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Andréia Quatrin

# CASCA DE JABUTICABA: METABOLIZAÇÃO E IMPLICAÇÕES NA PREVENÇÃO DAS COMPLICAÇÕES DO DIABETES

Tese

Santa Maria, RS
2019

**Andréia Quatrin**

# CASCA DE JABUTICABA: METABOLIZAÇÃO E IMPLICAÇÕES NA PREVENÇÃO DAS COMPLICAÇÕES DO DIABETES

Tese apresentada para o curso de doutorado do Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Ciência e Tecnologia dos Alimentos.**

Orientadora: Profª. Drª. Tatiana Emanuelli
Co-orientadora: Drª. Vivian Caetano Bochi

Santa Maria, RS
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 Endereço: Avenida Roraima, n. 1000, bairro Camobi, Santa Maria, RS. CEP: 97105900 Fone: (0xx) 55 32208547; email: deiaquatrin@hotmail.com

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**Aprovada em 31 de janeiro de 2019:**

**Tatiana Emanuelli**
(Presidente/Orientadora)

**Vivian Caetano Bochi (UFCSPA)**
(Co-orientador)

**Veridiana Vera de Rosso (UNIFESP)**

**Tiago Franco de Oliveira (UFCSPA)**

**Ângela Giovana Batista (UFSM)**

**Letícia Cruz (UFSM)**

Santa Maria, RS
2019

# DEDICATÓRIA

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## RESUMO

Tese de doutorado
Programa de Pós-graduação em Ciência e Tecnologia dos Alimentos
Universidade Federal de Santa Maria

# CASCA DE JABUTICABA: METABOLIZAÇÃO E IMPLICAÇÕES NA PREVENÇÃO DAS COMPLICAÇÕES DO DIABETES

AUTORA: ANDRÉIA QUATRIN
ORIENTADORA: TATIANA EMANUELLI

O objetivo deste trabalho foi avaliar a composição de polifenóis da casca de jabuticaba (PCJ), sua bioacessibilidade após simulação da digestão gastrointestinal e o envolvimento da microbiota intestinal na sua metabolização, bem como avaliar a ação do PCJ sobre complicações hepáticas e de estresse oxidativo em ratos com DM tipo 2. Verificou-se algumas diferenças no perfil de compostos fenólicos entre as duas espécies de jabuticabas (*M. trunciflora e M. jaboticaba*) analisados em HPLC-DAD-Q-TOF-MS/MS. O PCJ de *M. trunciflora* (PCJ-MT) apresentou maior teor compostos fenólicos livres sendo a classe predominante os taninos hidrolisáveis, enquanto que para o PCJ de *M. jaboticaba* (PCJ-MJ) foram as antocianinas. O estudo *in vitro* de bioacessibilidade após a simulação da digestão gastrointestinal humana do PCJ-MT evidenciou extensa hidrólise dos taninos hidrolisáveis, em especial na fase intestinal, resultando na liberação de monômeros dos ácidos gálico e elágico. As antocianinas sofreram degradação branda nas condições oral e gástrica, mas elevada degradação sob condição intestinal, resultando em baixa bioacessibilidade, enquanto que a maioria dos taninos hidrolisáveis e flavonois apresentaram maior bioacessibilidade em comparação com as antocianinas. Entretanto, devido ao elevado teor de antocianinas e taninos hidrolisáveis no PCJ, a cianidina-3-glicosídeo foi o composto fenólico mais abundante na fração bioacessível, seguida do ácido elágico (um dos produtos da hidrólise dos elagitaninos). Além disso, verificou-se através do estudo da fermentação com fezes humanas de voluntários saudáveis (incubação por até 48 h) com PCJ-MT previamente digerido pela simulação do trato gastrointestinal, que a microbiota intestinal desempenhou papel fundamental na metabolização dos compostos fenólicos do PCJ. A incubação com fezes humanas promoveu o catabolismo progressivo dos taninos hidrolisáveis e das antocianinas formando novos metabólitos de menor peso molecular, como as urolitinas (A, B, C, D, M5, M6 e M7), oriundas do metabolismo de elagitaninos, e o ácido protocatecuico, principal metabólito da cianidina 3-glicosídeo, que podem ser absorvidos e assim contribuir com os benefícios do consumo de PCJ. Adicionalmente, o PCJ forneceu substrato para o crescimento bacteriano seletivo, mantendo a viabilidade de *Bifidobacterium* e *Lactobacillus* e inibindo seletivamente a viabilidade de *Enterobacteria* após 48 h de fermentação. Além disso, verificou-se que a fermentação do PCJ aumentou a produção de ácido graxos de cadeia curta (AGCC) nas primeiras 2 h de fermentação e que possui relação com o conteúdo da fração solúvel presente no PCJ (fibra alimentar solúvel). Também foram observados os efeitos *in vivo* do consumo de PCJ-MJ, com redução do estresse oxidativo e melhora das complicações hepáticas decorrentes do DM tipo 2. Assim, verificou-se que o consumo de PCJ aumentou a síntese de glutationa e modulou o equilíbrio redox glutationa reduzida/glutationa oxidada (GSH/GSSG), bem como reduziu a hiperglicemia e a lesão hepática em ratos com DM tipo 2 induzida. Essas evidências sugerem que, embora apresente baixa bioacessibilidade após a simulação gastrointestinal, o elevado conteúdo de compostos fenólicos do PCJ confere proteção frente as complicações do DM tipo 2. Além disso, os produtos gerados pela metabolização microbiana do PCJ, como urolitinas e ácido protocatecuico provavelmente contribuem para os benefícios do consumo de PCJs, juntamente com a sua ação prebiótica e os altos níveis de AGCC produzidos durante a fermentação.



**Palavras-chave:** Antocianinas. Taninos hidrolisáveis. Diabetes mellitus tipo 2. Bioacessibilidade. Jabuticaba. Estresse oxidativo. Ácidos graxos de cadeia curta. Microbiota.

ABSTRACT
Doctoral Thesis Graduate
Program on Food Science and Technology
Federal University of Santa Maria

# JABOTICABA PEEL: METABOLIZATION AND PREVENTION OF DIABETES COMPLICATIONS

AUTHOR: ANDRÉIA QUATRIN
ADVISOR: TATIANA EMANUELLI

The aim of this study was to evaluate the profile of phenolic compounds from jaboticaba peel powder (JPP), their bioaccessibility after simulation of gastrointestinal digestion and the involvement of gut microbiota in phenolic metabolization. Additionally, the effects of JPP on oxidative stress and hepatic complications in a rat model of type 2 diabetes melittus (type 2 DM) were also evaluated. Two jaboticaba species (*M. trunciflora* and *M. jaboticaba*) were analyzed in HPLC-DAD-Q-TOF-MS/MS. Some differences were observed in the phenolic compounds profile between both JPP species. JPP from *M. trunciflora* (JPP-MT) had higher content of free phenolic compounds, the predominant class being hydrolyzable tannins, while anthocyanins were the major class in the JPP from *M. jaboticaba* (JPP-MJ). The *in vitro* bioaccessibility study after simulation of gastrointestinal digestion of PCJ-MT showed extensive hydrolysis of hydrolyzable tannins, especially in the intestinal step, releasing gallic and ellagic acid monomers. Anthocyanins had mild degradation in salivary and gastric conditions but extensive degradation in the intestinal condition, resulting in low bioaccessibility. Most hydrolyzable tannins and flavonols had greater bioaccessibility than anthocyanins in JPP. However, due to the high content of anthocyanins and hydrolyzable tannins in JPP matrix, cyanidin-3-glycoside was the most abundant phenolic compound in the bioaccessible fraction, followed by ellagic acid that was produced by the hydrolysis of ellagitannins. In addition, the fermentation study using JPP-MT previously digested by gastrointestinal simulation (JPP-IN) and feces from healthy human volunteers (up to 48 h of incubation) showed that gut microbiota plays a key role in the metabolism of the JPP phenolic compounds. JPP-IN incubation with human feces promoted the progressive catabolism of hydrolyzable tannins and anthocyanins yielding new metabolites of smaller molecular weight such as urolithins (A, B, C, D, M5, M6 and M7) originating from the metabolism of ellagitannins, and protocatechuic acid that originates from cyanidin 3-glycoside. All these metabolites can be absorbed and contribute to the health benefits of JPP. In addition, JPP provided substrate for selective bacterial growth, the counts of *Bifidobacterium* and *Lactobacillus* remained unchanged after 48 h of fermentation with JPP-IN, whereas *Enterobacteria* growth was decreased. JPP fermentation also increased short chain fatty acid (SCFA) production in the first 2 h of fermentation that is strongly related to the soluble fraction of JPP (soluble dietary fiber). The consumption of JPP-MJ in a rat model of type 2 DM reduced oxidative stress and improved hepatic complications. Thus, it was verified that the consumption of JPP increased the synthesis of glutathione and positively modulated the redox balance of GSH/GSSG, as well as reduced hyperglycemia and liver injury in rats with type 2 DM. This evidence suggest that despite their low bioaccessibility, the high content of phenolic compounds in JPPs contributes to the protection against type 2 DM complications. In addition, the microbial metabolism of JPP generates bioactive metabolites such as urolithins and protocatechuic acid that likely contribute to the health benefits of JPP along with its prebiotic action and the high levels of SCFA produced during JPP fermentation.

**Keywords:** Anthocyanins. Hydrolyzable tannins. Type 2 diabetes *mellitus*. Bioaccessibility. Jabuticaba. Oxidative stress. Short-chain fatty acids. Microbiota.

## LISTA DE ABREVIATURAS

PCJ – Pó da casca de jabuticaba

MT- *Myrciaria trunciflora*

MJ- *Myrciaria jabuticaba*

DM- Diabetes *mellitus*

HPLC- Cromatografia Líquida de Alta Eficiência

AGCC-Ácidos graxos de cadeia curta

GSH- Glutationa reduzida

GSSG - Glutationa oxidada

SHNP- Tióis não proteicos

HHDP- Hexahidroxidifenoil

EROS- Espécies reativas de oxigênio

EO-Estresse oxidativo

LDL-Colesterol de baixa densidade

HDL-Colesterol de alta densidade

STZ- Estreptozotocina

ALA-D- delta-aminolevulinato desidratase

GST-Glutationa-S-transferase

CAT-Catalase

SOD- Superóxido dismutase

TrxR-1-Tioredoxina redutase

ALT- Aspartato aminotrasferase

AST- Alanina aminotrasferase

QUICK- Índice de sensibilidade à glicose

FIRI- Índice de resistência à insulina

IN- Fração não bioacessível

OUT- Fração dialisável

PCJ-IN- Pó da casca de jabuticaba digerida

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# 1.INTRODUÇÃO

O diabetes mellitus (DM) representa uma das maiores preocupações de saúde pública devido ao grande avanço da doença no mundo. Em 2015, o DM afetou 415 milhões de adultos, cerca de 9,4% da população mundial (INTERNATIONAL DIABETES FEDERATION, 2016). No Brasil 11 milhões pessoas estão diagnosticadas com DM (8,7 % da população), e mais de 3 milhões de pessoas desconhecem que possuem a doença. Além disso, os gastos com saúde devido a esta patologia são enormes no Brasil. A IDF estimou um gasto de 1500 dólares por pessoa no ano de 2014 (INTERNATIONAL DIABETES FEDERATION, 2014).

Para o tratamento do DM são preconizados a mudança de estilo de vida e intervenção medicamentosa como o uso de hipoglicemiantes orais e insulina, que apesar do valor inquestionável podem provocar efeitos adversos consideráveis como maior risco em desenvolver insuficiência cardíaca (BERTHET et al., 2011), aumento de peso (YKI-JÄRVINEN, 2004) e até mesmo do risco de câncer (THAKKAR et al., 2013).

Desta maneira, torna-se importante a descoberta de substâncias naturais capazes de auxiliar na prevenção e no tratamento do DM tipo 2. O consumo de compostos bioativos presentes na casca da jabuticaba pode auxiliar tanto preventivamente quanto no tratamento do DM tipo 2. Existem alguns estudos com a casca de jabuticaba envolvendo capacidade antioxidante em modelo de obesidade e apenas um estudo em modelo de DM tipo 1 (ALEZANDRO; GRANATO; GENOVESE, 2013), entretanto os modelos já estudados não mimetizam as desordens metabólicas ocorridas no modelo proposto de DM tipo 2, que concentra todas as alterações ocorridas no desenvolvimento de DM tipo 2 em humanos (REUTER, 2007).

A biodisponibilidade e o envolvimento da microbiota intestinal na metabolização dos compostos fenólicos tem despertado bastante interesse, uma vez que a biodisponibilidade dos compostos *in natura* é bastante reduzida, entretanto são observados inúmeros benefícios após o consumo dos mesmos (ANHÊ et al., 2013). Acredita-se que os metabólitos fenólicos gerados durante a digestão e, em especial, na metabolização pela microbiota intestinal antes da absorção esteja contribuindo ou até mesmo promovendo os efeitos benéficos constantemente relatados na literatura, seja pelo maior teor ou, simplesmente, pela melhor absorção dos metabólitos.

Na tentativa de elucidar quais compostos realmente estão envolvidos com os efeitos observados *in vivo*, foi desenvolvido o estudo de bioacessibilidade *in vitro* com o objetivo de identificar os metabólitos que estão disponíveis para ser absorvidos após a simulação da digestão gastrointestinal, bem como observar o envolvimento da matriz alimentar na liberação e produção dos metabólitos. Embora a bioacessibilidade *in vitro* não mimetize todas as condições humanas, o estudo fornece resultados aproximados e não sofre com bloqueios éticos (CARBONELL-CAPELLA et al., 2014).

Além disso, o envolvimento da microbiota humana na metabolização de compostos fenólicos já foi consolidada para diferentes alimentos (MOSELE; MACIÀ; MOTILVA, 2015), entretanto ocorre algumas particularidades em função da matriz do alimento e dos tipos de compostos presente no mesmo (GUERGOLETTO et al., 2016; MOSELE et al., 2015, 2016). Ao mesmo tempo que os compostos fenólicos são metabolizados pelos microorganismos também promovem uma modulação na proliferação dos microorganismos (FARIA; FERNANDES; MATEUS, 2014). Além disso, os compostos fenólicos conferem uma seletividade no crescimento microbiano, com maior proliferação de microrganismos probióticos como *Bifidobacterium* e *Lactobacillus* que estão fortemente associados a melhora da saúde sistêmica, enquanto que a disbiose intestinal está relacionada com diversas doenças, como DM, obesidade e doença hepática gordurosa não alcoólica (ARON-WISNEWSKY et al., 2013; HE; SHAN; SONG, 2015). O controle da flora microbiana associado a alimentação, ou seja com o fornecimento de substratos fermentáveis, como fibras e compostos fenólicos, estão fortemente envolvidos no aumento da produção de ácidos graxos de cadeia curta (AGCC) (GUERGOLETTO et al., 2016), que por sua vez promovem tanto efeito local, melhorando a barreira intestinal, como efeito sistêmico com redução do processo inflamatório e melhora do controle glicêmico (TREMAROLI; BÄCKHED, 2012).

A casca da jabuticaba é bastante estudada devido ao elevado teor de compostos bioativos (BATISTA et al., 2018; DRAGANO et al., 2013; LAMAS et al., 2018), entretanto ainda não foi completamente elucidada a composição fenólica da sua casca, bem como, os metabólitos envolvidos com os benefícios evidenciados após o seu consumo. Existem apenas dois estudos envolvendo bioacessibilidade dos compostos fenólicos (DANTAS et al., 2018; PEIXOTO et al., 2016), porém nenhum estudo avalia o perfil de degradação dos compostos no decorrer da simulação gastrointestinal e o envolvimento da microbiota na metabolização dos compostos fenólicos.

## 1.1 ALIMENTAÇÃO RELACIONADA COM A SAÚDE

O grande número de casos de DM tipo 2 está fortemente relacionado com a alimentação incorreta, devido ao consumo de alimentos ricos em gorduras, principalmente gordura saturada oriunda de fast foods e carboidratos (WORLD HEALTH ORGANIZATION, 2015). Observou-se que o consumo de 2 frutas diárias de baixa caloria durante 3 meses melhorou o controle glicêmico, parâmetros antioxidantes e antropométricos em pacientes com DM tipo 2 (HEGDE et al., 2013). Além disso, dados da literatura relatam que o consumo de 2-3 porções de vegetais /dia e 2 frutas/dia é recomentado como uma forma de evitar o desenvolvimento do DM tipo 2 (WU et al., 2014). Dentre as frutas especialmente amoras, uvas e maçãs, estão associadas com um menor risco de desenvolvimento de DM tipo 2 (MURAKI et al., 2013).

O consumo de frutas e vegetais tem função nutricional e também funções antioxidante, anti-inflamatória e antidiabética, devido principalmente à presença de compostos bioativos como polifenóis (ANHÊ et al., 2013).

Os polifenóis são produzidos em plantas pelo metabolismo secundário desenvolvido como resposta a agentes estressores durante o cultivo. Os polifenóis compreendem um grupo de compostos bastante numerosos e amplamente distribuídos no reino vegetal, sendo que já foram observados em frutas, vegetais, grãos integrais, chocolate e bebidas como chás e vinhos (TSAO, 2010). Além disso os ácidos fenólicos nas frutas e vegetais encontram-se na sua maioria na forma livre, conjugados com diferentes unidades de açúcares e açúcares acilados, entretanto percentual considerável, cerca de 24% dos compostos fenólicos encontram-se ligadas à matriz (ACOSTA-ESTRADA; GUTIÉRREZ-URIBE; SERNA-SALDÍVAR, 2014).

Os polifenóis podem ser classificados conforme estruturas químicas das agliconas em 2 grandes grupos os flavonoides e os não flavonoides. Os não flavonoides compreendem dois tipos principais os derivados de ácido hidroxibenzóico e derivados de ácido hidroxicinamico cuja estrutura é baseada em ligações C1-C6 e C3-C6. Enquanto que os flavonoides possuem na estrutura dois anéis aromáticos ligados por três carbonos normalmente dispostos como um heterociclo oxigenado (C6-C3-C6). Estes são divididos em subclasses (Figura 1): antocianidinas, flavonóis, flavonas, flavanóis e isoflavonas (CROZIER; JAGANATH; CLIFFORD, 2009).

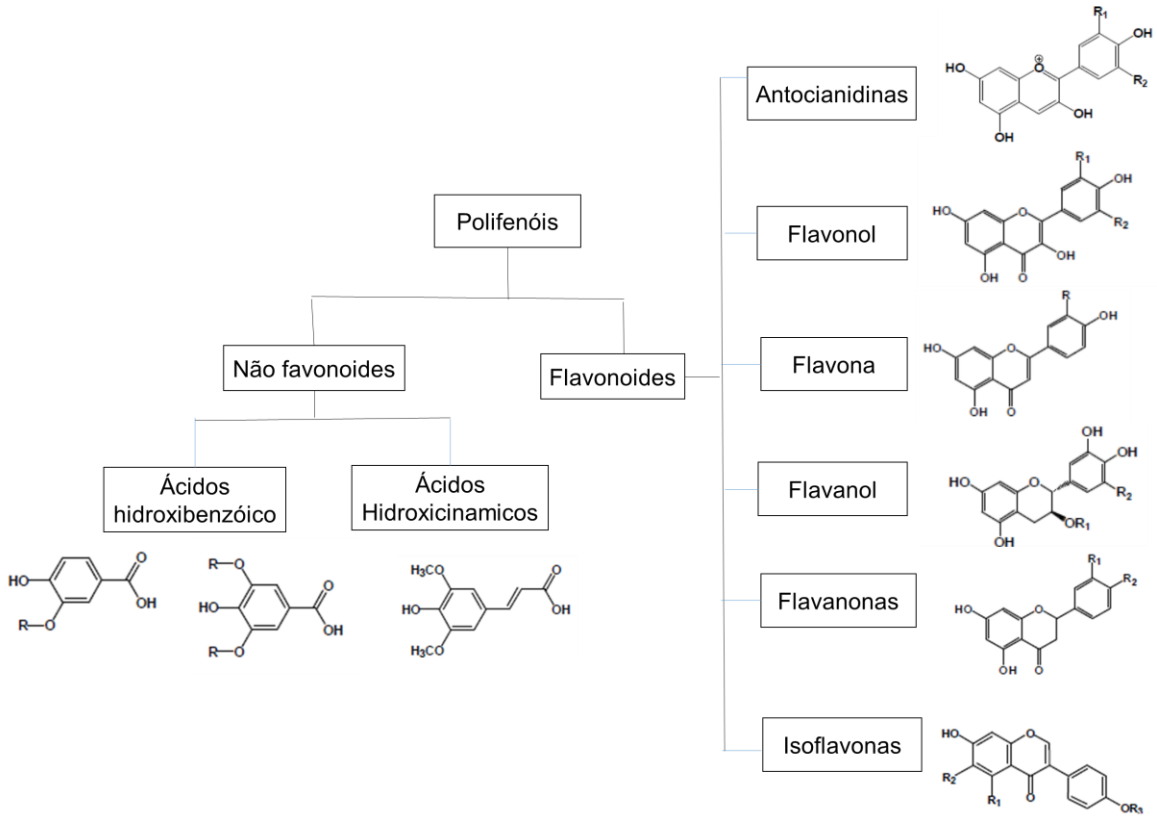


Figura 1: Classificação dos compostos fenólicos em diferentes subclasses.

Fonte: Autoria pessoal.

Os compostos fenólicos também podem ocorrer na forma de oligômeros e polímeros classificados como taninos condensados e taninos hidrolisáveis (VERMERRIS; NICHOLSON, 2006). Os taninos hidrolisáveis são compostos por um poliol central (normalmente glicose) acilado por um número variável de unidades de ácido gálico ou ácido elágico formando compostos fenólicos de alto peso molecular (Figura 2). Ainda, podem ser classificados em elagitaninos quando o açúcar central está ligado a unidades monoméricas de ácido elágico, sendo que unidades de hexahidroxidifenoil (HHDP) são formados por reações de oxidação e ligados ao açúcar central. Além disso, quando o ácido gálico está acilado ao açúcar central os taninos hidrolisáveis são chamados de galotaninos (ARAPITSAS, 2012; DOMÍNGUEZ-RODRÍGUEZ; MARINA; PLAZA, 2017).

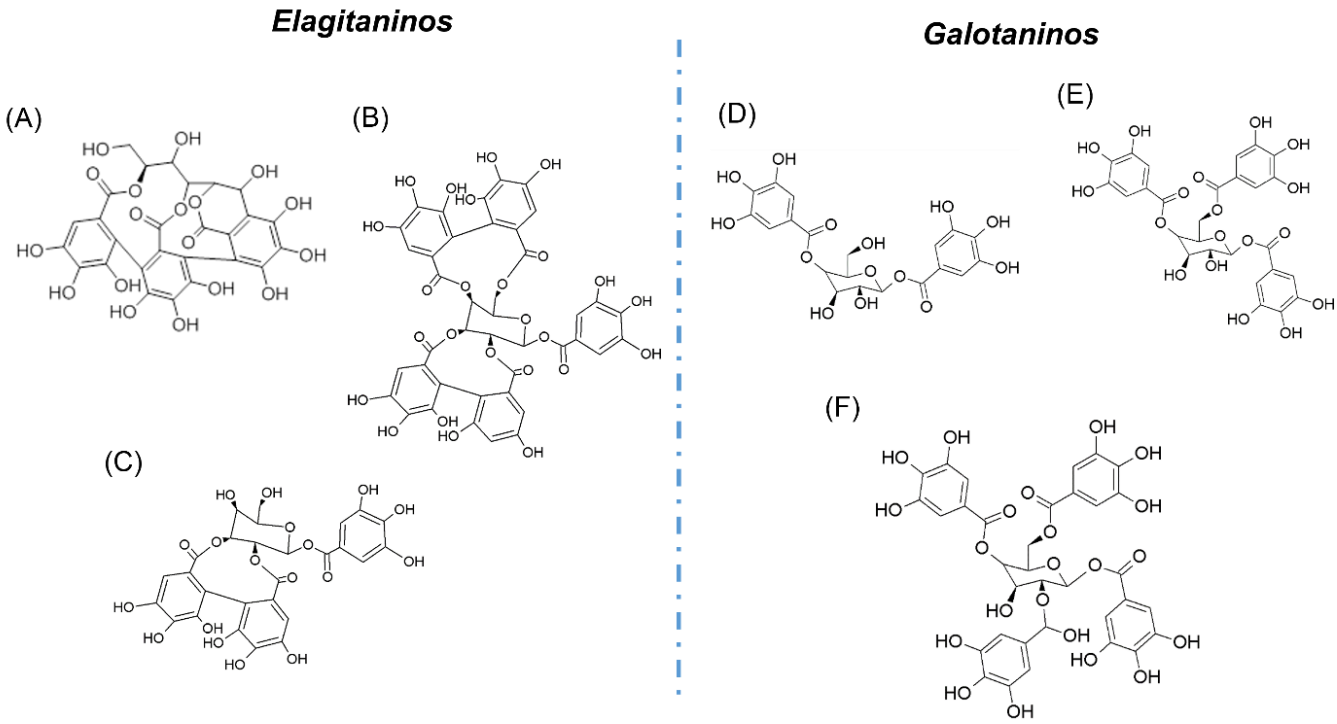


Figura 2: Alguns representantes dos taninos hidrolisáveis. Elagitaninos: (A) Castalina, (B) bis-HHDP-galoilglicose e (C) HHDP-galoilglicose. Galotaninos: (D) Digaloilglicose, (E) Trisgaloilglicose e (F) Tetragaloilglicose. HHDP= hexahidroxidifenoil.

Fonte: Autoria pessoal.

Os ellagitanninos são abundantemente encontrados na natureza, enquanto que os galotaninos possuem distribuição limitada (ARAPITSAS, 2012). Os elagitaninos apresentam estruturas complexas, porém bastante diversificada conferindo importante contribuição na cor, sabor adstringente e estabilidade para as frutas e bebidas como vinho e destilados (ZHIMIN; HOWARD, 2012). O consumo de elagitaninos também está fortemente associado a benefícios para a saúde relacionado com doenças crônicas como diabetes, obesidade e cardiovascular (GE et al., 2017; LADDHA; KULKARNI, 2019)

As antocianinas são considerados pigmentos vegetais, pois conferem coloração aos vegetais que variam da cor azul, roxa até vermelha. Podem ser encontradas em cereais, vinho tintos, raízes de vegetais e em maior proporção em frutas vermelhas que englobam cerejas, ameixas, amoras, framboesa, uvas, groselhas negras, jabuticaba e morango (ALEZANDRO et al., 2013; CLIFFORD, 2000). A análise das antocianinas tornou-se particularmente relevante devido aos numerosos estudos que tratam de seus potenciais efeitos sobre a saúde (ANHÊ et al., 2013; GOWD; JIA; CHEN, 2017).

## 1.2 JABUTICABA

A jabuticabeira é uma planta nativa da América do Sul, pertencente à família *Myrtaceae*, cuja a fruta se desenvolve no caule (Figura 3), quando madura apresenta aproximadamente 2 – 3 cm de diâmetro e a sua casca possui coloração roxa/azulada intensa e a polpa possui coloração branca (WU; LONG; KENNELLY, 2013).

Figura 3: Jabuticabeira com frutas jabuticabas maduras.

Fonte: Arquivo pessoal.

A jabuticaba possui grande potencial como alimento funcional, devido a elevada concentração de antocianinas, ácido elágico, taninos hidrolisáveis, especialmente na casca (ALEZANDRO et al., 2013; INADA et al., 2015; PEREIRA et al., 2017). Existem diferentes espécies de jabuticabas que se adaptaram as diferentes condições climáticas de diferentes regiões do Brasil. As espécies *Myrciaria jabuticaba* Vell. Berg. cultivada na região de São Paulo e a *Myrciaria cauliflora* cultivada mais na região de Minas Gerais são as espécies mais estudadas e com maior volume de produção anual (WU; LONG; KENNELLY, 2013). Ambas espécies apresentam cianidina 3-glicosídeo e delfinidina 3-glicosídeo como antocianinas

predominantes na casca (ALEZANDRO et al., 2013; LEITE et al., 2011). Além disso, a casca da espécie *M. jaboticaba* também apresenta quantidade considerável de taninos hidrolisáveis, especialmente elagitaninos, ainda é fonte de ácido elágico e quercetina (PEREIRA et al., 2017; PLAZA et al., 2016). A espécie *M. cauliflora* também é fonte de antocianinas e quercetina em maior proporção na casca, e proantocianidinas e ácido elágico na polpa e semente (ALEZANDRO et al., 2013). Na espécie *M. jaboticaba*, cultivada em São Paulo, foi encontrada quantidade considerável de ácido elágico quando comparado as frutas da mesma família Myrtaceae como cambuci, goiaba vermelha, camu-camu e pitanga (ABE; LAJOLO; GENOVESE, 2012). Porém, a espécie *M. trunciflora* que se adaptou ao clima do Rio Grande do Sul existe apenas um estudo que realizou a identificação dos compostos, sendo a cianidina 3-glicosídeo e kaempferol os compostos identificados na espécie (CALLONI et al., 2015).

Apesar da elevada quantidade de compostos bioativos acredita-se que a quantidade de compostos fenólicos presente na jabuticaba esteja sendo subestimada, devido à grande quantidade de polifenóis ligados as fibras e proteínas da matriz (não extraíveis) que não são quantificados, conforme observado em outros alimentos (ARRANZ et al., 2009). Estes polifenóis ligados podem estar contribuindo para os benefícios à saúde da jabuticaba, como já demonstrado para outras frutas (PÉREZ-JIMÉNEZ; DÍAZ-RUBIO; SAURA-CALIXTO, 2013).

A inclusão de 1 e 2% de pó da casca de jabuticaba (PCJ) na dieta aumentou a capacidade antioxidante de ratos saudáveis (LEITE et al., 2011). Além de reduzir a peroxidação lipídica e aumentar a atividade das enzimas superóxido dismutase e glutationa peroxidase no tecido hepático de ratos obesos (BATISTA et al., 2014). Também foi observado aumento da capacidade antioxidante renal pela metodologia de ORAC e TEAC em ratos obesos (BATISTA et al., 2014). O PCJ também pode atuar na prevenção e manutenção da saúde, evitando o ganho de peso, aumentando a excreção de triglicerideos, reduzindo a área de esteatose hepática e estímulando à produção de ácidos graxos de cadeia curta (AGCC) pela microbiota intestinal em modelo animal alimentados com dieta gordurosa (BATISTA et al., 2018). Acredita-se que o elevado teor de taninos desempenham um papel decisivo na redução da glicemia de jejum, níveis de colesterol total, e da hiperinsulinemia após o tratamento com extratos de jabuticaba (*M. jabuticaba*) em modelo de obesidade (MOURA et al., 2018).

Adicionalmente, os extratos de PCJ também apresentaram ação antiproliferativa contra células leucêmicas e contra células cancerígenas prostáticas (LEITE-LEGATTI et al., 2012).

O extrato de jabuticaba (*M. jaboticaba*) também promoveu a modulação anti-inflamatória, hipoglicêmica e lipídica, e ainda foi capaz de prevenir o pré-diabetes e esteatose hepática em camundongos idosos alimentados com dieta gordurosa (LAMAS et al., 2018).

Ademais, o consumo de PCJ reduziu a resistência à insulina e a resposta inflamatória em modelo de obesidade em roedores (DRAGANO et al., 2013; LENQUISTE et al., 2012) e aumentou os níveis de colesterol HDL após consumo de 2 e 4% de PCJ na dieta (LENQUISTE et al., 2012). Também aumentou a excreção fecal de triglicerídeos e reduziu a peroxidação hepática. O aumento da excreção de triglicerídeos pode estar relacionado com o consumo fibras solúvel (BATISTA et al., 2013), uma vez que o PCJ é considerado uma boa fonte de fibra alimentar solúvel e insolúvel (LEITE-LEGATTI et al., 2012; MARQUES et al., 2012; QUATRIN et al., 2018). PCJ também demonstrou ação antioxidante no plasma de animais com DM tipo 1 induzida (FRAP) e em humanos saudáveis (ORAC) após o consumo de PCJ (ALEZANDRO; GRANATO; GENOVESE, 2013; PLAZA et al., 2016). PCJ também melhorou a sensibilidade à insulina evidenciados com redução da glicemia e da insulina em humanos após o consumo de 27,6 g de PCJ equivalente a 1,25 g de compostos fenólicos totais (PLAZA et al., 2016).

## 1.3 METABOLIZAÇÃO INTESTINAL E BIODISPONIBILIDADE DOS COMPOSTOS FENÓLICOS

Ao analisar os compostos fenólicos como compostos antioxidantes um fator importante a ser considerado é a forma como são absorvidas e sua distribuição no organismo. Cerca de 20 a 25% (FELGINES et al., 2006; TALAVÉRA et al., 2003) da quantidade de antocianinas consumida é rapidamente absorvida no estômago, atingindo a concentração máxima no plasma de roedores 15-30 min após a ingestão de diferentes frutas (POJER et al., 2013). Uma hora após a administração oral de suco de groselha contendo 100 mg de antocianinas/kg de peso corporal, foram encontrados 2 µg de antocianinas/mL de plasma e após 8 h não foram mais detectadas antocianinas intactas no plasma (MATSUMOTO et al., 2006). Quinze min após a ingestão de extrato de mirtilo, contendo 67,3 µmol antocianinas/kg peso corporal, foi atingida a concentração plasmática máxima de antocianinas (1,18 nM), reduzindo para próximo de zero em 120 min (SAKAKIBARA et al., 2009).

HE et al.,(2009) demostraram que as antocianinas da framboesa preta resistem ao trato gastrointestinal superior em ratos sob jejum, com taxa de recuperação entre 75-79% da dose

administrada no estômago e intestino delgado após 30-120 min. Estes dados diferem um pouco dos encontrados em estudo de digestão in vitro, no qual cerca de 50% das antocianinas são perdidas na simulação da passagem gastrointestinal (BERMÚDEZ-SOTO; TOMÁS-BARBERAN; GARCÍA-CONESA, 2007).

Após a absorção os compostos fenólicos sofrem biotransformaçãointensa por enzimas de fase I e fase II nos enterócitos e fígado através de reações com o ácido glicurônico e metilação (TALAVÉRA, SÉVERINE FELGINES et al., 2005; WU et al., 2005), sendo no decorrer excretados na urina e bile. Esta última retorna para o intestino por meio da circulação entero-hepática (VAN DUYNHOVEN et al., 2011).

Além disso, a microflora intestinal desempenha um papel importante na biotransformação e degradação dos compostos fenólicos ao atingirem o cólon, seja quando eles não são absorvidos no estômago e intestino delgado ou quando são excretados pela bile após a absorção (KAY, 2006). Dessa forma, os compostos fenólicos estão sujeitos a modificações estruturais como hidrólise da porção açúcar e também da aglicona, os quais podem ser degradados a ácidos fenólicos (MOSELE et al., 2015) que podem ser absorvidos (VITAGLIONE et al., 2007). A hidrólise de glicosídeos e agliconas conjugadas promovem a formação de produtos de biotransformação com atividade biológica potencialmente mais ativa do que seus precursores (CHIOU et al., 2014). A metabolização da cianidina-3-glicosídeo resulta em vários produtos de biotransformação, sendo o principal o ácido protocatecuico (VITAGLIONE et al., 2007), enquanto o principal produto de biotransformação da pelargonidina-3-glicosídeo é o ácido 4-hidroxibenzóico identificado no plasma de humanos após ingestão de morangos (300 g da fruta), em virtude de ser a antocianina majoritária neste fruto (AZZINI et al., 2010).

Segundo Vitaglione et al. (2007) o ácido protocatecuico está presente no plasma 2 horas após a ingestão de antocianinas, e forma-se por biotransformação via microbiota intestinal, devido ao maior tempo para ser detectado na circulação sanguínea. Um estudo mais recente confirmou esta hipótese, já que a supressão da microbiota intestinal com antibióticos impediu o aparecimento deste produto de biotransformação (ácido protocatecuico) na circulação (WANG et al., 2012).

O principal produto de biotransformação dos elagitaninos são as urolitinas, produtos do metabolismo microbiano, e estão associados a efeitos anti-inflamatórios (PIWOWARSKI et al., 2014). Mas a produção de diferentes urolitinas dependem da microflora bacteriana de cada indivíduo, sendo que as urolitinas A, B, C, D e E foram relatadas, recentemente, em

humanos (ESPÍN; GONZÁLEZ-SARRÍAS; TOMÁS-BARBERÁN, 2017). A formação destes produtos de biotransformação demanda mais tempo para atingir o pico de concentração, cerca de 48 horas, após o início da fermentação *in vitro* utilizando fezes humanas de voluntários saudáveis (MOSELE et al., 2016).

Além disso, estudos demostram que os compostos fenólicos não absorvidos no estômago e intestino delgado podem ser metabolizados pela microflora intestinal antes de serem absorvidos o que resulta em efeitos benéficos a saúde, pois estimulam o crescimento e atividade microbiana promovendo assim efeito prebiótico (ANHÊ et al., 2013; FARIA; FERNANDES; MATEUS, 2014).

Entretanto, a biotransformação intestinal de nutrientes depende da constituição da flora intestinal que pode variar de indivíduo para indivíduo (TAPPENDEN; DEUTSCH, 2007) e está fortemente relacionada com os hábitos alimentares, que por sua vez, influenciam a biodisponibilidade das compostos fenólicos (CANI; DELZENNE, 2011). O mesmo estudo evidencia a importância dos prebióticos, pois modulam a composição da flora intestinal (aumento de *Bifidobacterium* spp.), uma vez que maus hábitos alimentares como consumo de dieta rica em lipídios e doenças como obesidade e DM tipo 2 promovem uma disbiose intestinal. Os prebióticos atuam diminuindo a permeabilidade do intestino a endotoxina metabólica (lipopolisacarídeo-LPS) e com isso aumentam a sensibilidade à insulina, reduzem a esteatose e a inflamação de baixo grau (CANI et al., 2007; CANI; DELZENNE, 2011).

Devido à intensa biotransformação e rápida excreção (TALAVÉRA et al., 2003), vários estudos relataram a baixa biodisponibilidade das antocianinas intactas (CRESPY et al., 2001; FELGINES et al., 2003). Entretanto, mesmo com baixa absorção as antocianinas ainda apresentam ação antioxidante (BITSCH et al., 2004; NAIN et al., 2012), que pode ser explicada pela deposição em diferentes órgãos como cérebro, fígado e olhos, por exemplo, observada em porcos após ingestão de mirtilo durante 4 semanas (KALT et al., 2008). Em ratos foi identificado maior acúmulo de cianidina 3-glicosídeo e delfinidina 3-glicosídeo no fígado do que nos tecidos renal e pulmonar após consumo de 67,3 µmol de antocianina/kg (SAKAKIBARA et al., 2009).

Após ingestão de 20 g de extrato de amora preta, composto de 85% de cianidina 3-glicosídeo/kg de dieta, foram detectadas antocianinas intactas em ordem decrescente de concentração nos seguintes tecidos: bexiga, próstata, tecido adiposo, testículos e coração, bem como também foi detectada cianidina-3-glicosídio no plasma (FELGINES et al., 2009). A detecção de antocianina intacta no plasma pode ser explicada porque no estudo de Felgines et al. (2009) os animais não estavam em jejum como observado no estudo de Kalt et al. (2008),

já que é sabido que as antocianinas são rapidamente metabolizadas e excretadas, e consequentemente não seria possível detectar no plasma após jejum prolongado.

## 1.4 DIABETES MELLITUS TIPO 2 E COMPLICAÇÕES

O DM é considerado uma doença metabólica crônica, caracterizada por hiperglicemia e intolerância à glicose, devido à deficiência de insulina, deficiência na ação da insulina ou ambos (WORLD HEALTH ORGANIZATION, 2015).

Com base na etiologia, o DM é classificado em quatro tipos, sendo o DM tipo 2 o principal, pois abrange cerca de 90-95% dos casos de DM atingindo principalmente adultos que apresentam obesidade ou maior porcentagem de gordura abdominal (AMERICAN DIABETES ASSOCIATION, 2014). O DM tipo 2 inicia com graus variáveis de resistência tecidual a insulina que progride com o tempo e leva ao dano pancreático de 50% das células β-pancreáticas (afetando cerca de 25% da função) promovendo uma deficiência relativa na secreção de insulina, que resulta em hiperglicemia (AMERICAN DIABETES ASSOCIATION, 2014; TAYLOR, 2013).

A resistência à insulina provoca alterações metabólicas, pois impede que a mesma suprima adequadamente a gliconeogênese e a glicogenólise, exacerbando a hiperglicemia e resulta em hiperinsulinemia compensatória (SALTIEL, 2001) até a exaustão progressiva das células β pancreáticas (LEAHY, 2005). Além disso, a insulina apresenta função importante na regulação do metabolismo lipídico (VERGÈS, 2005), o que pode explicar os altos níveis de lipoproteína de baixa densidade (LDL) e baixos níveis de lipoproteína de alta densidade (HDL) que correlacionam-se com o descontrole glicêmico e o tempo de duração do diabetes em pacientes com DM tipo 2 (KARIM et al., 2013).

A resistência à insulina estimula a lipólise resultando no aumento dos ácidos graxos livres circulantes, que após absorção serão oxidados no fígado, o que pode acarretar na formação de radicais livres que em excesso podem comprometer a função hepática (AHMADIEH; AZAR, 2014; SALTIEL, 2001). Doenças hepáticas não alcoólicas são fortemente relacionadas com o DM tipo 2 independente do tempo de diagnóstico do DM, sendo que mais de 40% dos pacientes com DM tipo 2 apresentam complicações hepáticas (WILLIAMSON et al., 2011).

O estresse oxidativo (EO) está envolvido no desenvolvimento do DM e de suas complicações (KASSAB; PIWOWAR, 2012; RAINS; JAIN, 2011). Acredita-se que uma das

principais fontes de radicais livres seja a oxidação da glicose, que resulta na geração exagerada de espécies reativas de oxigênio (EROs) na mitocôndria, devido ao aumento da glicose intracelular que induz uma superprodução de doadores de elétrons (KASSAB; PIWOWAR, 2012). Este aumento da produção de EROs somado a deficiência das defesas antioxidantes enzimáticas e não enzimáticas leva ao EO em vários tecidos (ROCHETTE et al., 2014). Além disso, a hiperglicemia e espécies reativas podem ativar vias sensíveis ao estresse tal como a via do fator nuclear-κB (NF-κB) o qual aumenta a expressão de citocinas pro-inflamatórias (EVANS et al., 2002), que por sua vez podem agravar a resistência à insulina (RAINS; JAIN, 2011).

## 1.5 PROPRIEDADES DOS COMPOSTOS FENÓLICOS RELACIONADOS AO DIABETES MELLITUS TIPO 2

Elevado número de estudos relatam a ação antioxidante dos compostos fenólicos tanto *in vitro* quanto *in vivo* (RAVICHANDIRAN; NIRMALA; AHAMED, 2012; XIAO et al., 2017). O tratamento com o flavonol, fisetina, restaura a atividade de enzimas antioxidantes e reduz a peroxidação lipídica no tecido hepático de ratos diabéticos (PRASATH; SUBRAMANIAN, 2013). Além disso, a cianidina-3-glicosídeo reduziu a produção de espécies reativas de oxigênio e aumentou a síntese de glutationa (GSH) em cultura de células hepáticas HepG2 expostas a hiperglicemia (ZHU et al., 2012).

Adicionalmente, as antocianinas do feijão preto (cerca de 68% de cianidina-3-glicosídeo, 25% de delfinidina-3-glicosídeo e 6% de petunidina-3-glicosídeo) reduzem o acúmulo de lipídios e diferenciação de adipócitos 3T3-L1 (via supressão da expressão de PPAR-γ) em cultura (KIM et al., 2012) auxiliando assim na melhora da sinalização da insulina e no controle do processo inflamatório, sintomas estes característicos na patologia do DM tipo 2.

Os taninos também são capazes de retardar ou diminuir as complicações do DM (LADDHA; KULKARNI, 2019), reduzindo a formação de produtos finais de glicação avançada em tecido cardíaco e reduzindo a anormalidade do metabolismo lipídico (PATEL; GOYAL, 2011). O extrato de *Myrica faya* rico em compostos fenólicos, em especial, antocianinas, flavonóis e elagitaninos inibe a atividade α-glucosidase, aldose redutase e reduz a glicação de proteínas (SPÍNOLA; LLORENT-MARTÍNEZ; CASTILHO, 2018). Os taninos da *Ficus racemosa* atenuam a hiperglicemia e restaura a atividade das enzimas antioxidantes

superóxido dismutase e catalase, bem como os níveis de colesterol total e triglicerídeos em ratos diabéticos após 30 dias de tratamento (RAVICHANDIRAN; NIRMALA; AHAMED, 2012).

Além dos polifenóis, a fibra alimentar presente nos cereais e frutas também pode auxiliar no tratamento do diabetes como demostrado por Prangthip et al., (2013) que observou redução nos níveis de colesterol total, colesterol de baixa densidade (LDL) e triglicerídeos em ratos diabéticos alimentados apenas com acréscimo de fibra alimentar (mesma proporção de fibra solúvel e insolúvel presentes no arroz preto), mas a ação antioxidante e anti-inflamatória foi observada somente nos grupos que receberam na ração arroz preto, devido a presença dos compostos bioativos (antocianinas, carotenoides, flavonoides) e não da fibra.

Estudos anteriores sugerem que a ação antioxidante dos polifenóis ocorre de forma indireta, já que os polifenóis intactos apresentam baixa biodisponibilidade. Ou seja, os polifenóis atuariam modulando a expressão gênica de enzimas antioxidantes (SPANIER et al., 2009) e também modulando a sinalização celular ou através da interação com receptores (FRAGA; OTEIZA, 2011). Entretanto, existe a possibilidade de que metabólitos, exerçam atividade antioxidante direta, já que ocorrem biologicamente em concentrações bem superiores a dos polifenóis que lhes deram origem (CHIOU et al., 2014).

Além disso, os benefícios à saúde podem ser explicados por uma liberação lenta e contínua de compostos fenólicos e metabólitos através do intestino para a corrente sanguínea (FERNANDES et al., 2014). Segundo Wang et al., (2012) o ácido protocatecuico, principal metabólito da cianidina-3-glicosideo, apresenta maior capacidade de promover o efluxo do colesterol do que a cianidina-3-glicosídeo em macrófagos. Ainda em estudo com células THP-1 humanas os metabólitos monometilados da cianidina-3-glicosídio e delfinidina-3-glicosídio apresentaram atividade antioxidante nos ensaios de DPPH e FRAP (FERNANDES et al., 2013).

Mas, poucos estudos avaliam a atividade dos metabólitos secundários gerados durante o processo de digestão, assim como a elucidação da biodisponibilidade e distribuição dos mesmo no organismo. No entanto existem vários estudos comprovando os benefícios do consumo ou tratamento com compostos fenólicos na patologia do DM (ANHÊ et al., 2013; SANCHO; PASTORE, 2012). Como observado por Roopchand et al., (2013) a suplementação na dieta com 40% de concentrado rico em compostos fenólicos (suco de mirtilo enriquecido com farinha de soja) durante 13 semanas reduziu o ganho de peso, melhorou a tolerância à glicose e a glicemia de jejum em modelo de ratos obesos

hiperglicêmicos. Além disso, extrato purificado de antocianinas de frutas vermelhas (*Aristotelia chilensis*) melhorou a tolerância à glicose em ratos com DM tipo 2 e reduziu a produção de glicose em células hepáticas de ratos H4IIE (ROJO et al., 2012).

Adicionalmente, extratos de amora preta e *rowanberry* (fruta nativa da América e Europa) auxiliam no controle glicêmico pós-prandial por meio da inibição da atividade da enzima α-glicosidase (BOATH; STEWART; MCDOUGALL, 2012), evitando assim picos de hiperglicemia pós-prandial que em pacientes com DM tipo 2, o qual representa maior risco para o desenvolvimento de complicações cardiovasculares do que a glicemia de jejum (BONORA; MUGGEO, 2001).

# 2. OBJETIVOS

## 2.1 OBJETIVO GERAL

Avaliar a composição e bioacessibilidade dos compostos fenólicos do pó da casca de jabuticaba (PCJ), bem como sua metabolização pela microbiota intestinal e seus efeitos em modelo de diabetes *mellitus* tipo 2 em ratos.

## 2.2 OBJETIVOS ESPECÍFICOS

- Determinar a composição de compostos fenólicos livres e ligados à matriz em PCJ de duas espécies (*M. jabotic*aba vs. *M. trunciflora*).
- Avaliar a bioacessibilidade e o perfil de biotransformação dos compostos fenólicos do PCJ (*M. trunciflora*) após simulação da digestão gastrointestinal humana *in vitro*.
- Avaliar *in vitro* a metabolização do PCJ (*M. trunciflora*) pela microbiota intestinal humana através da simulação da fermentação colônica utilizando fezes de voluntários saudáveis.
- Avaliar o efeito do tratamento com diferentes doses de PCJ (*M. jaboticaba*) sobre as complicações hepáticas e o estresse oxidativo em modelo de diabetes mellitus tipo 2 em ratos.

# 3.MATERIAL E MÉTODOS

O estudo foi dividido em quatro experimentos, sendo o Manuscrito 1: Análise de composição e quantificação dos compostos fenólicos das casca de jabuticaba de duas espécies (*M. jaboticaba* vs. *M. trunciflora)*; Manuscrito 2: Bioacessibilidade dos compostos fenólicos do PCJ (*M. trunciflora*) usando modelo de digestão gastrointestinal *in vitro*. Manuscrito 3: Análise do efeito da microbiota humana na metabolização de compostos fenólicos do PCJ (*M. trunciflora*) e implicações na modulação da microbiota e seus metabólitos. Manuscrito 4: Efeito do tratamento com PCJ (*M. jaboticaba*) sobre complicações do diabetes tipo 2 em modelo animal.

## 3.1 ANÁLISES DE COMPOSIÇÃO FENÓLICOS DE CASCAS DE JABUTICABAS

### 3.1.1 Preparo dos pós das cascas de jabuticabas

As jabuticabas foram obtidas no estágio de coloração característico de maturação completa da fruta. Frutas da espécie *Myrciaria trunciflora* foram colhidas no verão de 2014 (40 kg) na cidade de São Vicente do Sul, Rio Grande do Sul, Brasil. As plantas foram identificadas e depositadas no herbário do Departamento de Ciências Florestais (nº HDCF 7228) da UFSM. As frutas de *Myrciaria jaboticaba* foram coletadas no verão de 2012 (40 kg) na cidade de Casa Branca, São Paulo, Brasil. As frutas foram lavadas e a casca foi separada manualmente. Na sequência, foram secas em liofilizador (Terroni, São Paulo, Brasil), trituradas usando um micro triturador analítico (Marconi, MA-630, São Paulo, Brasil) e armazenadas a -80°C. A nomenclatura utilizada para o pó da casca de jabuticaba da espécie *M. trunciflora* foi PCJ-MT e para a espécie *M. jaboticaba* foi PCJ-MJ.

### 3.1.2 Extração, isolamento e quantificação dos compostos fenólicos

Os polifenóis da casca de jabuticaba liofilizada foram extraídos de forma exaustiva conforme WU et al.,(2004) com algumas modificações. PCJ (0,5 g) foi extraído com 7,5 mL de solução de metanol / água / ácido fórmico (85: 15: 0,5; v/v/v), agitado (30 s, 2000 rpm, Biomixer VTX-2500, São Paulo, Brasil) e sonicado por 5 min (134 W RMS, ULTRA Cleaner 1600, São Paulo, Brasil), após foi separado por centrifugação (2000 *x g*, 10 min) e o

sedimento foi usado em 5 procedimentos de re-extração com 5 mL da mesma solução. Todas as soluções extraídas foram reunidas e concentradas em um evaporador rotativo a 38ºC (Büchi, Essen, Alemanha) para remover o solvente orgânico. O extrato final foi ressuspenso em volume conhecido com solução de água acidificada com 0,35% de ácido fórmico (v/v). Os extratos brutos foram purificado e fracionado por extração em fase sólida (Solid Phase Extraction, SPE) utilizando fase reversa (C18) de acordo com Rodriguez-Saona e Wrolstad, (2001) modificado por Bochi et al., (2015). Em seguida, os solventes foram evaporados em evaporador rotativo e os compostos fenólicos foram recuperados em 0,6 mL de uma solução aquosa acidificada com 0,1% de ácido fórmico contendo de 5% de metanol. As antocianinas foram recuperadas em 2 mL com solução aquosa de 0,35% de ácido fórmico.

Para a quantificação de compostos fenólicos ligados à matriz celular, o resíduo obtido após a extração de compostos fenólicos livres foi seqüencialmente submetido à hidrólise alcalina e ácida, conforme descrito anteriormente por Mattila e Kumpulainen, (2002) com algumas modificações.

Para hidrólise alcalina, o resíduo foi imediatamente incubado com 12 mL de água e 5 mL de NaOH (10 M) à temperatura ambiente (25 ± 2ºC) durante 16 h sob agitação contínua em tubos protegidos da luz. Posteriormente, o pH foi ajustado para 2-3 com HCl 6 M e os tubos foram centrifugados (2.000 *x g*, 10 min) para recolher o sobrenadante. Os resíduos foram lavados duas vezes com 5 mL de água Milli-Q seguido de agitação (30 s, 2000 rpm, Biomixer VTX-2500, São Paulo, Brasil) e centrifugado (2000 *x g*, 10 min) para coleta os sobrenadantes. Todos os sobrenadantes foram combinados e purificados em cartuchos C-18 conforme Rodriguez-Saona e Wrolstad, (2001) modificado por Bochi et al., (2015).

Para a hidrólise ácida, os resíduos obtidos após a hidrólise alcalina foram adicionados 2,5 mL de HCl concentrado e incubado por 30 min, a 85 ± 3 ° C (MATTILA, KUMPULAINEN, 2002). Posteriormente, o pH foi ajustado para 2-3 com NaOH 10 M, as amostras foram centrifugadas (2 000 *x g*, 10 min) e o sobrenadante foi recolhido. Os resíduos foram lavados duas vezes com 5 mL de água Milli-Q seguido de agitação (30 s, 2000 rpm, Biomixer VTX-2500, São Paulo, Brasil). Todos os sobrenadantes foram combinados e purificados em cartuchos C-18 conforme Rodriguez-Saona e Wrolstad, (2001) modificado por Bochi et al., (2015).

*3.1.2.1 Identificação e quantificação de compostos fenólicos livres e ligados à matriz da casca da jabuticaba por HPLC-ESI-MS/MS*

A identificação de compostos fenólicos antociânicos e não antociânico foram realizadas em dois equipamentos: um sistema de HPLC conectado a um espectrômetro de massas com armadilha de íons equipado com uma fonte de ionização por eletrospray (ESI) (Esquire 6000, Bruker Daltonics, Billerica, MA, EUA). A análise foi realizada em modo iônico positivo para identificação de antocianinas e sob modo de iônico negativo para a identificação dos demais compostos fenólicos. As seguintes configurações foram usadas: voltagem capilar a 4500 V (positivo e negativo), temperatura do gás a 310 ° C, fluxo de gás de 11 L/min e gás nebulizador a 30 psi. Os experimentos de $MS^2$ foram realizados em uma faixa de varredura completa de 100 a 1800 m/z de todos os fragmentos, formados a partir de três íons principais. Também foi analisado em espectro de massa com analisador Q-TOF e fonte de ionização por eletrospray (ESI) (Bruker Daltonics, modelo micrOTOF-QIII, Bremen, Alemanha). Os parâmetros MS foram ajustados como segue: modo ion negativo, voltagem capilar a -4000 V, temperatura de gás a 310 ° C, gás nebulizador a 29 psi, vazão de 8 L/min. A identificação de todos os compostos fenólicos foi baseada no tempo de retenção e ordem de eluição na coluna de fase reversa, absorção máxima (UV-vis) e características do espectro de MS em comparação com os padrões analisados nas mesmas condições.

*3.1.2.2 Validação de método e quantificação de compostos fenólicos não antociânicos*

Compostos fenólicos livres extraídos dos PCJs e compostos fenólicos ligados à matriz foram analisados usando HPLC CBM-20A (Shimadzu, Kyoto, Japão) equipado com desgaseificador (DGU20A5, Shimadzu, Japão), forno de coluna (CTO-20A, Shimadzu, Japão) e acoplado a um detector DAD (SPDM-20A, Shimadzu, Japão). A separação foi realizada em coluna Hypersil Gold de fase reversa C-18 (5 µm de tamanho de partícula, 150 mm, 4,6 mm) a 38 °C. O volume de injeção foi de 20 µL e as fases móveis foram compostas por 5% (v/v) de metanol em água acidificada (0,1% (v/v) de ácido fórmico) como solvente (A) e de solução de acetonitrila acidificada com 0,1% (v/v) de ácido fórmico como solvente (B). Foi utilizado gradiente estabelecido como segue: 4% de B de 0 a 10 min; 4% B foi mantido até 21 min; 16% de B de 21,1 a 55 min; 50% de B de 55,1 a 70 min; 100% B de 70,1 a 72 min; 100% B foi mantido até 80 min; 0% B de 80,1 a 83 min e foi mantido até 92,1 min a uma taxa de fluxo de 1 mL/min.

Os espectros de absorção foram registados de 200 a 800 nm e os compostos fenólicos das amostras foram identificados por comparação com o tempo de retenção dos padrões

autênticos e os dados espectrais obtidos a partir dos espectros de absorção de UV-visível e espectros de massa. Para todos os compostos que não foram possíveis comparações com padrões autênticos, foi proposta uma tentativa de identificação baseada na ordem de eluição em cromatografia de fase reversa C-18, características espectrais UV/visível e padrão de fragmentação em análise de espectrometria de massa.

Os cromatogramas para fins de quantificação foram obtidos a 280 nm, para hidroxibenzoatos ou taninos, a 320 nm para derivados hidroxicinamicos e a 360 nm para flavonols. Os compostos que são derivados de um dos monômeros padrão foram quantificados por equivalência e os resultados foram expressos como mg/100 g de peso seco da amostra.

As condições de quantificação foram validadas como preconizado na Conferência Internacional sobre Diretrizes de Harmonização (ICH, 2005). Assim, a linearidade, exatidão, limite de detecção (LOD) e o limite de quantificação (LOQ) foram determinados para os treze padrões (ácido gálico, ácido protocatecuico, ácido 4-hidroxibenzóico, catequina, ácido cafeico, ácido vanílico, ácido siringico, ácido p-coumárico, ácido trans-ferúlico, ácido trans-cinâmico, ácido sinápico, miricetina e kaempferol-3Bd-glucopiranosídeo) em metanol. O desvio padrão residual da regressão linear (r) e a inclinação (m) das curvas de calibração foram usados para calcular os limites de quantificação (LOQ) e detecção (LOD). A precisão do método foi avaliada através da avaliação da repetibilidade (intra-dia) usando todos os padrões de compostos fenólicos em três repetições independentes no mesmo dia em três níveis (n = 3) baixa (LOQ), média (30 ppm) e alta concentração (60 ppm). A precisão intermediária (inter-dia) foi avaliada pelo mesmo analista em dez dias diferentes (n = 10) na concentração de 25 ppm para cada composto. A precisão do método foi expressa como o coeficiente de variação (CV) do tempo de retenção e valores da área de picos. Além disso, foram realizados testes de recuperação dos compostos fenólicos após a fortificação da amostra com três padrões (ácido siríngico, kaempferol-3βD-glucopiranosideo e ácido p-coumárico) em três diferentes concentrações correspondentes a 80, 100 ou 120% da concentração encontrada antes da fortificação. A precisão foi analisada após a contaminação da amostra, em triplicata, e a recuperação média foi determinada por comparação com a amostra não fortificada.

*3.1.2.3 Quantificação de antocianinas*

Para a quantificação de antocianinas foram injetadas amostras (20 µL) em uma coluna de fase reversa C-18 da coluna Core-Shell Kinetex (2,6 µm de tamanho de partícula, 100 mm, 4,6 mm) a 38 ° C. As fases móveis foram: solvente (A) uma solução de 3% de ácido fórmico em água (v/v) e solvente (B) 100% acetonitrila (J.T. Baker® ACS) a um fluxo de 0,9 mL/min. A separação foi realizada usando um gradiente linear de 0% a 8% B em 5 min, 20% de B foi mantido até 15 min. No final do gradiente, a coluna foi lavada aumentando B para 90%, mantendo-a durante 7 min e equilibrando com o condionamento inicial durante 7 min. Os cromatogramas foram obtidos a 520 nm para propósito de quantificação. Curvas de calibração variando de 0,022 a 100 mg/L foram realizadas em dias diferentes (n = 3) para quantificar cada antocianinas como mg de equivalentes de cianidina-3-glicosídeo/100 g de peso da amostra seca.

### 3.1.3 Análise estatística

A quantificação do conteúdo fenólico livre e ligados à matriz foram analisados utilizando três amostras independentes e os valores foram apresentados em média ± desvio padrão. As médias obtidas para cada composto fenólico identificado foram comparadas pelo teste de Student’s, com nível de confiança de 95%. Para as curvas padrão (n = 3) foram realizadas regressão simples e os modelos foram avaliados quanto à falta de ajuste pelo método dos mínimos quadrados utilizando software Statistica (versão 9.0, StatSoft Inc., Tulsa, OK).

## 3.2 BIOACESSIBILIDADE DOS COMPOSTOS FENÓLICOS DO PCJ

### 3.2.1 Preparo da jabuticaba

O pó da casca de jabuticaba da espécie *Myrciaria trunciflora* foi utilizado para o estudo. Os procedimentos para obtenção do PCJ foram os mesmos descritos no experimento 2.

### 3.2.2 Simulação da digestão gastrointestinal *in vitro* e quantificação dos compostos bioativos

A simulação da digestão ocorreu em 3 fases sequenciais: (1) simulação da digestão na boca, (2) no estômago (digestão gástrica) e (3) no intestino delgado (digestão duodenal) conforme padronização internacional de digestão *in vitro* (MINEKUS et al., 2014). Amostra de PCJ (5 g) foram usadas para simular as diferentes etapas da digestão humana. A etapa da boca (volume final de 10 mL) foi simulado usando saliva artificial (pH = 7,5) e concentração padronizada da enzima α-amilase (Sigma Aldrich, 75 U/mL) incubada a 37ºC por 2 min sob agitação. Após a etapa da boca, o pH do digerido foi ajustado para 3,0 pela adição da solução gástrica artificial. Não houve necessidade de usar HCl adicional para ajustar o pH. O digerido foi incubado (37ºC por 2 h sob agitação) sob condições gástricas pela adição de pepsina (2000 U / mL) e água para ajustar o volume final para 20 mL. Posteriormente, o pH do digerido gástrico foi ajustado para 7,0 com NaOH 1M e o digerido foi submetido à digestão intestinal. Pancreatina (100 U/mL) e sais biliares (10 mM) foram adicionados e o volume foi ajustado para 50 mL com água. O digerido foi colocado dentro da membrana de diálise (12000 Da, Sigma Aldrich, Brasil) que foi imerso em 500 mL de tampão fosfato (24,96 mM, pH 7,2) e incubado a 37ºC por 2 h sob agitação esporádica. Duas frações foram coletadas após a digestão intestinal, IN e OUT. A fração IN foi a que permanece dentro da membrana de diálise e corresponde ao digerido que permanece no intestino delgado e grosso, enquanto a fração OUT foi a fração capaz de atravessar a membrana de diálise e representa a fração bioacessível do PCJ.

#### *3.2.2.1 Extração dos compostos fenólicos*

As amostras de todas as etapas da simulação gastrointestinal foram congeladas com nitrogênio líquido e, na sequência, estocadas -80ºC até o procedimento de extração. A extração dos compostos fenólicos foi realizada conforme metodologia descrita para compostos fenólicos livres no Manuscrito 1 para as seguintes etapas de digestão gastrointestinal (boca, gástrica e intestino IN). Os extratos exaustivos obtidos nas etapas de digestão oral e gástrica foram concentrados em evaporador rotatório (Büchi, Alemanha), a 38ºC, por aproximadamente 10 min e ressuspendidos em volume conhecido (2 mL). Em seguida, 1 mL do extrato evaporado foi purificado usando o cartucho de SPE (cartucho SPE-C18, Strata C18-E, Phenomenex) conforme procedimento anteriormente descrito para o Manuscrito 1.

A fração intestinal IN foi centrifugada a 1559.6 *x g* durante 10 min e o sobrenadante (1 mL) foi imediatamente purificado por SPE como descrito no Manuscrito 1. O sedimento da porção IN foi extraído com metanol: água: ácido fórmico (85: 15: 0,5, v/v/v) conforme Manuscrito 1, o solvente foi evaporado e purificado por SPE. A quantidade de compostos fenólicos na fração IN foi calculada como a soma dos polifenóis encontrados no sobrenadante e no extrato do sedimento. Vinte e quatro mililitros da fração intestinal OUT foram submetidos a purificação em SPE como descrito no Manuscrito 1.

*3.2.2.2 Análise dos compostos fenólicos por HPLC-DAD-MS/MS*

Foram analisados os compostos fenólicos antociânicos e não antociânicos utilizando HPLC-DAD-Q-TOF-MS/MS conforme descrito no Manuscrito 1. Além disso o mesmo método validado foi utilizado para a quantificação e identificação dos compostos fenólicos em diferentes etapas da digestão.

### 3.2.3 Índice de bioacessibilidade

A bioacessibilidade foi calculada com base nos compostos fenólicos liberados da matriz do PCJ após digestão gastrintestinal *in vitro* e encontrada na fração OUT, usando a seguinte equação:

Bioacessibilidade (%) = (compostos fenólicos OUT / compostos fenólicos PCJ) * 100

### 3.2.4 Índice de recuperação

A porcentagem de recuperação é a quantidade de compostos fenólicos encontrados no digerido após digestão completa (frações OUT + IN) em relação aos compostos fenólicos, presentes no PCJ íntegro, calculada pela seguinte equação:

Índice de recuperação (%) = [(compostos fenólicos OUT + compostos fenólicos IN) / compostos fenólicos PCJ] * 100

### 3.2.5 Índice de digesta intestinal residual

Os compostos fenólicos residuais do digerido intestinal (RID%) são a fração de compostos fenólicos que permanecem intactas após a simulação da digestão oral, gástrica e intestinal, mas não são bioacessíveis. Essa fração representa os compostos fenólicos que podem atingir o cólon, calculada pela seguinte equação:

Índice de digesta intestinal residual (%) = [IN compostos fenólicos / compostos fenólicos PCJ] * 100

### 3.2.6 Capacidade antioxidante

A capacidade antioxidante foi avaliada pelo ensaio de capacidade de remoção de radicais livres de oxigênio (ORAC) (OU; HAMPSCH-WOODILL; PRIOR, 2001). Os extratos de antociânicos e de compostos fenólicos não antociânicos obtidos do PCJ (não digerido) e de cada fase das etapas digestiva [oral, gástrica e intestinal (IN e OUT)] foram analisados.

### 3.2.7 Análise estatística

Os dados foram expressos como médias ± erro padrão de quatro repetições para cada etapa de digestão. As percentagens de variação foram calculadas em relação ao PCJ não digerida. Os dados de capacidade antioxidante foram analisados por análise fatorial (5 fases de digestão x 2 frações de compostos antociânicos e não antociânicos) seguida de teste de Duncan quando apropriado. A análise de componentes principais (PCA) foi utilizada para resumir as mudanças na concentração dos metabólitos compostos fenólicos digeridos, e foi realizada com base em quatro repetições em cada fase de digestão usando programa Statistic versão 7 (StatSoft Inc., Tulsa, OK, EUA).

## 3.3 FERMENTAÇÃO UTILIZANDO MICROBIOTA HUMANA

Foi avaliada a metabolização dos compostos fenólicos do PCJ pela microbiota intestinal humana *in vitro* após simulação pela passagem gastrointestinal, investigando o envolvimento da composição da microbiota intestinal de voluntários considerados saudáveis.

O estudo foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal de Santa Maria (UFSM) sob o número de parecer 1.348.232.

### 3.3.1 Desenho do estudo

Para o desenvolvimento da pesquisa foi necessária a adesão de 20 voluntários de ambos os sexos, com idade entre 20-55 anos, saudáveis. O recrutamento dos voluntários saudáveis ocorreu entre servidores e alunos da UFSM nas dependências do Departamento de Tecnologia e Ciência dos Alimentos. No primeiro encontro os indivíduos receberam informações sobre os objetivos e risco da pesquisa e foram convidados a participar do estudo com a assinatura do Termo de Consentimento Livre e Esclarecido (TCLE) (Anexo A).

No segundo encontro com agendamento prévio os voluntários foram convidados a permanecer em jejum de 12 h e disponibilizar uma amostra de sangue que foi coletada por flebotomia do membro superior para realização dos exames laboratoriais (perfil lipídico, glicemia de jejum, uréia, creatinina e transaminases hepáticas) além da verificação da pressão sanguínea e peso corporal. A coleta do sangue foi realizada nas dependências do Laboratório NIDAL-UFSM. As análises foram custeadas pelo projeto, apenas os custos com deslocamento/transporte ficaram por conta dos voluntários.

Após adesão voluntária foi realizada a triagem inicial, na qual foram selecionados os voluntários após análise do questionário aplicado (Anexo B) juntamente com os exames e dados clínicos. No terceiro encontro os voluntários foram convidados a disponibilizar uma amostra de fezes fresca sólida, que foi coletada pelo próprio voluntário em sua residência para a condução do experimento de *in vitro*. Para a coleta das amostras de fezes os voluntários receberam recipientes estéreis adequados sem custo algum.

#### *3.3.1.2 Critérios de inclusão e exclusão*

Foram incluídos no estudo sujeitos de ambos os sexos, com idade entre 20-55 anos, sem nenhuma doença diagnosticada e foram excluídos do estudo voluntários que fizeram uso de qualquer antibiótico nos 6 meses anteriores a pesquisa, tabagistas, etilistas ou portadores de doenças intestinais crônicas ou agudas (diarreia) e portadores de doenças infecto contagiosas. Além disso, os voluntários que aderiram a pesquisa foram orientados a evitar o consumo de

qualquer bebida alcoólica e comida ricas em antocianinas (frutas e legumes com coloração vermelha e roxa) nas 48 h que antecedem a coleta de fezes.

### 3.3.2 Preparo da jabuticaba

As jabuticabas utilizadas foram da espécie *M. trunciflora* e o preparo do PCJ foi previamente descrito no Manuscrito 1.

### 3.3.3 Simulação da digestão gastrointestinal *in vitro*

A simulação *in vitro* da digestão gastrointestinal foi realizada de acordo com um procedimento padronizado (MINEKUS et al., 2014) com detalhes descritos anteriormente no Manuscrito 2. O PCJ (5,0 g) foi submetido a três etapas para mimetizar o processo digestivo: digestão oral, gástrica e intestinal (intestino delgado). A digestão intestinal foi realizada simultaneamente em um sistema de diálise, cuja fração IN, que permaneceu dentro do tubo de diálise, foi considerada aquela fração não absorvível que chega no cólon.

O procedimento foi repetido dez vezes para obter a quantidade necessária para desenvolver a fermentação. Toda a fração IN foi imediatamente congelada com $N_2$ líquido, liofilizada e pulverizada para fermentação colônica *in vitro*. O PCJ digerido que permaneceu no tubo de diálise (IN) após liofilização foi denominada de PCJ-IN.

### 3.3.4 Fermentação *in vitro* pela microbiota de fezes humanas

Os ensaios foram realizados utilizando um *pool* de amostras de fezes frescas fornecidas por quatro voluntários saudáveis (20 - 55 anos), de ambos os sexos. A cinética de fermentação da PCJ-IN foi avaliada pela técnica de produção de gases *in vitro*. A suspensão fecal foi preparada utilizando o tampão carbonato-fosfato anaeróbio, pH 6,5 (DURAND et al., 1988) na proporção de 0,5 g de amostras fecais e 10 mL de tampão (0,5: 10 w/v) (MOSELE et al., 2015). Após a homogeneização, a suspensão fecal foi filtrada sob condições anaeróbicas e distribuída em frascos (50 mL) com adição ou não de 0,5 g do digerido PCJ-IN. Dois controles foram conduzidos em paralelo: (1) Incubação de PCJ-IN com solução tampão sem fezes para determinar a possível degradação química e (2) Incubação da suspensão fecal sem PCJ-IN como um controle negativo. Para a fermentação foi utilizado o equipamento Ankom RF Gas Production System (Ankom Technology) a 37ºC em anaerobiose com

diferentes tempos de incubação 0, 2, 8, 24 e 48 horas. O equipamento Ankom utilizado consiste em 15 módulos automatizados de fermentação individual, acoplados a jarros apropriados para acomodação do material a ser fermentado e manutenção da anaerobiose, permite ainda o registro da temperatura e pressão cumulativa de cada módulo (a cada hora de incubação) com a finalidade de estimar a produção de gases.

Todas as incubações foram repetidas cinco vezes (n = 5) e foram utilizados um jarro para cada tempo de fermentação, no qual foram recolhidos uma alíquota de todos os tempos para determinados o pH. E somente para as incubações 0 e 48h foram separadas uma alíquota para análise de contagem microbiana. Na sequência, todo o conteúdo de cada jarro foi centrifugado a 1358,6 *x g* durante 10 min e o sobrenadante foi congelado por imersão em $N_2$ líquido e, em seguida, armazenado a -20 °C até a análise dos compostos fenólicos, amônia e AGCC.

A pressão de gás foi mensurada em intervalos de 1 h durante 48 h de incubação, totalizando n = 49 leituras por curva. As leituras de pressão (psi) foram convertidas para moles de gases usando a lei do gás 'ideal', e então para mililitros (mL) de gases através da Lei de Avogadro. Após, os resultados foram expressos em mililitro de gases produzidos por grama de matéria orgânica incubada. As curvas de produção cumulativa de gases observadas *in vitro* foram ajustadas pelo modelo logístico bicompartimental (SCHOFIELD; PITT; PELL, 1994), dado por:

$$V = \frac{V_1}{1 + e^{[2-4k_1(t-\lambda)]}} + \frac{V_2}{1 + e^{[2-4k_2(t-\lambda)]}} + \varepsilon$$

em que, $V$ = volume de gas (mL $g^{-1}$ MO incubada) no tempo $t$; $V_1$ = volume máximo de gás produzido pela degradação da fração solúvel de rápida digestão; $V_2$ = volume máximo de gás produzido pela degradação da fração insolúvel potencialmente degradável de lenta digestão; $V_T$ ($V_1 + V_2$) = volume total de gases produzidos; $k_1$ = taxa específica de produção de gás pela degradação da fração solúvel de rápida digestão; $k_2$ = taxa específica de produção de gás pela degradação da fração insolúvel potencialmente degradável de lenta digestão; $t$ = tempo de incubação ou degradação (fermentação); $e$ = exponencial; $\lambda$ = fase de latência ou período de hidratação, aderência e colonização microbiana (*lag time*); $\varepsilon$ = erro experimental associado a cada observação, suposto ~ NIID $(0; \sigma^2)$. Os parâmetros do modelo considerado foram estimados pelo algoritmo de Gauss-Newton modificado com o procedimento NLIN. O

coeficiente de determinação ($r^2$) foi expresso em relação à fonte tratamentos (regressão + falta de ajuste).

### 3.3.5 Quantificação dos compostos fenólicos

#### *3.3.5.1 Extração dos compostos fenólicos*

Os compostos fenólicos das amostras (6 mL) foram extraídos usando solução acidificada de acetona (0,35% de ácido fórmico, v/v; 7mL), para precipitação de impurezas. Após, foram homogeneizados por 1 min em agitador tipo vórtex, centrifugados 1083,1 *x g*, durante 10 min. O sobrenadante foi coletado e o solvente orgânico removido em evaporador rotatório (38 ± 2ºC) e todo o extrato (± 6mL) foi purificado usando cartuchos de extração em fase sólida (cartuchos SPE-C18, Strata C18-E, Phenomenex). O procedimento de purificação foi realizado conforme descrito anteriormente por Rodriguez-Saona e Wrolstad, (2001) com as modificações descritas por Bochi et al., (2015). Os fenólicos não antociânicos foram recolhidos usando solvente acetato de etila (grau de 99%) e por fim, as antocianinas foram recuperadas com solução de metanol acidificada (0,35% de ácido fórmico v/v). O acetato de etila foi evaporado em evaporador rotativo (38 ± 2ºC) e os compostos fenólicos não antociânicos foram recuperados em 1,0 mL com solução contendo 10% de metanol em água acidificada (0,1% de ácido fórmico, v/v). O metanol foi evaporado em evaporado rotativo (38 ± 2ºC) e as antocianinas foram recuperadas com 1 mL de água acidificada (0,35% de ácido fórmico, v / v).

#### *3.3.5.2 Identificação e quantificação dos compostos fenólicos*

Após a extração e purificação dos compostos fenólicos em SPE os extratos foram analisados em HPLC-DAD-Q-TOF-MS/MS utilizando os mesmos métodos e parâmetros anteriormente descritos no Manuscrito 1 tanto para compostos fenólicos não antociânicos como para as antocianinas.

### 3.3.6 Determinação de amônia

A amônia foi determinada no sobrenadante do fermentado de cada jarro utilizando solução de fenol e nitroprussiato de sódio (10:0,05 m/m) e solução de hidróxido de sódio e hipoclorito de sódio (2,5: 1,7 m /v) que catalisaram a reação de indofenol (CHANEY; MARBACH, 1962). O cloreto de amônio foi utilizado como padrão (0,03-0,15 umol) e os resultados foram expressos como umol $NH_4$/50mL. As amostras foram analisadas em duplicata.

### 3.3.7 Análise do potencial de hidrogenação (pH)

O pH foi determinado imediatamente após o término de cada tempo de fermentação com o potenciômetro digital PHOX P1000 (PHOX Equipamentos Científicos, Colombo, Paraná, Brasil).

### 3.3.8 Contagens microbiológicas

A contagem de bactérias foi realizada no fermentado (0 e 48h) usando diluições seriadas com água peptona (0,1 g/100 mL). A contagem de *Bifidobacterium* foi determinada em ágar MRS enriquecido com cloreto de lítio (0,1%) e l-cisteína (0,05%) (HANSEN, 1999) de acordo com as recomendações do fabricante, utilizando o método semeadura em profundidade. Enquanto as contagens de *Lactobacillus* e *Enterobacteria* foram determinadas em ágar MRS e Ágar Violet Red Bile Dextrose, respectivamente, usando o método de semeadura em profundidade.

As placas foram incubadas sob condições anaeróbias em frascos anaeróbios com o sistema Anaerobac (Probac, São Paulo, Brasil) a 37 °C por 72 h. Após o período de incubação, a células viáveis foram enumeradas e os resultados expressos em log CFU/mL.

### 3.3.9 Análise de ácidos graxos de cadeia curta

Os ácidos graxos de cadeia curta foram determinados em todos os tempos de fermentação, utilizando cromatógrafo gasoso Agilent Technologies (HP 6890 N) equipado com uma coluna capilar Nukol ™ (30 m x 0,25 mm; 0,25 µm Supelco, Bellefonte, PA, EUA) e detector de ionização de chama (FID) (ZHAO; NYMAN; JÖNSSON, 2006). As condições cromatográficas foram as seguintes: temperatura do injetor e do detector ajustada a 250 ºC,

volume injetado de 1 µL com razão de separação ajustada para 1:10, e o gás transportador foi nitrogênio a 1 mL/min. O forno foi programado para ser mantido a 100 ºC por 0,5 min, após aquecido a 8 ºC /min até 180 ºC, mantido por 1 min, aquecido por 20 ºC/min até 200 ºC, e mantido por 5 min. A temperatura do FID foi de 240ºC e a porta de injeção foi de 200ºC. Para a preparação das amostras foi necessário ajustar o pH para 2-3 utilizando a solução aquosa de HCl 2N. Após a amostra foi centrifugada a 1700 *x g* por 20min. O padrão interno foi adicionado no sobrenadante da amostra para atingir uma concentração final de 1 mM. Uma mistura padrão de ácidos graxos livres voláteis (46975-U, Sigma-Aldrich, St. Louis, MO, EUA) foi diluída em solução aquosa ácida (ácido fórmico 12%) e usada como padrão externo.

### 3.3.10 Análise estatística

Os dados foram expressos como médias ± erro padrão de cinco repetições para cada fermentação. As porcentagens de mudança foram calculadas em relação ao PCJ digerido liofilizado (PCJ-IN). Os ácidos graxos de cadeia curta (conteúdo de AGCC pela fermentação da PCJ-IN descontado o conteúdo de AGCC do Controle 2, no mesmo tempo de fermentação), conteúdo de amônia e pH foram analisados por ANOVA de uma via seguida pelo teste de Duncan. A contagem microbiana foi realizada utilizando ANOVA fatorial (2 tempos x 2 condições de fermentação com ou sem PCJ-IN) seguido de teste de Duncan utilizando programa Statistic versão 7 (StatSoft Inc., Tulsa, OK, EUA). A produção de gases foi analisada utilizando SAS® System for Windows ™ versão 9.4 (SAS Institute Inc., Cary-NC, EUA) ao nível de 5% de significância.

## 3.4 INDUÇÃO DO DIABETES E TRATAMENTO COM PCJ (*M. jaboticaba*)

O protocolo de estudo foi conduzido segundo as diretrizes do Conselho Nacional de Controle de Experiemntação Animal e aprovado pela Comissão de Ética no Uso de Animais (CEUA) sob o número 086/2013.

### 3.4.1 Preparo do pó da casca de jabuticaba

Para a preparação do PCJ foram utilizadas jabuticabas da espécie *Myrciaria jaboticaba* (Vell) Berg. cultivadas em Casa Branca, São Paulo, Brasil no ano de 2012. As

jabuticabas foram lavadas e descascadas manualmente e a casca congelada a -18°C. As cascas foram secas em liofilizador (Terroni, São Paulo, Brasil), trituradas usando um micro triturador analítico (Marconi, MA-630, São Paulo, Brasil) e armazenadas a -80°C.

### 3.4.2 Composição das dietas e do pó da casca de jabuticaba

Umidade, cinzas, proteína bruta, fibra alimentar solúvel e insolúvel foram determinados conforme AOAC, (2005). Os lipídios totais serão extraídos usando clorofórmio e metanol e quantificados por gravimetria (BLIGH; DYER, 1959). Os carboidratos não fibrosos foram determinados por diferença matemática.

### 3.4.3 Quantificação dos compostos bioativos do PCJ

#### *3.4.3.1 Extração e quantificação dos compostos fenólicos ligados do PCJ*

Os polifenóis da casca de jabuticaba liofilizada foram extraídos de forma exaustiva conforme Wu et al., (2004) com algumas modificações. Após, o extrato bruto de PCJ foi seco em evaporador rotatório e ressuspenso em volume conhecido para análise de antocianinas monoméricas totais utilizando a metodologia de pH diferencial (GIUSTI; WROLSTAD, 2001) e o teor de polifenóis totais pelo método colorimétrico de Folin-Ciocalteau (SINGLETON; ROSSI, 1965). Também foram quantificados os taninos condensados livres utilizando 4-dimetilaminocinamaldeído e curva de calibração de catequina (WALLACE; GIUSTI, 2010).

#### *3.4.3.2 Quantificação espectrofotométrica de compostos fenólicos ligados da casca de jabuticaba*

O resíduo da extração do compotos fenólicos livres do PCJ foi seco em estufa com circulação de ar a 35 ºC para posterior extração dos taninos condensados ligados através de tratamento químico com butanol/HCl/Fe por 60 min a 100 ºC (PORTER; HRSTICH; CHAN, 1986). A quantificação espectrofotométrica foi realizada utilizando curva de calibração de taninos condensados (ZURITA; DÍAZ-RUBIO; SAURA-CALIXTO, 2012). Na sequência

foram extraídos do resíduo (após evaporação do solvente a frio) os taninos hidrolisáveis após reação com metanol e ácido sulfúrico durante 20 h a 85ºC. A quantificação foi realizada após reação com iodeto de potássio a 525 nm (HARTZFELD et al., 2002).

*3.4.3.3 Quantificação espectrofotométrica de carotenóides*

Carotenóides foram exaustivamente extraídos do PCJ (0,5 g) com acetona, transferido para mistura éter de petróleo / dietil éter (1: 1, v/v), e o solvente foi evaporado sob fluxo de $N_2$ a temperatura ambiente. A amostra foi reconstituída em éter de petróleo para avaliar a concentração total de carotenóides utilizando o coeficiente de absorção de β-caroteno (A 1 cm 1% = 2396) (RODRIGUEZ-AMAYA, 2001).

### 3.4.4 Modelo de diabetes tipo 2 induzido em ratos

*3.4.4.1 Acondicionamento dos animais e dietas antes e após a indução de diabetes*

Foram utilizados ratos machos Wistar com peso entre 180 e 200 gramas provenientes do Biotério Central da UFSM. Os animais ficaram em gaiolas, sob um ciclo de claro/escuro de 12:12 h com acesso livre à água. O consumo de ração foi controlado (ofertado 30 g ração/dia/rato) durante todo o período experimental, onde os grupos controle não diabéticos receberam ração comercial convencional (Nuvital- Quimtia S/A, Colombo, PR) por 30 dias antes da administração de veículo (Tampão citrato), enquanto grupos diabéticos receberam uma ração hipercalórica (incorporação de 16% de banha suína e 10% de sacarose aos pellets da ração comercial convencional para ratos Nuvital) por 30 dias antes da administração com estreptozotocina (STZ) para indução de DM tipo 2. Estes dois grupos de animais continuaram recebendo as mesmas rações durante todo o período experimental, após a administração da STZ ou veículo.

*3.4.4.2 Indução do diabetes*

Considerando que cerca de 70% dos animais tratados com STZ desenvolvem hiperglicemia e diabetes, para obter os 32 animais diabéticos foram necessários 46 ratos

Wistar machos adultos para indução do diabetes. Além disso, foram utilizados 8 animais controle não diabéticos, totalizando 54 animais em todo o experimento. Nos 30 dias que antecedem a administração de STZ (Sigma-Aldrich), os animais foram alojados em gaiolas de polietileno com 4 animais cada, onde receberam dieta hipercalórica e água à vontade. Após permanecerem em jejum por 8 h, foi administrada estreptozotocina (diluída em tampão citrato 0,1 M, pH 4,4) na dose única de 35 mg/1mL/kg, via intraperitoneal (SRINIVASAN et al., 2005). Após uma semana, foi coletada uma gota de sangue da veia caudal, com lanceta, dos animais sob jejum de 8h, para avaliação da glicemia. Somente os animais com glicemia de jejum superior a 250 mg/dL foram considerados diabéticos tipo 2 e selecionados para o experimento.

*3.4.4.3 Tratamento*

Uma semana após a administração de STZ, os animais considerados diabéticos foram divididos em 6 grupos experimentais (n=8 por grupo) para tratamento com PCJ (*Myrciaria jaboticaba*) e veículo (solução de água com 0,5% de carboximetilcelulose). O PCJ ou veículo foram administrados na água de beber durante 60 dias. Durante a fase de tratamento o peso dos animais foi controlado a cada 3 dias e o consumo de PCJ ou veículo substituídos na água de beber foram controlados diariamente para estabelecer a dose diária de consumo por kg de peso corporal dos animais. Os grupos experimentais:

-**Grupo controle**: ratos não diabéticos, alimentados com ração balanceada e tratados com veículo na água de beber.
-**Grupo controle diabético**: ratos diabéticos, alimentados com ração hipercalórica e tratados com veículo na água de beber.
-**Grupo diabético + 2,7 g PCJ/L de água (PCJ-I)**: ratos diabéticos, alimentados com ração hipercalórica e tratados com jabuticaba na água de beber na concentração de 0,038 g de antocianina presente no PCJ/L de solução veículo.
-**Grupo diabético + 4,5 g PCJ /L de água (PCJ-II)**: ratos diabéticos, alimentados com ração hipercalórica e tratados com jabuticaba na água de beber na concentração de 0,076 g de antocianina presente no PCJ/L de solução de solução veículo.

**-Grupo diabético + 10,8 g PCJ /L de água (PCJ-III)**: ratos diabéticos, alimentados com ração hipercalórica e tratados com jabuticaba na água de beber na concentração de 0,152 g de antocianina presente no PCJ/L de solução veículo.

Depois de decorrido o período experimental do tratamento, os animais ficaram em jejum de 8 h para a coleta de uma gota de sangue da veia caudal, com lanceta, com a finalidade de verificar a glicemia. Imediatamente após os animais foram anestesiados com anestésico inalatório isoflurano (aplicado com vaporizador de cone nasal), o sangue foi coletado por punção cardíaca e após os animais foram sacrificados por exsanguinação. O sangue foi coletado em tubos sem anticoagulante para obtenção do soro. Uma porção do fígado foi homogeneizada em tampão fosfato (PBS), pH 7,4 para determinação da oxidação lipídica e proteica. Uma alíquota do homogeneizado foi centrifugada para obtenção do sobrenadante para a determinação da atividade das enzimas antioxidantes e os níveis de tióis não proteicos (SHNP).

### 3.4.5 Análises de marcadores inflamatórios, antioxidantes e bioquímicos

#### *3.4.5.1 Indicadores de estresse oxidativo*

A lipoperoxidação (OHKAWA; OHISHI; YAGI, 1979), oxidação proteica (LEVINE; OLIVER, 1990) foram determinadas no homogeneizado do tecido hepático. Os níveis de glutationa reduzida (GSH) e oxidada (GSSG) foram medidos pelo método fluorimétrico usando O-ftalaldeído (HISSIN; HILF, 1976). Os níveis de SHNP foram determinados conforme Ellman, (1959). No sobrenadante do homogeneizado do fígado foram determinadas as atividades das enzimas glutationa redutase (GR) (CARLBERG; MANNERVIK, 1979), glutationa S-transferase (GST) (HABIG; PABST; JAKOBY, 1974) catalase (CAT) (AEBI, 1984), superóxido dismutase (SOD) (MISRA; FRIDOVICH, 1972), tiorredoxina redutase (TrxR-1) (LUTHMAN; HOLMGREN, 1982) e delta-aminolevulinato desidratase (ALA-D) (SASSA, 1982).

#### *3.4.5.2 Análises bioquímicas*

As análises bioquímicas de glicose foram determinadas por glicosímetro (Roche) e os níveis de triglicerídeos, colesterol total, colesterol HDL, colesterol LDL direto e atividade das

enzimas aspartato aminotrasferase (ALT) e alanina aminotrasferase (AST) séricas foram realizadas por kit Doles ® (Goiânia, GO, Brasil). Além disso, os níveis de lipoproteína de muito baixa densidade (VLDL) foram calculados (Triglicerideos/5). A insulina sérica foi quantificada por radioimunoensaio. O índice de sensibilidade à glicose (QUICKI) (KATZ et al., 2000) e o índice de resistência à insulina (FIRI) foram calculados (DUNCAN et al., 1995):

QUICKI= 1/[LOG (insulina de jejum) + LOG (glicemia de jejum)]

FIRI = [insulina de jejum (mU/L) + glicemia de jejum (mmol/L)]/25

*3.1.5.3 Análise quantitativa de PCR em tempo real*

A expressão gênica de enzimas envolvendo a síntese de glutationa [Glutationa sintase (GS), Glutamato-cisteína ligase subunidade catalítica (GCLcs)] e marcadores de apoptose (Caspase 3 e Caspase 9) foram determinadas após a extração do RNA utilizando Trizol, seguindo as instruções do fabricante (Ludwing-Biotec, Brazil) e conforme metodologia descrita por Barbisan et al., (2014), no qual o gene da β-actina foi usado como *housekeeping* e os níveis de expressão foram usados como controle interno.

### 3.4.6 Análise estatística

Os dados foram analisados usando análise de variância de uma via (ANOVA) seguida do teste de múltiplas escolhas *post hoc* de Duncan ou teste de Dunnett (para dados de expressão gênica), quando necessário. Os dados que não atenderam às premissas da ANOVA foram submetidos à análise não paramétrica de Kruskal-Wallis, seguida de teste de comparações múltiplas. Os resultados foram expressos como a média ± erro padrão e as diferenças foram consideradas estatisticamente significativas quando $p < 0,05$. Os dados foram analisados utilizando o software Statistica® versão7 (StatSoft Inc., Tulsa, OK, EUA).

# 4. DESENVOLVIMENTO

## 4.1 MANUSCRITO 1:

CHARACTERIZATION AND QUANTIFICATION OF TANNINS, FLAVONOLS, ANTHOCYANINS AND MATRIX-BOUND POLYPHENOLS FROM JABOTICABA FRUIT PEEL: A COMPARISON BETWEEN *Myrciaria trunciflora* AND *M. jaboticaba*

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**Characterization and quantification of tannins, flavonols, anthocyanins and matrix-bound polyphenols from jaboticaba fruit peel: a comparison between *Myrciaria trunciflora* and *M. jaboticaba***

Quatrin, A.[1]; Pauletto, R.[1]; Maurer, L. H.[1,2]; Minuzzi, N[1]; Nichelle, S. M.[1]; Carvalho, J. F. C.[3]; Maróstica Junior, M. R.[4], Rodrigues, E.[5]; Bochi, V. C.[6]; Emanuelli, T[1*].

[1]Department of Food Technology and Science, Center of Rural Sciences, Federal University of Santa Maria, 97105-900, Santa Maria, RS, Brazil;

[2]Farroupilha Federal Institute, 97555-000, Alegrete, RS, Brasil;

[3]Farroupilha Federal Institute, 97420-000, São Vicente do Sul, RS, Brasil;

[4]School of Food Engineering, University of Campinas - UNICAMP, 13083-862 Campinas, SP, Brazil;

[5]Department of Food Science, Federal University of Rio Grande do Sul, 91501-970, Porto Alegre, RS, Brazil;

[6]Department of Nutrition, Federal University of Health Sciences of Porto Alegre (UFCSPA), 91501-970, Porto Alegre, RS, Brazil;

*Corresponding author Tel.: +55 55 3220 8547; fax: +55 55 3220 8353. E-mail address: tatiana.emanuelli@ufsm.br (Tatiana Emanuelli).

**Abstract**

Jaboticaba, a polyphenol-rich fruit, has numerous nutraceutical properties. Studies on jaboticaba composition have focused on solvent-extractable polyphenol compounds, whereas the matrix-bound polyphenols that can be delivered during digestion have been neglected. This study aimed to characterize the polyphenol profiling of matrix-bound and free phenolic compounds in Jaboticaba peel powder (JPP) using two fruit species, *Myrciaria jaboticaba* (JPP-MJ) and *M. trunciflora* (JPP-MT). An HPLC-DAD-MS/MS method was developed and validated to analyze JPP polyphenols. The total content of free phenolic compounds was 2.4 times higher in JPP-MT than in JPP-MJ and the profile of free polyphenols differed between the two species. JPP-MT had 60.3% tannins and 33% anthocyanins, whereas JPP-MJ had 34.7% and 50.7%, respectively. The content of matrix-bound phenolic compounds was higher in JPP-MJ than in JPP-MT (15.3% *vs.* 4.3% of total phenolic compounds). Besides cyanidin 3-glucoside, three other compounds were identified as major: a tetragalloylglucose isomer for both species, trigalloylglucose for JPP-MT, and delphinidin-3-glucoside for JPP-MT. Phenolic profiling of JPP revealed new compounds that may contribute to health benefits after JPP consumption. Despite differences in the phenolic profile between the jaboticaba species, both JPPs are rich sources of polyphenols and could be used for promoting health benefits.

**Keywords**: Jaboticaba peel, fruit, phenolic compounds, anthocyanins, HPLC-DAD-Q-TOF-MS/MS, identification/quantification.

## 1. Introduction

Jaboticaba (Myrtaceae family) is a tropical fruit native to Brazil and widely cultivated in the south and southeast of the country. It can be consumed as the fresh fruit or processed to yield juice, jam, and wine (Wu et al., 2013). However, the fruit's peel has the most remarkable potential health benefits and has attracted great attention (Dragano et al., 2013; Leite et al., 2011). The peel of most jaboticaba species, including *Myrciaria jaboticaba* and *M. cauliflora,* has a dark color and a higher content of anthocyanins and other free phenolic compounds such as ellagitannins and gallic acid derivatives when compared to other fruit parts (Alezandro et al., 2013; Inada et al., 2015; Plaza et al., 2016).

Among the different species, *M. cauliflora* (synonym *Plinia cauliflora*) is the most abundant (Wu et al., 2013), whereas *M. jaboticaba* (Vell.) Berg. (synonym *P. jaboticaba* (Vell.) Berg) is the most extensively studied species (Alezandro et al., 2013; Batista et al., 2018; Leite-legatti et al., 2012). *M. trunciflora* (synonym *P. peruviana* or *P. trunciflora*) is distributed in the southern region of Brazil and has been scarcely investigated in relation to its bioactive effects and phenolic compounds composition (Calloni et al., 2015).

Jaboticaba peel powder (JPP) has been shown to have strong antioxidant, anti-inflammatory, hypolipidemic and anti-diabetic effects in murine models of obesity (Batista et al., 2018; Lamas et al., 2018; Lenquiste et al., 2015; Moura et al., 2018). Moreover, JPP was recently named among the superfruits due to its relevant health-promoting effects (Chang et al., 2018) and has been shown to reduce postprandial glucose and insulin levels and increase antioxidant capacity in humans (Plaza et al., 2016). These effects of JPP have been attributed only to free phenolic compounds that are easily extracted by organic solvents. Nevertheless, insoluble or matrix-bound phenolic compounds can be delivered during digestion and they also contribute to the health benefits of several fruits (Pérez-Jiménez et al., 2013). To our knowledge, matrix-bound phenolic compounds have been scarcely investigated in jaboticaba

fruits and quantified only in *M. jaboticaba* without using mass spectrometry to confirm the compound identity (Inada et al., 2015).

Matrix-bound phenolic compounds can be extracted after chemical hydrolysis of bonds between phenolic compounds and cell-matrix constituents (protein and cell-wall constituents like cellulose, hemicellulose and others). While alkaline hydrolysis promotes the disruption of ether and ester bonds, acid hydrolysis promotes the disruption of glycosidic bonds (Acosta-Estrada et al., 2014; Shahidi and Yeo, 2016). Recent research has attributed strong health benefits to matrix-bound phenolic compounds (Pérez-Jiménez et al., 2013), mainly because, part of these compounds can become bioaccessible after the action of digestive enzymes, and the non-absorbed portion can be fermented by colonic microbiota yielding free bioactive compounds (Pérez-Jiménez et al., 2013). These compounds can also modulate the gut microbiota resulting in a healthier profile that could be a potential tool to counteract several chronic diseases related to intestinal dysbioses such as diabetes, obesity and inflammatory bowel diseases (Marchesi et al., 2016; Mosele et al., 2015; Pérez-Jiménez et al., 2013). Several studies have focused on evaluating the biological activity of JPP (Batista et al., 2018; Dragano et al., 2013; Lenquiste et al., 2015) but a limited number of the studies were addressed to elucidating the chemical composition of JPP from different species; in particular, the composition of matrix-bound phenolic compounds has been neglected.

The aim of this study was to provide a comprehensive polyphenol profiling of matrix-bound and free phenolic compounds in the peel of two jaboticaba fruit species, *M. trunciflora* and *M. jaboticaba*, the latter being the most studied for its bioactive properties for human health.

## 2. Materials and methods

## 2.1 Chemicals and standards

4-Hydroxybenzoic acid (99%), caffeic acid (98%), catechin (98%), gallic acid (98%), kaempferol-3βD-glucopyranoside (97%), myricetin (96%), p-coumaric acid (98%), protocatechuic acid (97%), quercetin (95%), sinapic acid (99%), syringic acid (95%), trans-cinnamic acid (99%), trans-ferulic acid (99%), vanillic acid (97%) and cyanidin 3-glucoside (95%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile from J.T. Baker® ACS (Center Valley, PA, USA) was used for the mobile phase. Formic acid (88% w/v) was obtained from J.T. Baker® ACS. All other chemicals used were of analytical grade. HPLC-grade water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Polytetrafluoroethylene syringe filter membrane (PTFE) and cartridges for reversed-phase solid phase extraction (SPE; Strata® C18-E) were from Phenomenex (Torrance, CA, USA).

## 2.2 Samples

Jaboticaba fruits were obtained at the mature turning color maturity state and randomly collected from at least three different trees to yield a representative pooled sample. *M. trunciflora* (MT) fruits (40 kg) were collected in the summer of 2014 in São Vicente do Sul city, Rio Grande do Sul, Brazil. Plants were identified, and a voucher specimen was deposited in the herbarium of the Department of Forestry Sciences (no. HDCF 7228) of UFSM. *M. jaboticaba* (MJ) fruits (40 kg) were collected in the summer of 2012 in Casa Branca city, São Paulo, Brazil. Fruits were washed and manually peeled at room temperature. Peels were immediately frozen at -18 °C, freeze-dried and powdered using an analytical micro grinder (Marconi, MA-630, São Paulo, Brazil) to yield the JPP that was stored at -80 °C until analysis. HPLC analysis was performed 3 months after obtaining JPPs. Due to the difference

in the collection time for both fruit species, *M. jaboticaba* samples were re-analyzed together with *M. trunciflora*, and no change in the polyphenol content was observed.

### 2.3 Free phenolic compound extraction

Free phenolic compounds were recovered from fruit as previously described by Wu et al. (2004) with some modifications for exhaustive extraction. JPP (0.5 g) was firstly extracted with 7.5 mL of methanol/water/formic acid solution (85:15:0.5; v/v), stirred (30 s, 2000 rpm, Biomixer VTX-2500, São Paulo, Brazil) and sonicated for 5 min (134 W, RMS, ULTRA Cleaner 1600, São Paulo, Brazil). Extract was separated by centrifuging (2000 × *g*, 10 min), and the residue was used in five re-extraction procedures with 5 mL of the solution described above. All extracted solutions were pooled and a 5-mL aliquot was concentrated in a rotary evaporator at 38 °C (Büchi, Essen, Germany) to remove the organic solvent. Final JPP extract was brought to a known volume (2 mL) with an aqueous solution of 3% formic acid in water (v/v). Three independent extractions were conducted for each sample and triplicate samples from each extract were taken for SPE fractionation of phenolic compounds followed by chromatographic analysis.

#### 2.3.1 SPE method for phenolic compounds fractionation

JPP extract (1.0 mL) was purified by SPE using C-18 reverse-phase cartridges (SPE-C18 cartridges, Strata C18-E, Phenomenex) as previously described by Rodriguez-Saona and Wrolstad (2001) with the same modifications reported by Bochi et al. (2014). The purified anthocyanin and non-anthocyanin phenolics were concentrated in a rotary evaporator (38 ± 2 °C) to remove organic solvents and made up to a known volume with 10% methanol in acidified water (0.1% formic acid, v/v) for non-anthocyanin phenolic compounds (0.6 mL) and with acidified water (0.35% formic acid, v/v) for anthocyanins (2 mL). A recovery test

was performed with phenolic standards from three different classes, as described in section 2.6.

## 2.4 Matrix-bound phenolic compounds extraction

### 2.4.1 Alkaline hydrolysis

For the quantification of phenolic compounds linked to the cell-matrix, the residue obtained after the extraction of free phenolic compounds (described in section 2.3) was sequentially submitted to alkaline and acid hydrolysis as previously described by Mattila and Kumpulainen (2002) with some modifications.

For alkaline hydrolysis, the residue was immediately incubated with 12 mL of water and 5 mL of NaOH (10 M) at room temperature (25 ± 2 °C) for 16 h under continuous agitation in light-protected tubes. Thereafter, the pH was adjusted to 2-3 with 6 M HCl and tubes were centrifuged (2000 × *g*, 10 min) to collect the supernatant. The residue was additionally extracted twice with 5 mL of Milli-Q water under agitation (30 s, 2000 rpm, Biomixer VTX-2500, São Paulo, Brazil) and centrifuged (2000 × *g*, 10 min) to collect the supernatants. All supernatants were combined and purified in C-18 cartridges prior to HPLC analysis. Purification was performed as described in section 2.4.3.

### 2.4.2 Acid hydrolysis

The residues obtained after alkaline hydrolysis were thereafter submitted to acid hydrolysis in the presence of 2.5 mL of concentrated HCl for 30 min, at 85 ± 3°C (Mattila and Kumpulainen, 2002). Thereafter, the pH was adjusted to 2-3 with 10 M NaOH, samples were centrifuged (2000 × *g*, 10 min) and the supernatant was collected. Residues were extracted twice with 5 mL Milli-Q water under stirring (30 s, 2000 rpm, Biomixer VTX-2500, São

Paulo, Brazil). The supernatants were combined and purified prior to HPLC analysis as described in section 2.4.3

### 2.4.3 SPE extraction procedure for sample clean-up

The combined supernatants of alkaline (8.0 mL) and acid (6.0 mL) hydrolysis were separately loaded into a C-18 SPE cartridge according to Bochi et al. (2014) with the modifications described below. Polar compounds were washed with three volumes of acidified water (0.35% formic acid, v/v) and total phenolic compounds were then eluted with acidified methanol (0.1% formic acid). The ethyl acetate step was removed, because after hydrolysis the extract showed clear appearance. The purified extracts were concentrated (rotary evaporator at 38 ± 2 °C) and recovered in 1.2 ml of 10% methanol in acidified water (0.1% formic acid, v/v). Prior to HPLC analysis, purified extracts were diluted in initial mobile phases and filtered through a 0.22 µm PTFE syringe filter (Millipore®, São Paulo, Brazil).

## 2.5 Identification of phenolic compounds by HPLC-ESI-MS/MS

Identification of anthocyanin and non-anthocyanin phenolic compounds was performed in two pieces of equipment. The first was an HPLC system connected to an ion-trap mass spectrometer equipped with an electrospray ionization source (ESI) (Esquire 6000, Bruker Daltonics, Billerica, MA, USA). The analysis was performed under positive ion mode for anthocyanin identification and under negative ion mode for the identification of other phenolic compounds. The following settings were used according to Rodrigues et al. (2013): capillary voltage at 4500 V (positive and negative), dry gas temperature at 310 °C, dry gas flow at 11 L $min^{-1}$, and nebulizer gas at 30 psi. The $MS^2$ experiments were performed in a full scan range of 100 to 1800 m/z of all fragments, formed from three major parent ions. The

other mass spectrometer used had a Q-TOF analyzer and ESI source (Bruker Daltonics, model micrOTOF-QIII, Bremen, Germany). The MS parameters were set according to Dal Magro et al. (2016): ESI source in negative ion mode, capillary voltage at −4000 V (negative), dry gas temperature at 310 °C, nebulizer gas at 29 psi, flow rate at 8 L $min^{-1}$. Identification of all phenolic compounds was based on the retention time and elution order in the reversed-phase column, maximum absorption (UV-vis) and MS spectra features compared to standards analyzed under the same conditions.

**2.6 Method validation for quantification of phenolic compounds from JPP by HPLC-DAD analysis**

Since the sample matrix composition is complex, the HPLC method was optimized to improve peak resolution of samples (Supplementary material, Figure S1). The method described by Stefanello et al. (2018) was the basis for the new method. The conditions for quantification were validated as preconized in the International Conference on Harmonization Guidelines (ICH, 2005) (See Supplemetary material, Table S1).

**2.7 Quantification of phenolic compounds by HPLC-DAD**

Free phenolic compounds were extracted from JPP as described in section 2.3, and matrix-bound phenolic compounds (sections 2.4.1 and 2.4.2) were purified by SPE (sections 2.3.1 and 2.4.3) and analyzed using a CBM-20A Prominence HPLC (Shimadzu, Kyoto, Japan) equipped with a degasser (DGU20A5 prominence, Shimadzu, Japan) and column oven (CTO-20A prominence, Shimadzu, Japan) and coupled to a DAD detector (SPDM-20A prominence, Shimadzu, Japan). Separation was performed in a reverse-phase C-18 Hypersil Gold column (5-µm particle size, 150 mm, 4.6 mm; Thermo Fisher Scientific, Massachusetts, USA) at 38 °C using the method optimized and validated as described in section 2.6. Injection

volume was 20 µL, and the mobile phases were composed of 5% (v/v) methanol in acidified water (0.1% (v/v) of formic acid) as solvent (A), and 0.1% (v/v) of formic acid in acetonitrile as solvent B. Different gradient elution conditions and flow rates were tested (see supplementary material, Figure S1), and the optimal gradient was set as follows: 4% B from 0 to 10 min; 4% B was kept until 21 min; 16% B from 21.1 to 55 min; 50% B from 55.1 to 70 min; 100% B from 70.1 to 72 min; 100% B was kept until 80 min; 0% B from 80.1 to 83 min and then kept until 92.1 min at a flow rate of 1 mL $min^{-1}$.

The absorption spectra were recorded from 200 to 800 nm, and phenolic compounds from samples were identified by comparison with the retention time of authentic standards (Figure 1) and the spectral data obtained from UV–visible absorption spectra and mass spectra. For all compounds for which comparison with authentic standards was not possible, a tentative identification was proposed based on elution order in the C-18 reverse-phase chromatogram, UV to visible spectral characteristics, and the fragmentation pattern in mass spectrometry analysis.

The chromatograms for quantification purposes were obtained at 280 nm for hydroxybenzoates and tannins, at 320 nm for hydroxycinnamates, and at 360 nm for flavonols. Calibration curves were constructed using stock solutions of 13 phenolic compounds (Supplementary material, Figure S2). Compounds that are derivative of one of the standard monomers were quantified by equivalence and results were expressed as mg per 100 g of dry sample weight.

For anthocyanin quantification, samples extracted as described in sections 2.3 and 2.3.1 were injected (20 µL) into a reverse-phase C-18 Core-Shell Kinetex column (2.6 µm particle size, 100 mm, 4.6 mm; Phenomenex, Torrance, CA) at 38 °C. The mobile phases were: solvent (A), a solution of 3% of formic acid in water (v/v) and solvent (B), 100% acetonitrile (J.T. Baker® ACS) at a flow rate of 0.9 mL $min^{-1}$. Separation was achieved using

a linear gradient from 0% to 8% B in 5 min, and 20% B was kept until 15 min. At the end of the gradient, the column was washed increasing B to 90%, keeping it for 7 min, and equilibrating to initial condition for 7 min. Chromatograms were obtained at 520 nm for quantification purposes. A calibration curve of cyanidin 3-glucoside was used to quantify each anthocyanin as mg of cyanidin 3-glucoside equivalents 100 $g^{-1}$ of dry sample weight (Supplementary material, Figure S3).

### 2.8 Statistical analysis

Statistica software (version 9.0, StatSoft Inc., Tulsa, OK) was used to perform statistical analysis. Simple regression was performed for standard curves (n=3). The models were evaluated for lack of fit by the least square method. The quantification of free and matrix-bound phenolic contents in the analyzed samples was performed in three independent samples and the values were presented as means ± standard deviation. Means obtained for each phenolic compound identified in both species were compared using Student's test, at 95% confidence level.

## 3. Results and discussion

### 3.1 Tentative identification of free non-anthocyanin phenolic compounds

Analysis of free non-anthocyanin phenolic compounds from JPP-MT revealed 81 peaks (Figure 2) among which 24 were tentatively identified, amounting to 46.2% of free non-anthocyanin phenolic content (Table 1). For the JPP-MJ extract, 25 peaks were tentatively identified out of 78 peaks detected, which amounted to 50.7% of the total content of free non-anthocyanin phenolic compounds (Figure 3).

The tentative identification of compounds was based on the analysis of their retention time, wavelength of maximum UV-vis absorption and mass spectral features of deprotonated molecule ($[M-H]^-$) and their major fragment ions, which are shown in Tables 2 and 3. These data were compared to authentic standards (Figure 1) and previously published studies; the exact mass obtained for the $[M-H]^-$ in LC-q-TOF-MS/MS was compared with literature data.

### 3.1.1 Tentative identification of free ellagitannins

All compounds identified as ellagitannins had a characteristic absorption maximum at 280 nm and exhibited the characteristic elevation of baseline due to the difficult separation of hydrolyzable tannins (Talcott and Krenek, 2012).

Hexahydroxydiphenic acid (HHDP)-galloylglucose and its isomers were identified in peaks 1A, 2A, 5A and 13A in both JPP species (Table 1 and Table 2) due to the detection of a deprotonated molecule ($[M-H]^-$) at *m/z* 633 and fragment ions at *m/z* 481 (neutral loss of a galloyl residue, 152 Da), 301 (ellagic acid), 275 and 249. The last two fragments are characteristic of the breakdown of HHDP, which also forms ellagic acid when hydrolyzed through elimination of water (Plaza et al., 2016). This tentative identification was corroborated by analysis of the exact mass obtained for these compounds. For example, peak 5A found in JPP-MT and JPP-MJ had $[M-H]^-$ at *m/z* 633.0731 (Table 1) and 633.0769 (Table 2), respectively, yielding a mass error lower than 5 ppm compared to previous data (Plaza et al., 2016). These compounds were HHDP-galloylglucosewhich has already been reported in jaboticaba and other fruits such as *Pouteria macrophylla* that also had several isomers of HHDP-galloylglucose (Gordon et al., 2011; Plaza et al., 2016). In a similar manner, peak 13A in both JPPs (Table 1 and 2) was identified as a co-elution of bis-HHDP-galloylglucose, also known as casuarinin, by $[M-H]^-$ at *m/z* 935 that was confirmed by their exact mass (Plaza et al., 2016). Peak 14A (Table 1 and 2) was identified as casuarictin, a casuarinin isomer. The

**Table 1:** Tentative identification of free and matrix-bound phenolic compounds in the peel powder of *Myrciaria trunciflora* (JPP-MT) by HPLC-DAD coupled to LC-TRAP-MS/MS and LC-q-TOF-MS/MS.

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Tentative identification |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| **FREE POLYPHENOLS** | | | | | | |
| **Hydroxybenzoate derivatives (HBD) or tannins** | | | | | | |
| 1A | 280 | 3.1 ± 0.1 | 216, 276 | | **331.0635:** 169.0096; 123.0051; 109.3878 | Monogalloyl glucose |
| | | | | **633**: 301, 275, 249 | **633.0728:** 300.997; 275.0186; 249.0389; 169.008 | HHDP-galloylglucose |
| 2A | 280 | 4.7 ± 0.1 | 226, 270 | **783**: 301, 481, 623, 543 | **783.0714:** 300.9973; 275.0179; 481.0635 | Bis-HHDP-glucose (Casuariin) [a] |
| | | | | **633:** 301, 275, 481 | **633.0747:** 300.9967; 249.0389 | HHDP-galloylglucose isomer |
| | | | | | **343.0647:** 191.0527 | 3-O-Galloyl quinic acid |
| 5A | 280 | 7.0 ± 0.1 | 279 | | **633.0731:** 300.9963; 169.0074 | HHDP-galloylglucose isomer |
| 8A | 280 | 9.2 ± 0.1 | 262 | **783:** 301, 481, 623 | **783.0719:** 300.997; 275.0179; 481.0648 | Bis-HHDP-glucose isomer (Pedunculagin) [a] |
| 9A | 280 | 9.9 ± 0.1 | 225, 280 | **951:** 907, 783 | **951.0802:** 300.9977 (2); 783.0689 | Trisgalloyl HHDP glucose |
| 10A | 280 | 12.1 ± 0.1 | 215, 272 | **785:** 301, 483, 633, 677, 569 | **785.0879:** 300.9971(2); 275.0178; 249.0376 | HHDP-digalloylglucose (Tellemagrandin I) [a] |
| | | | | **631:** 451 | **631.0556:** 450.9933 | Castalin |
| 12A | 280 | 14.4 ± 0.1 | 202, 277 | | **289.0677:** 109.0257; 121.0243; 261.5021 | (Epi)Catechin |
| | | | | | **631.0569:** 450.9920 | Castalin isomer |
| 13A | 280 | 16.0 ± 0.1 | 214, 268 | **935:** 917, 873 | **467.0359(2):** 169.0106; 249.0371; 275.0187; 301.0008 | Bis-HHDP-galloylglucose (Casuarinin) [a] |

Table 1 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Tentative identification |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| | | | | **633:** 301 | **633.0720:** 300.9968; 169.0084 | HHDP-galloylglucose isomer |
| 14A | 280 | 16.9 ± 0.1 | 215, 273 | **483:** 271 | **483.0765:** 271.0419; 169.0104 | Digalloylglucose |
| | | | | **935:** 917, 873 | **935.0655:** 633.0604; 300.9877; 481.0488; 571.0561 | Bis-HHDP-galloylglucose isomer (Casuarictin) [a] |
| 17A | 280 | 21.3 ± 0.1 | 216, 269 | **785:** 301, 483, 633, 691, 767 | **785.0881:** 300.9979(2); 249.0370; 275.0183 | HHDP-digalloylglucose isomer |
| | | | | **951:** 907, 783 | **475.0348 (2):** 391.0393(2); 275.0206; 300.9968(2) | Trisgalloyl-HHDP-glucose isomer |
| 19A | 280 | 31.2 ± 0.1 | 216, 275 | **635:** 465 | **635.0881:** 465.0518; 169.0019 | Trigalloylglucose |
| 24A | 280 | 37.4 ± 0.1 | | **933:** 451, 763 | **933.0728:** 450.9918(2); 650.1654; 275.0204; 300.9981(2) | Castalagin/Vescalagin[a] |
| 31A | 280 | 44.3 ± 0.1 | 213, 280 | **787:** 617,635 | **787.1066:** 617.0792; 465.0641; 169.0108 | Tetragalloylglucose |
| 34A | 280 | 47.1 ± 0.1 | 216, 275 | **787:** 617 | **787.1048:** 617.0779; 465.0620; 169.0098 | Tetragalloylglucose isomer |
| 39A | 280 | 52.7 ± 0.1 | 216, 278 | **1085:** 783, 633, 451 | **542.0366 (2):** 169.0111 | Galloyl-castalagin |
| | | | | **939:** 769 | **939.1158:** 769.0912; 617.0757; 169.0122 | Pentagalloyl glucose[a] |
| 47A | 280 | 61.4 ± 0.1 | 215, 279 | **1085:** 783, 633, 451 | **542.0374 (2):** 300.9984; 169.0111 | Galloyl-castalagin isomer |
| **Flavonol derivatives** | | | | | | |
| 9C | 360 | 39.7 ± 0.1 | 251, 353 | | **479.0874:** 316.0203; 317.0238 | Myricetin-hexoside |

Table 1 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Tentative identification |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 10C | 360 | 43.2 ± 0.1 | 252, 366 | **301:** 285, 284 | **300.9972:** 283.9919; 185.0212; 145.0260 | Ellagic acid[a] |
| 11C | 360 | 44.7 ± 0.1 | 261, 348 | **463:** 316 | **463.0885:** 316.0204; 317.0244 | Myricetin-3-rhamnoside[a] |
| 12C | 360 | 46.2 ± 0.1 | 251, 354 | | **463.0876:** 301.0294; 300.0254 | Quercetin-hexoside |
| 13C | 360 | 47.8 ± 0.1 | 255, 353 | **463:** 301 | **463.0878:** 301.0302; 300.0251 | Quercetin-hexoside |
| 15C | 360 | 50.7 ± 0.1 | 259, 353 | | **433.0767:** 300.0260; 301.0305 | Quercetin-pentoside |
| 16C | 360 | 52.0 ± 0.1 | 206, 348 | **433:** 301 | **433.0751:** 300.0252; 301.0309 | Quercetin-pentoside |
| 17C | 360 | 54.0 ± 0.1 | 254, 347 | **447:** 301 | **447.0915:** 301.0320; 300.0266 | Quercetin-rhamnoside |
| **Anthocyanins** | | | | | | |
| 1D | 520 | 11.2 ± 0.1 | 516 | **465:** 303 | **465.1011:** 303.0475 | Delphinidin-3-glucoside[b] |
| 2D | 520 | 12.2 ± 0.1 | 521 | **449:** 287 | **449.1052:** 287.0536 | Cyanidin-3-glucoside[a,b] |
| 3D | 520 | 13.2 ± 0.1 | 514 | **433:** 271 | **433.1096** | Pelargonidin-3-glucoside |
| **MATRIX-BOUND POLYPHENOLS- Alkaline Hydrolysis** | | | | | | |
| **Hydroxybenzoate derivatives (HBD)** | | | | | | |
| 4A | 280 | 3.8 ± 0.1 | 215, 270 | | **169.0140:** 124.008; 125.0174; 123.0084 | Gallic acid[b] |
| 7A | 280 | 7.6 ± 0.1 | 220, 272 | | **153.0177:** 108.0215; 109.0285 | Protocatechuic acid[b] |
| | | | | | **341.0199:** 165.0551; 137.058; 191.0354 | 4-Hydroxybenzoic acid derivative |

Table 1 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Tentative identification |
|---|---|---|---|---|---|---|
| | | | | **[M-H]$^-$:**MS$^2$ | **[M-H]$^-$:** MS$^2$ | |
| **Flavonol Derivatives** | | | | | | |
| 16C | 360 | 43.1 ± 0.1 | 252, 367 | | **301.0037:** 283.9986; 229.0165; 185.0247; 173.0242 | Ellagic acid[a] |
| **MATRIX-BOUND POLYPHENOLS-Acid Hydrolysis** | | | | | | |
| **Hydroxybenzoate derivatives (HBD)** | | | | | | |
| 2A | 280 | 3.8 ± 0.1 | 270 | | **169.0142:** 107.0592 | Gallic acid[b] |
| 5A | 280 | 7.3 ± 0.1 | 272 | | **153.0173:** 109.0281; 108.0188 | Protocatechuic acid[b] |
| **Flavonol Derivatives** | | | | | | |
| 6C | 360 | 43.2 ± 0.1 | 252, 366 | | **301.0026:** 185.0263; 201.0180 | Ellagic acid[a] |

*Peak numbers are shown in figures 2 (free phenolic compounds) and 4 (matrix-bound phenolic compounds), where peak letter indicates the figure panel. Peaks that were only detected in DAD but did not generate mass spectral signal or that generate mass spectral are not shown in the table. HHDP= hexahydroxydiphenic acid. RT= Retention time. SD=standard deviation. Bold number correspond to m/z [M-H]$^-$. [a] Previously reported in jaboticaba peel; [b] Positively identified by comparison with authentic standard

**Table 2:** Tentative identification of free and matrix-bound phenolic compounds in the peel powder of *Myrciaria jaboticaba* (JPP-MJ) by HPLC-DAD coupled to LC-TRAP-MS/MS and LC-q-TOF-MS/MS.

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Tentative identification |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| **FREE PHENOLIC COMPOUNDS** | | | | | | |
| **Hydroxybenzoate derivatives (HBD) or tannins** | | | | | | |
| 1A | 280 | 3.1 ± 0.1 | 217, 265 | **633:** 301,275,135,249,481 | **633.0765**: 300.9979; 275.0190; 249.0388 | HHDP-galloylglucose |
| 2A | 280 | 4.7 ± 0.1 | 270 | **783:** 301, 481, 603, 765 | **783.0757:** 300.998; 275.0193; 481.0653 | Bis-HHDP-glucose (Casuariin)[a] |
| | | | | | **343.0662:** 191.0541 | 3-O-Galloyl quinic acid |
| | | | | | **633.0764:** 300.998; 249.0394; 275.0190 | HHDP-galloylglucose isomer |
| 5A | 280 | 7.0 ± 0.1 | 260 | | **633.0769**: 300.9987; 275.0199; 481.0578 | HHDP-galloylglucose isomer |
| 7A | 280 | 9.1 ± 0.1 | 260 | **783:** 481, 301,765 | **783.0772**: 300.9984; 275.0184; 481.0634; 249.0396 | Bis-HHDP-glucose isomer (Pedunculagin)[a] |
| 8A | 280 | 9.8 ± 0.1 | 270 | **951:** 907, 783 | **951.0878**: 300.9982; 481.0635; 275.0186 | Trisgalloyl HHDP glucose |
| 9A | 280 | 12.0 ± 0.1 | 216, 274 | **785:** 483,301, 633 | **785.0906:** 300.9976(2); 249.0287; 275.0187; 331.3528 | HHDP-digalloylglucose (Tellemagrandin I)[a] |
| 11A | 280 | 14.3 ± 0.2 | 202, 277 | **631:** 451 | **631.0614:** 450.9944 | Castalin |
| | | | | **631:** 451, 509 | **631.0605:** 450.9947 | Castalin isomer |
| | | | 203, 271 | | **289.0698:** 109.0266; 123.0413; 149.0234; 221.0775 | (Epi)Catechin |

Table 2 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Tentative identification |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 13A | 280 | 15.9 ± 0.1 | 267, 214 | **935:** 917, 873, 783,855 | **467.0399:** 169.0105; 275.0196; 301.0008 | Bis-HHDP-galloylglucose (Casuarinin)[a] |
| | | | | **633:** 301 | **633.076:** 300.9985; 275.0185 | HHDP-galloylglucose isomer |
| 14A | 280 | 16.9 ± 0.1 | 215, 271 | **935:** 917, 633, 783, 855 | **467.0406:** 169.0106; 275.0187; 300.9982 | Bis-HHDP-galloylglucose isomer (Casuarictin)[a] |
| | | | | | **483.0786**: 271.0446; 169.0110 | Digalloylglucose |
| 16A | 280 | 21.2 ± 0.2 | 216, 263 | **785:** 765,301, 483, 633 | **785.0913:** 300.9984(2); 275.0193; 249.0396; 169.0134 | HHDP-digalloylglucose (Tellemagrandin I) isomer[a] |
| | | | 207, 271 | **951:** 907,783 | **951.0893:** 783.0813; 481.0606; 275.0175 | Trisgalloyl-HHDP-glucose isomer |
| 18A | 280 | 31.2 ± 0.2 | 215, 276 | **635:** 465 | **635.0909:** 465.0653; 169.011; 313.0493 | Trigalloylglucose |
| 20A | 280 | 33.0 ± 0.2 | 215, 263 | | **635.0904:** 169.0091; 465.065 | Trigalloylglucose isomer |
| 21A | 280 | 33.6 ± 0.2 | 218, 261 | **935:** 917, 633,301, 451 | **467.0255 (2):** 391.0209; 169.0032 (2); 275.0084 | Bis-HHDP-galloylglucose isomer |
| 23A | 280 | 37.4 ± 0.4 | 215, 270 | **933:** 451, 631, 763, 287 | **933.0672:** 450.9916; 300.9987; 765.0596; 631.0636 | Castalagin/Vescalagin[a] |
| 27A | 280 | 44.3 ± 0.1 | 215, 272 | **787:** 617 | **787.1041:** 617.0766; 169.012; 465.0637 | Tetragalloylglucose |

Table 2 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | **Tentative identification** |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 29A | 280 | 47.0 ± 0.1 | 216, 277 | **787:** 617 | **787.1046:** 617.0798; 169.0113; 456.0656 | Tetragalloylglucose isomer |
| 33A | 280 | 52.7 ± 0.1 | 216, 270 | **1085:** 783 | **542.0372(2):** 300.9991(2); 169.0121 | Galloyl-castalagin |
| | | | | **939:** 769 | **939.1191:** 769.0964; 617.0764; 447.0564; 169.0086 | Pentagalloylglucose[a] |
| 40A | 280 | 61.4 ± 0.1 | 216, 278 | **1085:** 783 | **542.0378(2):** 169.0089; 300.9977 | Galloyl-castalagin isomer |
| **Flavonol derivatives** | | | | | | |
| 6C | 360 | 37.3 ± 0.1 | 255, 361 | | **433.0333:** 299.9880; 300.9973 | Ellagic acid pentoside[a] |
| 9C | 360 | 42.0 ± 0.1 | 249, 359 | | **433.0407:** 300.9976; 299.988 | Ellagic acid pentoside[a] |
| 10C | 360 | 43.2 ± 0.1 | 252, 366 | | **300.9983:** 283.9934; 173.0229; 185.0133; 245.064; 257.0072 | Ellagic acid[a] |
| 11C | 360 | 44.7 ± 0.1 | 255, 349 | **463:** 316 | **463.0894:** 316.0205; 317.0264 | Myricetin-3-rhamnoside[a] |
| 12C | 360 | 46.2 ± 0.1 | 251, 353 | | **463.0884:** 300.0262; 301.0319 | Quercetin-hexoside |
| 13C | 360 | 47.8 ± 0.1 | 255, 353 | **463:** 301 | **463.0888:** 300.0276; 301.0311 | Quercetin-hexoside |
| 17C | 360 | 54.0 ± 0.1 | 255, 347 | **447:** 301 | **447.0922:** 300.0265; 301.0317 | Quercetin-3-rhamnoside |
| **Anthocyanins** | | | | | | |
| 1D | 520 | 11.2 ± 0.1 | 517 | **465:** 303 | **465.1011:** 303.0487 | Delphinidin-3-glucoside[a] |

Table 2 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Tentative identification |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 2D | 520 | 12.2 ± 0.1 | 520 | **449:** 287 | **449.1064:** 287.0541 | Cyanidin-3-glucoside[a, b] |
| 3D | 520 | 13.1 ± 0.1 | 516 | **433:** 271 | **433.1126:** | Pelargonidin-3-glucoside |
| 4D | 520 | 14.0 ± 0.1 | | **463:** 301 | **463.1200:** | Peonidin-3-glucoside[a] |
| **MATRIX-BOUND POLYPHENOLS- Alkaline hydrolysis** | | | | | | |
| **Hydroxybenzoate derivatives (HBD)** | | | | | | |
| 1A | 280 | 3.8 ± 0.1 | 215, 271 | | **169.0176**: 125.0274; 123.0103 | Gallic acid[b] |
| 3A | 280 | 7.4 ± 0.1 | 259, 293 | | **153.0186**: 109.0294; 108.0235 | Protocatechuic acid[b] |
| | | | | | **341.0241**: 165.0576; 137.0584; 191.0391 | 4-hidroxibenzoic acid derivative |
| **Flavonol derivatives** | | | | | | |
| 11C | 360 | 43.2 ± 0.1 | 252, 367 | | **301.0065:** 229.006; 185.0273; 173.0276 | Ellagic acid |
| **MATRIX-BOUND POLYPHENOLS- Acid hydrolysis** | | | | | | |
| **Hydroxybenzoate derivatives (HBD)** | | | | | | |
| 1A | 280 | 3.8 ± 0.1 | 270 | | **169.0164:** 124.0238 | Gallic acid[b] |
| 4A | 280 | 7.3 ± 0.1 | 259, 293 | | **153.0212**: 109.0304; 108.0211 | Protocatechuic acid[b] |
| **Flavonol derivatives** | | | | | | |
| 6C | 360 | 43.3 ± 0.1 | 252, 367 | | **301.0055:** 257.0119; 201.0056; 285.0053 | Ellagic acid |

*Peak numbers are shown in figures 3 (free phenolic compounds) and 5 (matrix-bound phenolic compounds), where peak letter indicates the figure panel. Peaks that were only detected in DAD but did not generate mass spectral signal or that generate mass spectral are not shown in the table. RT= Retention time. SD=standard deviation. HHDP= hexahydroxydiphenic acid. Bold number correspond to m/z [M-H]⁻. [a] Previously reported in jaboticaba peel; [b] Positively identified by comparison with authentic standard

fragmentation pattern of this compound was the same as previously reported (Plaza et al., 2016). Casuarinin and casuarictin have been previously identified in jaboticaba (*M. cauliflora* and *M. jaboticaba*) (Pereira et al., 2017; Plaza, et al., 2016).

The ellagitannin castalagin/vescalagin (Peak 24A in Table 1 and Peak 23A in Table 2) were identified based on their deprotonated molecule $[M-H]^-$ at *m/z* 933 and fragment ions at *m/z* 631 (loss of HHDP unit), 451 (cleavage of tri-galloyl unit and spontaneous lactonization) and 301 (ellagic acid residue) (Tables 1 and 2) as previously reported by Liberal et al. (2015). Castalin and its isomer (peaks 10A and 12A in Table 1 and peaks 9A and 11A in Table 2) were identified based on their deprotonated molecule $[M-H]^-$ at *m/z* 631 and fragment ions at *m/z* 451 (loss of the hexose yielding HHDP linked to one galloyl residue by a c-glycosyl bond) (Sun et al., 2014) and confirmed by q-TOF analysis. Peaks 10A in JPP-MT and 9A in JPP-MJ were identified as co-elutions of the ellagitannin HHDP-digalloylglucose, whereas peak 12A in JPP-MT (Table 1) and peak 11A in JPP-MJ (Table 2) were identified as co-elutions of (epi)catechin based on their mass spectral characteristics (Bascaran et al., 2016).

Casuariin and its isomer (pedunculagin) (peaks 2A and 8A in Table 1 and peaks 2A and 7A in Table 2) were identified based on their $[M-H]^-$ at *m/z* 783 and fragment ions at *m/z* 481 (loss of HHDP) and 301 (loss of hexose moiety yielding ellagic acid) and confirmed by q-TOF analysis and comparison with literature spectral data (Gordon et al., 2011; Plaza et al., 2016).

Peaks 9A and 17A of JPP-MT (Table 1) and peaks 8A and 16A of JPP-MJ (Table 2), which had $[M-H]^-$ at *m/z* 951 and fragment ions at *m/z* 907 and 783 (di-HHDP glucose after the neutral loss of galloyl residue with a C-C linkage to one of the HHDP molecules). LC-q-TOF-MS/MS analysis confirmed this pattern that was tentatively identified as trisgalloyl-HHDP glucose (Gordon et al., 2011).

Peaks 10A and 17A (Table 1) and peaks 9A and 16A (Table 2) had $[M-H]^-$ at *m/z* 785 and fragment ions at *m/z* 633 (loss of a galloyl group), 483 (loss of HHDP unit) and 301 (ellagic acid), confirmed by q-TOF analysis (Table 1 and 2). These fragments were characteristic of HHDP-digalloylglucose, also known as tellimagrandin I, previously reported in the peel of *M. jaboticaba* and *M. cauliflora* fruits (Plaza et al., 2016; Wu et al., 2012), but reported for the first time in JPP-MT.

Peaks 39A and 47A in Table 1 and peaks 33A and 40A in Table 2 had $[M-H]^-$ at *m/z* 1085 and fragment ions at *m/z* 783 (loss of the HHDP moiety) and 633 (neutral loss of the trisgalloyl group of 452 Da, yielding HHDP galloylglucose), confirmed by q-TOF analysis and tentatively identified as galloyl-castalagin (Gasperotti et al., 2013).

Peak 2A was tentatively identified as 3-O-galloylquinic acid in both JPP species due to the detection of $[M-H]^-$ at m/z 343.0647 (Table 1) and 343.0662 (Table 2) and fragment ions at m/z 191.0527 (Table 1) and 191.0541 (Table 2), equivalent to the neutral loss of galloyl residue (152 Da) yielding quinic acid (Gordon et al., 2011).

Six ellagitannin compounds were reported for the first time in the jaboticaba peel, namely trisgalloyl-HHDP-glucose and its isomer, 3-O-galloylquinic acid, castalin, and galloyl-castalagin and its isomers (Tables 1 and 2). All these compounds have already been reported in other fruit sources (Gordon et al., 2011; Fischer et al., 2011; Gasperotti et al., 2013).

### 3.1.2 Tentative identification of free gallotannins

JPP-MT had numerous compounds that had a mass spectral pattern suggestive of gallotannins containing galloylglucose (Peaks 39A, 34A, 31A, 19A, 14A, and 1A, Table 1). Peak 39A (Table 1) had a deprotonated molecule $[M-H]^-$ at *m/z* 939.1158 and characteristic fragments at *m/z* 769.0912 (loss of $H_2O$ and a galloyl group), 617.0757 (loss of galloyl group)

and 169.0122 (loss of two galloyl groups minus two $H_2O$, yielding gallic acid) (Plaza et al., 2016). Pentagalloylglucose has been previously reported in the peel of *M. jaboticaba* and in *M. cauliflora* (Pereira et al., 2017; Plaza et al., 2016).

Peaks 31A and 34A (Table 1) were tentatively identified as tetragalloylglucose and its isomer. They had $[M-H]^-$ at *m/z* 787 and fragment ions at 617 (loss of $H_2O$ and a galloyl group), 465 (loss of a galloyl residue) and 169 (loss of galloyl glucose minus two $H_2O$, yielding a gallic acid residue) (Gordon et al., 2011). Similarly, peak 19A that had $[M-H]^-$ at *m/z* 635.0881 and fragment ions at 465.0518, and 169.0019 was suggested to be trigalloylglucose (Sun et al., 2014; Wyrepkowski et al., 2014), which is reported for the first time in jaboticaba peel. Peak 14A was tentatively identified as digalloylglucose as it had $[M-H]^-$ at *m/z* 483.0765 and a fragment ion at 169.0104, which corresponds to the loss of 314 Da equivalent to the galloyl residue bound to hexose minus 18 Da ($H_2O$), yielding gallic acid (Wyrepkowski et al., 2014). Peak 1A (Table 1) had $[M-H]^-$ at m/z 331.0635 and fragment ions at 169.0096 (neutral loss of glucose, 162 Da, yielding gallic acid), 123.0051 and 109.3878, which can be related to the breakdown of the aglycone. This compound is suggested to be monogalloylglucose (Wyrepkowski et al., 2014) and was identified only in JPP-MT.

Identification of the gallotannins in JPP-MJ (Table 2) was similar to that in JPP-MT, as observed by tentative identification of pentagalloylglucose (peak 33A), tetragalloylglucose and its isomer (peaks 27A and 29A), trigalloylglucose (peak 18A) and digalloylglucose (peak 14A). Nevertheless, the trigalloylglucose isomer (peak 20A, Table 2) was only identified in JPP-MT, while monogalloylglucose was found only in JPP-MT.

### 3.1.3 Tentative identification of free (epi)catechin

Peaks 12A (Table 1) and 11A (Table 2) had a deprotonated molecule $[M-H]^-$ at *m/z* 289, characteristic of the presence of (epi)catechin, as observed previously in the extract of *M. cauliflora* fruit (Wu et al., 2016). However, this is the first report for (epi)catechin in *M. trunciflora* and *M. jaboticaba* species.

### 3.1.4 Tentative identification of free flavonols

The flavonols myricetin-hexoside (peak 9C) and myricetin-rhamnoside (peak 11C) were tentatively identified in JPP-MT (Table 1). These peaks had maximum UV absorption within the characteristic UV spectra of the flavonol class (Nollet and Toldrá, 2012). In addition, the deprotoned molecule and the $MS^2$ fragments obtained confirmed these structures with loss of hexosyl and rhamnosyl residues (162 and 146 Da), respectively, yielding the myricetin aglycone fragment at m/z 317 (Wu et al., 2012). On the other hand, in JPP-MJ it was only possible to identify myricetin-3-rhamnoside (peak 11C, Table 2), also known as myricitrin, which has not been previously reported in the peel of this species, but has been found in the juice of jaboticaba of the *M. cauliflora* species (Wu et al., 2012).

Other quercetin-derived flavonols (peaks 12C, 13C, 15C, 16C, and 17C) were identified by their maximum absorption at 360 nm and MS spectrum in JPP-MT. The fragments at *m/z* 301 and exact mass suggest the presence of quercetin. In addition, the neutral loss of the hexosyl (loss 162 Da; peaks 11C and 12C in Table 1), pentosyl (132 Da; peaks 15C and 16C in Table 1) and rhamnosyl (loss 176 Da; peak 17C in Table 1 and peak 17C in Table 2) residues suggests the identification of quercetin-hexoside (12C and 13C in Table 1) (Gordon et al., 2011), quercetin-pentoside (15C and 16C in Table 1) (Gordon et al., 2011) and quercetin-3-rhamnoside (17C in Table 1) (Ambigaipalan et al., 2017) in JPP-MT. Free ellagic acid ($[M-H]^-$ at *m/z* 300.9972, peak 10C in Table 1) was also observed in JPP-MT, as already reported for jaboticaba fruit (Wu et al., 2013) and peel (*M. jaboticaba*) (Plaza et al., 2016).

*M. jaboticaba* differed from *M. trunciflora* in relation to quercetin and ellagic acid derivatives; while JPP-MT had only free ellagic acid, JPP-MJ had free ellagic acid (peak 16C, Table 2) and two ellagic acid derivatives (peaks 6C and 9C, Table 2) because they had in common the fragment ion at *m/z* 301, suggested to be ellagic acid, and confirmed by UV spectra with maximum absorption at 360 nm. Peaks 6C and 9C had $[M-H]^-$ at *m/z* 433.0333 and 433.0407 and fragment ions at *m/z* 299.9880, 300.9973 (peak 6C), 300.9976 and 299.988 (peak 9C), confirming the presence of ellagic-pentoside structures with loss of the pentosyl residue (neutral loss 132 Da) (Plaza et al., 2016).

JPP-MJ had compounds tentatively identified as quercetin-hexoside (peaks 12C and 13C, Table 2) and quercetin-rhamnoside (peak 17C, Table 2), confirmed by the characteristic molecular ion and fragments as previously described for JPP-MT (Table 1). However, the profile quercetin derivatives of *M. jaboticaba* differed from those of *M. Trunciflora*; while JPP-MT had two peaks of quercetin-pentoside (peaks 15C and 16C in Table 1), JPP-MJ had no characteristic peak of quercetin-pentoside.

### 3.2 Tentative identification of free anthocyanins

Three anthocyanins were found in JPP-MT (Figure 2D and Table 1), and four in JPP-MJ (Figure 3D and Table 2). The major anthocyanin compounds in both fruit species were delphinidin-3-glucoside (molecular ion $[M]^+$ at m/z 465 and main fragment at m/z 303; Peak 1, Figure 2D and 3D) and cyanidin 3-glucoside ($[M]^+$ at m/z 449 and fragment at m/z 287; Peak 2, Figure 2D and 3D). These major anthocyanins were confirmed by TOF-analysis (Table 1 and 2), and their presence in the Myrciaria genus has been extensively reported (Alezandro et al., 2013; Calloni et al., 2015; Pereira et al., 2017; Plaza et al., 2016). The other minor anthocyanin compounds were identified as pelargonidin-3-glucoside ($[M]^+$ at m/z 433 and fragment ion at 271) (Fischer et al. 2011; Jin et al. 2017) that was found in JPP of both

species studied, and peonidin-3-glucoside ($[M]^+$ at m/z 463.1200 and fragment at m/z 301) (Sun et al., 2014) that was identified only in the JPP-MJ (Table 2) .

The present study brings new information concerning the identification of free phenolic compounds from jaboticaba peel, mainly by firstly providing data for *M. trunciflora*, and by identifying novel compounds from *M. jaboticaba*. Some free phenolic compounds were identified for the first time in jaboticaba, namely trisgalloyl-HHDP-glucose, 3-O-galloylquinic acid, castalin and galloyl-castalagin and its isomers, as well as monogalloylgucose, digalloylglucose, trigalloylglucose, tetragalloylglucose, myricetin-hexoside, quercetin-pentoside, and quercetin-hexoside.

### 3.3 Tentative identification of matrix-bound phenolic compounds

Phenolic compounds linked to the cellular matrix by ester and ether bonds can be analyzed after alkaline hydrolysis, whereas acid hydrolysis releases phenolic compounds linked to the cellular matrix through glycosidic bonds (Shahidi and Yeo, 2016). Figures 4–7 show the chromatograms obtained after the alkaline and acid hydrolysis of JPP-MT and JPP-MJ samples. Three compounds were tentatively identified out of the 42 peaks detected in JPP-MT after alkaline hydrolysis (Figure 4, Table 1), which corresponds to 27.1% of total matrix-bound polyphenols. In JPP-MJ, 3 compounds were tentatively identified out of 44 peaks observed after alkaline hydrolysis, which corresponds to 24.6% of total matrix-bound polyphenols (Figure 6, Table 2). After acid hydrolysis, three phenolic compounds were tentatively identified for both JPPs (Tables 1 and 2), amounting to 1.6% of total matrix-bound polyphenols for JPP-MT and 1.3% for JPP-MJ (21 peaks).

Peaks 4A (Table 1) and 1A (Table 2) were identified as gallic acid by comparison with an authentic standard. Similarly, peaks 7A (Table 1) and 3A (Table 2) were identified as protocatechuic acid by matching with the authentic standard. Peak 7A (Table 1) comprised a

co-elution with a compound that had $[M-H]^-$ at *m/z* 341.0199 and fragment ions at *m/z* 137.0580 (4-hydroxybenzoic acid), 191.0354 and 165.0551 suggestive of the presence of a 4-hydroxybenzoic acid derivative. Gallic and protocatechuic acids bound to the cell wall by ester and ether-links have also been observed in apple fruit pulp (Baskaran et al., 2016).

Gallic acid (peak 2A in Table 1 and peak 1A in Table 2), protocatechuic acid (peak 5A in Table 1 and peak 4A in Table 2) and ellagic acid (peak 6C in Table 1 and peak 6C in Table 2) were also identified in both JPPs after acid hydrolysis suggesting that they were also linked to cell-matrix components by glycosidic bonds since acid hydrolysis generally leaves ester bonds intact (Shahidi and Yeo, 2016).

These data provide for the first time the profile of matrix-bound phenolic compounds (gallic acid, protocatechuic acid and ellagic acid), which so far had not been studied in jaboticaba using mass analysis.

### 3.4 Quantification of free phenolic compounds

The total content of free phenolic compounds (sum of all compounds separately quantified according to their subclass) was higher for JPP-MT cultivated in the south of Brazil (5500.6 mg 100 $g^{-1}$ peel) compared to JPP-MJ cultivated in the southeastern region of Brazil (2272.4 mg 100 $g^{-1}$ peel), suggesting that the *M. trunciflora* species has a higher content of phenolic compounds than *M. jaboticaba* (Table 3).

Hydroxybenzoate derivatives (HBD) or tannins were the major group of phenolics found in JPP-MT (60.3% of all free phenolic compounds), whereas JPP-MJ had 34.7% of HBD or tannins in relation to the total free phenolic compound content (Table 3).

Hydrolyzable tannins were the most abundant free phenolic compounds in JPP-MT, and JPP-MT also had a higher content of all hydrolyzable tannins identified, compared to JPP-MJ ($p<0.05$, Table 3). The major differences were observed for trigalloylglucose, the co-

**Table 3:** The quantification of the free and matrix-bound phenolic compounds.

| λ (nm) | Tentative identification | Peak no | Quantification mg/100g JPP-MT[#] | Peak no | Quantification mg/100g JPP-MJ[#] |
|---|---|---|---|---|---|
| | **FREE PHENOLIC COMPOUNDS** | | | | |
| **Hydroxybenzoate derivatives (HBD) or tannins*** | | | | | |
| 280 | Monogalloyl glucose + HHDP-galloylglucose | 1A | 9.9 ± 4.5[A] | 1A | 3.7 ± 0.8[A] |
| 280 | Casuariin + HHDP-galloylglucose + 3-O-galloyl quinic acid | 2A | 168 ± 21[A] | 2A | 45.7 ± 3.6[B] |
| 280 | HHDP-galloylglucose isomer | 5A | 14.5 ± 4.1[A] | 5A | 3.1 ± 0.3[B] |
| 280 | Pedunculagin | 8A | 93.0 ± 9.0[A] | 7A | 30.4 ± 3.4[B] |
| 280 | Trisgalloyl HHDP glucose | 9A | 18.8 ± 1.3[A] | 8A | 11.2 ± 0.8[B] |
| 280 | Tellimagrandin I + Castalin | 10A | 91.4 ± 7.8[A] | 9A | 9.8 ± 1.0[B] |
| 280 | Catechin or Epicatechin + Castalin isomer | 12A | 37.8 ± 6.4[A] | 11A | 5.6 ± 0.5[B] |
| 280 | Casuarinin + HHDP-galloylglucose | 13A | 89.6 ± 4.1[A] | 13A | 18.1 ± 1.0[B] |
| 280 | Digalloylglucose + Casuarictin | 14A | 127 ± 6[A] | 14A | 33.4 ± 1.7[B] |
| 280 | HHDP-digalloylglucose isomer + Trisgalloyl-HHDP-glucose isomer | 17A | 115 ± 2[A] | 16A | 14.8 ± 0.8[B] |
| 280 | Trigalloylglucose | 19A | 217 ± 2[A] | 18A | 18.8 ± 0.7[B] |
| 280 | Trigalloylglucose isomer | | | 20A | 5.8 ± 0.4 |
| 280 | Bis-HHDP-galloylglucose isomer | | | 21A | 15.7 ± 1.0 |
| 280 | Castalagin/Vescalagin | 24A | 60.8 ± 6.1[A] | 23A | 15.3 ± 0.3[B] |
| 280 | Tetragalloylglucose | 31A | 40.9 ± 3.3[A] | 27A | 24.7 ± 2.4[B] |
| 280 | Tetragalloylglucose isomer | 34A | 244 ± 18[A] | 29A | 71.8 ± 4.3[B] |
| 280 | Galloyl-castalagin + Pentagalloyl glucose | 39A | 142 ± 12[A] | 33A | 89.0 ± 3.7[B] |
| 280 | Galloyl-castalagin isomer | 47A | 44.9 ± 4.8[A] | 40A | 17.4 ± 1.0[B] |
| 280 | Sum of non identified HBD or tannins | ## | 1799.9 | ## | 361.2 |
| | **Total HBD or tannins** | | **3315.4** | | **788.5** |
| **Hydroxycinnamate derivatives (HCD)**** | | | | | |
| 320 | Sum of non identified HCD | ## | 15.7 | ## | 12.8 |
| | **Total HCD** | | **15.7** | | **12.8** |
| **Flavonol derivatives***** | | | | | |

Table 3 (*Continued*)

| λ (nm) | Tentative identification | Peak no | Quantification mg/100g JPP-MT# | Peak no | Quantification mg/100g JPP-MJ# |
|---|---|---|---|---|---|
| 360 | Ellagic acid pentoside | | | 6C | 9.7 ± 0.1 |
| 360 | Ellagic acid pentoside | | | 9C | 9.3 ± 0.1 |
| 360 | Ellagic acid | 10C | 59.4 ± 4.1[A] | 10C | 53.9 ± 1.9[A] |
| 360 | Myricetin-rhamnoside | 11C | 24.4 ± 1.3[A] | 11C | 15.7 ± 0.3[B] |
| 360 | Quercetin-hexoside | 12C | 16.5 ± 0.6[A] | 12C | 12.0 ± 0.1[B] |
| 360 | Quercetin-hexoside | 13C | 21.8 ± 1.1[A] | 13C | 13.8 ± 0.2[B] |
| 360 | Quercetin-pentoside | 15C | 12 ± 0.2 | | |
| 360 | Quercetin-pentoside | 16C | 14.5 ± 0.4 | | |
| 360 | Quercetin-rhamnoside | 17C | 25.4 ± 1.2[A] | 17C | 18.9 ± 0.4[B] |
| 360 | Sum of non identified flavonol | ## | 169.7 | ## | 184.9 |
| | **Total flavonol** | | **356.5** | | **318.1** |
| **Anthocyanins****** | | | | | |
| 520 | Delphinidin-3-glucoside | 1D | 176 ± 7.5[A] | 1D | 108.1 ± 3.7[B] |
| 520 | Cyanidin-3-glucoside | 2D | 1632 ± 69.1[A] | 2D | 1039.1 ± 47.1[B] |
| 520 | Pelargonidin-3-glucoside | 3D | 4.7 ± 0.2[A] | 3D | 1.9 ± 0.03[B] |
| 520 | Peonidin-3-glucoside | | | 4D | 3.5 ± 0.1 |
| 520 | Sum of non identified anthocyanins | | 0.0 | | 0.0 |
| | **Total anthocyanins** | | **1813** | | **1153** |
| | **Total free phenolic compounds** | | **5500.6** | | **2272.4** |
| | **MATRIX-BOUND PHENOLIC COMPOUNDS - Alkaline Hydrolysis** | | | | |
| **Hydroxybenzoate derivatives (HBD) or tannins*** | | | | | |
| 280 | Gallic acid | 4A | 8.0 ± 0.5[B] | 1A | 38.4 ± 12.8[A] |
| 280 | Protocatechuic acid | 7A | 12.2 ± 1.2[B] | 3A | 33.9 ± 5.0[A] |
| 280 | Sum of non identified HBD or tannins | ## | 23.6 | ## | 23.6 |
| | **Total HBD or tannins** | | **43.8** | | **95.9** |
| **Hydroxycinnamate derivatives (HCD)**** | | | | | |
| 320 | Sum of non identified HCD | ## | **28.1** | ## | **49.8** |
| | **Total HCD** | | **28.1** | | **49.8** |

Table 3 (*Continued*)

| λ (nm) | Tentative identification | Peak no | Quantification mg/100g JPP-MT[#] | Peak no | Quantification mg/100g JPP-MJ[#] |
|---|---|---|---|---|---|
| **Flavonol derivatives*** ** | | | | | |
| 360 | Ellagic acid | 16C | 46.6 ± 3.2[A] | 11C | 32.8 ± 6.3[B] |
| 360 | Sum of non identified flavonol | ## | 108.3 | ## | 187.4 |
| | **Total Flavonol** | | **155.0** | | **220.2** |
| | **Total matrix-bound- alkaline hydrolysis** | | **226.9** | | **365.9** |
| | **MATRIX-BOUND POLYPHENOLS-Acid Hydrolysis** | | | | |
| **Hydroxybenzoate derivatives (HBD) or tannins*** | | | | | |
| 280 | Gallic acid | 2A | 1.3 ± 0.2[A] | 1A | 2.3 ± 1.4[A] |
| 280 | Protocatechuic acid | 5A | 0.2 ± 0.1[B] | 4A | 0.5 ± 0.02[A] |
| 280 | Sum of non identified HBD or tannins | ## | 8.7 | ## | 39.5 |
| | **Total HBD or tannins** | | **10.2** | | **42.3** |
| **Hydroxycinnamate derivatives (HCD)** ** | | | | | |
| 320 | Sum of non identified HCD | ## | 2.6 | ## | 3.9 |
| | **Total HCD** | | **2.6** | | **3.9** |
| **Flavonol derivatives*** ** | | | | | |
| 360 | Ellagic acid | 6C | 2.5 ± 0.4[A] | 6C | 2.6 ± 0.6[A] |
| **360** | Sum of non identified flavonol | ## | 4.3 | ## | 12.5 |
| | **Total flavonol** | | **6.8** | | **15.1** |
| | **Total matrix-bound- acid hydrolysis** | | **19.6** | | **61.3** |
| | **Total matrix-bound phenolic compounds** | | **246.5** | | **427.2** |

[#] Mean ± standard deviation; HHDP= hexahydroxydiphenic acid. Different letters are significantly different $p < 0.05$. * Hydroxybenzoate derivatives (HBD) were quantified as equivalent of catechin; ** Hydroxycinnamate derivatives (HCD)** were quantified as equivalent of p-coumaric acid *** Flavonol derivatives were quantified as equivalent of Kaempferol-3βD-glucopyranoside; **** The anthocyanins were quantified as equivalent of cyanidin-3-glucoside. The compounds identified by HPLC-MS/MS were quantified by its corresponding standard. ## Individual quantification of the all not identified compounds can be seen in Supplementary Material, Tables S4 and S5.

elution tellimagrandin I + castalin, and the co-elution tellimagrandin I isomer + trisgalloyl-HHDP-glucose isomer that were respectively 12, 9 and 8 times higher in JPP-MT than in JPP-MJ (Table 3). Even with the two co-elutions of tellimagrandin I and its isomer, their content in JPP-MJ (peak 9A and 16A, Table 2) was lower than reported by Plaza et al. (2016) in the same species (9.8 *vs* 51.3 and 14.8 *vs* 74.8 mg 100 $g^{-1}$).

The compounds that were identified for the first time in JPPs exhibited small differences between the species studied, such as tetragalloylglucose and tetragalloylglucose isomer that had a content 1.6 and 3.4 times higher in JPP-MT compared to JPP-MJ, respectively ($p<0.05$, Table 3). These differences in content may be inherent to the species or climate and soil conditions, as the fruits used for extraction were fully ripe. Despite the differences between species, both JPPs had an elevated content of hydrolyzable tannins when compared to other fruits such as strawberries and the skin of pomegranate (Ambigaipalan et al., 2016; Gasperotti et al., 2013). The expressive content of hydrolyzable tannins in JPP can contribute to better antioxidant effects, since hydrolyzable tannins have been shown to present higher antioxidant capacity than anthocyanins in an HPLC-DAD-ECD-CAD assay that allowed the simultaneous quantification of individual phenolic compounds and assessment of their antioxidant capacity (electrochemical detection) (Plaza et al., 2016). Thus, these compounds are likely implicated in increased serum antioxidant capacity (removal of peroxyl radicals) after JPP intake by humans (Plaza et al., 2016). Besides that, tetragalloylglucose has important inhibitory effects against adhesion of mouse lung carcinoma cells, preventing tumor cell invasion *in vitro* (Saeki et al., 1999).Anthocyanin was the major class of free phenolic compounds in JPP-MJ (50.7% of total free phenolic compounds), which is in agreement with previous data on *M. jaboticaba* and *M. cauliflora* (Alezandro et al., 2013; Inada et al., 2015; Pereira et al., 2017; Plaza et al., 2016). In contrast, JPP-MT had a higher content of

hydrolyzable tannins than anthocyanins (50.3 *vs* 33% of total free phenolic compounds, respectively) (Table 3).

This is the first full report for the anthocyanin composition of *M. trunciflora* species but previous studies on *M. jaboticaba* peel revealed a higher cyanidin 3-glucoside content (2866 and 1964 mg 100 $g^{-1}$) (Leite et al., 2011; Plaza et al., 2016). The content of total anthocyanins differed significantly between the JPPs not only due to the cyanidin 3-glucoside content, but also due to the higher content of delphinidin-3-glucoside and pelargonidin-3-glucoside in JPP-MT compared to JPP-MJ ($p<0.05$, Table 3). Delphinidin-3-glucoside has already been found in jaboticaba (Wu et al., 2013). Despite its minor content, pelargonidin-3-glucoside was identified for the first time in the JPP of both species.

*M. trunciflora* is also a good source of anthocyanins. Cyanidin 3-glucoside, has already been identified in *M. trunciflora* species but not quantified, and no other anthocyanin had been previously reported in this species (Calloni et al., 2015). JPPs have a very elevated content of anthocyanins when compared to other fruit and peel such as pomegranate peel, grape peel and jussara (Caldas et al., 2018; Fischer et al., 2011; Inada et al., 2015). Thus, JPP can reduce the risk of diabetes and obesity, due to the well-documented antioxidative and anti-inflammatory properties of anthocyanins (Guo and Ling, 2015).

The flavonols class amounted to 6.5% and 14% of the total content of free phenolic compounds in JPP-MT and JPP-MJ, respectively. The major compound was ellagic acid that was found at similar amounts in both JPP species (Table 3). A higher content of ellagic acid had been previously reported for *M. jaboticaba* peel (142.8 mg 100 $g^{-1}$ dry weight) (Plaza et al., 2016). Discrepancies may be related to climate differences during fruit growing.

Ellagic acid pentoside was found only in JPP-MJ (19 mg $100g^{-1}$, Table 3), as already reported for *M. jaboticaba* peel and juice (Plaza et al., 2016; Wu et al., 2013). In contrast, other flavonols were identified at higher amounts in JPP-MT when compared to JPP-MJ,

namely myricetin-rhamnoside, quercetin-hexoside and quercetin-rhamnoside ($p<0.05$, Table 3).

### 3.5 Quantification of matrix-bound phenolic compounds

Fruits, in general, have an appreciable amount of matrix-bound phenolic compounds, ranging from 6.5–76% of their total phenolic compound content (Acosta-Estrada et al., 2014). JPP-MT had 246.5 mg of matrix-bound phenolic compounds 100 $g^{-1}$ of peel powder, which amounts to 4.3% of total phenolic compounds (free + bound, Figures 4 and 5, Table 3). Compared to JPP-MT, JPP-MJ had a higher proportion of matrix-bound phenolic compounds (427.2 mg of matrix-bound phenolic compounds 100 $g^{-1}$; Figures 6 and 7, Table 3), which amounted to 15.8% of total phenolic compounds, and is higher than the amount previously reported for jaboticaba peel (11% of bound phenolic compounds) (Inada et al., 2015). This difference can be associated to differences in the cell-matrix composition among different jaboticaba species and to the pectin content because phenolic compounds have a greater affinity for the cell wall (Pinelo et al., 2006).

Most matrix-bound phenolic compounds of JPP-MT and JPP-MJ were linked to cell-wall constituents by ester or ether bonds (92% and 85.7%, respectively) and were observed after alkaline hydrolysis. The total phenolic compound content found after alkaline hydrolysis in JPP-MT was similar to that in a previous report on the peel of *M. jaboticaba* (Inada et al., 2015), whereas JPP-MJ had a content of matrix-bound phenolics about 1.7 times greater than that of JPP-MT (Table 3).

The profile of matrix-bound phenolics released from JPP after alkaline hydrolysis was different to that in a previous study on *M. jaboticaba* (Inada et al., 2015). The discrepancy may be explained because these authors used only DAD detection for compound identification. Only three compounds were identified after alkaline hydrolysis of JPP-MT, the

two major peaks being ellagic acid followed by the co-elution of protocatechuic acid and 4-hydroxybenzoic acid derivative. Gallic acid and ellagic acid have been already reported as matrix-bound phenolics in *M. jaboticaba* (Inada et al., 2015). However, we did not find matrix-bound myricetin and trans-cinnamic acid as previously reported for *M. jaboticaba* (Inada et al., 2015). The same compounds were identified in JPP-MJ after alkaline hydrolysis but the content differed between the species studied (Table 3). While in JPP-MT the major compound was ellagic acid, in JPP-MJ gallic acid was the major one (Table 3). The content of gallic acid and protocatechuic acid in JPP-MJ was 4.8 and 2.3 times higher than in JPP-MT, respectively. The gallic acid content in JPP-MJ was lower than previously reported for this species (Inada et al., 2015). However, JPP-MT had a higher ellagic acid content than JPP-MJ.

The matrix-bound phenolic compounds linked by glycosidic bonds, observed after acid hydrolysis of JPPs, were mostly classified as HBD ($p<0.05$ Table 3). The content of gallic and ellagic acid bound to the matrix through glycosidic bonds was similar in JPP of both species ($p>0.05$, Table 3) but JPP-MT had a lower content of protocatechuic acid than JPP-MJ.

The amount of matrix-bound phenolic compounds was much larger in JPP-MJ than in JPP-MT; however, the compounds identified were the same in both fruit species. Among the phenolic compounds linked by ether or ester bonds, ellagic acid was the major one in JPP-MT. Moreover, ellagic acid was also linked to the cell-matrix by glycosidic bonds since this compound was observed after acid hydrolysis. JPP-MT had a greater amount of ellagic acid linked to the cell matrix through ester and ether bonds than JPP-MJ, which can be associated to interspecies differences in the cell matrix composition.

Matrix-bound phenolic compounds have been pointed out as macromolecular dietary antioxidants as they can reach the lower gastrointestinal tract almost intact and exert direct antioxidant properties before or after being catabolized by colonic microbiota, besides

modulating this microbiota (Mosele et al., 2015; Pérez-Jiménez et al., 2013). Thus, JPP-MJ that had a greater content of matrix-bound phenolic compounds would have greater potential to promote colonic homeostasis, whereas JPP-MT that had greater content of free phenolic compounds, would have greater fraction of bioaccessible phenolics to exert systemic bioactive effects.

## 1. Conclusion

In conclusion, both jaboticaba species had a great diversity of phenolic compounds, and some new compounds were clearly identified for the first time in jaboticaba peel. Among the ellagitannin class, the compounds identified for the first time in jaboticaba peel were trisgalloyl-HHDP-glucose, castalin, galloyl-castalagin and its isomers (Figure 8). Monogalloylgucose, digalloylglucose, trigalloylglucose and tetragalloylglucose, which belong to the gallotannin class, as well as myricetin-hexoside, quercetin-pentoside and quercetin-hexoside, were also identified for the first time in this fruit (Figure 8). *M. jaboticaba* had a greater content of matrix-bound polyphenols than *M. trunciflora* (15.3% vs. 4.3% of total phenolic compounds). We have identified three monomers, namely ellagic acid, gallic acid and protocatechuic acid that were linked to the JPP matrix by ester/ether and glycosidic bonds. Besides that, *M. trunciflora* peel had higher free phenolic compound content than that of *M. jaboticaba*. Hydrolyzable tannins were the major free phenolic compounds of *M. trunciflora*, followed by anthocyanins, whereas anthocyanins were the major ones of *M. trunciflora* followed by hydrolyzable tannins. Cyanidin 3-glucoside was the major free phenolic compound identified in both species, followed by tetragalloylglucose isomer, besides trigalloylglucose for *M. trunciflora*, and delphinidin 3-glucoside for *M. jaboticaba*. The large diversity and content of free and matrix-bound phenolic compounds observed in JPPs can contribute to health maintenance. However, it remains to be determined whether the

differences in polyphenol composition between the two jaboticaba species would result in different health promoting effects.

**Acknowledgements**

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**Figure captions**

**Figure 1:** HPLC-PDA chromatogram of phenolic compound standards at 280 nm. (1) gallic acid, (2) protocatechuic acid, (3) 4-hydroxybenzoic acid, (4) catechin, (5) vanillic acid, (6) caffeic acid, (7) syringic acid, (8) p-coumaric acid, (9) t-ferulic acid, (10) synapic acid, (11) myricetin, (12) kaempferol-3βD-glucopyranoside, (13) t-cinnamic acid and (14) quercetin.

**Figure 2:** Chromatograms of the free phenolic compounds fraction from the peel powder of *Myrciaria trunciflora* at 280 nm to assess the hydroxybenzoate derivatives (A), at 320 nm to assess hydroxycinnamate derivatives (B), at 360 nm to assess flavonols (C) and at 520 nm to assess anthocyanins (D).

**Figure 3:** Chromatograms of the free phenolic compounds fraction from the peel powder of *Myrciaria jaboticaba* at 280 nm to assess the hydroxybenzoate derivatives (A), at 320 nm to assess hydroxycinnamate derivatives (B), at 360 nm to assess flavonols (C) and at 520 nm to assess anthocyanins (D).

**Figure 4:** Chromatograms obtained after alkaline hydrolysis of the matrix-bound phenolic compounds fraction from the peel powder of *Myrciaria trunciflora* adquired at 280 nm (A), 320 nm (B) and 360 nm (C).

**Figure 5:** Chromatograms obtained after acid hydrolysis of the matrix-bound phenolic compounds fraction from the peel powder of *Myrciaria trunciflora* adquired at 280 nm (A), 320 nm (B) and 360 nm (C).

**Figure 6:** Chromatograms obtained after alkaline hydrolysis of the matrix-bound phenolic compounds fraction from *Myrciaria jaboticaba* adquired at 280 nm (A), 320 nm (B) and 360 nm (C).

**Figure 7:** Chromatograms obtained after acid hydrolysis of the matrix-bound phenolic compounds fraction from *Myrciaria jaboticaba* adquired at 280 nm (A) 320 nm (B) and 360 nm (C).

**Figure 8:** New phenolic compounds found in jaboticaba peel (*M. jaboticaba* and *M. trunciflora*).

Figure 1

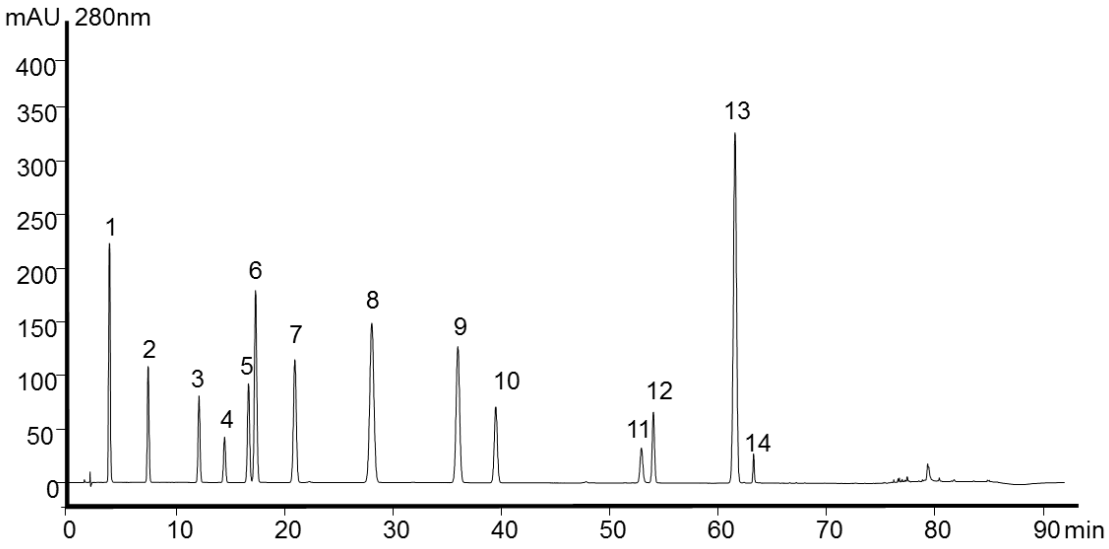
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Figure 2

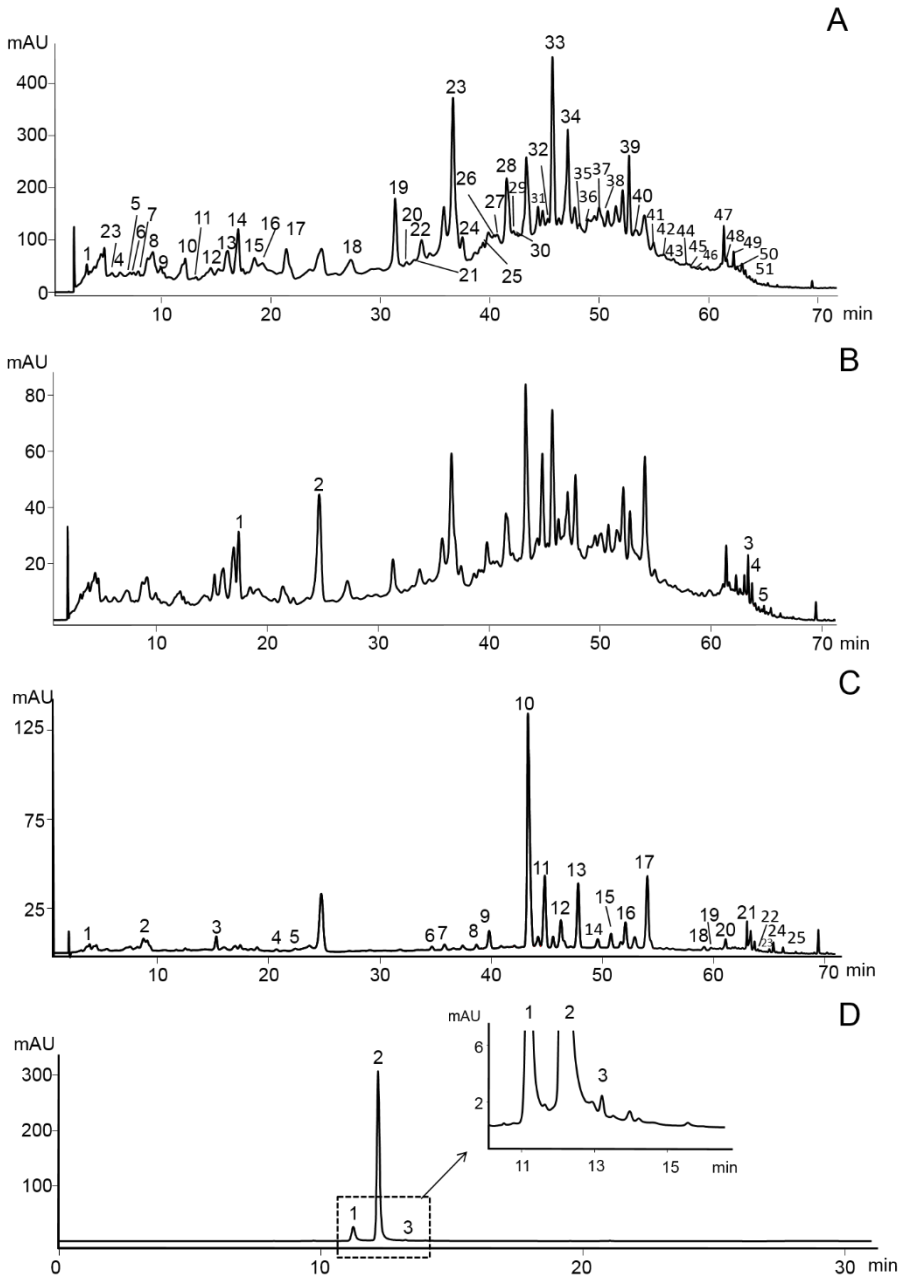

Figure 3

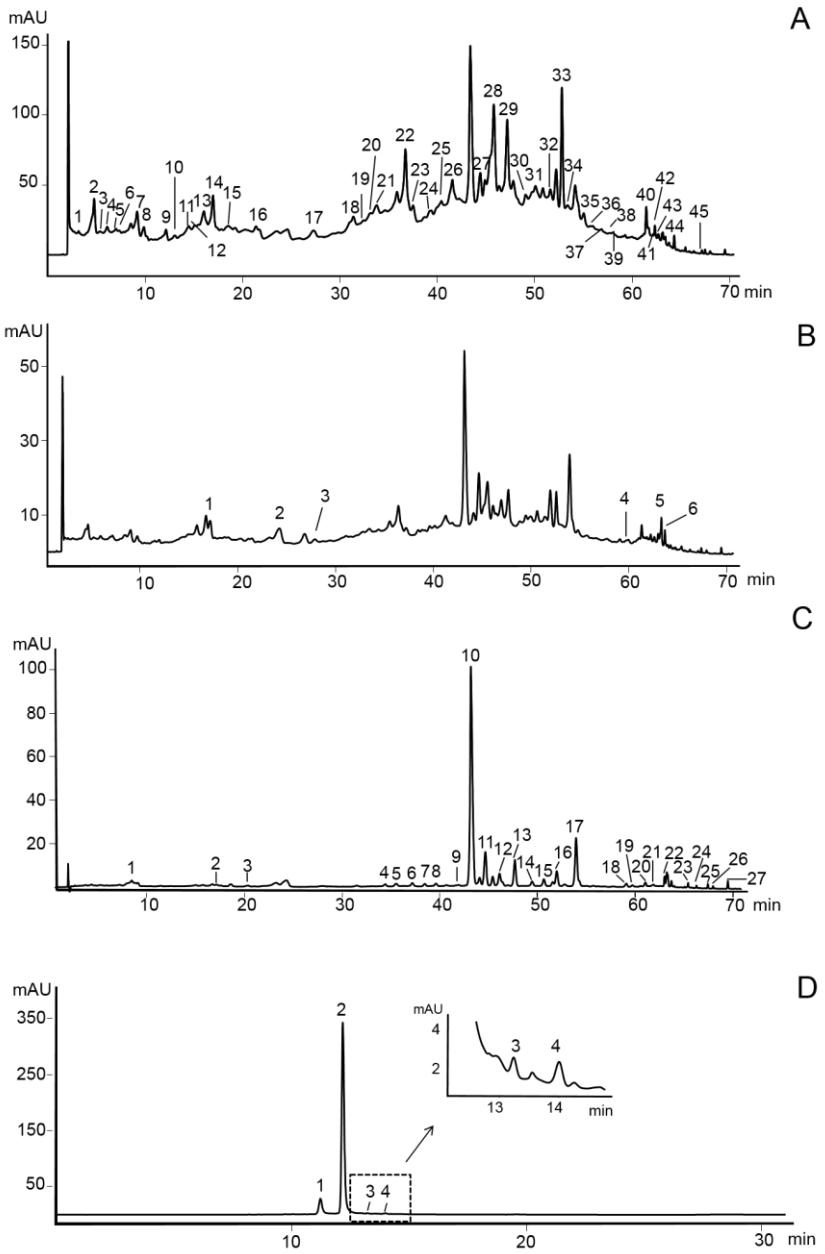
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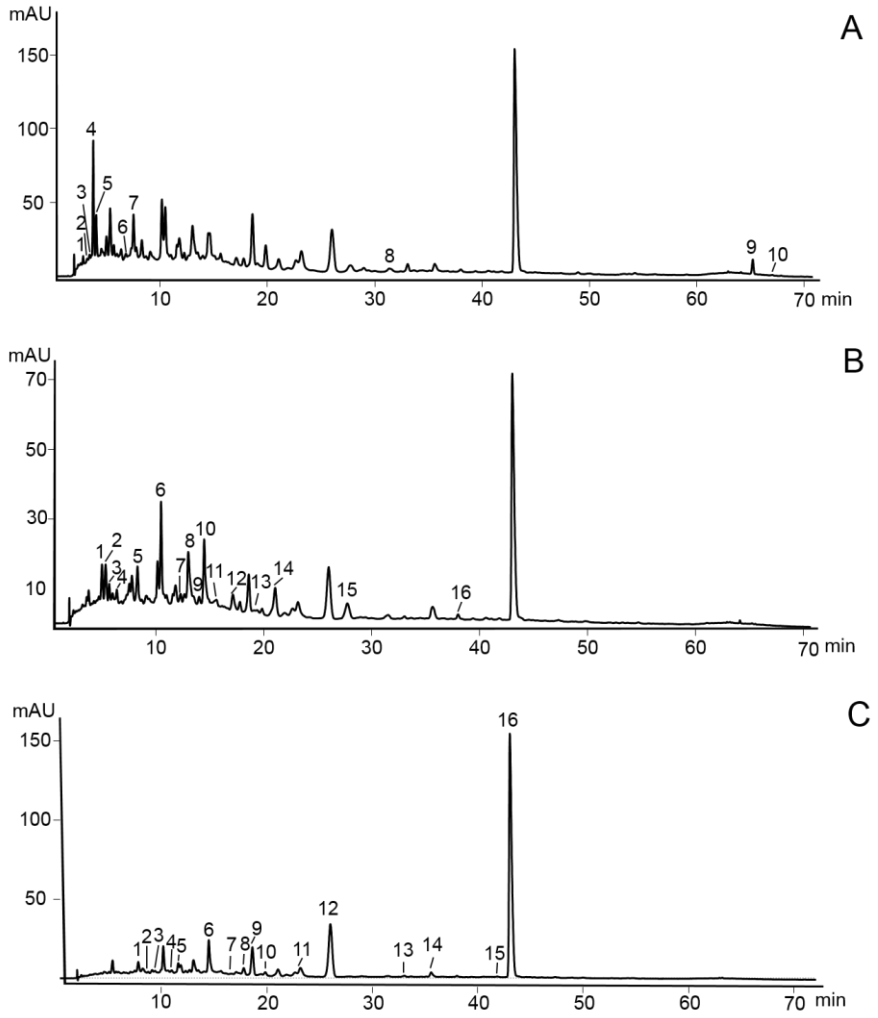
Figure 4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30
40
50
60
70 min

Figure 5

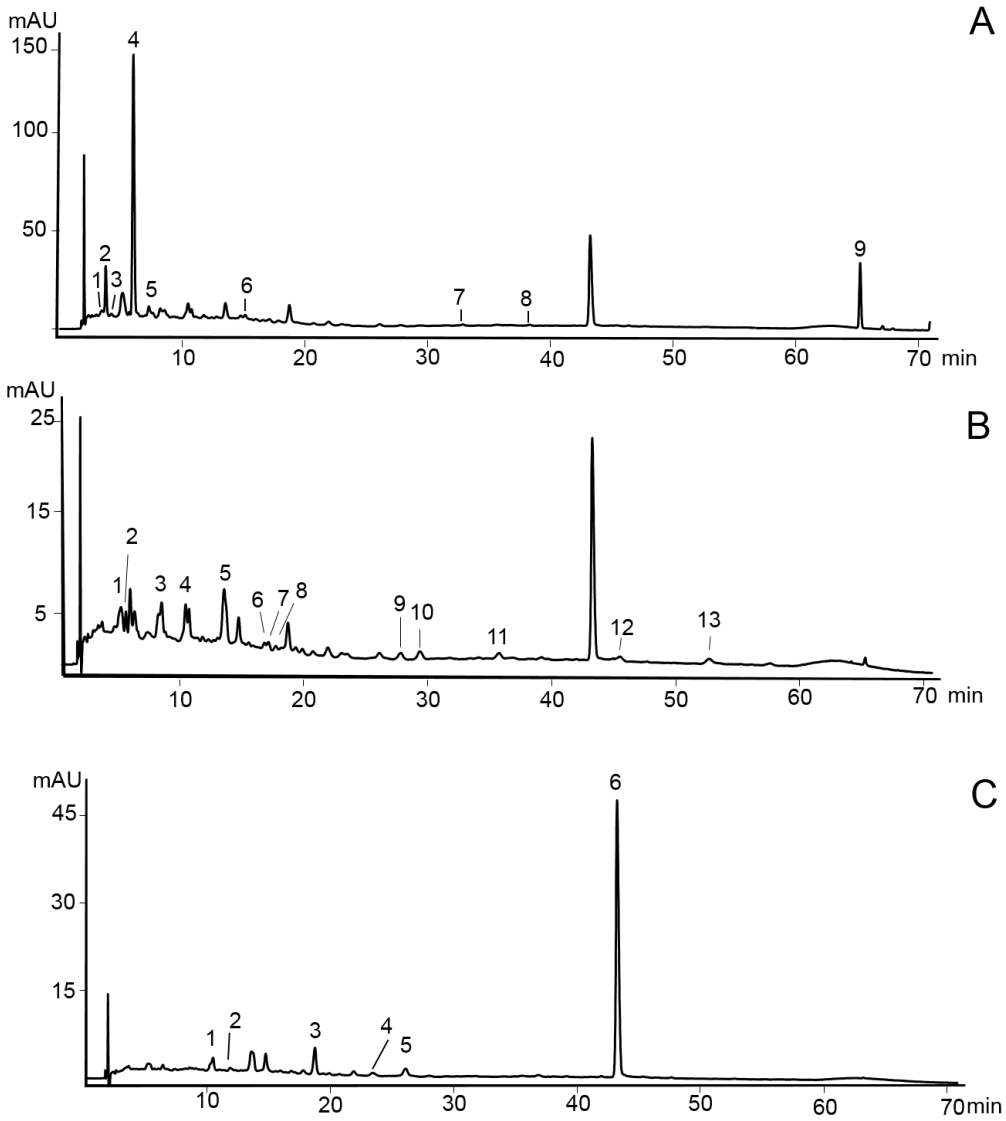

A
mAU
150
100
50
1
2
3
4
5
6
7
8
9
10
20
30
40
50
60
70
min
B
mAU
25
15
5
1
2
3
4
5
6
7
8
9
10
11
12
13
10
20
30
40
50
60
70
min
C
mAU
45
30
15
1
2
3
4
5
6
10
20
30
40
50
60
70
min

Figure 6

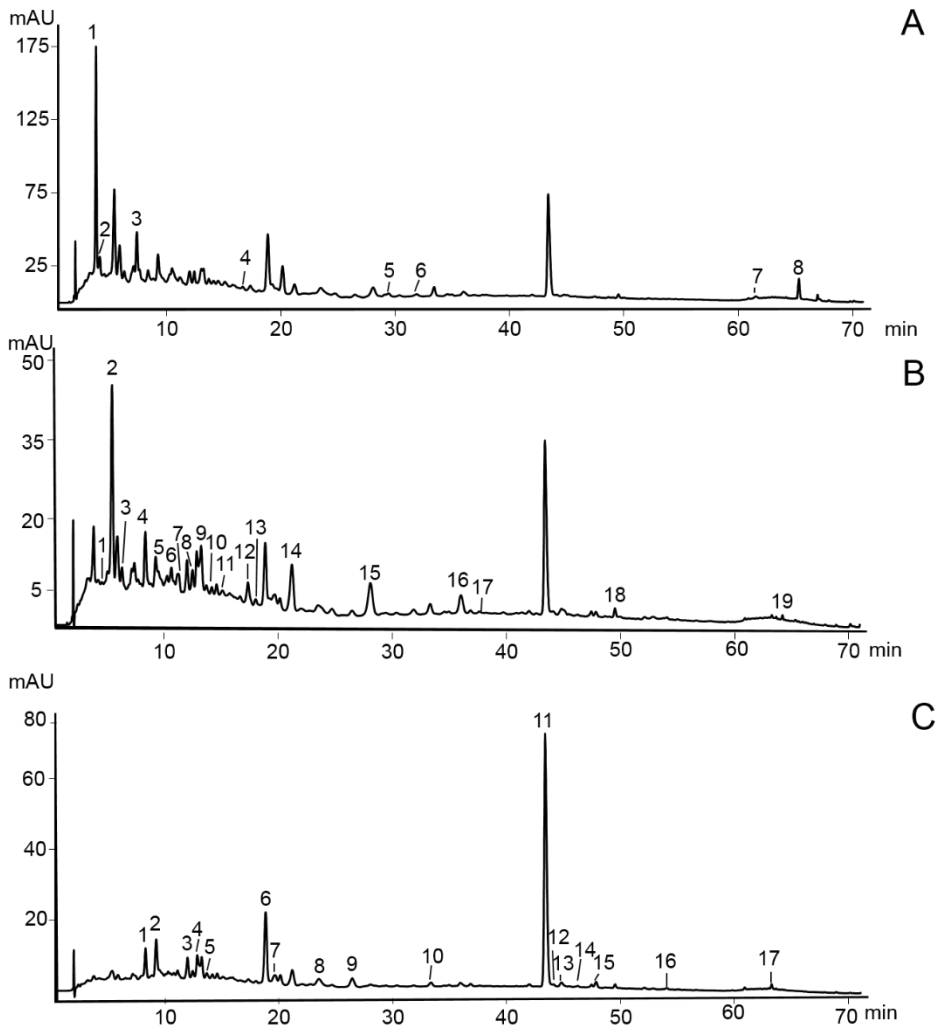

A
mAU
175
125
75
25
1
2
3
4
5
6
7
8
10
20
30
40
50
60
70
min
B
mAU
50
35
20
5
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
10
20
30
40
50
60
70
min
C
mAU
80
60
40
20
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
10
20
30
40
50
60
70
min

Figure 7

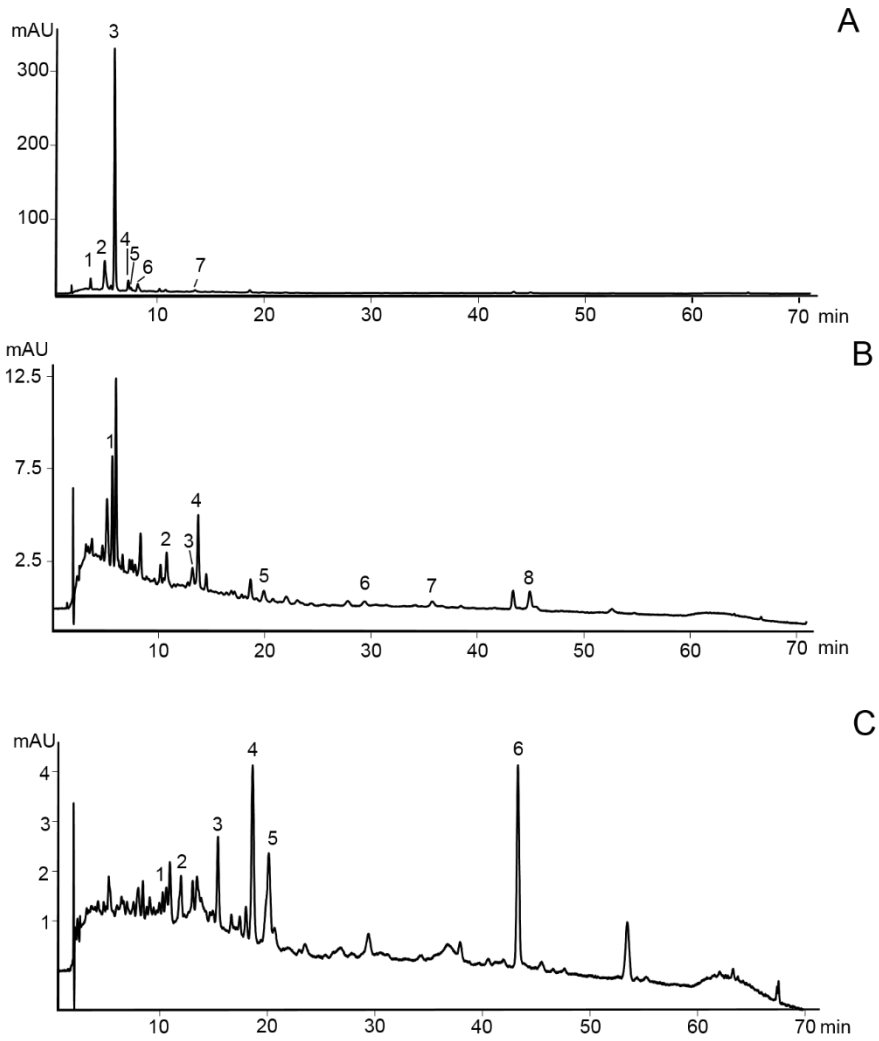
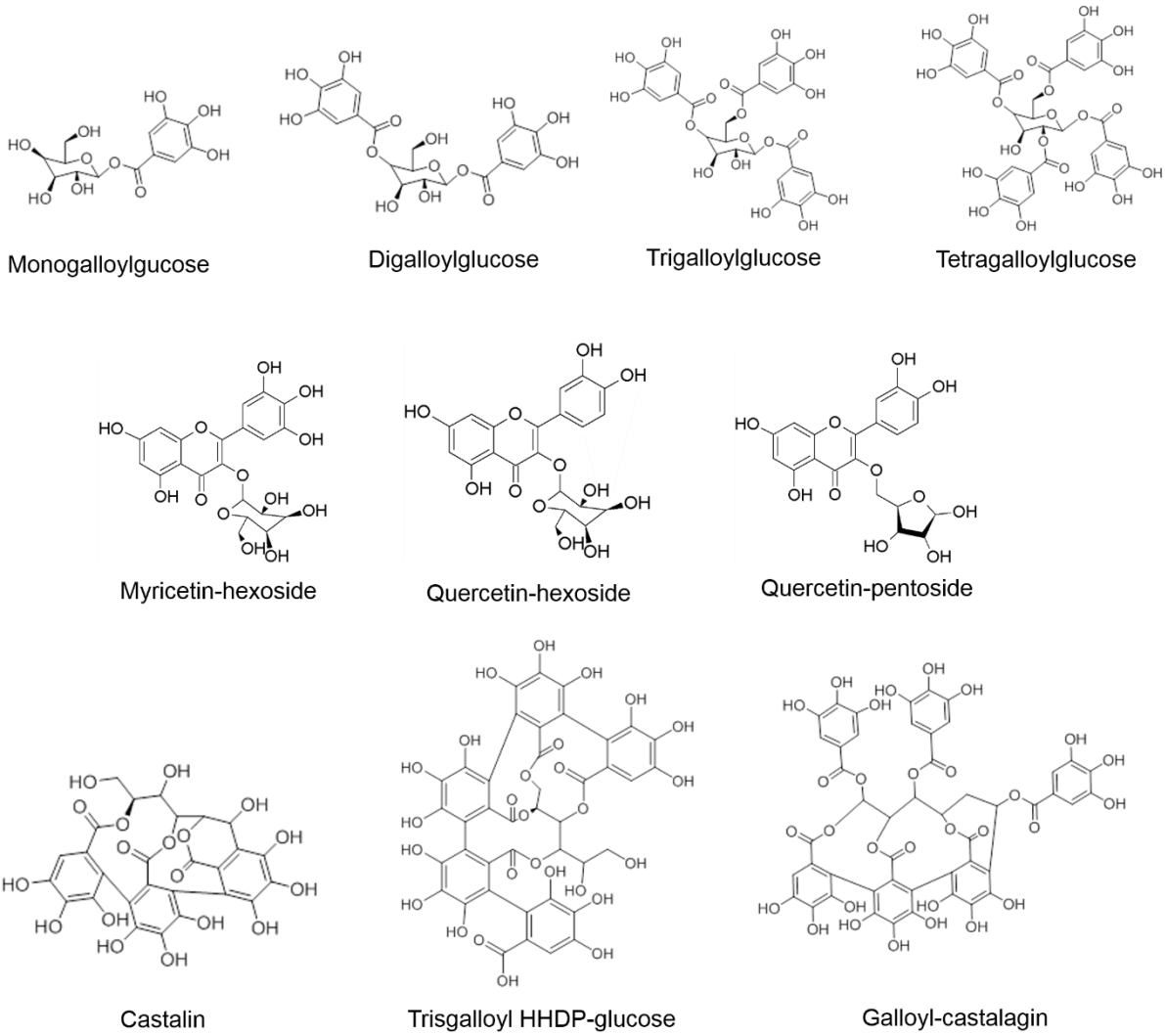
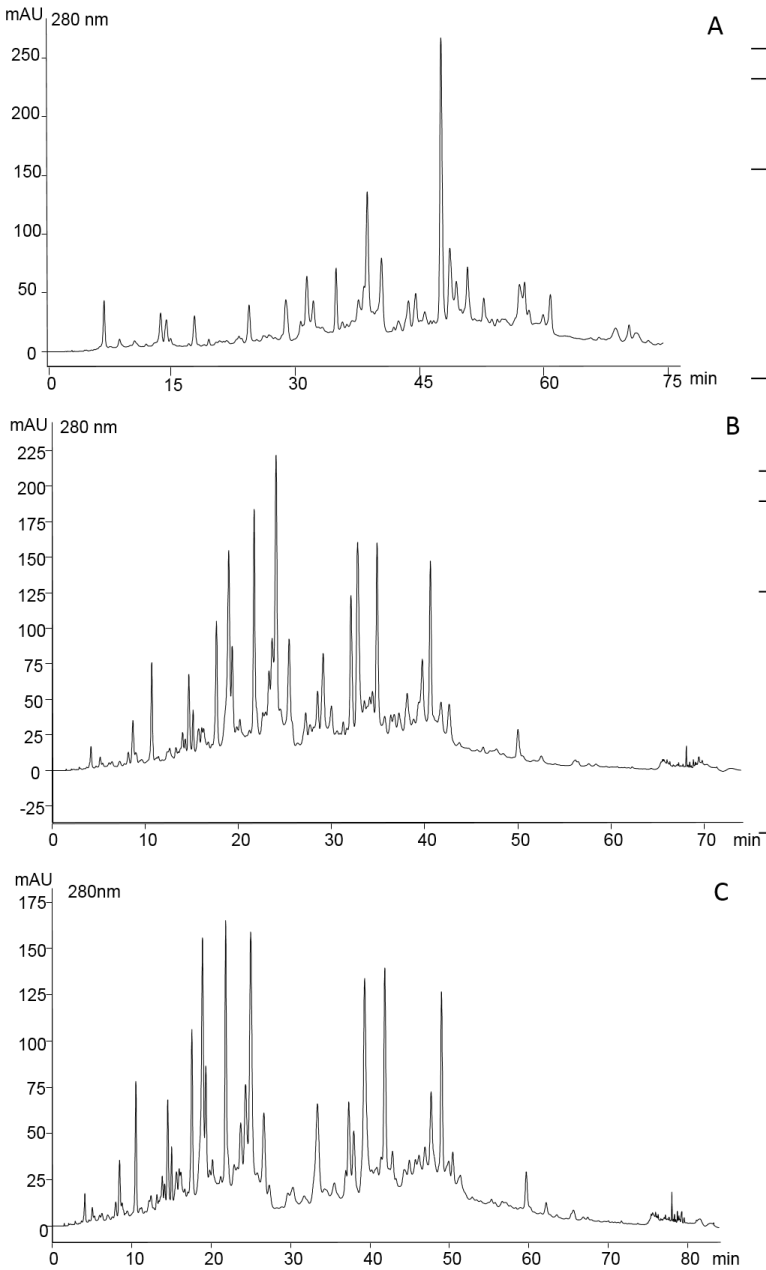

A
mAU
300
200
100
1
2
3
4
5
6
7
10
20
30
40
50
60
70 min
B
mAU
12.5
7.5
2.5
1
2
3
4
5
6
7
8
10
20
30
40
50
60
70 min
C
mAU
4
3
2
1
1
2
3
4
5
6
10
20
30
40
50
60
70 min

Figure 8


Monogalloylgucose
Digalloylglucose
Trigalloylglucose
Tetragalloylglucose
Myricetin-hexoside
Quercetin-hexoside
Quercetin-pentoside
Castalin
Trisgalloyl HHDP-glucose
Galloyl-castalagin

## Supplementary material




| Method 1 | |
|---|---|
| Time (min) | Mobile phase B (%) |
| 40 | 16 |
| 60 | 30 |
| 62 | 75 |
| 67 | 75 |
| 69 | 0 |
| 74 | 0 |

| Method 2 | |
|---|---|
| Time (min) | Mobile phase B (%) |
| 15 | 6 |
| 50 | 16 |
| 60 | 25 |
| 62 | 80 |
| 67 | 80 |
| 69 | 0 |
| 74 | 0 |

| Method 3 | |
|---|---|
| Time (min) | Mobile phase B (%) |
| 15 | 6 |
| 25 | 6 |
| 60 | 16 |
| 70 | 25 |
| 72 | 80 |
| 77 | 80 |
| 79 | 0 |
| 84 | 0 |

**Figure S1**: Different gradients used to select the method that best separates phenolic compounds from JPPs. Mobile phase A was composed of 5% (v/v) methanol in acidified water (0.1% (v/v) of formic acid) and mobile phase B was 0.1% (v/v) of formic acid in acetonitrile.

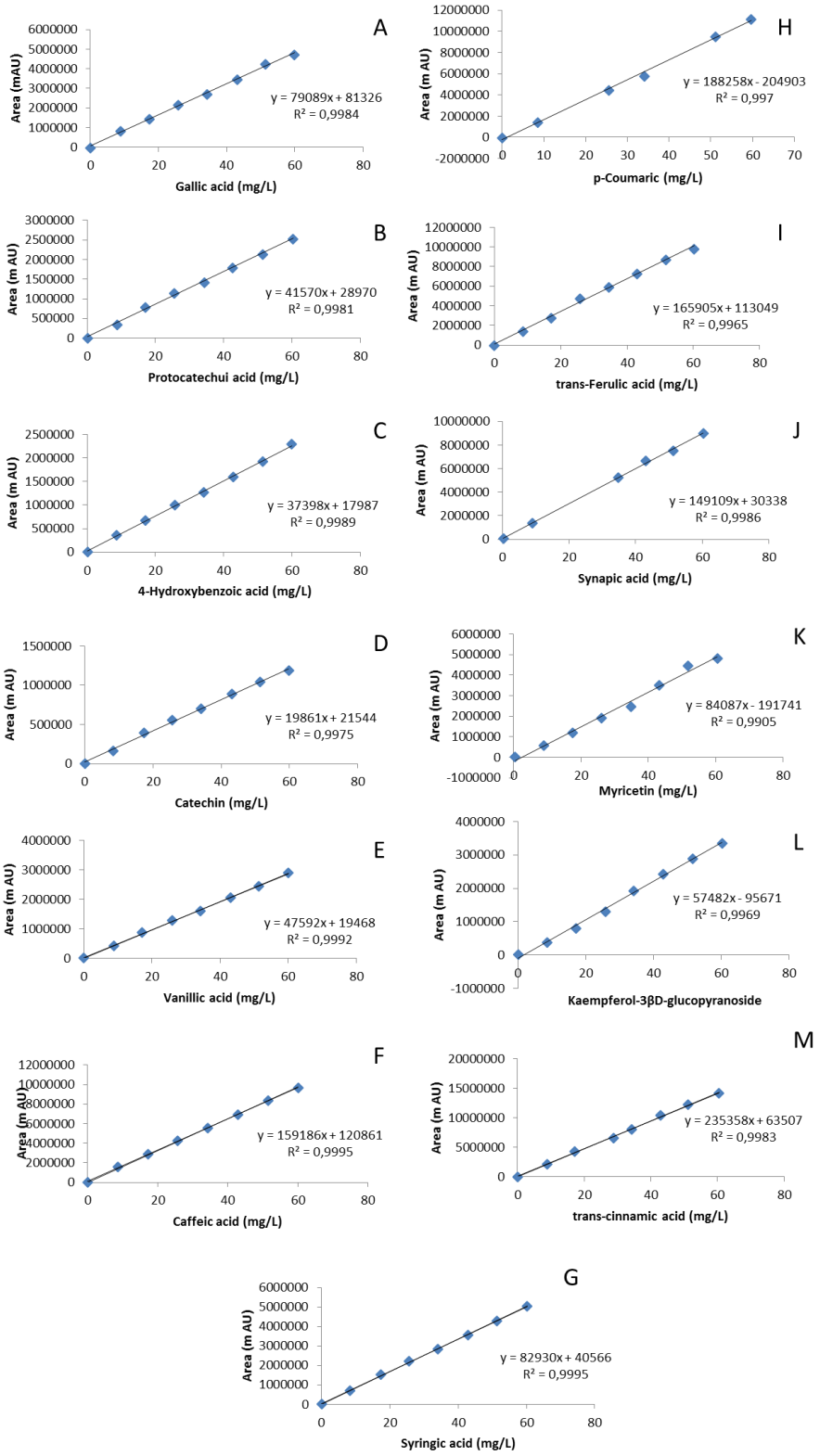



**Figure S2:** Calibration curves for phenolic compounds. (A) Gallic acid, (B) Protocatechuic acid, (C) 4-Hydroxybenzoic acid, (D) Catechin, (E) Vanillic acid, (F) Caffeic acid, (G) Syringic acid, (H) p-Coumaric acid, (I) trans-Ferulic acid, (J) Synapic acid, (K) Myricetin, (L) Kaempferol-3βD-glucopyranoside, (M) trans-Cinnamic acid.

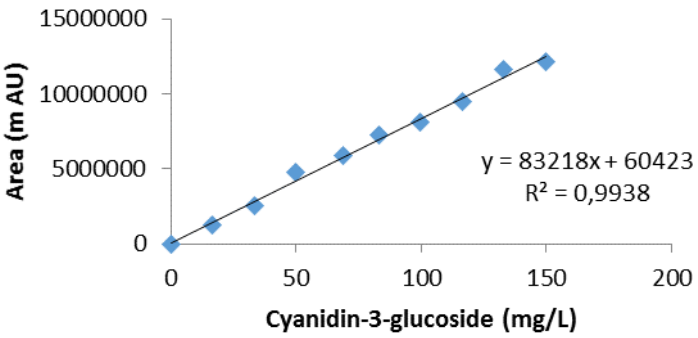



**Figure S3:** Calibration curve for cyanidin-3-glucoside.

**Table S1:** Parameters used for method validation as preconized in the International Conference on Harmonization Guidelines.

| Standard | LoQ | LoD | Precision* | | | | Accuracy | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Repeatability (intraday) n=3 | | | Precision (inter-day) n=10 | Fortified at three different concentrations (n=3)** | | |
| | | | Low (LoQ) | Medium (30 ppm) | High (60 ppm) | Intermediate (25 ppm) | 80% | 100% | 120% |
| Gallic acid | x | x | x | x | x | x | | | |
| Protocatechuic acid | x | x | x | x | x | x | | | |
| 4-hydroxybenzoic acid | x | x | x | x | x | x | | | |
| Catechin | x | x | x | x | x | x | | | |
| Cafeic acid | x | x | x | x | x | x | | | |
| Vanillic acid | x | x | x | x | x | x | | | |
| Syringic acid | x | x | x | x | x | x | x | x | x |
| p-coumaric acid | x | x | x | x | x | x | x | x | x |
| Trans-ferulic acid | x | x | x | x | x | x | | | |
| Trans-cinnamic acid | x | x | x | x | x | x | | | |
| Synapic acid | x | x | x | x | x | x | | | |
| Myricetin | x | x | x | x | x | x | | | |
| Kaempferol-3Bd-glucopyranoside | x | x | x | x | x | x | x | x | x |

*Method precision was expressed as the coefficient of variation (CV) from retention time and peaks area values. **Accuracy was expressed as the percentage of phenolic recovery after sample fortification with three standards (syringic acid, kaempferol-3βD-glucopyranoside, and p-coumaric acid). The accuracy was analyzed after sample contamination, in triplicate, and the average recovery was determined by comparison with the non-fortified sample.

**Table S2:** Validation data for the method to analyze fruit phenolic compounds.

| Phenolic compound | λ (nm) | RT (min) (n =3) | R* (n=3) | Linear range (mg/L) | Regression equation | p** | $R^2$ | Instrumental*** | | Method# | | Repeatability intra-day precision, CV (%) | | | | | | Intermediate inter-day precision, CV (%) n = 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Low level (n=3) | | Medium level (n=3) | | High level (n=3) | | | |
| | | | | | | | | LOD | LOQ | LOD | LOQ | RT | Peak area | RT | Peak area | RT | Peak area | RT | Peak area |
| GA | 280 | 3.80 | -- | 0.04 - 60 | y = 79089x + 81326 | 0.81 | 0.998 | 0.012 | 0.037 | 0.019 | 0.058 | 0.50 | 4.84 | 0.41 | 0.36 | 0.21 | 1.80 | 0.46 | 6.19 |
| Pro | 280 | 7.34 | 13.21 | 0.08 - 60 | y = 41570x + 28970 | 0.06 | 0.997 | 0.027 | 0.083 | 0.042 | 0.129 | 0.47 | 5.41 | 0.40 | 0.18 | 0.20 | 0.87 | 0.41 | 6.42 |
| 4-HBA | 280 | 12.02 | 14.91 | 0.06 - 60 | y = 37398x + 17987 | 0.10 | 0.999 | 0.020 | 0.062 | 0.031 | 0.096 | 0.16 | 3.89 | 0.20 | 0.42 | 0.09 | 0.72 | 0.37 | 4.14 |
| Cat | 280 | 14.37 | 6.84 | 0.08 - 60 | y = 19861x + 21544 | 0.21 | 0.997 | 0.026 | 0.078 | 0.040 | 0.122 | 0.23 | 3.92 | 0.18 | 0.33 | 0.08 | 3.51 | 0.28 | 2.82 |
| Van | 280 | 16.58 | 5.95 | 0.03 - 60 | y = 47592x + 19468 | 0.12 | 0.999 | 0.008 | 0.025 | 0.012 | 0.039 | 0.17 | 6.82 | 0.12 | 0.34 | 0.05 | 0.69 | 0.21 | 0.66 |
| Caf | 320 | 17.23 | -- | 0.02 - 60 | y = 159186x + 120861 | 0.96 | 0.999 | 0.006 | 0.017 | 0.009 | 0.026 | 0.04 | 6.46 | 0.12 | 0.38 | 0.05 | 0.93 | 0.24 | 6.21 |
| Syr | 280 | 20.85 | 9.5 | 0.02 - 60 | y = 82930x + 40566 | 0.24 | 0.999 | 0.008 | 0.024 | 0.012 | 0.037 | 0.19 | 2.43 | 0.13 | 0.44 | 0.07 | 0.64 | 0.29 | 4.80 |
| p-Cou | 320 | 27.93 | 18.97 | 0.03 - 60 | y = 188258x - 204903 | 0.06 | 0.997 | 0.009 | 0.029 | 0.014 | 0.045 | 0.18 | 5.03 | 0.07 | 0.45 | 0.06 | 0.78 | 0.37 | 1.25 |
| trans-fer | 320 | 35.90 | 12.01 | 0.03 - 60 | y = 165905x + 113049 | 0.53 | 0.996 | 0.011 | 0.033 | 0.017 | 0.051 | 0,14 | 9.17 | 0.03 | 0.23 | 0.07 | 0.76 | 0.18 | 4.13 |
| Syn | 320 | 39.41 | 6.10 | 0.26 - 60 | y = 149109x + 30338 | 0.26 | 0.998 | 0.085 | 0.258 | 0.132 | 0.402 | 0.04 | 3.83 | 0.02 | 0.24 | 0.07 | 0.83 | 0.23 | 2.80 |
| Myr | 360 | 52.87 | -- | 0.50 - 60 | y = 84087x - 191741 | 0.86 | 0.990 | 0.166 | 0.503 | 0.258 | 0.785 | 0.03 | 2.96 | 0.02 | 2.09 | 0.06 | 0.68 | 0.15 | 5.66 |
| Kae-3DGlp | 360 | 54.01 | 2.49 | 0.08 - 60 | y = 57482x - 95671 | 0.67 | 0.996 | 0.028 | 0.084 | 0.043 | 0.131 | 0.01 | 4.71 | 0.02 | 0.45 | 0.05 | 0.89 | 0.13 | 5.27 |
| trans-cin | 280 | 61.57 | 76.88 | 0.03 - 60 | y = 235358x + 63507 | 0.75 | 0.998 | 0.010 | 0.032 | 0.015 | 0.049 | 0.004 | 3.69 | 0.02 | 0.37 | 0.04 | 0.70 | 0.14 | 1.84 |

*Peak resolution; **p value for the lack of fitness; *** LOD and LOQ for the phenolic standard solution (mg/L), #LOD and LOQ analysis of phenolic compounds in jaboticaba peel samples (mg/100 g dry weight of the jaboticaba peel). RT= retention time; CV= coefficient of variation ; GA=gallic acid; Pro= Protocatechuic acid; 4-HBA= 4-Hydroxybenzoic acid; Cat= catechin; Van= Vanillic acid; Caf= Caffeic acid; Syr= Syringic acid; p-Cou= p-coumaric acid; trans-fer= trans-ferulic acid; Syn= Synapic acid; Myr= Myricitin; Kae-3DGlp= Kaempferol-3βD-glucopyranoside; trans-cin= trans-cinnamic acid; Low level= LOQ of the each compounds; Medium level= 30 ppm for all compounds; High level = 60 ppm for all compounds.

**Table S3:** Accuracy test for the method to analyze fruit phenolic compounds.

| Phenolic compounds | Accuracy | | |
|---|---|---|---|
| | Average recovery low level (%) * | Average recovery medium level (%)** | Average recovery high level (%) *** |
| Syringic acid | 109 | 109 | 103 |
| p-Coumaric acid | 87 | 80 | 85 |
| Kae-3DGlp | 104 | 108 | 94 |

*The low level was the LOQ of each compound; ** The medium level was fortified with 80% of the polyphenols present in the sample; ** The medium level was the was fortified with 100% of the polyphenols present in the sample ; *** The high level was fortified with 120% of the polyphenols present in the sample; Kae-3DGlp= Kaempferol-3βD-glucopyranoside

**Table S4:** Compound not identified in free and matrix-bound phenolic compounds in the peel powder of *Myrciaria trunciflora* (JPP-MT) by HPLC-DAD coupled to LC-TRAP-MS/MS and LC-q-TOF-MS/MS.

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| **FREE POLYPHENOLS** | | | | | | |
| **Hydroxybenzoate derivatives (HBD) or tannins** | | | | | | |
| 1A | 280 | 3.1 ± 0.1 | 216, 276 | **951**: 907, 783, 605 | | # |
| 3A | 280 | 5.4 ± 0.05 | 216, 269 | | | 5.8 ± 1.7 |
| 4A | 280 | 6.1 ± 0.04 | 216, 271 | | | 11.1 ± 1.2 |
| 6A | 280 | 7.3 ± 0.1 | 262, 293 | **797:** 725 | **797.0500:** 300.9974 (2); 275.018 | 10.9 ± 7.5 |
| 7A | 280 | 7.8 ± 0.05 | 216, 276 | | | 7.4 ± 1.9 |
| 11A | 280 | 13.0 ± 0.04 | 258 | | | 2.7 ± 0.6 |
| 15A | 280 | 18.3 ± 0.04 | 215, 275 | | | 60.3 ± 8.0 |
| 16A | 280 | 19.0 ± 0.19 | 204, 283 | | | 40.9 ± 9.3 |
| 18A | 280 | 27.2 ± 0.1 | 215, 270 | **784:** 765, 301 | **784.0840 (2):** 765.0613; 300.9969(2) | 105 ± 7.9 |
| 20A | 280 | 32.29 ± 0.04 | 216, 275 | | | 6.3 ± 1.1 |
| 21A | 280 | 33.0 ± 0.05 | 216, 257 | | | 7.7 ± 1.2 |
| 22A | 280 | 33.7 ± 0.04 | 214, 260 | | | 73.1 ± 6.0 |
| 23A | 280 | 36.6 ± 0.1 | 215, 268 | **784:** 765, 631, 301 | **784.0859 (2):** 765.0615; 300.9972 | 594 ± 32 |
| 25A | 280 | 39.2 ± 0.3 | 216, 268 | | | 19.9 ± 3.6 |
| 26A | 280 | 40.3 ± 0.03 | 216, 277 | | | 2.9 ± 0.8 |
| 27A | 280 | 40.6 ± 0.1 | 216, 275 | **783:** 765, 481 | | 13.9 ± 2.0 |
| 28A | 280 | 41.5 ± 0.1 | 216, 278 | **859:** 785, 765 | **859.0811(2):** 785.0841; 450.9926 | 217 ± 16 |
| 29A | 280 | 42.0 ± 0.11 | 216, 271 | | | 11.8 ± 2.1 |

Table S4 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 30A | 280 | 42.4 ± 0.1 | 216, 275 | **937:** 893, 767, 741, 681, 301 | | 2.9 ± 0.3 |
| 32A | 280 | 45.2 ± 0.1 | 216, 270 | | | 14 ± 3.9 |
| 33A | 280 | 45.7 ± 0.1 | 218, 273 | **860:** 785, 765, 466 | **860.0915(2):** 765.0605; 300.9971(2); 169.0096; 785.0451 | 411 ± 6 |
| 35A | 280 | 48.1 ± 0.02 | 218, 277 | | | 4.4 ± 0.3 |
| 36A | 280 | 48.9 ± 0.02 | 216, 269 | | | 14.6 ± 1.6 |
| 37A | 280 | 49.9 ± 0.1 | 217, 275 | **860:** 785, 765, 275,195 | | 41.9 ± 3.4 |
| 38A | 280 | 51.5 ± 0.1 | 217, 260 | **933:** 451, 301 | | 39.9 ± 2.8 |
| 40A | 280 | 53.2 ± 0.01 | 217, 264 | | | 13.9 ± 1.4 |
| 41A | 280 | 54.9 ± 0.01 | 217,278 | | | 20.4 ± 2.3 |
| 42A | 280 | 55.9 ± 0.02 | 217,269 | | | 9.4 ± 0.3 |
| 43A | 280 | 56.7 ± 0.02 | 217,278 | | | 3.0 ± 0.2 |
| 44A | 280 | 57.9 ± 0.02 | 217,258 | | | 4.1 ± 0.3 |
| 45A | 280 | 58.2 ± 0.02 | 217,280 | | | 1.7 ± 0.4 |
| 46A | 280 | 58.6 ± 0.1 | 218, 282 | | **489.0681:** 299.9876; 300.9963 | 0.4 ± 0.1 |
| 48A | 280 | 61.6 ± 0.1 | 217, 278 | **609:** 463, 301 | **489.0673:** 299.9884; 300.9964 | 7.5 ± 0.3 |
| 49A | 280 | 62.3 ± 0.01 | 217,280 | | | 15.7 ± 0.5 |
| 50A | 280 | 62.6 ± 0.01 | 215,269 | | | 3.2 ± 0.3 |
| 51A | 280 | 64.2 ± 0.01 | 216,278 | | | 1.2 ± 0.1 |
| **Hydroxycinnamate derivatives (HCD)** | | | | | | |

Table S4 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 1B | 320 | 17.3 ± 0.04 | 238, 310 | | | 3.9 ± 0.2 |
| 2B | 320 | 24.5 ± 0.08 | 288, 337 | **933:** 451, 763, 632, 301 | **933.0727:** 450.9933; 650.1633; 300.9980 | 5.5 ± 1.1 |
| 3B | 320 | 63.3 ± 0.01 | 266, 313 | | | 2.4 ± 0.1 |
| 4B | 320 | 63.7 ± 0.01 | 258, 316 | | | 2.1 ± 0.1 |
| 5B | 320 | 64.8 ± 0.01 | 235,313 | | | 1.8 ± 0.01 |
| **Flavonol derivatives** | | | | | | |
| 1C | 360 | 3.88 ± 0.23 | 332 | | | 9.6 ± 0.3 |
| 2C | 360 | 8.6 ± 0.1 | 232, 370 | | **299.0742:** 155.8719 and **257.0971:** 117.0524 | 12.4 ± 0.62 |
| 3C | 360 | 15.1 ± 0.1 | 204, 339 | | | 9.9 ± 0.1 |
| 4C | 360 | 20.5 ± 0.06 | 340 | | | 9.4 ± 0.01 |
| 5C | 360 | 22.2 ± 0.04 | 335 | | | 9.4 ± 0.02 |
| 6C | 360 | 34.6 ± 0.04 | 252, 361 | | | 9.8 ± 0.1 |
| 7C | 360 | 35.7 ± 0.03 | 265, 370 | | | 10.3 ± 0.1 |
| 8C | 360 | 38.6 ± 0.04 | 254,356 | | | 9.9 ± 0.1 |
| 14C | 360 | 49.5 ± 0.1 | 251, 353 | | **433.0757:** 300.0249; 301.0291 | 10.9 ± 0.2 |
| 18C | 360 | 59.1 ± 0.02 | 253, 360 | | | 9.7 ± 0.05 |
| 19C | 360 | 59.7 ± 0.02 | 369 | | | 9.3 ± 0.02 |
| 20C | 360 | 61.1 ± 0.01 | 250,372 | | | 10.5 ± 0.1 |
| 21C | 360 | 63.0 ± 0.01 | 253,361 | | | 10.8 ± 0.1 |
| 22C | 360 | 64.0 ± 0.01 | 290,357 | | | 9.2 ± 0.01 |

Table S4 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 23C | 360 | 65.0 ± 0.01 | 250,360 | | | 9.3 ± 0.02 |
| 24C | 360 | 65.4 ± 0,01 | 253,360 | | | 9.8 ± 0.1 |
| 25C | 360 | 66.3 ± 0,01 | 253,360 | | | 9.5 ± 0.04 |
| **MATRIX-BOUND POLYPHENOLS- Alkaline Hydrolysis** | | | | | | |
| **Hydroxybenzoate derivatives (HBD)** | | | | | | |
| 1A | 280 | 2.9 ± 0.02 | 195, 253 | | | 1.7 ± 0.2 |
| 2A | 280 | 3.2 ± 0.1 | 225, 285 | | | 0.6 ± 0.1 |
| 3A | 280 | 3.5 ± 0.1 | 198, 257 | | | 1.1 ± 0.8 |
| 5A | 280 | 4.1 ± 0.1 | 195, 254 | | **362.0537:** 211.0019; 150.0414; 133.0149 and **153.0189:** | 11.9 ± 2.0 |
| 6A | 280 | 6.9 ± 0.1 | 198,268 | | | 1.8 ± 0.8 |
| 8A | 280 | 31.6 ± 0.1 | 233, 275 | | | 1.7 ± 0.3 |
| 9A | 280 | 65.2 ± 0.03 | 223, 280 | | | 4.5 ± 3.9 |
| 10A | 280 | 67.0 ± 0.1 | 229, 276 | | | 0.3 ± 0.1 |
| **Hydroxycinnamate derivatives (HCD)** | | | | | | |
| 1B | 320 | 5.1 ± 0.1 | 277, 312 | | | 1.7 ± 0.1 |
| 2B | 320 | 5.4 ± 0.04 | 310 | | | 1.9 ± 0.3 |
| 3B | 320 | 5.7 ± 0.1 | 259, 308 | | **491.0172:** 163.0399; 161.0237; 123.0072; 191.0349 | 1.3 ± 0.04 |
| 4B | 320 | 6.4 ± 0.1 | 271, 311 | | **463.0227:** 161.0306; 207.0306; 123.0084 | 1.4 ± 0.05 |

Table S4 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 5B | 320 | 8.4 ± 0.1 | 276, 307 | | **477.002:** 207.0206; 245.0384; 163.0308 and **235.0264:** 135.0432; 119.047; 157.0512 | 2.4 ± 0.3 |
| 6B | 320 | 10.6 ± 0.1 | 199, 273, 310 | | **463.0228:** 246.0195; 247.0237 and **207.0311:** 135.0445 and **251.0212:** 135.0444; 117.0336; 145.0261 | 3.2 ± 0.4 |
| 7B | 320 | 12.3 ± 0.04 | 208,256, 310 | | | 1.2 ± 0.02 |
| 8B | 320 | 13.1 ± 0.03 | 256, 315 | | | 3.0 ± 0.6 |
| 9B | 320 | 14.1 ± 0.05 | 252, 333 | | | 1.2 ± 0.1 |
| 10B | 320 | 15.1 ± 0.11 | 277, 336 | | | 1.2 ± 0.05 |
| 11B | 320 | 15.7 ± 0.1 | 297, 341 | | **277.001:** 149.0237; 177.0203; 133.0282 | 1.2 ± 0.05 |
| 12B | 320 | 17.2 ± 0.04 | 297, 320 | | | 1.6 ± 0.1 |
| 13B | 320 | 19.4 ± 0.06 | 251, 319 | | | 1.2 ± 0.04 |
| 14B | 320 | 21.1 ± 0.05 | 252, 322 | | | 2.5 ± 0.5 |
| 15B | 320 | 27.8 ± 0.1 | 216, 307 | | | 1.9 ± 0.2 |
| 16B | 320 | 38.1 ± 0.1 | 255, 305 | | | 1.2 ± 0.05 |
| **Flavonol Derivatives** | | | | | | |
| 1C | 360 | 7.9 ± 0.1 | 245, 350 | | | 6.5 ± 0.1 |
| 2C | 360 | 8.8 ± 0.1 | 275, 365 | | | 6.0 ± 0.04 |

Table S4 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 3C | 360 | 9.2 ± 0.1 | 254, 280, 360 | | **611.0075:** 217.0153; 261.0065; 189.0197 and **261.0067:** 161.0241; 133.0283; 189.0202 | 6.5 ± 0.3 |
| 4C | 360 | 11.0 ± 0.06 | 273, 341 | | | 6.3 ± 0.1 |
| 5C | 360 | 11.9 ± 0.1 | 268, 342 | | **304.9983:** 161.0245; 133.0284; 189.0202 | 7.2 ± 0.2 |
| 6C | 360 | 14.5 ± 0.1 | 251, 347 | | **477.0008:** 261.0057; 259.9966; 217.0145 | 8.6 ± 1.2 |
| 7C | 360 | 16.5 ± 0.3 | 207, 359 | | | 6.1 ± 0.1 |
| 8C | 360 | 17.8 ± 0.1 | 208, 336 | | | 6.3 ± 0.2 |
| 9C | 360 | 18.7 ± 0.1 | 276, 353 | | **467.0219:** 207.0309; 163.0406 and **291.0173:** 247.0262; 191.0348; 119.0516 | 11.6 ± 1.6 |
| 10C | 360 | 19.9 ± 0.1 | 282, 380 | | | 6.4 ± 0.3 |
| 11C | 360 | 23.3 ± 0.1 | 263, 352 | | | 8.5 ± 1.9 |
| 12C | 360 | 26.1 ± 0.1 | 256, 378 | | **277.0014:** 177.0204; 149.0234; 133.0282 | 9.5 ± 1.0 |
| 13C | 360 | 33.1 ± 0.1 | 281, 383 | | | 6.2 ± 0.1 |
| 14C | 360 | 35.6 ± 0.1 | 257, 376 | | | 6.6 ± 0.6 |
| 15C | 360 | 41.8 ± 0.1 | 249, 366 | | | 6.0 ± 0.1 |
| **MATRIX-BOUND POLYPHENOLS-Acid Hydrolysis** | | | | | | |
| **Hydroxybenzoate derivatives (HBD)** | | | | | | |

Table S4 (*Continued*)

| **Peak*** | **λ (nm)** | **RT ± SD** | **λ max (nm)** | **LC-TRAP-MS/MS** | **LC-q-TOF-MS/MS** | **Quantification mg/100g** |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 1A | 280 | 3.5 ± 0.02 | 256 | | | 0.1 ± 0.03 |
| 3A | 280 | 4.3 ± 0.04 | 272 | | | 0.1 ± 0.01 |
| 4A | 280 | 6.0 ± 0.1 | 283 | | **153.0176:** 129.0386 and **675.2127:** 193.0358 | 7.4 ± 1.4 |
| 5A | 280 | 7.33 ± 0.1 | 272 | | **341.0199:** 165.0576 | # |
| 6A | 280 | 15.1 ± 0.02 | 229, 280 | | | 0.1 ± 0.04 |
| 7A | 280 | 32.9 ± 0.04 | 196, 273 | | | 0.1 ± 0.004 |
| 8A | 280 | 38.3 ± 0.1 | 196, 271 | | | 0.1 ± 0.01 |
| 9A | 280 | 65.2 ± 0.02 | 223, 281 | | | 0.8 ± 1.1 |
| **Hydroxycinnamate derivatives (HCD)** | | | | | | |
| 1B | 320 | 5.2 ± 0.02 | 284, 328 | | | 0.2 ± 0.01 |
| 2B | 320 | 5.7 ± 0.02 | 303 | | | 0.2 ± 0.003 |
| 3B | 320 | 8.4 ± 0.1 | 231, 314 | | **279.0162:** 163.0400; 135.0450; 145.0287; 117.0329 | 0.2 ± 0.004 |
| 4B | 320 | 10.5 ± 0.1 | 248, 293 | | **261.0069:** 133.0281 and **251.0218:** 135.0459 | 0.2 ± 0.01 |
| 5B | 320 | 13.5 ± 0.05 | 278, 334 | | | 0.2 ± 0.01 |
| 6B | 320 | 16.8 ± 0.1 | 280, 339 | | **183.0306:** 129.055 | 0.2 ± 0.004 |
| 7B | 320 | 17.2 ± 0.03 | 279, 324 | | | 0.2 ± 0.002 |
| 8B | 320 | 19.2 ± 1.8 | 274,312 | | | 0.2 ± 0.001 |
| 9B | 320 | 27.8 ± 0.02 | 204, 308 | | | 0.2 ± 0.002 |
| 10B | 320 | 29.4 ± 0.02 | 231, 310 | | | 0.2 ± 0.004 |

Table S4 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 11B | 320 | 35.7 ± 0.03 | 317 | | | 0.2 ± 0.001 |
| 12B | 320 | 45.5 ± 0.02 | 321 | | | 0.2 ± 0.001 |
| 13B | 320 | 52.6 ± 0.01 | 316 | | | 0.2 ± 0.001 |
| **Flavonol Derivatives** | | | | | | |
| 1C | 360 | 11.9 ± 0.04 | 271, 341 | | | 0.8 ± 0.01 |
| 2C | 360 | 14.7 ± 0.1 | 253, 347 | | **304.9975:** 233.0017; 161.0246 | 0.9 ± 0.02 |
| 3C | 360 | 18.8 ± 0.1 | 276, 356 | | **291.0162:** 247.0283 | 1.0 ± 0.02 |
| 4C | 360 | 23.5 ± 0.1 | 253, 382 | | | 0.8 ± 0.004 |
| 5C | 360 | 26.2 ± 0.3 | 257, 378 | | | 0.8 ± 0.01 |

*Peak numbers are shown in figures 2 (free phenolic compounds) and 4 (matrix-bound phenolic compounds), where peak letter indicates the figure panel. Peaks that were only detected in DAD but did not generate mass spectral signal are not shown in the table. RT= Retention time, SD=standard deviation Bold number correspond to m/z $[M-H]^-$.[#] Not identified compound co-eluted with identified compound.

**Table S5:** Compound not identified in free and matrix-bound phenolic compounds in the peel powder of *Myrciaria jaboticaba* (JPP-MJ) by HPLC-DAD coupled to LC-TRAP-MS/MS and LC-q-TOF-MS/MS.

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| **FREE PHENOLIC COMPOUNDS** | | | | | | |
| **Hydroxybenzoate derivatives (HBD) or tannins** | | | | | | |
| 2A | 280 | 4.7 ± 0.1 | 270 | | **191.0172:** 169.1591 | # |
| 3A | 280 | 5.3 ± 0.1 | 204, 254 | | | 3.7 ± 0.8 |
| 4A | 280 | 6.0 ± 0.1 | 276 | | | 10.6 ± 2.2 |
| 6A | 280 | 7.3 ± 0.1 | 256(sh), 293(sh) | | | 1.2 ± 0.2 |
| 10A | 280 | 12.9 ± 0.1 | 259 | | | 2.4 ± 0.2 |
| 12A | 280 | 14.9 ± 0.3 | 254 | | | 1.7 ± 0.2 |
| 15A | 280 | 18.4 ± 0.2 | 215, 274 | **953:** 907, 935, 783 | | 7.7 ± 1.0 |
| 17A | 280 | 27.1 ± 0.3 | 212, 271 | **784:** 765, 633, 301, 481 | **784.0644 (2):** 300.9870; 765.0442; 169.0003 | 15.0 ± 0.6 |
| 19A | 280 | 32.3 ± 0.2 | 215, 269 | | | 1.4 ± 0.1 |
| 22A | 280 | 36.6 ± 0.15 | 216, 270 | **784:** 765,287 | **784.0864(2):** 765.0608; 650.1234; 300.9977; 483.0783 | 101.0 ± 2.2 |
| 24A | 280 | 39.2 ± 0.1 | 215, 277 | | | 5.1 ± 0.6 |
| 25A | 280 | 40.3 ± 0.1 | 218, 257 | **783:** 765, 301, 481, 631 | | 12.3 ± 1.2 |
| 26A | 280 | 41.4 ± 0.1 | 217, 268 | **859:** 785, 689 | | 30.2 ± 0.6 |
| 28A | 280 | 45.7 ± 0.1 | 217, 274 | **860:** 765 | **860.0960 (2):** 765.06; 169.0105; 300.9981; 785.085 | 115 ± 8 |

Table S5 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 29A | 280 | 47.1 ± 0.1 | 216, 277 | | **393.0473(2):** 169.0117 | # |
| 30A | 280 | 48.9 ± 0.1 | 215, 276 | | | 4.9 ± 0.1 |
| 31A | 280 | 49.9 ± 0.1 | 217, 277 | | | 7.6 ± 0.9 |
| 32A | 280 | 51.4 ± 0.1 | 217, 258 | | | 7.3 ± 0.2 |
| 34A | 280 | 53.2 ± 0.1 | 218, 265 | | | 2.4 ± 1.0 |
| 35A | 280 | 54.9 ± 0.1 | 215, 278 | | | 6.4 ± 0.2 |
| 36A | 280 | 55.6 ± 0.1 | 214, 285 | | | 1.2 ± 0.1 |
| 37A | 280 | 56.7 ± 0.1 | 213, 270 | | | 1.5 ± 0.1 |
| 38A | 280 | 57.5 ± 0.1 | 211, 280 | | | 1.3 ± 0.1 |
| 39A | 280 | 57.9 ± 0.1 | 214, 279 | | | 2.2 ± 0.2 |
| 41A | 280 | 62.1 ± 0.1 | 217, 278 | | | 0.8 ± 0.1 |
| 42A | 280 | 62.3 ± 0.1 | 216, 270 | | | 5.1 ± 0.2 |
| 43A | 280 | 62.6 ± 0.1 | 214, 277 | | | 2.8 ± 0.1 |
| 44A | 280 | 64.2 ± 0.1 | 217, 278 | | | 5.2 ± 0.2 |
| 45A | 280 | 67.1 ± 0.1 | 208, 276 | **573:** 300 | **573.0912:** 299.9898; 300.9942 | 1.4 ± 0.2 |
| **Hydroxycinnamate derivatives (HCD)** | | | | | | |
| 1B | 320 | 17.2 ± 0.1 | 252, 322 | | | 2.1 ± 0.2 |
| 2B | 320 | 24.4 ± 0.2 | 288, 337 | **933:** 451, 631, 745, 301 | **933.0727:** 450.9929; 300.9981(2); 631.0665 | 2.9 ± 0.5 |

Table S5 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 3B | 320 | 27.9 ± 0.1 | 202, 315 | | | 1.9 ± 0.02 |
| 4B | 320 | 60.0 ± 0.1 | 204, 313 | | | 1.9 ± 0.01 |
| 5B | 320 | 63.4 ± 0.03 | 256(sh), 310 | | | 2.2 ± 0.02 |
| 6B | 320 | 63.7 ± 0.03 | 257, 316 | | | 2.0 ± 0.01 |
| **Flavonol derivatives** | | | | | | |
| 1C | 360 | 8.5 ± 0.1 | 253, 363 | | **423.0402:** 247.0130; 273.0279 | 11 ± 0.06 |
| 2C | 360 | 15.1 ± 0.1 | 200, 353 | | | 9.2 ± 0.1 |
| 3C | 360 | 18.7 ± 0.1 | 274, 358 | | | 9.5 ± 0.05 |
| 4C | 360 | 34.5 ± 0.1 | 252, 360 | | | 9.5 ± 0.1 |
| 5C | 360 | 35.6 ± 0.1 | 256, 373 | | | 9.7 ± 0.1 |
| 7C | 360 | 38.6 ± 0.1 | 257, 357 | | | 9.5 ± 0.01 |
| 8C | 360 | 39.7 ± 0.1 | 232, 351 | | | 9.5 ± 0.03 |
| 14C | 360 | 49.5 ± 0.1 | 264, 353 | | | 9.9 ± 0.03 |
| 15C | 360 | 50.7 ± 0.1 | 256, 354 | | | 10.3 ± 0.1 |
| 16C | 360 | 52.0 ± 0.1 | 251, 351 | | | 11.9 ± 0.1 |
| 18C | 360 | 59.1 ± 0.1 | 254,350 | | | 9.6 ± 0.04 |
| 19C | 360 | 59.8 ± 0.1 | 251, 368 | | | 9.3 ± 0.02 |

Table S5 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 20C | 360 | 61.1 ± 0.1 | 250, 371 | | | 9.5 ± 0.02 |
| 21C | 360 | 61.7 ± 0.1 | 252, 350 | **609:** 463, 301 | **609.1275:** 463.0898; 300.027; 301.0323 | NQ |
| 22C | 360 | 63.0 ± 0.1 | 253, 361 | | | 9.8 ± 0.05 |
| 23C | 360 | 65.4 ± 0.1 | 253, 361 | | | 9.4 ± 0.04 |
| 24C | 360 | 66.3 ± 0.1 | 253, 362 | | | 9.2 ± 0.02 |
| 25C | 360 | 67.4 ± 0.1 | 263,357 | | | 9.3 ± 0.01 |
| 26C | 360 | 67.9 ± 0.1 | 266,355 | | | 9.2 ± 0.01 |
| 27C | 360 | 69.5 ± 0.1 | 253, 360 | | | 9.6 ± 0.03 |
| **MATRIX-BOUND POLYPHENOLS- Alkaline hydrolysis** | | | | | | |
| **Hydroxybenzoate derivatives (HBD)** | | | | | | |
| 2A | 280 | 4.4 ± 0.4 | 197, 253 | | **362.0604**: 150.0436; 211.0054; 133.0167 | 10.1 ± 3.1 |
| 4A | 280 | 16.4 ± 0.2 | 266 | | | 3.9 ± 1.5 |
| 5A | 280 | 29.6 ± 0.4 | 283 | | | 2.7 ± 0.5 |
| 6A | 280 | 31.7 ± 0.1 | 232, 269 | | | 3.0 ± 0.8 |
| 7A | 280 | 61.4 ± 0.1 | 273 | | | 0.9 ± 0.2 |
| 8A | 280 | 65.3 ± 0.1 | 223, 280 | | | 3.0 ± 1.6 |
| **Hydroxycinnamate derivatives (HCD)** | | | | | | |

Table S5 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 1B | 320 | 4.6 ± 0.3 | 233, 269, 315 | | **437.085**: 317.0376; 347.0525 | 2.0 ± 0.5 |
| 2B | 320 | 5.6 ± 0.12 | 215, 260, 306 | | **491.0249:** 191.0382; 163:0420; 161.0338; 123.0029 and **435.0322:** 163.0421 and **235.029:** 135.00464; 117.0344 | 1.6 ± 0.4 |
| 3B | 320 | 6.2 ± 0.1 | 276, 303 | | **463.0284:** 161.0272; 207.0327; 123.0099 | 2.2 ± 0.3 |
| 4B | 320 | 8.3 ± 0.1 | 276, 329 | | **235.029:** 135.0464; 117.0344 | 3.6 ± 1.0 |
| 5B | 320 | 9.4 ± 0.2 | 335 | | | 1.8 ± 0.1 |
| 6B | 320 | 10.6 ± 0.1 | 268, 311 | | **295.0174**: 163.0424; 135.0463 | 5.5 ± 0.2 |
| 7B | 320 | 11.2 ± 0.1 | 237, 327 | | | 2.1 ± 0.1 |
| 8B | 320 | 12.5 ± 0.1 | 283, 322 | | | 1.9 ± 0.1 |
| 9B | 320 | 13.1 ± 0.1 | 278, 326 | | | 3.7 ± 1.9 |
| 10B | 320 | 14.1 ± 0.1 | 254, 325 | | | 1.9 ± 0.3 |
| 11B | 320 | 15.2 ± 0.1 | 255, 326 | | | 1.8 ± 0.2 |
| 12B | 320 | 17.2 ± 0.1 | 320 | | **495.0202:** 163.0414 | 2.3 ± 0.2 |
| 13B | 320 | 17.8 ± 0.2 | 329 | | | 1.8 ± 0.1 |
| 14B | 320 | 21.03 ± 0.13 | 252,320 | | | 5.9 ± 1.5 |
| 15B | 320 | 27.9 ± 0.1 | 287, 309 | | **277.0066:** 177.0213; 133.0319 | 3.5 ± 0.8 |

Table S5 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 16B | 320 | 35.8 ± 0.1 | 257,310 | | | 2.8 ± 0.4 |
| 17B | 320 | 38.0 ± 0.1 | 334 | | | 1.0 ± 0.3 |
| 18B | 320 | 49.7 ± 0.1 | 257, 310 | | | 1.8 ± 0.1 |
| 19B | 320 | 64.1 ± 0.1 | 241,336 | | | 1.7 ± 0.1 |
| **Flavonol derivatives** | | | | | | |
| 1C | 360 | 7.8 ± 0.1 | 248, 351 | | **473.0144:** 260.0024; 217.018 | 13.1 ± 4.6 |
| 2C | 360 | 9.1 ± 0.1 | 254, 370 | | **463.0287:** 246.0224; 278.0142 | 11.5 ± 2.8 |
| 3C | 360 | 10.2 ± 0.1 | 249, 356 | | **261.0109:** 161.0272; 133.0308 | 12.9 ± 3.8 |
| 4C | 360 | 11.9 ± 0.1 | 266, 342 | | | 10.5 ± 0.6 |
| 5C | 360 | 14.5 ± 0.1 | 250, 340 | | **305.0018:** 161.0274; 133.0301 | 14.9 ± 1.4 |
| 6C | 360 | 18.7 ± 0.1 | 276, 354 | | | 17.6 ± 0.5 |
| 7C | 360 | 19.7 ± 0.1 | 250, 346 | | **291.0214:** 247.0302; 191.0379; 219.0336; 173.0271 | 12.5 ± 3.3 |
| 8C | 360 | 23.3 ± 0.2 | 263, 355 | | | 16.0 ± 8.0 |
| 9C | 360 | 26.2 ± 0.2 | 257, 378 | | | 11.4 ± 2.0 |
| 10C | 360 | 33.1 ± 0.2 | 282, 383 | | | 10.4 ± 1.2 |
| 12C | 360 | 43.9 ± 0.1 | 298, 355 | | | 10.1 ± 1.3 |
| 13C | 360 | 44.7 ± 0.1 | 253, 349 | | | 9.4 ± 0.3 |

Table S5 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 14C | 360 | 46.0 ± 0.1 | 256, 353 | | | 9.0 ± 0.5 |
| 15C | 360 | 47.7 ± 0.1 | 255, 363 | | | 9.9 ± 0.4 |
| 16C | 360 | 53.9 ± 0.1 | 253, 342 | | | 9.0 ± 0.4 |
| 17C | 360 | 63.2 ± 0.1 | 255, 369 | | | 9.2 ± 0.1 |
| **MATRIX-BOUND POLYPHENOLS- Acid hydrolysis** | | | | | | |
| **Hydroxybenzoate derivatives (HBD)** | | | | | | |
| 2A | 280 | 5.1 ± 0.1 | 233, 270 | | **273.0081** | 11.1 ± 8.2 |
| 3A | 280 | 6.1 ± 0.1 | 229, 280 | | **153.0212**: 107.8839; 124.0076 | 25.9 ± 12.1 |
| 5A | 280 | 7.6 ± 0.1 | 273 | | **187.1116 and 231.1394**: 130.0893; 181.3678 | 0.1 ± 0.04 |
| 6A | 280 | 8.3 ± 0.1 | 251 | | **221.0969**: 179.0293 | 2.1 ± 0.7 |
| 7A | 280 | 15.2 ± 0.1 | 274 | | **231.1374**: 130.0863 | 0.3 ± 0.1 |
| **Hydroxycinnamate derivatives (HCD)** | | | | | | |
| 1B | 320 | 5.7 ± 0.1 | 214, 304 | | | 0.4 ± 0.1 |
| 2B | 320 | 10.8 ± 0.1 | 264, 330 | | **235.0200**: 135.0467; 127.6591 and **279.0168**: 135.0451 | 0.5 ± 0.2 |
| 3B | 320 | 13.3 ± 0.1 | 241, 332 | | | 0.6 ± 0.2 |
| 4B | 320 | 13.7 ± 0.2 | 326 | | **183.032:** | 0.5 ± 0.1 |

Table S5 (*Continued*)

| Peak* | λ (nm) | RT ± SD | λ max (nm) | LC-TRAP-MS/MS | LC-q-TOF-MS/MS | Quantification mg/100g |
|---|---|---|---|---|---|---|
| | | | | **[M-H]⁻:** $MS^2$ | **[M-H]⁻:** $MS^2$ | |
| 5B | 320 | 19.4 ± 0.1 | 232, 320 | | | 0.5 ± 0.1 |
| 6B | 320 | 29.4 ± 0.1 | 321 | | | 0.6 ± 0.2 |
| 7B | 320 | 35.8 ± 0.1 | 320 | | | 0.4 ± 0.1 |
| 8B | 320 | 45.5 ± 0.1 | 311 | | | 0.4 ± 0.1 |
| **Flavonol derivatives** | | | | | | |
| 1C | 360 | 10.3 ± 0.1 | 250, 293, 348 | | **261,0086:** 133,0314 | 2.3 ± 0.8 |
| 2C | 360 | 11.9 ± 0.1 | 253, 340 | | **221.0966:** | 2.6 ± 0.5 |
| 3C | 360 | 14.8 ± 0.4 | 346 | | | 2.8 ± 0.6 |
| 4C | 360 | 18.7 ± 0.1 | 276, 353 | | | 2.6 ± 0.8 |
| 5C | 360 | 20.0 ± 0.1 | 340 | | **291.0079:** 147.0366; 219.0210; 191.027 | 2.2 ± 0.6 |

*Peak numbers are shown in figures 3 (free phenolic compounds) and 5 (matrix-bound phenolic compounds), where peak letter indicates the figure panel. Peaks that were only detected in DAD but did not generate mass spectral signal are not shown in the table. RT= Retention time, SD=standard deviation, Bold number correspond to m/z [M-H]⁻. NQ= not quantified (area < LOQ).# Not identified compound co-eluted with identified compound.

## 4.2 MANUSCRITO 2:

*In vitro* GASTROINTESTINAL DIGESTION OF JABOTICABA PEEL POWDER: BIOACCESSIBILITY AND ANTIOXIDANT CAPACITY OF BIOACCESSIBLE PHENOLIC COMPOUNDS

**Manuscrito em fase de revisão para submissão à Revista Food Reseach International**

(Configurado conforme normas da revista)

**_In vitro_ gastrointestinal digestion of jaboticaba peel powder: bioaccessibility and antioxidant capacity of bioaccessible phenolic compounds**

Quatrin, A.[1]; Pauletto, R.[1]; Maurer, L. H.[2]; Nicheli, S. M.[1]; Rodrigues, E.[3]; Klein, B.[1]; Maróstica Junior, M. R.[4]; Bochi, V. C.[5]; Emanuelli, T.[1]

[1]Department of Food Technology and Science, Center of Rural Sciences, Federal University of Santa Maria, 97105-900, Santa Maria, RS, Brazil;

[2]Farroupilha Federal Institute, 97555-000, Alegrete, RS, Brasil;

[3]Department of Food Science, Federal University of Rio Grande do Sul, 91501-970, Porto Alegre, RS, Brazil;

[4]School of Food Engineering, University of Campinas - UNICAMP, 13083-862 Campinas, SP, Brazil;

[5]Department of Nutrition, Federal University of Health Sciences of Porto Alegre (UFCSPA), 91501-970, Porto Alegre, RS, Brazil;

*Corresponding author Tel.: +55 55 3220 8547; fax: +55 55 3220 8353. E-mail address: tatiana.emanuelli@ufsm.br (Tatiana Emanuelli).

**Abstract**

Jaboticaba peel powder (JPP) is polyphenol-rich food that has several health benefits. Fruit composition may not reflect the compounds that will be responsible for the biological effects as polyphenols may be extensively modified during digestion. The objective of this study was to evaluate the bioaccessibility and antioxidant capacity of JPP polyphenols using an *in vitro* model of gastrointestinal digestion. Salivary and gastric digestion promoted the degradation of non-anthocyanins polyphenols, mainly of hydrolyzable tannins but had small effects on anthocyanins. Intestinal digestion promoted extensive hydrolysis of ellagitannins and gallotannins that resulted in increase of gallic and ellagic acids. Anthocyanin had low bioaccessibility (0.08-2.3%), whereas most hydrolyzable tannins (1.2-166.0%) and flavonols (0-36.8%) had greater bioaccessibility than anthocyanins. Despite their low bioaccessibility, anthocyanins were the most abundant polyphenols in the bioaccessible intestinal fraction and had a major contribution to the oxygen scavenging capacity of the polyphenols found in this fraction. The intestinal bioaccessible fraction of jaboticaba peel was also rich in ellagic acid and hydrolyzable tannins, namely the co-elutions monogalloyl glucose with HHDP-galloylglucose and casuariin with galloylquinic acid and HHDP-galloylglucose.

**Keywords:** Jaboticaba peel, phenolic compounds, gastrointestinal digestion, polyphenols bioaccessibility, HPLC-DAD-Q-TOF-MS/MS, antioxidant capacity

## 1. Introduction

Jaboticaba, also known as “Brazilian grape tree”, is native to Brazil but can be cultivated in all territory of the South America (S.-B. Wu, Long, & Kennelly, 2013). The fruits grow attached to the trunk and have spherical shape with diameter of 2.05 ± 0.28 cm. Fruit peel contributes to the greatest amount of fruit phenolic compounds, which are mostly composed by anthocyanins that are responsible for their characteristic dark purple color (Pereira, Barbosa, Ribeiro Da Silva, Ferri, & Santos, 2017; Plaza et al., 2016).

Jaboticaba peel (JPP) is a promising source of bioactive compounds and has been demonstrated to be effective in the treatment and prevention (Leite et al., 2011; Plaza et al., 2016) of chronic diseases in several animal models (Alezandro, Granato, & Genovese, 2013; Â. G. Batista et al., 2018). Such effects have been attributed to its content of phenolic compounds (Alezandro, Dubé, Desjardins, Lajolo, & Genovese, 2013; Inada et al., 2015).

The major extractable phenolic compounds found in jaboticaba peel are cyanidin-3-glucoside, ellagitannins (HHDPs) and gallotannins (ALEZANDRO et al., 2013; INADA et al., 2015; PLAZA et al., 2016). The JPP ellagitannins and gallotannins has high antioxidant capacity even with lower content when compared to anthocyanins (Plaza et al., 2016) and it can be responsible for elevate antioxidant effects (A. G. Batista et al., 2014; Lamas et al., 2018; Leite-legatti et al., 2012). Food polyphenols may undergo intense transformation during human digestion. Parent polyphenols may be metabolized into smaller compounds due to the action of different enzymes in the mouth, stomach and small intestine. Therefore, phenolic composition and antioxidant activities of jaboticaba peel food matrix may not reflect the compounds that will be accessible for gastrointestinal absorption, being responsible for the biological effects.

Hydrolyzable tannins, also known as ellagitannins and gallotannins, as well as anthocyanins have been demonstrated to be poorly bioacessible for absortion in the intestinal digestion (Mosele, Macià, Romero, & Motilva, 2016). Moreover, the stability of polyphenols during digestion is remarkably affected by the food matrix yielding different bioavailability for polyphenols and their metabolites

(Mosele, Macià, Romero, Motilva, & Rubió, 2015; Mosele et al., 2016). Soluble and insoluble fiber can interact with anthocyanins and increase their stability during digestion (Mosele, Macià, Romero, et al., 2015), whereas pectin makes difficult the solubilization of the some phenolic compounds during gastric digestion (Mosele et al., 2016).

The compounds that can be responsible for the recently reported beneficial effects of JPP in murine models of obesity and diabetes (Alezandro, Granato, et al., 2013; A. G. Batista et al., 2014; Dragano et al., 2013; Quatrin et al., 2018) have not been described. Only two studies have been carried out to investigate the bioaccessibility of jaboticaba polyphenols but the stability and catabolism of polyphenols in each digestion step has not been described separately (Dantas et al., 2018; Peixoto et al., 2016). Besides, Peixoto et al. (2016) had studied only anthocyanin bioaccessibility at gastric and intestinal steps using epithelium absorptive models. This is important because jaboticaba polyphenols had direct antioxidant effects in intestinal cells in a rat model of colitis (Da Silva, 2017).

This study was aimed to evaluating the bioaccessibility of polyphenols from jaboticaba peel (*Myrciaria trunciflora*) using a static model for human gastrointestinal digestion and to evaluate the changes in antioxidant capacity in each phase of digestion.

## 2. Materials and method

### 2.1 Preparation of the jaboticaba peel powder

Jaboticaba fruits were collected in São Vicente do Sul city, at Rio Grande do Sul State, Brazil. Plant specimen was identified by the Forest Engineer Mauricio Figueira as *Myrciaria trunciflora* and had an exsiccate deposited in the herbarium of the Department of Forestry Sciences (nº HDCF 7228) of UFSM. Fruits were washed and the peels were separated from the pulp. Peels were freeze-dried (Terroni, São Paulo, Brazil) and triturated using an analytical micro grinder (Marconi, MA-630, São Paulo, Brazil) to yield jaboticaba peel powder (JPP) that was stored at -80°C until analysis. Particle

size of JPP was: 14.8% ≥ 300 µm; 300 µm > 40.8% ≥ 106 µm; 106 µm > 21.5% ≥ 50 µm; 50 µm > 10.2% ≥ 12 µm and 12.6% < 12 µm.

### *2.2 In vitro* simulation of gastrointestinal digestion

The standardized static *in vitro* digestion method (Minekus et al., 2014) was used to investigate the biaccessibility of anthocyanin and non-anthocyanin phenolic compounds from JPP. JPP samples (5 g) was used to simulate the different steps of human digestion: mouth, gastric and small intestine conditions (Figure 1). Mouth step (10 mL final volume) was simulated using artificial saliva (pH= 7.5) and standardized concentration of the α-amylase enzyme (Sigma Aldrich, Brazil, 75 U/mL) incubated at 37ºC for 2 min under agitation. After the mouth step, the pH of the digesta was adjusted to 3.0 by adding the artificial gastric solution. There was no need to use additional HCl to adjust the pH of JPP sample. The digesta was incubated (37ºC for 2 h under agitation) under gastric conditions by adding pepsin (2000 U/mL) and water to adjust the final volume to 20 mL. Thereafter, the pH of gastric digesta was adjusted to 7.0 with 1 M NaOH and the digesta was submitted to intestinal digestion. Pancreatin (100 U/mL) and bile salts (10 mM) were added and the volume was adjusted 50 mL with water. Digesta was put inside the dialysis membrane (12000 Da, Sigma Aldrich, Brazil) that was immersed in 500 mL phosphate buffer (24.96 mM, pH 7.2) and incubated at 37ºC for 2 h under sporadic agitation. Two fractions were collected after the intestinal digestion, IN and OUT. The IN fraction was the one that remains inside the dialysis membrane and corresponds to the digesta that will reach the colon, while the OUT fraction was fraction that was able to cross the dialysis membrane and represents the bioaccessible fraction of JPP.

Aliquots were withdrawn at the end of each digestive phase and immediately frozen in liquid $N_2$ and stored at -80ºC until chromatographic analysis.

### 2.3 Extraction of phenolic compounds

Extraction of the phenolic compounds was performed as described previously (X. Wu, Gu, Prior, & McKay, 2004) with modifications except that the extraction was exhaustive using methanol:water:formic acid solvent solution (85:15:0.5, v/v) according the proportion 1:65 (m/v) sample content of each gastrointestinal digestion step (mouth, gastric and intestinal fractions IN).

The exhaustive extracts obtained from the mouth and gastric digestion steps were concentrated in a rotary evaporator (Büchi, Germany), at 38ºC for no more than 10 min and resuspended in known volume (2 mL). Then, 1 mL of the evaporated extract was purified using SPE cartridge (SPE-C18 cartridges, Strata C18-E, Phenomenex) according to (Rodriguez-Saona & Wrolstad, 2001) as modified by (Bochi, Godoy, & Giusti, 2015). The phenolic compounds were recovered in ethyl acetate and anthocyanins were eluted in methanol solution containing 0.35% formic acid. Then, solvents were evaporated in a rotary evaporator and phenolic compounds were recovered in 0.6 mL of an aqueous solution of 5% methanol containing 0.1% formic acid. Anthocyanins were recovered in 2 mL of an aqueous solution of 0.35% formic acid.

The IN intestinal fraction was centrifuged at 1559.6 *x g* for 10 min and the supernatant was immediately purified by SPE as described above. The pellet was extracted with methanol:water:formic acid (85:15:0.5, v/v/v), the solvent was evaporated and purified by SPE as described above. The amount of polyphenols in the IN fraction was calculated as the sum of polyphenols found in the supernatant and pellet extract. Twenty four mL of the OUT intestinal fraction were submitted to SPE purification as described above.

**2.4 Identification and quantification of JPP polyphenols by HPLC-DAD-Q-TOF-MS/MS**

The identification of anthocyanin and non-anthocyanin phenolic compounds was performed in equipment a HPLC system connected to mass spectrometer equipped with spectrometer used was a Q-TOF analyzer and electrospray ionization (ESI) source (Bruker Daltonics, model micrOTOF-QIII, Bremen, Germany). The MS parameters were ESI source in negative ion mode, capillary voltage at −4000 V (negative) for non-anthocyanin phenolic compounds and ESI source in positive ion mode

with the capillary voltage at +4000 V (positive), dry gas temperature at 310°C, nebulizer gas at 29 psi, flow rate at 8 L $min^{-1}$. The tentative identification was proposed based on elution order in C-18 reverse phase chromatogram, UV to visible spectral characteristics, and the fragmentation pattern in mass spectrometry analysis.

The quantification of JPP polyphenols were analyzed using a HPLC (CBM-20A prominence,Shimadzu, Kyoto, Japan) equipped with degasser (DGU20A5 prominence, Shimadzu, Japan), column oven (CTO-20A prominence, Shimadzu, Japan) and coupled to a DAD detector (SPDM-20A prominence, Shimadzu, Japan). The non-anthocyanin polyphenols separation was performed in a reverse-phase C-18 Hypersil Gold column (5 µm particle size, 150 mm, 4.6 mm) at 38°C. Injection volume was 20 µL and the mobile phases were composed of 5% (v/v) methanol in acidified water (0.1% (v/v) of formic acid) as solvent (A) and 0.1% (v/v) of formic acid in acetonitrile as solvent B. Gradient was set as follows: 4% B from 0 to 10 min; 4% B was kept until 21 min; 16% B from 21.1 to 55 min; 50% B from 55.1 to 70 min; 100% B from 70.1 to 72 min; 100% B was kept until 80 min; 0% B from 80.1 to 83 min and was kept until 92.1 min at a flow rate of 1 mL/min.

The chromatograms for quantification purposes were obtained at 280 nm, for hydroxybenzoates and tannins and at 360 nm for flavonols. Calibration curves were constructed using stock solutions of thirteen phenolic compounds (gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, catechin, cafeic acid, vanillic acid, syringic acid, p-coumaric acid, trans-ferulic acid, trans-cinnamic acid, synapic acid, myricetin and kaempferol-3Bd-glucopyranoside) previously validated for our research group. Compounds that were derivative of one of the standard monomers were quantified by equivalence and results were expressed as mg per 5 g of JPP weight in digestion.

For the anthocyanin quantification (purified fraction) samples were injected (20 µL) in a reverse-phase column C-18 Core-Shell Kinetex column (2.6 µm particle size, 100 mm, 4.6 mm) at 38°C. The mobile phases were: solvent (A) a solution of 3% of formic acid in water (v/v) and solvent (B) 100% acetonitrile (J.T. Baker® ACS) at a flow rate of 0.9 mL/min. Separation was achieved using a linear gradient from 0% to 8% B in 5 min, 20% of B was kept until 15 min. At the end of the gradient, the column was washed increasing B to 90%, keeping it for 7 min, and equilibrating to initial condition for 7 min. Chromatograms were obtained at 520 nm for quantification purposes. Calibration

curves was constructed using stock solutions of cyanidin 3-glucoside and results were expressed as mg of cyanidin 3-glucoside equivalents 5 $g^{-1}$ of JPP weight in digestion.

**2.5 Bioaccessibility index**

The bioaccessibility was calculated based on the phenolic compounds released from the JPP matrix after *in vitro* gastrointestinal digestion and found in the OUT fraction, using the following equation:

Bioaccessibility (%) = (OUT polyphenols /JPP polyphenols)*100

**2.6 Recovery index**

The percentage of recovery is the amount of polyphenols found in the complete digesta (OUT plus IN fractions) after intestinal digestion of JPP and was calculated by the following equation:

Recovery index (%) = [(OUT polyphenols + IN polyphenols)/ JPP polyphenols]*100

**2.7 Residual intestinal digesta index**

The residual polyphenols of the intestinal digesta (RID %) is the fraction of polyphenols that remains intact after mouth, gastric and intestinal simulation but are not bioaccessible. This fraction, which represents polyphenols that can reach the colon, was calculated by the following equation:

Residual intestinal digesta index (%) = [IN polyphenols/ JPP polyphenols]*100

**2.8 Antioxidant capacity**

The antioxidant capacity was evaluated by the oxygen radical absorbance capacity assay (ORAC) as previously described (Ou, Hampsch-woodill, & Prior, 2001). The anthocyanin and non-

anthocyanin phenolic extracts obtained from the JPP (undigested) and each digestive phase [mouth, gastric and intestinal phase (IN and OUT)] were analyzed.

### 2.9 Statistical analysis

Data were expressed as mean ± standard error of four replicates for each digestion step. The percentages of change were calculated in relation to the undigested JPP matrix. The antioxidant capacity data was analyzed by factorial analyses of variance (5 digestion phase x 2 polyphenol fraction) followed by Duncan's test when appropriate. Principal component analysis (PCA) was used to summarize changes in the concentration of digestive polyphenol metabolites, and was performed based on four replicates at each digestion phase using Statistic version 7 (StatSoft Inc., Tulsa, OK, USA).

## 3. Results and discussion

### 3.1 Anthocyanin digestion and bioaccessibility

JPP had high content of anthocyanins (90.6 mg/5 g of JPP), among which cyanidin-3-glucoside was the predominant one as previously demonstrated (Inada et al., 2015; Plaza et al., 2016). The bioaccessibility of phenolic compounds from JPP was studied in a static model that simulates the oral, gastric and intestinal (duodenal) phase of human digestion coupled to a dialysis membrane to separate the bioaccessible compounds. Oral digestion caused small decrease in the content of anthocyanins (83.8 mg/5 g of JPP), in special for cyanidin-3-glucoside and peonidin-3-glucoside (-8.3 and 9.7% vs. JPP, respectively). However, delphinidin-3-glucoside was relatively stable to the salivary conditions (+0.7% vs. JPP; Figure 3, Table 1).

Anthocyanins have elevated stability in acid medium because of the flavilium cation form; as previously demonstrated the recuperation of blueberry and mulberry anthocyanins submitted only to gastric digestion amounted to 96.6% and 98.4% , respectively (Correa-betanzo et al., 2014; Liang et

al., 2012). However, when sequentially submitted to salivary and gastric digestion the content of anthocyanins from red grape and strawberry tree (*Arbutus unedo*) were decreased about 45% and 20%, respectively (Lingua, Wunderlin, & Baroni, 2018; Mosele et al., 2016). Similar results were observed for the JPP that was sequentially submitted to salivary and gastric digestion and had a small decrease in the content of anthocyanins (75.7 mg/5 g gastric digestion; range 16-29% anthocyanin loss) even at pH 3 (Figure 3, Table 1). These data suggest that anthocyanins in JPP are susceptible to degradation during gastric digestion compared to pomegranate extract that had been shown to undergo 6.3% decrease (Mosele, Macià, Romero, et al., 2015). Besides the matrix composition, the processing food canmodify the microstructure of the food matrix that changes the matrix protection as observed for flavonoids (Dutra et al., 2017). The evaluation of JPP anthocyanin stability during the gastrointestinal digestion, in special for salivary and gastric digestion, were the first time related in literature, since the jaboticaba studies focus only in polyphenol bioacessibility. Despite of the decrease in JPP anthocyanins up to the gastric phase of digestion (about 20%), enough amount of anthocyanins was available for absorption in the stomach (Table 1) and can be responsible for the beneficial health effects of JPP as previously demonstrated in other studies (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Peixoto et al., 2016) .

The intestinal digestion caused an important decrease in the recovery of anthocyanins (sum of anthocyanins IN + OUT: 10.69 mg/ 5 g JPP; Table 1) due to the alkaline conditions of the medium, which converts the colored flavilium cation to the colorless chalcone pseudobase. Thus, only 10.3% of JPP anthocyanins were found in intestine, which represents the amount that will remain after complete duodenal digestion (Table 1). Only 1.3% of JPP anthocyanins were able to cross the cellulose dialysis membrane, corresponding to fraction available for absorption (Table 2). Thus, intact anthocyanins of JPP had very low bioaccessibility after intestinal digestion, similar to recently reported for strawberry whose major anthocyanin is pelargonidin-3-glucoside (Ariza et al., 2018). The bioavailability of cyanidin-3-glucoside has been demonstrated to be very low, as only 0.02% of the ingested dose was recovered in human plasma (Vitaglione et al., 2007). However, due to the high content of cyanidin-3-

glucoside in the JPP matrix, even exhibiting low bioaccessibility, cyanidin-3-glucoside was the phenolic compound found at the highest concentration in the OUT fraction(Table 1 and 2).

Most part of soluble anthocyanins recovered in the intestinal phase remain inside the dialysis tube (9%) (Table 2), which corresponds to the fraction remains in the gut and will reach the colon, where it can be metabolized by gut microbiota (Mosele, Macià, & Motilva, 2015). However, study suggests that phenolic compounds that undergo colonic fermentation can also contribute to the health benefits of dietary polyphenols (Mosele, Macià, & Motilva, 2015).

**3.2 Hydrolyzable tannins digestion and bioaccessibility**

Hydrolyzable tannins were the second most abundant class of phenolic compounds found in JPP (65.3 mg/5g JPP), after anthocyanins (Table 1). Ellagitannins and gallotannins were more prone to degradation into minor compounds in the first digestion step (salivary), when compared to anthocyanins (Table 1).

The co-elution casuariin with galloylquinic acid and HHDP-galloylglucose isomer (Peak 2) HHDP-galloylglucose isomer (Peak 3), trisgalloyl HHDP-glucose (Peak 6) and the co-elution of bis-HHDP-galloylglucose and HHDP-galloylglucose (Peak 9) were the hydrolyzable tannins that had the largest decrease after salivary digestion (>74% decrease) and likely had the greatest contribution for increasing gallic acid content (Table 1). Besides, more the 80-90% these compounds were degraded after gastric digestion (Table 1 and Figure 2).

The decrease in trigalloylglucose (peaks 12, 13 and 16) and tetragalloylglucose isomer (peak 18) content can be also associated to the increase of gallic acid. Besides, the increase in tetragalloylglucose (peak 17) content after salivary and gastric digestion may be partially associated to the degradation of pentagalloylglucose (co-eluted with galloyl-castalagin; peak 19) with the release of one gallic acid molecule or the conditions salivary facilitated the release of bound phenolic compounds. Nevertheless, the content of tetragalloylglucose isomer (peak 18) progressively decreased through the salivary to intestinal digestion steps (4.75 mg/5 g JPP for the sum of IN + OUT fractions). Similar behavior was observed for trigalloylglucose and its isomer (peaks 12, 13 and 16). This

degradation of gallotannins is likely to contribute to increase gallic acid release as recently observed for *Arbutus unedo*, submitted to gastric and intestinal conditions (Mosele et al., 2016).

The recovery of gallotannin trigalloylglucose and its isomers (Figure 2 peaks 12, 13 e 16 and Table 2) after intestinal digestion ranged from 16.5 to 61%, while for tetragalloylglucose and its isomer (Figure 2, peaks 17 and 18) the recovery ranged from 48 to 96.6%. This data indicates that tetragalloylglucose has greater stability during JPP digestion than trigalloylglucose. Despite the lower recovery, trigalloylglucose and its isomers had higher bioccessibility (2.3 to 8.5%) than tetragalloylglucose and its isomer (0 to 1.1%), probably due the lower molecular weight of trigalloylglucose that facilitates crossing dialysis membrane. On the other hand, tetragalloylglucose, which remains in the gut, will likely promote health benefits by modulating colonic microbiota and generating bioactive catabolites after colonic fermentation (Faria, Fernandes, & Mateus, 2014; Mosele, Macià, & Motilva, 2015).

Ellagic acid appears to be susceptible to hydrolysis in the salivary and gastric digestion conditions as their content decreased in these steps even with the degradation of JPP ellagitannins (peaks 1 ,2 ,3 ,5 ,6 ,7 ,8 ,9 ,10 ,11 ,14 ,15, 19 and 20) and likely release of ellagic acid molecules (Table 1). On the other hand, ellagic acid content was increased by 92% in after intestinal digestion (IN + OUT intestinal content: 4.66 mg/5 g JPP) when compared to the undigested JPP (Table 1). This finding can be due to the release of matrix as already observed in jaboticaba peel (*Myrciaria jaboticaba*) linked ester/ether bonds after alkaline hydrolysis (Inada et al., 2015). Ellagitannins can be hydrolyzed and release ellagic acid when exposed to acid or basic conditions as in the gastric and intestinal digestion simulation, respectively (Alminger et al., 2014). However, the extent of ellagitannin degradation and ellagic acid release appears to depend on the food matrix, as ellagic acid content was increase after gastric digestion of *Arbutus unedo* (Mosele et al., 2016), while for JPP the increase of ellagic acid content occurred only after intestinal digestion. Besides, ellagic acid had 193% recovery index after the complete digestion, one of the highest recovery rates among JPP phenolic compounds, being only lower than the co-elution of monogalloyl glucose and HHDP-galloylglucose, whose recovery index amounted to 352.5% (Table 2). For these compounds, the increase in the

recovery index occurred in after intestinal digestion and were parallel to the decrease in the content of ellagitannins.

Additionally, the bioaccessibility of most ellagitannins was very low (0.9 to 7.7%), except for HHDP-galloylglucose isomer (Table 2, Figure 2 peak 3) and the co-elution of bis-HHDP-glucose, galloylquinic acid and HHDP-galloylglucose (Table 2, Figure 2 Peak 2), whose bioaccessibility was greater than 10%. The low bioaccessibility of complex ellagitannins has been previously reported for different food matrices, such as *Rubus fruticosus L.* and *Arbutus unedo* that also had elevated bioaccessibility of ellagic acid (14.9 and 20.4%, respectively) (Mosele et al., 2016; Van de Velde, Pirovani, & Drago, 2018) as observed for JPP in our study (32.4%). In contrast, the ellagitannin HHDP-galloylglucose co-elution with monogalloyl glucose (peak 1) had elevated bioacessibility (175.9%). Monogalloyl glucose is likely the major contributor of this co-elution due its low molecular weight that would be expected to result in greater facility to permeate the dialysis tube.

The salivary and gastric conditions reduced flavonol content probably due to the sensitivity to pH conditions and proteolytic enzymes, or by complexion with other constituents. But, the flavonol class had high recovery index (average 107.3%) after complete digestion of JPP. This high flavonol recovery can be related to matrix release after intestinal conditions as observed after alkaline hydrolysis of jaboticaba with release of the flavonol that linked to matrix with glycosidic bonds (Inada et al., 2015). Flavonols also had elevated bioaccessibility, ranging between 18.4 - 36.8%. However, flavonol content in JPP is much lower than hydrolyzable tannins or anthocyanins, and therefore, the amount of flavonols after complete digestion is lower than for ellagitannins and anthocyanins. As observed for phenolic acids, anthocyanins and ellagitannins, only a small fraction of the flavonols that reach intestine are bioaccessible (OUT vs. IN fraction). Myricetin-hexoside had a distinct behavior compared to the other flavonols, as it was not able to cross the dialysis membrane at all, indicating that myricetin-hexoside is less likely to be absorbed in the intestine and would be entirely delivered for fermentation by gut microbiota.

**3.3 Antioxidant capacity of JPP during simulated digestion**

The antioxidant capacity of the anthocyanin and non-anthocyanin polyphenols of JPP before digestion and during the *in vitro* digestion process were determined by the ORAC assay (Table 3). The undigested JPP extracts had the highest antioxidant capacity, and the values were similar for the anthocyanin and non-anthocyanin fractions of polyphenols. However, even though JPP had higher content of anthocyanins than non-anthocyanin polyphenols (90.6 vs. 73.4 mg/5 g JPP), the last ones, namely hydrolyzable tannins, flavonols and phenolic acids, had proportionaly greater potential to remove peroxyl radicals than anthocyanins (Table 3). This result corroborates data from jaboticaba peel pointing to a greater antioxidant capacity of tannins compared to other polyphenols (Plaza et al., 2016).

JPP digestion promotes accentuated decrease in the content of non-anthocyanin polyphenols that was parallel to the decrease in the antioxidant capacity of the non-anthocyanin phenolic fraction. For anthocyanins, the decrease in the content along digestion was slower and it was not directly associated to the decrease in antioxidant capacity. While anthocyanin and non-anthocyanin polyphenols equally contributed to the antioxidant capacity of JPP, the major contributor to the antioxidant capacity in the salivary, gastric and intestinal step was anthocyanin. (Table 3).

The antioxidant capacity in the intestinal step was lower compared to the gastric, salivary and undigested JPP (Table 3). The IN fraction had greater antioxidant capacity than the OUT fraction (bioaccessible) as already expected due the lower content of anthocyanin and non-anthocyanin phenolic compounds in the bioaccessible fraction (OUT). The decrease in the antioxidant capacity already been reported for another fruit matrix using different digestion methods (Correa-betanzo et al., 2014; Dutra et al., 2017; Liang et al., 2012; Schulz et al., 2017). Besides, the anthocyanin found in the intestinal IN fraction had similar capacity to remove peroxil radicals than observed in the undigested JPP and salivary step (Table 3). In contrast, non-anthocyanin polyphenol metabolites generated during digestion had 76% lower antioxidant capacity than the undigested JPP. Thus intact non-anthocyanin polyphenols of JPP had greater capacity to remove the peroxyl radical than their metabolites generated after gastrointestinal digestion.

The intestinal bioaccessible fraction (OUT) had elevated antioxidant capacity even at reduced content of anthocyanins (1.19 mg/5 g JPP) when compared to non-anthocyanin polyphenols (5.69 mg/5 g JPP). However, when the OUT fraction was compared to the undigested JPP or salivary, gastric and intestinal IN digestion steps the antioxidant capacity decreased to 92%, 88.7%, 90% and 87.8%, respectively.

The compounds that most contributed to the antioxidant activity of JPP during digestion were cyanidin-3-glucoside, ellagic acid, the co-elution monogalloyl glucose with HHDP-galloylglucose and the co-elution casuariin with galloylquinic acid and HHDP-galloylglucose due to its major content in the OUT fraction.

### 3.4 PCA analysis of JPP phenolic compounds during digestion

A multivariate exploratory analysis, principal component analysis (PCA), was used to determine the metabolic pattern of phenolic compounds of JPP under the influence of digestion (undigested, and salivary, gastric and intestinal steps). The two major components accounted for 85.5% of the total variance (Figure 4). Principal component I separated undigested JPP from the OUT intestinal fraction, which represents the bioaccessible phenolic compounds (Figure 4A). Phenolic compounds 1 (co-elution of monogalloyl glucose with HHDP-galloyl glucose) and 31 (gallic acid) were correlated with the OUT intestinal fraction (Figure 4C), whereas JPP was mainly associated to co-elution HHDP-digalloylglucose isomer with trisgalloyl-HHDP-glucose isomer (peak 11), tetragalloylglucose isomer (peak 18), trigalloylglucose isomer (peak 16), co-elution tellemagrandin with castalin (peak 7), myricetin rhamnoside (peak 24), myricetin-hexoside (peak 22).

Principal Component II discriminated the IN intestinal fraction from the other digestion steps and the undigested JPP (Figure 4A). The IN intestinal digestion had higher concentration of the phenolic acids, namely ellagic (23) and gallic acid (31), as well as flavonol compounds, namely quercetin-pentoside (27, 28 and 29), quercetin-hexoside (25, 26) and quercetin- rhamnoside (30).

## 4. Conclusion

Jaboticaba peel had high content of polyphenols, mostly anthocyanins and hydrolyzable tannins in the undigested matrix. Jaboticaba peel polyphenols had greater stability under salivary and gastric conditions than in the intestinal conditions. Most jaboticaba flavonols had elevated bioaccessibility. The content of anthocyanins and hydrolyzable tannins was remarkably decreased in the intestinal conditions, whereas gallic and ellagic acid were increased due to the partial hydrolysis and release from fruit matrix. Despite their low bioaccessibility, anthocyanins were the most abundant polyphenols found in the bioaccessible intestinal fraction and a major contributor for the oxygen scavenging capacity in the intestinal fraction. In addition, the intestinal bioaccessible fraction of jaboticaba peel was also rich in ellagic acid and hydrolyzable tannins, namely the co-elutions monogalloyl glucose with HHDP-galloylglucose and casuariin with galloylquinic acid and HHDP-galloylglucose.

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**Figure caption**

**Figure 1:** Scheme of the *in vitro* gastrointestinal digestion simulating three steps of digestion (salivary, gastric and intestinal). IN = fraction that remains inside the dialysis tube and corresponds to the digesta that will reach the colon. OUT= fraction that crosses the dialysis membrane and represents the bioaccessible fraction.

**Figure 2:** Chromatograms representative of non-anthocyanin (280 nm) phenolic compounds during simulated gastrointestinal digestion of jabuticaba peel powder (JPP). Non-anthocyanin phenolic compounds in the undigested JPP matrix (A) and after salivary (B), gastric (C) and intestinal IN (D) and OUT (E) digestion steps.

**Figure 3:** Chromatograms representative of anthocyanin (520 nm) phenolic compounds during simulated gastrointestinal digestion of jabuticaba peel powder (JPP). Anthocyanins in the undigested JPP matrix (A) and after salivary (B), gastric (C) and intestinal IN (D) and OUT (E) digestion steps

**Figure 4:** Scores (A and B) and correlation loading (C and D) plots showing the three major principal components for the phenolic compounds content of the undigested jabuticaba peel powder (JPP) and after digestion steps of JPP (salivary, gastric and intestinal IN and OU

**Table 1:** Content of phenolic compounds in the undigested jaboticaba peel powder (JPP) and after salivary, gastric and intestinal (IN and OUT) steps of the *in vitro* gastrointestinal digestion of JPP.

| Peak | Compounds | Undigested JPP | Digestion step | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Salivary | | Gastric | | Intestinal | | | |
| | | | | | | | IN | | OUT | |
| | | Content (mg/5 g JPP)* | Content (mg/5 g JPP)* | VAR % | Content (mg/5 g JPP)* | VAR % | Content (mg/5 g JPP)* | VAR% | Content (mg/5 g JPP)* | VAR% |
| | | | | | **Phenolic acids** | | | | | |
| 31 | Gallic acid # | Nd | 0.12 ± 0.02 | Nc | 0.14 ± 0.03 | + 23.2 | 1.04 ± 0.13 | +820.0 | 0.06 ± 0.01 | -44.5 |
| 4 | Protocatechuic acid | 0.21 ± 0.04 | 0.19 ± 0.02 | -9.5 | 0.18 ± 0.03 | -12.9 | 0.17 ± 0.01 | -28.9 | 0.07 ± 0.01 | -72.9 |
| 23 | Ellagic acid | 2.41 ± 0.10 | 1.93 ± 0.05 | -19.8 | 2.17 ± 0.04 | -9.6 | 3.87 ± 0.13 | +60.9 | 0.78 ± 0.04 | -67.6 |
| | **Sum of phenolic acids** | **2.62** | **2.24** | **-14.5** | **2.49** | **+5.0** | **5.08** | **+93.9** | **0.91** | **-65.3** |
| | | | | | **Hydrolyzable tannins** | | | | | |
| 1 | Monogalloyl glucose + HHDP-galloylglucose | 0.47 ± 0.03 | 0.25 ± 0.03 | -47.6 | 0.20 ± 0.03 | -56.2 | 0.83 ± 0.05 | 79.7 | 0.84 ± 0.25 | 75.4 |
| 2 | Casuariin + Galloylquinic acid + HHDP-galloylglucose | 6.80 ± 0.33 | 1.73 ± 0.33 | -74.9 | 0.93 ± 0.1 | -86.4 | 0.22 ± 0.02 | -96.8 | 0.76 ± 0.09 | -88.6 |
| 3 | HHDP-galloylglucose isomer | 0.59 ± 0.07 | 0.12 ± 0.01 | -78.4 | 0.04 ± 0.01 | -93.8 | 0.07 ± 0.004 | -88.3 | 0.07 ± 0.01 | -87.4 |
| 5 | Pedunculagin | 4.11 ± 0.34 | 1.55 ± 0.18 | -62.5 | 1.09 ± 0.05 | -72.9 | 0.47 ± 0.003 | -88.2 | 0.20 ± 0.01 | -94.9 |
| 6 | Trisgalloyl- HHDP glucose | 0.77 ± 0.02 | 0.12 ± 0.01 | -83.8 | 0.02 ± 0.01 | -97.4 | nd | -100 | 0.03 ± 0.01 | -96.5 |

Table 1 (Continued)

| Peak | Compounds | Undigested JPP | Digestion step | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Salivary | | Gastric | | Intestinal | | | |
| | | | | | | | IN | | OUT | |
| | | Content (mg/5 g JPP)* | Content (mg/5 g JPP)* | VAR % | Content (mg/5 g JPP)* | VAR % | Content (mg/5 g JPP)* | VAR% | Content (mg/5 g JPP)* | VAR% |
| 7 | Tellemagrandin + Castalin | 3.71 ± 0.15 | 1.73 ± 0.16 | -53.1 | 1.37 ± 0.11 | -62.9 | 2.0 ± 0.25 | -45.0 | 0.29 ± 0.02 | -92.2 |
| 8 | (Epi)catechin + Castalin isomer | 1.53 ± 0.13 | 0.63 ± 0.05 | -58.5 | 0.65 ± 0.06 | -57.2 | 0.33 ± 0.02 | -77.6 | 0.07 ± 0.01 | -94.9 |
| 9 | Bis-HHDP-galloylglucose (Casuarinin) + HHDP-galloylglucose | 3.30 ± 0.33 | 0.71 ± 0.24 | -78.8 | 0.07 ± 0.03 | -97.5 | 0.16 ± 0.03 | -95.2 | 0.04 ± 0.01 | -98.8 |
| 10 | Digalloylglucose + Casuarictin | 4.86 ± 0.31 | 2.01 ± 0.33 | -58.7 | 3.18 ± 0.09 | -33.6 | 1.85 ± 0.17 | -60.7 | 0.46 ± 0.05 | -90,5 |
| 11 | HHDP-digalloylglucose isomer + trisgalloyl-HHDP-glucose isomer | 4.48 ± 0.2 | 2.22 ± 0.09 | -50.3 | 1.6 ± 0.11 | -63.8 | 1.73 ± 0.21 | -61.5 | 0.13 ± 0.02 | -97.1 |
| 12 | Trigalloylglucose | 8.84 ± 0.34 | 6.19 ± 0.29 | -29.5 | 2.72 ± 0.13 | -69.2 | 1.26 ± 0.04 | -85.7 | 0.20 ± 0.01 | -97.7 |
| 13 | Trigalloylglucose isomer | 0.26 ± 0.02 | 0.19 ± 0.01 | -29.2 | 0.16 ± 0.01 | -36.5 | 0.07 ± 0.004 | -73.1 | 0.01 ± 0.002 | -95.5 |
| 14 | HHDP-glucose | 2.97 ± 0.12 | 2.18 ± 0.10 | -36.5 | 1.21 ± 0.02 | -59.1 | 0.29 ± 0.03 | -90.1 | 0.06 ± 0.01 | 97.9 |
| 15 | Vescalagin/castalin | 2.47 ± 0.12 | 0.82 ± 0.13 | -66.9 | 0.45 ± 0.03 | -81.6 | 0.30 ± 0.02 | -87.5 | 0.05 ± 0.002 | -98.1 |
| 16 | Trigalloylglucose isomer | 0.80 ± 0.06 | 0.33 ± 0.05 | -58.2 | 0.33 ± 0.04 | -58.7 | 0.42 ± 0.03 | -47.6 | 0.07 ± 0.01 | -91.5 |

Table 1 (*Continued*)

| Peak | Compounds | Undigested JPP | Digestion step | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Salivary | | Gastric | | Intestinal | | | |
| | | | | | | | IN | | OUT | |
| | | Content (mg/5 g JPP)* | Content (mg/5 g JPP)* | VAR % | Content (mg/5 g JPP)* | VAR % | Content (mg/5 g JPP)* | VAR% | Content (mg/5 g JPP)* | VAR% |
| 17 | Tetragalloylglucose | 1.66 ± 0.07 | 1.94 ± 0.11 | 18.2 | 2.47 ± 0.14 | 48.8 | 1.56 ± 0.05 | -5.0 | Nd | -100 |
| 18 | Tetragalloylglucose isomer | 9.94 ± 0.37 | 7.00 ± 0.23 | -29.2 | 5.28 ± 0.29 | -47.0 | 4.64 ± 0.06 | -53.1 | 0.11 ± 0.01 | -98.8 |
| 19 | Galloyl-castalagin + Pentagalloyl glucose | 5.77 ± 0.26 | 4.9 ± 0.18 | -14.5 | 4.48 ± 0.24 | -22.4 | 3.36 ± 0.12 | -41.2 | 0.05 ± 0.004 | -99.1 |
| 20 | Galloyl-castalagin isomer | 1.93± 0.02 | 1.55 ± 0.06 | -19.6 | 1.06 ± 0.13 | -45.4 | 0.74 ± 0.01 | -61.8 | 0.10 ± 0.01 | -94.6 |
| | **Sum of hydrolyzable tannins** | **65.26** | **36.17** | **-44.6** | **27.31** | **-58.2** | **20.30** | **-68.9** | **3.54** | **-94.6** |
| | | | | **Flavonols** | | | | | | |
| 21 | Myricetin-hexoside | 0.40 ± 0.003 | 0.17 ± 0.003 | -57.3 | 0.32 ± 0.004 | -21.3 | 0.06 ± 0.004 | -84.1 | Nd | -100 |
| 22 | Myricetin-hexoside | 0.52 ± 0.01 | 0.26 ± 0.001 | -49.7 | 0.37 ± 0.01 | -28.6 | 0.34 ± 0.001 | -34.4 | Nd | -100 |
| 24 | Myricetin rhamnoside | 0.99 ±0.03 | 0.66 ± 0.01 | -32.9 | 0.75 ± 0.02 | -24.5 | 0.66 ±0.02 | -32.8 | 0.18 ± 0.004 | -81.6 |
| 25 | Quercetin-hexoside | 0.67 ± 0.01 | 0.36 ± 0.01 | -46.0 | 0.48 ± 0.01 | -28.1 | 0.68 ± 0.003 | +1.14 | 0.17 ± 0.001 | -73.8 |
| 26 | Quercetin-hexoside | 0.88 ± 0.02 | 0.58 ± 0.01 | -33.9 | 0.68 ± 0.02 | -23.6 | 0.87 ± 0.01 | -1.6 | 0.21 ± 0.003 | -76.4 |
| 27 | Quercetin-pentoside | 0.44 ± 0.01 | 0.21 ± 0.005 | -51.6 | 0.36 ± 0.003 | -18.9 | 0.58 ± 0.003 | +31.0 | 0.16 ± 0.003 | -63.1 |
| 28 | Quercetin-pentoside | 0.49 ± 0.001 | 0.25 ± 0.003 | -50.3 | 0.38 ± 0.004 | -22.4 | 0.61 ± 0.004 | +24.7 | 0.17 ± 0.001 | -66.3 |

Table 1 (*Continued*)

| Peak | Compounds | Undigested JPP | Digestion step | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Salivary | | Gastric | | Intestinal | | | |
| | | | | | | | IN | | OUT | |
| | | Content (mg/5 g JPP)* | Content (mg/5 g JPP)* | VAR % | Content (mg/5 g JPP)* | VAR % | Content (mg/5 g JPP)* | VAR% | Content (mg/5 g JPP)* | VAR% |
| 29 | Quercetin-pentoside | 0.59 ± 0.01 | 0.32 ± 0.004 | -44.6 | 0.46 ± 0.01 | -21.4 | 0.69 ± 0.01 | +17.2 | 0.18 ± 0.001 | -69.9 |
| 30 | Quercetin- rhamnoside | 1.03 ± 0.03 | 0.72 ± 0.02 | -30.5 | 0.84 ± 0.02 | -18.1 | 1.01 ± 0.01 | -4.6 | 0.22 ± 0.003 | -78.6 |
| | **Sum of flavonols** | **6.01** | **3.53** | **-41.3** | **4.64** | **-22.8** | **5.50** | **-8.5** | **1.29** | **-78.5** |
| | **Sum of non-anthocyanin phenolics** | **73.96** | **41.85** | **-43.4** | **34.33** | **-53.5** | **29.94** | **-59.5** | **5.69** | **-92.3** |
| | **Anthocyanins** | | | | | | | | | |
| 32 | Delphinidin-3-glucoside | 8.81 ± 0.19 | 8.86 ± 0.19 | +0.6 | 6.25 ± 0.02 | -29.0 | 0.21 ± 0.01 | -97.6 | 0.01 ± 0.002 | -99.9 |
| 33 | Cyanidin-3-glucoside | 81.61 ± 1.73 | 74.7 ± 0.78 | -8.3 | 69.27 ± 1.54 | -14.9 | 9.26 ± 0.37 | -88.6 | 1.18 ± 0.14 | -98.6 |
| 34 | Peonidin-3-glucoside | 0.23 ± 0.005 | 0.21 ±0.004 | -9.7 | 0.19 ± 0.004 | -16.1 | 0.03 ± 0.001 | -86.7 | 0.005 ± 0.0001 | -97.7 |
| | **Sum of anthocyanins** | **90.65** | **83.79** | **-7.6** | **75.72** | **-16.5** | **9.50** | **-89.5** | **1.19** | **-98.7** |

*Mean ± SE VAR% = percent change relative to the undigested JPP; HHDP= hexahydroxydiphenic acid. Nd: not detected. Nc: not calculated because the compound was not detected at the JPP undigested.# The compounds that there was no content at JPP undigested was performed the calculation with the content of salivary step.

**Table 2:** Estimation of the intestinal bioaccessibility (%), recovery index after complete digestion (%) and residual intestinal digesta index (RID %) for phenolic compounds of jabuticaba peel powder (JPP).

| Peak | Phenolic compounds | Recovery index (%) | Bioaccessibility (%) | RID index (%) |
|---|---|---|---|---|
| **Phenolic acid** | | | | |
| 31 | Gallic acid | 110.9 ± 12.9 | 6.7 ± 0.8 | 104.3 ± 13.4 |
| 4 | Protocatechuic acid | 97.6 ± 5.0 | 27.1± 2.4 | 71.1 ± 5.6 |
| 23 | Ellagic acid | 193.3 ± 7.3 | 32.4 ± 2.0 | 160.9 ± 5.4 |
| | **Average of phenolic acid (%)** | **133.93** | **22.07** | **112.10** |
| **Hydrolyzable tannins** | | | | |
| 1 | Monogalloyl glucose + HHDP-galloylglucose | 355.1 ± 46.0 | 175.9 ± 51.5 | 179.7 ± 22. |
| 2 | Bis-HHDP-glucose (Casuariin) + Galloylquinic acid + HHDP-galloylglucose | 14.6 ± 1.7 | 11.3 ± 1.6 | 3.2 ± 0.4 |
| 3 | HHDP-galloylglucose isomer | 24.2 ± 1.6 | 12.5 ± 0.6 | 11.7 ± 1.5 |
| 5 | Pedunculagin | 16.87± 1.6 | 5.1 ± 0.7 | 11.8 ± 0.9 |
| 6 | Trisgalloyl HHDP-glucose | 3.4 ± 1.2 | 3.4 ± 1.2 | 0.0 |
| 7 | Tellemagrandin I + castalin | 62.7 ± 8.2 | 7.7 ± 0.4 | 54.9 ± 8.5 |
| 8 | (Epi)catechin + Castalin Isomer | 27.4 ± 2.9 | 5.0 ± 0.8 | 22.4 ± 2.1 |
| 9 | Bis-HHDP-galloylglucose (Casuarinin) + HHDP-galloylglucose | 5.9 ± 0.9 | 1.1 ± 0.1 | 4.8 ± 0.8 |
| 10 | Digalloylglucose + Casuarictin | 48.8 ± 6.2 | 9.4 ± 0.4 | 39.3 ± 6.6 |
| 11 | HHDP-digalloylglucose isomer + trisgalloyl-HHDP-glucose isomer | 41.4 ± 3.4 | 2.9 ± 0.4 | 38.5 ± 3.6 |
| 12 | Trigalloylglucose | 16.5 ± 0.4 | 2.2 ± 0.1 | 14.2 ± 0.5 |
| 13 | Trigalloylglucose isomer | 31.3 ± 3.8 | 4.5 ± 0.7 | 26.9 ± 3.7 |
| 14 | HHDP-glucose | 12.0 ± 1.6 | 2.1 ± 0.4 | 9.9 ± 1.2 |
| 15 | Castalin/Vescalagin | 14.4 ± 1.5 | 1.9 ± 0.1 | 12.5 ± 1.4 |
| 16 | Trigalloylglucose isomer | 60.9 ± 6.0 | 8.5 ± 2.0 | 52.4 ± 5.4 |

Table 2 (*Continued*)

| Peak | Phenolic compounds | Recovery index (%) | Bioaccessibility (%) | RID index (%) |
|---|---|---|---|---|
| 17 | Tetragalloylglucose | 96.6 ± 1.7 | 0.00 | 96.6 ± 1.7 |
| 18 | Tetragalloylglucose isomer | 48.0 ± 2.1 | 1.1 ± 0.1 | 46.9 ± 2.1 |
| 19 | Galloyl-castalagin + Pentagalloyl glucose | 59.7 ± 4.4 | 0.9 ± 0.1 | 58.8 ± 4.3 |
| 20 | Galloyl-castalagin isomer | 43.5 ± 1.1 | 5.3 ± 0.6 | 38.1 ± 0.8 |
| **Average of hydrolyzable tannins (%)** | | **51.7** | **13.7** | **38.0** |
| **Flavonol** | | | | |
| 21 | Myricetin-hexoside | 15.9 ± 0.2 | 0.0 | 15.9 ± 0.2 |
| 22 | Myricetin-hexoside | 65.5 ± 1.1 | 0.0 | 65.5 ± 1.1 |
| 24 | Myricetin-rhamnoside | 85.6 ± 3.2 | 18.4 ± 0.6 | 67.2 ± 2.9 |
| 25 | Quercetin-hexoside | 127.2± 1.3 | 26.1 ± 0.3 | 101.1 ± 1.6 |
| 26 | Quercetin-hexoside | 121.9 ± 4.9 | 23.5 ± 1.0 | 98.4 ± 3.8 |
| 27 | Quercetin-pentoside | 167.9 ± 2.6 | 36.8 ± 0.5 | 131.0 ± 2.1 |
| 28 | Quercetin-pentoside | 158.4 ± 0.6 | 33.6 ± 0.1 | 124.7 ± 0.6 |
| 29 | Quercetina-rhamnoside | 115.9 ± 1.4 | 21.3 ± 0.9 | 95.4 ± 0.9 |
| | **Average of flavonols** | **107.3** | **19.9** | **87.4** |
| **Anthocyanin (%)** | | | | |
| 1 | Delfinidin-3-glucoside | 2.4 ± 0.1 | 0.1 ± 0.02 | 2.3 ± 0.1 |
| 2 | Cyanidin-3-glucoside | 12.8 ± 0.5 | 1.4 ± 0.1 | 11.4 ± 0.6 |
| 3 | Peonidin-3-glucoside | 15.6 ± 0.8 | 2.3 ± 0.1 | 13.3 ± 0.7 |
| **Average of anthocyanin phenolics (%)** | | **10.3** | **1.3** | **9.0** |

Recovery index (%) = percentage of recovery of phenolic compounds in the complete digesta (OUT + IN fractions) after intestinal digestion of JPP; Bioaccessibility (%) = percentage of phenolic compounds found in the OUT fraction relative to undigested JPP; RID (%) = residual polyphenols of the intestinal digesta were the fraction of phenolic compounds found in the IN phase and represents compounds that will reach the colon. HHDP= hexahydroxydiphenic acid.

**Table 3:** Antioxidant capacity of the non-anthocyanin and anthocyanin fractions from undigested jaboticaba peel powder (JPP) and after salivary, gastric and duodenal (IN and OUT) steps of the *in vitro* gastrointestinal digestion of JPP.

| Digestion step | Non-anthocyanin phenolic fraction (mmol eq. trolox/5 g of JPP) | Anthocyanin fraction (mmol eq. trolox/5 g of JPP) | Sum of non-anthocyanin and anthocyanin fractions (mmol eq. trolox/5 g of JPP) |
|---|---|---|---|
| Undigested JPP | $22.32 \pm 1.63^{bA}$ | $21.69 \pm 0.35^{bB}$ | $44.00 \pm 1.78^{aA}$ |
| Salivary | $8.75 \pm 0.27^{cB}$ | $20.95 \pm 0.20^{bB}$ | $29.69 \pm 0.07^{aB}$ |
| Gastric | $9.55 \pm 0.20^{cB}$ | $24.44 \pm 0.56^{bA}$ | $33.98 \pm 0.56^{aC}$ |
| IN- Intestinal digestion | $5.31 \pm 0.31^{cC}$ | $21.84 \pm 1.11^{bB}$ | $27.62 \pm 0.66^{aB}$ |
| OUT - Intestinal digestion | $1.42 \pm 0.05^{cD}$ | $1.94 \pm 0.06^{bC}$ | $3.36 \pm 0.11^{aE}$ |

Data are mean ± SE. Lowercase letters indicate statistical difference within the same line, whereas uppercase letters indicate differences within the same column. JPP= jaboticaba peel powder, IN= fraction that stay in dialysis tube, OUT= the fraction bioaccessible.

Figure 1

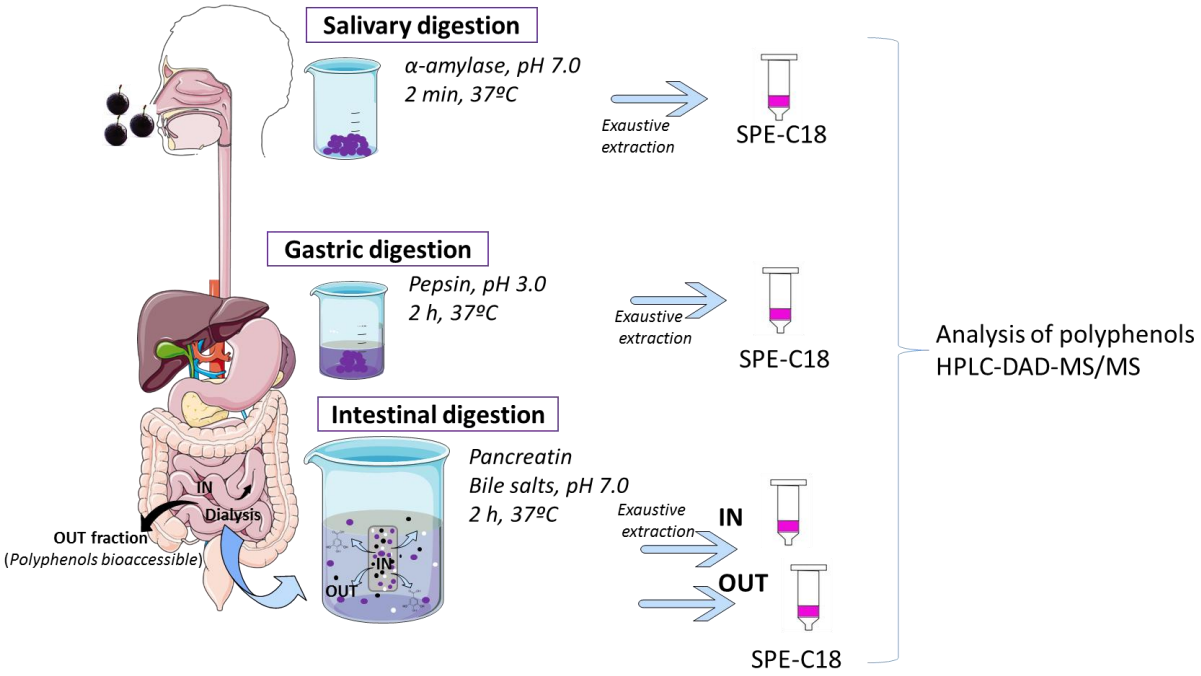

Salivary digestion
α-amylase, pH 7.0
2 min, 37ºC
Exaustive extraction
SPE-C18
Gastric digestion
Pepsin, pH 3.0
2 h, 37ºC
Exaustive extraction
SPE-C18
Intestinal digestion
Pancreatin
Bile salts, pH 7.0
2 h, 37ºC
Exaustive extraction
IN
OUT
SPE-C18
IN
Dialysis
OUT fraction
(Polyphenols bioaccessible)
IN
OUT
Analysis of polyphenols
HPLC-DAD-MS/MS

Figure 2

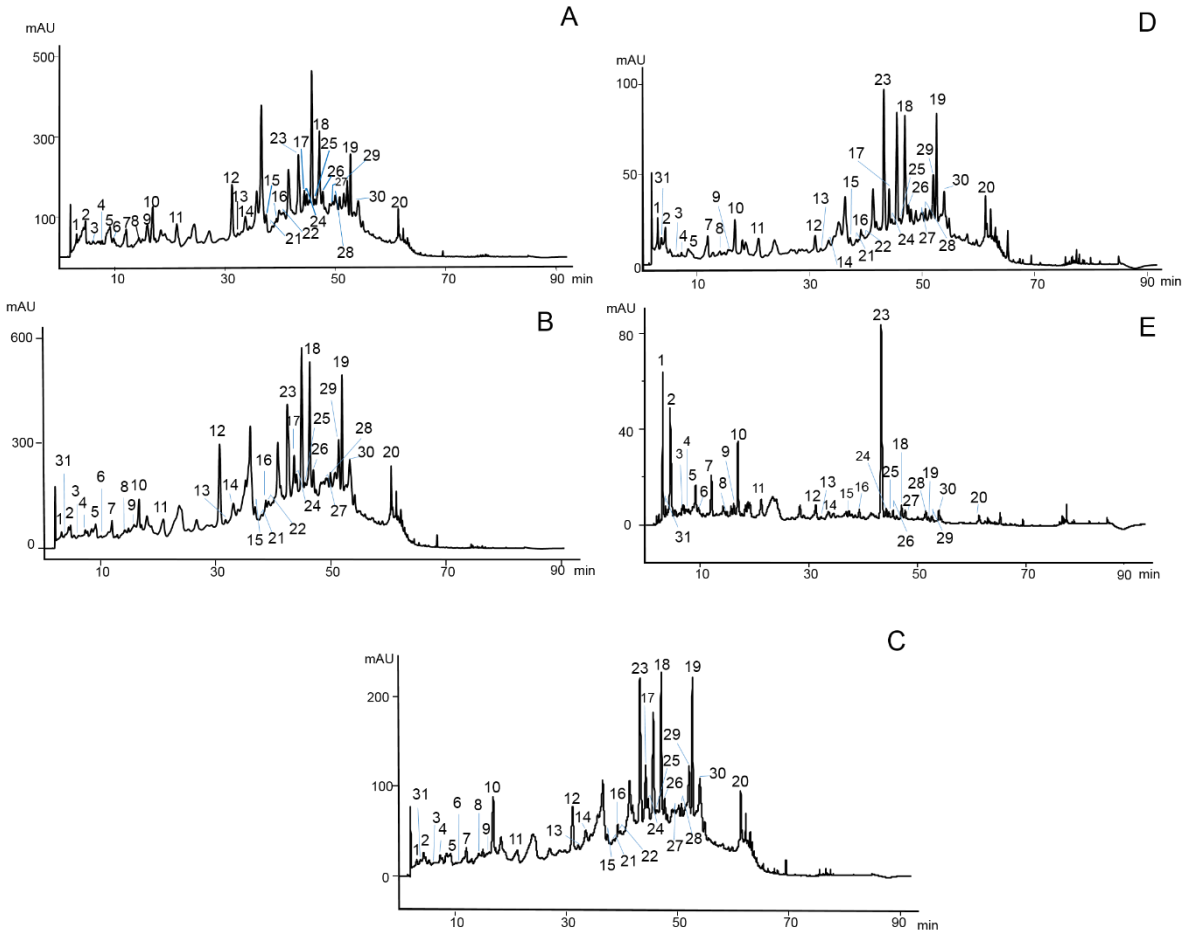
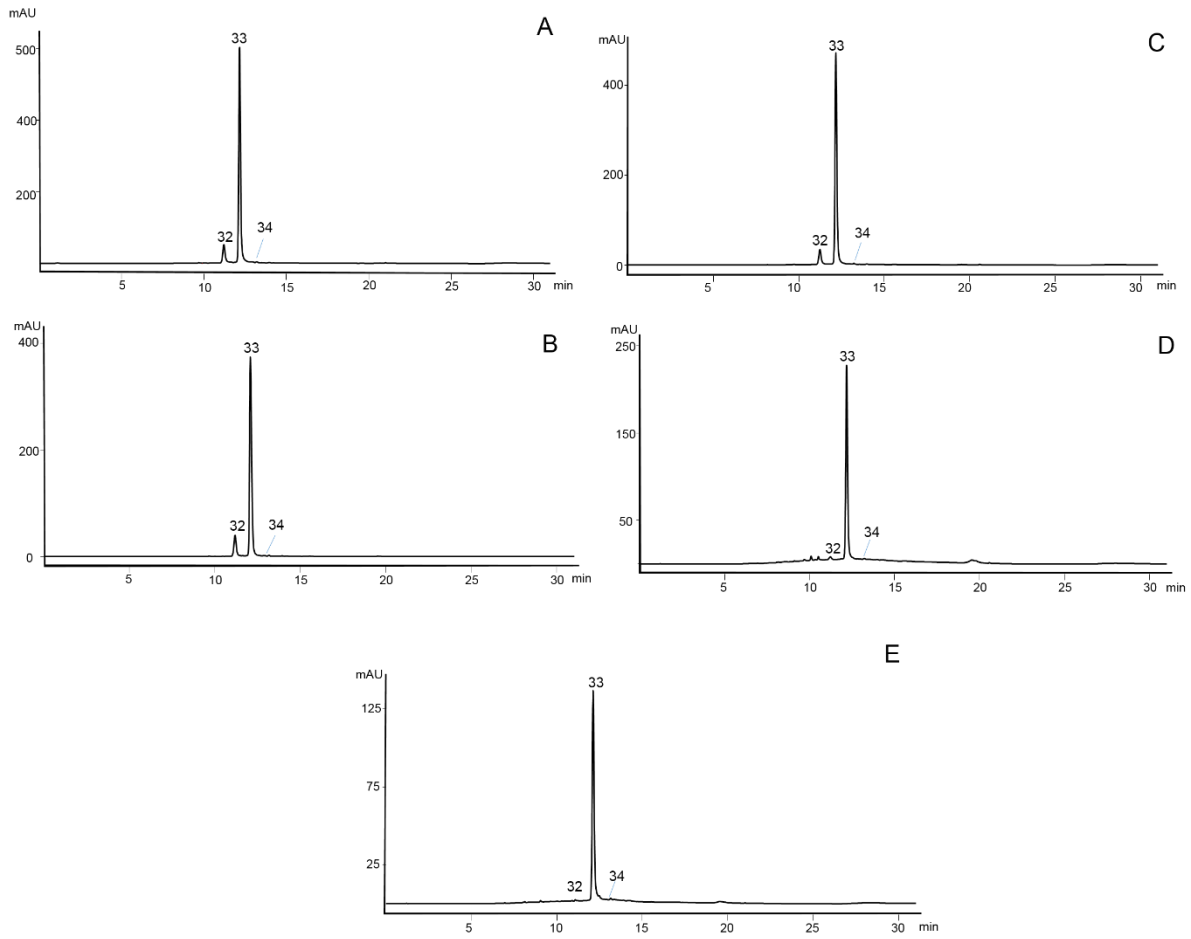


Figure 3


A
mAU
500
400
200
33
32
34
5
10
15
20
25
30
min
C
mAU
400
200
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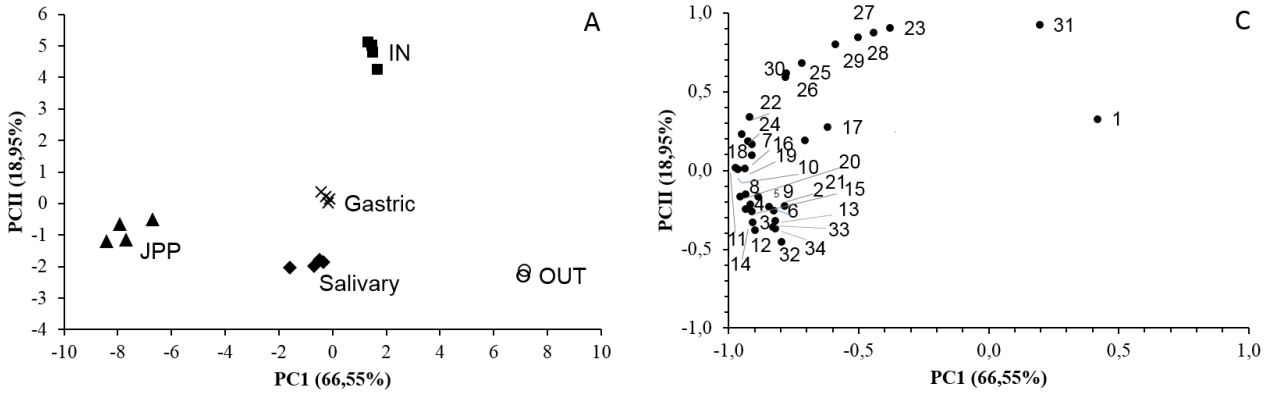
33
32
34
5
10
15
20
25
30
min
B
mAU
400
200
0
33
32
34
5
10
15
20
25
30
min
D
mAU
250
150
50
33
32
34
5
10
15
20
25
30
min
E
mAU
125
75
25
33
32
34
5
10
15
20
25
30
min

Figure 4




1. Monogalloyl glucose + HHDP-galloylglucose
2. Casuariin + Galloylquinic acid + HHDP-galloylglucose
3. HHDP-galloylglucose isomer
4. Protocatechuic acid
5. Pedunculagin
6. Trisgalloyl- HHDP glucose
7. Tellemagrandin + Castalin
8. (Epi)catechin + Castalin
9. Bis-HHDP-galloylglucose (Casuarinin) + HHDP-galloylglucose
10. Digalloylglucose + Casuarictin
11. HHDP-digalloylglucose isomer + trisgalloyl-HHDP-glucose isomer
12. Trigalloylglucose
13. Trigalloylglucose isomer
14. HHDP-glucose
15. Vescalagin/castalin
16. Trigalloyl glucose isomer
17. Tetragalloyl glucose
18. Tetragalloyl glucose isomer
19. Galloyl-castalagin + Pentagalloyl glucose
20. Galloyl-castalagin isomer
21. Myricetin-hexoside
22. Myricetin-hexoside
23. Ellagic acid
24. Myricetin rhamnoside
25. Quercetin-hexoside
26. Quercetin-hexoside
27. Quercetin-pentoside
28. Quercetin-pentoside
29. Quercetin-pentoside
30. Quercetin- rhamnoside
31. Gallic acid
32. Delphinidin-3-glucoside
33. Cyanidin-3-glucoside
34. Peonidin-3-glucoside

## 4.3 MANUSCRITO 3:

# METABOLISM OF JABOTICABA PEEL BY HUMAN GUT MICROBIOTA: INFLUENCE ON GUT BACTERIAL GROWTH

**Manuscrito em fase de revisão para submissão ao periódico Food Chemistry**

(Configurado conforme normas da revista)

# Metabolism of jaboticaba peel by human gut microbiota: Influence on gut bacterial growth

**Quatrin, A.[1]; Rampelotto, C. [1]; Rodrigues, R. F. [1]; Menezes, C. R. [1]; Fonseca, B. S. [1]; Mello, R.O.[1]; Rodrigues, E.[2]; Bochi, V.C.[3]; Emanuelli, T.[1*]**

[1]Department of Food Technology and Science, Center of Rural Sciences, Federal University of Santa Maria, 97105-900, Santa Maria, RS, Brazil;

[2]Department of Food Science, Federal University of Rio Grande do Sul, 91501-970, Porto Alegre, RS, Brazil;

[3]Department of Nutrition, Federal University of Health Sciences of Porto Alegre (UFCSPA), 91501-970, Porto Alegre, RS, Brazil;

*Corresponding author Tel.: +55 55 3220 8547; fax: +55 55 3220 8353. E-mail address: tatiana.emanuelli@ufsm.br (Tatiana Emanuelli).

**Abstract**

Microbial fermentation plays a crucial role in the metabolism of polyphenols and in modulation of gut microbiota. The phenolic metabolites and potential fermentation properties of jaboticaba peel (JPP) were investigated using an *in vitro* colonic fermentation assay with human feces. JPP was previously submitted to in vitro simulation of mouth, stomach and small intestine digestion yielding an undigested fraction (JPP-IN) that was used for the colonic fermentation assay under anaerobic conditions. The influence on microbiota growth was monitored after 48 h of fermentation. Phenolic compounds metabolites and short-chain fatty acids (SCFA) were monitored at 0, 2, 8, 24 and 48 h during fermentation by HPLC-DAD-Q-TOF-MS/MS and GC-FID, respectively. JPP-IN had a prebiotic effect promoting decreased the pathogenic bacteria count and shifts in the production of SCFA. The increases in acetic, propionic and lactic acid production at 2 h of fermentation were associated to soluble fiber content in JPP-IN. JPP-IN had large content of hydrolyzable tannins that undergo progressive catabolism forming new metabolites of smaller size as urolithins (A, B, C, D, M5, M6 and M7) from ellagitannins. Protocatechuic acid was the major metabolite from cyanidin-3-glucoside or even from gallic acid or gallotannins. This is the first study reporting the colonic catabolism and prebiotic effect of polyphenols from JPP. Urolithins and protocatechuic acid were the major catabolites found and their increase was parallel to the modulation of microbiota growth and increase of SCFA and gas production.

**Keywords:** Ellagitannins, anthocyanins, glut fermentation, short-chain fatty acids, human feces, urolithins, protocatechuic acid, dietary fiber.

## 1.Introduction

Fruit consumption has beneficial effects for reducing the risk of chronic diseases (Kim & Kim, 2018; Y. Wu, Zhang, Jiang, & Jiang, 2014). Red–black berries have attracted more interest due to their high content of bioactive compounds, namely phytochemicals and dietary fiber, as well as their antioxidant effects (Costa, Garcia-Diaz, Jimenez, & Silva, 2013). Blueberries and grapes were associated with a lower risk of development of type 2 diabetes development (Muraki et al., 2013).

Jaboticaba is an emerging fruit crop from Brazil that exhibits promising functional properties to reduce the risk of chronic diseases and was recently named amond the superfruits (Chang, Alasalvar, & Shahidi, 2018; S.-B. Wu, Long, & Kennelly, 2013). Such effects are attributed to the elevated content and diversity of the phenolic compounds found in jaboticaba species (Alezandro, Dubé, Desjardins, Lajolo, & Genovese, 2013; Inada et al., 2015). Hydrolyzable tannins and anthocyanins are among the major polyphenols in the fruit peel, which is also rich in quercetin and ellagic acid derivatives (Pereira, Barbosa, Ribeiro Da Silva, Ferri, & Santos, 2017; Plaza et al., 2016). Besides, jaboticaba peel has an important nutritional value, being rich in dietary fiber and soluble sugars (Leite-legatti et al., 2012; Leite et al., 2011).

JPP consumption have shown high antioxidant capacity (Leite et al., 2011; Plaza et al., 2016), reduced insulin resistance and oxidative stress in humans (Plaza et al., 2016) in animal model of obesity (A. G. Batista et al., 2014; Dragano et al., 2013; Lenquiste, Batista, Marineli, Dragano, & Maróstica, 2012). In addition, JPP consumptions has been shown to improve glutathione regulation in type 2 diabetes model (Quatrin et al., 2018) and plasma lipid profile in type 1 diabetes model (Alezandro, Granato, & Genovese, 2013).

These nutraceutical properties of JPP have been attributed to their polyphenols. However, the bioaccessibility of polyphenols from JPP have been scarcely investigated (Dantas et al., 2018; Peixoto et al., 2016). In general, the polyphenols bioaccessibility of jaboticaba is very low (Dantas et al., 2018) and the matrix constitution seems to influence the percentage of bioaccessibility, that varied 0-10% for anthocyanins of the jaboticaba frozen pulp and JPP, respectively (Peixoto et al., 2016). These findings are in agreement with most studies on the digestibility of polyphenols, which demonstrated that only 5-10% of the phenolic compounds from different fruits are bioavailable in the small intestine, whereas approximately 90% of phenolic compounds reach the large intestine, where they are catabolized by the gut microbiota yielding new bioactive compounds (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013; Pojer, Mattivi, Johnson, & Stockley, 2013). These secondary metabolites produced are easier to be absorbed (Espín, González-Sarrías, & Tomás-Barberán, 2017) and are probably the true responsible for the beneficial effects of polyphenol intake (Vitaglione et al., 2007). Additionally, the polyphenols could also promotes prebiotic effects modulating the bacterial growth with predominance of beneficial bacteria as *Bifidobacteria* and *Lactobacillus* (Faria, Fernandes, & Mateus, 2014).

In addition, fermentable carbohydrates that arrive intact in the colon as dietary fiber are catabolized into short chain fatty acids (SCFA) that also have prebiotic activity (Hugenholtz, Mullaney, Kleerebezem, Smidt, & Rosendale, 2013) and have been shown to improve local and systemic health (Mosele, Macià, & Motilva, 2015).

*In vitro* simulation of human colonic fermentation has been used to uncover the involvement of gut microbiota in the metabolism of different food matrices (Guergoletto, Costabile, Flores, Gracia, & Gibson, 2016; Mosele, Macià, Romero, & Motilva, 2016). This innovative approach allows to identify effects in gut microbiota, as well as the generated phenolic catabolites that are likely implicated in the known healthy benefits of polyphenol-

rich fruits. Although the *in vitro* study approach does not encompass all interactions involved in the human digestion it has been widely used for initial screening (Payne, Zihler, Chassard, & Lacroix, 2012), especially because each food matrix may exhibit different behavior (Mosele, Macià, Romero, Motilva, & Rubió, 2015). Although the initial digestion steps of jaboticaba have been recently studied, their colonic fermentation has not been explored.

Thus, the objective of this study was to investigate the polyphenol catabolites produced during the *in vitro* colonic fermentation of jaboticaba peel powder (JPP) with human feces. In addition, the influence of JPP on gut bacterial growth, SCFA and gas production was also investigated.

## 2. Material and methods

### 2.1 Sample preparation

Jaboticaba fruits (*Myrciaria trunciflora*) were collected in São Vicente do Sul (Rio Grande do Sul, Brazil) with exsiccate deposited in the herbarium of the Department of Forestry Sciences (HDCF nº 7228) of UFSM. The peels were manually separated, freeze-dried (Terroni, São Paulo, Brazil) and powdered using an analytical micro grinder (Marconi, MA-630, São Paulo, Brazil) to yield the jaboticaba peel powder (JPP) that was stored at -80ºC until analysis.

The *in vitro* simulation of gastrointestinal digestion was conducted according to a standardized procedure (Minekus et al., 2014). JPP (5.0 g) was submitted to three sequential steps that mimic the digestive process: mouth, gastric and intestinal (small intestine) digestion. The intestinal digestion was performed simultaneously in a dialysis system, where the fraction inside the dialysis tube (IN fraction) was considered that non-absorbable fraction

that will be able to reach the colon. The procedure was repeated ten times for to obtain the amount of sample necessary to conduct the colonic fermentation assay. The IN fraction, named JPP-IN, was immediately frozen under liquid $N_2$, lyophilized, powdered and stored at -20$^{o}$C for not more than 10 days before use in the colonic fermentation assay.

**2.2 Proximate composition of jaboticaba peel powder**

Moisture, ash, protein and dietary fiber were determined according to (AOAC, 2005). Lipids were extracted with chloroform and methanol and quantified by the gravimetric method (Bligh & Dyer, 1959). The carbohydrate content was calculated by difference.

**2.3 *In vitro* colonic fermentation**

Seventeen healthy volunteers were included as fecal donors in this study (20 – 53 years, 8 men, 9 women). The exclusion criteria were chronic, infectious or gastrointestinal diseases or having received antibiotic treatment during the previous 6 months. Serum biochemical analysis of volunteers confirmed that their total cholesterol, HDL cholesterol, triglycerides, transaminases activity, fasting glucose and creatinine levels were within normal values (data not shown). Each assay was preformed using a pool of fresh fecal samples collected from four healthy volunteers. Feces were used within 2 h from defecation and maintained at room temperature in anaerobic conditions until the moment of the experiment.

The kinetics of JPP-IN fermentation were evaluated by the *in vitro* gas production technique (Figure 1). The pooled fecal samples (2.5 g) were suspended in 50 mL of anaerobic carbonate-phosphate buffer, pH 6.5 (Durand, Dumay, Beaumatin, & Morel, 1988; Mosele,

Macià, Romero, et al., 2015), homogenized, filtered using sterile gauze and maintained under anaerobic conditions using $CO_2$ gas for remove the $O_2$ of the fecal suspension.

JPP-IN (0.5 g) were incubated in glass bottles containing 50 mL of fecal suspension. The individual glass bottles were sealed with AnkomRF 18 Gas Production System (Ankom Technology, Macedon - NY, USA), in a system coupled to a computer equipped with Gas Pressure Monitor application (Ankom Technology, Macedon - NY, USA) and automated metabolic gas metering radiofrequency wireless signal, and incubated at 37 ± 1°C for 0, 2, 8, 24 and 48 hours. Separate bottles were used for each fermentation time.

Two controls were run in parallel: (1) JPP-IN was incubated in buffer solution without fecal sample to assess the chemical degradation of phenolic compounds, and (2) the fecal suspension was incubated without the JPP-IN to determine the presence of basal phenolic compounds in the fecal samples.

pH was determined at all incubation times, whereas microbiota analysis was assessed only at 0 and 48 h of incubation. After incubation all glass bottles were centrifuged at 1400 *x g* for 10 min and the supernatant was immediately frozen under liquid $N_2$ and stored at −20°C until the analysis of phenolic compounds, ammonia and SCFA.

The gas pressure was measured at 1 h intervals up to 48 h of incubation, totalizing 49 readings per curve. Pressure readings (psi) were converted into moles of gases using the 'ideal' gas law, and then into mL of gas using the Avogadro's Law. Data were expressed as mL of gas produced per gram of incubated organic matter (OM).

The cumulative gas production curves observed *in vitro* were adjusted by the bicompartmental logistic model (Schofield, Pitt, & Pell, 1994), given by:

$$V = \frac{V_1}{1 + e^{[2-4k_1(t-\lambda)]}} + \frac{V_2}{1 + e^{[2-4k_2(t-\lambda)]}} + \varepsilon$$

V = gases volume (mL/g OM incubated) at time t; $V_1$ = maximum volume of gases produced by the degradation of the soluble fraction of fast digestion; $V_2$ = maximum volume of gases produced by the degradation of the potentially degradable insoluble fraction of slow digestion; VT ($V_1$ + $V_2$) = total volume of gases produced; $k_1$ = specific rate of gas production by the degradation of the soluble fraction of fast digestion; $k_2$ = specific rate of gas production by the degradation of the potentially degradable insoluble fraction of slow digestion; t = incubation or degradation time (fermentation); e = exponential; $\lambda$ = latency phase or period of hydration, adherence and microbial colonization (lag time); $\varepsilon$ = experimental error associated with each observation, assumed ~ NIID (0; $\sigma^2$). The parameters of the model considered were estimated by the Gauss-Newton algorithm modified with the NLIN procedure. The coefficient of determination ($r^2$) was expressed in relation to the source treatments (regression + lack of fit).

**2.4 Ammonia analysis**

The ammonia was determined in supernatant of the samples using the phenol and sodium nitroprusside solution (10:0.05 w/w) and sodium hydroxide and sodium hypochlorite solution (2.5:1.7 w/v) that catalyzed indophenol reaction according to (Chaney & Marbach, 1962). Ammonium chloride was used as standard (0.03 - 0.15 µmol) and results were expressed as µmol $NH_4$/50 mL. Samples were analyzed in duplicate.

**2.5 Analysis of pH**

pH value was determined immediately after finishing each incubation time using a digital potentiometer PHOX P1000 (PHOX Equipamentos Científicos, Colombo, Paraná, Brazil).

### 2.6 Microbiological counts

Bacteria counts were carried out after serial dilution of samples with peptone water (0.1 g/100 mL). *Bifidobacteria* counts was determined in MRS agar enriched with lithium chloride (0.1%) and L-cysteine (0.05%), according to manufacturer recommendations (Hansen, 1999) using the pour-plate method. Lactobacillus and *Enterobacteria* counts were determined in MRS agar and Violet Red Bile Dextrose Agar, respectively, using the pour-plate method.

Plates were incubated in anaerobic jars with Anaerobac system (Probac, Sao Paulo, Brazil) at 37°C for 72 h, viable cells were enumerated and results were expressed as log CFU/mL.

### 2.7 Short-chain fatty acid analysis

SCFA were determined according (Zhao, Nyman, & Jönsson, 2006). Supernatant samples obtained after fermentation had their pH adjusted to 2-3 using the aqueous solution of 2 N HCl. Then samples were centrifuged at 1700 *x g* for 20 min, the supernatant was collected, the internal standard was added (1 mM). Samples were injected in an Agilent Technologies gas chromatograph (HP 6890 N) equipped with a capillary column Nukol ™ (30 m x 0.25 mm; 0.25 µm Supelco, Bellefonte, PA, US) and flame ionization detector (FID). The chromatographic conditions were as follows: injector and detector temperatures set at

250ºC, injected volume 1 µL at 1:10 split ratio, and the carrier gas was nitrogen at 1 mL/min. The column oven was programmed for kept at 100ºC for 0.5 min, then heated at 8ºC/min until 180ºC, kept for 1 min, heated at 20ºC/min until 200ºC, and kept for 5 min. The temperature of the FID was 240ºC and the injection port was 200°C. A standard mix of volatile free fatty acids (46975-U, Sigma-Aldrich, St. Louis, MO, USA) were diluted in aqueous acid solution (formic acid 12%) and used as external standard.

### 2.8 Phenolic compound extraction

Aliquots of supernatant samples obtained after fermentation (6 mL) were extracted using an acidified acetone solution (0.35% formic acid, v/v; 7 mL) for impurities precipitation. After homogenization for 1 min in vortex mixer, samples were centrifuged at 1100 *x g* for 10 min to collect the supernatant. The organic solvent was removed in a rotary evaporator (38 ± 2ºC) and the extract was purified using reversed-phase solid phase extraction (SPE-C18 cartridges, Strata C18-E, Phenomenex), as previously described (Rodriguez-Saona & Wrolstad, 2001) with the same modifications described by (Bochi, Godoy, & Giusti, 2015). The purified phenolic fractions obtained were dried in a rotary evaporator (38 ± 2ºC) and made up to known volumes (1.0 mL) with 10% methanol in acidified water (0.1% formic acid, v/v) (non-anthocyanin phenolic fraction) or acidic water (0.35% formic acid, v/v) (anthocyanin fraction).

### 2.9 Identification and quantification of JPP polyphenols by HPLC-DAD-Q-TOF-MS/MS

The identification of anthocyanin and non-anthocyanin phenolic compounds was performed in a HPLC system connected to electrospray ionization (ESI) source and a mass spectrometer Q-TOF analyzer (Bruker Daltonics, model micrOTOF-QIII, Bremen, Germany). The MS parameters were ESI source in negative ion mode, capillary voltage at negative mode (−4000 V) for non-anthocyanin phenolic compounds and positive mode (+4000 V) for anthocyanins, dry gas temperature at 310°C, nebulizer gas at 29 psi, flow rate at 8 L $min^{-1}$. The tentative identification was proposed based on the elution order in C-18 reverse phase chromatogram, UV to visible spectral characteristics, and the fragmentation pattern in mass spectrometry analysis.

The quantification of JPP polyphenols were analyzed using a CBM-20A Prominence HPLC (Shimadzu, Kyoto, Japan) equipped with degasser (DGU20A5 prominence, Shimadzu, Japan), column oven (CTO-20A prominence, Shimadzu, Japan) and coupled to a DAD detector (SPDM-20A prominence, Shimadzu, Japan). The non-anthocyanin polyphenols separation was performed in a reverse-phase C-18 Hypersil Gold column (5 µm particle size, 150 mm, 4.6 mm) at 38°C. Injection volume was 20 µL and the mobile phases were composed of 5% (v/v) methanol in acidified water (0.1% (v/v) of formic acid) as solvent (A) and 0.1% (v/v) of formic acid in acetonitrile as solvent B. Gradient was set as follows: 4% B from 0 to 10 min; 4% B was kept until 21 min; 16% B from 21.1 to 55 min; 50% B from 55.1 to 70 min; 100% B from 70.1 to 72 min; 100% B was kept until 80 min; 0% B from 80.1 to 83 min and was kept until 92.1 min at a flow rate of 1 mL/min.

The chromatograms for quantification purposes were obtained at 280 (hydroxybenzoates, tannins and phenolic acid) and 360 nm (flavonols and urolithins). Calibration curves were constructed using stock solutions of thirteen phenolic compounds (gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, catechin, cafeic acid, vanillic acid, syringic acid, p-coumaric acid, trans-ferulic acid, trans-cinnamic acid, synapic acid, myricetin

and kaempferol-3β D-glucopyranoside). The method was previously validated by our research group. Compounds that are derivative of one of the standard monomers were quantified by equivalence and results were expressed as mg per g of JPP-IN.

The anthocyanin purified fractions were injected (20 µL) in a reverse-phase column C-18 Core-Shell Kinetex column (2.6 µm particle size, 100 mm, 4.6 mm) at 38°C. The mobile phases were: solvent (A) a solution of 3% of formic acid in water (v/v) and solvent (B) 100% acetonitrile (J.T. Baker® ACS) at a flow rate of 0.9 mL/min. Separation was achieved using a linear gradient from 0% to 8% B in 5 min, 20% of B was kept until 15 min and then the column was washed by increasing B to 90% for 1 min, keeping it for 7 min, and equilibrating to initial condition for 7 min. Chromatograms were obtained at 520 nm. Calibration curves was constructed using stock solutions of cyanidin 3-glucoside and results were expressed as mg of cyanidin 3-glucoside equivalents per g of JPP-IN.

### 2.10 Statistical analysis

Data were expressed as the means ± standard error of five replicates for each fermentation condition. The percentages of change in phenolic content were calculated in relation to the sample used for the fermentation assay, which was the lyophilized sample of digested JPP (JPP-IN). SCFA (calculated as $SCFA_{JPP\text{-}IN} - SCFA_{CONTROL\ 2}$), ammonium content and pH data were analyzed by one-way ANOVA followed by Duncan's test. Microbiological counts were analyzed by factorial analyses of variance (with or without JPP-IN x incubation time) followed by Duncan's test when appropriate. The production of gases were analyzed in the SAS® System for Windows ™ version 9.4 (SAS Institute Inc., Cary - NC, USA) application at the level of significance of 5%.

## 3.Results and discussion

### 3.1 Metabolites phenolic compounds formed during the colonic fermentation of JPP-IN

The sample of JPP was previously submitted to in vitro gastrointestinal digestion yielding a non-dialyzable digested fraction (JPP-IN) that was used in the colonic fermentation assay. The tentative identification by HPLC-DAD-Q-TOF-MS/MS of the parent phenolic compounds found in the JPP-IN, as well as their metabolites formed during the *in vitro* fermentation assays are shown in Table 1. The profile of phenolic compounds in the JPP-IN before the fermentation is shown in Table 2. JPP-IN had expressive content of phenolic compounds (3.53 mg/g) with predominance of hydrolyzable tannins (52.6%) (Table 2 at 0 h, Figure 2A). The major hydrolyzable tannins were HHDP-galloylglucose + monogalloyl glucose (Peak 1), HHDP-galloylglucose + bis-HHDP-glucose (Peak 3), digalloylglucose + gallic acid (Peak 2) and digalloylglucose isomer (Peak 11). Besides, JPP-IN had high content of anthocyanins, amounting to 33% of total phenolic content, cyanidin-3-glucoside being the major anthocyanin found (98.7%, Table 2). Flavonols were the third most abundant class of phenolics found in JPP-IN, amounting to 12.2 % of total phenolic content, ellagic acid was the predominant one (Peak 28, Table 2). Besides, the JPP-IN had low content of the phenolic acids and urolithins, about 1.8 and 0.7%, respectively.

The content of phenolic compounds along the fermentation of JPP-IN were corrected to eliminate the interference of phenolic compounds already present in the feces (control 2) and the major phenolic compounds found as well as their variation along the fermentation are shown in Table 2. The phenolic compounds of JPP-IN incubated with feces exhibited significantly different trends from the control 1 (JPP-IN incubated only with buffer, data not shown). Most hydrolyzable tannins were rapidly fermented by fecal microbiota, within 2 h.

Galloyl-castalagin (Figure 2B, Peak 20; Table 2) and the co-elution of HHDP-galloylglucose with monogalloyl glucose (Peak 1, Figure 2B and Table 2) were the compounds that showed the greatest decrease.

The gallotannins tetragalloylglucose and its isomer (Peak 17 and 18, respectively) and pentagalloylglucose (Peak 19, Table 2) also had more than 80% decrease in their content within 2 h. There was a parallel increase in digalloylglucose isomer (Peak 11) (45% within 2 h) indicating the catabolism of complex gallotannins into simpler ones. The pronounced catabolism of gallotannins is expected to increase gallic acid content as observed during the fermentation of *Arbutus unedo* fruit (Mosele et al., 2016). However, we observed a progressive catabolism of gallic acid, -38% within 2 h and -86% within 8 h (Table 2). Gallic acid was likely consumed as a substrate for maintenance of bacteria fermentation or was catabolized into protocatechuic acid by dehydroxylation reaction (Mosele, Macià, & Motilva, 2015). Indeed, we observed a remarkable increase in the content of protocatechuic acid in the start of fermentation (~400% within 2 h). However, gallic acid was quantified as a co-elution with digalloylglucose, which may have masked its changes.

There was an increase of bis-HHDP-glucose isomer (Peak 6) within 2 and 8 h of fermentation (43 and 33% vs. 2 h, respectively), which is suggestive of the catabolism of ellagitannins such as castalagin/vescalagin (Peak 5) and galloyl-castalagin (Peak 20) which decreased within 2 h (27. and 93.8%, respectively) and 8 h of incubation (100% for both compounds)..

The disappearance of hydrolyzable tannins, mainly ellagitannins, during fermentation was parallel to the appearance of urolithins. Urolithin M6, which was not detected in the JPP-IN before fermentation, was the major content at 2 hours, suggesting that these is one of the first metabolites produced during the fermentation of JPP-IN by human feces. The fermentation of JPP-IN also confirmed the production of the urolithin intermediates (D, C and

M7) resulting from the ellagitannins and ellagic acid catabolism as previously reported for pomegranate and *Arbutus unedo* fruits (García-Villalba et al., 2017; Mosele et al., 2016). The final products of phenolics catabolism, urolithin-B sulfate and urolithin A, increased during fermentation specially from 8 and 24 h onwards, while the content of iso-urolithin A remained low. This indicates that the production of urolithin-B was likely produced from iso-urolithin-A, as observed for pomegranate fermentation with human feces (García-Villalba et al., 2017). The intermediate catabolites, urolithins M5, M6 and M7, which showed great increase after 24 and 48 h of JPP-IN fermentation, have been already observed in human feces hereafter of the ellagic acid consumption (García-Villalba, Beltrán, Espín, Selma, & Tomás-Barberán, 2013). M6 and M5 were the major urolithins found in the end of JPP-IN fermentation (48 h) followed by urolithin A and urolithin B sulfate (Table 2).

Urolithins can be absorbed and contribute for the systemic biological effects of ellagitannins once urolithin metabolites were found in the plasma and urine of humans after ellagitannin consumption (García-Villalba, Espín, & Tomás-Barberán, 2016; Seeram et al., 2006). The cardiovascular effects of urolithins C and association of urolithin A+B were observed in cell culture with reduction of the accumulation of cholesterol in macrophages (THP-1) (Mele et al., 2016). The cardiovascular effects also observed *in vivo* with the urolithin A consumption that attenuate myocardial infarct size and cell death in mice after ischemia/reperfusionits injury (Tang et al., 2017).

The profile of urolithin production during polyphenol fermentation is greatly affected by the composition of gut microbiota and not all urolothins are produced under some fermentative conditions (Tomás-Barberán, García-Villalba, González-Sarrías, Selma, & Espín, 2014). Urolithin production is strongly favored by the proliferation of Gordonibacter in human feces (García-Villalba et al., 2017). Although we have not assessed the viability of this

bacteria in our assay, the great number of urolithin metabolites found during JPP-IN fermentation suggests that it is a good substrate for Gordonibacter proliferation.

Anthocyanins, mainly cyanidin-3-glucoside, were the second major class present in JPP-IN. However, they were largely degraded after 2 h and almost completely degraded after 8 h of fermentation. This decrease was parallel to the increase in the content of protocatechuic acid, which is the main metabolite of cyanidin-3-glucoside and has been strong associated with the beneficial health effects of anthocyanin consumption (Wang et al., 2012; Wang, Wei, Yan, Jin, & Ling, 2010). Besides, hydroxyphenylpropionic acid, that was increased after 8 h of JPP-IN fermentation can be another intermediate of cyanidin-3-glucoside catabolism as observed for *Arbutus unedo* fruit after simulation of gastrointestinal digestion (Mosele et al., 2016).

**3.2 Changes in microbial counts and metabolism**

The impact of the JPP-IN on the composition of human gut microbiota was analyzed by assessing plate counts of *Bifidobacteria*, *Enterobacteria* and *Lactobacillus* (Figure 3), as well as SCFA (Figure 4), gas production (Figure 5 and Table 4) and ammonia content (Supplementary material) in collected from the fermentation media of JPP-IN. JPP-IN had no direct effect on fecal bacterial growth (*Lactobacillus*, *Bifidobacteria* and *Enterobacteria*), once a similar plate counts were observed for the control (only feces) and JPP-IN + feces at the start of fermentation (0 h). After 48 h of fermentation the counts of *Lactobacillus* and *Bifidobacteria* remained constant and were not affected by the presence of JPP-IN. However, the selective influence of JPP-IN on proliferation of beneficial bacteria on other fermentation times cannot be ruled out, because of other evidence of beneficial bacteria growth as the increased production of SCFA (Figure 4) and gas production (control data not shown) and the

decreased ammonia content (Supplementary material). In addition, the fermentation of juçara pulp (*Euterpe edulis*) that bears similar polyphenol content to jaboticaba has been shown to increase *Bifidobacteria* counts after 24 h of incubation (Guergoletto et al., 2016). Additionally, pomegranate has been shown to increase *Bifidobacteria* counts during *in vitro* fermentation with human feces *in vivo* in mice after extract consumption (Bialonska et al., 2010; Neyrinck et al., 2013). Although there was no change in the counts of Lactobacillus and *Bifidobacteria*, JPP-IN demonstrated a selective effect against pathogenic bacteria (*Enterobacteria*) since JPP inhibited its growth compared to the control at 48 h of fermentation. The inhibitory effect can be associated to presence of phenolic compounds in JPP-IN. Similar results were observed for red wine extract that reduced *Clostridium histolyticum* counts without effect on beneficial bacteria (Sánchez-Patán et al., 2012). These discrepant results among fruits can be related to the content and types of phenolic compounds from subtract (Parkar, Trower, & Stevenson, 2013). The human colon contains a wide range of bacterial communities, the balance between beneficial and pathogenic bacteria play an important role in health maintenance, since dietary habits and diseases such as obesity and diabetes are strongly associated with intestinal dysbiosis (Ojeda, Bobe, Dolan, Leone, & Kristina, 2016; Sommer & Bäckhed, 2013).

After gastrointestinal digestion JPP still had available substrate for gut bacteria metabolism other than polyphenols as it had high content of dietary fiber and non-fibrous carbohydrates (35.1 and 34.6 g/100g JPP-IN, respectively) (Table 3). Carbohydrate fermentation is the main energy source for gut microbiota yielding SCFA, which are associated to the decrease in pH values, and gas (Danneskiold-Samsøe et al., 2019). Then, gut microbiota also use secondary substrates as proteins for energy, that resulting in fermentation end-products as SCFA (Koecher et al., 2014). Additionally, polyphenol compounds have also

been demonstrated to be able to promote the growth of *Bifidobacteria* and *Lactobacillus* (Guergoletto et al., 2016; Hidalgo et al., 2012).

The kinetic analysis of gas production confirmed the involvement of the soluble fraction initially with the maximum production of gases at 2 h of fermentation contributing to 28.7 mL/g of organic matter incubated (Table 4 and Figure 5). The metabolites HHDP-digalloylglucose isomer and dihydroxyphenyl-γ-valerolactone (Peak 13, Table 3) and protocatechuic acid (Peak 5) had major content at 2 h of fermentation. Therefore, the degradation of polyphenols into these compounds, as well as the degradation of non-fibrous carbohydrates and soluble fiber likely support this early gas production. In fact, these polyphenols have been. previously demonstrated to promote the growth of *Bifidobacteria* and *Lactobacillus*, and SCFA production (Aura et al., 2005; Sun, Chen, Cheng, Zhang, & Zheng, 2017). On the other hand, the fermentation of the insoluble portion of JPP-IN produced 35.9 mL of gas/g of organic matter, and reached the maximum rate of gas production later at 8 h of fermentation (Table 4 and Figure 5). Ellagic acid (peak 28), the co-elution of HHDP-digalloylglucose isomer with dihydroxyphenyl-γ-valerolactone (peak 13), protocatechuic acid (peak 5), and hydroxyphenylpropionic acid (peak 9) had high content at 8 h of fermentation and therefore their formation was likely associated to the later gas production, together with the insoluble fiber content of the JPP-IN (Table 3).

In addition to gas production, JPP-IN fermentation also increased SCFA production compared to the time 0h (Figure 4). Acetic, propionic and butyric acids, where the major SCFA found during fermentation of JPP-IN, and were increased after the first 2 h of fermentation (Figure 4) concomitant with the pH reduction (Supplementary material). The increase of valeric, caproic and heptanoic acid production occurred only after 8 h of fermentation and did not cause additional change in pH value.

SCFA production was likely associated to the fermentation of the soluble fraction of JPP-IN that was demonstrated to contribute to gas production at the same fermentation time (Figure 5). Similar effects have been found in an animal model of obesity, where increased butyric and acetic acid were found in the feces after JPP consumption (Â. G. Batista et al., 2018). Acetic and propionic acid production by gut bacteria involves the metabolism of polysaccharides, that are likely related to *Bifidobacteria* proliferation (not assessed at 2 h of fermentation), that produces acetate mainly through the fructose-6-phosphate phosphoketolase pathway (Miller & Wolin, 1996).

Besides, the greatest concentration of valeric, heptanoic and caproic acids were recorded after 8 h of JPP-IN fermentation and remained unchanged up to 48 h. This can be associated to the degradation of insoluble fiber, which was the major contributor for gas production at this time (Figure 5). SCFA have a major role in the maintenance of body functions, acetate, propionate and butyric acids plays a role in maintaining the integrity and energy source for colonocytes (butyric acid), and peripheral tissues (acetate and propionate), besides modulating inflammatory response (Morrison & Preston, 2016; Sivaprakasam, Prasad, & Singh, 2017). Thus, the prevalence of beneficial bacteria (mainly *Bifidobacteria* and *Lactobacillus*), as well as the elevate SCFA content are associates with health maintenance, while dysbioses and low SCFA production are strongly associated to disease (Cani et al., 2007; Puddu, Sanguineti, Montecucco, & Viviani, 2014; Unger et al., 2016).

## 3. Conclusion

This study provides for the first time insights on the gut microbial metabolism of phenolic compounds from jaboticaba peel powder. The large content of ellagitannins and anthocyanins in JPP-IN yielded numerous phenolic metabolites during fecal fermentation,

which were parallel to the increase in SCFA and decrease in the viability of pathogenic bacteria (*Enterobacteria*). Ellagitannins were catabolized into several urolithins (A, B, C, D, M5, M6 and M7) that were formed in the first 2 h of fermentation. Gallic acid and gallotannins were likely catabolized into protocatechuic acid, whereas anthocyanins (cyanidin-3-glucoside) were mostly catabolized into protocatechuic acid and hydroxyphenylpropionic acid. JPP-IN has been proven to generate bioactive phenolic catabolites that can be bioavailable, as well as to support SCFA production and decrease *Enterobacteria* viability when incubated with human feces. These mechanisms may underlie the beneficial health effects of JPP, which were largely demonstrated in animal models. More human studies are required to confirm such effects.

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**Figure caption**

**Figure 1:** Simulation of JPP fermentation by human gut microbiota in vitro, a representative scheme.

**Figure 2:** Representative chromatogram of the non-anthocyanin phenolic compounds at 280 nm during JPP-IN fermentation. Panels show JPP-IN digested incubated without feces for 0 h (A), and JPP-IN digested incubated with feces for 2 h (B), 8 h (C), 24 h (D), and 48 h (E).

**Figure 3:** Viability of fecal Bifidobacteria (A), Lactobacillus (B) and Enterobacteria (C) during fermentation of JPP-IN with human feces. Different lowercase letters indicate significant difference between JPP-IN and control within the same time and different uppercase letters indicate significant difference between fermentation times ($p < 0.05$).

**Figure 4:** Short-chain fatty acids (SCFAs) production during in vitro fermentation of JPP-IN with human feces. (A) Major SCFAs (B) Minor SCFAs. Data are the SCFA content of JPP-IN fermentation with human feces minus SCFA produced in the control 2 condition. One-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test. * Different from 0 h.

**Figure 5:** Gas production during the fermentation of JPP-IN. The stunned circle is equivalent to the total volume of gases produced (degradation of soluble and insoluble fraction). The triangle represents the volume of gases produced by the degradation of the soluble fraction of

fast digestion. The square represents the volume of gases produced by the degradation of the potentially degradable insoluble fraction of slow diges

**Table 1:** Tentative identification of phenolic compounds and their metabolites found in jaboticaba peel after *in vitro* digestion and fermentation

| **Tentative identification** | **RT** | **MS**/MS |
|---|---|---|
| HHDP-galloylglucose and Monogalloyl glucose | 3.15 | **633.0761:** 301.0025, 275.0226, 249.0437 and **331.0701:** 169.0169 |
| Digalloylglucose and Gallic acid | 3.79 | **483.0806:** 169.0177 and 169.0174: 125.0263 |
| HHDP-galloylglucose and bis-HHDP-glucose | 4.49 | **633.0746:** 301.0021, 275.0225, 249.0433 and **783.0673:** 301.0030, 275.0236 |
| Digalloylglucose isomer | 6.79 | **483.0808:** 169.0177 |
| Protocatechuic acid | 7.35 | **153.0221:** 109.0315, 108.0233 |
| Bis-HHDP-glucose isomer | 9.24 | **783.0638:** 301.0011, 275.0214 |
| Castalin and HHDP-digalloylglucose | 12.09 | **631.0558:** 450.9974, and **785.0857:** 301.0010, 275.0214,249.0422, 169.0161 |
| Castalin isomer and (Epi)catechin | 14.82 | **631.0546:** 450.9974 and **289.0728:** |
| HHDP-galloylglucose | 16.20 | **633.0710:** 301.0021 |
| Digalloylglucose isomer | 17.05 | **483.0776:** 169.0151, 271.0464 |
| Trigalloylglucose | 18.35 | **635.0876:** 169.0162, 465.0676, |
| HHDP-digalloylglucose isomer and Dihydroxyphenyl-γ-valerolactone | 21.39 | **785.0807:** 300.9995, 275.0226 and **207.0646:** 163.0748 |
| Trigalloylglucose isomer | 31.49 | **635.0876:** 169.0162, 465.0676, |
| Castalin/Vescalagin | 38.61 | **466.0207(2):** |
| Trigalloylglucose isomer | 39.48 | **635.0857:** 169.0153 |
| Tetragalloylglucose | 41.62 | **787.0899:** 465.0679, 169.0159, 233.6043 |
| Tetragalloylglucose isomer | 47.24 | **787.0899:** 465.0679, 169.0159, 233.6043 |
| Pentagalloyl glucose | 52.84 | **469.0506 (2):** 169.0141 |
| Galloyl-castalagin | 61.43 | **542.0300(2):** |
| Ellagic acid hexoside | 35.03 | **463.0497:** 300.9993, 299.9928 |
| Ellagic acid pentoside | 37.75 | **433.0410:** 300.9986, 299.9922 |
| Ellagic acid pentoside and Ellagic acid | 43.59 | **433.0418:** 300.9998, 299.9920 and **300.9991:** |

Table 1 (*Continued*)

| **Tentative identification** | **RT** | **MS**/MS |
|---|---|---|
| Myricetin-rhamnoside | 44.52 | **463.0835:** 316.0232, 317.0274 |
| Quercetin-hexoside | 45.87 | **463.0846:** 300.0260, 301.0315 |
| Quercetin-hexoside | 47.94 | **463.0844:** 300.0261, 301.0329 |
| Quercetin-pentoside | 49.72 | **433.0744:** 300.0264, 301.0322 |
| Quercetin-pentoside | 50.91 | **433.0736:** 300.0257, 301.0331 |
| Quercetin-pentoside | 52.21 | **433.0742:** 300.0254, 301.0334 |
| Quercetin-rhaminoside | 53.19 | **447.0931:** 300.0252,301.0337 |
| Delphinidin-3-glucoside | 11.31 | **465.0878:**303.0412 |
| Cyanidin-3-glucoside | 12.28 | **449.095**0:287.0470 |
| Peonidin-3-glucoside | 13.32 | **463.1073:** |
| Urolithin B sulfate | 18.65 | **291.0113:**247.0232,191.0333 |
| Urolithin M5 | 30.50 | **275.0163:**257.0060, 229.0106, 219.0272, 203.0325 |
| Urolithin D | 36.79 | **259.0226:**213.0186, 242.0197 |
| Urolithin D isomer | 38.49 | **259.0226:**213.0186 |
| Urolithin M6 | 43.10 | **259.0226:**213.0186, 159.0426, 241.0099 |
| Urolithin C | 46.85 | **243.0283:**187.0381, 171.0440, 199.0374 |
| Urolithin M7 | 50.84 | **243.0279:**198.0302, 147.0445 |
| Isourolithin A | 57.98 | **227.0333:** 171.0446, 183.0422,159.0423 |
| Urolithin A | 58.86 | **227.0329:**198.0292,182.0352 |

RT= retention time; HHDP= hexahydroxydiphenic acid

**Table 2**: Changes in phenolic compounds (mg compound/g of jaboticaba peel powder digested) during the fermentation of JPP-IN with human feces

| Peak | Phenolic compounds | 0h | 2h | | 8h | | 24h | | 48h | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Content (mg/g JPP-IN) | Content (mg/g JPP-IN) | %VAR | Content (mg/g JPP-IN) | %VAR | Content (mg/g JPP-IN) | %VAR | Content (mg/g JPP-IN) | %VAR |
| **Hydrolyzable tannins** | | | | | | | | | | |
| 1 | HHDP-galloylglucose and Monogalloyl glucose | 0.475±0.276 | 0.087± 0.046 | -81.7 | 0.022±0.009 | -95.4 | nd | -100.0 | Nd | -100.0 |
| 3 | HHDP-galloylglucose and bis-HHDP-glucose | 0.231±0.108 | 0.208± 0.072 | -9.7 | 0.027±0.005 | -88.3 | nd | -100.0 | Nd | -100.0 |
| 6 | Bis-HHDP-glucose isomer | 0.033±0.005 | 0.047 ± 0.013 | 43.3 | 0.044±0.023 | 32.8 | 0.017±0.008 | -48.7 | 0.041±0.014 | 23.7 |
| 7 | Castalin and HHDP-digalloylglucose | 0.128±0.032 | 0.106 ± 0.015 | -16.9 | 0.048±0.028 | -62.6 | nd | -100.0 | 0.018±0.005 | -86.0 |
| 8 | Castalin isomer and (Epi)catechin | 0.014±0.002 | 0.0161 ± 0.009 | 11.6 | 0.029±0.016 | 101.1 | nd | -100.0 | Nd | -100.0 |
| 10 | HHDP-galloylglucose | 0.042±0.004 | 0.020 ± 0.003 | -52.2 | nd | -100.0 | nd | -100.0 | Nd | -100.0 |
| 15 | Castalin/Vescalagin | 0.022±0.003 | 0.016 ± 0.002 | -27.3 | nd | -100.0 | nd | -100.0 | Nd | -100.0 |
| 13 | HHDP-digalloylglucose isomer and Dihydroxyphenyl-γ-valerolactone | 0.104±0.029 | 0.665 ± 0.038 | 538.8 | 0.257±0.157 | 146.9 | 0.107±0.026 | 2.8 | Nd | -100.0 |
| 20 | Galloyl-castalagin | 0.045±0.009 | 0.003 ± 0.001 | -93.8 | nd | -100.0 | 0.028±0.027 | -38.1 | 0.031±0.008 | -31.3 |
| 2 | Digalloylglucose and Gallic acid | 0.219±0.07 | 0.136 ± 0.084 | -38.0 | 0.031±0.005 | -85.9 | nd | -100.0 | Nd | -100.0 |
| 4 | Digalloylglucose isomer | 0.018±0.005 | Nd | -100.0 | Nd | -100.0 | nd | -100.0 | Nd | -100.0 |
| 11 | Digalloylglucose isomer | 0.211±0.040 | 0.307± 0.012 | 45.3 | 0.028±0.029 | -86.7 | 0.034±0.029 | -83.9 | Nd | -100.0 |
| 12 | Trigalloylglucose | 0.033±0.005 | 0.020± 0.005 | -37.3 | nd | -100.0 | nd | -100.0 | Nd | -100.0 |
| 14 | Trigalloylglucose isomer | 0.059±0.010 | 0.014± 0.003 | -76.2 | nd | -100.0 | nd | -100.0 | Nd | -100.0 |
| 16 | Trigalloylglucose isomer | 0.030±0.004 | 0.011± 0.002 | -64.3 | nd | -100.0 | nd | -100.0 | Nd | -100.0 |
| 17 | Tetragalloylglucose | 0.025±0.003 | 0.005± 0.001 | -81.2 | 0.004±0.003 | -84.0 | nd | -100.0 | Nd | -100.0 |

Table 2 (*Continued*)

| Peak | Phenolic compounds | 0h | 2h | | 8h | | 24h | | 48h | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Content (mg/g JPP-IN) | Content (mg/g JPP-IN) | %VAR | Content (mg/g JPP-IN) | %VAR | Content (mg/g JPP-IN) | %VAR | Content (mg/g JPP-IN) | %VAR |
| 18 | Tetragalloylglucose isomer | 0.115±0.009 | Nd | -100.0 | 0.005±0.001 | -95.6 | nd | -100.0 | Nd | -100.0 |
| 19 | Pentagalloyl glucose | 0.050±0.006 | Nd | -100.0 | nd | -100.0 | nd | -100.0 | Nd | -100.0 |
| | **∑ Hydrolyzable tannins** | **1.854** | **1.666** | | **0.495** | | **0.186** | | **0.090** | |
| **Phenolic acids** | | | | | | | | | | |
| 5 | Protocatechuic acid | 0.029±0.012 | 0.143± 0.071 | 398.0 | 0.109±0.016 | 278.0 | 0.020±0.008 | -30.6 | 0.025±0.009 | -13.3 |
| 9 | Hydroxyphenylpropionic acid | 0.034±0.006 | 0.032± 0.023 | -5.6 | 0.065±0.068 | 91.1 | nd | -100.0 | nd | -100.0 |
| | **∑ Phenolic acids** | **0.063** | **0.176** | | **0.174** | | **0.020** | | **0.025** | |
| **Flavonols** | | | | | | | | | | |
| 23 | Ellagic acid hexoside | 0.005±0.001 | nd | -100.0 | 0.001±0.001 | -81.1 | 0.004±0.002 | -24.2 | 0.002±0.0006 | -47.9 |
| 25 | Ellagic acid pentoside | 0.005±0.001 | nd | -100.0 | 0.003±0.001 | -40.0 | 0.003±0.001 | -40.0 | 0.009±0.002 | 80.0 |
| 28 | Ellagic acid pentoside and Ellagic acid | 0.342±0.031 | 0.218 ± 0.024 | -36.2 | 0.404±0.147 | 18.2 | 0.118±0.0625 | -65.5 | 0.057±0.006 | -83.3 |
| 29 | Myricetin-rhamnoside | 0.007±0.001 | 0.002± 0.0004 | -73.8 | nd | -100.0 | nd | -100.0 | nd | -100.0 |
| 30 | Quercetin-hexoside | 0.010±0.001 | 0.006± 0.001 | -42.3 | nd | -100.0 | nd | -100.0 | nd | -100.0 |
| 32 | Quercetin-hexoside | 0.035±0.005 | 0.004± 0.004 | -75.5 | nd | -100.0 | nd | -100.0 | nd | -100.0 |
| 33 | Quercetin-pentoside | 0.005±0.001 | nd | -100.0 | nd | -100.0 | nd | -100.0 | nd | -100.0 |
| 35 | Quercetin-pentoside | 0.018±0.002 | 0.003 ± 0.0007 | -83.3 | nd | -100.0 | 0.001± 0.001 | -94.4 | 0.003±0.0008 | -83.3 |
| 36 | Quercetin-rhamnoside | 0.0017±0.0009 | 0.002± 0.0002 | 5.9 | nd | -100.0 | nd | -100.0 | nd | -100.0 |
| | **∑ Flavonols** | **0.429** | **0.235** | | **0.408** | | **0.126** | | **0.071** | |
| **Urolithins** | | | | | | | | | | |
| 21 | Urolithin B sulfate | 0.014±0.002 | 0.022± 0.001 | 59.7 | 0.049±0.006 | 258.9 | 0.057±0.031 | 317.4 | 0.102±0.016 | 829.8 |

Table 2 (Continued)

| Peak | Phenolic compounds | 0h | 2h | | 8h | | 24h | | 48h | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Content (mg/g JPP-IN) | Content (mg/g JPP-IN) | %VAR | Content (mg/g JPP-IN) | %VAR | Content (mg/g JPP-IN) | %VAR | Content (mg/g JPP-IN) | %VAR |
| 22 | Urolithin M5* | nd | 0.007± 0.001 | Nc | 0.017±0.003 | 136.1 | nd | -100.0 | 0.236±0.125 | 3177.7 |
| 24 | Urolithin D | 0.002±0.001 | nd | -100 | nd | -100.0 | nd | -100.0 | 0.005±0.0011 | 150.0 |
| 26 | Urolithin D isomer | nd | nd | Nc | nd | nc | 0.008±0.002 | nc | 0.011±0.0024 | nc |
| 27 | Urolithin M6* | nd | 0.0496± 0.095 | Nc | 0.055±0.015 | 10.9 | 0.185±0.092 | 273.0 | 0.287±0.0592 | 478.6 |
| 31 | Urolithin C * | nd | 0.004± 0.004 | Nc | 0.012±0.015 | 166.7 | 0.067±0.051 | 1388.9 | 0.044±0.0233 | 394.5 |
| 34 | Urolithin M7 | 0.009±0.001 | Nd | -100.0 | nd | -100.0 | 0.044± 0.015 | 417.3 | 0.080±0.0040 | 1440.0 |
| 37 | Isourolithin A | nd | Nd | Nc | 0.002±0.0005 | nc | 0.003± 0.001 | nc | 0.001±0.0002 | nc |
| 38 | Urolithin A* | Nd | 0.023± 0.0001 | Nc | nd | -100.0 | 0.054± 0.036 | 134.8 | 0.121±0.0243 | 426.1 |
| | **∑ Urolithins** | **0.025** | **0.106** | | **0.135** | | **0.418** | | **0.887** | |
| | **∑ Non-anthocyanin polyphenols** | **2.371** | **2.183** | | **1.212** | | **0.750** | | **1.073** | |
| **Anthocyanins** | | | | | | | | | | |
| # | Delphinidin-3-glucoside | 0.012 ± 0.005 | 0.003±0.001 | -75.0 | 0.0001±0.00006 | -98.8 | 0.001±0.0005 | -98.8 | 0.00006±0.00004 | -99.5 |
| # | Cyanidin-3-glucoside | 1.142 ± 0.530 | 0.158 ± 0.046 | -86.1 | 0.013±0.002 | -98.8 | Nd | -98.8 | 0.001±0.00005 | -93.3 |
| # | Peonidin-3-glucoside | 0.003 ± 0.001 | 0.001 ±0.0008 | -66.6 | nd | -100.0 | Nd | -100.0 | nd | -100.0 |
| | **∑ Anthocyanins** | **1.157** | **0.162** | | **0.013** | | **0.0012** | | **0.0009** | |

Content data are expressed as the content found in JPP-IN incubated with human feces minus the content found in human feces incubated without JPP-IN (control 2). nd: lower than the detection limit (<LOD); %VAR: percentual change in the content at different fermentation times relative to the content at 0 h. * For the compounds that were not detected at 0 h the %VAR was calculated relative to the content at 2 h. #The anthocyanin chromatograms were not shown. nc: not calculated because the compound was not detected at the initial times (0 and 2 h).

**Table 3:** Composition of the lyophilized digested JPP used in the fermentation assay.

| Proximate composition | JPP-IN (g/100 g) |
|---|---|
| Moisture | 6.0 ± 0.5 |
| Ashes | 14.8 ± 0.03 |
| Crude protein | 6.9 ± 0.1 |
| Lipid | 2.4 ± 0.02 |
| Total fiber | 35.2 ± 1.40 |
| Soluble fiber | 14.6 ± 0.1 |
| Insoluble fiber | 20.6 ± 1.3 |
| Non-fibrous carbohydrates* | 34.7 ± 1.2 |

*Calculated by difference.

**Table 4:** Estimation of the parameters of gas production curve during the fermentation of JPP-IN, and their respective asymptotic standard deviation (DPA) and coefficient of determination ($r^2$).

| Parâmetros | JPP-IN |
|---|---|
| $\hat{V}_1$, mL $g^{-1}$ organic material incubated | 28.7 |
| $\hat{V}_2$, mL $g^{-1}$ organic material incubated | 35.9 |
| $\hat{k}_1$, $h^{-1}$ | 0.778 |
| $\hat{k}_2$, $h^{-1}$ | 0.124 |
| $\hat{\lambda}$, h | 1.3 |
| DPA | 2.8 |
| $r^2$ | 0.96 |

$\hat{V}_1$ = maximum volume of gases produced by the degradation of the soluble fraction of fast digestion;

$\hat{V}_2$ = maximum volume of gases produced by the degradation of the potentially degradable insoluble fraction of slow digestion;

$\hat{k}_1$ = specific rate of gas production by the degradation of the soluble fraction;

$\hat{k}_2$ = specific rate of gas production by the degradation of the potentially degradable insoluble fraction;

$\hat{\lambda}$ = latency phase or hydration time, adhesion and microbial colonization (*lag time*).

Figure 1

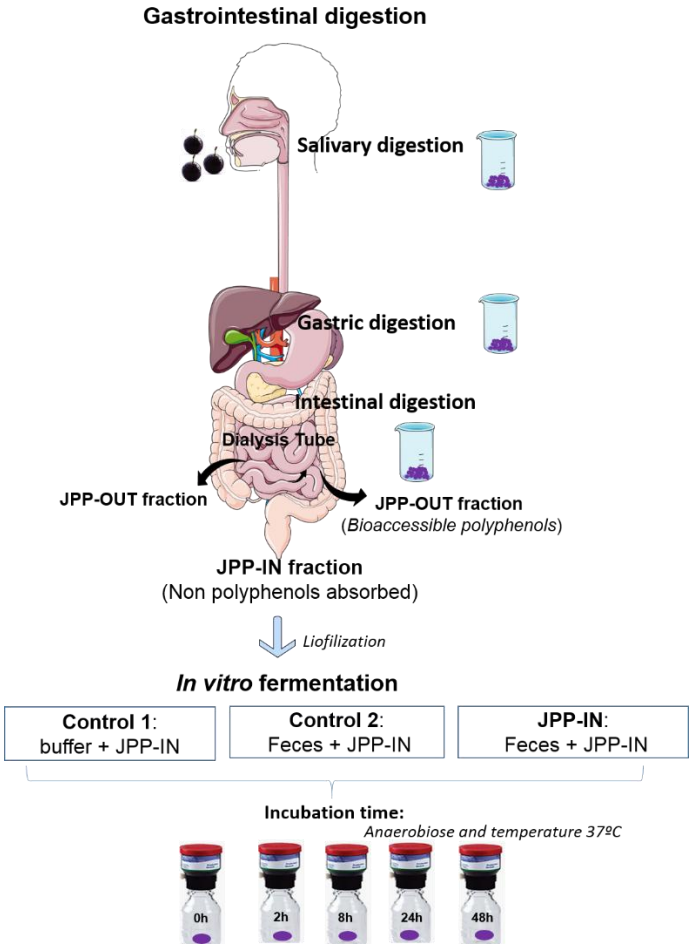

Gastrointestinal digestion
Salivary digestion
Gastric digestion
Intestinal digestion
Dialysis Tube
JPP-OUT fraction
JPP-OUT fraction
(Bioaccessible polyphenols)
JPP-IN fraction
(Non polyphenols absorbed)
Liofilization
In vitro fermentation
Control 1:
buffer + JPP-IN
Control 2:
Feces + JPP-IN
JPP-IN:
Feces + JPP-IN
Incubation time:
Anaerobiose and temperature 37ºC
0h
2h
8h
24h
48h

Figure 2

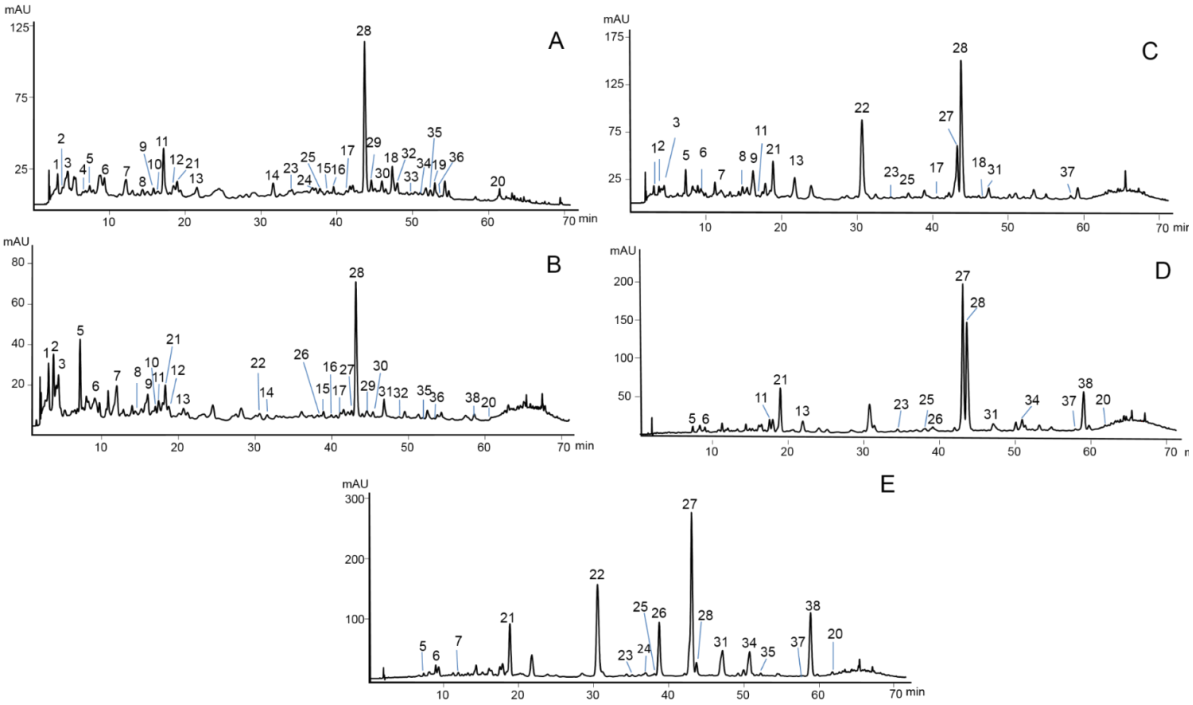


Figure 3

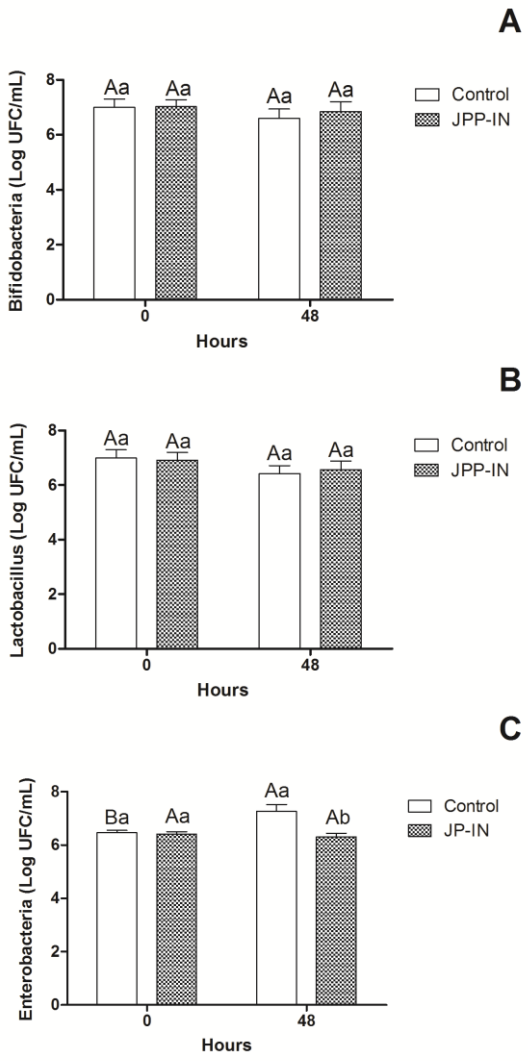

A
Bifidobacteria (Log UFC/mL)
8
6
4
2
0
Aa
Aa
Aa
Aa
0
48
Hours
Control
JPP-IN
B
Lactobacillus (Log UFC/mL)
8
6
4
2
0
Aa
Aa
Aa
Aa
0
48
Hours
Control
JPP-IN
C
Enterobacteria (Log UFC/mL)
8
6
4
2
0
Ba
Aa
Aa
Ab
0
48
Hours
Control
JP-IN

Figure 4

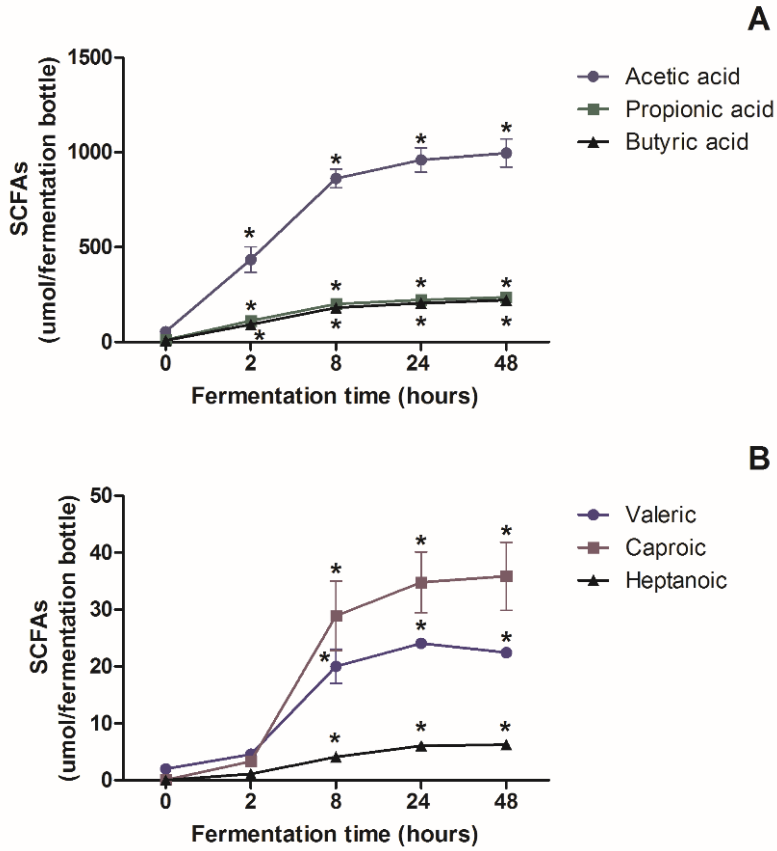
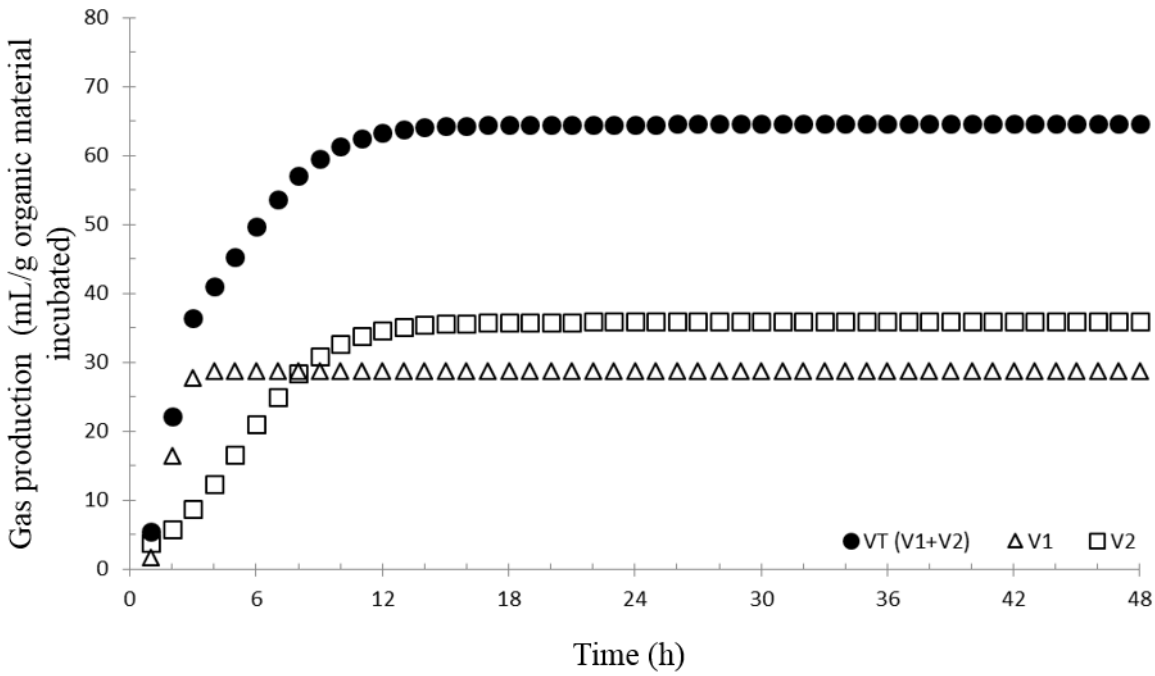

A
SCFAs
(umol/fermentation bottle)
1500
1000
500
0
0
2
8
24
48
Fermentation time (hours)
Acetic acid
Propionic acid
Butyric acid
B
SCFAs
(umol/fermentation bottle)
50
40
30
20
10
0
0
2
8
24
48
Fermentation time (hours)
Valeric
Caproic
Heptanoic

Figure 5


Gas production (mL/g organic material incubated)
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
36
42
48
Time (h)
VT (V1+V2)
V1
V2

## SUPPLEMENTARY MATERIAL

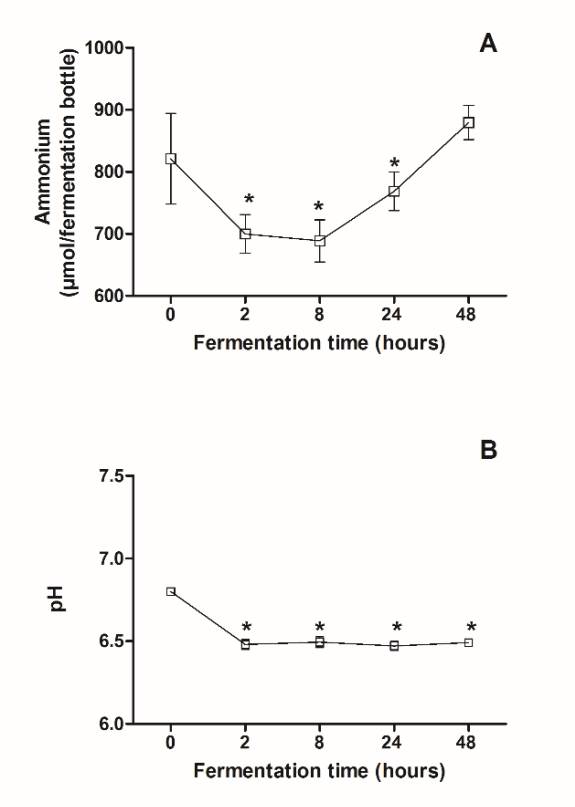



**Figure 1S:** Changes in ammonium content (A) and pH (B) during JPP-IN fermentation with human feces. *differs significantly to initial time (0 hours).

## 4.4 MANUSCRITO 4:

THE HEPATOPROTECTIVE EFFECT OF JABOTICABA PEEL POWDER IN A RAT MODEL OF TYPE 2 DIABETES *MELLITUS* INVOLVES THE MODULATION OF THIOL/DISULFIDE REDOX STATE THROUGH THE UPREGULATION OF GLUTATHIONE SYNTHESIS

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https://www.hindawi.com/journals/jnme/2018/9794629/

**The hepatoprotective effect of jaboticaba peel powder in a rat model of type 2 diabetes mellitus involves the modulation of thiol/disulfide redox state through the upregulation of glutathione synthesis**

Andréia Quatrin [a,b], Lisiane Conte [b,c], Dariane Trivisiol da Silva [b,c], Cassieli Gehlen Figueiredo[b], Sabrina Somacal [b], Miguel Roehrs [b], Cibele Ferreira Teixeira [d], Fernanda Barbisan [c,d], Paula Rossini Augusti [e], Mário Roberto Maróstica Júnior [f], Ivana Beatrice Mânica da Cruz [e], Tatiana Emanuelli [a,b]*

[a]*Graduate Program Food Science and Technology, Centre of Rural Sciences, Federal University of Santa Maria, 97105-900, Santa Maria, RS, Brazil;*

[b]*Integrated Centre for Laboratory Analysis Development (NIDAL), Department of Food Technology and Science, Centre of Rural Sciences, Federal University of Santa Maria, 97105-900, Santa Maria, RS, Brazil;*

[c]*Graduate Program on Pharmacology, Centre of Health Sciences, Federal University of Santa Maria, 97105-900, Santa Maria, RS, Brazil;*

[d]*Biogenomic Laboratory, Department of Morphology, Centre of Health Sciences, Federal University of Santa Maria, Santa Maria, Brazil;*

[e]*Department of Food Science, Federal University of Rio Grande do Sul, Porto Alegre, RS 91501-970, Brazil;*

[f]*School of Food Engineering, University of Campinas, 13083-862 Campinas, SP, Brazil;*

**Corresponding author Tel.: +55 55 3220 8547; fax: +55 55 3220 8353. E-mail address: tatiana.emanuelli@ufsm.br (Tatiana Emanuelli).*

**The hepatoprotective effect of jaboticaba peel powder in a rat model of type 2 diabetes mellitus involves the modulation of thiol/disulfide redox state through the upregulation of glutathione synthesis**

Jaboticaba peel powder (JPP) is rich in bioactive compounds, mainly soluble and insoluble polyphenols with great antioxidant properties. The aim of this study is to evaluate the effects of JPP supplementation on the oxidative stress and hepatic damage in a rat model of type 2 diabetes mellitus (T2DM). Diabetic rats received vehicle or JPP at 2.7 (JPP-I), 5.4 (JPP-II) or 10.8 (JPP-III) g/L in drinking water during 8 weeks. JPP-III attenuated hyperglycaemia and dyslipidemia, increased by 86% the liver content of non-protein thiol groups and by 90% the GSH/GSSG ratio by activating glutathione synthesis. Accordingly, JPP supplementation prevented the loss of activity of the sulfhydryl-dependent enzyme δ-aminolaevulinic acid dehydratase and attenuated hepatic injury assessed by the reduction of serum aspartate aminotransferase activity and liver hypertrophy. Our results support that JPP supplementation to T2DM rats decreases hepatic damage most likely by increasing glutathione synthesis and modulating the thiol/disulfide redox balance.

**Keywords:** Jaboticaba peel powder; oxidative stress, hepatic damage, glutathione, type-2 diabetes

## Introduction

Diabetes is a major public health problem that affected about 387 million persons worldwide in 2014. This figure is expected to reach 592 million by 2035 [1]. Type 2 diabetes (T2DM) is characterized by hyperglycemia and a variable degree of insulin resistance in tissues, as well as, disturbances in the metabolism of lipids, protein and carbohydrates [2]. Chronic hyperglycemia contributes to oxidative stress, which has been hypothesized as a key component in the development of diabetic macrovascular complications [3] and hepatic damage [4].

Liver is a crucial organ for both lipid and carbohydrate metabolism [4]. Lipid and protein oxidation as well as the depletion of endogenous enzymatic and non-enzymatic antioxidants have been shown to contribute to liver membrane damage in diabetic models [5]. The tripeptide glutathione is a

major non-enzymatic antioxidant that plays a key role in the maintenance of hepatic redox balance and protection against diabetes-induced liver damage [6,7]. In addition, the accumulation of free fatty acids (FFA) in T2DM increases the hepatic production of very low-density lipoprotein (VLDL) and impairs insulin signalling [8]. Such events contribute to the nonalcoholic fatty liver disease [4] and dyslipidemia, which increase the risk of cardiovascular disease in diabetic patients [9].

Oral hypoglycemic drugs and insulin are used to treat T2DM but are not effective against the development of macrovascular complications and usually cause side-effects, such as hypoglycemia, weight gain and gastrointestinal intolerance [10]. Thus, nutritional strategies to attenuate diabetic complications would be of great benefit.

Jaboticaba tree (Myrciaria jaboticaba (Vell.) Berg.) is native from Brazil, belonging to the Myrtaceae family [11]. Jaboticaba fruit is very tasty and can be consumed fresh or as juice, liqueurs and jams [11]. It has attracted attention due to the high content of phenolic compounds, mainly anthocyanins and ellagitannins that are mostly found in the fruit peel [11,12]. Jaboticaba also contains insoluble phenolic compounds that are covalently bound to cell wall structural components such as fibre and structural proteins [13]. The consumption of jaboticaba peel powder (JPP) has been shown to yield antioxidant effects in the liver, kidney and plasma of obese rats [14] and improve insulin sensitivity in a mouse model of obesity [15]. In addition, JPP treatment has increased fecal triglycerides and attenuated hepatic lipid oxidation but has not protected against hepatic steatosis in obese rats [16]. The treatment with jaboticaba extract has been shown to reduce triacylglycerol and cholesterol plasma levels but not the hepatic lipid peroxidation in a rat model of type 1 diabetes (T1DM) [17]. T1DM model is characterized only by hyperglycemia but does not mimic the insulin resistance that occurs in T2DM and plays a pivotal role in the disease complications. T2DM responds for 90% of DM cases worldwide, however, JPP effects have not been explored in T2DM models.

This work was aimed to evaluate the effects of dietary supplementation with JPP on the dyslipidemia, oxidative stress and hepatic damage in a rat model of T2DM.

**Materials and methods**

**Jaboticaba peel powder**

Jaboticaba fruits (Myrciaria jaboticaba Vell Berg) were collected, washed and manually peeled in Campinas (São Paulo, Brazil). The peels were frozen at -18 ºC and then freeze-dried in a Liobras (São Carlos, São Paulo, Brazil) freeze-dryer to obtain the JPP, which was stored at -80 ºC until the experimental protocol.

Moisture, ash and protein content were determined according to [18]. Lipid content was determined according to the Bligh & Dyer (1959) method [19]. Total, soluble and insoluble dietary fibre content were determined according to AOAC (2005) [18]. The content of non-fibrous carbohydrate was calculated by subtracting the above percentages from 100%.

The soluble polyphenols of JPP were extracted as described by [20] with some modifications. JPP (1.0 g) was first extracted with 15 mL of a methanol/water/formic acid (85:15:0.5 v/v) solution, stirred for 30 s in vortex and submitted to ultrasound for 5 min (ULTRA Cleaner 1600, São Paulo, Brazil; 135 W RMS). The extract was centrifuged (2000 x g, 10 min) and the pellet was exhaustively extracted as described above. Supernatant fractions were combined and used to determine total monomeric anthocyanins by the pH-differential method [21]. Total monomeric anthocyanin content was calculated using the molecular weight (MW) and molar absorptivity (ε) of cyanidin-3-glucoside, 449.2 and 26.900, respectively. Results were expressed as cyanidin-3-glucoside equivalents. Supernatant fractions were also used to determine soluble polyphenols [22], using a calibration curve of gallic acid, and total soluble proanthocyanidins using a calibration curve of catechin [23].

After drying at 30°C, the pellet (0.2 g) was treated with 10 mL of butanol/HCl (97.5:2.5 v/v) and 0.7 g of FeCl3 at 100ºC for 1 h. The supernatant obtained was used to determine insoluble polyphenols by measuring the anthocyanidin concentration by assessing the sum of absorbances at 555 and 450 nm and using a calibration curve of a carob pod tannin concentrate [24].

Carotenoids were exhaustively extracted from the JPP (0.5 g) with acetone, transferred to petroleum ether/diethyl ether (1:1, v/v), the solvent was evaporated under N2 flux and sample was

reconstituted in petroleum ether to assess the total carotenoid concentration using the specific absorption coefficient (A1 cm1% = 2396) of β-carotene [25].

The composition of soluble polyphenols of the JPP used in this study was assessed by high performance liquid chromatography coupled to photodiode array and mass spectrometry detectors and comprises 52% anthocyanins and 48% non-anthocyanin phenolics.

**Animals and diabetes induction**

Forty male Wistar rats (eight-weeks-old, 150-200 g) were supplied by the Central Animal House of the Federal University of Santa Maria (UFSM, RS, Brazil). All procedures were approved by Committee on Care and Use of Experimental Animal Resources/UFSM (protocol no.: 086/2013). Animals were housed in standard polypropylene cages (four rats/cage) and maintained under controlled room temperature (22 ± 2°C) and humidity (55 ± 5%) with 12:12 h light and dark cycle with access to feed and water ad libitum.

After a one-week acclimation period, rats were randomly divided in two dietary regimens with access to 30 g diet/day/rat and water ad libitum. The non-diabetic control group received a commercial chow diet (Nuvital CR1, Quimtia, Colombo, Paraná, Brazil) and diabetic groups were fed a high-fat diet (HFD), containing 74% commercial chow (Nuvital CR1, Quimtia, Colombo, Paraná, Brazil), 16% lard and 10% sucrose (w/w). After 30 days of dietary manipulation, overnight fasted animals were intraperitoneally injected with a freshly prepared solution of streptozotocin (STZ) (1 mL/kg b.w., 35 mg/kg b.w.) in 0.1 M citrate buffer (pH 4.4) [26] or only 0.1 M citrate buffer (1mL/kg b.w.). Seven days after STZ administration, glycemia was measured and only rats that had glycemia higher than 250 mg/dL were assigned to the diabetic groups.

Rats were then divided into 5 groups (8 animals/group) for an 8-week treatment with JPP as detailed in Table 1. During this period, rats received drinking solutions ad libitum, control rats continued to receive the commercial chow diet (30 g/day/rat) and diabetic rats continued to receive the HFD (30 g/day/rat).

Food and water intake was recorded daily. Body weight was measured every 3 days. At the end of the experiment, rats were fasted for 8 h and then blood was collected from the caudal vein and glucose levels were determined with an automatic analyser donated by Roche® of Brazil (Active, Boehringer Mannheim, Indianapolis, Indiana, USA). Subsequently, rats were anaesthetise with isofluorane and the blood was collected by cardiac puncture into tubes with no additives. Serum was obtained after blood centrifugation at 2000 x g for 15 min, and was then stored at -20°C until biochemical analyses.

A liver portion was homogenized in phosphate buffered saline (PBS) pH 7.4 (1/8, w/v). Liver homogenate was immediately used to determine thiobarbituric acid reactive substances (TBARS). A fraction of liver homogenate was centrifuged at 2000 x g for 15 min to yield a supernatant that was used to determine the activity of antioxidant enzymes and the contents of non-protein thiol groups (NPSH) and protein carbonyl groups.

**Serum biochemical assays**

Insulin was measured in serum by radioimmunoassay using commercial Immunotech kit (Beckman Coulter Company, Marseille/France). The quantitative insulin sensitivity check index (QUICKI) [27] and the fasting insulin resistance index (FIRI) [28] were calculated as follows:

QUICKI= 1/[log (fasting insulin level) + log (fasting glycemia)] (1)

FIRI = [fasting insulin level (mU/L) + fasting glycemia (mmol/L)]/25 (2)

The serum levels of total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol, glucose, as well as the activities of aspartate aminotransferase (ALT) and alanine aminotransferase (AST) were determined by enzymatic methods, using commercial kits (Doles, Goiania, GO, Brazil). The serum level of very low density lipoprotein cholesterol (VLDL) was calculated by the Friedewald's equation [29]:

VLDL= TG/5 (3)

The atherogenic index of plasma (AIP) was calculated conform [30] with the formula:

$$AIP = Log\ (TG/HDL) \quad (4)$$

**Markes of oxidative stress**

Lipid peroxidation in liver homogenate was estimated colorimetrically by measuring TBARS [31]. Hepatic protein carbonyl groups was determined by reaction with 2,4-dinitrophenylhydrazine (DNPH) at 240 nm [32]. Hepatic NPSH levels were measured as described by [33] after sample deproteinization with 4% trichloroacetic acid. The levels of reduced glutathione (GSH) and oxidised glutathione (GSSG) were measured by the fluorimetric
method using O-phthalaldehyde [34] and results were expressed as the GSH/GSSG ratio.

**Antioxidant enzymes activities**

Superoxide dismutase (SOD) activity was determined at 480 nm [35]. Catalase (CAT) activity was measured at 240 nm [36]. Thioredoxin reductase-1 (TrxR-1) activity was determined at 412 nm [37].

Glutathione peroxidase (GPx) activity was determined at 340 nm [38]. Glutathione reductase (GR) activity was determined according to [39]. Glutathione S-transferase (GST) activity was determined at 340 nm [40].

Hepatic δ–aminolaevulinic acid dehydratase (δ-ALA-D) activity was assayed by measuring the rate of porphobilinogen (PBG) formation using Ehrlich's reagent at 555 nm after sample incubation for 1 h at 37$^{o}$C [41].

The protein content of liver tissue supernatant was measured using bovine serum albumin as standard [42] and used to calculate enzyme activities in liver tissue.

**Inflammatory cytokine level**

TNF-α concentration in serum samples were analysed by ELISA (eBIOSCIENCE®, San Diego, CA, USA).

**Quantitative real time RT-PCR (Qrt-PCR) analysis**

The gene expression of enzymes involved in the glutathione synthesis and apoptosis markers were assessed in liver samples. The RNA extraction was performed using Trizol reagent following the manufacturer's instructions (Ludwing-Biotec, São Paulo, Brazil). The RNA extracted was measured by a Thermo Scientific NanoDropTM 1000 spectrophotometer. To perform reverse transcription, RNA was added to the samples of RNA (1000 ng/ul) with 0,2ul of DNAase (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37ºC for 5 minutes followed by heating at 65ºC for 10 minutes. The cDNA was generated with Iscript cDNA and Mix Iscript (Bio-Rad Laboratories, Hercules, CA-USA). qRT-PCR was conducted in the Rotor-Gene Q 5plex HRM System (Qiagen Biotechnology, Germany) with 2x QuantiFast SYBR Green PCR Master Mix (Qiagen Biotechnology, Germany). qRT-PCR reactions were run in triplicate, using 1 µM of each primer, 1000 ng/µL of cDNA, RNAase-free water and 2x QuantiFast SYBR® Green PCR Master Mix (QIAGEN Biotechnology, Germany). β-actin was used as the housekeeping gene and its expression level was used as an internal control. The relative gene expression was calculated using the comparative cytosine-thymine (Ct) method and was expressed as the fold expression compared to the control. The specific primer pairs used were described in Table 2.

**Statistical analyses**

Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test or Dunnett's test (for gene expression data), when necessary. Data that did not meet the ANOVA assumptions were submitted to the non-parametric Kruskal Wallis analysis, followed by a multiple comparison test. Results were expressed as the mean ± SEM and differences

were considered statistically significant when $p<0.05$. Data were analysed using the Statistica ® 9.1 software system (Statsoft Inc., 2004).

**Results**

JPP contains important phytochemicals such as polyphenols and carotenoids (Table 3). Among these phytochemicals, soluble polyphenols are found at the greatest concentration, being composed mostly by cyanidin-3-glucoside, ellagitanins, gallic acid derivatives, delphinidin-3-glucoside and quercetin (Table 4). JPP is also a good source of insoluble polyphenols, as well as carotenoids. Besides, JPP has considerable amount of total dietary fiber (24.4%), being 9.3% soluble dietary fibre (Table 3).

The average drinking intake for diabetic rats amounted to 265 mL/day/kg b.w. and did not differ among all diabetic groups during the treatment (data not shown).

Before treatment with JPP, rats that received high-fat diet and low doses of STZ to induce T2DM had increased glycemia when compared to the control group (Table 5; $p < 0.05$). After 8-week treatment, only rats that received the highest dose of JPP (JPP-III) had lower glycemia (19%) than the diabetic-vehicle group. T2DM induction did not alter fasting serum insulin levels. However, the diabetic JPP-I group had lower insulin level than the diabetic-vehicle group (Table 5; $p<0.05$). T2DM induction increased insulin resistance (FIRI index) and decreased insulin sensitivity (QUICKI index) when compared to the control group (Table 5; $p < 0.05$). JPP treatment reduced to control levels the insulin resistance and improved insulin sensitivity only at the lowest dose (JPP-I; Table 5; $p < 0.05$).

T2DM induction increased the levels of TC, TG, LDL-cholesterol and VLDL-cholesterol and the atherogenic index in comparison to the control group (Figure 1A, 1B, 1C and 1E; $p < 0.05$), whereas the HDL-cholesterol levels did not differ among groups (Figure 1D; $p > 0.05$). JPP treatment did not affect HDL-cholesterol levels (Figure 1D) but reduced the total cholesterol levels in diabetic rats at all doses tested (Figure 1A; $p < 0.05$). In addition, treatment with JPP-II and JPP-III, also reduced TG, LDL-cholesterol and VLDL-cholesterol levels (Figures 1B, 1C and 1E; $p < 0.05$) and

attenuated the cardiovascular risk (AIP, Figure 1F; $p < 0.05$) to values similar to the control levels but JPP-I had no effect.

The diabetic-vehicle group had higher liver weight (31.7%) and serum levels of the inflammatory marker TNF-α (54.9%) and ALT activity (125.2%) than the control group (Figure 2A, 2B and 2C; $p < 0.05$), although AST serum activity did not differ among groups (Figure 2C, $p > 0.05$). JPP treatment at the highest dose (JPP-III) prevented the increase of liver weight (Figure 2A), TNF-α levels (Figure 2B) and ALT activity (Figure 2C) in diabetic rats ($p < 0.05$).

Hepatic gene expression of caspase-3 increased after induction of T2DM when compared to control group, whereas caspase-9 gene expression decreased with induction of T2DM (Figures 2D and 2E; $p < 0.05$). Only JPP-I treatment was able to prevent the increase in caspase-3 expression (Figure 2D; $p < 0.05$) but no treatment was able to restore caspase-9 gene expression (Figure 2E; $p > 0.05$).

T2DM induction increased hepatic lipid oxidation assessed by TBARS levels (82.9%) and protein oxidation assessed by the content of protein carbonyl groups (65.4%) in comparison to the control group (Figures 3A and 3B; $p < 0.05$). JPP treatment did not prevent the increase in lipid or protein oxidation (Figures 3A and 3B; $p > 0.05$).

T2DM induction also reduced NPSH levels (49.8%), the GSH/GSSG ratio (47.0%) and the activity of the sulfhydryl-containing enzyme δ-ALA-D (26.1%) in liver compared to the control group (Figures 3C, 3Dand 3E; $p < 0.05$). After 8 weeks of JPP treatment, the depletion of NPSH groups in liver was partially prevented by JPP-I and JPP-II, whereas JPP-III completely restored NPSH levels (Figure 3C; $p < 0.05$). The GSH/GSSG ratio was partially recovered by JPP-II and completely recovered by JPP-III supplementation (Figure 3D; $p < 0.05$). ALA-D activity was also recovered by JPP treatment but only at the highest dose (Figure 3E; $p < 0.05$).

Additionally, T2DM reduced gene expression of GCLcs (25.3%) and GS (12.3%), two enzymes involved in GSH synthesis, in comparison to the control group (Figures 3F and 3G; $p < 0.05$). The treatment with JPP-II and JPP-III recovered gene expression of GCLcs up to control level (Figure 3F; $p < 0.05$). On the other hand, the treatment with JPP-I and JPP-II increased gene

expression of GS expression above control levels ($p < 0.05$) but the treatment with JPP-III failed to do so (Figure 3G; $p < 0.05$).

T2DM reduced the activities of hepatic antioxidant enzymes SOD (31.2%), CAT (48.5%), TrxR-1 (48.2%), GPx (57.5%) and GST (20.6%), but not GR when compared to the control group (Table 6, $p < 0.05$). JPP-III supplementation decreased GR activity (Table 6, $p < 0.05$). No JPP doses restored SOD, CAT, TrxR-1, GPx or GST activities impaired by T2DM (Table 6, $p > 0.05$).

**Discussion**

Currently much attention has been focused on the potential of fruit phytochemicals, especially polyphenols, to prevent and treat chronic diseases such as T2DM [43]. Extractable polyphenols and anthocyanins were the main bioactive compounds found in JPP and they have been associated to the improvement of oxidative stress, insulin resistance and lipid profile in a rat model of obesity [14,15,44].

The content of JPP's insoluble polyphenols, which refers to the polyphenols bound to fibres and protein, was higher than the amount found in other freeze-dried fruits, as cherry, white grape, strawberry and apple with peel [45]. Insoluble polyphenols can be hydrolysed by gut microbiota yielding phenolic metabolites that can be absorbed promoting beneficial health effects [46]. Additionally, the JPP used in this study had higher content of carotenoids (1.78 mg/100 g JPP) than the jaboticaba fruit of the same species cultivated in Minas Gerais (Brazil) (0.87 mg β-carotene/100 g dry weight basis) [13].

We have demonstrated that the consumption of JPP for 8 weeks reduced glycaemia, dyslipidemia and hepatic complications in a rat model of T2DM, providing evidence that JPP treatment has beneficial protective effects even after diabetes and insulin resistance are installed. Distinct mechanisms seem to underline the protective effects of JPP at the different doses tested. While the highest JPP dose decreased end glycaemia, the lowest JPP dose decreased insulin levels. Most protective effects of JPP had a linear dose-response behavior and were more evident at the highest dose of JPP, namely the decrease of liver hypertrophy and plasma alanine aminotransferase

activity (a marker of liver damage) and the increase of liver thiol levels (increased NPSH levels, GSH/GSSG ratio) and ALA-D activity. Similar dose-response behavior was observed for the protective effects of JPP against diabetes-induced dyslipidemia. On the other hand, the beneficial effects of JPP by improving insulin signalling and decreasing the expression of caspase-3 did not obey a linear dose-response behavior as they were observed only at the lowest dose of JPP. These effects seem to be better explained by a hormetic response [47].

Anthocyanins likely contributed to the antidiabetic effect of JPP as the intake of cyanidin 3-glucoside either purified or from Queen Garnet plum juice (7.4-7.6 mg anthocyanin/kg b.w./day) have been shown to decrease insulinemia and improve glucose tolerance in diet-induced metabolic syndrome in rats [48]. In the present study, we found similar results after the intake of JPP-I (daily average intake of 10.3 mg anthocyanins/kg b.w.). This lowest dose of JPP improved insulin signalling as indicated by the increased insulin sensitivity (QUICKI) and decreased insulin resistance (FIRI). These effects were caused by the ability of JPP-I to remarkably reduce serum insulin levels, while keeping glycaemia levels similar to the vehicle group. The decrease of insulin levels triggered by JPP disappeared at the highest JPP doses indicating a U-shaped effect characteristic of hormetic responses [47]. This biphasic dose response model has been shown to be better than the linearity and threshold-response models to explain the behavior of various toxic and therapeutic drugs [49] and appears to explain the effect of JPP on insulin signalling. On the other hand, the hypoglycaemic effect of JPP exhibits a linear dose-response behaviour (observed at the highest dose, JPP-III; daily average intake of 37.5 mg anthocyanins/kg b.w.). This effect may be related to low intestinal glucose absorption either due to dietary fibre or due to the inhibition of digestive α-amylase and α-glucosidase activities by phenolic compounds as recently reported for jaboticaba extracts [50].

Dyslipidemia can result in cardiovascular complications in T2DM patients [9] and the atherogenic index can predict future cardiovascular disease even before the development of diabetes (prediabetes) [51]. Accordingly, we found an increased atherogenic index in the diabetic-vehicle group. JPP treatment improved the lipid profile, mainly by decreasing triglycerides and LDL levels, which consequently attenuated the atherogenic index. Soluble dietary fibre probably contributed to the

lipid-lowering effect of JPP, because it increases the viscosity of the intestinal content and limits fat absorption by impeding the action of bile salts and enzymes or by sequestering bile salts [52]. Accordingly, the hypocholesterolemic and hypotriglyceridemic effects of riceberry in a rat model of T2DM have been attributed to the presence of fibre (soluble and insoluble) and not to the polyphenols [5].

The consumption of fat diet along with the insulin resistance can further increase circulating FFA, which promotes lipotoxicity by increasing VLDL and triglycerides synthesis in hepatic tissue. Thus, fat liver accumulation in non-treated T2DM patients can result in hepatic steatosis or nonalcoholic fatty liver disease (NAFLD), when fat accumulation is associated with inflammatory process [4]. Accordingly, the rat model of T2DM exhibited dyslipidemia, insulin resistance, inflammatory response (TNF-α), as well as hepatic damage indicated by increased activity of serum transaminases and liver hypertrophy and apoptosis (increased gene expression of caspase-3). JPP-III treatment was able to improve the dyslipidemia, most likely by reducing lipid accumulation in the liver of T2DM rats, which may be associated with the decrease of liver weight. JPP-III treatment attenuated hepatic damage as indicated by the reduced serum ALT activity and liver hypertrophy. These protective effects of JPP were not associated to the prevention of hepatic lipid oxidation or apoptosis. Excess glucose promotes an imbalance between reactive species and antioxidant defences being responsibly for tissue damage in the T2DM [52]. Accordingly, we found increased lipid and protein oxidation with concomitant reduction of enzymatic antioxidant defences (SOD, CAT, TrxR-1, GPx and GST activities) in the liver of diabetic rats. T2DM induction also depleted non-enzymatic antioxidant defences assessed by the hepatic content of NPSH and the GSH/GSSG ratio. GSH, which is the major contributor to the hepatic content of NPSH, can modulate cell death by regulating the redox state of specific thiol residues of proteins, such as caspases [54]. The depletion of NPSH levels and the decrease of GSH/GSSG ratio in T2DM rats were not associated to an increase in GST activity, which uses GSH to detoxify xenobiotics. Also the depletion of GSH was not associated to an increase in GPx activity, which uses GSH to remove peroxides, or to a decreased GR activity, which reduces oxidized GSH. GSH depletion could be possibly associated to a failure in the conversion the oxidized gluthatione into GSH by the thioredoxin protein or thioredoxin system [55], confirmed by the decrease

in TrxR-1 activity with T2DM induction. We observed that the depletion of NPSH and GSH (GSH/GSSG ratio) content was accompanied by decreased activity of δ-ALA-D. The sulfhydryl-containing enzyme δ-ALA-D was inhibited upon oxidation of its sulfhydryl groups and has been suggested to be a sensor for oxidative stress [56]. Thus, GSH depletion in diabetic rats was functionally relevant and indicates an imbalance in the thiol/disulfide redox state that probably contributed to the hepatic oxidative stress and tissue damage.

Despite restoring GSH levels (NPSH and GSH/GSSG ratio) and the activity of δ-ALA-D in diabetic rats, JPP treatment was not able to decrease the hepatic oxidative damage in lipids or protein oxidation assessed by protein carbonylation. The improvement of NPSH levels and GSH/GSSG ratio caused by JPP-III were most likely caused by increased GSH synthesis due to recovered gene expression of GCLcs, which is the rate-limiting step in GSH biosynthesis [54]. Cyanidin-3-glucoside has been shown to increase hepatic GSH synthesis in a culture of liver human cells (HepG2) by inducing GCLcs expression via the PKA–CREB signalling pathway [57]. The decrease in the expression of GS observed in the present study for the JPP-III group (0.4 times that of control and vehicle group) will not be expected to decrease GSH synthesis because the activity of GS, which is upstream GCL in glutathione synthesis, is normally 2 to 4 times higher than GCL [54]. Accordingly, NPSH levels and GSH/GSSG ratio were linearly increased with the increase in JPP dose as observed for the gene expression of GCL.

The beneficial effect of JPP on δ-ALA-D activity is very important, since this is the rate-limiting enzyme for the biosynthesis of heme, the prostetic group of haemoglobin. We can speculate that the protective effect of JPP occurred by preventing the oxidation of sulfhydryl groups in the active site of δ-ALA-D. This antioxidant mechanism is likely caused by the increased synthesis of GSH due to the upregulation of gene expression of GCLcs, the main regulatory enzyme for glutathione biosynthesis in liver.

Liver complications were observed in T2DM rats as indicated by the tissue hypertrophy and increased activity of ALT in the serum, which is a better marker of liver damage than AST activity that remained unchanged. ALT activity has been long considered as a specific indicator of hepatic

damage, because it is found at higher concentration in liver than in other tissues [58]. Lipid and protein oxidation reveals hepatic oxidative stress in our T2DM model, which has been shown to alter transport function and membrane permeability in hepatocytes [53] and could contribute to the increase in serum transaminase [59]. The hepatic damage was confirmed by the activation of the apoptotic pathway as we observed an increase in gene expression of caspase-3 in the diabetic-vehicle group. Caspase-3 was most likely activated by the extrinsic pathway (caspases 8 and 10) as the gene expression of caspase 9, which belongs to the intrinsic pathway was indeed reduced in diabetic rats. Additionally, a decrease in the redox status of GSH and thioredoxin protein (Trx) can induce JNK-dependent apoptosis [61]. The oxidation of Trx triggers signalling for the expression of proapoptotic factors, such as TNFα, FasL and Bak [60]. In addition, apoptosis can be activated by a caspase-independent pathway when the GSH levels decline, via activation of the apoptosis inducing factor (AIF) that causes direct DNA fragmentation [61]. Hyperglycemia has been shown to increase the intrinsic apoptosis pathway/mitochondrial through oxidative stress and Bax protein expression in diabetes models [62]. However, our T2DM model was characterized by increased dietary fat content and inflammatory process (elevated TNF-α levels), which can activate the extrinsic apoptosis. In fact, the activation of TRL-4 and TNF-α receptors in liver has been shown to activate the extrinsic apoptosis pathway with activation of caspase 8 and 10 in a rat model of T1DM [63].

Liver necrosis can occur simultaneously with apoptosis, and both mechanisms of cell death involve caspase activation. Necrosis is a mechanism of unregulated cell death, which involves an exacerbated inflammatory process and the loss of cellular permeability [64]. The rupture of cellular membrane releases cytosolic transaminases into serum and triggers cellular events causing cell swelling. The hepatic accumulation of triglycerides together with the activation of cell death by necrosis can contribute to the hepatic hypertrophy in T2DM rats. Thus, our T2DM model was likely associated to necrosis as indicated by the release of ALT into serum, the inflammatory process and the increased gene expression of caspase-3.

Although JPP treatment at the highest doses (II and III) was not able to decrease caspase-3 expression, the consumption of JPP at the highest dose promoted an initial recovery of hepatic damage by attenuating the inflammatory response and preventing changes in liver weight and serum ALT

activity, which is likely related to the preservation of hepatocyte cell membrane integrity. On the other hand, only the lowest dose of JPP was able to decrease gene expression of caspase-3. The decrease of caspase-3 expression in JPP-I may be explained by the reduction in insulin levels, since insulin can induce apoptosis by activating caspase-3 via phosphatidylinositol 3-kinase (PI3-kinase) pathway [65].

Additionally, JPP treatment recovered T2DM dyslipidemia in a dose-dependent manner and restored non-enzymatic antioxidant defences (NPSH and GSH/GSSG ratio), without changes in the enzymatic antioxidant defences (SOD, CAT, TrxR-1, GPx and GST activities). The highest dose of JPP was particularly effective for recovering the sulfhydryl reducing status of liver tissue as it yielded the greatest increase in NPSH levels, GSH/GSSG ratio and prevented δ-ALA-D inhibition. Thus, our data suggest that the modulation of thiol/disulfide redox state, rather than modulation of the apoptosis pathway, is the major mechanism responsible for the protective effect of JPP against hepatic damage in T2DM.

Our data indicate that JPP had a relevant protective role by preserving thiol/disulfide redox balance and protecting sensitive protein thiols from irreversible oxidation triggered by diabetes-induced oxidative conditions. During oxidative stress, protein cysteine residues (Prot-SH) can be oxidized to sulfenic acid (Prot-SOH) , which can react with GSH to form protein mixed disulfides Prot-SDG (glutathionylation), which in turn can be reduced back to Prot-SH via glutaredoxin (Grx) or sulfiredoxin (Srx) [54]. This is a mechanism to protect sensitive protein thiols from irreversible oxidation [54].

In conclusion, the present study showed that JPP treatment at the highest dose (JPP-III) attenuated hyperglycaemia, insulin resistance, hyperlipidemia and hepatic complications in T2DM model. Hepatic protection was likely mediated by the increase in GSH synthesis that restored NPSH levels and probably prevented the loss of activity of sulfhydryl-containing enzymes.

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**Disclosure interest**

The authors report no conflicts of interest.

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Table 1. Treatment of experimental groups.

| Groups | Diet | Injection (i.p.) | Drinking solution |
|---|---|---|---|
| Control (non-diabetic) | commercial chow diet | vehicle* | vehicle[#] |
| Diabetic-vehicle | HFD | STZ | vehicle[#] |
| Diabetic JPP-I | HFD | STZ | 2.7 g JPP/L of vehicle |
| Diabetic JPP-II | HFD | STZ | 5.4 g JPP/L of vehicle |
| Diabetic JPP-III | HFD | STZ | 10.8 g JPP/L of vehicle |

*STZ vehicle was 0.1 M citrate buffer, pH 4.4. [#] Water containing 0.5% carboxymethyl cellulose was used as vehicle to stabilize the drinking suspension of JPP. STZ:streptozotocin. HFD: high-fat diet.

Table 2. Primer sequences.

| Primers | Forward | Reverse |
|---|---|---|
| Caspase-3 | GAGACAGACAGTGGAACTGACGATG | GGCGCAAAGTGACTGGATGA |
| Caspase-9 | CTGAGCCAGATGCTGTCCCATA | GACACCATCCAAGGTCTCGATGTA |
| Glutamate-cysteine ligase, catalytic subunit (GCLcs) | GTGGACACCCGATGCAGTAT | TCATCCACCTGGCAACAGTC |
| Glutathione synthase (GS) | GCAGGAACTGAGCAGGGTG | GCTTCAGCACAAAGTGGCTAG |
| β-actin | GCAGGAGTACGATGAGTCCG | ACGCAGCTCAGTAACAGTCC |

Table 3. Composition of freeze-dried jaboticaba peel powder

| | Amount (mean ± SD) |
|---|---|
| *Proximate composition* | |
| Moisture (%) | 17.1 ± 0.2 |
| Ash (%) | 3.3 ± 1.0 |
| Protein (%) | 5.6 ± 0.0 |
| Lipids (%) | 1.3 ± 0.2 |
| Total dietary fiber (%) | 24.4 ± 1.0 |
| Soluble dietary fiber (%) | 9.3 ± 0.9 |
| Insoluble dietary fiber (%) | 15.1 ± 0.1 |
| Non-fibrous carbohydrates (%) | 48.3 ± 0.4 |
| *Phytochemicals* | |
| Soluble polyphenols (g gallic acid equivalents/100 g JPP) | 9.67 ± 0.42 |
| Insoluble polyphenols (g condensed tannins/100 g JPP) | 0.73 ± 0.15 |
| Carotenoids (mg β-carotene equivalents/100 g JPP) | 1.78 ± 0.13 |

Table 4. Composition of soluble polyphenols of freeze-dried jaboticaba peel powder evaluated by LC-PDA-MS$^n$

| | Amount (%) |
|---|---|
| *Anthocyanins* | *52* |
| Cyanidin-3-glucoside | 90.15 |
| Delphinidin-3-glucoside | 9.38 |
| Pelargonidin-3-glucoside | 0.17 |
| Peonidin-3-glucoside | 0.31 |
| *Non-anthocyanin phenolics* | *48* |
| Ellagitannins and Gallotannins | 69.57 |
| Quercetin-hexoside | 2.35 |
| Ellagic acid | 4.95 |
| Myricetin-rhamnoside | 1.44 |

Table 5. Effect of JPP treatment on the glycaemia, insulinemia, insulin sensitivity and insulin resistance of control and diabetic rats.

| | | Diabetic | | | |
|---|---|---|---|---|---|
| | Control | Vehicle | JPP-I | JPP-II | JPP-III |
| Onset glucose (mg/dL) | 102.3 ± 1.5 | 353.3 ± 29.9* | 366.7 ± 20.6* | 362.0 ± 16.7* | 369.7 ± 42.2* |
| End glucose (mg/dL) | 96.7 ± 1.9 | 469.0 ± 15.6* | 443.3 ± 20.1* | 422.5 ± 15.1* | 379.5 ± 36.4*# |
| Insulin (mU/L) | 5.12 ± 0.6 | 7.39 ± 1.4 | 3.64 ± 0.7 # | 10.10 ± 2.7 | 8.45 ± 2.5 |
| QUICKI | 0.71 ± 0.02 | 0.46 ± 0.02* | 0.53 ± 0.02*# | 0.44 ± 0.02* | 0.49 ± 0.03* |
| FIRI | 0.56 ± 0.05 | 0.82 ± 0.09* | 0.58 ± 0.06# | 0.86 ± 0.08* | 0.79 ± 0.08* |

Glycaemia was assessed before (onset) and after (end) JPP treatment, whereas the other measures were only made after JPP treatment. Data are presented as means ± SEM (n=8). *Different from the control group, #Different from the diabetic-vehicle group (ANOVA followed by Duncan's test, $p<0.05$). QUICKI: insulin sensitivity index; FIRI: fasting insulin resistance index.

Table 6. Effect of JPP treatment on the activity of antioxidant enzymes in the liver of control and diabetic rats.

| | | Diabetic | | | |
|---|---|---|---|---|---|
| | Control | Vehicle | JPP-I | JPP-II | JPP-III |
| SOD (U/mg ptn) | 27.2 ± 0.7 | 18.7 ± 0.7* | 21.0 ± 1.3 | 19.3 ±0.9* | 20.4 ± 1.7* |
| CAT (K/mg ptn) | 32.6 ± 1.8 | 16.8 ± 0.9* | 17.3 ± 0.9* | 19.2 ± 1.0* | 20.1 ± 3.3 |
| TrxR-1 (nmol DTNB/min/mg ptn) | 14.1 ± 0.6 | 7.3 ± 0.7* | 9.8 ± 0.9 | 8.8 ± 0.9* | 9.0 ± 1.1 |
| GPx (nmol NADPH/min/mg ptn) | 4.0 ± 0.8 | 1.7 ± 0.6* | 1.1 ± 0.3* | 1.1 ± 0.2* | 1.9 ± 0.4* |
| GR (nmol NADPH/min/mg ptn) | 13.3 ± 1.1 | 9.6 ± 2.4 | 9.9 ± 0.7 | 13.3 ± 1.6 | 3.8 ± 0.7*# |
| GST (nmol CDNB/min/mg ptn) | 504.1 ± 13.6 | 400.3 ± 28.4* | 344.5 ± 16.7* | 377.7 ± 22.8* | 356.3 ± 44.3* |

Data are presented as means ± SEM (n=8). *Different from the control group (CAT, SOD and TrxR-1: Kruskal-Wallis-Multiples comparisons' Test and GST, GPx and GR: ANOVA-Duncan's Test, $p<0.05$). JPP: jaboticaba peel powder, SOD: superoxide dismutase, CAT: catalase, TrxR-1: thioredoxin reductase-1, GPx: glutathione peroxidase, GR: glutathione reductase, GST: glutathione S-transferase, ptn: protein, DTNB: 5,5'-ditiobis(2-nitrobenzoic acid), NADPH: nicotinamide adenine dinucleotide phosphate reduced, CDNB: 1-chloro-2,4-dinitrobenzene.

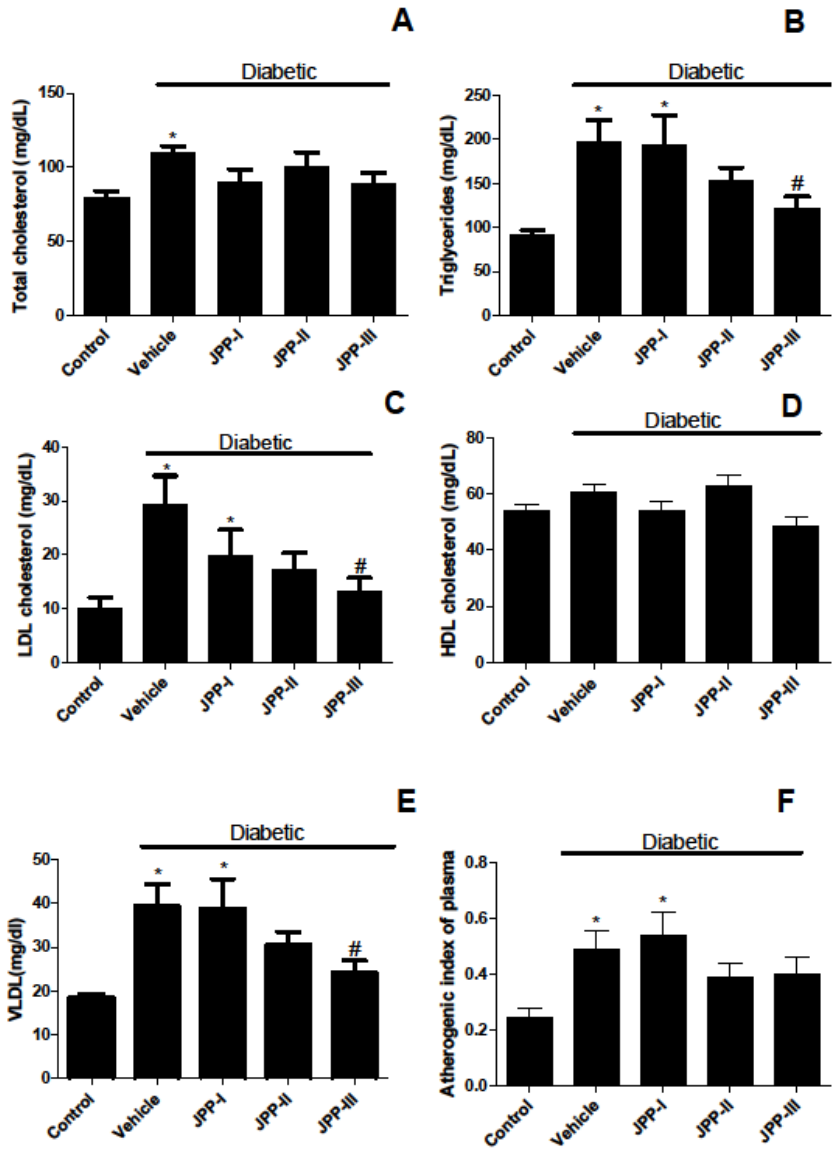


**Figure 1**. Serum levels of total cholesterol (A), triglycerides (B), LDL (C), HDL (D) and VLDL (E) and the atherogenic index (F) of diabetic rats fed high-fat diet and treated with JPP or vehicle for 8 weeks (means ± SEM, n=8). *Different from the control group ($p<0.05$), #Different from the diabetic-vehicle group (Triglycerides, cholesterol, VLDL: ANOVA-Duncan's test and HDL, LDL: Kruskal-Wallis-Multiple comparisons' test $p<0.05$). JPP: Jaboticaba peel powder, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, HDL: high-density lipoprotein.

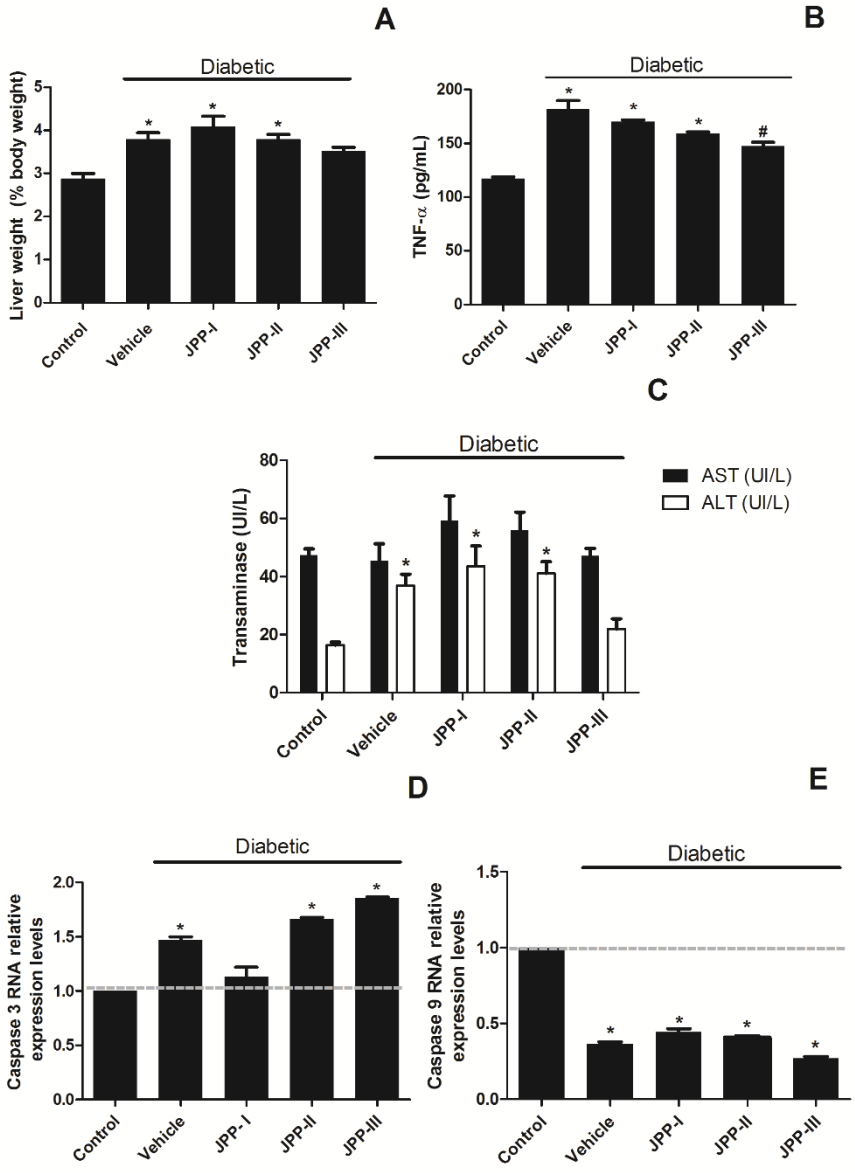


**Figure 2**. Liver weight (A), serum level of TNF-α (B), serum transaminases activity (C), hepatic expression of caspase-3 (D), and hepatic expression of caspase-9 (E) in diabetic rats fed high-fat diet and treated with JPP or vehicle for 8 weeks (means ± SEM, n=8). *Different from the control group, #Different from the diabetic-vehicle group (ALT, liver histology: ANOVA-Duncan's test; Liver weight: Kruskal-Wallis-Multiple comparisons' test, caspase-3 and caspase-9: ANOVA-Dunnet's test, $p<0.05$). The gene expression data were normalized by β-actin gene. Mean ± SD are obtained from three independent repetitions. ALT: aspartate aminotransferase, AST: alanine aminotransferase, JPP: jaboticaba peel powder, TNF-α: tumour necrosis factor alfa.

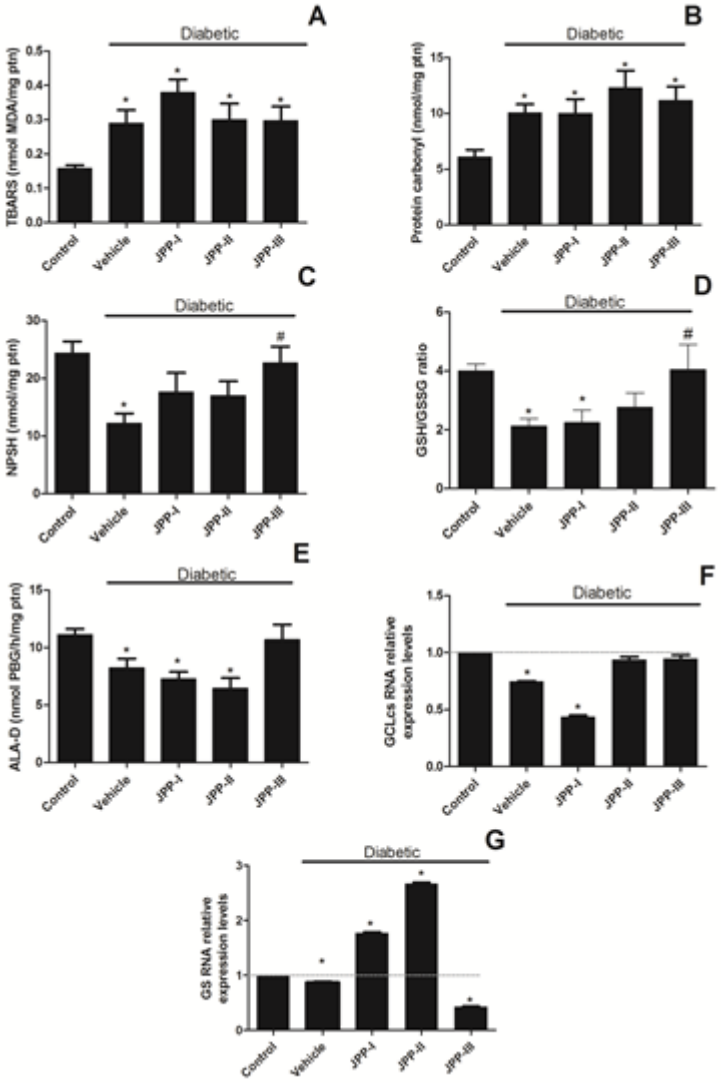


**Figure 3.** TBARS (A), protein carbonyl (B) and NPSH (C) levels, GSH/ GSSG ratio (D), δ-ALA-D activity (E) and gene expression of GCLcs (F) and GS (G) in the liver of diabetic rats fed high-fat diet and treated with jaboticaba peel powder or vehicle for 8 weeks (means ± SEM, n=8). *Different from the control group ($p<0.05$), #Different from the diabetic-vehicle group (TBARS, protein carbonyl, NPSH, GSH/GSSG ratio and δ-ALA-D: ANOVA-Duncan's test; GCLcs and GS: ANOVA-Dunnet test; $p<0.05$). The gene expression data were normalized by β-actin gene expression and mean ± SD were obtained from three independent repetitions. TBARS= thiobarbituric acid reactive substances, NPSH= non-protein thiol groups, GSH = reduced glutathione, GSSG = oxidized glutathione, δ-ALA-D = δ -aminolaevulinic acid dehydratase, GCLcs= glutamate-cysteine ligase, catalytic subunit, GS= glutathione synthase.

## 5. DISCUSSÃO

A jabuticaba vem despertando bastante interesse como alternativa para o tratamento e ou prevenção de doenças crônicas como o diabetes e a obesidade (ALEZANDRO; GRANATO; GENOVESE, 2013; BATISTA et al., 2018; LAMAS et al., 2018; MOURA et al., 2018), porque sua matriz é composta por elevado teor de compostos fenólicos, especialmente a casca (ALEZANDRO et al., 2013; INADA et al., 2015). A composição fenólica da casca da jabuticaba ainda não foi totalmente elucidada, mas pode ser considerada fonte de taninos hidrolisáveis e antocianinas (PLAZA et al., 2016). Além de apresentar elevado teor de fibra alimentar solúvel e insolúvel (LEITE-LEGATTI et al., 2012; LEITE et al., 2011).

Em vista disso o efeito do PCJ tem sido explorado em estudos de obesidade induzida com dieta gordurosa em animais (BATISTA et al., 2014; LEITE et al., 2011), onde tem apresentado ação antioxidante, anti-inflamatória e de regulação do metabolismo de lipídeos, que estão de acordo com os resultados observados no modelo de DM tipo 2 explorados no Manuscrito 4. O tratamento com PCJ (*M. jaboticaba*), dose de 10,8 g PCJ/L, durante 8 semanas foi capaz de atenuar a dislipidemia e a hiperglicemia, e principalmente restaurou em 86% os níveis de SHNP do tecido hepático, bem como restaurou em 90% a relação GSH/GSSG favorecendo o estado reduzido. Estes efeitos foram fundamentais para reestabelecer o desbalanço oxidativo observado com a indução do DM tipo 2. Os mecanismos envolvidos neste efeito compreendem o aumento da síntese de GSH endógena via regulação positiva da expressão gênica da GCLcs, enzima limitante da síntese de GSH. O PCJ também restaurou a atividade da enzima ALA-D, principal enzima envolvida na síntese do grupo heme, que compõe a hemoglobina. Acredita-se que o PCJ inibe a oxidação de grupamentos sulfidrila do sítio ativo da ALA-D, mantendo assim a sua atividade. Estes efeitos estão fortemente relacionados ao conteúdo e perfil de compostos fenólicos do PCJ como observado no Manuscrito 1, bem como os metabólitos gerados durando o processo digestivo (Manuscrito 2) e pela fermentação da microbiota (Manuscrito 3), pois os mesmos podem ser absorvidos (VITAGLIONE et al., 2007).

Considerando-se que a administração de PCJ foi realizada através da água de beber, e os animais apresentaram um consumo médio diário de 265 mL da solução contendo PCJ/kg de peso corporal, a dose de PCJ que promoveu efeitos benéficos nos ratos (10,8 g PCJ/L de água) correponde ao consumo de 0,2862 g de PCJ por kg de peso corporal do animal.

Utilizando o fator de conversão de 6,2 preconizado por Nair e Jacob (2016) para levar em consideração a diferença de porte que resulta em menor taxa metabólica nos humanos em comparação com os ratos, a dose é equivalente ao consumo de 0,046 g de PCJ por kg de peso corporal humano, o que corresponde a 3,23 g de PCJ por dia para um humano adulto, pesando 70 kg. Seria viável adicionar esta quantidade de PCJ como suplemento na dieta. Segundo estudos prévios as formas de adicionar o PCJ na dieta com boa aceitação e sem perdas quanto ao conteúdo de compostos fenólicos foram em iogurte, na forma de suco e barra de cereal (BOESSO, 2014; CARVALHO, 2013; RIBEIRO et al., 2016). Por outro lado, se a opção fosse consumir a fruta fresca, esta massa de PCJ (3,23 g) corresponde ao consumo diário de aproximadamente 10 unidades de jaboticaba com casca, considerando as seguintes características do fruto: peso médio fresco 5,63 g, 28,9% de casca sobre o peso fresco e rendimento de 20,5% durante a liofilização da casca para obtenção do PCJ.

O PCJ de ambas as espécies estudadas (Manuscrito 1) apresenta constituição bastante semelhante em relação aos compostos fenólicos identificados, porém o teor dos compostos/classes são distintos. O PCJ (*M. trunciflora*) possui maior teor total de compostos fenólicos livres quando comparado ao PCJ (*M. jaboticaba*) (5500,6 mg $100g^{-1}$ PCJ *vs.* 2272,4 mg 100 $g^{-1}$ PCJ). Também foi observado que a *M. trunciflora* apresenta maior percentual de taninos hidrolisáveis do que antocianinas, enquanto que na espécie *M. jaboticaba* ocorre o inverso, ou seja, maior teor de antocianinas do que de taninos hidrolisáveis. As diferenças entre as espécies reforçam a importância do consumo de jabuticaba, com interesse especial pela espécie *M. trunciflora* encontrada no Rio Grande do Sul, devido ao elevado conteúdo de compostos fenólicos. Estes resultados são bastante relevantes, pois podem estimular o cultivo regional de jabuticabeira (Rio Grande do Sul), uma vez que, a fruta é produzida 2 vezes ao ano, e a produção média de uma árvore equivale a 200 kg/ano, desta forma pode gerar uma renda extra aos produtores.

As antocianinas majoritárias identificadas são as mesmas, cianidina-3-glicosídeo e delfinidina-3-glicosídeo, para ambas as espécies. Porém, em relação às antocianinas minoritárias apenas a *M. jaboticaba* apresenta peonidina-3-glicosídeo, enquanto ambas apresentam pelargonidina-3-glicosídeo. Além disso, foram identificados alguns taninos hidrolisáveis foram identificados pela primeira vez nas espécies estudadas de jabuticabas (Manuscrito 1), dentre eles trisgaloil-HHDP-glicose, castalina, galoil-castalagina, monogaloilglicose, digaloilglicose, trigaloilglicose e tetragaloilglicose. Estes compostos podem contribuir para os efeitos antioxidantes observados no tratamento do DM tipo 2 (Manuscrito 4), uma vez que os taninos hidrolisáveis apresentam maior capacidade

antioxidante direta em comparação com as antocianinas quando analisada a capacidade antioxidante individual dos compostos por HPLC (PLAZA et al., 2016).

Os diferentes métodos de extração dos compotos bioativos e diferentes métodos de ionização em MS/MS, bem como, o padrão de energia de colisão utilizados em diferentes estudos podem ser as causas de ainda não ter sido relatado na literatura, em especial para a espécie *M. jaboticaba* (mais estudada), os novos compostos identificados no Manuscrito 1.Apesar das diferenças consideráveis no teor dos compostos fenólicos entre as espécies, ambas podem ser consideradas boas fontes de compostos fenólicos, uma vez que em comparação com outros alimentos bastante estudados como a casca de diferentes variedades de uvas (PANTELIĆ et al., 2016), o PCJ da espécie *M. jaboticaba* possui cerca de 2 vezes mais composto fenólicos.

Entretanto, geralmente, os compostos íntegros presentes nos alimentos nem sempre são os responsáveis pelos benefícios do consumo dos mesmos. É necessário considerar as alterações causadas pelo processo digestivo, que resultam em novos compostos (metabólitos), bem como a biodisponibilidade dos mesmos (GOWD et al., 2018). Em vista disso foi analisado a cinética de degradação e a bioacessibilidade dos compostos fenólicos do PCJ (*M. trunciflora*) no Manuscrito 2. A simulação do processo digestivo promoveu branda degradação das antocianinas na boca, cerca de 10% em relação à PCJ integra. Além disso, observou-se degradação das antocianinas do PCJ em condições gástricas, variando entre 16-26% de degradação. Estes resultados contrariam os achados em estudos prévios, visto que as antocianinas apresentaram elevada estabilidade frente as condições gástricas (CORREA-BETANZO et al., 2014; LIANG et al., 2012). Entretanto, quando comparado aos taninos hidrolisáveis, as antocianinas apresentam maior estabilidade frente as condições salivar e gástrica, uma vez que os taninos hidrolisáveis sofreram degradação de mais de 74% na boca e entre 80-90% em condições gástricas. A menor degradação das antocianinas no estômago é bastante interessante, pois cerca de 20% dos compostos (FERNANDES et al., 2014) são absorvidas no estômago e, portanto, podem ser responsáveis pela ação antioxidante observada no Manuscrito 4, uma vez que não existe diferença substancial entre as duas espécies, diferindo apenas no teor dos compostos. Adicionalmente, corrobora com a elevada capacidade antioxidante (ORAC) das antocianinas em relação aos compostos não antociânicos observada nas fases oral e gástrica (Manuscrito 2).

Os taninos hidrolisáveis sofrem degradação desde a etapa da boca, sendo que a maior degradação ocorre sob as condições intestinais, resultando principalmente na liberação de

monômeros de ácido elágico (aumento de 92% em relação ao PCJ íntegro) na fase intestinal, resultado da hidrólise parcial da matriz e também da degradação dos elagitaninos. As antocianinas também sofrem elevada degradação sob as condições alcalinas da fase intestinal, mas, devido ao elevado teor na matriz (PCJ), a cianidina-3-glicosídeo é o composto fenólico com maior concentração bioacessível, seguida pelo ácido elágico, principal metabólito dos elagitaninos.

Além disso, a maioria dos elagitaninos também apresentam baixa bioacessibilidade intestinal, enquanto os flavonois apresentam maior índice de recuperação intestinal e elevada bioacessibilidade, porém devido ao teor reduzido na matriz, a quantidade de flavonois bioacessível é pouco expressiva e, provavelmente, pode estar contribuindo minimamente com os benefícios do PCJ frente ao DM tipo 2 observados no Manuscrito 4.

Entretanto, a maior proporção dos taninos hidrolisáveis e antocianinas permaneceu no intestino grosso, e pode sofrer metabolização pela microbiota intestinal (Manuscrito 3), com subsequente absorção dos metabólitos gerados, que também podem contribuir com os benefícios à saúde observado frente ao DM tipo 2 (Manuscrito 4). Puupponen-Pimi et al., (2013) ressaltou a importância da microbiota para a metabolização de elagitaninos e a relação dos metabólitos gerados, urolitinas, com a melhora de parâmetros de perfil lipídico em pacientes com síndrome metabólica.

Os metabólitos urolitinas já foram encontrados no plasma de humanos, comprovando assim que os metabólitos são absorvidos no intestino grosso (GARCÍA-VILLALBA et al., 2013; GARCÍA-VILLALBA; ESPÍN; TOMÁS-BARBERÁN, 2016). Eles desempenham diversas funções anti-inflamatórias tanto local (intestino grosso) quanto sistêmica, e ainda ação antioxidante e no controle da disfunção muscular em decorrência da hiperglicemia (GIMÉNEZ-BASTIDA et al., 2012; ISHIMOTO et al., 2011; LARROSA et al., 2010; ZHOU et al., 2018). Em vista disso, possivelmente as urolitinas produzidas com a metabolização dos elagitaninos (Manuscrito 3) também podem contribuir para os efeitos benéficos do consumo de PCJ observados no Manuscrito 4, devido ao elevado teor encontrado após 48 horas de fermentação (Figura 4). Além disso, o principal metabólito da cianidina-3-glicosídeo, o ácido protocatecuico, pode estar associado aos efeitos do PCJ, contribuindo para um efeito mais precoce do que as urolitinas, uma vez que o pico de concentração do ácido protocatecuico ocorreu entre 2-8 horas de fermentação enquanto para as urolitinas ocorreu em 48 horas (Figura 4).

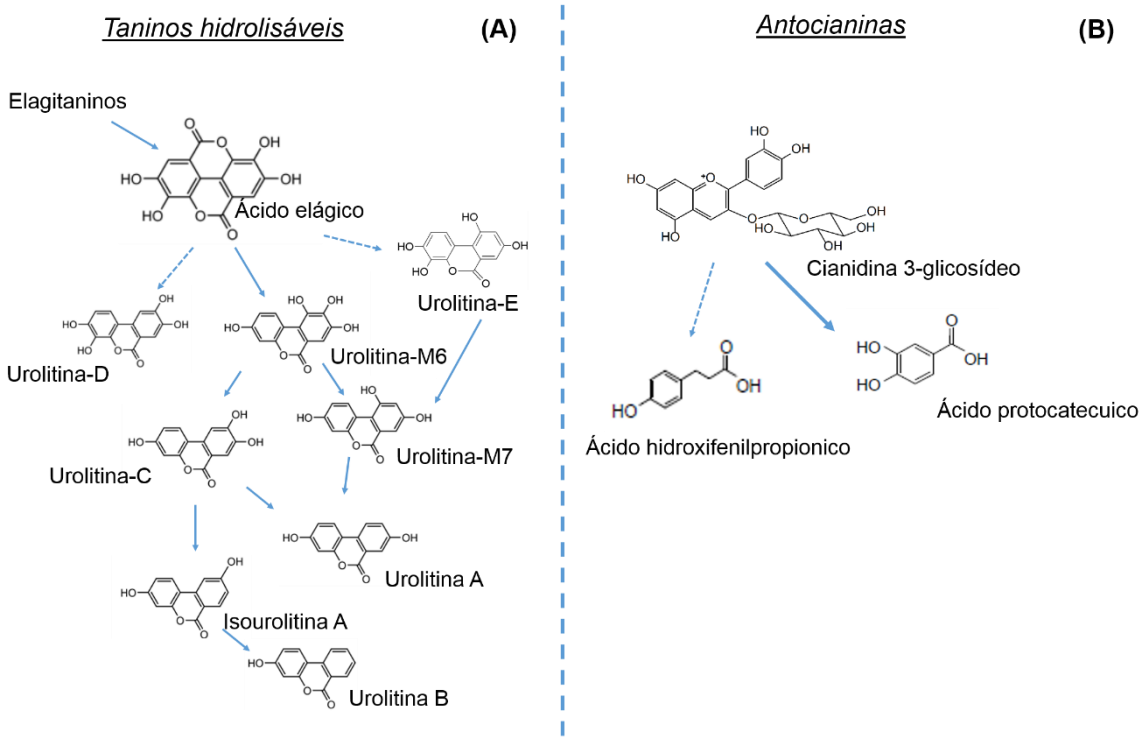


Figura 4: Metabólitos da microbiota intestinal oriundos do catabolismo dos (A) elagitaninos/ácido elágico e (B) antocianinas.

Fonte: Autoria pessoal

Adicionalmente, o PCJ (rico em fibra alimentar e compostos fenólicos mesmo após sofrer digestão gastrointestinal) modulou o crescimento microbiano observado após 48 horas de fermentação (Manuscrito 3) com manutenção da contagem de bactérias probióticas (*Bifidobacterium* e *Lactobacillus*) e redução do crescimento de bactéria patogênica (*Enterobacteria*). Além disso, a fermentação do PCJ também aumentou a produção AGCC nas primeiras horas de fermentação, elevando o teor de acetato, butirato e propionato. Este aumento parece ter associação com o conteúdo de fibra alimentar solúvel no PCJ, enquanto que o aumento na formação dos ácidos valérico, heptanóico e capróico ocorreu após 8 horas e possui associação com o conteúdo de fibra alimentar insolúvel do PCJ. Resultados semelhantes foram observados, previamente, *in vivo* após consumo de PCJ (*M. jaboticaba*) e foram indicados como um dos mecanismos pelo qual o PCJ atuou frente a obesidade, reduzindo o acúmulo de adipócitos, a área de esteatose hepática, o ganho de peso e o processo inflamatório (BATISTA et al., 2018).

Em vista disso, o consumo de alimentos que aumentam a produção de AGCC é de fundamental interesse para o tratamento do DM tipo 2, uma vez que todas as alterações

observadas na obesidade culminam no desenvolvimento do DM tipo 2 em decorrência da inflamação de baixo grau, resistência à insulina e alterações no metabolismo lipídico (BOLES; KANDIMALLA; REDDY, 2017; JAACKS et al., 2016). Além disso, a modulação da microbiota intestinal como observado na fermentação com PCJ (Manuscrito 3) também ressalta o interesse para contra-atacar tanto a obesidade quanto o DM, tendo em vista que ambas patologias estão relacionadas com a disbiose intestinal (SOMMER; BÄCKHED, 2013). Portanto, o controle da microbiota possibilita melhora do controle da homeostasia energética e da imunidade via produção de metabólitos como AGCC que podem ser produzidos tanto pela fermentação de carboidratos complexos como por compostos fenólicos (DANNESKIOLD-SAMSØE et al., 2019; SUN et al., 2017; TOMÁS-BARBERÁN; SELMA; ESPÍN, 2016).

## 6. CONCLUSÃO

Foram encontradas diferenças na composição de fenólicos entre as duas espécies de PCJ estudadas. Os compostos fenólicos mais abundantes nos PCJs estudados (*M. jaboticaba* e *M. trunciflora*) são os taninos hidrolisáveis e antocianinas, sendo que o teor de compostos fenólicos totais (livres e ligados à matriz) é 2,4 vezes maior no PCJ (*M. trunciflora*) em comparação com o PCJ (*M. jaboticaba*). Por outro lado, o teor de compostos fenólicos ligados à matriz foi 1,6 vezes maior no PCJ (*M. jaboticaba*) do que na espécie *M. trunciflora*. Além disso, as espécies diferem, em relação ao teor dos compostos fenólicos livres, enquanto que a espécie *M. trunciflora* apresenta maior teor de taninos hidrolisáveis, a espécie *M. jaboticaba* apresenta maior teor de antocianinas.

A simulação do processo digestivo do PCJ promoveu degradação dos compostos fenólicos, principalmente de taninos hidrolisáveis, mas teve pequeno efeito sobre as antocianinas nas etapas salivar e gástrica. Porém, na etapa intestinal (intestino delgado) os teores de antocianinas e taninos hidrolisáveis foram notavelmente diminuídos, com aumento concomitante nos teores de ácidos gálico e elágico, devido à hidrólise parcial dos taninos hidrolisáveis e liberação da matriz do fruto.

As antocianinas do PCJ apresentaram baixa bioacessibilidade, variando entre 0,08-2,3%, enquanto que a maioria dos taninos hidrolisáveis e flavonóis apresentaram maior bioacessibilidade em comparação com as antocianinas. Entretanto, apesar de sua baixa bioacessibilidade, as antocianinas foram os compostos fenólicos mais abundantes na fração intestinal bioacessível seguida pelo ácido elágico e taninos hidrolisáveis.

A microbiota intestinal desempenha expressivo efeito sobre a metabolização dos compostos presentes na matriz do PCJ previamente digerido, em especial, na formação de metabólitos urolitinas, resultantes da degradação dos elagitaninos, e ácido protocatecuico, principal metabólito da cianidina-3-glicosídeo. Além disso, a presença de fibra alimentar solúvel e insolúvel presente na matriz promoveu aumento substancial da produção de AGCC e auxiliou, juntamente com os polifenois e seus metabólitos, na modulação da microbiota intestinal inibindo o crescimento de bactéria patogênica (*Enterobacteria*).

O consumo de PCJ, maior dose (10,8 g PCJ/L), foi eficaz para a retomada do balanço oxidativo, bem como, redução da hiperglicemia, dislipidemia e dano hepático em modelo de DM 2 em ratos, sendo que o principal mecanismo envolvido foi o aumento da síntese de

glutationa e a modulação do equilíbrio redox GSH/GSSG possivelmente relacionados com os teores de compostos fenólicos totais (livres e ligados à matriz) e fibra alimentar do PCJ.

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## ANEXO A – TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DOS ALIMENTOS

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Título do projeto: **Efeitos biológicos da casca de jabuticaba: metabolização e implicações na prevenção do diabetes**
Pesquisador responsável: Dra. Tatiana Emanuelli
Instituição/Departamento: Universidade Federal de Santa Maria/Departamento de Tecnologia e Ciência dos Alimentos
Telefone para contato: (55) 3220 8547
Pesquisadores participantes: Tatiana Emanuelli, Andréia Quatrin, Luana H. Maurer e Lisiane Conte.
Telefones para contato: (55) 3220-8547 e (55) 81401633
Endereço eletrônico: quatrinandreia@yahoo.com.br

Você está sendo convidado(a) para participar, como voluntário, em uma pesquisa. Você precisa decidir se quer participar ou não. Por favor, não se apresse em tomar a decisão. Leia cuidadosamente o que se segue e pergunte ao responsável pelo estudo qualquer dúvida que você tiver. Após ser esclarecido(a) sobre as informações a seguir, no caso de aceitar fazer parte do estudo, assine ao final deste documento, que está em duas vias. Uma delas é sua e a outra é do pesquisador responsável. Em caso de recusa você não será penalizado(a) de forma alguma.

♦Você está sendo convidado a participar de um projeto de pesquisa intitulado "**Efeitos biológicos da casca de jabuticaba: metabolização e implicações na prevenção do diabetes**" que tem como objetivo avaliar se a casca da jabuticaba é aproveitada de forma diferente após o consumo por pessoas obesas ou com diabetes em comparação com pessoas não diabéticas e sem obesidade. Os voluntários diabéticos deverão se deslocar até o HUSM em três ocasiões, sendo os custos de transporte até o HUSM de responsabilidade dos voluntários. Na primeira ocasião, após consulta com médico no Ambulatório de Endocrinologia, você será convidado a responder o questionário sobre hábitos alimentares. Na segunda ocasião, na reconsulta com o médico, previamente agendada para a realização da coleta de sangue para avaliação do estado de saúde geral. As análises de sangue não acarretarão custo algum aos voluntários. Na terceira ocasião para a entrega das fezes frescas de consistência sólida. Os resultados da análise de sangue serão disponibilizados individualmente aos voluntários por correspondência e se houver qualquer alteração nos exames o voluntário será avisado e encaminhado a uma avaliação médica.

Os voluntários saudáveis deverão se deslocar até o laboratório Núcleo Integrado de Desenvolvimento de Análises Laboratoriais (NIDAL)-UFSM em três ocasiões seguindo os mesmos procedimentos descritos acima para os voluntários diabéticos. O custo de transporte até o laboratório será de responsabilidade dos voluntários, mas as análises sanguíneas não terão custo para os voluntários. Os resultados das análises de sangue serão disponibilizados individualmente aos voluntários e se eles indicarem alterações no quadro de saúde do voluntário, este será encaminhado a

uma avaliação médica. Fica, também, garantida indenização em casos de danos comprovadamente decorrentes da participação na pesquisa.



**Riscos individuais e possibilidade de exclusão**

O voluntário que se sentir constrangido ao responder ao questionário ou alguma pergunta específica do questionário poderá neste momento abandonar este estudo sem prejuízo algum a ambas as partes.

A coleta de sangue é um procedimento de baixo risco e desconforto, mas em alguns casos, pode ocorrer formação de manchas roxas no local da coleta, que será tratado através da realização de massagem local com gel ou pomada contendo medicamento para remover as manchas, sem nenhum custo para os voluntários da pesquisa. Também existe risco de que ocorra redução dos níveis de glicose no sangue devido ao jejum de 12 h que antecederá a coleta de sangue, sendo que nestes casos a pessoa receberá todos os cuidados necessários e será excluída automaticamente do trabalho.

Além disso, você poderá se sentir constrangido durante a coleta das fezes e também no transporte das mesmas podendo desistir de participar do estudo neste momento.

Não há benefício direto para o participante. Trata-se de estudo experimental simulando a digestão da casca da jabuticaba. Somente no final do estudo poderemos concluir a presença de algum benefício.

**Confidencialidade**

Se você concordar em participar do estudo, seu nome e identidade serão mantidos em sigilo. A menos que requerido por lei ou por sua solicitação, somente o pesquisador, a equipe do estudo, o Comitê de Ética independente terão acesso a suas informações para verificar as informações do estudo.

O material coletado e os seus dados serão utilizados somente para esta pesquisa e ficarão guardados com o pesquisador pelo período de cinco anos, após o qual serão destruídos.

**Período de participação**

Sua participação na pesquisa terá duração de dois meses. Você poderá retirar-se da pesquisa em qualquer momento, antes ou durante a mesma, sem penalidades ou prejuízo.

**Garantia de acesso**

Em qualquer etapa do estudo, você terá acesso aos profissionais responsáveis pela pesquisa para esclarecimento de eventuais dúvidas.

Consentimento da participação da pessoa como sujeito

Eu, _____, portador do RG_____ abaixo assinado, concordo em participar do presente estudo, como voluntário. Fui suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo "EFEITOS BIOLÓGICOS DA CASCA DE JABUTICABA: METABOLIZAÇÃO E IMPLICAÇÕES NA PREVENÇÃO DO DIABETES". Eu discuti com os pesquisadores sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia do acesso a tratamento hospitalar quando necessário. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo ou perda de qualquer benefício que eu possa ter adquirido, ou no meu acompanhamento/ assistência/tratamento neste serviço.

Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato: Comitê de Ética em Pesquisa – UFSM - Cidade Universitária - Bairro Camobi, Av. Roraima, nº1000 - CEP: 97.105.900 Santa Maria – RS. Telefone: (55) 3220-9362 – Fax: (55)3220- 9362. Email: cep.ufsm@gmail.com
.. Web: www.ufsm.br/cep

Local e data _____

Nome e Assinatura do sujeito ou responsável:

_____

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste voluntário de pesquisa ou representante legal para a participação neste estudo.

Santa Maria ____, de _____ de 20___

_____
Pesquisador responsável

Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato: Comitê de Ética em Pesquisa – UFSM - Cidade Universitária - Bairro Camobi, Av. Roraima, nº1000 - CEP: 97.105.900 Santa Maria – RS. Telefone: (55) 3220-9362 – Fax: (55)3220- 9362. Email: cep.ufsm@gmail.com
.. Web: www.ufsm.br/cep

**ANEXO B – FICHA DE COLETA DE DADOS DOS VOLUNTÁRIOS**

Nome completo:
Endereço:
Cidade: Estado: CEP:
Telefone pra contato:

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1. Data de Nascimento: Sexo:
2. Altura: Peso:
3. Pressão arterial:
4. Consome bebida alcoólica:
( ) NÃO (nunca consome) ( ) ESPORADICAMENTE (1 vez por semana)
( ) REGULARMENTE (2 ou 3 vezes por semana)
5. Pratica exercício físico:
( ) NÃO (nenhuma vez na semana) ( ) ESPORADICAMENTE ( 1 vez ao mês)
( ) REGULARMENTE (2 ou 3 vezes por semana)
6. Você é fumante:
( ) NÃO ( ) SIM
7. Consome frutas :
( ) NÃO (nunca consome) ( ) ESPORADICAMENTE (algumas vezes na semana)
( ) REGULARMENTE (todos os dias)
Frutas vermelhas (morango, figo, uva, amora, jabuticaba, cereja, framboesa)
( )SIM ( )NÃO
QUAL(is)?.....
Frequências (nº frutas por dia)?.....
8. Consome vegetais e legumes?
( ) NÃO (nunca consome) ( ) ESPORADICAMENTE (algumas vezes na semana)
( ) REGULARMENTE (todos os dias)
Repolho roxo, alface roxa, rabanete, cebola roxa e berinjela
( )SIM ( )NÃO
QUAL(is)?.....
Frequências (nº vegetais/legumes por dia)?.....
9.Usou de antibiótico nos últimos 6 meses:
( ) NÃO ( ) SIM.....QUAL?.....
10. Você tem alguma doença diagnosticada:
( ) NÃO ( ) SIM QUAL(is)?.....
DATA do primeiro diagnóstico?.....
11. Uso de medicamento para o diabetes:
( ) NÃO ( )SIM
QUAL?..... TEMPO de uso?.....
12. Uso de outros medicamentos:
( ) NÃO ( ) SIM QUAL(is)?.....
13. Distúrbios intestinais nos últimos meses (diarreia):
( ) NÃO ( ) SIM
14. Portador de doença infecto contagiosa: ( ) NÃO ( ) SIM
QUAL(is)?.....

# ANEXO C – APROVAÇÃO PELO COMITÊ DE ÉTICA EM PESQUISA-UFSM

UNIVERSIDADE FEDERAL DE SANTA MARIA/ PRÓ-REITORIA DE PÓS-GRADUAÇÃO E

## PARECER CONSUBSTANCIADO DO CEP

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** EFEITOS BIOLÓGICOS DA CASCA DE JABUTICABA: METABOLIZAÇÃO E IMPLICAÇÕES NA PREVENÇÃO DO DIABETES

**Pesquisador:** Tatiana Emanuelli

**Área Temática:**

**Versão:** 2

**CAAE:** 50151015.6.0000.5346

**Instituição Proponente:** Universidade Federal de Santa Maria/ Pró-Reitoria de Pós-Graduação e

**Patrocinador Principal:** MINISTERIO DA CIENCIA, TECNOLOGIA E INOVACAO

**DADOS DO PARECER**

**Número do Parecer:** 1.348.232

**Apresentação do Projeto:**

A hiperglicemia é uma das causas da geração excessiva de espécies reativas de oxigênio (EROs) que leva ao estresse oxidativo, e está associada ao diabetes mellitus (DM). Existe uma grande preocupação com o avanço do DM em todo o mundo, e com os efeitos adversos dos medicamentos sintéticos utilizados atualmente. Como alternativa o uso de compostos naturais vem crescendo, pela possibilidade de atuar tanto na prevenção quanto no tratamento do DM, com risco diminuido de efeitos adversos. A jabuticaba [Myrciaria jaboticaba (Vell.) Berg.] é uma fruta, cuja casca apresenta alta capacidade antioxidante devido à presença de antocianinas e polifenois. Entretanto, ainda não está elucidada a sua metabolização, distribuição e principalmente o envolvimento da microbiota na metabolização das antocianinas. Sendo assim, o presente projeto tem como objetivo avaliar a absorção, metabolização e distribuição dos polifenóis e seus metabólitos no soro, urina, fezes e tecidos hepático e pancreático de ratos Wistar adultos alimentados com dieta padrão acrescida ou não de 2% de pó da casca da jabuticaba (PCJ) durante 15 dias por HPLC-DAD-MS2 e também verificar o envolvimento da microbiota através da supressão da microbiota com coquetel de antibióticos. Também será avaliada a ação dos polifenois do PCJ e frações (polifenois extraíveis ou fibras do PCJ) adicionados na dieta durante 30 dias sobre parâmetros de estresse oxidativo e disfunção mitocondrial dos tecidos pancreáticos e

**Endereço:** Av. Roraima, 1000 - prédio da Reitoria - 2º andar
**Bairro:** Camobi **CEP:** 97.105-970
**UF:** RS **Município:** SANTA MARIA
**Telefone:** (55)3220-9362 **E-mail:** cep.ufsm@gmail.com

hepático, além de análises bioquímicas sérica em modelo de diabetes mellitus tipo 2 (DM2) induzida por baixa dose de estreptozotocina e dieta hipercalórica em ratos. Além disso, será avaliada a as diferenças na metabolização das antocianinas do PCJ pela microbiota intestinal humana associada a indivíduos com DM2 obesos ou não em comparação com indivíduos saudáveis não obesos após simulação pela passagem gastrointestinal in vitro. Espera-se com os resultados deste estudo elucidar a biodisponibilidade dos polifenois do PCJ e verificar se a metabolização das antocianinas é depende da microbiota, demonstrar quais os compostos bioativos estão envolvido com os efeitos terapêuticos PCJ frente ao DM2.

**Objetivo da Pesquisa:**

GERAL: avaliar a absorção, distribuição e excreção dos polifenóis e seus metabólitos após ingestão do PCJ, bem como a ação dos polifenois e fibras do PCJ sobre marcadores bioquímicos, de estresse oxidativo e inflamatórios em ratos com DM2.

ESPECÍFICOS:

- Avaliar a absorção, distribuição e metabolização das antocianinas em ratos após ingestão de PCJ, investigando a importância da microbiota intestinal nestes eventos utilizando a estratégia de supressão da microbiota com coquetel de antibióticos.
- Avaliar os efeitos do PCJ e frações (polifenois extraíveis ou fibras ligada a polifenois não extraíveis) sobre a digestibilidade de nutrientes (amido, proteínas e lipídios) e a resposta glicêmica pós-prandial em modelo de DM 2 em ratos.
- Avaliar os efeitos do PCJ sobre o estresse oxidativo e disfunção mitocondrial no fígado e pâncreas de ratos com DM 2, identificando as frações do PCJ (polifenóis ou fibra) responsáveis pelos efeitos.
- Avaliar o efeito do PCJ e dos flavonoides e antocianinas do PCJ (extratos purificados) sobre a atividade de enzimas digestivas in vitro.
- Avaliar in vitro a metabolização das antocianinas do PCJ pela microbiota intestinal humana após simulação pela passagem gastrointestinal, avaliando se existem diferenças na metabolização pela microbiota associada a indivíduos obesos ou portadores de diabetes tipo 2 em comparação com indivíduos saudáveis não obesos.

**Avaliação dos Riscos e Benefícios:**

Foram avaliados corretamente.

**Comentários e Considerações sobre a Pesquisa:**

A pesquisa apresenta justificativa contundente. Os pesquisadores responsáveis tem experiência no tema e o tamanho da amostra foi justificado.

**Considerações sobre os Termos de apresentação obrigatória:**

Todos apresentados adequadamente.

**Recomendações:**

Veja no site do CEP - http://w3.ufsm.br/nucleodecomites/index.php/cep - na aba "orientações gerais", modelos e orientações para apresentação dos documentos. Acompanhe as orientações disponíveis, evite pendências e agilize a tramitação do seu projeto.

**Conclusões ou Pendências e Lista de Inadequações:**

O projeto não apresenta pendências ou inadequações.

**Considerações Finais a critério do CEP:**

**Este parecer foi elaborado baseado nos documentos abaixo relacionados:**

| Tipo Documento | Arquivo | Postagem | Autor | Situação |
|---|---|---|---|---|
| Informações Básicas do Projeto | PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_607459.pdf | 01/12/2015 11:42:59 | | Aceito |
| TCLE / Termos de Assentimento / Justificativa de Ausência | TCLE.pdf | 01/12/2015 11:41:26 | Tatiana Emanuelli | Aceito |
| Outros | Tatiana_autorizacao.pdf | 01/12/2015 11:41:12 | Tatiana Emanuelli | Aceito |
| Outros | Resposta_Pendencias_projeto.pdf | 01/12/2015 11:40:33 | Tatiana Emanuelli | Aceito |
| Projeto Detalhado / Brochura Investigador | Projeto_doutorado_CEP_UFSM.docx | 01/12/2015 11:34:19 | Tatiana Emanuelli | Aceito |
| Folha de Rosto | Tatiana_folha_de_rosto.pdf | 01/12/2015 11:33:21 | Tatiana Emanuelli | Aceito |
| Outros | Autoriza_DEPE_HUSM.jpg | 12/10/2015 19:08:49 | Tatiana Emanuelli | Aceito |

| | | | | |
|---|---|---|---|---|
| Outros | Registro_GAP_com_HUSM.pdf | 12/10/2015 19:05:15 | Tatiana Emanuelli | Aceito |
| Outros | Termo_confidencialidade_final.pdf | 12/10/2015 18:56:59 | Tatiana Emanuelli | Aceito |
| Orçamento | termo_Concessao_Universal2014.pdf | 12/10/2015 18:54:08 | Tatiana Emanuelli | Aceito |

**Situação do Parecer:**
Aprovado

**Necessita Apreciação da CONEP:**
Não

SANTA MARIA, 02 de Dezembro de 2015

**Assinado por:**
**CLAUDEMIR DE QUADROS**
**(Coordenador)**

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