Mundial da Saúde desenvolveu o projeto "*Global Action Plan for the Prevention and Control of NCDs 2013-2020*" com o objetivo de reduzir a mortalidade por doenças não transmissíveis, entre elas as DCVs, em 25% até 2025. A hipertensão foi identificada como uma medida fundamental para alcançar o objetivo do projeto, sendo que a redução da prevalência da hipertensão e o controle da pressão arterial (PA) foram citados em uma das nove metas do Plano de Ação Global da OMS (WHO, 2013).

No ano de 2017, uma nova Diretriz de Hipertensão foi criada pela Associação Americana do Coração com o objetivo de auxiliar na prevenção, detecção, avaliação e no tratamento de pressão alta em adultos. Essa nova diretriz estabeleceu novas categorias de PA (tabela 1) para implementação nos Sistemas de Saúde Pública e facilitar a conduta clínica do profissional da saúde. Nota-se que houve uma mudança na definição da HA, que agora é considerada como qualquer medida de PA sistólica de ≥ 130 milímetros de mercúrio (mm Hg) ou qualquer medida de PA diastólica ≥ 80 mm Hg (AHA, 2017).

Tabela 1. Categorias de Pressão Arterial.

Categoria	Pressão sistólica		Pressão diastólica
Normal	< 120 mm Hg	*e*	< 80 mm Hg
Elevada	120-129 mm Hg	*e*	< 80 mm Hg
Hipertensão: estágio 1	130-139 mm Hg	*ou*	80-89 mm Hg
Hipertensão: estágio 2	≥ 140 mm Hg	*ou*	≥ 90 mm Hg

Fonte: American Heart Association (2017)

Além de ser considerado um fator de risco para o desenvolvimento de outras doenças, a HA se caracteriza por ser uma doença crônica de caráter multifatorial, que provoca alteração na homeostase dos vasos, promovendo um desequilíbrio entre a vasodilatação e a vasoconstrição. Tendo conhecimento de que HA é um problema de saúde pública, reconhecer os fatores associados ao seu desenvolvimento é de grande importância para que se possa atuar de forma adequada na prevenção e tratamento dessa doença.

2. SISTEMA CARDIOVASCULAR

O sistema circulatório é responsável pela circulação do sangue por todo o organismo. Os vasos sanguíneos funcionam como ductos de transporte de sangue e nutrientes, sendo a principal ligação entre o coração e os tecidos do corpo (Sandoo, Veldhuijzen van Zanten, Metsios, Carroll, & Kitas, 2010).

A parede vascular de artérias e veias é composta de três camadas: (1) A túnica íntima é a camada mais interna formada por células endoteliais, e está em contato direto com o sangue; (2) a túnica média é uma camada intermediária formada por células musculares lisas, inseridas em uma matriz de colágeno, elastina e glicoproteínas; (3) a túnica adventícia é a camada mais externa, formada por tecido conjuntivo rico em fibras colágenas, elásticas, fibroblastos e macrófagos, como ilustrado na figura 2 (Sandoo et al., 2010).

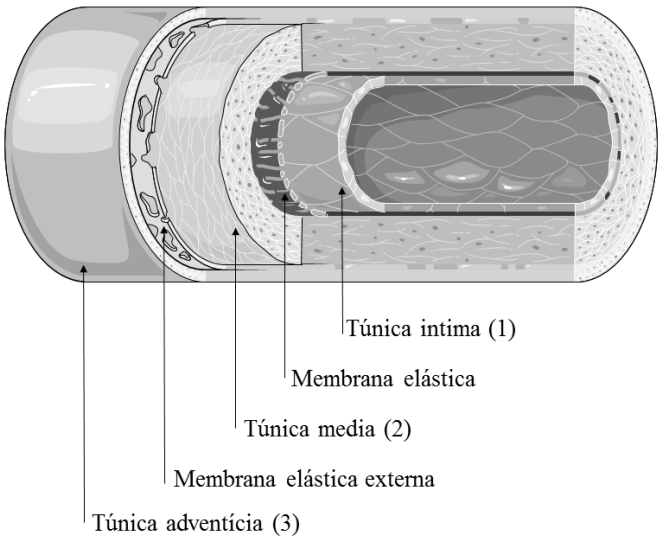


Figura 2. Imagem representativa das três camadas da parece vascular: íntima, média e adventícia. Fonte: Adaptado de Servier Medical Art – Powerpoint Image Bank.

A integridade do endotélio vascular é essencial à homeostase vascular, uma vez que atua não só como uma barreira entre a circulação sanguínea e a parede vascular, como também exerce um papel fundamental na regulação do tônus vascular (Batlouni, 2001; Sandoo et al., 2010).

O endotélio é responsável pela síntese e liberação de diversas substâncias vasoativas, que agem nas células musculares lisas vasculares levando a vasodilatação ou

vasoconstrição. As substâncias liberadas pelo endotélio são conhecidas como fatores de relaxamento dependente do endotélio (EDRFs) e fatores de constrição derivados do endotélio (EDCFs). O óxido nítrico (NO) é considerado um dos principais EDRFs, enquanto que a angiotensina II é um importante EDCFs (Bleakley, Hamilton, Pumb, Harbinson, & Mcveigh, 2015).

A acetilcolina (Ach) é uma substância que induz a vasodilatação dependente do endotélio via produção de fatores endoteliais, como o NO. Fisiologicamente, a Ach é um agonistas que interage com receptores muscarínicos (M3) da célula endotelial intacta, gerando um aumento de cálcio intracelular (Ca^{2+}), que se liga a calmodulina (CaM) para formar o complexo Ca^{2+}-CaM, que por sua vez atua na ativação da enzima oxido nítrico-sintase (NOS) para produzir NO (González, 2014; Sandoo et al., 2010).

A NOS atua na conversão do aminoácido *L*-arginina em NO e citrulina. Existem diferentes isoformas da enzima NOS, classificadas de acordo com seu tecido de origem. A eNOS localiza-se nas células endoteliais, sendo responsável pela produção de NO nos vasos sanguíneos (Cerqueira & Yoshida, 2002; Sandoo et al., 2010). O NO desempenha os inúmeros efeitos fisiológicos vasculares, atuando na inibição da inflamação por inibir a adesão de leucócitos no endotélio vascular, além de controlar a migração e proliferação de células da musculatura lisa vascular (VSMC - *vascular smooth muscle cells*). O NO também exerce papel importante na coagulação sanguínea por controlar a agregação plaquetária. Além disso, atua no controle da pressão arterial por agir nas vias de regulação do tônus vascular, antagoniza os efeitos vasoconstritores da angiotensina II e controla a síntese da enzima conversora de angiotensina (ECA). Assim, além de ser um potente vasodilatador, o NO desempenha um papel essencial na manutenção da homeostase vascular (Bleakley et al., 2015; Ferroni, Basili, Paoletti, & Davì, 2006; González, 2014; Vasconcelos, Goulart, Silva, & Gomes, 2007).

Uma vez sintetizado no endotélio, o NO se difunde até o músculo liso adjacente, ativando a enzima guanilato ciclase solúvel (GCs), que por sua vez catalisa a conversão da guanosina trifosfatada (GTP) em guanosina monofosfatada cíclica (GMPc). O aumento dos níveis de cGMP no citoplasma da célula muscular lisa desencadeia uma cascata de reações que reduzem a concentração de Ca^{2+} intracelular ($[Ca^{2+}]_i$), levando ao relaxamento do vaso, como ilustrado na figura 3 (Bleakley et al., 2015; Cerqueira & Yoshida, 2002; González, 2014; Sandoo et al., 2010).

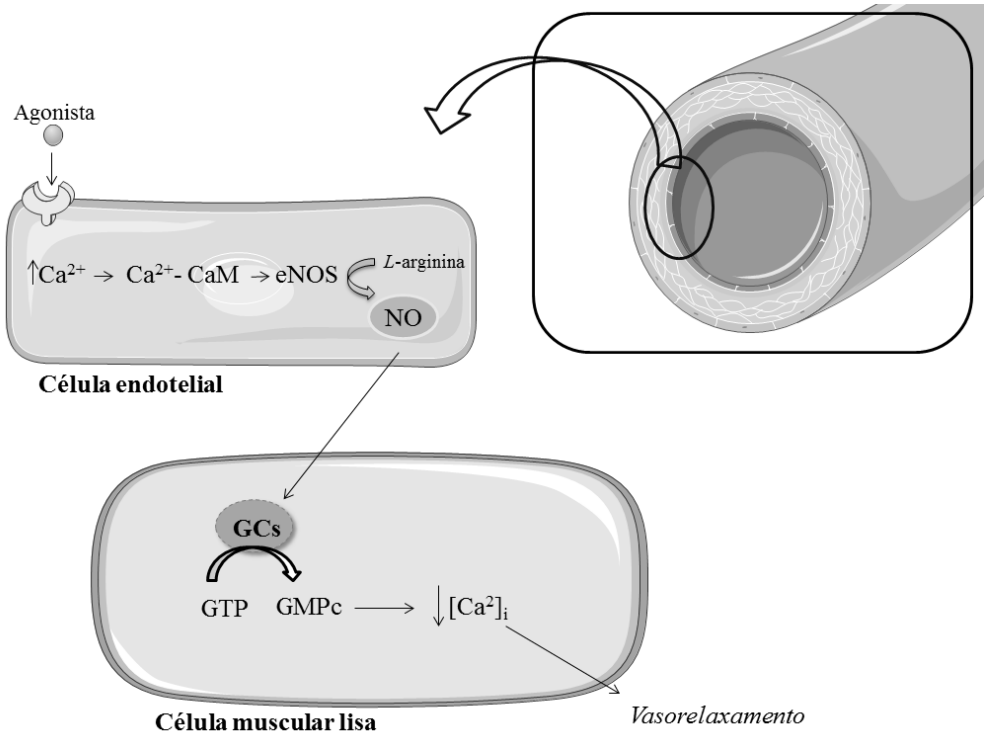


Figura 3. Esquema ilustrativo da produção de óxido nítrico endotelial, liberação para o músculo liso e ação vasodilatadora no sistema vascular. Fonte: Adaptado de Sandoo et al. (2010) e Servier Medical Art – Powerpoint Image Bank.

3. HIPERTENSÃO ARTERIAL

Muitos estudos evidenciam que a HA é uma condição que se caracteriza por uma disfunção endotelial associada ao estresse oxidativo, levando a alterações estruturais e funcionais do sistema vascular que reduzem a biodisponibilidade e a bioatividade do NO na parede vascular. Essa depleção de NO é considerada prejudicial à saúde vascular devido ao seu papel fundamental na manutenção da homeostase vascular (Bleakley et al., 2015; González, 2014; Vasconcelos et al., 2007).

As alterações estruturais e funcionais do sistema vascular em resposta às mudanças hemodinâmicas para o controle da pressão arterial envolvem o aumento da resistência vascular, devido à hipertrofia da parede arterial, e à redução do lúmen das artérias. Esses eventos provocam uma inflamação associada à produção excessiva de radicais livres no meio vascular, os quais interferem na síntese e liberação de fatores vasoativos, comprometendo principalmente os efeitos fisiológicos do NO (Bleakley et al., 2015; González, 2014; Renna, De Las Heras, & Miatello, 2013).

O estresse oxidativo se caracteriza pelo desequilíbrio entre agentes oxidantes e antioxidantes, levando ao excesso de produção de espécies reativas de oxigênio (EROs). Essas espécies oxidantes incluem radicais livres como $O2^{\bullet-}$ (ânion superóxido), $ONOO^{\bullet-}$ (peroxinitrito) e $OH^{\bullet}$ (radical hidroxila), além de moléculas não radicais, como H_2O_2 (peróxido de hidrogênio) (Siti, Kamisah, & Kamsiah, 2015; Wen, Gwathmey, & Xie, 2012).

Em condições fisiológicas, os ROS são gerados naturalmente como subproduto no metabolismo do oxigênio e desempenham papéis importantes na sinalização celular. (Wen et al., 2012). Entretanto, a produção excessiva de ROS está relacionada com a disfunção de vários tecidos, inclusive do coração e de vasos sanguíneos, exercendo um papel importante na disfunção endotelial, no remodelamento vascular, na hipóxia, no envelhecimento e no desenvolvimento da hipertensão.

No tecido vascular, o estresse oxidativo provoca um aumento na produção de $O2^{\bullet}$, que reage com o NO e forma o $ONOO^{\bullet-}$, reduzindo a biodisponibilidade de NO na parede arterial. Essa menor biodisponibilidade de NO favorece a ação de substâncias vasoconstritoras, como a angiotensina II, e promove crescimento de células endoteliais. Esses efeitos levam à disfunção endotelial e contribuem para a o desenvolvimento da HA (Ferroni et al., 2006; González, 2014; Vasconcelos et al., 2007; Wen et al., 2012).

Diferentes vias estão envolvidas na produção de ROS no sistema vascular. A via não enzimática envolve o stress do fluxo sanguíneo turbulento e o estiramento vascular devido a maior pressão no lúmen vascular. Em relação às vias enzimáticas, a própria eNOS, responsável pela produção endotelial de NO, pode gerar radias livres quando há deficiência do substrato L-arginina. Esse fenômeno tem sido referido como desacoplamento de eNOS. Contudo, o sistema enzimático NAD(P)H oxidase é considerado a maior responsável pela geração de $O2^{\bullet-}$ nas membranas das células endoteliais e musculares lisas, uma vez que catalisa a redução do oxigênio molecular utilizando NAD(P)H como doador de elétrons, gerando assim o ânion superóxido (Figura 4) (González, 2014; Virdis, Duranti, & Taddei, 2011; Wen et al., 2012). Esse complexo também pode atuar como fonte inicial para geração adicional de ROS por outras fontes, como nas mitocôndrias (Wen et al., 2012).

O complexo NAD(P)H oxidase pode ser estimulado por diferentes hormônios, sendo que algumas evidências científicas indicam que angiotensina II está fortemente envolvida na regulação e ativação desse sistema, induzindo a geração de ROS na parede celular (Ferroni et al., 2006; González, 2014; Virdis et al., 2011).

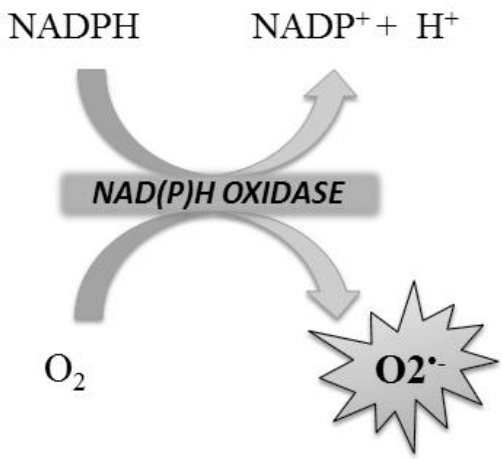


Figura 4. Esquema simplificado da geração do ânion superóxido pelo complexo NAD(P)H oxidase. Fonte: Autor

A angiotensina II é um potente vasoconstritor que faz parte do sistema renina-angiotensina (SRAA), conhecido por atuar no controle da pressão arterial e da homeostase cardiovascular. A produção de angiotensina II resulta de uma cascata enzimática que inicia quando a renina é liberada pelos rins para a corrente sanguínea devido à redução da PA. A renina, uma protease, cliva o angiotensinogênio formando o decapeptídeo angiotensina I, que é clivado pela enzima conversora de angiotensina (ECA), uma dipeptidil carboxipeptidase, produzindo o octapeptídeo angiotensina-II, que é o componente do SRA farmacologicamente ativo. Os efeitos da angiotensina II são mediados principalmente por sua ação direta nos receptores de tipo I de angiotensina II (AT-1) presente em vários tecidos do sistema cardiovascular, como nos rins e nos vasos sanguíneos, que leva a vasoconstrição. Além disso, a angiotensina II estimula a produção e liberação de aldosterona das glândulas suprarrenais, aumentando a retenção de sódio nos rins. Como resultado dos efeitos da angiotensina II, ocorre o aumento da pressão arterial (Figura 5) (Lavoie & Sigmund, 2003; Wen et al., 2012).

Considera-se que a angiotensina II exerce um papel crítico no controle da homeostase vascular, uma vez que age nos receptores AT1 não só provocando vasoconstrição, como também ativando o complexo NAD(P)H oxidase. A ativação desse sistema resulta no aumento de ROS, que contribuem para redução da biodisponibilidade de NO no meio vascular, desencadeia o processo de crescimento celular, já que as ROS geradas ativam cascatas proliferativas do músculo liso vascular, e ativa a cascata da coagulação no sistema vascular (Ferroni et al., 2006; González, 2014; Vasconcelos et al., 2007; Wen et al., 2012).

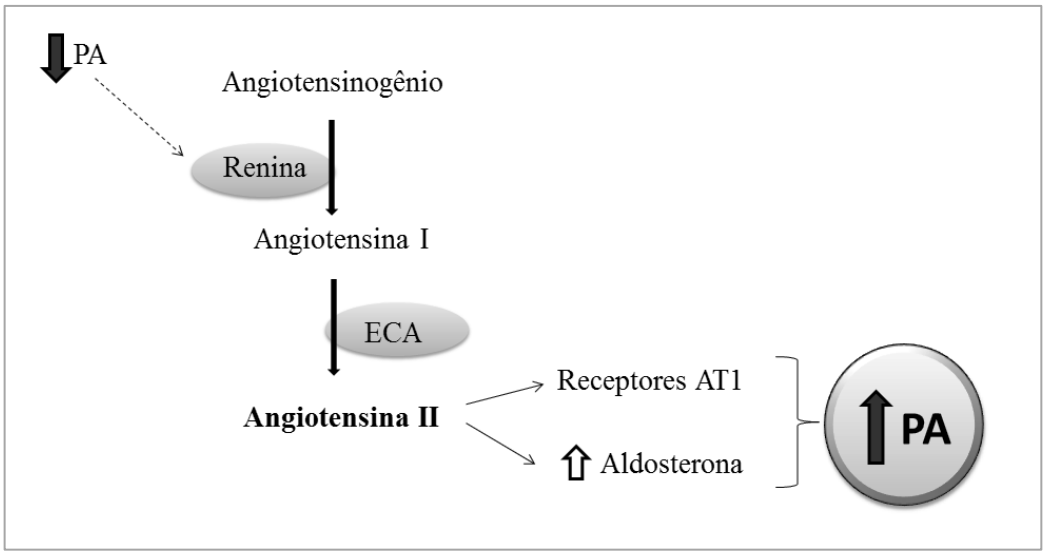


Figura 5. Esquema clássico do sistema renina angiotensina-aldosterona (SRAA). A redução da pressão arterial (PA) leva a liberação de renina, que converte angiotensinogênio em angiotensina I, que é clivada em angiotensina II pela enzima conversora de angiotensina (ECA). A angiotensina II age nos recepetores AT1 nos rins levando a retenção de sódio, além de estimular a produção e liberação de aldosterona das glândulas suprarrenais. Ambos os estímulos levam ao aumento da PA. Fonte: Adaptado de LAVOIE; SIGMUND (2003).

Entre as estratégias para o controle da hipertensão e de seus efeitos paralelos no organismo, destaca-se o uso de terapias medicamentosas. Por exemplo, as drogas *inibidoras da ECA* têm como objetivo de bloquear a produção de angiotensina II e melhorar o fluxo sanguíneo, com discutido anteriormente. Já os *beta-bloqueadores* são uma classe de fármacos com capacidade de bloquear os receptores β da noradrenalina, reduzindo a contractilidade e da velocidade das contrações cardíacas, o que resulta na diminuição a frequência cardíaca e na melhora do fluxo sanguíneo. Os fármacos *bloqueadores dos canais de cálcio* levam a redução da concentração de cálcio intracelular, provocando o relaxando do músculo liso arterial e consequentemente, a redução da pressão arterial. Fármacos diuréticos também são usados no tratamento da hipertensão, uma vez que promovem a eliminação de eletrólitos como o sódio e o potássio no rins, além de aumentar o volume e o fluxo urinário (IFPMA, 2016).

Atualmente, a criação de Políticas Públicas garante o maior acesso à população aos medicados para o tratamento da hipertensão. Entretanto, algumas barreiras dificultam que o tratamento seja efetivo, como a falta de manejo adequado dos medicamentos para cada paciente, a baixa adesão ao tratamento pelo paciente e a falta de consciência de que é necessária uma mudança no estilo de vida que incluem a prática de atividade física e mudança de hábitos alimentares (Mengue et al., 2016).

Nesse contexto, há uma crescente evidência de que o consumo de alimentos naturais ricos em compostos fenólicos é capaz de melhorar a função vascular, proteger contra danos oxidativos e reduzir o risco de desenvolvimento de DCVs (Oak et al., 2018; Siti et al., 2015). Esses efeitos são atribuídos aos efeitos bioativos dos polifenóis, que incluem a capacidade antioxidante (Woodman & Chan, 2004), ação anti-inflamatória, inibição da agregação plaquetária (Lescano et al., 2018), relaxamento de vasos (Van Rymenant et al., 2017) e inibição da ECA (Guerrero et al., 2012).

4. COMPOSTOS FENÓLICOS

Os compostos fenólicos, também denominados de polifenóis, são considerados metabólitos secundários envolvidos na defesa química das plantas, sendo produzidos em condições de estresse. Assim, são moléculas encontradas naturalmente em frutas, vegetais, grãos, em seus subprodutos e derivados (Claudine Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Oak et al., 2018).

Quimicamente, os compostos fenólicos são definidos como moléculas que possuem pelo menos um anel aromático ligado a uma ou mais hidroxilas, sendo divididos em diferentes classes conforme o número de anéis aromáticos presentes na estrutura química e com os elementos estruturais que ligam estes anéis entre si. Entre as principais classes de polifenóis encontram-se os ácidos fenólicos, álcoois fenólicos, flavonoides, estilbenos, lignanas e taninos (Escarpa & Gonzalez, 2001; Manach et al., 2004).

Os flavonoides são considerados os polifenois mais abundantes da dieta humana. São compostos por um esqueleto de 15 carbonos (C6-C3-C6), com dois anéis aromáticos (A e B) ligados por uma ponte de carbono formando um heterociclo oxigenado (anel C) (figura 6). Além disso, compreendem o maior número de compostos e podem ser classificados nas subclasses de acordo com o grau de oxidação do anel C em flavonas, flavanonas, flavonois, flavanolois, isoflavonas, flavanois e antocianidinas (Heim, Tagliaferro, & Bobilya, 2002; Manach et al., 2004; Oak et al., 2018).

Nas plantas, os flavonoides estão presentes na forma glicosilada, ou seja, associados a moléculas de açúcar. A glicosilação ocorre geralmente na posição C7 da aglicona, sendo a glicose e a ramnose os monossacarídeos mais comuns. De acordo com o padrão de ligação glicosídica, os glicosídeos podem ser denominados O-glicosídeos, formados pela ligação do açúcar ao grupo hidroxila da aglicona, ou C-glicosídeos, que tem o

açúcar conectado a aglicona com uma ligação carbono-carbono (Claudine Manach et al., 2004; Yang, Liu, Yang, Gupta, & Jiang, 2018).

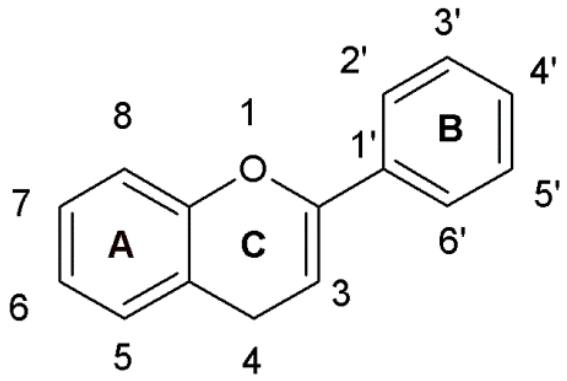


Figura 6. Estrutura química dos flavonoides. Fonte: Adaptado de HEIM; TAGLIAFERRO; BOBILYA (2002).

Cada espécie vegetal apresenta um perfil fenólico único, o que justifica o estudo destes compostos e de suas propriedades bioativas em diferentes tipos de matrizes.

Na medicina popular, medicamentos de origem vegetal são utilizados para melhorar a circulação sanguínea, desempenhando um papel importante no tratamento de doenças circulatórias. Os medicamentos mais comuns incluem as sementes de castanha da índia (*Aesculus hippocastanum L.*) ou a escina isolada, assim como os flavonoides hesperidina, diosmina, rutina e seus derivados (Dudek-Makuch & Studzińska-Sroka, 2015)

As sementes de castanha da índia contem uma mistura complexa de compostos bioativos, sendo a quercetina, o caempferol e a proantocianidina os principais flavonoides isolados da semente. Entretanto, o efeito terapêutico das sementes de castanha da índia é atribuído principalmente à saponina chamada escina (Dudek-Makuch & Studzińska-Sroka, 2015; Frishman, Sinatra, & Moizuddin, 2004). Na Europa, por exemplo, extratos preparados a partir das sementes de castanha da índia são comumente utilizados para o tratamento de insuficiência venosa crônica (Dudek-Makuch & Studzińska-Sroka, 2015).

Medicamentos a base de hesperidina também são empregados no tratamento de doenças vasculares, como o Diosmin® e Perivasc®. Diferentes estudos demonstram que a hesperidina possui ação antioxidante, anti-inflamatória e efeitos analgésicos (Chanet, Milenkovic, Manach, Mazur, & Morand, 2012; Roohbakhsh, Parhiz, Soltani, Rezaee, &

Iranshahi, 2015). Além disso, contribui para a saúde vascular por reduzir o estresse oxidativo (Di Majo et al., 2005), promover vasorelaxamento (Calderone et al., 2004; López-carreras, Castillo, Muguerza, & Aleixandre, 2015), regular o tônus vascular e reduzir a pressão arterial (Kang, Ryu, Lee, Kim, & Hee, 2016). A hesperidina é a principal flavanona isolada de frutas cítricas (Khan, Zill-E-Huma, & Dangles, 2014), tornando frutas como a laranja alvo de estudos para a extração de compostos fenólicos bioativos com efeito na proteção do sistema cardiovascular.

5. FRUTAS CÍTRICAS E SUBPRODUTOS DA INDÚSTRIA

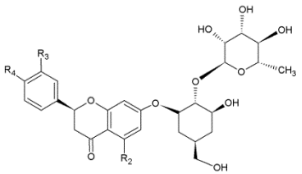
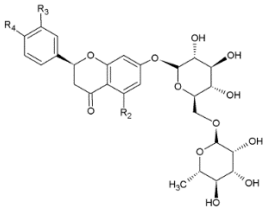
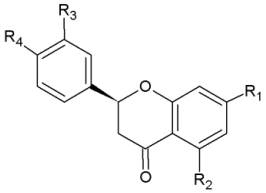
5.1 Composição fenólica e efeitos bioativos

As frutas cítricas são ricas em compostos fenólicos, sendo os flavonoides e ácidos fenólicos os principais representantes desse grupo. A maior concentração dos flavonoides pertence ao grupo das flavanonas, entre as quais de destacam a hesperidina, naringina e narirutina (Di Majo et al., 2005; Khan et al., 2014). Como os demais flavonoides, as flavanonas podem ocorrer na forma glicosilada e, raramente, na forma aglicona. Na forma aglicona essas moléculas são denominadas hesperitina e naringenina, respectivamente (KHAN et al., 2014). As características estruturais das principais flavanonas encontradas nas frutas cítricas podem ser observadas na Tabela 2.

Outra classe minoritária de compostos bioativos encontrados nas frutas cítricas são os ácidos hidroxibenzoicos, como o ácido elágico, e as flavanonas metoxiladas, como tangeritina e diosmetina (Barbosa, Ruviaro, & Macedo, 2018; Khan et al., 2014).

A concentração de flavonoides nas frutas cítricas varia de acordo com alguns fatores, com estação do ano, variedade da fruta e parte do fruto (Ghasemi, Ghasemi, & Ebrahimzadeh, 2009). Sabe-se que o suco de laranja é considerado uma fonte rica de compostos antioxidantes (FERREIRA et al., 2013; USDA, 2017). Entretanto, os estudos realizados por ESCOBEDO-AVELLANEDA et al. (2014) mostraram que os subprodutos provenientes da extração do suco de laranja continham maior teor de compostos fenólicos e maior atividade antioxidante do que o suco. Outras evidências reforçam a informação de que as flavanonas estão presentes principalmente na casca e ligados à matriz celular, em comparação com o suco e outras partes da fruta (Abeysinghe et al., 2007; Yinshi Sun et al., 2010).

.Tabela 2. Características estruturais das principais flavononas encontradas em frutas cítricas.

Composto fenólico	**Característica estrutural**	**Sacarídeo**	**Nome oficial**
Flavanona glicosilada			
			
Naringina	R2 = OH; R3 = H; R4 = OH	neohesperidose	naringenina-7-O-neohesperidose
Neohesperidina	R2 = OH; R3 = OH; R4 = OCH3		hesperetina-7-O-neohesperidose
			
Narirutina	R3=H; R4 = OH	rutinose	naringenina-7-O-rutinose
Hesperidina	R3 = OH; R4 = OCH3		hesperetina-7-O-rutinose
Flavanona aglicona			
			
Naringenina	R1 = OH; R2 = OH; R3=H; R4 = OH	-	Naringenina
Hesperetina	R1 = OH; R2 = OH; R3 = OH; R4 = OCH3	-	Hesperetina

Fonte: (Khan et al., 2014; Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007)

Em geral, compostos fenólicos de frutos cítricos são associados a uma variedade de efeitos benéficos a saúde devido aos seus efeitos antioxidantes (Barbosa et al., 2018; Zou, Xi, Hu, Nie, & Zhou, 2016), anti-inflamatório (Benavente-García & Castillo, 2008; Nakajima et al., 2017), antiobesogênico (Nakajima, Macedo, & Macedo, 2014), antimicrobiano (Iranshahi, Rezaee, Parhiz, Roohbakhsh, & Soltani, 2015), anticâncer (Benavente-García & Castillo, 2008), antitrombótico e anti-hipertensivo (Chanet et al., 2012; Roohbakhsh et al., 2015). Assim, poderiam ser utilizados como terapia auxiliar na prevenção e tratamento de doenças, como nas doenças vasculares.

SUN et al. (2013) avaliou a atividade antioxidante de seis espécies diferentes de frutas cítricas. Os autores mostraram que atividade antioxidante estava relacionada com o perfil fenólico das amostras avaliadas, sendo maior nas frutas com maior concentração de hesperidina e naringina. KIM & KIM (2016) mostram que extratos preparados de resíduos da indústria processadora de citrus apresentam atividade antioxidante devido a sua capacidade de remoção de radicais livreis como $O2^{\bullet-}$ e $ONOO^{\bullet-}$. A capacidade antioxidante e a habilidade de remoção do radical peroxinitrito estão associadas aos efeitos cardioprotetores dos frutos cítricos (Ho, Su, & Lin, 2013)

YON et al. (2011) atribuíram à hesperidina e à naringenina o efeito inibidor da agregação plaquetária de extratos cítricos. PEREZ et al. (2010) demonstraram que extratos preparados de folhas de frutos cítricos, ricos em hesperidina e naringina, antagonizaram os efeitos da angiotensina II, contribuído para a redução da pressão arterial em ratos. LÓPEZ-CARRERAS et al. (2015) evidenciaram o efeito relaxante de extratos de cítricos na aorta de ratos hipertensos, sugerindo que a presença de hesperidina e naringina foram os principais responsáveis por esse efeito.

5.2 Agliconas

Apesar de todos os estudos evidenciarem a ação antioxidante e de proteção cardiovascular das flavanonas glicosilados naringina e hesperidina, diversos autores tem sugerido que as formas agliconas é que apresentam maior bioatividade. Estudos *in vitro* mostraram que a hesperetina e a naringenina apresentam uma atividade antioxidante significativamente maior quando comparadas as suas respectivas formas glicosiladas (Cao, Chen, Jassbi, & Xiao, 2015; Ferreira et al., 2013; J. V. Madeira & Macedo, 2015). CAVIA-SAIZ et al. (2010) demonstraram que a remoção da rutinose no C7 da naringina aumentou seu

efeito antioxidante, destacando mais uma vez a maior bioatividade da aglicona em relação ao glicosídeo. Da mesma forma, CALDERONE et al. (2004) e ORALLO; ÁLVAREZ; BASARAN (2004) demonstraram que a glicosilação no C7 da flavanona leva a redução de seu efeito farmacológico. Tanto a hesperetina quando a naringenina foram mais eficazes em promover o relaxamento de vasos de ratos *in vitro* quando comparadas à hesperidina e naringina, respectivamente (Orallo et al., 2004; Orallo & Camiæa, 2005).

Os efeitos biológicos dos compostos fenólicos dependem de sua biodisponibilidade no organismo. Estudos sugerem que os glicosídeos, como a hesperidina, apresentam bioacessibilidade limitada, e precisam ser previamente hidrolisadas para então serem absorvidos no intestino (Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003; Vallejo et al., 2010). Entretanto, os mecanismos envolvidos na absorção dos flavonoides ainda são muito discutidos por diversos autores (Roohbakhsh, Parhiz, Soltani, Rezaee, & Iranshahi, 2014).

É importante entender que a bioacessibilidade de um composto está relacionada com o que chega ao intestino e está disponível para ser absorvido, enquanto que a biodisponibilidade refere-se ao que foi absorvido e passou para a corrente sanguínea.

Estudos realizados por Vallejo et al. (2010) em humanos demonstraram que as flavanonas glicosiladas e as agliconas não são absorvidas no estômago ou no intestino delgado, e chegam até o cólon de forma intacta. No cólon, os glicosídeos são hidrolisados para liberar as agliconas, que são absorvidas e levadas para a corrente sanguínea.

A etapa de deglicosilação das flavanonas no cólon intestinal é mediada por enzimas intestinais de origem bacteriana, tais como α-ramnosidases e β-glicosidases (Erlund, 2004; Nielsen et al., 2006; Vallejo et al., 2010).

Em um estudo de revisão, Manach, Williamson, Morand, Scalbert, & Rémésy (2005) destacaram que as flavanonas agliconas são absorvidas mais rapidamente do que as moléculas glicosídicas, sugerindo que as agliconas apresentam maior bioacessibilidade. Londoño-Londoño et al. (2010) demonstraram que a remoção da rutinose ou da ramnose das flavanonas glicosiladas aumentou a biodisponibilidade dessas moléculas, contribuindo para sua maior bioatividade. De acordo com estudos *in vivo* realizados por De Souza et al. (2016), os monoglucuronídeos de hesperitina foram as principais formas presente no plasma de ratos após a ingestão de hesperitina e de seus glicosídeos.

Ao avaliar os parâmetros farmacocinéticos das agliconas hesperetina e naringenina no plasma e na urina de seres humanos, Kanaze, Bounartzi, Georgarakis, & Niopas (2007) observaram que hesperetina e a naringenina foram mais rapidamente

absorvidas do que seus glicosídeos. Além disso, a hesperetina e a naringenina (e não hesperidina e naringina) foram detectadas em fluídos biológicos, indicando que a forma ativa no organismo são as agliconas (González-Sarrías, Espín, & Tomás-Barberán, 2017; Vallejo et al., 2010).

De Souza et al. (2016) realizaram estudos *in vitro* e *in vivo*, comparando os efeitos antioxidantes da hesperitina e de suas formas glicosiladas. Os resultados demonstraram que aglicona hesperetina possui atividade inibidora da xantina oxidase (enzima responsável pela geração de ROS) *in vitro* mais potente em relação aos seus glicosídeos. Para o estudo *in vivo*, ratos *Wistar* foram suplementados com hesperetina, hesperidina ou G-hesperidina durante 30 dias. Os resultados mostraram que a suplementação com a hesperitina aumentou o status antioxidante do fígado dos animais, sugerindo um efeito ligado à maior biodisponibilidade da aglicona.

Sabendo dos efeitos biológicos da hesperetina e naringenina em relação aos seus glicosídeos, o interesse em desenvolver produtos ricos nessas agliconas se tornou crescente. Entretanto, as agliconas são de difícil obtenção, e os estudos que avaliaram os efeitos desses compostos costumam utilizar padrões analíticos de alto custo. Levando em consideração que as flavonas são encontradas principalmente na casca e nos resíduos do processamento da indústria cítrica, esses resíduos de baixo valor agregado podem ser considerados uma importante fonte de extração de compostos fenólicos de maior bioatividade.

5.3 Subprodutos

A laranja é uma fruta cítrica de destaque no Brasil, sendo considerado o maior produtor mundial. De acordo com dados estatísticos, o Brasil produziu mais de 17 milhões de toneladas da fruta na última safra (FAOSTAT, 2018), o que corresponde a 34% da produção mundial de laranja (CitrusBR, 2017). A maior parte da parcela da produção é destinada a indústria processadora quando comparado ao consumo *in natura* da fruta (Neves & Trombin, 2017).

O principal destino da produção de laranja é a produção de suco, sendo que o Brasil é responsável por mais de 80% do total de exportações de suco de laranja no mundo (Franco, 2016; Neves & Trombin, 2017). Para a produção de suco, o máximo de suco é extraído de cada fruto e os resíduos sólidos remanescentes são utilizados para fabricação de subprodutos (Bampidis & Robinson, 2006; Mamma & Christakopoulos, 2008). De acordo

com a Associação Nacional dos Exportadores de Sucos Cítricos, a cada 1000 kg de laranja são extraídos 553 kg de suco, sendo que o restante é considerado subproduto (CitrusBR, 2010). Como consequência, milhões de toneladas de resíduos são gerados (aproximadamente 12,8 milhões), sendo destinados principalmente para a extração de pectina, óleo essencial e alimentação animal (Mamma & Christakopoulos, 2008, 2014). A Tabela 3 destaca os produtos e subprodutos originados da produção de laranja.

Tabela 3. Produtos e subprodutos da laranja.

Produto/ subprodutos	Componentes	Destino
Produtos principais		
Fruta fresca	Fruta	Consumo *in natura* ou indústria processadora de suco
Suco de laranja	1) Suco concentrado congelado (FCOJ[1]), cuja água é retirada do suco natural e pode ser armazenado por longos períodos; 2) Suco não concentrado (NFC[2]), suco pasteurizado sem a retirada de água.	Mercados locais, nacionais e para exportação
Subprodutos		
Comminuted Citrus Base	Resultante da moagem da fruta inteira ou de um pouco de suco concentrado misturado à casca moída	Ingrediente para bebidas à base de frutas devido seu alto *flavor*
Polpa	Gomos de suco rompidos e paredes internas do fruto que sobram após o processo de extração do suco	Pode ser readicionada ao suco para dar aparência natural ao produto
Suco extraído da polpa	Suco obtido após a lavagem da polpa, contendo sólidos	Pode ser usado em bebidas à base de frutas ou como fonte

	provenientes da fruta	de açúcares. Utilizado como agente de turvação para dar corpo à bebida
Essências	Compostos voláteis separados pelo processo de evaporação, sendo separados em fase aquosa e oleosa	Matéria-prima para indústria de alimentos e bebidas
Óleo da casca de laranja (Cold-Pressed Oil)	Óleo essencial extraído da casca de laranja	Utilizado na produção de compostos para bebidas, cosméticos e produtos químicos
D-Limoneno	Principal componente do óleo da casca da laranja	Utilizado nas indústrias de plásticos como matéria-prima para a fabricação de resinas sintéticas e adesivos
Pellets de polpa cítrica	Resultante do processamento do suco, formado a partir dos resíduos úmidos do fruto: casca, bagaço, polpa não utilizada e sementes. Passam por processo de secagem e formam uma forragem concentrada transformada em Pellets.	Alimentação fibrosa de ovelhas e gado
Pectina	Obtido da casca da laranja	Usado na fabricação de geléias, marmelada e gelatinas
Álcool	A prensagem do bagaço de laranja produz um líquido cuja fermentação resulta em álcool	

Fonte: Bampidis & Robinson (2006); Mamma & Christakopoulos, (2008) CitrusBR, (sd).
[1]Sigla em inglês Frozen Concentrate Orange Juice. [2]Sigla em inglês Not-from-concentrate

Muitos estudos evidenciaram o perfil fenólico dos diferentes resíduos da indústria processadora de citrus, destacando o alto teor de compostos fenólicos nesses subprodutos de baixo valor agregado, sendo a hesperidina, naringina e narirutina as principais flavanonas encontradas (Barbosa et al., 2018; Kim & Kim, 2016; Mamma & Christakopoulos, 2008; Pereira et al., 2017; Ruviaro, Barbosa, & Macedo, 2018). Assim, o reaproveitamento de resíduos da indústria cítrica para o desenvolvimento de ingredientes e produtos ricos em compostos bioativos tem sido reconhecido pela indústria alimentícia e farmacêutica como uma alternativa viável, tanto do ponto de vista econômico, quanto na questão ambiental pela redução no descarte de resíduos no meio-ambiente (Mahato, Sharma, Sinha, & Cho, 2018; Mamma & Christakopoulos, 2014; Sharma, Mahato, Cho, & Lee, 2016).

Entre as alternativas de reaproveitamento desses resíduos agroindustriais, a biotransformação enzimática merece destaque, uma vez que tem se mostrado eficiente na extração de compostos fenólicos de diferentes matrizes, além de aumentar a concentração de compostos de maior bioatividade, como a hesperetina e a naringenina.

6. BIOTRANSFORMAÇÃO ENZIMÁTICA

Processos enzimáticos podem ser utilizados para obtenção de compostos fenólicos em matrizes alimentares. Muitos estudos têm demonstrado que tratamentos enzimáticos melhoram as propriedades biológicas de extratos produzidos a partir de diferentes matrizes alimentícias, uma vez que facilitam a liberação de compostos fenólicos da parede celular, além de promover mudanças na estrutura molecular dos fenólicos (Ferreira et al., 2013; Kessy, Wang, Zhao, Zhou, & Hu, 2018; Kitrytė, Kraujalienė, Šulniūtė, Pukalskas, & Venskutonis, 2017; Martins, Roberto, Blumberg, Chen, & Macedo, 2016).

A biotransformação enzimática se caracteriza pelo uso de enzimas exógenas produzidas por fungos ou bactérias, que apresentam diferentes atividades hidrolíticas e catalíticas. A biotransformação enzimática tem sido bastante empregada para a valorização de resíduos agroindustriais, que possuem baixo valor comercial. Vários fatores influenciam no produto final gerado pela reação enzimática, como concentração da enzima, pH, tempo de reação e tipo de substrato utilizado (Madeira, Teixeira, & Macedo, 2015).

Enzimas como β-glicosidase (β-D-glicosídeo glicohidrolase EC 3.2.1.21) hidrolisam a glicose a partir de glicosídeos fenólicos em diferentes tipos de substratos, aumentando a concentração de agliconas (Kessy et al., 2018; Ruviaro et al., 2018; Shin, Nam,

& Oh, 2013). Da mesma forma, a tanase (tanil acil hidrolase EC 3.1.1.20) é uma enzima induzida produzida por microrganismos, que pode atuar em complexos de polifenóis, hidrolisando ligações éster e ligação depsídica de compostos fenólicos (Madeira & Macedo, 2015; Martins et al., 2016; Roberto et al., 2016; Ruviaro et al., 2018). Outra classe de enzimas, as ramnosidases (α-ramnosidase EC 3.2.1.40), reconhecem a ligação entre as duas moléculas de açúcar e clivam a molécula ramnose (Céliz, Rodriguez, Soria, & Daz, 2015). A diglicosidase α-ramnosil-β-glicosidase (EC 3.2.1.168) é empregada no bioprocessamento devido a ambas as atividades de ramnosidases e β-glucosidase (Mazzaferro & Breccia, 2012; Piñuel, Breccia, Guisán, & López-Gallego, 2013). Este coquetel enzimático é frequentemente comercializado como naringinase e/ ou hesperidinase.

A biotransformação enzimática da hesperidina para produzir hesperetina envolve diferentes mecanismos que variam de acordo com a enzima utilizada (Figura 7).

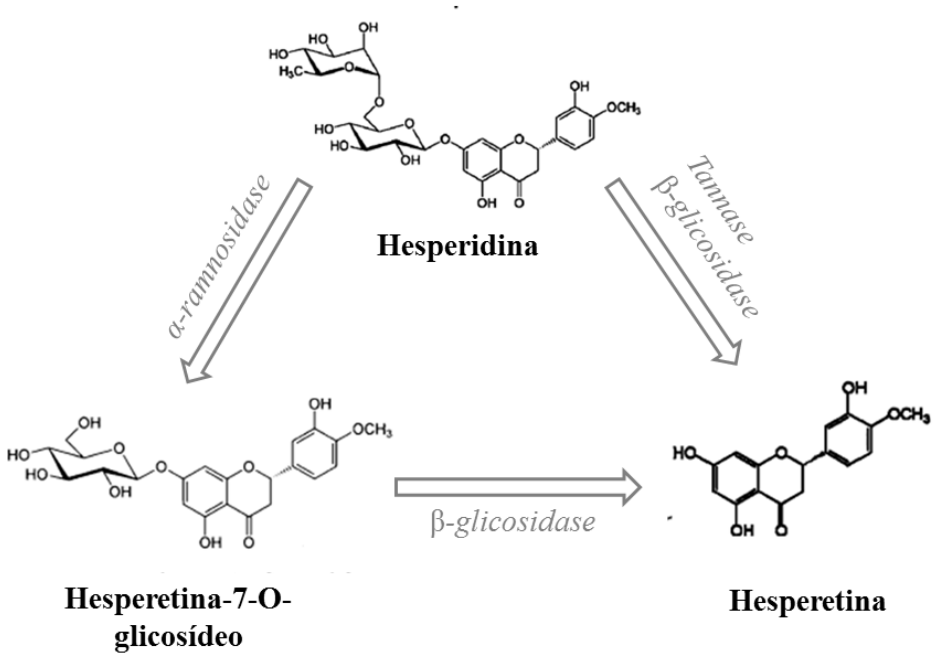


Figura 7. Reação de biotransformação do glicosídeo hesperidina em hesperetina empregando diferentes enzimas. Fonte: Adaptado de CÉLIZ et al. (2015) e SHIN; NAM; OH (2013).

O emprego de coquetéis enzimático contendo α-ramnosidase e β-glucosidase envolvem dois passos de reação. Na primeira etapa a enzima α-ramnosidase produz hesperetina-7-O-glicosídeo pela remoção da ramnose. Na segunda etapa, a β-glicosidase converte hesperitina-7-O-glicosídeo em hesperetina devido à remoção da glicose. Com a utilização de enzimas isoladas como a β-glicosidase e a tanase, hesperetina pode ser

produzida por meio de uma reação de uma única etapa (Céliz et al., 2015; Shin et al., 2013). O mesmo mecanismo de reação pode ser usado para entender a conversão de naringina e narirutina na aglicona naringenina.

O tratamento enzimático tem sido empregado com sucesso em diferentes tipos de resíduos agroindustriais. Esse processamento não só reduz o descarte de resíduos no meio ambiente, como também aumenta a concentração de fenólicos livres das matrizes alimentares. Consequentemente, a biotransformação enzimática é capaz de extrair compostos com maior atividade antioxidante e produzir extratos com maior bioatividade, que podem ser utilizados pela indústria farmacêutica e alimentícia para o desenvolvimento de produtos cosméticos e nutracêuticos (Madeira et al., 2015; Radenkovs, Juhnevica-Radenkova, Górnaś, & Seglina, 2018)

Outra vantagem da biotransformação enzimática é ser considerado um processo amigável para o meio ambiente, uma vez que é capaz e extrair fenólicos de diferentes matrizes alimentares sem utilização de solventes orgânicos ou outros produtos químicos tóxicos (Madeira et al., 2015; Radenkovs, Juhnevica-Radenkova, Górnaś, & Seglina, 2018).

7. CONSIDERAÇÕES FINAIS

A biotransformação enzimática de resíduos de citrus pode ser um processo alternativo viável para o reaproveitamento desses resíduos, pois além de reduzir o descarte no meio ambiente, aumenta o valor econômico agregado produzindo extratos com alto teor de compostos fenólicos de maior atividade biológica, como a hesperetina e naringenina. Essas agliconas apresentam maior potencial bioativo e de proteção cardiovascular aos compostos naturalmente presentes em frutos cítricos. Assim sendo, os subprodutos provenientes da indústria processadora de citrus são considerados uma importante fonte de compostos bioativos que podem ser empregados na indústria farmacêutica, química, cosmética e alimentícia para o desenvolvimento de ingredientes com propriedades de proteção contra danos oxidativos e de proteção vascular.

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CAPÍTULO II. COMPARISON OF DIFFERENT BRAZILIAN CITRUS BY-PRODUCTS AS SOURCE OF NATURAL ANTIOXIDANTS

(Original research paper published by Journal of Functional Foods - https://doi.org/10.1007/s10068-018-0383-4, 2018)

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ABSTRACT

Significant amounts of citrus by-products remain after juice processing, which is used for pectin obtainment. At pectin industry, the problem faced is the generation of a new waste. Until now, no study had characterized this residue or suggested applications for it. In this field, the main goal of this study was to compare citrus industrial residues, remained after juice (CJB) and pectin (CPB) extraction, aiming the obtainment of bioactive compounds. The residues were evaluated about their chemical composition, antioxidant capacity, and polyphenols content. CJB had 2-fold higher total phenols than CPB. Also, CJB exhibited higher antioxidant capacity than CPB. Nine polyphenols were detected; hesperidin was the main compound on both residues. CPB presented higher content of polyphenols than CJB, being attributed to the industry procedure of pectin extraction. Thus, this study provides support for the reuse of CPB for the obtainment of nutraceutical compounds, converting waste into added-value products.

KEYWORDS: Agroindustrial residues; Polyphenols; Flavanones; Phenolic acids; Methoxylated flavones.

1. INTRODUCTION

Oranges are the world's largest fruit crop, reaching a production of ~71 million tons in 2014, and Brazil is its main producer (~17 million tons) (1). About 40% of this world production is addressed to juice industry (2). As a consequence of this, millions of tons of residues are also generated, yielding approximately 45% of the original whole fruit mass, and ~12.8 million tons of by-products. Citrus peel is the primary waste product from juice processing, which is usually dried and either sold as raw material for pectin extraction or pelletized for animal feeding. Nowadays, because of its several functional components, as carotenoids, polyphenols and limonoids, citrus peel became an important raw material for

chemical, food, and pharmaceutical industries (3,4). Thus, all possible value from the fruit can be obtained.

Among all the bio-compounds obtained from citrus by-products, pectin is widely used as functional ingredient by food, pharmaceutical and cosmetic industries. Its utilization is mainly based on its gelling and stabilizer properties, e.g. used for the production of jams, jellies, fruit juice, yogurts, milk drinks, and gels/pastes in cosmetic field. Due the innumerable products with pectin as ingredient, the worldwide consumption of this compound is estimated to be around 45 million kg per year, with a global market value of ~400 million Euros (4). Currently, the major sources of pectin are citrus and apple juice residues, which yield in citrus peel is about 25% of the dry matter (5). Thus, the main problem faced by pectin industry is the generation of a considerable volume of a new waste. In this field, pectin industries are commercially interested to achieve low environmental impact applications of their residues, being able to convert waste into added-value products. However, for this, it is necessary to characterize this residue composition, with particular regards to its bioactive molecules.

It is well known that citrus by-products represent an important inexpensive source of polyphenols, as flavonoids and phenolic acids. According to Bocco et al. (3), orange peels contain about 13.5 g of flavonoids per Kg of dry matter. The main flavonoids found in citrus are the glycosilated flavanones, as hesperidin, narirutin, and naringin, which are unique from citrus. Among the aglycone forms, naringenin and hesperetin are the most important ones (6,7). Other important compounds found in citrus are hydroxybenzoic acids, and methoxylated flavones, e.g. ellagic acid, tangeritin and diosmetin (8). In general, citrus phenolic compounds are associated with many health benefic effects, as anti-inflammatory, antimicrobial, cancer prevention, inhibition of human platelet aggregation, and hypocholesterolemic effects (9,10). In this context, there is a growing interest in these substances obtainment, supplied as natural food components (replacing synthetic antioxidants and antimicrobials) or as specific preventive pharmaceuticals and nutraceuticals. Thus, the sustainable exploration of citrus pectin waste for recovery these valuable compounds will reduce amounts of residue disposal and may be even more economically interesting source than citrus juice by-products.

Until now, studies on citrus bioactive compounds have focused in citrus juice and its by-products (CJB). However, to the best of author's knowledge, the waste remained after the obtainment of citrus pectin (CPB) has not been previously characterized. For the reasons presented above, the aim of this study was to investigate the potential use of this new residue CPB for the obtainment of valuable compounds. For this, CJB and CPB residues were

investigated about its chemical composition, antioxidant effectiveness and polyphenols content. Also, the residues were compared to each other to better understand the effect of pectin obtainment industry process on citrus bioactive compounds.

2. MATERIAL AND METHODS

2.1 Materials

Gallic acid, narirutin, hesperidin, hesperetin, naringin, naringenin, ellagic acid, tangeritin, diosmetin, 2,2'-azobis(2-methylpropionamidine) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein, and trolox were purchased from Sigma–Aldrich (St. Louis, MO, USA). Folin Ciocalteu's reagent, monobasic and dibasic sodium phosphate, sodium carbonate, and formic acid were purchased from Dinâmica Química Contemporânea (Diadema, Brazil). LC grade methanol was purchased from JT Baker (Center valley, PA, USA). All other chemicals were used in analytical grade.

2.2 Citrus by-products

Two residues of citrus were used in this study. They were supplied by CP Kelco Industry Headquarters (Limeira, Brazil), specialized in pectin production. One, the citrus juice by-products (CJB), is derived from orange juice production (flavedo, albedo and seeds from *Citrus latifolia* and four cultivars from *Citrus sinensis*: Hamlin, Valencia, Pera riu and Pera Natal); the other, the citrus pectin by-products (CPB) from pectin industry, remained after the citrus pectin extraction. Both residues were dried, crushed in Blender (OXY, Brazil), and sieved at 0.80 mm (Bertel Metallurgical Industries Ltda, Brazil).

2.3 Residues chemical composition

All chemical analyses of citrus residues were performed according to the Association of Official Analytical Chemists (11). Moisture and fat content were determined gravimetrically. Fat in the samples were extracted following the method of Bligh and Dyer (12). The total protein was determined by Kjeldahl nitrogen determination, using the factor 6.25 to protein conversion. Ash content was determined by mineralization of the samples at

450 ºC. The content of carbohydrate was calculated based on difference (100 – moisture – fat – protein – ash).

2.4 Polyphenols extraction

The phenolic compounds of the citrus by-products were extracted with aqueous-ethanol solution (1:1) according to Nakajima et al. (13). One gram of the dried citrus residues was added to 25 mL of water-ethanol mixture. Then, the samples were treated for 15 min in ultrasonic bath at 30 °C, and in shaker for 15 min at 200 rpm. After that, the samples were filtered on Whatman paper (number 1) and the products obtained were concentrated on a rotary evaporator at 40 °C for 15 minutes to remove the ethanol. The aqueous solutions were freeze-dried and frozen until the analyses.

2.5 Total polyphenols content (TPC)

The TPC of the lyophilized citrus extracts was measured by Folin–Ciocalteu method, according to Singleton et al. (14). Gallic acid standard or test samples were mixed with Folin–Ciocalteu phenol reagent in the dark. After 3 minutes, Na_2CO_3 was added and mixed. Then, the mixtures were kept in the dark at room temperature for 2 hours and the assay was carried out on a NovoStar Microplate reader (BMG LABTECH, Germany) with absorbance filters of 725 nm wavelength. Gallic acid was used as a standard to calibration curve construction, its concentration ranged from 15 to 300 µg mL^{-1}. Results were expressed as mg of gallic acid equivalents in 100 g of dry matter (GAE $100g^{-1}$ DM).

2.6 Antioxidant capacity

DPPH radical scavenging activities of liofilized citrus extracts were determined following the method of Macedo et al. (15). The reaction mixtures containing the standard trolox or test samples were added to DPPH solution. The decolorizing process was recorded during 90 min of reaction and the assay was carried out in microplate reader with absorbance filters of 520 nm wavelength. The DPPH radical-scavenging activity was evaluated by trolox calibration curve. The results were expressed as µmol of trolox equivalent per g of DM (µmol TE g^{-1} DM).

ORAC assay was performed using the fluorescent probe fluorescein (FL), as described by Ferreira et al. (16). Trolox standard or test samples were distributed in black-walled 96-well plate, followed by the addition of fluorescein. The reaction was initiated by addition of the AAPH solution. The fluorescence was monitored every 56 s during 75 min in a microplate reader at 37 °C with excitation filter of 485 nm and emission filter of 520 nm. All reagents and samples preparation was done in sodium phosphate buffer 75 mM (pH 7.4). Results were calculated by the difference between the area under the FL decay curve of the samples and the blank (net AUC). Regression equations between net AUC and samples concentration were made. Trolox was used as standard to construct calibration curve and the results were expressed as µmol of Trolox equivalent per g of DM (µmol TE g^{-1} DM).

2.7 Identification and quantification of main citrus polyphenols by HPLC-DAD

Citrus extracts and standards were combined with 70% methanol solution. All phenolic compounds were quantified using a Dionex UltiMate 3000 (Dreieich, Germany) liquid chromatography system, equipped with a C-18 Acclaim 120 column (Dionex, 3 um, 4.6 x 150 mm) maintained at 30 °C. The method was adapted by Madeira Jr. and Macedo (8). The mobile phases were A, H_2O (0.1% of formic acid); and B, Methanol (0.1% of formic acid). The gradient elution was 90% A (0–5 min), 20% A (5–80 min), 90% A (80–85 min), and 90% A, with a flow rate of 0.6 mL min^{-1}. The detection was carried out at 280 nm using a diode array detector (DAD-3000). Individual flavonoids were identified by comparison with retention time and UV-VIS spectra of the standard compounds naringin, naringenin, hesperidin, hesperitin, tangeritin, gallic acid, ellagic acid, and diosmetin. These compounds contents in the citrus by-products extracts were calculated using the standards calibration curves.

2.8 Statistical analysis

All measurements were performed in triplicate, and the results are presented as mean ± standard deviation (SD). The statistical difference between the samples was analyzed using t-test ($P \leq 0.05$). All statistical analyses were performed using Minitab 16.1.1.

3. RESULTS AND DISCUSSION

3.1 Chemical composition

The citrus industrial by-products remained after juice obtainment (CJB) and after pectin extraction (CPB) were studied and compared to each other about their chemical composition, antioxidant activity, and phenolic content. Table 1 illustrates the chemical composition of CJB and CPB. The results demonstrate that the residues had similar composition of fat and protein, slightly differed in moisture and carbohydrate concentration, and the main difference was the minerals content, indicated by ash values. The residue CJB presented mineral composition >4-fold higher than CPB. The hypothesis for this observation can be related to pectin capacity to absorb and maintain other substances e.g. minerals (17), so the procedure of pectin obtainment also extracts minerals together, and the residue left after pectin extraction CPB present low ash content.

Table 1. Chemical composition of different citrus by-products.

Components (g/100 g)	Citrus by-products		*P*-value
	CJB	CPB	
Moisture	7.92 ± 0.09 [a]	7.23 ± 0.10 [b]	0.003
Literature*	8.51 - 10.55	-	
Ash	2.81 ± 0.01 [a]	0.56 ± 0.02 [b]	0.0001
Literature*	2.52 – 3.8	-	
Protein	6.67 ± 0.08 [a]	6.76 ± 0.40 [a]	0.741
Literature*	6.0 - 9.2	-	
Fat	3.81 ± 0.11 [a]	4.15 ± 0.45 [a]	0.331
Literature*	2.27 – 2.8	-	
Carbohydrate	78.79 ± 0.52 [b]	81.30 ± 0.06 [a]	0.015
Literature*	75.69 – 77.78	-	

Abbreviations: CJB (Citrus Juice by-product), CPB (Citrus pectin by-product), DM (dry matter).
[a, b] Values (mean ± SD) within each analyte with different letters significantly differ ($P \leq 0.05$), tested using t-test.
* O'Shea et al. (18) and Senevirathne et al. (19).

Many studies have already evaluated CJB residue, reporting its composition, reuse and new methods for obtainment of pectin and its bioactive compounds. O'Shea et al. (18) and Senevirathne et al. (19) studied the chemical composition of citrus juice by-products from

Ireland and Korea, respectively. The results reported by the authors (Table 1) were similar to the composition obtained in our Brazilian CJB, even with the differences of citrus growing climates, locations and agriculture managements.

About the CPB residue, to the best of authors' knowledge, the study of Aravantinos-Zafiris et al. (20) is the only literature that reports the use of CPB. According to them, the chemical Composition of CPB in dry basis was 87% of carbohydrate, 6.9% of protein, 3.3% of ash, and 0.4% of fat. They also determined that the carbohydrate fraction mainly corresponded to fibers (83.9% of dry matter) and Ca, Mg, P, and K were the predominant minerals. Their results mainly differed from ours in fat and ash content. The reason can be attributed to differences in citrus growing conditions, like soil characteristics, maturity of the fruits, or because of the process of pectin extraction utilized by the authors, which may influenced the final residue composition.

At Aravantinos-Zafiris et al. (20) study the CPB proved to be an useful source of fiber for food or feed ingredient. However, its bioactive potential was not taken into account, and is still unknown. Thus, the study of CPB phenolic composition and its antioxidant potential can give greater added value to this residue.

3.2 Total polyphenols content and Antioxidant capacity

The total phenols content and the antioxidant properties of the citrus by-products were assessed using the assays of TPC by Folin-Ciocalteu method, DPPH radical scavenging capacity and oxygen radical absorption capacity, ORAC. Table 2 shows the results for TPC, DPPH and ORAC assays compared with literature. The results indicated that CJB is approximately 2-fold higher in TPC than CPB (Table 2). Also, our TPC results exceeded the values in orange peels obtained by Anagnostopoulou et al. (21) (3.63–254 mg GAE/100 g DM) and in fresh orange peels by Casquete el al. (22) (284 mg GAE/100 g). However, when compared to flavedo extracts, our results presented lower content than those found by Escobedo-Avellaneda et al. (6) (588.6 – 679.9 mg GAE/100 g DM). According to Abeysinghe et al. (23), the content and composition of phenolic compounds vary in different citrus tissues. In this context, the residue CJB used in this study contains peel (flavedo and albedo), pulp (juice sac residue), rag (membranes and cores), seeds, and pectin. Thus, the different residues composition may difficult the comparison of our results with other data.

Usually, phenolic compounds extraction is made with organic solvent, as methanol and acetone. However, in industry, the methanol application is unfeasible due its

toxicity. According to Dahmoune et al. (24), the type of solvent significantly influences the TPC results and aqueous-ethanol is the best extractive solution for polyphenols. Results obtained by Nakajima et al. (13) confirmed the potential of aqueous-ethanol (1:1) solution for phenolics extractions. In this study, we choose to use the same solution, aiming a higher polyphenols obtainment, lower solvent use, and less harmful to health solvent.

Table 2. Total phenolic content and antioxidant capacity of citrus by-products extracts CJB and CPB.

	Citrus by-product		*P*-value
	CJB	CPB	
TPC (mg GAE/ 100 g DM)	386.99 ± 23.29[a]	170.04 ± 10.89 [b]	0.0001
DPPH (µmol TE / g DM)	11,035.86 ± 549.04[a]	2,571.84 ± 146.23[b]	0.0001
Literature*	9,188 ±187	-	-
ORAC (µmol TE/ g DM)	91,570.62 ± 12153.77[a]	37,588.87 ± 6207.17[b]	0.002
Literature*	90,075 ± 9132	-	-

Abbreviations: CJB: citrus juice byproducts; CPB: citrus pectin by-products; DM: dry matter; GAE: gallic acid equivalent; TE: trolox equivalent.
a,b Values (mean ± SD) within different letters in the line significantly differ by t-test ($P \leq 0.05$).
* Madeira Jr and Macedo (8).

Previous studies reported that the high presence of phenolic compounds contributes for stronger antioxidant activity in fruits. Also, it was demonstrated a significantly positive correlation between TPC and antioxidant activity in citrus fruits (17, 25). This observation is consistent with our results, which CJB exhibited higher antioxidant activity and higher TPC, when compared to CPB (Table 1). In addition, the CJB values of ORAC and DPPH were similar to those reported by Madeira Jr. and Macedo (8) with citrus juice residues (Table 1).

In citrus peel, Xi et al. (26) obtained values from 9.10 to 19.75 µmol TE g^{-1} of DW in DPPH and 126.6 – 331.29 µmol TE g^{-1} of DW in ORAC. Regarding DPPH, Casquete et al. (22) found 102.39 mg TE 100 g^{-1} of fresh orange peels. In this context, CJB and CPB appear to have higher and stronger antioxidant capacity.

According to Ferreira et al. (16), the orange juice is recognized as a rich source of antioxidants. However, it appears to have lower TPC and antioxidant activity than its by-products (6). At Ferreira et al. (16) study, they found orange juice TPC values of 6.7 mg GAE 100 mL^{-1}, and antioxidant activity equivalent to 5,319 µmol TE L^{-1} (DPPH assay) and 18,750

μmol TE L^{-1} (ORAC assay). Also, according to the USDA (27) database, orange juice presents TPC of 67 mg GAE 100 g^{-1} and antioxidant activity of 726 μmol TE 100 g^{-1} (ORAC assay). In fact, the data found in this study indicates that citrus and pectin by-products can be considered important and higher sources of polyphenols and antioxidant compounds than orange juice.

3.3 Polyphenolic composition

Some of the most important citrus polyphenols compounds were detected and quantified by HPLC–DAD in CJB and CPB, and the results are summarized in Table 3. The major polyphenols present in both citrus by-products was the glycoside flavanone hesperidin, followed by narirutin (Figure 1). Besides flavanones, CJB presented high content of ellagic acid, and tangeretin was predominantly found in CPB. Gallic acid, naringin, naringenin, hesperetin, and diosmetin were found in both samples, however, in low amounts.

The obtained results are consistent with previous finding, which reveals hesperidin, narirutin, naringin and eriocitrin the main flavonoids found in citrus, the first one being the most abundant (9). At Khan et al. (9) study, similar techniques were used to extract phenolics from orange peel. The content of hesperidin obtained from our citrus by-products was similar and quite higher to those obtained in Khan et al. (9) study (205.2 mg/100 g FW). On the other hand, the naringin content obtained in CJB and CPB was lower than expected. The absence and low content of naringin in the samples can be explained by Coll et al. (28) study, who observed that peels and other solid residues of lemon, e.g., predominantly had the flavonoids hesperidin and eriocitrin, while naringin was mainly found at liquid waste. Also, in a previous study of our group, a low content of naringin was observed in Brazilian citrus residues (13). In addition, Chinapongtitiwat et al. (29) showed that the composition and the amount of flavonoids in citrus is highly linked to species, and factors such growth conditions, harvest time and type of processing. About the other phenolic compounds, the aglycones and phenolic acids were found in low concentrations (Table 3), in agreement with other studies (7,8,10).

Comparing CJB and CPB, all the quantified phenolic compounds were significantly higher ($P \leq 0.05$) in CPB, except narirutin, elagic acid, and hesperetin. Also, the sum of the individual levels of the polyphenols was calculated, and CPB presented the highest amount of polyphenols. At industry, usually, the raw materials used for pectin obtainment are CJB and apple pomace. The extraction of pectin is accomplished by pH decrease (around 1.2–

1.6), using mineral acids (hydrochloric or nitric acid), and high temperatures (around 80 ºC) (30). Previously studies indicated that phenolic compounds could be released by simple heat treatment from citrus peel (17, 31). Also, acidified extraction of orange peel polyphenols yield high content of these compounds (32). Based on these, the industry procedure of pectin

Table 3. Content of polyphenols (mg/100 g DM) in citrus by-products, quantified by HPLC-DAD.

Peak	Polyphenol		*Citrus* by-product		*P*-value
			CJB	CPB	
1		Galic acid	0.57 ± 0.02 [b]	1.07 ± 0.01[a]	0.02
	Hydroxybenzoic		-	-	
5	*acids*	Elagic acid	10.97 ± 0.08 [a]	0.27 ± 0.05 [b]	0.004
		Literature[*]	17.25[*]	-	
2		Narirutin	29.34 ± 0.43 [a]	17.50 ± 0.41 [b]	0.022
		Literature[*]	85.54[*]	-	
3	*Glycoside*	Naringin	1.02 ± 0.05 [b]	3.11 ± 0.08 [a]	0.021
	flavanones	Literature[*]	5.1 - 70.30[*]	-	
4		Hesperidin	232.65 ± 10.47 [b]	314.44 ± 18.82 [a]	0.033
		Literature[*]	205.20 – 258.38[*]	-	
6		Naringenin	0.47 ± 0.01 [b]	1.00 ± 0.09 [a]	0.015
	Aglycone	Literature[*]	0.13[*]	-	
7	*flavanones*	Hesperetin	1.05 ± 0,04 [a]	0.91 ± 0,05 [a]	0.108
		Literature[*]	0.13[*]	-	
8		Diosmetin	Tr	0.32 ± 0.13	-
			-	-	
9	*Methoxyflavones*	Tangeretin	1.41 ± 0,04 [b]	6.07 ± 0.36 [a]	0.003
		Literature[*]	6.52	-	
		Total	277.46 ± 9.90	344.68 ± 19.12	

Abbreviations: CJB (*Citrus* Juice by-product), CPB (*Citrus* pectin by-product), DM (dry matter), tr (traces)

[a, b] Values (mean ± SD) within each analyte with different letters significantly differ ($P \leq 0.05$), tested using t-test

[*] Madeira Jr and Macedo (8), Khan et al. (9) and Senevirathne et al. (19).

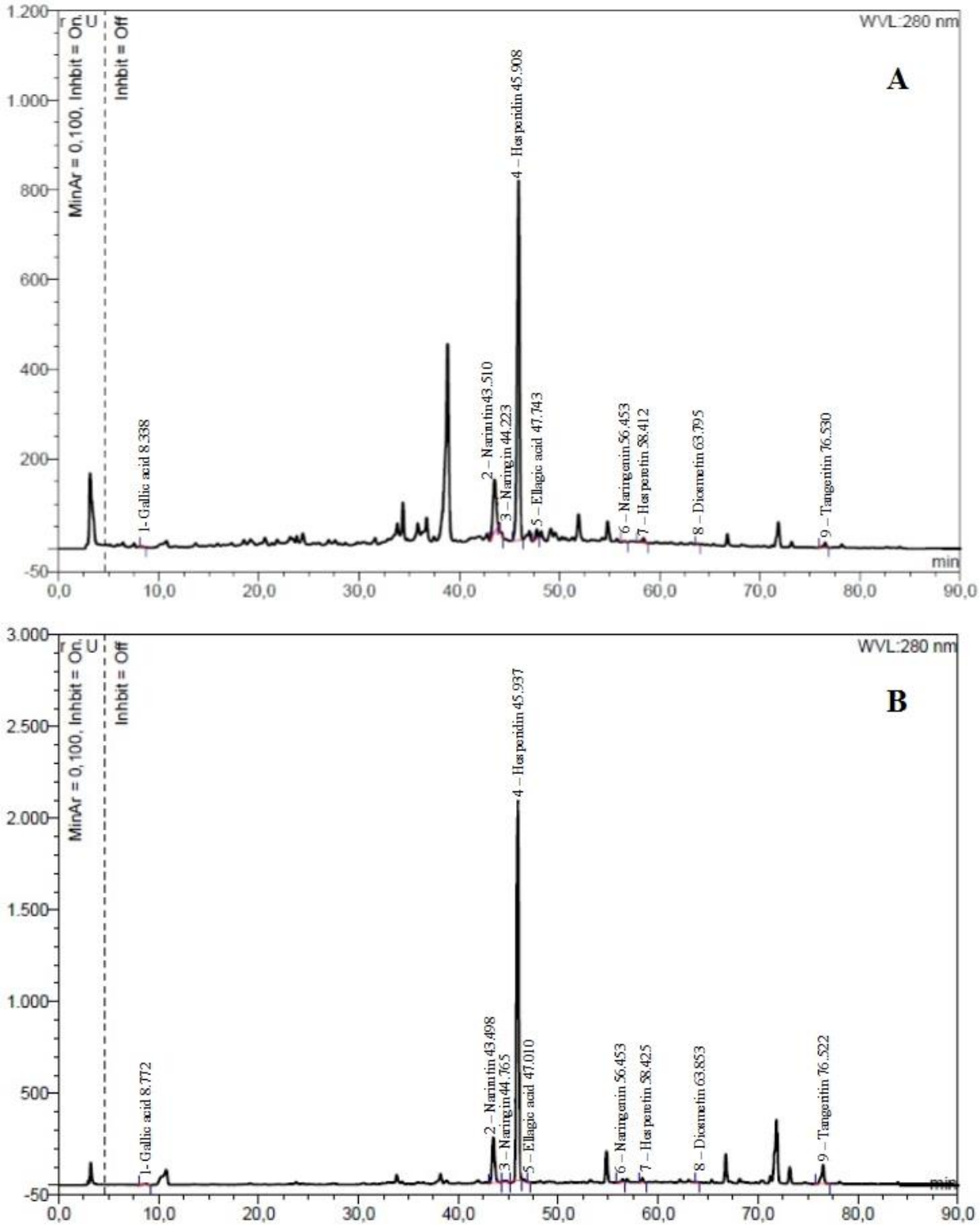

Figure 1. Chromatogram of the citrus residues CJB (A) and CPB (B) polyphenols obtained using HPLC-DAD.

extraction may release the polyphenols compounds from the matrix, justifying the better results obtained in CPB.

In general, polyphenols compounds are considered potent antioxidant molecules. In agreement with this, a high positive correlation was observed between TPC and DPPH/ORAC results (Table 2). However, the comparison between the quantified polyphenols content (Table 3) and the samples antioxidant capacity did not show any clear relationship. These results demonstrate that not only the detected polyphenos, but other compounds with reducing power must be responsible for the major bioactivity of CJB. In addition, the hypothesis is that these compounds might have been lost in the pectin-extraction process, which reduced CPB antioxidant power.

According to Russo et al. (33), the primary product of orange industry, the juice, has the lowest amount of bioactive compounds and polyphenols. Nakajima et al. (10) observed that the main flavonoids of *C. sinensis* juice are hesperidin (28.6 mg 100 mL^{-1}) and narirutin (5.2 mg 100 mL^{-1}). However, they were found in minor concentration when compared to the residues CJB and CPB. This data reinforces the advantage health benefits of CJB and CPB.

Once the studied residues are obtained from industrial waste and commonly employed for animal feed, their use to the extraction of citrus bioactive compounds is a possibility to increase these residues commercial added-value in profitable applications. Especially polyphenols, they are related to many functional and nutraceutical activities, e.g. antioxidant, inhibition of juice browning, antimicrobial action, and protection against inflammatory process (10, 34). Moreover, other compounds can be investigated and exploited in these by-products, like enzymes (lipases, proteases, and peroxidases), dietary fiber, and vitamins (2, 4). Thus, this study may provide significant information for a future profitable utilization of both residues as natural antioxidants, and for polyphenols and other bioactive compounds obtainment.

In conclusion, as already known, a significant amount of citrus by-products remain after juice processing. Previous studies have shown the high presence of phenolic compounds in this residue. However, to the best of authors knowledge, there are no studies on residues remained after citrus pectin extraction or industry application for it. Our results may prove that the treatment applied to the extraction of pectin does not promote polyphenols degradation, and the by-product obtained after this industry procedure contains even higher content of the quantified phenolic compounds. These results provide a support for the reuse of both studied residues, which can be in the future applied as ingredients to functional foods

development, natural antioxidants in food to prevent oxidative alterations (replacing synthetic antioxidants), and to the extraction of molecules with therapeutic action.

4. ACKNOWLEDGMENTS

Acknowledgements for the financial support of FAPESP (grant number 2015/04555-2). The authors also appreciated CNPq scholarships.

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CAPÍTULO III. ENZYME-ASSISTED BIOTRANSFORMATION INCREASES HESPERETIN CONTENT IN CITRUS JUICE BY-PRODUCTS

(Original research paper published by Food Research International - https://doi.org/10.1016/j.foodres.2018.05.004, 2018)

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With permission from Ruviaro, A.R.; Barbosa, P.P.M.; Macedo, G.A. Enzyme-Assisted Biotransformation Increases Hesperetin Content In Citrus Juice By-Products, Food Research International, in press, Elsevier, 2018.

ABSTRACT

Juice extraction from citrus fruits generates large amounts of residues, which account for 50% of the fruit weight. Citrus juice by-products (CJBs) are a rich source of phenolic glycosides. The enzyme-assisted extraction and biotransformation of phenolic compounds from CJBs were investigated. Pectinase, cellulase, tannase and β-glucosidase were used individually or in combination. The effects of enzymes to improve the release and bioconversion of phenolics from citrus residues were evaluated. Enzymes facilitated the extraction of phenolics from CJB and promoted their hydrolysis from sugar residues, resulting in changes in the phenolic profile and higher antioxidant activity. The results indicated that the optimum condition for hesperetin and naringenin production was 24 h of reaction using β-glucosidase at 20 U g^{-1}. Our results provide a basis for the production of extracts rich in bioactive compounds from CJB, which may be used as food and pharmaceutical applications.

KEYWORDS: citrus waste; phenolic compounds; enzymatic reaction; hesperetin; bioconversion; aglycones; tannase

1 INTRODUCTION

Brazil is the world's largest producer of oranges, having produced approximately 18 million tonnes in 2016/2017 (USDA, 2017), most of which is destined to the citrus juice industry. One of the steps of juice processing involves using a cold press to extract the maximum amount of juice. The remaining solids are considered waste residues, accounting for about 50% of the fruit weight (Delgado & Fleuri, 2016; Forster-Carneiro et al., 2013;

Mamma & Christakopoulos, 2008). The by-products obtained from these residues are used for different purposes: pectin and essential oils are widely employed in cosmetic, pharmaceutical, and food industries, whereas pellets are used for cattle feed. In general, the material discarded by the citrus processing industry includes many parts of the fruit, such as peels (flavedo and albedo), rags or pulp residues (juice sacs, segment walls, and cores), and seeds (Bampidis & Robinson, 2006; Mamma & Christakopoulos, 2008). Interestingly, studies have shown that citrus juice by-products (CJBs) have biological activities as a result of their phenolic composition, linked to a variety of health benefits (Khan, Zill-E-Huma, & Dangles, 2014). The glycosides hesperidin (hesperetin-7-O-rutinoside), narirutin (naringenin-7-O-rutinoside), and naringin (naringenin-7-O-neohesperidoside) are naturally present in varying concentrations in different citrus fruits (Khan et al., 2014; Pereira et al., 2017). Other important compounds present in citrus fruits are polymethoxylated flavones, e.g., tangeritin and diosmetin (Pereira et al., 2017; Yujing Sun, Shen, Liu, & Ye, 2015). Flavanones are reported to have different biological effects, such as cardiovascular protection, prevention of cancer and obesity as well as antioxidant, anti-inflammatory, and antimicrobial properties (Khan et al., 2014; Zou, Xi, Hu, Nie, & Zhou, 2016). Phenolics are commonly complexed with the plant cell wall and linked to polysaccharides (Singh et al. 2016; Madeira et al., 2015b). For this reason, enzymes such as pectinases and cellulases are often used to disrupt the integrity of cell walls and improve the extraction of phenolic compounds (Laroze, Soto, & Zúñiga, 2010; Li, Smith, & Hossain, 2006b). The chemical structure of phenolic compounds influences their biological activities (Di Majo et al., 2005; Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007). A study conducted by Cavia-Saiz et al. (2010) demonstrated that deglycosylation of phenolics in citrus extracts led to an enhanced antioxidant activity. Moreover, many studies have shown that enzymatic treatments can improve the biological properties of fruit extracts by facilitating the release of phenolic compounds from the cell wall and promoting changes in the molecular structure of phenolics (Ferreira, Macedo, Ribeiro, & Macedo, 2013; Martins, Roberto, Blumberg, Chen, & Macedo, 2016; Wilkins, Widmer, Grohmann, & Cameron, 2007). Enzymes such as β-glucosidase and tannin acyl hydrolase (tannase) have been exploited to increase the concentration of bioactive compounds in inexpensive agro-industrial residues by converting glycosylated compounds, phenolics linked to a glycoside, into their respective aglycones (Ahmed, Nasim, Batool, & Bibi, 2017; Madeira & Macedo, 2015; Zheng & Shetty, 2000). A tannase-mediated treatment improved the bioactivity and chemopreventive potential of orange juice through production of the aglycones hesperetin and naringenin (Ferreira et al., 2013). In addition, hydrolysis of ester

bonds from phenolics mediated by tannase led to a release of catechins from the fruit matrix and increased the antioxidant activity of grape pomace (Chamorro et al., 2012; Martins et al., 2016). The combination of tannase and cellulase increased hesperetin and naringenin concentration, improving the biological activity of citrus by-products (Madeira & Macedo, 2015). Likewise, cellulolytic enzymes facilitated the effect of β-glucosidases in olive oil mill wastewater treatment (Dammak et al., 2016). Thus, enzymatic treatment is considered an alternative process to promote the release and bioconversion of phenolic compounds from agro-industrial by-products, increasing the bioactive properties of extracts (Madeira et al., 2015b) and offering an alternative solution to the reuse of wastes from the largest orange industry in the world.

Considering the abundance of CJBs and their high concentration of bioactive substances, enzymatic hydrolysis of CJBs is a sustainable method for obtaining extracts rich in phenolic compounds. The objectives of this study were to evaluate the effects of cellulase, pectinase, tannase, and β-glucosidase on the release of phenolic compounds from CJBs, the conversion of phenolic glycosides into aglycones, and the antioxidant activity of CJB extracts after enzymatic treatments.

2 MATERIALS AND METHODS

2.1 Chemicals

Gallic acid, narirutin, naringin, naringenin, hesperidin, hesperetin, ellagic acid, tangeritin, diosmetin, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein, and Trolox® were purchased from Sigma–Aldrich (St. Louis, MO, USA). Folin–Ciocalteu's reagent, monobasic and dibasic sodium phosphate, sodium carbonate, and formic acid were purchased from Dinâmica Química Contemporânea (Diadema, SP, Brazil). Tannic acid was purchased from Ajinomoto OmniChem Division (Wetteren, Belgium). Liquid chromatography grade methanol was purchased from JT Baker (Center Valley, PA, USA). All other chemicals were analytical grade.

2.2 Preparation of citrus juice by-products

Dried CJB was supplied by CP Kelco Industry (Limeira, SP, Brazil). The material was crushed and sieved to a particle size of 1.86 mm (10 mesh, Bertel Metallurgical Industries LT).

CJB is a complex matrix composed of different varieties and parts of citrus fruits, such as peels (flavedo and albedo), pulp residues (juice sacs and segment walls), seeds and pectin. The proximate composition of the citrus residue used in the present study was previously determined by our research group (Barbosa et al., 2018).

2.3 Enzymes

Tannase (EC 3.1.1.20) from *Paecilomyces variotii* was produced at the Bioprocesses Laboratory of the School of Food Engineering, UNICAMP, by semi-solid fermentation (Battestin and Macedo, 2007). For the fermentation process, flasks containing 10 g of wheat bran, 10 mL of distilled water, and 10% (w/w) tannic acid were sterilized at 120 °C for 20 min. Subsequently, a *P. variotii* suspension (1.6×10^7 spores mL^{-1}) was inoculated and incubated at 30 °C for 120 h. After fermentation, 160 mL of acetate buffer (2mM, pH 5.0) was added and shaken at 200 rpm for 1 h (Fermentation Design Incorporated, Allentown, USA). The solution was filtered and centrifuged (9650g, 30 min) at 4 °C (Beckman J2-21 centrifuge, Beckman-Coulter, Inc. Fullerton, CA, USA). The supernatant was treated with solid ammonium sulfate (80% saturation) at 4 °C overnight. The precipitate was collected after centrifugation (9650g, 30 min), resuspended, and dialyzed against distilled water for 48 h. The dialyzed preparation was used as crude freeze-dried tannase for the enzymatic treatments.

β-glucosidase (naringinase from *Penicillium decumbens*) was purchased from Sigma–Aldrich (St. Louis, MO, USA). The enzymes cellulase (Celluclast 1.5L) and pectinase (Novozym 33095) were obtained from Novozymes (Latin America, Brazil). Tannase activity was evaluated using gallic acid as standard, according to Sharma et al. (2000), but with tannic acid as the substrate. The enzymatic activity of β-glucosidase was evaluated using p-nitrophenol as a standard (Matsuura et al., 1995). Cellulase and pectinase activities were determined by the DNS method, employing glucose (Wood and Garcia-Campayo, 1990) and D-*galacturonic acid* as standards (Couri and Farias, 1995), respectively. Activities were evaluated spectrophotometrically using the calibration curves of the standard compounds and were expressed as U mg^{-1} of protein capable of generating 1 μmol of product in 1 min.

2.4 Enzyme treatments

The enzymatic reaction medium consisted of 2 g of CJB and 25 mL of sodium acetate buffer (20 mM, pH 5.0), incubated with different enzymes for 6, 12, or 24 h at 40 °C under shaking (300 rpm), according to the procedures described by Madeira and Macedo (2015). Single or combined enzyme treatments were employed: C (5 U cellulase g^{-1} dry matter, DM), P (5 U pectinase g^{-1} DM), T (5 U tannase g^{-1} DM), B (5 U β-glucosidase g^{-1} DM), CP (5 U cellulase and 5 U pectinase g^{-1} DM), TCP (5 U tannase, 5 U cellulase, and 5 U pectinase g^{-1} DM), and BCP (5 U β-glucosidase, 5 U cellulase, and 5 U pectinase g^{-1} DM). The hydrolysis reaction was stopped by placing the samples in an ice-water bath for 15 min. Control samples were treated under the same conditions but without the addition of enzymes.

The phenolic extracts were prepared according to Nakajima et al. (2016). Samples were mixed with ethanol (25 mL), resulting in a 1:1 (v/v) ethanol-water mixture, placed in an ultrasonic bath (30 °C, 15 min), and shaken (15 min, 2000 rpm). After, ethanol was removed from the extracts by rotary evaporation (40 °C, 30 min). The aqueous solution was centrifuged, and the supernatant was kept frozen until analysis.

2.5 Effect of enzyme concentration on phenolic compounds

The enzymatic hydrolysis of phenolic compounds from CJB was performed using different enzyme concentrations: T5 (5 U tannase g^{-1} DM), T10 (10 U tannase g^{-1} DM), T20 (20 U tannase g^{-1} DM), B5 (5 U β-glucosidase g^{-1} DM), B10 (10 U β-glucosidase g^{-1} DM), B20 (20 U β-glucosidase g^{-1} DM), and 10T10B (10 U tannase and 10 U β-glucosidase g^{-1} DM). A control treatment was performed without the addition of enzymes.

2.6 Phenolic profile by HPLC

CJB extracts were diluted in 70% (v/v) methanol and filtered through a 0.45 µm Millex filter (HV PVDF, Millipore, Massachusetts, USA) before injection. Phenolic compounds were determined using a Dionex UltiMate 3000 (Dreieich, Germany) high performance liquid chromatography system equipped with a C18 Acclaim® 120 column (Dionex, 3 µm, 4.6 × 150 mm) at 30 °C. Detection was carried out using a UV/VIS detector (DAD-3000), and chromatograms were processed at 280 nm as adapted from Caridi et al. (2007) and de Mejía et al. (2010). The mobile phases were A (water/formic acid, 99.9:0.1 v/v) and B (methanol/formic acid, 99.9:0.1 v/v), injected at a flow rate of 0.6 mL min^{-1}.

Flavonoids (naringin, narirutin, naringenin, hesperidin, hesperetin, tangeritin, and diosmetin) were identified by comparison of their retention times and UV-VIS spectra to those of authenticated standards.

2.7 Total phenols and antioxidant potential

Total phenolic content (TPC) was measured using the Folin–Ciocalteu method according to Singleton et al. (1999). A gallic acid calibration curve was plotted, and TPC was expressed as mg gallic acid equivalents (GAE) 100 g^{-1} DM. The antioxidant potential (DPPH) was assessed using the DPPH radical scavenging activity as described by Macedo et al. (2011), and the oxygen radical absorbance capacity (ORAC) was measured as described by Dávalos et al. (2004) and adapted by Ferreira et al. (2013). Final values were expressed as μmol Trolox equivalents (TE) g^{-1} DM.

2.8 Statistical analysis

Results were expressed as mean ± standard deviation (SD). All measurements were performed in triplicate. The statistical difference between groups was analyzed by analysis of variance (ANOVA). Post-hoc comparison was performed by the Tukey's test. Differences were considered significant when $p \leq 0.05$. Statistical analyses were performed using Minitab 16.1.1.

3 RESULTS AND DISCUSSION

3.1 Enzymatic hydrolysis of phenolic compounds

CJB samples were treated with individual enzymes (Table 1) or enzyme combinations (Table 2) for 24 h to promote the release of phenolic compounds and increase the concentration of bioactive molecules.

Hesperidin was the most abundant polyphenol detected by HPLC-DAD in the control CJB, followed by narirutin and tangeritin. After 24 h of incubation, the concentration of narirutin and hesperidin decreased significantly (~1.4- and ~1.8-fold decrease, respectively) (Table 1). The degradation of these glycosides may have occurred through the oxidation or hydrolysis of hydroxyl groups (Madeira and Macedo, 2015).

Similar to our results, the glycoside hesperidin was the major compound detected in extracts of citrus by-products by Kim and Kim (2016),

Table 1. Phenolic profile (mg 100 g^{-1} DM) of CJB treated with individual enzymes.

Phenolic Compound (mg 100 g^{-1} DM)	**RT** (h)	**Enzyme Treatment***				
		Control	**Cellulase (C)**	**Pectinase (P)**	**Tannase (T)**	**β-glucosidase (B)**
Narirutin	6	10.2 ± 1.1 ^{aC}	47.5 ± 2.3 ^{aA}	23.1 ± 5.3 ^{aB}	11.9 ± 2.0 ^{aC}	10.4 ± 2.9 ^{aC}
	12	8.8 ± 3.5 ^{abC}	50.9 ± 4.5 ^{aA}	27.1 ± 0.2 ^{aB}	9.6 ± 1.5 ^{aC}	8.1 ± 0.9 ^{aC}
	24	7.4 ± 0.7 ^{bC}	49.2 ± 2.7 ^{aA}	22.4 ± 2.8 ^{aB}	10.5 ± 0.9 ^{aC}	10.2 ± 0.9 ^{aC}
Naringin	6	Tr	Tr	Tr	Tr	Tr
	12	Tr	Tr	Tr	Tr	Tr
	24	Tr	Tr	Tr	Tr	Tr
Hesperidin	6	106.6 ± 2.0 ^{aB}	255.2 ± 6.9 ^{aA}	104.6 ± 1.2 ^{bB}	109.3 ± 26.9 ^{aB}	129.4 ± 8.7 ^{aB}
	12	116.8 ± 10.3 ^{aB}	228.9 ± 7.0 ^{bA}	117.3 ± 1.6 ^{aB}	99.7 ± 7.4 ^{aBC}	88.3 ± 5.8 ^{bC}
	24	58.7 ± 21.0 ^{bC}	246.3 ± 7.8 ^{abA}	76.2 ± 4.2 ^{cBC}	89.4 ± 6.5 ^{aB}	71.0 ± 5.1 ^{cBC}
Naringenin	6	n.d.	n.d.	14.8 ± 0.2 ^{bB}	12.1 ± 0.1 ^{bC}	16.8 ± 0.5 ^{bA}
	12	n.d.	n.d.	19.5 ± 0.6 ^{aA}	22.6 ± 0.6 $^{a.A}$	21.1 ± 2.6 ^{aA}
	24	n.d.	n.d.	19.6 ± 0.9 ^{aA}	21.1 ± 0.9 $^{a.A}$	21.2 ± 1.6 ^{aA}
Hesperetin	6	n.d.	n.d.	26.5 ± 0.5 ^{cB}	32.3 ± 0.4 ^{cAB}	38.0 ± 6.0 ^{cA}
	12	n.d.	n.d.	51.8 ± 2.1 ^{bB}	80.8 ± 13.7 ^{bA}	82.5 ± 11.9 ^{bA}
	24	n.d.	n.d.	69.6 ± 4.1 ^{aB}	110.9 ± 5.8 ^{aA}	111.3 ± 12.5 ^{aA}
Tangeritin	6	1.1 ± 0.2 ^{aB}	1.5 ± 0.1 ^{aAB}	1.1 ± 0.1 ^{bb}	1.3 ± 0.2 ^{aAB}	1.6 ± 0.1 ^{aA}
	12	1.0 ± 0.1 ^{aC}	1.1 ± 0.1 ^{cC}	1.3 ± 0.1 ^{aBC}	1.7 ± 0.2 ^{aA}	1.6 ± 0.1 ^{aAB}
	24	0.9 ± 0.1 ^{aB}	1.3 ± 0.1 ^{bA}	1.0 ± 0.1^{cB}	1.4 ± 0.1 ^{aA}	1.3 ± 0.1 ^{bA}
Diosmetin	6	n.d.	n.d.	Tr	Tr	Tr
	12	n.d.	n.d.	Tr	Tr	Tr
	24	n.d.	n.d.	Tr	Tr	Tr

Abbreviations: DM (dry matter), RT (reaction time), n.d. (not detected), tr (traces). *Reaction conditions: 130 rpm, 40 °C, 24 h, 5 U g^{-1} of each enzyme. ^{a,b,c} Values (mean ± SD) with different letters in the same column differ significantly ($P \leq 0.05$). Tested using two-way ANOVA, followed by post-hoc Tukey's test. ^{A,B,C} Values (mean ± SD) with different letters in the same line differ significantly ($P \leq 0.05$). Tested using two-way ANOVA, followed by post-hoc

Table 2. Phenolic profile (mg 100 g^{-1} DM) of CJB treated with combinations of enzymes.

Phenolic Compound (mg 100 g^{-1} DM)	**RT** (h)	**Enzyme Treatment***			
		Control	**CP**	**TCP**	**BCP**
Narirutin	6	10.2 ± 1.1 ^{aB}	13.0 ± 2.4 ^{bAB}	16. ± 2.3 ^{aA}	11.7 ± 0.1 ^{aB}
	12	8.8 ± 3.5 ^{abB}	18.0 ± 1.7 ^{aA}	14.7 ± 0.9 ^{aA}	9.9 ± 2.7 ^{aB}
	24	7.4 ± 0.7 ^{bC}	19.4 ± 0.8 ^{aA}	15.8 ± 2.8 ^{aAB}	13.031 ± 2.4 ^{aB}
Naringin	6	Tr	Tr	Tr	tr
	12	Tr	Tr	Tr	tr
	24	Tr	Tr	Tr	tr
Hesperidin	6	106.6 ± 2.5 ^{aB}	120.6 ± 1.0 ^{aA}	103.9 ± 4.3 ^{aB}	27.9 ± 0.9 ^{aC}
	12	116.8 ± 10.3 ^{aA}	100.4 ± 1.5 ^{cB}	109.3 ± 2.9 ^{aAB}	27.1 ± 0.5 ^{aC}
	24	58.7 ± 21.0 ^{bB}	112.3 ± 2.9 ^{bA}	102.9 ± 8.2 ^{aA}	23.8 ± 1.4 ^{bC}
Naringenin	6	n.d.	13.7 ± 0.1 ^{bB}	22.3 ± 0.9 ^{aA}	22.5 ± 0.3 ^{aA}
	12	n.d.	16.3 ± 0.2 $^{a.B}$	21.5 ± 0.8 ^{aA}	17.7 ± 1.4 ^{bB}
	24	n.d.	15.7 ± 0.5 $^{a.B}$	22.3 ± 0.8 ^{aA}	24.2 ± 0.9 ^{aA}
Hesperetin	6	n.d.	24.9 ± 0.1 ^{cC}	65.6 ± 2.9 ^{cB}	102.9 ± 1.5 ^{bA}
	12	n.d.	47.3 ± 0.3 ^{bB}	77.3 ± 3.7 ^{bA}	83.9 ± 8.9 ^{cA}
	24	n.d.	54.2 ± 2.2 ^{aC}	96.7 ± 4.8 ^{aB}	148.7 ± 6.8 ^{aA}
Tangeritin	6	1.2 ± 0.2 ^{aAB}	0.9 ± 0.1 ^{bC}	1.3 ± 0.1 ^{aA}	1.0 ± 0.01 ^{aBC}
	12	1.0 ± 0.02 ^{aA}	1.0 ± 0.1 ^{aA}	1.1 ± 0.1 ^{bA}	0.7 ± 0.05 ^{bB}
	24	0.9 ± 0.1 ^{aA}	0.8 ± 0.1 ^{bB}	0.9 ± 0.1 ^{cAB}	n.d.
Diosmetin	6	n.d.	Tr	Tr	tr
	12	n.d.	Tr	Tr	tr
	24	n.d.	Tr	Tr	tr

Abbreviations: DM (dry matter), RT (reaction time), T (tannase), B (β-glucosidase), C (cellulase), P (pectinase), n.d. (not detected), tr (traces). *Reaction conditions: 130 rpm, 40 °C, 24 h, 5 U g^{-1} of each enzyme. ^{a,b,c} Values (mean ± SD) with different letters in the same column differ significantly ($P \leq 0.05$). Tested using two-way ANOVA, followed by post-hoc Tukey's test. ^{A,B,C} Values (mean ± SD) with different letters in the same line differ significantly ($P \leq 0.05$). Tested using two-way ANOVA, followed by post-hoc Tukey's test.

Nakajima et al. (2016), and Pereira et al. (2017). Kim and Kim (2016) found 104.00 ± 0.05 mg 100 g^{-1} of hesperidin in citrus juice residues, similar to the amounts of hesperidin in the studied CJB. However, the authors found narirutin concentrations 82% higher than those found in the present study. TPC may change according to citrus variety, type of industrial waste, and the extraction method employed.

Pereira et al. (2017) extracted flavonoids from different Brazilian orange juice residues using dimethyl sulfoxide (DMSO) and reported hesperidin concentrations ranging from 11.49 to 44.23 mg g^{-1}, tangeritin from 0 to 1.93 mg g^{-1}, and, instead of narirutin, the authors found naringin concentrations ranging from 0.96 to 5.80 mg g^{-1}.

Cellulase was able to release large amounts of narirutin (~4.7-fold increase in comparison with the control) and hesperidin (~2.4-fold increase) after 6 h of reaction (Table 1). These data confirm the ability of cellulase to release phenolics from plant sources. Thus, the extraction of phenolic compounds is more efficient when cellulase is employed (Li et al., 2006b; Madeira and Macedo, 2015). The time of reaction for C treatment did not influence the amount of glycosides liberated. This may be due to the enzymatic inhibition caused by cellobiose, glucose, and phenolic compounds liberated in the reaction medium after the action of cellulase (Oberoi, Sandhu, & Vadlani, 2012; Ximenes, Kim, Mosier, Dien, & Ladisch, 2010).

Similarly, pectinase facilitated the release of phenolics in comparison with the control. P treatment resulted in a significant production of the aglycones naringenin and hesperetin during the incubation time. A previous report showed that pectinase was more effective than cellulase in hydrolyzing polysaccharides from grapefruit peels (Wilkins et al., 2007) and contributed to the release of monosaccharides and extraction of phenolic compounds from grape pomace in a study by Chamorro et al. (2012). The authors reported that the phenolic profile of grape pomace could be modified by pectinase. Commercial pectinolytic enzyme preparations may exhibit a glucosidase side activity (Pricelius et al., 2009), promoting glucose hydrolysis from various glycosidic forms, resulting in aglycone moieties.

The changes observed in the phenolic profile of CJB after T and B treatments indicated that the enzymatic extraction and conversion of phenolic compounds occurred simultaneously with the use of these enzymes. Regarding hesperetin production, T and B treatments were similar and significantly the most effective in all incubation times. As expected, time was an important factor for the production of aglycones. After 24 h of enzyme reaction, hesperetin production was increased by approximately 110-fold with T, 111-fold

with B, and 70-fold with P in comparison with the control (Table 1). As shown by Madeira and Macedo (2015), a 24-hour reaction is ideal to promote the bioconversion of phenolic compounds from citrus juice residues.

The results of the release and conversion of phenols after the combined enzyme treatments are shown in Table 2. All treatments increased the release of narirutin in comparison with the control. However, TCP and BCP treatments produced significantly higher amounts of naringenin in comparison with CP (~1.6-fold increase). This result did not change with different periods of incubation. Hesperidin contents varied between treatments; this flavanone is probably more affected by enzymes because it is present in higher amounts in the CJB matrix. Hesperetin had a significantly higher increase after BCP treatment, followed by TCP and CP treatments, at all times evaluated. Among the combined enzyme treatments, BCP seemed to be the most effective enzymatic cocktail for conversion of glycosylated phenolics, as it greatly decreased hesperidin concentration and increased hesperetin concentration at all incubation times. At 24 h of incubation, hesperetin recovery with BCP treatment was 1.5-fold higher than with TCP and 2.7-fold higher than with CP (Table 2).

The phenolic profile of CJB may be affected by factors such as type of enzyme, enzyme dosage, and interactions between enzyme and matrix compounds (Li et al., 2006b).

Enzymes such as β-glucosidase act on different types of glycoside substrates and may be used to hydrolyze glucose from glycosidic structures, including flavonoids (Ahmed et al., 2017; Zheng & Shetty, 2000b). Results reported by Zheng and Shetty, (2000) indicated that β-glucosidase played an important role in the conversion of free phenolic acids from cranberry pomace. Tannase is able to hydrolyze the ester bond of phenolic compounds (Chamorro et al., 2012; Ferreira et al., 2013) as well as the depside bond (Lekha and Lonsane, 1997). Ferreira et al. (2013) showed that tannase from *P. variotii* was able to remove glycosides from naringin and hesperidin, increasing the contents of hesperetin and naringenin. As in our study, previous reports have demonstrated that *P. variotti* tannase is able to modify the phenolic profile of complex food matrices, e.g., tea extracts (Macedo et al., 2011; Roberto et al., 2016), orange juice (Ferreira et al., 2013), citrus by-products (Madeira and Macedo, 2015), and grape pomace (Martins et al., 2016).

Madeira and Macedo (2015) and Martins et al. (2016) reported that pectinase or cellulase alone was less effective than enzyme mixtures, probably because of synergistic effects. Cellulolytic and pectinolytic enzymes are able to release phenolic compounds bound to several sites of the cell wall, facilitating the action of tannase and β-glucosidase in the

conversion of phenolics (Baik et al., 2014a; Chamorro et al., 2012; Dammak et al., 2016; Madeira & Macedo, 2015). Baik et al. (2014a) reported that simultaneous treatment with pectinase and tannase enhanced the extraction and conversion of bioactive components from green tea extracts. Similarly, Madeira and Macedo (2015) demonstrated that the combination of cellulase and tannase produced great amounts of naringenin and hesperetin after 24 h of reaction.

It is important to emphasize that both TCP and BCP treatments used 15 U mg^{-1} of enzyme and CP used 10 U mg^{-1} of enzyme, whereas the single-enzyme treatments contained only 5 U mg^{-1} of enzyme. In general, higher enzyme concentrations have a more pronounced effect on phenolic composition (Li et al., 2006b). Conversely, our results suggested that PC treatment did not display an important synergistic effect on the release of phenolics. When the TCP and BCP cocktails were used (Table 2), higher amounts of naringenin (~1.8- and ~2.1-fold increase) and hesperetin (~1.3- and 2.7-fold increase) were obtained after 6 h of incubation in comparison with T and B alone (Table 1). That is, the production of bioactive compounds from CJB was faster with TCP and BCP treatments. However, T and B treatments appeared to have effects comparable to those of TCP and BCP treatments on the bioconversion of phenolics after 12 and 24 h of reaction. Thus, individually, tannase and β-glucosidase may be sufficient to promote phenolic hydrolysis and conversion in CJB. Chamorro et al. (2012) showed that tannase did not contribute to cell wall degradation and phenol release. In contrast, Madeira and Macedo (2015) showed the significant effect of tannase on the extraction of phenolic compounds from citrus juice residues, as was observed in our study.

CJBs are a rich source of phenolic compounds, particularly of the glycosides hesperidin and naringin. Studies have reported the phenolic profile of peels from different citrus varieties and of different residues from the orange juice processing industry. Notably, none of the evaluated residues presented aglycones in their composition (Pereira et al., 2017). Reinforcing this information, hesperetin and naringenin were not detected in the control CJB at any of the incubation times (Table 1) but were detected in the enzyme-treated CJB. Thus, enzymatic treatment offers the advantage of increasing the concentration of bioactive compounds, such as deglycosylated flavanones in food matrices. At present, studies that test aglycone forms generally have to use high cost analytical standards, as there are no commercial sources available for extraction of hesperetin and naringenin. The enzymatic process presented in this study may be interesting to produce a commercial source of these

compounds. Thus, the reuse of citrus by-products through enzymatic biotransformation can be considered a viable and economically interesting industrial alternative.

Low amounts of tangeritin were detected in CJB, and higher values were found after T and B treatments in comparison with the control (Tables 1 and 2). Traces of diosmetin were detected in CJB after the enzymatic treatments. According to Pereira et al. (2017), tangeritin is more abundant in Murcott than in other citrus varieties, even though it is present in low concentrations. This flavone contributes to the anti-inflammatory activity of orange peel extracts (Chen et al., 2017) and shows an important antioxidant activity.

3.2 Total phenols and antioxidant potential

The results of the determination of TPC and antioxidant potential of CJB before and after enzyme treatments are shown in Fig. 1. Previous research has shown that citrus by-products represent an important source of phenolic compounds with high antioxidant activity and that enzymatic treatment can improve these properties (Li et al., 2006b; Wilkins et al., 2007).

According to Anagnostopoulou et al. (2006), TPC values of orange peels varied from 3.63 to 254 mg GAE 100 g^{-1} DM. Our results for the control CJB exceeded these values (~90% higher) but were similar to the TPC values that Hernández-Carranza et al. (2016) obtained for citrus peels (391–514 mg GAE 100 g^{-1} DM). As expected, the tested enzymes significantly increased the TPC of CJB at all incubation times in comparison with the control (Figs. 1A1 and 1B1).

DPPH is currently used to evaluate the free-radical scavenging capacity of antioxidant compounds extracted from food matrices. In general, enzymatic treatments containing pectinase, tannase, and β-glucosidase improved the DPPH values of CJB extracts (Fig. 1A2 and 1B2). However, our results showed that antioxidant activity by the DPPH assay significantly increased after T and BCP treatments (~18% and ~23%, respectively) at all times. These values were similar to those found in treated citrus residues (10.737 ± 156 μmol g^{-1}) by Madeira and Macedo (2015) after 24 h of enzymatic reaction.

Both before and after the enzymatic treatments, CJB showed a higher (>27%) antioxidant activity by the ORAC method than the treated citrus juice residues evaluated by Madeira and Macedo (2015). As observed in our results, ORAC values of CJB treated with tannase had a significant increase of approximately 30% after 12 h of incubation in comparison with the control. This increase was comparable to the effect promoted by the combined enzyme treatments (~30%) after 6, 12, and 24 h of incubation (Fig. 1A3 and 1B3).

Li et al. (2006b) observed that the integrity of cell walls was disrupted after enzymatic treatment, improving the recovery of TPC. However, other materials may have been released from the cells, forming complexes with phenolic compounds. Phenolics are potent antioxidants, a characteristic attributed to their hydrogen-donating ability. This property is affected by their molecular structure (Tripoli et al., 2007; Di Majo et al., 2005). Di Majo et al. (2005) showed that flavanones glycosylated with rutinose, such as hesperidin and narirutin, have an antioxidant capacity comparable to that of their aglycones. Moreover, the antioxidant power may be influenced by other types of compounds that were not identified in this study.

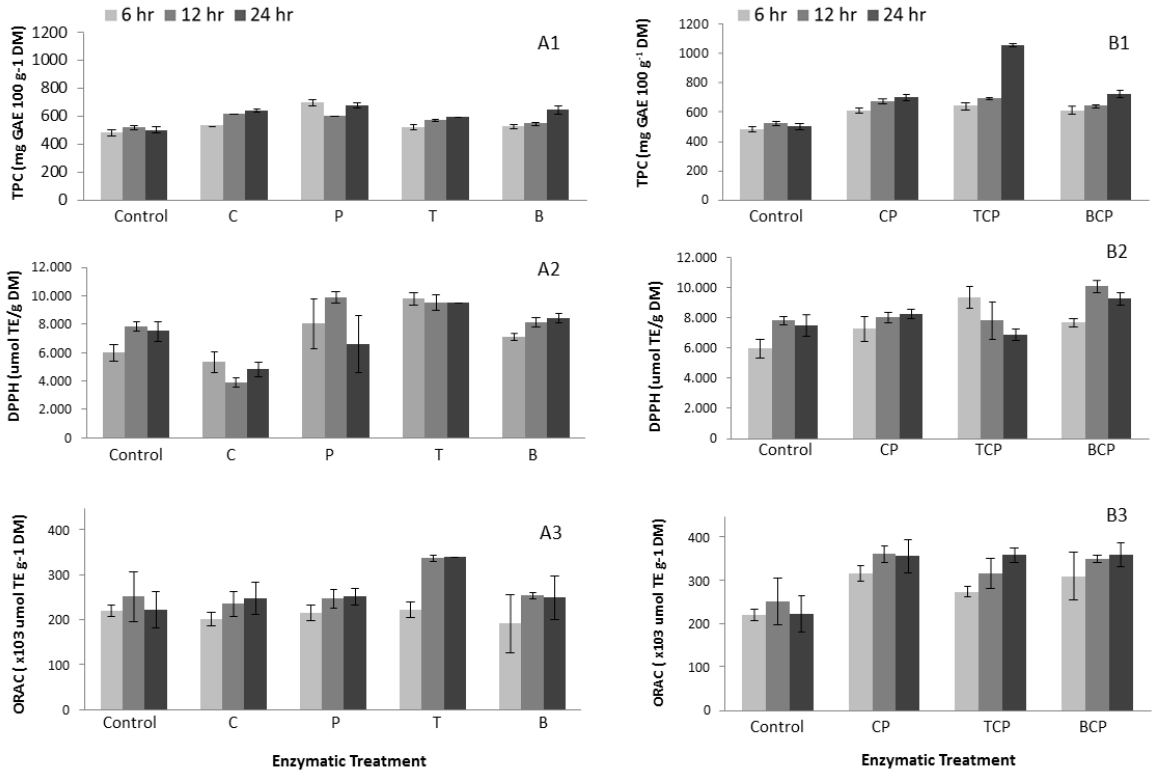

Figure 1. Total phenolic compounds (TPC), DPPH, and ORAC activities of CJB treated with single (A1, A2, and A3) or combined enzymes (B1, B2, and B3). Abbreviations: DM (dry matter), T (tannase), B (β-glucosidase), C (cellulase), P (pectinase), GAE (gallic acid equivalent), TE (Trolox equivalent), hr (hours). Results are presented as means±SD.

Our results of TPC and antioxidant potential can diverge from those of other studies, as CJB is a complex matrix formed by different citrus varieties and tissues (peels, pulp residues, seeds, and pectin). In addition, the conditions of the extraction method, such as the type of solvent used and the solid-liquid ratio, are important factors that influence the results (Li et al. 2006a). While the extraction of phenols was performed with solvents such as

acetone, methanol, and DMSO in some studies (Anagnostopoulou et al., 2006; Chamorro et al., 2012; Pereira et al., 2017), the extraction of phenolic compounds from CJB was performed with an ethanol/water solution (1:1) in the present study. The use of ethanol is justified, as it presents less toxicity, being considered a food-grade solvent. Nakajima et al. (2016) had already reported that the recovery of TPC from citrus residues was similar when using ethanol or methanol in the same concentrations, and preferred a 50% ethanol solution as the extraction solvent. Other authors used ethanol concentrations in the range of 70–80% for extraction of phenolics from citrus residues (Li et al., 2006a).

According to Chemat et al. (2017), a green method has a low processing time, low energy and water consumption, allows the recycling of by-products, and generates safe products. Currently, phenolic compounds are obtained by chemical synthesis or extraction, requiring the use of organic solvents and heating apparatus. Enzymatic biotransformation is a noteworthy process for obtaining phenolic compounds, as it eliminates the need for toxic organic solvents. In enzymatic biotransformation, bioactive compounds can be obtained from natural sources by the action of exogenous enzymes produced by microorganisms. In addition, the use of CJB is interesting because of its wide availability, low cost, and composition, which allows obtaining different bioactive phenolic compounds.

3.3 Effect of enzyme concentration on phenolic profile

The effect of enzyme concentration was analyzed after 24 h of enzymatic reaction using tannase and β-glucosidase at different concentrations.

The results shown in Table 3 indicate that T and B treatments were able to release phenolic compounds from raw CJB and promote the conversion of glycosides into their aglycone forms. Furthermore, enzyme concentration and type of enzyme used in the reaction medium influenced the release of phenols. The conversion into aglycone forms promoted by T appeared to have reached its peak with 10 U g^{-1} DM. Even at higher enzyme concentrations, the concentration of aglycones remained the same. Conversely, the conversion of phenolic glycosides by B treatment increased in a dose-dependent manner. Naringenin and hesperetin amounts after B treatment were significantly higher than after T treatment at the highest tested concentrations: an approximate 1.2-fold increase with 10 U g^{-1} DM and 1.8-fold increase with 20 g^{-1} DM (Table 3).

Hamza et al. (2011) investigated the effect of β-glucosidase concentration (0 to 1125 IU mL^{-1}) on the content of bioactive phenolic compounds of olive mill wastewater and

showed that the concentration of bioactive compounds increased as enzyme concentration increased in the reaction medium, as in our study.

The inhibition of T treatment may have been caused by the increase of free phenolic compounds in the reaction medium. Baik et al. (2014b) showed that treatment of green tea polyphenols using tannase (500 U g^{-1}) promoted the conversion of epigallocatechin gallate into gallic acid, but the enzyme was inhibited at higher concentrations of catechins (substrate). In comparison, the effect of commercial tannase at high concentrations (2000 and 4000 U g^{-1} DM) was evaluated in grape pomace during 24 h of incubation by Chamorro et al. (2012). The results showed that tannase hydrolyzed ester bonds of complex phenolic compounds and released smaller compounds, e.g., catechins and gallic acid. In addition, the amount of gallic acid incred as the enzyme concentration increased.

P. variotti tannase is considered a stable and efficient enzyme (Madeira et al. 2015a). However, more studies are necessary to investigate the activity of this enzyme and determine the ideal enzyme-to-substrate ratio and its mechanism of inhibition in order to improve its action. Moreover, as a crude extract of tannase was used in our study, the purity of the enzyme is a factor that needs to be taken into consideration, for it can affect enzyme activity. Significantly higher amounts of naringenin, hesperetin, and diosmetin were obtained with B20, and the amounts of hesperetin were similar to those obtained with TB treatment. The concentrations of naringenin, hesperetin, and diosmetin were approximately 26, 44, and 47% higher, respectively, with TB than with T20 (Table 3). These results indicate a possible synergistic effect between β-glucosidase and tannase to promote the conversion of phenols, increasing the concentration of aglycones. The main phenolic compounds identified in the control CJB and in CJB treated with TB are shown in Fig. 2.

Previously, Madeira and Macedo (2015) demonstrated that the combination of cellulase and tannase produced optimal naringenin (80 mg 100 g^{-1}) and hesperetin (120 mg 100 g^{-1}) amounts. Interestingly, the results of the present study demonstrated that B20 and TB treatments led to 4-fold higher hesperetin content than that reported by Madeira and Macedo (2015). These results reinforcing the information that the enzymatic treatment of citrus residues can be economically interesting for the industry in order to produce extracts rich in bioactive compounds while reduce the volume of waste to be disposed.

Enzymatic extraction is a viable, green process with great potential to provide extracts with large amounts of bioactive phenolic compounds, such as hesperetin, naringenin, and diosmetin. This way, biotransformed CJB appears promising for use in the development of functional and nutraceutical applications.

Table 3. Effect of enzyme concentration on the phenolic profile of CJB.

Phenolic Compound (mg 100 g^{-1} DM)	Treatment							
	Control	Tannase (T)			β-glucosidase (B)			Mixture (TB)
		5T	10T	20T	5B	10B	20B	10T+10B
Narirutin	7.4 ± 0.7^{C}	10.5 ± 0.9^{B}	10.6 ± 0.3^{B}	10.8 ± 0.8^{B}	10.2 ± 0.9^{B}	12.2 ± 1.5^{B}	11.1 ± 1.5^{B}	17.4 ± 0.4^{A}
Naringin	Tr	tr	5.6 ± 0.1^{A}	3.2 ± 0.6^{B}	Tr	Tr	tr	5.5 ± 0.2^{A}
Hesperidin	58.7 ± 21.0^{BCD}	89.4 ± 6.5^{A}	65.9 ± 3.3^{BC}	44.4 ± 1.8^{CD}	71.0 ± 5.1^{AB}	54.9 ± 2.6^{BCD}	56.6 ± 3.2^{BCD}	36.4 ± 0.9^{D}
Naringenin	n.d.	21.1 ± 0.9^{E}	34.1 ± 2.2^{CD}	30.7 ± 1.2^{D}	21.2 ± 1.6^{E}	38.9 ± 1.2^{BC}	53.1 ± 3.4^{A}	41.8 ± 0.5^{B}
Hesperetin	n.d.	110.9 ± 5.8^{D}	222.4 ± 16.7^{C}	247.5 ± 13.4^{BC}	111.3 ± 12.5^{D}	271.0 ± 9.1^{B}	462.2 ± 31.7^{A}	439.3 ± 3.0^{A}
Diosmetin	n.d.	tr	9.14 ± 0.6^{C}	8.6 ± 0.3^{C}	Tr	9.9 ± 1.2^{C}	26.5 ± 1.2^{A}	15.9 ± 1.3^{B}
Tangeritin	1.0 ± 0.1^{A}	1.4 ± 0.1^{A}	1.5 ± 0.1^{A}	1.5 ± 0.1^{A}	1.3 ± 0.1^{A}	2.1 ± 0.1^{A}	1.8 ± 1.2^{A}	1.8 ± 0.1^{A}

Abbreviations: DM (dry matter), T (tannase), B (β-glucosidase), n.d. (not detected), tr (traces).
^{A,B,C} Values (mean ± SD) with different letters in the same column differ significantly ($P \leq 0.05$). Tested using two-way ANOVA, followed by post-hoc Tukey's test.
*Reaction conditions: 130 rpm, 40 °C, 24 h.

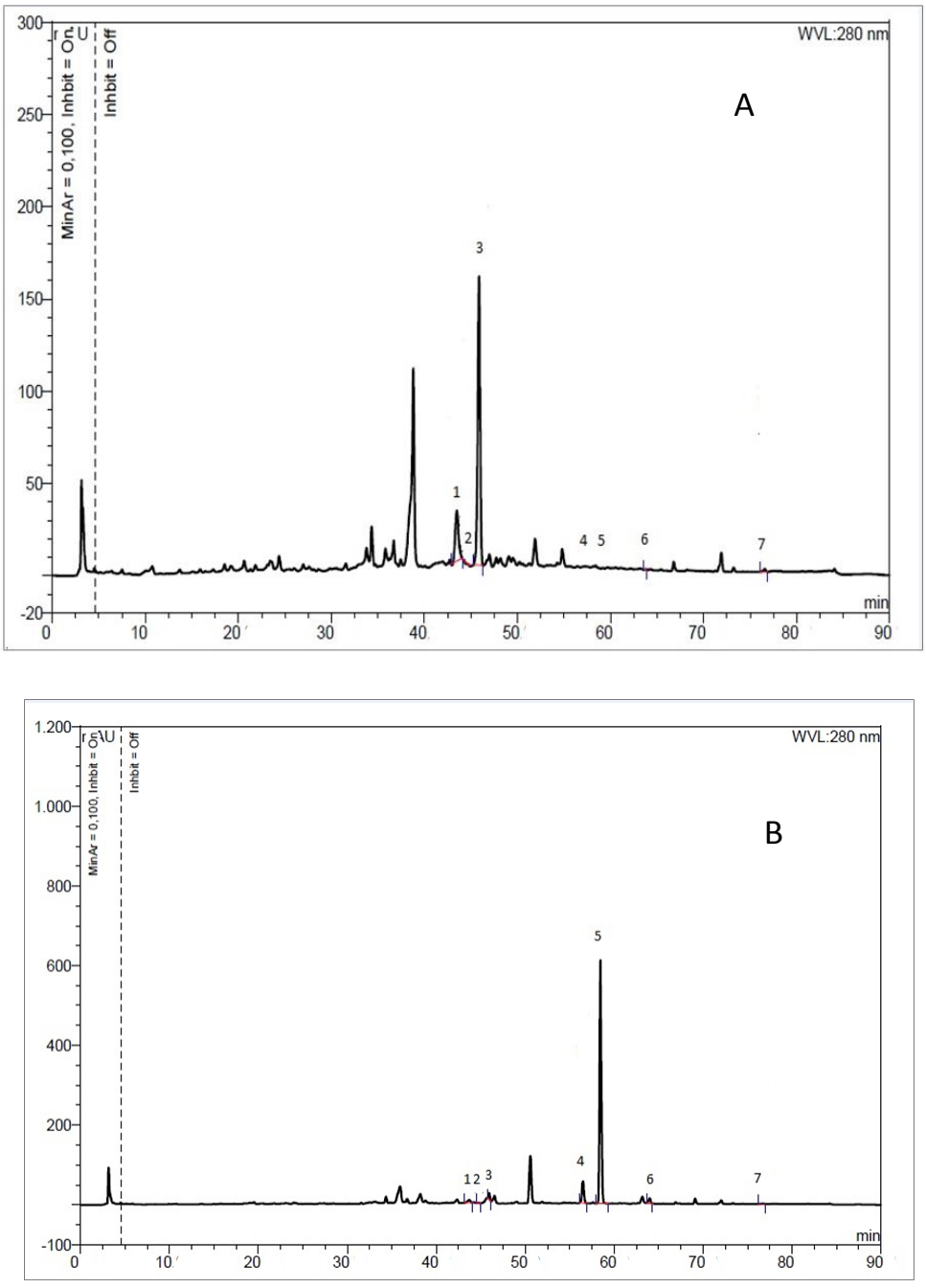

Figure 2. Chromatogram of polyphenols obtained using HPLC-DAD for the control - CJB with no enzymatic treatment (A) and after TB Treatment - CJB incubated with 10 U tannase and 10 U β-glucosidase g^{-1} DM (B). Reaction conditions: 130 rpm, 40oC, 24 hr. 1 – Narirutin; 2 – Naringin; 3 – Hesperidin; 4– Naringenin; 5 – Hesperetin; 6 – Diosmetin; 7 – Tangeritin.

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4 CONCLUSIONS

This current study demonstrated that enzymatic treatment is a viable method to enhance the bioactive potential of CJB, maximizing the applications of citrus fruits and

reducing the industrial waste. CJB are natural sources of bioactive phenolics compounds. Parameters such as type of enzyme, enzyme concentration, and time of reaction affected the phenolic profile of the CJB. Pectinase, tannase, and β-glucosidase, individually or combined, improved the extraction of phenolic compounds from the CJB while promoting the conversion of these compounds into aglycones. Of the enzymatic treatments evaluated, 20 U β-glucosidase g^{-1} DM (B20) was the most effective in promoting phenolic conversion after 24 h of reaction, mainly for hesperetin. In addition, the cocktail containing β-glucosidase and tannase was efficient in providing extracts with large amounts of bioactive compounds as hesperetin, naringenin and diosmetin. Our results provide evidence that biotransformation of CJB can be used to obtain products rich in bioactive compounds, which, in turn, may be used as ingredients for functional foods, as natural antioxidants, and for the extraction of bioactive molecules for food and pharmaceutical applications.

5 CONFLICT OF INTEREST

The authors declare no conflict of interest.

6 ACKNOWLEDGMENTS

The authors thank the São Paulo Research Foundation (FAPESP) and the Brazilian National Council for Scientific and Technological Development (CNPq) for the financial support.

7 FUNDING

This study was supported by the São Paulo Research Foundation (FAPESP, grant no. 2015/04555-2) and *the* Brazilian National Council for Scientific and Technological Development (*CNPq*, Ph.D. grant no. 140537/2015-5).

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CAPÍTULO IV. FLAVANONES BIOTRANSFORMATION FROM CITRUS BY-PRODUCTS IMPROVES ANTIOXIDANT AND ACE INHIBITORY ACTIVITIES IN VITRO

(Original research paper submitted to Journal of Functional Foods)

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ABSTRACT

We investigated enzymatic biotransformation effects (tannase and β-glucosidase) on phenolic profile, antioxidant capacity, and angiotensin-converting enzyme (ACE) inhibitory activity of extracts from two citrus industry wastes. Hesperidin and hesperetin ACE inhibitory activity was also evaluated. The major phenolic in crude extracts was hesperidin, whereas hesperetin in biotransformed. Antioxidant capacity and ACE inhibitory activity of enzyme-treated extracts were significantly higher. Citrus pectin residue extract treated with enzymes (CPRTB) had higher antioxidant and anti-ACE activities (FRAP = 297.8±3.1μmolTE mg^{-1}, DPPH = 231.8±7.8μmolTE mg^{-1}, IC_{50} = 0.21mg mL^{-1}, E_{max} = 100%), and higher hesperetin content (42.7mg g^{-1}). Hesperetin standard solution exhibited higher anti-ACE activity than hesperidin. Despite the lower flavanones concentration in comparison to analytical standards solution, CPRTB presented the same anti-ACE activity of hesperitin 1000μM, suggesting that the citrus extracts bioactivity is due to the presence of glycosides and aglycones. Enzymatic biotransformation allowed industrial wastes reuse, affording extracts with promising potential against hypertension.

KEYWORDS: anti-ACE; bioactivity; blood pressure; citrus waste; hesperetin; hypertension.

1. Introduction

Cardiovascular diseases are the leading cause of death worldwide, and hypertension is considered an important risk factor for the development of coronary heart disease and stroke (IFPMA, 2016). Blood pressure and cardiovascular homeostasis are controlled by the renin-angiotensin-aldosterone system, in which angiotensin II has a critical function. The system is activated by the release of renin into the bloodstream. Renin cleaves

angiotensinogen into angiotensin I, and angiotensin I is then hydrolyzed by angiotensin-converting enzyme (ACE) into angiotensin II, which induces vasoconstriction and, consequently, increases blood pressure (Lavoie & Sigmund, 2003; Wen, Gwathmey, & Xie, 2012).

Angiotensin II is also involved in the production of reactive oxygen species (ROS) in vascular tissues via the NAD(P)H oxidase pathway. In healthy conditions, free radicals are produced in a controlled manner, at low concentrations. In pathological conditions, such as in hypertension, there is an increase in ROS production, creating a state of oxidative stress. Thus, angiotensin II can have deleterious effects on blood vessels, causing functional and structural damage (Virdis, Duranti, & Taddei, 2011; Wen et al., 2012).

A strategy to control hypertension is the use of ACE inhibitors to prevent angiotensin II production (IFPMA, 2016). Many natural products have been studied as sources of ACE inhibitors (Balasuriya & Rupasinghe, 2011), and a previous study demonstrated that phenolic compounds are able to inhibit ACE (Guerrero et al., 2012). Complex extracts obtained from agro-industrial residues have been studied as sources of phenolics in an effort to reuse the widely available by-products and wastes generated by the food industry, such as, for instance, litchi pericarps (Kessy, Wang, Zhao, Zhou, & Hu, 2018), grape seeds (Afonso, Passos, Coimbra, Silva, & Soares-da-Silva, 2013), apple peels (Balasuriya & Rupasinghe, 2011), and citrus leaves (Perez, Jimenez-ferrer, Alonso, Botello-amaro, & Zamilpa, 2010).

Brazil is the largest orange producer in the world, with approximately 17 million tonnes of fruit produced in the last harvest (FAOSTAT, 2018). These fruits are mainly destined for orange juice production. After juice extraction, the remaining solids (about 50% of the fruit weight) are discarded, resulting in large amounts of waste (Delgado & Fleuri, 2016). Several studies have shown that citrus residues are rich in phenolics and bioactive molecules, compounds associated with beneficial health effects, including anti-obesity, antioxidant, anti-inflammatory, antimicrobial, and cardiovascular protective properties (Khan et al., 2014; Zou et al., 2016).

Hesperidin (hesperetin-7-O-rutinoside) and narirutin (naringenin-7-O-rutinoside) are glycosides flavanones naturally present in citrus fruits in higher concentrations. However, these phenolics exhibit higher reactivity and bioactivity in the aglycone form, as hesperitin and naringenin (Cavia-Saiz et al., 2010). Madeira & Macedo (2015) showed that hesperetin and naringenin have significantly higher *in vitro* antioxidant activity than their glycosides. Naringenin seems to be a stronger metallic ions chelator and presents higher antioxidant and

antiradical capacities than its glycoside. These effects have been attributed to the elimination of rutinoside at the C7 of narirutin (Cavia-Saiz et al., 2010). Phenolic glycosides can be submitted to enzymatic or microbial hydrolysis to improve bioaccessibility and bioavailability (González-Sarrías, Espín, & Tomás-Barberán, 2017; J. V. Madeira & Macedo, 2015; Radenkovs, Juhnevica-Radenkova, Górnaś, & Seglina, 2018; Ruviaro, Barbosa, & Macedo, 2018). Biotransformation of polyphenols by tannase and β-glucosidase improved the antioxidant capacity of citrus extracts (Ruviaro et al., 2018) and litchi pericarp extracts (Kessy et al., 2018). Thus, it is possible to increase the yield of bioactive compounds obtained from natural sources by the action of exogenous enzymes produced by microorganisms. In addition, enzymatic biotransformation is considered a safe and green process, as it eliminates the need for toxic organic solvents (Madeira et al., 2015; Radenkovs et al., 2018).

On the basis of literature data and our previous positive results with phenolic biotransformation, we aimed to compare the phenolic profile of citrus extracts biotransformed using tannase and β-glucosidase and evaluate the effect of biotransformation on the antioxidant activity and ACE inhibitory activity of extracts.

2. Materials and Methods

2.1 *Chemicals*

Diosmetin, gallic acid, hesperidin, hesperetin, narirutin, naringenin, tangeritin, Trolox, fluorescein, 2,2'-azobis(2-methylpropionamidine)dihydrochloride (AAPH), tripyridyltriazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hippuryl-L-histidyl-L-leucine H1635 (HHL), and rabbit lung ACE (A6778) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Folin–Ciocalteu's reagent, monobasic and dibasic sodium phosphate, sodium carbonate, and formic acid were purchased from Dinâmica Química Contemporânea (Diadema, SP, Brazil). Tannic acid was purchased from Ajinomoto OmniChem Division (Wetteren, Belgium). Liquid chromatography grade methanol was purchased from JT Baker (Center Valley, PA, USA). All other chemicals were analytical grade.

2.2 Citrus by-products

Two citrus by-products were used in this study: CJR (citrus juice residue), resulting from the extraction of orange juice, and CPR (citrus pectin residue), resulting from the extraction of pectin from CJR.

These materials were obtained directly from an industry specialized in citrus pectin extraction (CP Kelko, SP, Brazil). Dried samples were ground with a portable laboratory grinder (particle size of 1.86 mm). The chemical composition of these residues was previously described by our research group (Barbosa, Ruviaro, & Macedo, 2018).

2.3 Enzymes

Penicillium decumbens β-glucosidase was purchased from Sigma–Aldrich (St. Louis, MO, USA). *Paecilomyces variotii* tannase (EC 3.1.1.20) was produced by semi-solid fermentation at the Bioprocesses Laboratory of the School of Food Engineering, UNICAMP, according to the method previously described by Battestin and Macedo (2007).

β-glucosidase activity was determined using ρ-nitrophenol as standard (Matsuura et al., 1995). Tannase activity was measured using a calibration curve of gallic acid (Sharma et al., 2000) but with tannic acid as substrate. Activities were evaluated spectrophotometrically using standard curves and were expressed as U mg^{-1} of protein capable of generating 1 μmol of product in 1 min.

2.4 Enzymatic treatment of citrus residues

Citrus residues (2 g) were incubated with an enzyme cocktail (TB) in 25 mL of sodium acetate buffer (20 mM, pH 5.0) for 24 h at 40 °C under agitation of 300 rpm, according to the procedures of Madeira & Macedo (2015).

TB was composed of 10 U tannase and 10 U β-glucosidase per gram of substrate. These concentrations were based on the results obtained by Ruviaro, Barbosa, & Macedo (2018). At the end of the reaction time, the mixture was chilled in an ice bath for 15 min to inhibit enzyme activity. Control samples were submitted to the same conditions but without the addition of enzymes.

Lastly, samples were mixed with 25 mL of ethanol, resulting in a 1:1 (v/v) ethanol/water mixture. After 60 min of shaking (2000 rpm), samples were sonicated for 30 min in an ultrasonic bath and centrifuged for 15 min at 9000 g and 4 °C. The supernatant was collected and filtered through Whatman No. 1 filter paper. Then, ethanol was removed from the extracts by rotary evaporation at 40 °C, and the resulting aqueous solution was frozen and freeze-dried (adapted from Nakajima, Madeira, Macedo, & Macedo, 2016).

Four phenolic extracts were prepared, two using crude residues and two using biotransformed residues: i) CJR extract (citrus juice residue extract), ii) CPR extract (citrus pectin residue extract), iii) CJRTB extract (citrus juice residue extract treated with tannase

and β-glucosidase), and iv) CPRTB extract (citrus pectin residue extract treated with tannase and β-glucosidase).

2.5 HPLC-DAD analysis

Individual phenolic compounds were quantified using a Dionex UltiMate 3000 (Dreieich, Germany) high-performance liquid chromatography system equipped with a C18 Acclaim® 120 column (Dionex, 3 µm, 4.6 × 150 mm) at 30 °C. Citrus extracts were diluted in 70% (v/v) methanol and filtered through a 0.45 µm Millex filter (HV PVDF, Millipore, Massachusetts, USA) before injection. Detection was carried out using a UV/VIS detector (DAD-3000), and chromatograms were processed at 280 nm, as adapted from Caridi et al. (2007) and de Mejía et al. (2010). The mobile phases were A (water/formic acid, 99.9:0.1 v/v) and B (methanol/formic acid, 99.9:0.1 v/v) at a flow rate of 0.6 mL min^{-1}. Flavonoids (narirutin, naringenin, hesperidin, hesperetin, diosmetin, and tangeritin) were identified by comparison of their retention times and UV-VIS spectra with those of authenticated standards.

2.6 Total phenolic content (TPC)

Total phenolic content was determined as described by Singleton et al. (1999) by the Folin–Ciocalteu method. Results are expressed as mg gallic acid equivalents (GAE) per mg of lyophilized extract (LE).

2.7 Antioxidant capacity

The antioxidant capacity of citrus extracts was assessed using the DPPH radical scavenging activity (DPPH), oxygen radical absorbance capacity (ORAC), and ferric reducing antioxidant power (FRAP) assays (Benzie & Strain, 1996; Brand-Williams, Cuvelier, & Berset, 1995; Macedo et al., 2012; Prior et al., 2003). Values are expressed as µmol Trolox equivalents (TE) per mg of LE.

2.8 ACE inhibitory activity in vitro

This protocol is based on the hydrolysis of HHL by ACE to form hippuric acid (HA) (Cushman & Cheung, 1971, with modifications). Briefly, 50 µL of diluted samples were mixed with 50 µL of ACE solution (25 mU mL^{-1}) and incubated at 37 °C for 10 min. The enzymatic reaction was initiated by adding 150 µL of the substrate (4.15 mM HHL in borate buffer containing 0.3 M NaCl, pH 8.3) to the mixture. After incubation for 30 min at 37 °C, the reaction was stopped by adding 500 µL of HCl (1 M). Ethyl acetate (1.5 mL) was

added to the sample for HA extraction. The resulting mixture was vortexed for 1 min and left to stand for 5 min. Then, 800 µL of the ethyl acetate layer was taken and evaporated in a drying oven at 80 °C. The residue was dissolved in 1 mL of distilled water, and absorbance was measured at 228 nm using a spectrophotometer.

The following samples were tested for ACE inhibitory activity: a negative control (without inhibitors, containing 50 µL of distilled water to replace the volume of extract), CJR extract, CPR extract, CJRTB and CPRTB extracts (0.25 to 1.0 mg mL^{-1}), and pure hesperidin and hesperetin (100 to 1000 µM). ACE inhibition percentage was calculated by the following formula:

$$\%\text{ACE inhibition} = (A_{\text{negative control}} - A_{\text{sample}}) \times 100/A_{\text{negative control}} \quad (1)$$

where $A_{\text{negative control}}$ is the absorbance of the negative control and A_{sample} is the absorbance of the sample. A blank was used for each sample to correct readings.

A concentration–response curve was constructed for each extract. The IC_{50} value was determined as the concentration of extract required for 50% inhibition of ACE activity. The inhibitory efficacy of extracts was measured by the maximum effect (E_{max}) and expressed as percentage of inhibition (%).

2.9 Statistical analysis

Results are expressed as mean ± standard deviation (SD). Measurements were performed in triplicate. Statistical difference between groups was analyzed by analysis of variance (ANOVA). *Post hoc* comparison was performed by Tukey's test. The correlations between flavanone concentration determined by HPLC and ACE inhibition percentage and between ACE inhibition percentage and antioxidant activity were analyzed using Pearson's correlation test. Differences were considered significant when $p \leq 0.05$. Statistical analyses were performed in Minitab 16.1.1.

3. Results and Discussion

3.1 Effect of enzymatic treatment on phenolic profile

Fig. 1 shows that the concentration of polyphenols varied widely among extracts. Of the quantified flavanones, the glycoside hesperidin was predominant in both crude

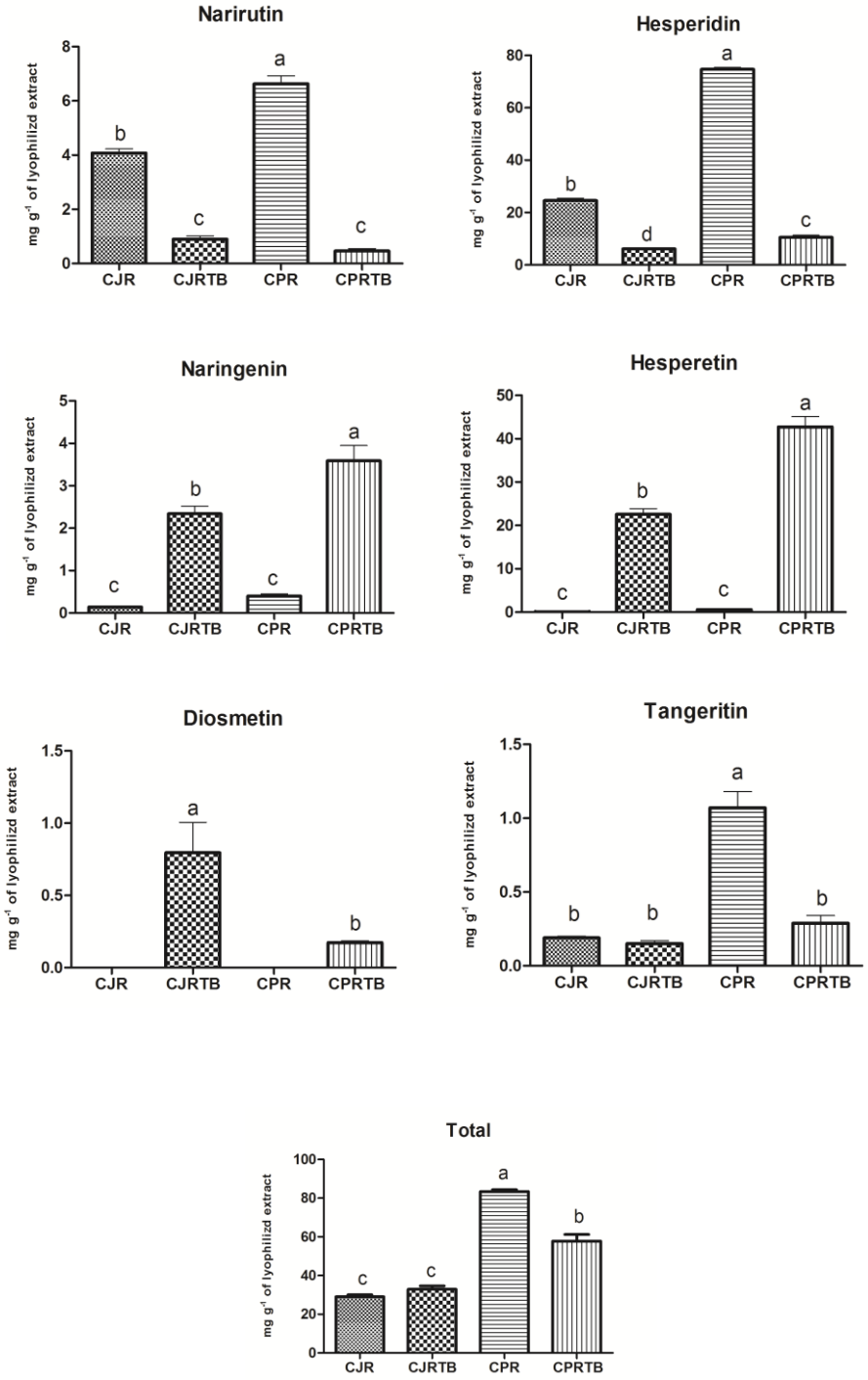

Fig. 1. Concentration (mg g^{-1} of lyophilized extract) of phenolic compounds in citrus extracts quantified by HPLC. Results are expressed as mean ± SD. Different letters in each graphical indicate significant differences by Tukey's test ($p \leq 0.05$). CJR: citrus juice residue; CJRTB: citrus juice residue treated with tannase and β-glucosidase; CPR: citrus pectin residue; and CPRTB: citrus pectin residue treated with tannase and β-glucosidase

extracts, but hesperidin concentration was three times higher in CPR (74.7 mg g^{-1}) than in CJR (24.6 mg g^{-1}). Similarly, narirutin concentration was 1.6-fold higher in CPR than in CJR (6.6 and 4.1 mg g^{-1}, respectively).

The citrus residues analyzed in this study are derived from a mixture of cultivars and differ in composition. Whereas CJR is composed of peels, pulp waste, seeds, and pectin, CPR results from industrial pectin extraction from CJR in acidic medium. Barbosa et al. (2018) suggested that the acidic extraction process used for pectin production might improve the release of phenolic compounds from CPR, thereby explaining the best results obtained with this sample. Previous studies have shown that the phenolic profile of citrus residues is influenced by a wide range of factors, from citrus varieties to the type of industrial waste (Barbosa et al., 2018; Escobedo-Avellaneda, Gutiérrez-Uribe, Valdez-Fragoso, Torres, & Welti-Chanes, 2014; Pereira et al., 2017). Similar to our findings, other studies reported that hesperidin was the most abundant polyphenol in citrus juice by-products, followed by narirutin (J. V. Madeira & Macedo, 2015; Pereira et al., 2017; Ruviaro et al., 2018).

HPLC analysis confirmed that enzymatic treatment promoted phenolic conversion, significantly increasing aglycone concentration while decreasing the concentration of glycosidic phenolics (Fig. 1). CJRTB had a hesperetin content of 22.5 mg g^{-1}, whereas CJR had a hesperetin content of 0.1 mg g^{-1}, which corresponds to a 225-fold increase promoted by enzymatic treatment. In contrast, hesperidin content decreased from 24.6 mg g^{-1} in CJR to 6.1 mg g^{-1} in CJRTB. CPRTB had a 70 times higher hesperetin concentration (42.7 mg g^{-1}) than CPR (0.62 mg g^{-1}). Enzymatic treatment decreased hesperidin concentration from 74.7 mg g^{-1} in CPR to 10.5 mg g^{-1} in CPRTB. Naringenin concentration was 17 times higher in CJRTB (2.3 mg g^{-1}) and 9 times higher in CPRTB (3.6 mg g^{-1}) than in their respective crude extracts ($p < 0.05$).

Hesperetin was the major aglycone in both biotransformed citrus extracts (Fig. 1). However, hesperetin and naringenin amounts were 1.9 and 1.5 times greater in CPRTB than in CJRTB, respectively.

Low concentrations of diosmetin and tangeritin were detected in citrus extracts (Fig. 1). Diosmetin is a methoxyflavone, the aglycone form of diosmin. Enzymatic treatment significantly increased diosmetin concentration in citrus extracts through TB-catalyzed deglycosylation. Tangeritin concentration was highest in CPR. According to Pereira et al. (2017), tangeritin concentration varies among citrus varieties, being higher in Murcott.Previous study reported that the polymethoxylated flavone (PMF) as tangeritin

expressed greater anti-inflammatory potential than flavone glycosides, hesperidin and narirutin (Ho & Lin, 2008).

Few studies have investigated the use of enzymes on complex food matrices such as agro-industrial wastes. Because of their structure, phenolic compounds have the ability to bind to polysaccharides and proteins at several sites of the cell wall. Enzyme combinations are often employed to decompose the cell-wall structure, increase the effect of enzymes, and improve the release of phenolic compounds from the matrix (Madeira et al., 2015; Pinelo, Arnous, & Meyer, 2006; Radenkovs et al., 2018). García-Conesa, Østergaard, Kauppinen, & Williamson (2001) reported that tannase might contribute to plant cell wall degradation. Chamorro, Viveros, Alvarez, Vega, & Brenes, (2012) emphasized that tannase had a similar behavior to pectinase in the release of phenolic compounds from grape pomace. Likewise, Ruviaro et al. (2018) showed that tannase and β-glucosidase alone or in combination were able to release phenols attached to the cell matrix of citrus by-products.

The activity of tannase on polyphenol complexes has been described by previous studies. This enzyme is able to hydrolyze ester bonds between gallic acid and glucose, and hydrolyze depside linkages (between two gallic acid units) present in hydrolyzable tannins or gallic esters, such as epigallocatechin O-gallate or epicatechin O-gallate, releasing gallic acid and glucose (Lekha & Lonsane, 1997; Madeira et al., 2015). In this manner, tannase can act on natural subtracts such as grape pomace (Martins et al., 2016) and tea (Macedo et al., 2011; Roberto et al., 2016). Tannase also modified the phenolic profile of orange juice by promoting the hydrolysis of naringin and hesperidin, thereby increasing the concentration of aglycones due to this diglicosidase activity (Ferreira et al., 2013). β-glucosidase catalyzes the hydrolysis of disaccharide glycosides, cleaving disaccharide units from the non-reducing end (Madeira et al., 2015). Many authors reported that β-glucosidase can hydrolyze glycosidic linkages of conjugated flavonoids to release aglycones, playing an important role in the release of phenolic compounds from different food matrices (Kessy et al., 2018; Ruviaro et al., 2018; Zheng & Shetty, 2000a).

Céliz, Rodriguez, Soria, & Daz (2015) and Shin, Nam, & Oh (2013) demonstrated that hesperetin can be produced via a one-step reaction using enzymes such as β-glucosidase and tannase. According to results obtained by Ruviaro et al. (2018), tannase and β-glucosidase can act synergistically in the extraction and conversion of phenolics from citrus residues, significantly increasing naringenin and hesperetin concentrations in extracts. Tannase and β-glucosidase can hydrolyze phenolics with high molecular weight, transforming them into simple compounds, as observed in the present study.

The use of a cocktail containing tannase and beta-glucosidase is justified by the combination of the activities of these enzymes, which can produce rich-phenolic extracts with a variable composition.

Madeira (2015) treated agro-industrial citrus residues with enzymes to increase the release of phenols. Treatment of residues with a mixture of tannase and cellulase for 24 h at 40 °C yielded the best results: an increase in hesperetin concentration from 1.25 to 130 μg g^{-1} of substrate. In comparison with these results, the tannase and β-glucosidase mixture used in the present study afforded much higher hesperetin yields, 3044 and 5338 μg g^{-1} of substrate for CJRTB and CPRTB extracts, respectively.

The biological properties of phenolics are largely influenced by their chemical structure. Studies conducted by Ferreira et al. (2016) in mice, showed that supplementation with hesperidin had protective effects against inflammation and oxidative stress caused by high-fat diet in mice, and may therefore prevent metabolic alterations associated with the development of cardiovascular diseases. In contrast, De Souza et al. (2016) performed in vitro and in vivo studies comparing the antioxidant effects of hesperitin and its glycosylated forms. The results demonstrated that aglycone hesperetin has more potent in vitro xanthine oxidase inhibitory activity (enzyme responsible for generating ROS) in relation to its glycosides. For the in vivo study, Wistar rats were supplemented with hesperetin, hesperidin or G-hesperidin for 30 days. The results showed that supplementation with hesperitin increased the antioxidant status of the liver of the animals, suggesting an effect linked to the greater bioavailability of the aglycone. Thus, the conversion of glycosidic flavanones to aglycones is interesting, as hesperetin and naringenin exhibit an array of beneficial health properties.

Hesperetin and naringenin are found naturally at low concentrations in citrus fruits and by-products (Khan et al., 2014; J. V. Madeira & Macedo, 2015), as shown in Fig. 1. Because of the difficulty in obtaining these compounds, studies on the effects of aglycones usually use high-cost analytical standards. This fact reinforces the importance and advantage of enzymatic biotransformation, as, through this process, extracts with high concentrations of hesperetin and naringenin can be obtained from agro-industrial residues. Therefore, enzymatic biotransformation enables the reuse of citrus residues, adding value to these products and representing an advantageous commercial alternative for citrus industries.

3.2 Effect of enzymatic treatment on the TPC of citrus residues

Although CJR had a significantly higher TPC than CPR by the Folin–Ciocalteu assay (table 1), HPLC analysis showed that CJR had lower glycoside content than CPR (Fig. 1). TPC varies among citrus tissues (Abeysinghe et al., 2007; Barbosa et al., 2018; Yinshi Sun et al., 2010), as observed in the present study. Because of the complexity of agro-industrial by-product matrices, many non-phenolic compounds can affect phenolic recovery from residues, such as proteins, cellulose, and pectic polysaccharides (Radenkovs et al., 2018). The TPC of crude extracts (30.93 ± 0.35 and 34.15 ± 2.35 mg GAE mg^{-1} of LE) was similar to the obtained by Nakajima et al. (2016), 33.31 to 36.2 mg GAE mg^{-1} of LE using ethanol–water (50%) as solvent.

Table 1. Total phenolic content (TPC) and antioxidant activity of citrus residue extracts.

Extract	TPC	FRAP	DPPH	ORAC
	mg GAE mg^{-1} of LE	*μmol TE mg^{-1} of LE*		
CJR	34.15 ± 2.35^{b}	208.6 ± 3.7^{c}	141.1 ± 1.2^{c}	798.1 ± 22.7^{a}
CJRTB	35.4 ± 0.5^{b}	235.6 ± 2.6^{b}	174.8 ± 9.5^{b}	819.2 ± 14.5^{a}
CPR	30.93 ± 0.35^{c}	253.3 ± 19.2^{b}	163.4 ± 6.6^{b}	817.4 ± 21.2^{a}
CPRTB	39.6 ± 1.3^{a}	297.8 ± 3.1^{a}	231.8 ± 7.8^{a}	813.4 ± 29.3^{a}

Results are presented as mean ± SD. ^{a,b,c} Means followed by different letters in the same column differ significantly ($p \leq 0.05$) by Tukey's test. CJR: citrus juice residue; CJRTB: citrus juice residue treated with tannase and β-glucosidase; CPR: citrus pectin residue; CPRTB: citrus pectin residue treated with tannase and β-glucosidase; GAE: gallic acid equivalents; TE: Trolox equivalents; and LE: lyophilized extract.

No differences were observed between the TPC of CJR and CJRTB extracts by the Folin–Ciocalteu assay (Table 1). However, HPLC analysis revealed differences between samples after enzyme treatment. Whereas CJR extract had higher levels of glycosidic flavanones, CJRTB had a higher content of naringenin and hesperetin (Fig. 1).

The TPC of CPRTB was 28.03% higher than that of CPR ($p < 0.05$) (Table 1). As shown by HPLC analysis, the glycoside content in CPR was significantly higher than the aglycone content in CPRTB. These results suggest that aglycones in this biotransformed extract have greater reducing potential than glycosides in the crude extract.

It is important to note that the Folin–Ciocalteu reagent is not specific for phenolic compounds, as the assay measures the reducing ability of a solution. Thus, the reagent may be subject to interference from other reducing compounds present in the matrix, such as vitamin C and polysaccharides (Huang, Boxin, & Prior, 2005; Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2009).

One of the strengths of enzymatic hydrolysis lies in the ability of enzymes to reduce particle size by increasing the contact area between particles and solvent, consequently releasing phenols attached to the matrix (Kessy et al., 2018). However, when the cell wall is disintegrated, other materials are released from cells and form complexes with phenolic compounds, which may affect phenolic extraction and TPC quantification (Li et al., 2006b).

Factors such as extraction method, type of solvent, solvent concentration, and extraction temperature can influence the TPC of an extract (Li et al., 2006a; M'hiri, Ioannou, Ghoul, & Boudhrioua, 2014). Of the solvents traditionally used for phenolic extraction, ethanol is the most suitable for food and pharmaceutical applications because of its efficiency and safety (M'hiri et al., 2014). Therefore, in the present study, we used 50% ethanol as the extraction solvent.

3.3 Effect of enzymatic treatment on the antioxidant capacity of citrus residues

The antioxidant capacity of CPR was approximately 1.2-fold higher than that of CJR ($p < 0.05$), according to FRAP and DPPH assays (Table 1). This result was expected, as the total amount of phenolics (quantified by HPLC) was 2.3 times higher in CPR than in CJR, with hesperidin representing 89% of the total content (Fig. 1).

Kim & Kim (2016) reported that citrus juice waste exhibits strong antioxidant activity by different mechanisms, such as by donating hydrogen ions to scavenge free radicals (DPPH assay) and by reducing ferric ion (FRAP assay). Many studies have reported that citrus juice residues are high-value by-products because of the antioxidant potential of their bioactive components. The results obtained in this study indicate that CPR, the residue obtained after pectin extraction from citrus juice residues, also has high biological value.

Treatment of CJR and CPR with tannase and β-glucosidase increased FRAP values by 13 and 24% and DPPH values by 18 and 42%, respectively ($p < 0.05$). HPLC results (Fig. 1) clearly show a change in the phenolic profile of extracts after enzymatic biotransformation. These results indicate that extracts rich in aglycones, mainly hesperetin,

present higher antioxidant activity than crude extracts, which contain higher amounts of glycosides.

Comparing biotransformed extracts, the FRAP and DPPH values of CPRTB were approximately 1.3-fold higher than those of CJRTB (Table 1). As shown in Fig. 1, hesperetin levels were 89% higher in CPRTB than in CJRTB, reinforcing the hypothesis that aglycones contributed strongly to the antioxidant activity of biotransformed extracts.

Previous studies compared the *in vitro* antioxidant effect of hesperetin and naringenin with that of their respective glycosides (Ferreira et al., 2013; J. V. Madeira & Macedo, 2015), concluding that the superior antioxidant capacity of hesperetin over hesperidin was due to the removal of esterified sugar molecules from the phenolic moiety. Cavia-Saiz et al. (2010) showed that the removal of rutinoside at C7 in flavanones increased the antioxidant and antiradical capacities of polyphenols. In addition, Ferreira et al. (2013) showed that tannase-catalyzed biotransformation of citrus juice led to a significant increase in antioxidant capacity. Similar effects of hydrolytic enzymes on the antioxidant capacity of grape pomace (Martins et al., 2016), tea (Roberto et al., 2016), and litchi pericarp (Kessy et al., 2018) were reported. In agreement, our findings showed that the increase in aglycones, such as hesperetin and naringenin, contributed to the significantly stronger antioxidant capacity of the biotransformed extract.

No significant differences were observed between the ORAC values of treated and crude extracts (Table 1). The ORAC assay is based on the hydrogen atom transfer reaction and measures the chain-breaking antioxidant capacity by peroxyl radical scavenging. DPPH and FRAP assays are based on the electron-transfer reaction and test the reducing power of antioxidants (Huang et al., 2005). Results may vary according to the method employed to evaluate antioxidant capacity (Tabart et al., 2009). Our data show that the biotransformed extracts were effective in donating electrons or hydrogen to stabilize free radicals, contributing to a significant antioxidant effect.

Our findings confirmed the increase in antioxidant capacity of extracts after biotransformation using tannase and β-glucosidase as a result of the sugar moieties removal from phenolics.

3.4 Effect of enzymatic treatment on ACE inhibitory activity

Fig. 2A shows the concentration–response curves for the ACE inhibitory activity of citrus extracts. The inhibitory effect occurred in a concentration-dependent manner.

CPRTB produced the strongest effect, having an IC_{50} of 0.21 mg mL^{-1}. The IC_{50} of CPR, CJRTB, and CJR extracts were 0.34, 0.37, and 1.7 mg mL^{-1}, respectively. CPRTB had the highest inhibitory effect (E_{max}) at the highest concentration tested (1 mg mL^{-1}), followed by CPR, CJRTB, and CJR (Fig. 2B). In comparison, to obtain approximately 50% ACE inhibitory activity, 0.01 mg/mL of captopril were needed (Yoshie-Stark, Bez, Wada, & W€asche, 2004).

Of the crude extracts, CPR exhibited the highest anti-ACE and antioxidant activities (Fig. 2, Table 1) and had significantly higher concentrations of phenolic compounds (Fig. 1) than CJR. Pearson's correlation test revealed a high correlation between hesperidin concentration and the E_{max} of crude extracts ($r = 0.847$, $p < 0.05$), suggesting that this glycoside might be involved in the ACE inhibitory activity of crude extracts.

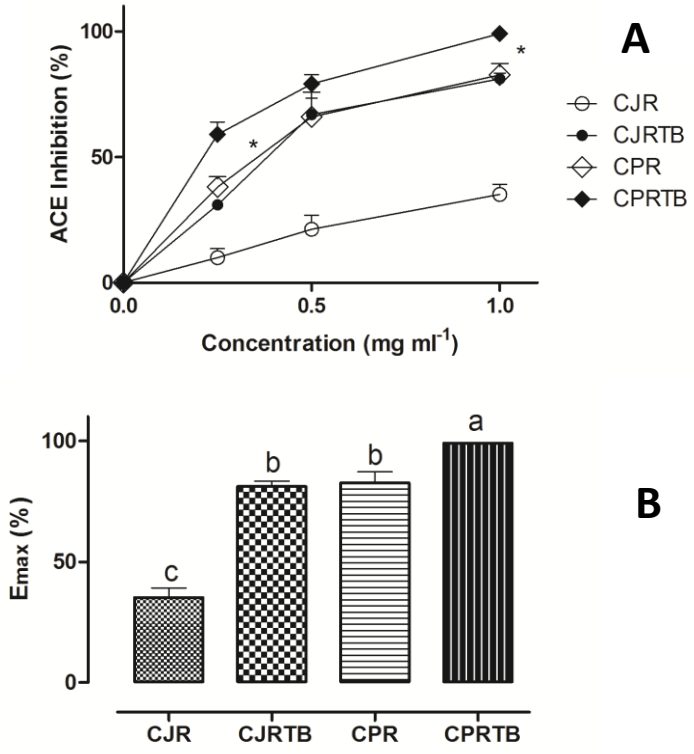

Fig. 2. (A) Concentration–response curves of angiotensin-converting enzyme inhibitory activity and (B) maximum effect (E_{max}) of citrus residue extracts. Results are presented as mean ± SD. Different letters indicate significant differences by Tukey's test ($p \leq 0.05$). IC_{50} = 1.7 mg mL^{-1} for CJR, 0.37 mg mL^{-1} for CJRTB, 0.34 mg mL^{-1} for CPR, and 0.21 mg mL^{-1} for CPRTB. CJR: citrus juice residue; CJRTB: citrus juice residue treated with tannase and β-glucosidase; CPR: citrus pectin residue; and CPRTB: citrus pectin residue treated with tannase and β-glucosidase.

The IC_{50} of CPRTB was approximately 2-fold lower than that of CJRTB. As CPRTB presented higher levels of aglycones than CJRTB, this result may be attributed to differences in aglycone concentration between extracts. Hesperetin accounted for more than 70% of the total phenolics present in both extracts after tannase–β-glucosidase treatment (Fig. 1). Hesperetin contents were highly correlated with the E_{max} of biotransformed extracts ($r = 0.977$, $p < 0.001$). Thus, it can be assumed that hesperetin is mainly responsible for the increased bioactivity of biotransformed extracts.

The ACE inhibitory effect of extracts was expressed in terms of their IC_{50} and maximum effect (E_{max}). Undoubtedly, the changes in phenolic profile caused by enzymatic hydrolysis (Fig. 1) significantly increased the anti-ACE activity of biotransformed citrus extracts in comparison with crude extracts (Fig. 2).

Noteworthy, the total flavanones per gram of lyophilized extract of CJR was similar to that of CJRTB. Still, CPRTB had a lower content of flavanones than CPR (Fig. 1). Despite these, CJRTB and CPRTB had greater bioactivity than CJR and CPR, respectively. These results suggest that aglycones in biotransformed extracts are more bioactive than glycosides in crude extracts, which explains the higher anti-ACE activity of biotransformed extracts.

For a better understanding of the ACE inhibitory activity of flavanones, hesperidin and hesperetin standard solutions were tested at different concentrations (Fig. 3). ACE inhibition percentages (62–76%) of hesperidin at different concentrations did not differ significantly (Fig. 3A). ACE inhibition by hesperetin ranged from 82.8% to 100% in a concentration-dependent manner ($p < 0.05$) (Fig. 3B). The ACE inhibitory activity of hesperetin was approximately 1.5 times higher than that of hesperidin at all concentrations.

These results confirm the high bioactivity of hesperetin in comparison with its glycoside form, a result also reported by Guerrero et al. (2012). The structural formulas of hesperetin and hesperidin are depicted in Fig. 3. The main difference between these molecules is the presence of a sugar unit at the C7 of ring A in hesperidin.

The physiological effect of phenolic compounds depends on their bioavailability. To be absorbed by the organism, flavanone glycosides must first be hydrolyzed by gut microbiota into aglycones, which are considered the active forms (Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003; Vallejo et al., 2010). De Souza et al. (2016) reported that hesperetin supplementation increased the organism’s antioxidant defenses in rats, suggesting an association between hesperetin bioavailability and antioxidant defense. These data reinforce the importance of producing extracts rich in aglycones.

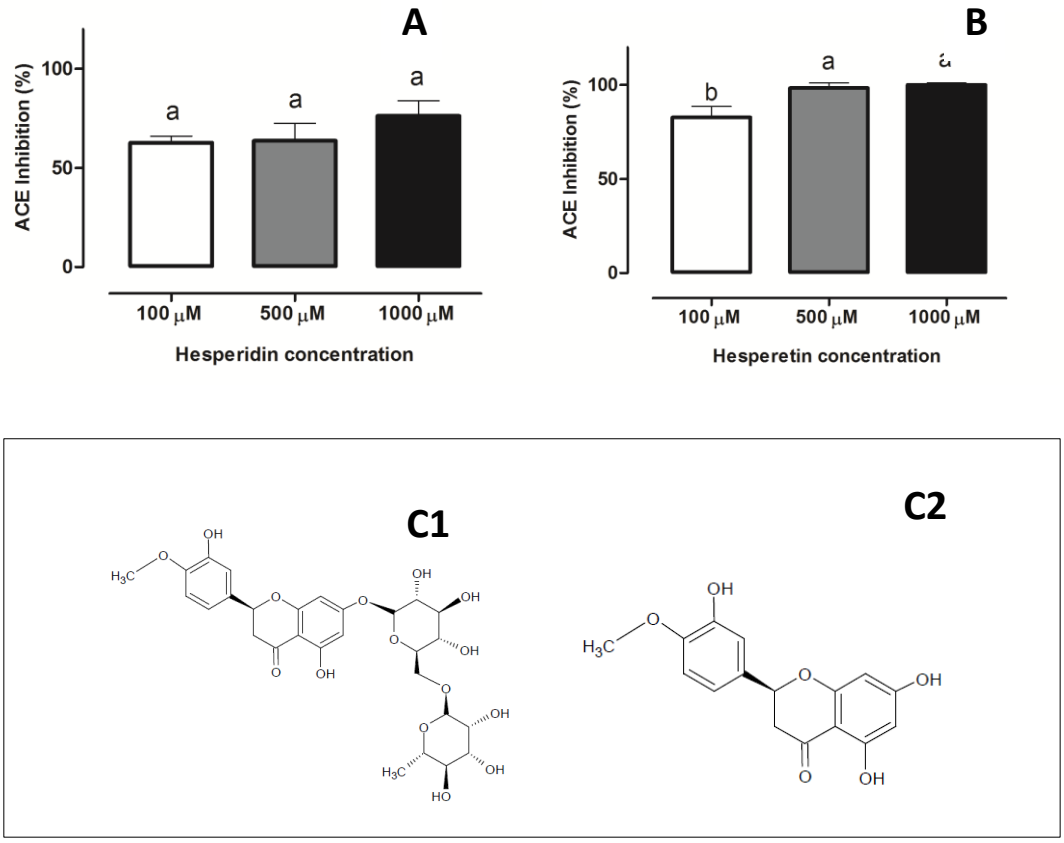

Fig. 3. Fig. 3. ACE inhibitory activity of analytical standards (A) hesperidin and (B) hesperetin and structural formula of the main flavanones found in citrus residue extracts: (C1) hesperidin (hesperetin-7-O-rutinoside) and (C2) its aglycone, hesperetin. Results are expressed as mean ± SD. Different letters indicate significant differences by Tukey's test ($p \leq 0.05$).

Nakajima, Madeira, Macedo, & Macedo (2016) showed the potential of aglycone-rich citrus extracts for treatment of obesity. An expressive reduction in lipid accumulation in cells occurred as a result of the lipolytic activity of hesperetin and naringenin. Nakajima et al. (2017) reported the anti-inflammatory effects of an aglycone-rich citrus extract in a co-culture of adipocytes and macrophages. Ruviaro et al. (2018) revealed that extracts rich in hesperetin and naringenin have high antioxidant potential. Ferreira et al. (2013) showed that the chemopreventive potential of orange juice can be increased by increasing the amount of aglycones by enzymatic treatment. The results of the present study show that citrus extracts rich in aglycones can be considered for hypertension treatment because of their ACE inhibitory and antioxidant activities.

Table 2 shows the relationship between ACE inhibitory activity and hesperidin and hesperetin concentrations in analytical standards and citrus extracts.. The maximum inhibition (E_{max}) promoted by CPR extract was similar to that promoted by 1000 µM hesperidin. However, hesperidin concentration in CPR was lower than 1000 µM. The ACE inhibition percentage of CJRTB was similar to that of 100 µM hesperetin. Interestingly, CPRTB promoted 100% ACE-inhibition, as did hesperetin at 500 and 1000 µM, but with a significantly lower concentration of this aglycone than that in hesperetin standard solutions

Table 2. Flavanone concentration (mg mL^{-1}) and ACE inhibition percentage of commercial standards and citrus residue extracts.

Sample	Concentration	Flavanone concentration		ACE inhibition
		hesperidin	*hesperetin*	(%)
*Pure flavanones**				
	100 µM	0.06	-	62.7
Hesperidin	500 µM	0.31	-	63.7
	1000 µM	0.61	-	76.3
	100 µM	-	0.03	82.8
Hesperetin	500 µM	-	0.14	98.4
	1000 µM	-	0.30	100
*Extracts***				
CJR	1 mg mL^{-1}	0.02	-	48.1
CJRTB	1 mg mL^{-1}	0.01	0.02	81.1
CPR	1 mg mL^{-1}	0.07	-	79.2
CPRTB	1 mg mL^{-1}	0.01	0.04	100

*Analytical standards. **1 mg mL^{-1} corresponds to the concentration that produced the maximum effect (E_{max}). CJR: citrus juice residue; CJRTB: citrus juice residue treated with tannase and β-glucosidase; CPR: citrus pectin residue; CPRTB: citrus pectin residue treated with tannase and β-glucosidase; and ACE: angiotensin-converting enzyme.

These results suggest a significant bioactive potential of citrus extracts over pure phenolics. Afonso et al. (2013) showed that flavanol-rich extracts from grape seeds exhibit a much higher ACE inhibitory activity than pure flavanol.

Some studies have investigated the potential of citrus extracts for treatment of hypertension (Oboh & Ademosun, 2011; Oboh, Bello, & Ademosun, 2014; Perez et al., 2010). For the first time, however, we report promising results obtained with an aglycone-rich extract produced from citrus residues. We emphasize that enzymatic treatment afforded

extracts rich in hesperetin and naringenin and with low concentrations of diosmetin and tangentin (Fig. 1). The greater anti-ACE activity of biotransformed samples might be attributed to the rich phenolic composition and synergistic effect of the several bioactive compounds present in this citrus extract. Similar to the results of our study, Kessy et al. (2018) and Fernández et al. (2015) observed that enzymatic treatment significantly increased the ACE inhibitory activity of litchi pericarps and grape seeds, respectively. However, it should be noted that the two studies mentioned above used bark and seeds acquired especially for the research, whereas in the present study, extracts of agro-industrial wastes that would be discarded or sold at low prices were used, representing a new use for food industry by-products. The pectin residue had the best results and can be considered a source of bioactive compounds to be exploited by the industry.

ACE inhibition is an important therapeutic intervention in the treatment of hypertension, as ACE catalyzes the production of angiotensin II, a potent vasoconstrictor. In addition, angiotensin II can double the amount of oxidants present in vascular tissues by producing ROS (Virdis et al., 2011; Wen et al., 2012). Our findings evidenced that there was a highly positive correlation between the E_{max} of citrus extracts and their DPPH ($r = 0.93$, $p \leq 0.0001$) and FRAP antioxidant capacities ($r = 0.91$ $p \leq 0.0001$). These results reinforce the importance of biotransformation to increase the concentration of aglycones in citrus extracts and enhance their bioactivity. The high antioxidant capacity and strong ACE inhibitory effects of citrus residues treated with enzymes represent a promising step toward the use of citrus waste to obtain natural extracts for the treatment of hypertension and other endothelial dysfunctions induced by oxidative stress.

4. Conclusions

Citrus industry by-products are an alternative source of bioactive compounds for the treatment of cardiovascular diseases, considering their low cost and availability. The crude citrus extracts studied are an interesting source of hesperidin and present antioxidant and ACE inhibitory activities. Still, treatment with tannase and β-glucosidase allowed the production of extracts rich in aglycones, such as hesperetin, naringenin, and diosmetin, with significantly improved antioxidant and anti-ACE activities. Thus, of all extracts tested, CPRTB presented the highest antioxidant and ACE inhibitory activities, comparable to that of analytical standards.

Our results evidence the potential of these under-utilized agro-industrial residues as a source of natural extracts, contributing to the development of new products with antioxidant and antihypertensive properties for the treatment or prevention of cardiovascular disorders.

5. Conflict of Interest

The authors declare no conflict of interest.

6. Acknowledgments

The authors thank the São Paulo Research Foundation (FAPESP) and the Brazilian National Council for Scientific and Technological Development (CNPq) for the financial support.

7. Funding

This study was supported by the São Paulo Research Foundation (FAPESP, grant no. 2015/04555-2) and *the* Brazilian National Council for Scientific and Technological Development (*CNPq*, Ph.D. grant no. 140537/2015-5).

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CAPÍTULO V. AGLYCONE-RICH EXTRACTS FROM CITRUS BY-PRODUCTS INDUCED ENDOTHELIUM-INDEPENDENT RELAXATION IN ISOLATED ARTERIES

(Original research paper to be summited to Food Chemistry)

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ABSTRACT

Studies showed that aglycone flavanones could have greater bioactivity than glycosidic forms. Based on this, this study evaluated the effects of enzyme biotransformation on antioxidant and vasorelaxant capacity of polyphenolic rich-extracts from citrus waste. Enzymatic treatment was carried out by tannase and β-glucosidase enzymes. Phenolic profile was evaluated by HPLC-DAD. Antioxidant capacity was evaluated by DPPH and FRAP assays. Vasorelaxant pathways were investigated in rat iliac arteries. Tests were conducted also with commercial drugs for the same purpose. Enzymatic treatment produces extracts with high hesperetin concentrations and higher antioxidant capacity. Citrus extracts caused relaxation on a concentration-dependent manner in intact and denuded endothelium. Citrus pectin extract increase the sCG activity, as BAY 58-2667. The present work showed that extracts from citrus could have importance in the treatment of vessels disorder by chronic-diseased, contributing to the viable reuse of food residues as added value ingredients in foods and pharmaceutical products.

KEYWORDS: bioactivity, citrus waste, hypertension, hesperitin, relaxation, vascular protection

1. Introduction

Hypertension and diseases that affect the vascular system are estimated to cause 7.5 million deaths worldwide (WHO, 2011; Opas, 2017). High blood pressure and vascular diseases are associated with endothelial dysfunction and vascular oxidative stress. The vascular endothelium is responsible for synthesis and release of several vasorelaxing substances, which NO acts as the most important one. Once synthesized by enzyme endothelial NO synthase (eNOS), NO diffuses across endothelial cells and activate the soluble guanylyl cyclase (sGC) in vascular smooth muscle cells (VSMCs). In turn, the sGC catalyzes

the formation of the second messenger cyclic guanosine monophosphate (cGMP), inducing the vascular relaxation (Bleakley et al., 2015; Oak et al., 2018; Sandoo, Veldhuijzen van Zanten, Metsios, Carroll, & Kitas, 2010).

In pathological conditions, the overproduction of reactive oxygen species (ROS) is related to dysfunction of many tissues, such as, heart and vessels. In particular, oxidative stress can lead to NO bioavailability reduction, resulting in superoxide anion production. Consequently, may occur vascular remodeling, endothelial dysfunction and hypoxia (Gallo et al., 2018; González, 2014)

There are increasing evidence that polyphenolic-rich products are able to improve the vascular function (Oak et al., 2018; Siti, Kamisah, & Kamsiah, 2015). Their bioactivity has been associated to antioxidant activities (Woodman & Chan, 2004), inhibition of platelet aggregation (Lescano et al., 2018), vascular relaxation(López-carreras, Castillo, Muguerza, & Aleixandre, 2015) and anti-inflammatory effects (Lescano et al., 2018; Siti et al., 2015), being a potential alternative for hypertension prevention and/or auxiliary treatment.

In this context, Brazil is the world's largest producer of oranges, with a total production of ~18 million tonnes in 2016/2017 (USDA, 2017). Since only about 50% the fruit is juice, the remainder results in an abundant supply of waste material, which consist basically of peels, pulp residues, and seeds. Usually, citrus juice industry dry this material to use for cattle feed as pellets or for pectin extraction. The main problem faced by pectin extraction is the generation of a considerable volume of a new waste (Mahato, Sharma, Sinha, & Cho, 2018; Mamma & Christakopoulos, 2008). As a result, the millions of tons of residues generated from citrus process represent a problem for the industry.

It is noteworthy that these wastes contain a considerable amount of bioactive phenolic compounds which make citrus by-products a promising potential as a functional food or nutraceuticals (Abeysinghe et al., 2007; Mahato et al., 2018). The glycosides hesperidin and naringin are the main polyphenols in citrus by-products, which rarely presented significant levels of flavanones in the aglycone form (Barbosa et al., 2018; Pereira et al., 2017).

López-carreras, Castillo, Muguerza, & Aleixandre (2015) demonstrated the relaxing ability of the citrus extracts composed predominantly by hesperidin and naringin. However, some evidences showed that aglycones form have greater antioxidant capacity (Ferreira et al., 2013; J. V. Madeira & Macedo, 2015) and vasorelaxant effects (Calderone et al., 2004; Orallo, Álvarez, & Basaran, 2004; Orallo & Camiæa, 2005) in comparison to glycosides. Since aglycones are difficult to obtain, these studies commonly use high cost

analytical standards, and researches about aglycone-rich extracts from citrus by-products and their vascular effects have not been reported until now.

Based on this, the aim of the present study was to evaluate the effects of enzyme-mediated biotransformation on antioxidant capacity and vasorelaxant ability of rich polyphenols extracts from citrus by-products.

We speculate that enzymatic biotransformation produces aglycones-rich extracts with greater bioactivity due to antioxidant and vasorelaxant properties. These effects could be important to the development of functional food products or ingredients that can be used in hypertension for prevention and/or auxiliary treatment of vascular diseases, representing an innovation with commercial interest.

2. Material and Methods

2.1 Chemicals

Gallic acid, narirutin, naringin, naringenin, hesperidin, hesperetin, ellagic acid, tangeritin, diosmetin, Trolox, 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), (R)-(-)phenylephrine hydrochloride (PE), acetylcholine chloride (Ach), sodium nitroprusside (SNP) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 4-[[(4-Carboxybutyl)[2-[2-[[4-(2-phenylethyl)phenyl]methoxy]phenyl]ethyl]amino]methyl] benzoic acid hydrochloride (BAY 58-2667) was purchase from Adipogen (San Diego, USA). Ferric chloride and ferric ammonium sulfate were purchased from Vetec Química (São Paulo, Brazil). Folin-Ciocalteu's reagent, monobasic and dibasic sodium phosphate, sodium carbonate, and formic acid were purchased from Dinâmica Química Contemporânea (Diadema, SP, Brazil). Tannic acid was purchased from Ajinomoto OmniChem Division (Wetteren, Belgium). Liquid chromatography grade methanol was purchased from JT Baker (Center Valley, PA, USA). All other chemicals were analytical grade.

2.2 *Samples and enzymes*

Two kinds of citrus residue were used in this study: The citrus juice by-products (JUI) are the residues from the production of orange juice; and the citrus pectin by-products (PEC) are the residues remaining after pectin extraction from JUI. Both residues were donated by the company CP Kelco Industry Headquarters (Limeira, Brazil). Dried samples were

crushed in Blender (OXY, Brazil) and passed through a 10-mesh sieve (Bertel Metallurgical Industries Ltda, Brazil).

Tannase (EC 3.1.1.20) from *Paecilomyces variotii* was produced in our laboratory (Bioprocesses Laboratory of the School of Food Engineering, UNICAMP) using a semi-solid fermentation (Battestin e Macedo 2007). β-glucosidase (naringinase from *Penicillium decumbens*) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Tannase activity was evaluated using gallic acid as a standard, according to Sharma et al. (2000), but with tannic acid as the substrate. The enzymatic activity of β-glucosidase was evaluated using p-nitrophenol as a standard (Matsuura et al., 1995). Activities were evaluated spectrophotometrically using the calibration curves of the standard compounds and were expressed as U/ mg of protein capable of generating 1 μmol of product in 1 minute.

2.3 Enzymatic treatment of citrus by-products

The enzyme-assisted extraction and biotransformation of phenolic compounds from citrus by-products were carried out according to a procedure adapted from Madeira e Macedo 2015. 2 g of citrus by-products was incubated with a combined enzyme treatment containing tannase and β-glucosidase (at concentration of 10 U of each enzyme per g of substrate) in 25 mL of 0.02 M sodium acetate buffer (pH 5.0) for 24 h at 40°C in an orbital shaker water bath agitating at 300 r min^{-1}. The reaction was stopped by placing the samples in an ice-water bath for 15 min. The choice of enzyme concentration was based on our previous published work (Ruviaro et al., 2018). Control samples were treated under the same conditions without the addition of enzymes.

The extracts were prepared according to a process adapted from Nakajima et al. (2016). At the end of biotransformation, the samples were mixed with 25 mL of ethanol, resulting in a 1:1 (v/v) ethanol-acetate buffer solution. After 60 min of shaking (200 rpm), the mixture was sonicated for 30 min. Then, the samples were centrifuged (9000 ×g at 4°C) for 15 min and filtrated through No. 1 Whatman paper.

Four extracts were produced: two crude extract from citrus by-products (JUI and PEC), and other two from the biotransformed citrus by-products (JUIE and PECE). Lastly, ethanol was removed from the extracts by rotary evaporation (40 °C for 15 min) and then the aqueous solution was frozen and freeze-dried for the analyses.

2.4 Detection and quantification of polyphenolic compounds (HPLC-DAD) of citrus extracts

Dried citrus extracts and standards were combined with 70% methanol solution and filtered through a 0.45 µm Millex filter (HV PVDF, Millipore, Massachusetts, USA) before injection. The main phenolic compounds were detected and quantified by Dionex UltiMate 3000 (Dreieich, Germany) Liquid Chromatography system, equipped with a C18 Acclaim® 120 column (Dionex, 3 µm, 4.6 x 150 mm) at 30 °C. The detection was carried out at 280 nm using a diode array detector (DAD-3000) (Adapted from Caridi et al. 2007 and de Mejía et al. 2010). Individual flavonoids (narirutin, naringenin, hesperidin, hesperetin, tangeritin and diosmetin) were calculated by comparison of their retention times and UV-VIS spectra to that of authenticated standards.

2.5 In vitro Antioxidant capacity of citrus extracts

Antioxidant capacity of the citrus extracts was assessed by DPPH radical scavenging activity (Brand-Williams et al., 1995; Macedo et al., 2011). The ability to scavenge the DPPH radical was calculated using a Trolox standard curve and the results were expressed as µmol Trolox equivalents (TE) per mg of lyophilized extract (LE).

The Ferric Reducing Antioxidant Power (FRAP) assay was carried out according to the procedure of Benzie & Strain (1996). The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated using a $FeSO_4$ standard curve and the results were expressed as mM Fe^{2+} equivalents per mg of LE.

2.6 Animals

All animal procedures and experimental protocols were approved by the Institutional Committee for Ethics in Animal Research of the University of Campinas (CEUA: 4519/1-2017). This study is in compliance with the ARRIVE guidelines (Kilkenny et al, 2010; McGrath and Lilley, 2015).

Male Wistar rats (200-250 g) were provide by the central animal facility of the Central Animal House Services of the University of Campinas CEMIB-UNICAMP (São Paulo, Brazil). The animals were housed three per cage on a 12-hour light/dark cycle and received filtered water and standard food *ad libitum*.

2.7 Preparation of rat isolated iliac artery

Rats were killed by overdose of isoflurane and exsanguination was performed to confirm the euthanasia.

Iliac arteries were carefully removed and cut into rings (3 mm). Prepared rings were mounted in 5-ml organ individual baths containing Krebs-Henseleit solution (mM: 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11 glucose). The solution was maintained at pH 7.4 and gassed with 95% O_2 and 5% CO_2 at 37°C. Each iliac ring was connected to a force transducer and was equilibrated for 45 min under a resting tension (10 mN) prior to initiating experiments. Changes in isometric force were recorded using a PowerLab system, version 7 (ADInstrumentsInc, Sidney, Australia). After the equilibration period, depolarization was induced by the addition of KCl (80 mM) and when the contraction had reached the steady state (approximately 15 min after the administration) the preparations were washed until the basal tension was recovered. Endothelium integrity was assessed by the pre-contraction of tissues with PE (1μM) followed by relaxation promoted by ACh (1μM). For studies of endothelium-intact vessels, only the tissue which relaxation was over 75% of the pre-contraction was eligible. For studies of endothelium-denuded vessels, the endothelium was mechanically removed. No relaxation in response to ACh in the denuded preparation indicated a removal's effectiveness of the endothelium.

2.8 ***In vitro functional assays***

The vascular relaxing effect of citrus by-products extracts was evaluated in iliac rings with intact endothelium. For this, the dried citrus extracts were diluted in KH solution prior to initiating experiments. After the stabilization at baseline tension, the iliac rings were pre-contracted with PE (1 μM) and cumulative increasing concentrations of citrus extracts were added (0.01 to 3 mg mL^{-1}). In a second set of experiments, the same procedures were also carried out in endothelium-denuded vessels.

Next, the effects of the analytical standards of flavanones were evaluated in intact endothelium iliac rings. Cumulative concentration–response curves to hesperidin (Hd) and hesperetin (Ht) at concentrations range 0.0001 to 1 mM were conducted in iliac rings precontracted with PE (1 μM). The results were compared with those obtained with iliac rings pre-incubated incubated with DMSO (vehicle used to dissolve flavanones). The final concentration of DMSO in solution was less than 0.1%.

To further investigate the relaxation mechanism induced by citrus extracts and pure flavanones, endothelium-intact rings were pre-incubated with ODQ (30 μM, 30 min), a

selective soluble guanylate cyclase (sGC) inhibitor. Then, iliac rings were contracted with PE (1 µM) and cumulative relaxing concentration–response curves for citrus extracts (0.01 to 3 mg mL^{-1}), pure flavanones (0.0001 to 1 mM) and BAY 58-2667 (0.0001 to 1 uM) were constructed. ODQ is known to be a selective inhibitor of sGC by oxidation. BAY 58-2667 is a potent sGC activator. The activation of sGC by BAY 58-2667 is strongest when the enzyme is oxidized.

The concentration–response curves were constructed using non-linear regression using GraphPad Prism (GraphPad Software, San Diego, CA). The vasorelaxing efficacy was evaluated as the maximal response effect (E_{max}) and was expressed as the percentage (%) of the contractile tone induced by PE (1 µM). The parameter of potency was presented as the negative logarithm of the concentration of the test compounds (pEC_{50}) and as the concentration of the test compounds in mg per ml (EC_{50}). This parameter evoking the concentration required to achieve a half-maximal response. The pEC50 value can not be calculated for those compounds that efficacy parameter was lower than (or close to) 50%. Therefore, the values given for these compounds are an estimate.

2.8 Determination of cGMP Levels

After the animals were killed, the iliac arteries were immediately excised and equilibrated for 45 min in an oxygenated *Krebs*–Henseleit (KH) solution. The tissues were pre-incubated for 30 min with ODQ (30 µM) and then SNP (30 µM), a relaxing agent, was added for 10 min. The same procedure was applied for the citrus extracts (1 mg ml^{-1}), but without the addition of ODQ. The tissue content of cGMP levels was presented as pmol mL^{-1}. After this, the arteries were immediately frozen in liquid nitrogen. Tissues were pulverized and subsequently processed for cGMP (cyclic guanosine monophosphate) measurement using an enzyme linked immunosorbent assay kit according to the manufacturer's protocol (cyclic GMP ELISA kit; Cayman Chemical, Ann Arbor, MI). Six iliac arteries were used to constitute each experimental n (n=6).

2.9 Statistical analysis

The results of citrus by-products extracts were expressed as mean ± SEM. All measurements were performed in triplicate. The results of isometric tension were shown as the percentage of relaxation, expressed as mean ± SEM of at least five experiments (n = 5-6) obtained from different animals.

Statistical analyses were performed using GraphPad Prism software (GraphPad Software). The statistical difference between the groups was analyzed by ANOVA and Tukey post-test. Student's unpaired t test was also used when appropriate. $P < 0.05$ was accepted as significant.

3. Results and Discussion

3.1 Enzyme treatment effect on citrus extracts

HPLC analyses showed that crude extracts of citrus by-products had higher amounts of glycosides hesperidin and narirutin, while the biotransformed samples presented higher levels of aglycones hesperetin and naringenin, as well as methoxyflavone diosmetin in a lower proportion (Table 1). These results proved that enzymatic treatment caused the biotransformation of the polyphenols, producing extracts with higher contents of phenolic compounds in free form.

The results in Table 1 also show that enzymatic treatment promoted a significantly increase of 19 and 15% in the antioxidant capacity by DPPH method as compared to JUI and PEC crude extracts, respectively. Enzyme treatment increase FRAP by 8 and 26% comparing with JUI and PEC crude extracts, respectively.

The crude extracts of citrus juice by-products and pectin by-products had phenolic compositions consistent with literature data (Barbosa et al., 2018; Ruviaro et al., 2018). Barbosa et al. (2018) and Pereira et al. (2017) showed that the amount of flavonoids vary according to the type of industrial waste. Anyway, hesperidin predominated, corresponding to the typical phenolic composition of extracts derived from citrus (Barbosa et al., 2018; Pereira et al., 2017).

The modification on polyphenolic content and the increase in antioxidant activity of agro-industrial residues promoted by enzymatic biotransformation using tannase and β-glucosidase have been evidenced by many authors. For example, Kessy, Wang, Zhao, Zhou, & Hu (2018) showed that tannase, b-glucosidase and pectinase treatment of litchi pericarps promoted the liberation and biotransformation of phenolic compounds, as well as boosted the antioxidant capacity of extracts.

Table 1. Enzyme treatment changes polyphenols content and antioxidant capacity in citrus by-products extracts.

	JUI	*JUIE*	*PEC*	*PECE*
Phenolic Compound (mg g^{-1} LE)				
Narirutin	3.96 ±0.10^{b}	0.83±0.03^{c}	6.73±0.21^{a}	0.52±0.01^{c}
Hesperidin	25.62±0.44^{b}	6.08±0.09^{c}	81.17±3.01^{a}	11.11±0.39^{c}
Naringenin	0.13±0.01^{c}	2.23±0.07^{b}	0.33±0.01^{c}	3.49±0.10^{a}
Hesperetin	0.00 ±0.06^{c}	22.02±0.48^{b}	0.62±0.07^{c}	43.70±0.79^{a}
Diosmetin	0.00±0.02^{c}	1.03±0.06^{a}	0.00±0.01^{c}	0.18±0.01^{b}
Tangeritin	0.20±0.01^{c}	0.14±0.01^{c}	1.28±0.08^{a}	0.37±0.02^{b}
Antioxidant capacity				
DPPH (*µmol TE mg^{-1} LE)*	98.71±1.76^{c}	117.97±3.60^{b}	114.12±1.69^{b}	131.44±2.06^{a}
FRAP (*mM Fe^{2+} equivalent mg^{-1} LE)*	3.98±0.16^{c}	4.31±0.09^{c}	5.00±0.04^{b}	6.36±0.08^{a}

Values are presented as mean ± SEM (n=3). ^{a,b,c} Different letters in the same column significantly differ ($p \leq 0.05$) by ANOVA, followed by Tukey test.
Abbreviations: JUI: crude extract of citrus juice by-products, JUIE: biotransformed extract of citrus juice by-products, and PEC: crude extract of citrus pectin by-products, PECE: biotransformed extract of citrus pectin by-products, TE: trolox equivalent, LE: lyophilized extract.

Zheng & Shetty (2000) reported that β-glucosidase was responsible for bioconversion of phenolic glucosides present in cranberry pomace, releasing free phenolic acids. Martins et al. (2017) evidenced that tannase displayed important hydrolytic efficacy, being able to liberated monomeric polyphenolics and aglycones from corresponding polymers, enhancing particularly gallic acid in grape pomace. The author also found that tannase also augmented antiradical (DPPH) activity by 57% in grape extract. Macedo et al. (2011) demonstrated that antioxidant capacity of tea increased after tannase treatment, and the authors attributed this increase to the hydrolysis of the epigallocatechin gallate to epigallocatechin and gallic acid. We previously reported that hesperetin and naringenin were only detected in citrus extracts after the β-glucosidase and tannase treatment, confirming the deglycosidic action of these enzymes (Ruviaro et al., 2018). Tannase also increased bioactivity of citrus extracts by augment of aglycones hesperetin and naringenin (Madeira & Macedo, 2015; Ruviaro et al., 2018). We report here the increase of aglycones in both citrus by-products, with emphasis on hesperetin amounts, after the tannase and b-glucosidase treatment, as well as the increase of the antioxidant capacity of the extracts (table 1).

The increased in antioxidant capacity of biotransformed extracts could be associated to the conversion of phenols present in the substrate into aglycones during the enzymatic process. According previously reports, the aglycone form of hesperidin and naringin molecules have more important effects on bioactivity due to much higher antioxidant potential than the respective glycoside (Ferreira et al., 2013; Madeira & Macedo, 2015).

Several vascular diseases display an impairment of antioxidant capacity due to depletion of antioxidants compounds that may be synthesized in the body or acquired from diet. This pathological condition may lead to oxidative stress and endothelial dysfunction (Gallo et al., 2018; González, 2014). Oxidative stress has been defined as an imbalance between the production of reactive-oxygen species and antioxidants (Oak et al., 2018). In a state of vascular oxidative stress occur an excessive production of reactive oxygen species (ROS) in vascular tissues, predominately the superoxide anion (O_2•), which reduces NO bioavailability in the arterial wall (Bleakley et al., 2015; Oak et al., 2018).

Antioxidants are molecules that show free radical scavenging activity by hydrogen atom and/or electron transfers, being able to stop the chain reaction of oxidative stress (Oak et al., 2018; Siti et al., 2015). Previously studies suggested that extracts rich in polyphenols are able to improve the vascular function due to their antioxidant molecules (Oak et al., 2018). As showed here, other works also demonstrated the ability of citrus by-products components to donate electrons or hydrogen to stabilize free radicals by FRAP and DPPH

assays, contributing to a significant reducing effect (Barbosa et al., 2018; Gironés-Vilaplana, Moreno, & García-Viguera, 2014).

Notably, the present study showed the higher antioxidant capacity of aglycones-rich extracts witch, consequently, may provide greater protection against oxidative stress caused by endothelial dysfunction.

3.2 Enzyme treatment effect on the relaxing ability of citrus extracts on isolated arteries

Figure 1A shows that all of the citrus extracts caused relaxation on a concentration-dependent manner in endothelium-intact iliac rings.

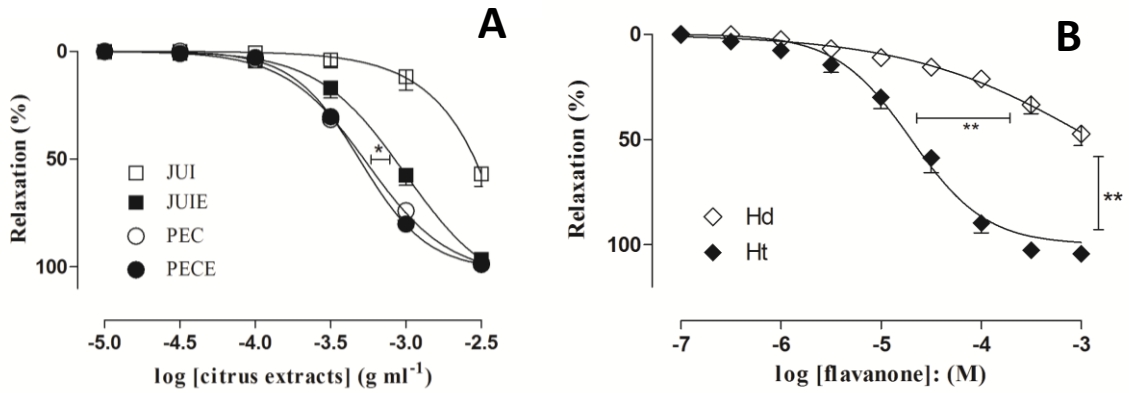

Figure 1. Cumulative concentration-response curves showing the vasorelaxant effect of (A) extracts of citrus by-products (0.01 – 3 mg ml^{-1}) and (B) pure flavanones (0.0001 - 1 mM) on iliac rings with intact endothelium. Values are presented as mean ±SEM (n=5-6). *$p \leq 0.05$ (ANOVA followed by Tukey test). ** $p \leq 0.001$ (Student's t-test).

Abbreviations: JUI: crude extract of citrus juice by-products, JUIE: biotransformed extract of citrus juice by-products, PEC: crude extract of citrus pectin by-products, PECE: biotransformed extract of citrus pectin by-products, Hd: hesperidin, Ht: hesperetin.

Considering that flavanones hesperidin and hesperetin were the major constituents of citrus extracts, representing 70 to 90% of the total quantified by HPLC (table 1), it is suggested that these flavanones are involved in the vasorelaxant effects of the samples. Reinforcing this information, when comparing the crude extracts, PEC had 3.3-fold higher hesperidin (table 1) and presented significantly higher maximal response (E_{max}) and potency (pEC_{50}) on relaxing the intact iliac rings than JUI (table 2).

Comparing the biotransformed extracts, PECE had ~ 2-fold higher hesperetin levels compared to JUIE (table 1). Although both biotransformed extracts made ~100% relaxation in iliac rings, the relaxation curve of PECE was shifted to the left in comparison to JUIE (figure 1A), which means that PECE presented a higher potency than the JUIE (table 2).

After JUI enzymatic treatment, the concentration-response curve of JUIE achieved maximal response (figure 1A). Also, the enzymatic treatment enhanced the pEC_{50} of JUIE extract comparing as crude JUI extract (table 2). The required concentration of JUIE extract (0.79 mg ml^{-1}) to achieve EC_{50} was ~3-fold lower compared to JUI extract (2.35 mg ml^{-1}). Interestingly, HPLC analyses showed that hesperidin amounts in crude JUI extract were similar to hesperetin contents in biotransformed JUIE extract (table 1).

Table 2. Maximal responses (Emax) and potency (pEC_{50}) derived from concentration–response curves to extracts of citrus by-products (0.01 – 3 mg ml^{-1}) and pure flavanones (0.0001 - 1 mM) in iliac rings with intact endothelium.

	Emax (%)	*pEC_{50}*
Citrus by-products		
JUI	-	2.62 ± 0.03^{c}
JUIE	96.5 ± 1.0^{a}	3.00 ± 0.07^{b}
PEC	98.3 ± 0.5^{a}	3.26 ± 0.03^{a}
PECE	98.9 ± 0.7^{a}	3.30 ± 0.02^{a}
Pure flavanones		
Hd	47.4±5.5	2.88±0.08
Ht	$104.3\pm1.12^{**}$	$4.70\pm0.04^{**}$

Values are presented as mean ± SEM (n = 5-6). ^{a,b,c} Citrus by-products with different letters in the same column significantly differ ($p \leq 0.05$) by ANOVA followed by Tukey test. **Pure flavanone significantly differ ($p \leq 0.001$) by Student's t-test.
Abbreviations: JUI: crude extract of citrus juice by-products, JUIE: biotransformed extract of citrus juice by-products, and PEC: crude extract of citrus pectin by-products, PECE: biotransformed extract of citrus pectin by-products, Hd: hesperidin, Ht: hesperitin.

Both PEC and PECE extract displayed a similar E_{max} and pEC_{50} in the relaxing effect in intact rat isolated iliac rings (table 2). The required concentration of PEC and PECE extract to achieve EC_{50} was 0.50 and 0.47 mg ml^{-1}, respectively. However, the HPLC analysis evidenced difference between the samples, since hesperidin contents in crude PEC were ~2-fold higher than hesperetin amounts in biotransformed PECE (table 1).

Based on the phenolic profile of the citrus extracts and the vasorelaxation induced by each type of extract, our results indicated that the aglycones present in the biotransformed extracts present greater relaxation ability than the glycosides present in the crude extracts. To verify these affirmations, analytical standards hesperidin and hesperetin were also investigated for relaxing effects on endothelium-intact iliac rings.

The results in figure 1 showed that flavanones exhibited concentration-dependent relaxation. Hesperidin exhibited only a partial vasorelaxant effect at the highest concentration tested (1 mM), whereas hesperetin showed a full relaxation at 300 μM (figure 1). The potency of aglycone hesperetin was significantly higher than that glycoside hesperidin (table 2), presenting EC_{50} values of 0.02 mM and 1.3 mM, respectively, which reinforces our assumptions above.

In agreement with our results, Calderone et al. (2004) demonstrated that hesperetin (E_{max} = 92%±4; pEC_{50} = 4.86±0.06) had higher efficacy and potency than hesperidin (E_{max} = 57%±17) to relax intact aorta rings pre-contracted with KCl (20 mM). Orallo, Álvarez, & Basaran (2004) also showed that the cumulative addition of the hesperetin (1 μM - 0.3 mM) completely relax NA (1 μM)-induced contractions in rat thoracic aortic rings, whereas the respective glycosides hesperidin did not promote any relaxation. Takumi et al. (2012) showed that hesperetin has better bioavailability than hesperidin, evidencing that this aglycone is a potent vasodilator, in addition to promoting vascular protection.

The biological properties of phenolic compounds are influenced by their chemical structure. Comparing the chemical structure of hesperidin (hesperetin-7-O-rutinoside) with the respective hesperetin aglycone, the results indicated that the glycosylation of the C7 atom may lead to the loss of its pharmacological activity (Orallo et al., 2004; Orallo & Camiæa, 2005) and reduces its vasorelaxant efficacy (Calderone et al., 2004; Orallo et al., 2004). These information help to explain the greater vasorelaxant efficacy and potency of aglycones-rich extracts produced by enzyme biotransformation comparing to crude citrus extracts (figure 1, table 2).

By relating the potency and the hesperidin concentrations in analytical standard and crude citrus extracts, our findings suggest a significant bioactive potential of extracts over pure phenolic. The hesperidin concentration in EC_{50} values of crude extracts ranged to 0.41 to 0.57 mg ml^{-1}, whereas analytical hesperidin was 0.79 mg ml^{-1}. Although the hesperetin concentration in citrus biotransformed extracts (0.017 and 0.021 mg ml-1) was higher than in pure hesperetin (0.006 mg mL^{-1}) at the EC_{50} values, the E_{max} were similar.

In this regard, it is important to emphasize that the aglycones are difficult to obtain and studies on the effects of hesperetin usually use high-cost analytical standards. In addition, previous reports had already shown the endothelium-dependent relaxing ability of citrus extracts rich in glycosylated flavanones (López-carreras et al., 2015). However, the present investigation represents the first effort to evaluate the effect of an aglycone-rich extract produced from citrus industrial waste in the vasorelaxant effect in isolated rat iliac artery.

It is important to note that enzymatic treatment afforded extracts rich in hesperetin and naringenin and low amounts of diosmetin and tangentin. The greater relaxing effects of biotransformed samples might be attributed to the rich phenolic composition and synergistic effect of the several bioactive compounds present in this citrus extract. Thus, these findings demonstrated the importance of biotransformation to produce extracts rich in bioactive compounds from inexpensive agro-industrial residues.

Interestingly, the extracts from pectin by-products presented the greater results in antioxidant potential and vasorelaxant effects, representing a new use for this devalued waste. Due to this, both pectin by-products extracts were chosen to evaluate the pathways involved in relaxant effect: the crude extract rich in glycosides (PEC) and the biotransformed extract rich in aglycones (PECE).

3.3 Influence of the endothelium in artery relaxation

To determine the role of endothelium in the vasorelaxant effects of crude and biotransformed citrus pectin extracts, we mechanically removed endothelial cells from iliac rings.

Figure 2 showed that citrus pectin extracts produced concentration-dependent vasorelaxation in both endothelium-intact and endothelium-denuded iliac rings. In addition, the relaxation response to each extract in the presence and absence of endothelium were comparable, with Emax values ranging from 98.3 to 102.0%, and EC_{50} values ranging from 0.4 to 0.6 mg ml^{-1} of extract. These findings suggested that the removal of the functional endothelium had no significant influence on intrinsic vasorelaxation of citrus extracts.

Many disorders process can cause to endothelial dysfunction resulting in loss of normal function, such as diabetes, hypertension, cardiovascular, inflammatory diseases and ageing (Bleakley et al., 2015; Oak et al., 2018; Sandoo et al., 2010). Therefore, our results also suggest that the effects of citrus extracts would not be compromised in diseased vessels.

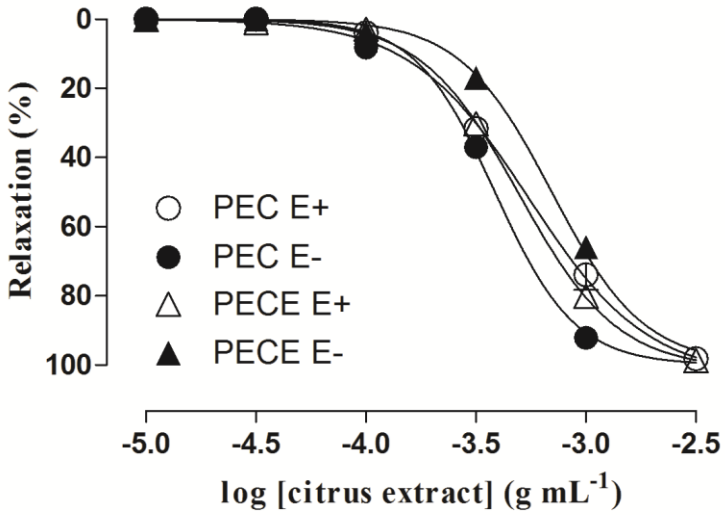

Figure 2. Cumulative concentration-response curves of extracts of citrus by-products (0.01 to 3 mg/ml) on iliac rings with endothelium-intact (E+) and endothelium-denuded (E−). Values are presented as mean ±SEM (n=5).
Abbreviations: PEC: crude extract of citrus pectin by-products, PECE: biotransformed extract of citrus pectin by-products

3.4 NO-GMPc pathway evaluation

In order to clarify the mechanism involved of the vasorelaxation effect of PEC and PECE extract on endothelium-dependent pathways, endothelium-intact iliac arteries were pre-incubated with ODQ (30 μM) before being contracted with PE (1 μM). Surprisingly, pre incubation with ODQ increased significantly the relaxation induced by crude and biotransformed citrus pectin extracts (figure 3. A), achieving the Emax in a minor concentration than respective control (vehicle). ODQ also increased significantly the pEC_{50} of both extracts (table 3). The vehicle DMSO alone had no significantly effect on relaxation.

BAY 58-2667, a sGC activator, promoted a concentration-dependent relaxation in iliac rings, which were rather potentiated by ODQ. As expected, the pre-incubation with ODQ increased significantly the efficacy of the drug and shifted relaxation curve to the left (figure 3B, table 3). Alexandre et al. (2014) previously showed that incubation with ODQ potentiated the relaxations induced by the sGC activator in lean mice.

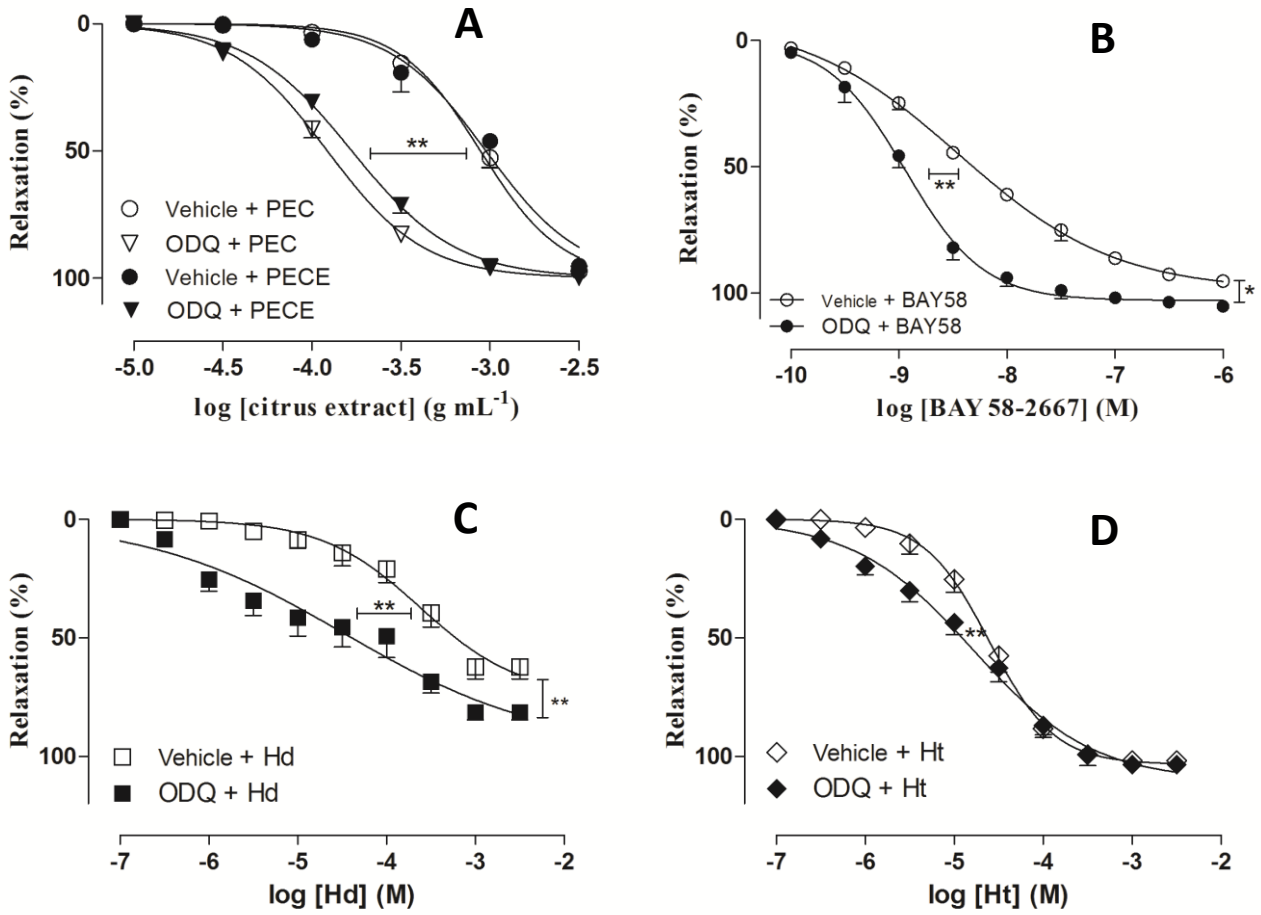

Figure 3. Cumulative concentration-response curves of (A) extracts of citrus by-products (0.01 – 3 mg ml^{-1}), (B) BAY 58-2667 (0.0001 - 1uM), (C) hesperidin (0.0001 - 1 mM) and (D) hesperetin (0.0001 - 1 mM) on iliac rings with intact endothelium in the absence or presence of ODQ (30 mM).Values are presented as mean±SEM (n=5-6). * $p<0.01$ and ** $p<0.0001$(Student's t-test). The vehicle DMSO alone had no significantly effect on relaxation. Abbreviations: PEC: crude extract of citrus pectin by-products, PECE: biotransformed extract of citrus pectin by-products, Hd: hesperidin, Ht: hesperetin.

Table 3. Maximal responses (E_{max}) and potency (pEC_{50}) values derived from concentration–response curves to BAY58 (0.0001 - 1uM), extracts of citrus by-products (0.01 – 3 mg ml-1) and pure flavanones (0.0001 - 1 mM) in iliac rings with intact endothelium.

	E_{max} (%)	*pEC_{50}*
Citrus by-products		
Vehicle + PEC	97.3±1.4	3.06±0.02
ODQ + PEC	98.3±0.8	3.92±0.02**
Vehicle + PECE	95.9±2.5	3.03±0.05
ODQ + PECE	100±1.2	3.78±0.02**
Drug		
Vehicle + BAY58	95.3±1.4	8.27±0.03
ODQ + BAY58	105±1.4*	8.97±0.03**
Pure flavanones		
Vehicle + Hd	62.3±4.99	3.13±0.07
ODQ + Hd	81.34±3.4**	4.37±0.11**
Vehicle + Ht	101±0.79	4.63±0.03
ODQ + Ht	103±2.8	4.95±0.05**

Values are presented as mean ± SEM (n = 5-6). * $p<0.01$ and ** $p<0.0001$ significantly differ by Student's t-test comparing with respective vehicle.
Abbreviations: PEC: crude extract of citrus pectin by-products, PECE: biotransformed extract of citrus pectin by-products Hd: hesperidin, Ht: hesperitin.

We also investigated the pure flavanones behavior in the presence of ODQ. Although hesperidin did not reach full relaxation, E_{max} and pEC_{50} of the flavanone was significantly higher in the presence of ODQ (figure 3C, table 3). Pre-incubation with ODQ did not modify the E_{max} of hesperetin, but increased significantly the potency of the aglycone (Figure 3D, table 3).

ODQ is known to be a selective inhibitor of sGC, the smooth muscle target of endothelial-derived NO. To be activated by NO, sGC requires the presence of the heme prosthetic group, which can be in the reduced (Fe^{2+}) or the oxidized (Fe^{3+}) form. ODQ oxidizes the sGC heme group, leading to a lower NO affinity and abolishing the vasorelaxation effects (Mónica & Antunes, 2018; Zhao et al., 2000).

There are only two classes of compounds able to increase sGC activity: stimulators and activators. The sGC stimulators depend of the presence of Fe^{2+}, whereas sGC activators act primarily when the heme group is in an oxidized state (Fe^{3+} instead Fe^{3+} or even when the heme group is missing. This way, sGC activators are highly effective at restoring

sGC expression and activity in disorders associated with increased oxidative stress, presenting importance in the treatment of chronic-diseased blood vessels, such as hypertension, atherosclerosis, diabetes mellitus and heart failure (Mónica & Antunes, 2018; Nossaman, Pankey, & Kadowitz, 2012; Petrova, 2018).

There are only two compounds NO- and heme-independent sGC activators. Among their, BAY 58-2667 is the most potent one due to its ability to activate sGC with EC_{50} values in the nanomolar range. Thus, activator as BAY 58-2667 can enhance cGMP levels by directly acting on sGC independently of NO and preferentially in diseased vessels (Mónica & Antunes, 2018; Nossaman et al., 2012).

Considering that ODQ is a selective inhibitor of sGC, we speculate that vasorelaxation induced by citrus pectin extracts are involved with this enzyme. In addition, we observed that relaxation promoted by PEC and PECE was potentiated in the presence of ODQ. Due to this, we suggest that vasorelaxation induced by both extracts occurs even when the sGC enzyme is oxidized due to oxidative stress in the vasculature, as BAY 58-2667. Also, we speculated that extracts from citrus pectin by-products also may increase the cGMP in iliac rings.

3.5 Levels of cGMP in iliac rings

In order to evaluate the levels in cGMP in iliac rings, the preparations were incubated with relaxing agents in the presence or absence of ODQ (30mM).

Incubation of iliac rings with SNP elevated by 5.7-fold the cGMP levels above basal levels (12.8±1.6 and 2.23±0.29 *p*mol ml^{-1}, respectively). The incubation with ODQ before the stimulation with SNP decreased significantly the cGMP levels (1.38±0.53 *p*mol ml^{-1}) in comparison with basal levels, confirming effective inactivation of sGC. The vehicle DMSO alone had no effect on the intracellular levels of cGMP in any condition (figure 4).

SNP is a NO donor. This way, SNP activate sGC via NO to increase cGMP production and induce relaxation (Nossaman et al., 2012). The increase of cGMP levels in the presence of SNP was observed in the present study. As previously discussed, ODQ oxidizes the sGC heme, abolishing it's NO sensitivity and inhibiting cGMP production. Thus, the pre-incubation with ODQ inhibit the production of cGMP by SNP, as observed in the present work.

Incubation of iliac rings with PEC and PECE extracts elevated by approximately ~2.2-fold the cGMP levels (5.6±1.0 and 4.66±0.53 *p*mol ml^{-1}, respectively) above basal levels

(figure 4). Based on the results found above, we postulate that pre-incubation with ODQ could further increased the cGMP levels in iliac preparation incubated with citrus extracts. Taken together, these data indicate that both citrus pectin extracts may activate the sGC, resulting in the production of cGMP and activation of its associated signaling pathways. However, further studies may better clarify the pathways that are involved in these effects

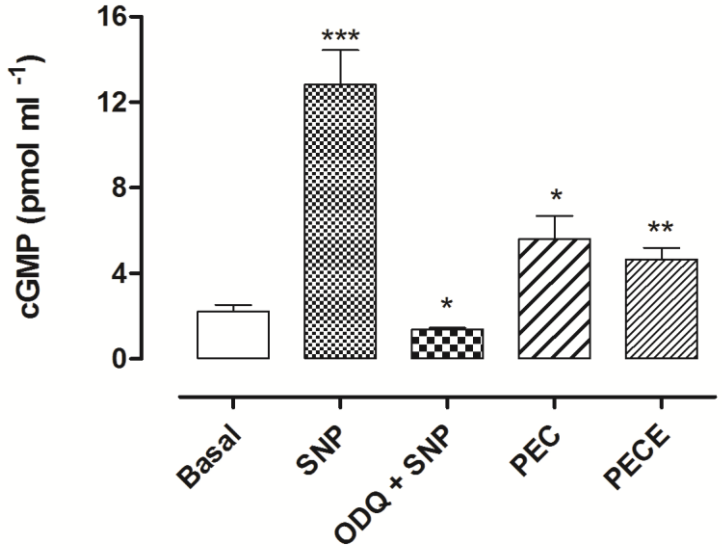

Figure 4. Cyclic GMP levels in iliac ring with intact endothelium. Tissues were stimulated with SNP (30 uM) and citrus extracts (1 mg ml^{-1}) alone or in the presence of ODQ (30 uM). Values are presented as mean ± SEM (n=6). ****p*<0.001, ** p< 0.01, *p <0.05 significantly different compared with Basal (Student's *t*-test). Abbreviations: PEC: crude extract of citrus pectin by-products, PECE: biotransformed extract of citrus pectin by-products. cGMP: cyclic guanosine monophosphate.

The use of class of drugs that activate sGC had benefits in contrast to conventional NO-donor therapy in chronic-diseased by reduce the common side effects. In addition, the sGC activators presenting importance in the treatment of many disorders associated with increased oxidative stress, since the ROS generation may promote changes in conformational state of the enzyme and inhibit their vasorelaxant effects.

In this context, we showed here several bioactive effects of citrus extracts, especially from citrus pectin by-products (PEC and PECE). The high antioxidant capacity of citrus extracts could contribute to prevent the oxidative stress development. Also, the relaxant effects of extracts in the presence or absence of endothelium, as well as the strong relaxation even with the sGC oxidized, could have importance in the treatment of vessels disorder by

chronic-diseased. Therefore, this work not only presents a novel biotechnological tool for the development of products with greater antihypertensive properties but also contributes to the reuse of food residues as ingredients as functional foods and nutraceutical products.

4. Conclusion

The present study shows that enzyme biotransformation of citrus by-products produced extracts rich in hesperetin and naringenin amounts, which increased bioactivity compared to crude extracts. The highest vasorelaxation potential of hesperetin than hesperidin was confirmed, explaining the greater results of aglycone-rich extract. Extracts from citrus pectin by-products (PEC and PECE) induced endothelium-dependent and -independent relaxation in rat iliac arteries in a concentration-dependent manner. The relaxation induced by both PEC and PECE extracts was rather potentiated in the presence of ODQ, as observed in the relaxation induced by BAY 58-2667 with ODQ. These results suggest that the vasorelaxation induced by citrus pectin extracts occurs even in the presence of endothelial dysfunction or when the sGC enzyme is oxidized due to oxidative stress in the vasculature. So, these relaxant effects may involve the antioxidant capacity of extracts and sGC expression.

Therefore, our work highlights the possibility of targeting the citrus by-products biotransformation to develop new products with anti-hypertensive properties, that could be used to prevent and/or auxiliary the treatment of oxidative stress related endothelium dysfunction.

5. Conflict of interest

The authors declare no conflict of interest.

6. Acknowledgments

The authors thank the São Paulo Research Foundation (FAPESP) and the Brazilian National Council for Scientific and Technological Development (CNPq) for the financial support.

7. Funding

This study was supported by the São Paulo Research Foundation (*FAPESP*, grant no. 2015/04555-2) and *the* Brazilian National Council for Scientific and Technological Development (*CNPq*, Ph.D. grant no. 140537/2015-5).

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DISCUSSÃO GERAL

De acordo com os dados estatísticos da Organização das Nações Unidas para a Alimentação e a Agricultura, o Brasil produziu aproximadamente 17 milhões de toneladas de laranja na última safra, sendo considerado o maior produtor mundial da fruta (FAOSTAT, 2018). Considerando que maior parte desse total é destinada à produção do suco de laranja, os sólidos remanescentes da extração de suco são considerados resíduos dessa indústria e correspondem a aproximadamente 50% do total da massa da fruta, o que significa um total de aproximadamente 13 milhões de toneladas de resíduos. Esses resíduos, por sua vez, são destinados a diferentes propósitos, como para a extração de pectina a partir da casca, como ingrediente para ração animal ou ainda, são descartados no meio ambiente (Bampidis & Robinson, 2006; Mamma & Christakopoulos, 2008).

Assim, a geração de resíduos tanto pela indústria processadora de suco quanto pela indústria processadora de pectina refletem um problema social, ambiental e econômico para a indústria. Considerando que esses resíduos são de fácil obtenção e possuem baixo valor agregado, encontrar alternativas para sua reutilização, evidenciando seus efeitos bioativos, poderia contribuir para solucionar as questões da maior indústria cítrica do mundo.

1. Caracterização dos resíduos cítricos

Dois tipos de resíduos foram estudados: resíduos da indústria de suco de laranja e resíduos da indústria processadora de pectina, como o objetivo de destacar sua composição química e fenólica.

Nossos resultados de composição química revelaram que os dois tipos de resíduos tinham composição similar de gordura e proteína e diferem ligeiramente na umidade e concentração de carboidratos. A principal diferença observada foi no conteúdo mineral, indicado pelos maiores valores de cinzas encontrados nos resíduos de suco de citrus. Essa diferença pode ser explicada apela presença de pectina nos resíduos de suco, que tem capacidade de absorver e manter outras substâncias, como os minerais (Xu et al., 2008).

Estudos vêm demonstrando que os resíduos da indústria de suco de laranja são ricos em compostos fenólicos (Pereira et al., 2017). Entretanto, existem poucos estudos descrevendo o perfil fenólico dos resíduos da extração de pectina.

A análise de HPLC-DAD mostrou que os principais compostos fenólicos encontrados nos resíduos cítricos avaliados nesse estudo foram as flavononas glicosiladas

hesperidina, narirutina e naringina. Entre eles, a hesperidina foi o composto majoritário. Outros polifenóis também foram detectados em menores concentrações, como a diosmetina, tangeritina, ácido gálico e ácido elágico. Nossos resultados vão ao encontro de estudos anteriores que descrevem o perfil fenólico de frutos cítricos e seus derivados (Khan, Zill-E-Huma, & Dangles, 2014; Kim & Kim, 2016; Madeira & Macedo, 2015; Pereira et al., 2017; Sun et al., 2010).

De um modo geral, a concentração de fenólicos foi significativamente maior nos resíduos da indústria de pectina do que nos resíduos da indústria de suco, especialmente a concentração de **hesperidina** (314,44 ± 18,82 e 232,65 ± 10,47 mg por 100 g de massa seca, respectivamente). As maiores concentrações de fenólicos podem ser explicadas pelo processo de extração de pectina na indústria, que utiliza soluções ácidas, o que pode contribuir para a liberação dos fenólicos presos a matriz celular da fruta (Hegde, Agrawal, & Gupta, 2015).

Assim, nossos resultados confirmam que os resíduos cítricos são uma importante fonte natural de compostos fenólicos, que podem ser explorados pela indústria farmacêutica e alimentícia para o desenvolvimento de produtos com propriedades biológicas e nutracêuticas. Os resíduos do processamento de pectina merecem destaque, já que estudos ainda não realizados para avaliar o potencial de reutilização desses resíduos.

2. Biotransformação enzimática

Diferentes tratamentos enzimáticos foram utilizados para aumentar a extração e a biotransformação das flavononas com maior potencial bioativo a partir dos resíduos da indústria de suco.

Para o processo enzimático, quatro enzimas foram utilizadas, de forma isolada ou combinadas: celulase, pectinase, β-glucosidase e tanase. A quantificação dos fenólicos por HPLC foi o método selecionado para a escolha do melhor tratamento enzimático, com o objetivo de aumentar a concentração das agliconas (hesperetina e naringenina) nos extratos cítricos.

Para a escolha do tratamento enzimático, trabalhamos com os resíduos da indústria processadora de suco. Após isso, o melhor tratamento enzimático foi aplicado nos resíduos de suco e nos resíduos de pectina com o objetivo de avaliar o perfil fenólico e as propriedades bioativas dos extratos biotransformados.

Os resultados que obtivemos mostraram que os **glicosídeos hesperidina e narirutina** foram os compostos majoritários encontrados nos extratos **cítricos sem**

tratamento enzimático. Como esperado, houve um aumento na concentração dessas flavononas nos extratos tratados com celulase e pectinase.

As mudanças no perfil das flavononas após as reações enzimáticas que continham β-glicosidase e tanase foram claramente demonstradas pela análise do HPLC. **Após reação enzimática com β-glicosidase e tanase,** a concentração de **agliconas** dos extratos cítricos aumentou significativamente, dando destaque para a concentração de **hesperetina e naringenina**. Esse aumento foi mais pronunciado após 24 horas de reação.

A celulase é uma enzima capaz de quebrar estruturas de hemicelulose e celulose da parece celular das plantas, e a pectinase atua na degradação da pectina. Por contribuírem para a degradação de estruturas da parece celular da planta, essas enzimas facilitam a extração dos fenólicos da matriz (Madeira et al., 2015). A ação da β-glicosidase e da tanase na quebra da ligação entre sacarídeos e a estrutura aglicona de diferentes matrizes já foi demonstrada anteriormente (Ferreira et al., 2013; Kessy, Wang, Zhao, Zhou, & Hu, 2018; Shin, Nam, & Oh, 2013), justificando o aumento da concentração de agliconas nos extratos cítricos desse estudo.

Estudos relaram que o uso de combinações enzimáticas com diferentes atividades contribui para a extração e conversão de fenólicos em matrizes alimentares. Por exemplo, as enzimas pectinolíticas e celulotícas liberariam os fenólicos ligados a estruturas da célula vegetal, facilitando a ação da β-glicosidase e da tanase na conversão desses fenólicos (Baik et al., 2014; Chamorro, Viveros, Alvarez, Vega, & Brenes, 2012).

Contudo, ao avaliar a concentração das agliconas hesperetina e naringenina nos extratos cítricos, nossos resultados demonstraram que os tratamentos enzimáticos contendo somente β-glicosidase ou tanase foram comparáveis aos tratamentos que combinavam essas enzimas com a celulase e a pectinase. Assim, sugere-se que a β-glicosidase e tanase *per se* são enzimas capazes de facilitar a extração dos fenólicos da matriz celular e converter esses fenólicos em agliconas. Isso nos levou a estudar efeito da concentração da β-glicosidase e da tanase na produção de extratos ricos em flavononas agliconas.

As maiores concentrações de **hesperetina** foram encontradas em extratos cítricos após o tratamento empregando 20 U/ g (de substrato) de tanase (247,5 ± 13,4 mg 100 g/ MS), 20 U/ g de β-glicosidase (462,2 ± 31,7 mg 100 g/ MS), e a combinação de 10 U/ g de tanase + 10 U/ g β-glicosidase (439,3 ± 3,0 mg 100 g/ MS).

A escolha do tratamento enzimático foi baseada em alguns fatores, como a composição dos fenólicos dos extratos biotransformados e as enzimas estudadas. A β-glicosidase é uma enzima que catalisam a hidrólise de ligações β-glicosícas. A tanase é uma

enzima capaz de atuar em ligações éster e depsídica, além de apresentar atividade de diglicosidase. Assim, o uso combinado das duas enzimas pode levar a produção de extratos mais ricos na composição fenólica.

Diante do exposto, **o tratamento escolhido** para processo de biotransformação enzimática dos resíduos da indústria suco e da indsústria pectina foi o tratamento combinado de tanase e β-glicosidase (10 U/ g de substrato de cada enzima), **denominado TB**.

2.1 Tratamento enzimático TB

A análise por HPLC mostrou que o tratamento enzimático TB promoveu a conversão das flavonas glicosiladas em suas respectivas agliconas, aumentando a concentração de hesperetina e naringenina nos extratos de citrus biotransformados.

O tratamento enzimático TB levou a produção de **3.044 e 5.338 μg de hesperitina por grama de substrato** a partir dos **resíduos da extração de suco e de pectina**, respectivamente. Em comparação, Madeira & Macedo (2015) obtiveram 120 μg/ g de hesperetina aplicando uma combinação enzimática de tanase (5 U/g de substrato) e celulase (5 U/g de substrato) para biotransformação de resíduos cítricos nas mesmas condições de tempo e temperatura. Nossos resultados representam um aumento de aproximadamente 25 e 44 vezes na produção de hesperetina em relação Madeira & Macedo (2015). Além disso, é importante destacar que a biotransformação enzimática aplicando TB deu origem extratos ricos em composto fenólicos de outras classes, como tangeritina e diosmetina.

É importante enfatizar que não existem fontes comerciais para extração de agliconas, como as hesperetina e naringenina. Esses compostos raramente são encontrados em frutos cítricos, seus derivados ou em resíduos agroindustriais de citrus (Abeysinghe et al., 2007; Pereira et al., 2017; Sun et al., 2010). Por serem de difícil obtenção, os trabalhos de pesquisa costuma utilizar padrões analíticos de alto custo. Nosso trabalho mostrou que a biotransformação enzimática é um método biotecnológico que aumenta significativamente a concentração das agliconas em extratos cítricos, tornando os resíduos industriais de citrus uma fonte comercial interessante para extração desses compostos, dando destaque principalmente para os resíduos do processamento de pectina, que deram origem a extratos com maior concentração de hesperetina.

Outra vantagem relacionada à biotransformação enzimática, é que esse processo minimiza o uso de solventes tóxicos na extração dos fenólicos de interesse, sendo considerado seguro para o manipulador e para o meio-ambiente.

Efeitos bioativos

O presente estudo avaliou o efeito da biotransformação enzimática TB no potencial antioxidante, na inibição da enzima conversora de angiotensina e no relaxamento de vasos *in vitro* dos extratos dos resíduos industriais de cítricos. Aqui, vale destacar a concentração de hesperidina e hesperetina dos extratos cítricos, já que representaram mais de 70% da composição fenólica.

Entre os extratos que **não receberam tratamento enzimático**, os extratos dos resíduos da indústria de pectina apresentaram aproximadamente 3 vezes mais **hesperidina** que os extratos dos resíduos da indústria de suco.

Entre os **extratos biotransformados**, os extratos dos resíduos da indústria de pectina apresentaram cerca de 2 vezes mais **hesperetina** que os extratos dos resíduos da indústria de suco.

A concentração de **hesperidina** os extratos dos resíduos da indústria de **suco sem tratamento** foi **similar** a concentração de **hesperetina** dos extratos dos resíduos da indústria de **suco biotransformado.**

A concentração de **hesperidina** nos extratos dos resíduos da indústria de **pectina sem tratamento** foi cerca de **2 vezes maior** que a concentração de **hesperetina** nos extratos dos resíduos da indústria de **pectina biotransformado**.

3.1 Potencial antioxidante

Nossos resultados demonstraram que os extratos dos resíduos da indústria de pectina sem tratamento apresentaram maior atividade antioxidante em relação aos extratos dos resíduos da indústria de suco sem tratamento. O tratamento enzimático aumentou significativamente o potencial antioxidante de ambos os extratos cítricos. Entre todos os extratos estudados, os extratos dos resíduos da indústria de pectina biotransformados apresentaram os melhores resultados.

O aumento da capacidade antioxidante de extratos de diferentes matrizes alimentares ricas em polifenóis modificados por processos de biotransformação enzimática também foi observado em estudos anteriores de nosso grupo de pesquisa, como em bagaço de uva (Martins, Roberto, Blumberg, Chen, & Macedo, 2016), em chás (Macedo, Battestin, Ribeiro, & Macedo, 2011; Roberto et al., 2016) e em extratos de soja (de Ávila et al., 2018).

Nossos resultados indicam a capacidade antioxidante está relacionada com a concentração e com o conteúdo de flavononas dos extratos cítricos, sendo maior nos extratos ricos em agliconas.

Estudos anteriores que compararam a atividade antioxidante *in vitro* das flavononas destacaram a maior bioatividade das agliconas em relação aos seus glicosídeos (Ferreira et al., 2013; Madeira & Macedo, 2015), e atribuíram esse efeito à remoção da molécula de açúcar da estrutura glicosídica. Cavia-Saiz et al. (2010) também demostraram que a remoção da rutinose do C7 de flavononas aumentou seu potencial antioxidante. Ferreira et al. (2013) reportaram que a biotransformação de suco de laranja catalisada por tanase levou a um aumento significativo na capacidade antioxidante, atribuindo esse efeito ao aumento da concentração de flavononas agliconas. Esses dados da literatura reforçam nossa teoria de que as agliconas hesperetina e naringenina contribuíram para a atividade antioxidante significativamente maior dos extratos cítricos biotransformados.

Muitas doenças do sistema vascular apresentam uma redução da capacidade antioxidante devido à depleção de compostos antioxidantes que são sintetizados no organismo ou adquiridos a partir da dieta. Como resultado, há um aumento na produção de radicais livres que levam a um estado de estresse oxidativo no meio vascular (Bleakley et al., 2015; González, 2014). Antioxidantes são moléculas que apresentam capacidade de remoção de radicais livres devido à capacidade de doação de elétrons e/ou hidrogênio, sendo capazes de impedir ou parar a reação em cadeia do estresse oxidativo (Oak et al., 2018; Siti, Kamisah, & Kamsiah, 2015). Estudos têm demonstrado que extratos ricos em compostos fenólicos são capazes de melhorar a função vascular devido as suas propriedades antioxidantes (Benavente-García & Castillo, 2008; Oak et al., 2018).

Em nosso estudo, evidenciamos o poder redutor dos extratos de citrus pelo método de *Folin–Ciocalteu*. Além disso, demonstramos que os extratos cítricos apresentaram capacidade de doar elétrons e/ou hidrogênio pelos métodos de FRAP e DPPH, contribuindo significativamente para seu poder antioxidante. De maneira notável, os extratos biotransformados apresentaram maior atividade antioxidante, podendo oferecer maior proteção contra estresse oxidativo causado em situações patológicas, como na hipertensão e em doenças vasculares.

3.2 Inibição da enzima conversora de angiotensina

Curvas de concentração-resposta foram construídas para avaliar o efeito dos extratos dos resíduos industriais de cítrus na inibição da ECA. Com essas curvas, obtivemos duas respostas farmacológicas que refletem a eficácia dos extratos em inibir a ECA. O efeito máximo ($E_{máx}$) corresponde à inibição máxima obtida para cada extrato. A potência (IC_{50}) corresponde à concentração necessária para reduzir o efeito máximo em 50%.

Os extratos cítricos inibiram a atividade da ECA de forma concentração-dependente.

Ao avaliar os extratos cítricos que não receberam tratamento enzimático, nossos resultados mostraram que o extrato dos resíduos da indústria pectina foi mais eficaz (E_{max} = 79,2% e IC_{50} = 0,34 mg/ml) em inibir a atividade da ECA do que os extratos dos resíduos da indústria de suco (E_{max} = 48,1 % e IC_{50} = 1,7 mg/ml)

Como esperado, a biotransformação enzimática aumentou significativamente a potência dos extratos biotransformados em relação aos extratos que não receberam nenhum tratamento enzimático. O extrato dos resíduos da indústria de suco biotransformado apresentou valores de E_{max} = 81,1% e IC_{50} = 0,37 mg/ml. O extrato biotransformado proveniente dos resíduos industriais de pectina foi o mais eficaz em inibir a atividade da ECA, apresentando $E_{máx}$ de 100% e menor IC_{50} (0,21 mg/ ml).

A correlação de Pearson revelou uma correlação positiva entre a concentração flavononas dos extratos de citrus e a inibição da ECA. Assim, o efeito inibitório da enzima foi atribuído principalmente à hesperidina (nos extratos sem tratamento) e à hesperetina (nos extratos biotransformados). Devido aos resultados de composição fenólica, já discutidos anteriormente, nossos resultados sugeriram que a hesperetina apresenta maior potencial anti-ACE do que seu glicosídeo. Para verificar essas afirmações, investigou-se o efeito dessas flavononas puras da inibição da atividade da ECA.

Nossos resultados confirmam a **maior atividade da hesperetina**, que apresentou um efeito inibitório da atividade da ECA de 82,8% a 100%, enquanto a hesperidina inibiu a atividade da enzima em 62 a 76%.

O efeito anti-ACE de diferentes compostos fenólicos foi demonstrado por Guerrero et al. (2012). Inclusive, alguns estudos evidenciaram o efeito de extratos cítricos ricos em compostos fenólicos na inibição da ECA. Entretanto, esse é o primeiro estudo que avalia extratos cítricos biotransformados ricos em hesperetina e naringenina na inibição da ECA.

A ECA faz parte do SRA, um dos principais sistemas responsáveis pelo controle da pressão arterial e da homeostase vascular. Essa enzima é responsável pela conversão da angiotensina I em angiotensina II, um potente vasoconstritor que aumenta a pressão arterial (Lavoie & Sigmund, 2003; Wen, Gwathmey, & Xie, 2012). Em estados patológicos, como na hipertensão, o efeito vasoconstritor da angiotensina II é potencializado, levando ao aumento da pressão sanguínea nos vasos, ao excesso de produção de radicais livres e à disfunção endotelial. A angiotensina II leva ao aumento na produção de radicais livres pela ativação do sistema NAD(P)H oxidase, contribuindo para a instauração de um estado de estresse oxidativo vascular (González, 2014; Virdis, Duranti, & Taddei, 2011; Wen et al., 2012). Desse modo, a inibição da ECA é uma importante intervenção terapêutica no tratamento da hipertensão.

Diante disso, nossos resultados demonstram que extratos ricos em agliconas obtidos pela biotransformação enzimática de resíduos industriais de citrus apresentaram um importante efeito anti-ACE. Esses resultados destacam a biotransformação como uma alternativa para a reutilização de resíduos industriais de citrus como fonte de compostos naturais com propriedades anti-hipertensivas.

3.3 Relaxamento de vasos

Curvas de concentração-resposta foram construídas para avaliar efeito dos extratos dos resíduos industriais cítricos no relaxamento de anéis de artéria ilíaca com endotélio íntegro de ratos. Com essas curvas, duas respostas farmacológicas foram obtidas: O efeito máximo ($E_{máx}$) corresponde ao relaxamento máximo obtido para cada extrato, e a potência (EC_{50}) corresponde à concentração necessária para atingir o efeito máximo em 50%. Esses parâmetros refletem a eficácia dos extratos como vasorelaxantes.

As curvas concentração-resposta mostraram que todos os extratos provocaram relaxamento dos vasos de forma concentração-dependente. A biotransformação enzimática dos extratos dos resíduos de suco deslocou para a esquerda a curva de relaxamento. Isso significa que a biotransformação aumentou significativamente a potência (EC_{50}) do extrato.

Os extratos dos resíduos da indústria de pectina sem tratamento e o biotransformado apresentaram potência similar (EC_{50} = 0,50 e 0,47 mg/ml, respectivamente).

Baseado no perfil fenólico desses extratos (mostrado anteriormente) e no vasorelaxamento induzido por cada tipo de extrato, os resultados obtidos nesse trabalho sugeriram que os principais responsáveis por esse efeito são as flavononas hesperidina e

hesperetina. Para confirmar essa hipótese, testamos o relaxamento induzido pelos padrões analíticos hesperidina e hesperetina nos anéis de artéria ilíaca com endotélio íntegro.

Como esperado, os dados obtidos nesse trabalho mostraram que a forma aglicona ($E_{máx}$ = 104% e EC_{50} = 0,02 mM) apresentou maior efeito vasorelaxante do que a forma glicosilada ($E_{máx}$ = 47,4% e EC_{50} = 1,3 mM). Esse resultado pode ser atribuído à remoção da glicose do C7, levando ao aumento da atividade farmacológica da flavona aglicona (Calderone et al., 2004; Orallo et al., 2004; Orallo & Camiæa, 2005).

Como o objetivo do nosso estudo também era investigar os mecanismos envolvidos no relaxamento vascular promovido pelos extratos cítricos de diferentes composições, selecionamos dois extratos que promoveram relaxamento máximo. Um extrato rico em glicosídeos (extrato dos resíduos da indústria de pectina sem tratamento) e um extrato rico em agliconas (extrato dos resíduos da indústria de pectina biotransformado).

Na primeira etapa, avaliou-se o papel do endotélio no efeito relaxante dos extratos cítricos. Para isso, as células endoteliais dos anéis ilíacos foram removidas mecanicamente. Os resultados obtidos em nosso estudo mostraram que a ação vasorelaxante dos extratos dos resíduos da indústria de pectina não foi alterada após a remoção do endotélio vascular.

O endotélio vascular é um dos principais responsáveis pela homeostase vascular, especialmente pela produção de oxido nítrico. O óxido nítrico é um gás produzido nas células endoteliais, que ativa a enzima GCs presente nas células musculares lisas vasculares, levando a produção de GMPc, que promove o relaxamento vascular (Bleakley et al., 2015; Sandoo, Veldhuijzen van Zanten, Metsios, Carroll, & Kitas, 2010).

Doenças como diabetes, hipertensão, doenças cardiovasculares, doenças inflamatórias e o próprio envelhecimento natural podem causar uma disfunção endotelial levando a perda da sua função normal, e consequentemente, comprometendo a homeostase vascular (Bleakley et al., 2015; Sandoo et al., 2010). Além disso, essas doenças costumam provocar um estresse oxidativo no meio vascular pelo aumento na concentração de radicais livres. Esses radicais livres, por sua vez, oxidam a enzima GCs. Como consequência, a enzima GCs fica menos sensível ao NO, e toda a cascata de relaxamento é prejudicada.

Diante disso, na segunda etapa nós investigamos os mecanismos envolvidos no relaxamento dos extratos pelas vias dependentes do endotélio íntegro. Para isso, os anéis ilíacos foram incubados com ODQ, uma droga que oxida a enzima GCs, inibindo a produção de GMPc e o relaxamento dos vasos.

Atualmente, só existem dois compostos capazes de ativar a enzima GCs quando ela está oxidada, sendo o BAY 58-2667 o mais potente. Isso significa que o vasorelaxamento

induzido por BAY 58-2667 ocorre mesmo na presença da disfunção endotelial ou quando a enzima GCs está oxidada devido ao estresse oxidativo (Mónica & Antunes, 2018; Nossaman, Pankey, & Kadowitz, 2012; Zhao et al., 2000). Em nosso estudo observamos que o efeito relaxante do BAY 58-2667 foi potencializado quando a enzima GCs estava oxidada pelo ODQ, como era esperado. O BAY 58-2667 é uma droga comercial produzido pela Indústria Farmacêutica Bayer que está em fase de testes e ainda não foi liberada comercialmente.

Os resultados obtidos nesse estudo mostraram que o efeito vasorelaxante dos extratos cítricos também foi potencializado na presença de ODQ. Esse efeito também foi observado no relaxamento induzido pelos padrões analíticos de hesperidina e hesperetina na presença de ODQ, sendo a aglicona o mais potente. Levando em consideração esses resultados e a composição fenólica dos extratos dos resíduos de pectina, evidencia-se mais uma vez a maior bioatividade dos extratos biotransformados ricos em agliconas em relação aos extratos ricos em fenólicos glicosilados.

Diante disso, mostramos que a ação vasorelaxante dos extratos dos resíduos da indústria de pectina foi independente do endotélio. Além disso, o efeito relaxante dos extratos foi potencializado quando a enzima GCs estava oxidada. Esses resultados sugerem que os extratos dos resíduos de pectina são capazes de induzir relaxamento mesmo na presença de uma disfunção endotelial ou quando a enzima GCs está oxidada devido ao estresse oxidativo vascular. Desse modo, os extratos dos resíduos da indústria de pectina podem ser utilizados não só na prevenção de doenças vasculares devido aos seus efeitos relaxantes, como também podem ser usados no tratamento auxiliar dessas doenças, já que seus efeitos não foram comprometidos pela ausência do endotélio vascular e nem pela oxidação da enzima GCs.

CONCLUSÃO GERAL

Os extratos cítricos são fontes importantes de compostos fenólicos, principalmente hesperidina e narirutina.

O tratamento TB (tanase + *b*-glicosidase) modificou o perfil fenólico dos resíduos de citrus, produzindo extratos ricos em agliconas hesperetina e naringenina.

Os extratos dos resíduos cítricos apresentaram propriedades antioxidantes, anti-ECA e vasorelaxante. A biotransformação enzimática TB melhorou estas atividades biológicas *in vitro* dos extratos.

A maior atividade da hesperetina em relação à hesperidina foi confirmada nos testes inibição da ECA e nos testes de relaxamento de vasos *in vitro*, justificando os melhores resultados dos extratos biotransformados.

Os resíduos da indústria extratora de pectina apresentaram maior concentração fenólica e maior bioatividade *in vitro,* com destaque para o extrato biotransformado PECE

Considerando que as doenças vasculares envolvem diversos fatores como estresse oxidativo vascular e disfunção endotelial, podemos sugerir que os extratos cítricos poderiam contribuir para o desenvolvimento de produtos com propriedades de proteção vascular.

Assim, esse trabalho de tese oferece uma alternativa para reutilização de resíduos agroindustriais de baixo custo, sugerindo uma solução para maior indústria Cítrica do mundo, além de contribuir para o desenvolvimento de produtos com o objetivo de promoção da saúde e prevenção de doenças

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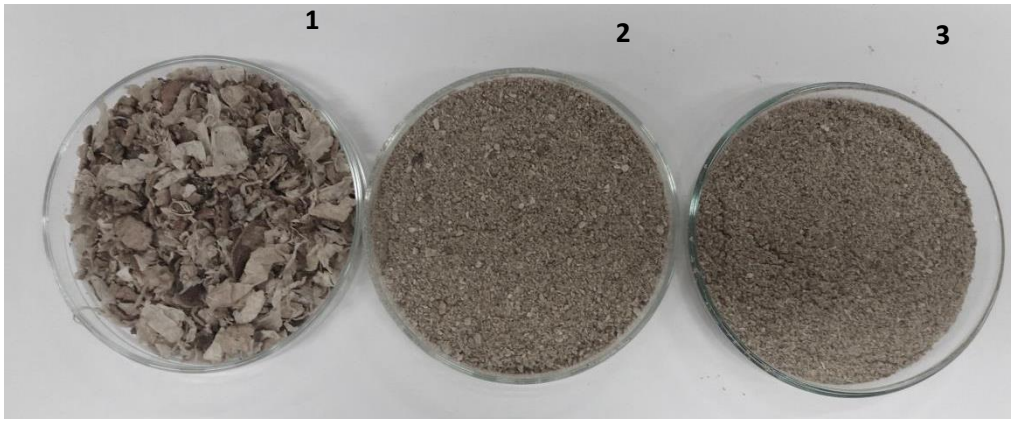
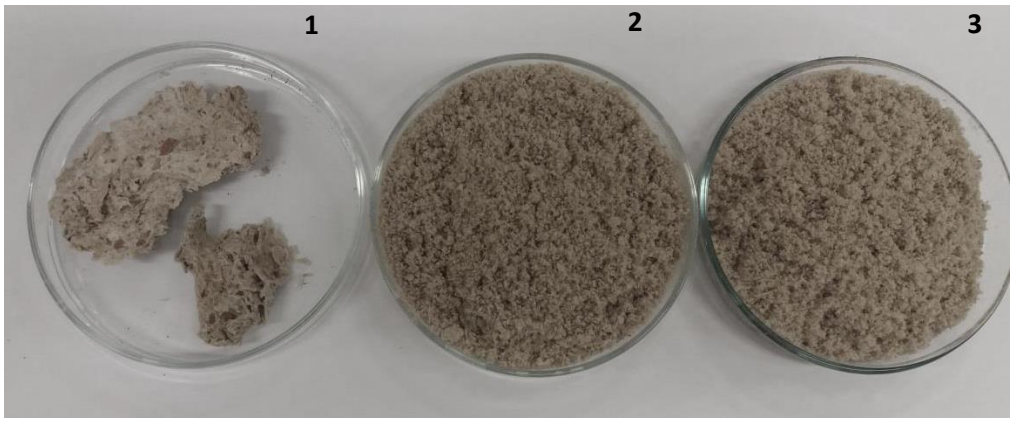
APÊNDICES

APÊNDICE 1 – FOTO DOS RESÍDUOS CÍTRICOS



Resíduo proveniente da extração de suco de laranja



Resíduo proveniente da extração de pectina

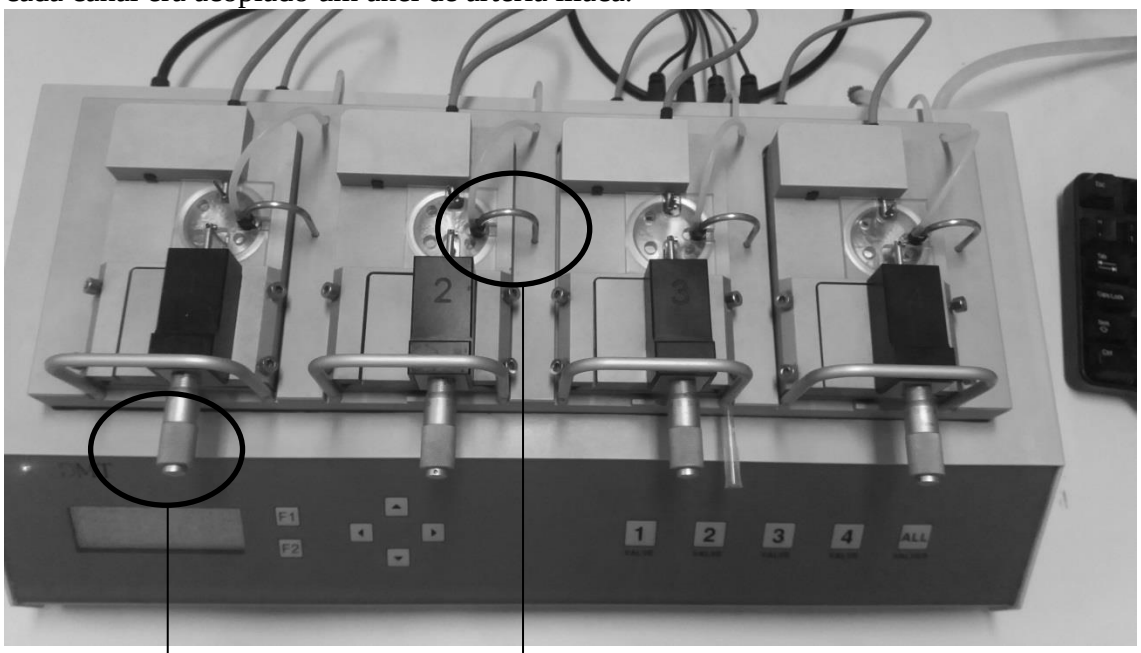
Legenda
Resíduo recebido da indústria
Resíduo processado em Blender OBL10/02 (Marchesoni)
Resíduo tamisado em peneira de 1.86mm – utilizado para preparo dos extratos.

APÊNDICE 2 – FOTO DA ARTÉRIA ILÍACA DE RATO EM MICROSCÓPIO

A artéria direita (maior) foi cortada em anéis de aproximadamente 3mm que foram usados nos experimentos de relaxamento de vasos.

APÊNDICE 3 – FOTO DO MIÓGRAFO

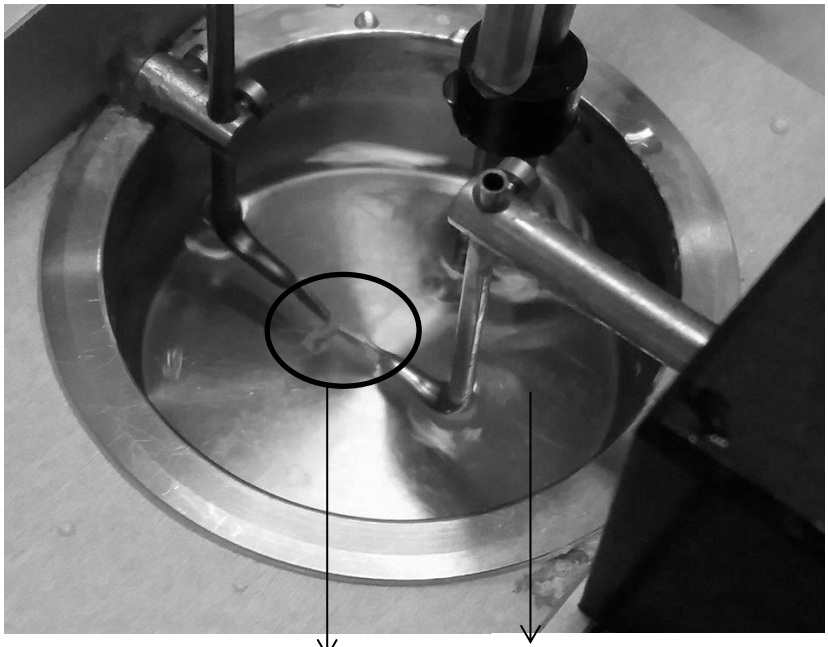
Miógrafo multicanais para pequenos vasos (modelo 610M, DMT/A/S, NA, Dinamarca). Em cada canal era acoplado um anel de artéria ilíaca.



Ajuste individual da tensão para 10 mN

Abastecimento de gás constante (95% O_2 e 5% CO_2)

Foto aproximada de um canal



Anel da artéria ilíaca

5 ml de Solução de Krebs-Henseleit pH 7,4 gaseificada

APÊNDICE 4 - LISTA DE ARTIGOS ORIGINAIS PUBLICADOS PELO GRUPO DE TRABALHO

Paula de Paula Menezes Barbosa, Amanda Roggia Ruviaro, Gabriela Alves Macedo. Comparison of different Brazilian citrus by-products as source of natural antioxidants. Food Science and Biotechnology, 2018. https://doi.org/10.1007/s10068-018-0383-4.
Disponível em: https://link.springer.com/article/10.1007/s10068-018-0383-4

Amanda Roggia Ruviaro, Paula de Paula Menezes Barbosa, Gabriela Alves Macedo. Enzyme-assisted biotransformation increases hesperetin content in citrus juice by-products. Food Research International, 2018. https://doi.org/10.1016/j.foodres.2018.05.004.
Disponível em: https://www.sciencedirect.com/science/article/pii/S0963996918303600

Francisco Manuel Barrales, Paula Silveira, Paula de Paula Menezes Barbosa, Amanda Roggia Ruviaro, Bruno Nicolau Paulino, Glaucia Maria Pastore, Gabriela Alves Macedo, Julian artinez. Recovery of phenolic compounds from citrus by-products using pressurized liquids - an application to orange peel. Food and Bioproducts Processing, 2018. https://doi.org/10.1016/j.fbp.2018.08.006.
Disponível em: https://www.sciencedirect.com/science/article/pii/S0960308518305534

ANEXOS

ANEXO 1- AUTORIZAÇÃO DA EDITORA SPRINGER PARA A INCLUSÃO NA TESE DO ARTIGO DE PESQUISA PUBLICADO

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Amanda Roggia Ruviaro <amandarruviaro@gmail.com>

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Use in doctoral thesis

4 mensagens

Amanda Roggia Ruviaro <amandarruviaro@gmail.com> 28 de junho de 2018 14:11
Para: permissionshelpdesk@elsevier.com

Dear editor,

My name is Amanda Roggia Ruviaro and I am co-author of the manucript entitled "Enzyme-assisted biotransformation increases hesperetin content in citrus
juice by-products", published in "Food Research International".

I am finishing my doctoral thesis, and I would like to include this manuscript, as an appendix. For that, I need an authorization of the Elsevier for the inclusion. Could you give me this permission?

Best regards,

Amanda Ruviaro

--
Amanda Roggia Ruviaro
Doutoranda em Alimentos e Nutrição - Faculdade de Engenharia de Alimentos - Unicamp
Mestre em Ciência e Tecnologia de Alimentos - UFSM
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Title: Enzyme-assisted biotransformation increases hesperetin content in citrus juice by-products

Author: Amanda Roggia Ruviaro,Paula de Paula Menezes Barbosa,Gabriela Alves Macedo

Publication: Food Research International

Publisher: Elsevier

Date: Available online 4 May 2018

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CERTIFICADO

Certificamos que a proposta intitulada **Efeito da biotransformação de resíduos de citrus na proteção cardiovascular**, registrada com o nº **4519-1/2017**, sob a responsabilidade de **Prof. Dr. Gabriela Alves Macedo, Prof. Dr. Edson Antunes, Amanda Roggia Ruviaro e Gláucia Coelho de Mello**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em **24 de abril de 2017**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	**01/05/2017-29/03/2019**
Vigência da autorização para manipulação animal:	**01/05/2017-29/03/2019**
Espécie / linhagem/ raça:	**Rato heterogênico / HanUnib: WH (Wistar)**
No. de animais:	**132**
Peso / Idade:	**06 semanas / 120g**
Sexo:	**machos**
Origem:	**CEMIB/UNICAMP**

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA, SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, 24 de abril de 2017.

Profa. Dra. Liana Maria Cardoso Verinaud
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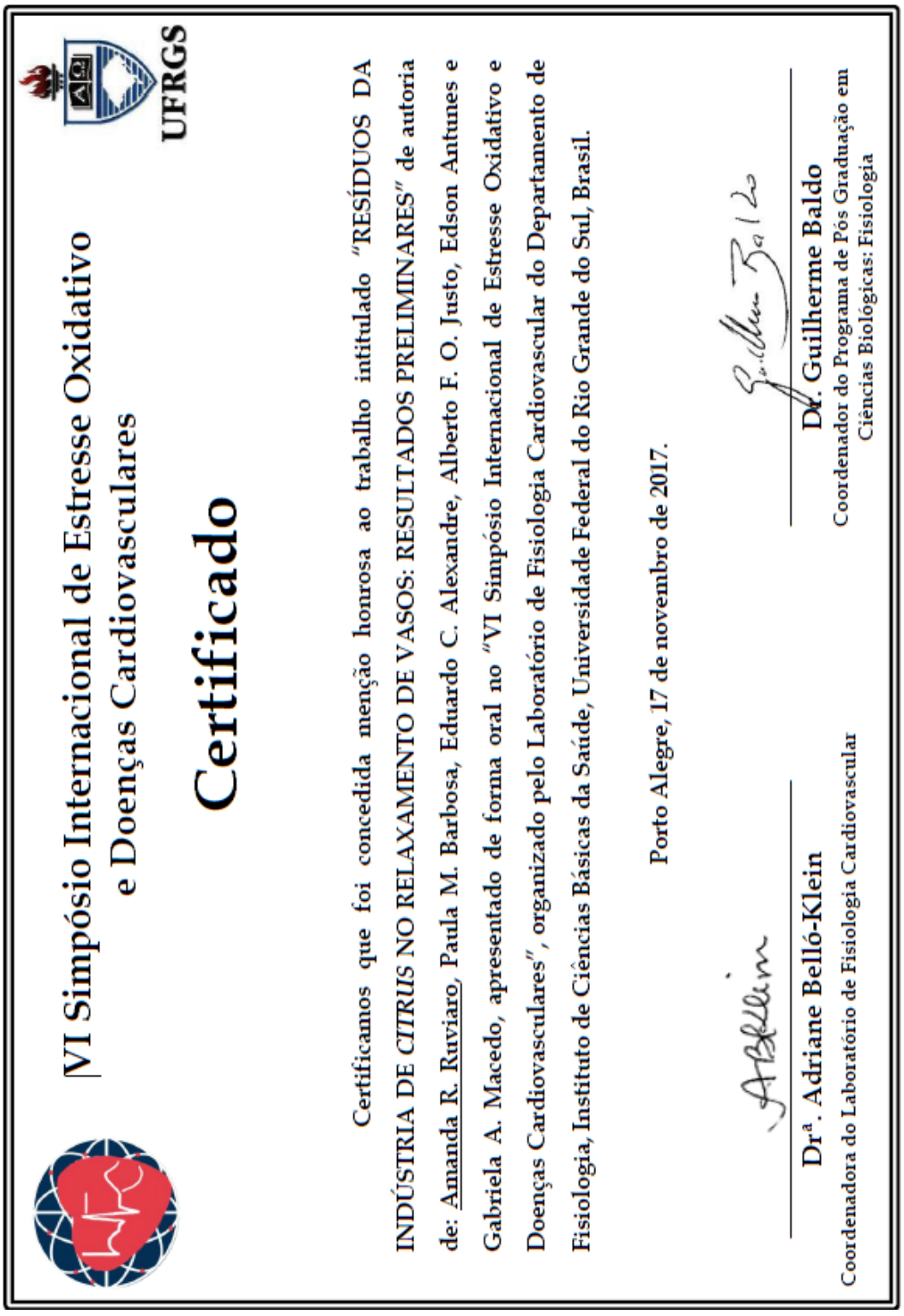
Secretária Executiva

ANEXO 4 – MENSÃO HONROSA AO TRABALHO APRESENTADO EM EVETO INTERNACIONAL



VI Simpósio Internacional de Estresse Oxidativo e Doenças Cardiovasculares

UFRGS

Certificado

Certificamos que foi concedida menção honrosa ao trabalho intitulado "RESÍDUOS DA INDÚSTRIA DE *CITRUS* NO RELAXAMENTO DE VASOS: RESULTADOS PRELIMINARES" de autoria de: Amanda R. Ruviaro, Paula M. Barbosa, Eduardo C. Alexandre, Alberto F. O. Justo, Edson Antunes e Gabriela A. Macedo, apresentado de forma oral no "VI Simpósio Internacional de Estresse Oxidativo e Doenças Cardiovasculares", organizado pelo Laboratório de Fisiologia Cardiovascular do Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Brasil.

Porto Alegre, 17 de novembro de 2017.

Drª. Adriane Belló-Klein
Coordenadora do Laboratório de Fisiologia Cardiovascular

Dr. Guilherme Baldo
Coordenador do Programa de Pós Graduação em Ciências Biológicas: Fisiologia

ANEXO 5 – PREMIAÇÃO DE TRABALHO APRESENTADO EM EVETO INTERNACIONAL – 2º lugar no Prêmio "Professor Antônio Belló"

Resumos indicados para apresentação oral - Concorrentes ao prêmio "Prof. Antonio Belló"/ Abstracts selected for oral presentation - "Prof. Antonio Belló" award competitors 11

Hidrolisado da clara do ovo previne a disfunção vascular e o aumento da pressão arterial após a exposição crônica ao aluminio em ratos/ Egg white hydrolysate prevents the vascular dysfunction and the increased blood pressure after long-term aluminum exposure in rats **12**

Resíduos da indústria de *citrus* no relaxamento de vasos: resultados preliminares/ Citrus industry by-products in vascular relaxing effects: preliminary results **16**

Aumento dos parâmetros oxidativos e diminuição dos níveis de citocinas em um modelo de transtorno de défict de atenção e hiperatividade/ Increased oxidative parameters and decreased cytokine levels in an animal model of attention-deficit/hyperactivity disorder **19**

RESÍDUOS DA INDÚSTRIA DE *CITRUS* NO RELAXAMENTO DE VASOS: RESULTADOS PRELIMINARES

★ *Segundo lugar – Prêmio "Professor Antonio Belló"* ★

Amanda R. Ruviaro[1], Paula M.Barbosa[2], Eduardo C. Alexandre[3], Alberto F. O. Justo[3], Edson Antunes[3], Gabriela A. Macedo[1]

1 Departamento de Alimentos e Nutrição, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil.

2 Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil.

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e-mail: amandarruviaro@gmail.com

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