UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA DOS
ALIMENTOS

HELENA RUDGE DE MORAES BARROS

**Efeito dos compostos fenólicos do camu-camu e do cupuaçu
no desenvolvimento da obesidade e diabetes mellitus tipo 2**

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Tese apresentada ao Programa de Pós-Graduação em Ciência dos Alimentos da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo para obtenção do título de Doutor.

Orientador: Prof Dr Maria Inés Genovese

SÃO PAULO
2015

Ficha Catalográfica

Elaborada pela Divisão de Biblioteca e
Documentação do Conjunto das Químicas da USP.

B277e	<p>Barros, Helena Rudge de Moraes</p> <p>Efeito dos compostos fenólicos do camu-camu e do cupuaçu no desenvolvimento da obesidade e diabetes mellitus tipo 2 / Helena Rudge de Moraes Barros. - São Paulo, 2015. 165p.</p> <p>Tese (doutorado) - Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. Departamento de Alimentos e Nutrição Experimental</p> <p>Orientador : Genovese, Maria Ines</p> <p>1. Alimento : Composto fenólico : Ciência dos alimentos 2. Frutas 3. Diabetes Mellitus 4. Obesidade I. T. II. Genovese, Maria Ines, orientador.</p> <p>641 CDD</p>
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Comissão Julgadora

Prof. Dr. Maria Inés Genovese
Orientador/Presidente

1º Examinador

2º Examinador

3º Examinador

4º Examinador

São Paulo, ____ de _____

AGRADECIMENTOS

Aqui, tenho a oportunidade de rapidamente tomar ciência do processo pelo qual passei e quantas foram as pessoas envolvidas nele. Os agradecimentos aqui envolvem muito mais do que simples obrigados.

Agradeço à prof. Dra Maria Inés Genovese pela oportunidade, orientação, ensinamentos, confiança, conversas e amizade. Se hoje sou um pouquinho melhor que há 4 anos atrás, com certeza você foi a maior contribuinte!

Ao prof. Dr. William Festuccia por ter disponibilizado seu laboratório e compartilhado seu tempo e seu conhecimento do início ao fim deste projeto. Este trabalho, provavelmente, não teria sido desenvolvido sem seu apoio.

Ao Thiago Belchior de Oliveira pela paciência, ensinamentos e ajuda em muitas etapas deste trabalho.

Ao prof. Dr. Francisco Tomás-Barberán pela oportunidade, disponibilidade e conversas; à querida Rocio por todo o conhecimento dividido; e aos pesquisadores (María Romo, María Angeles, David, Maka e Alice) do *Centro de Edafología e Biología Aplicada del Segura* - CEBAS que fizeram da minha estadia na Espanha mais feliz.

Ao meu marido Edgard, por seu amor, sua compressão e seu apoio em todas as fases deste trabalho. Você foi a pessoa que mais acreditou desde o começo, e seu incentivo foi fundamental! Obrigada por mudar sua vida para poder realizar um sonho meu!

Aos meus pais Marina e Luiz Carlos e à minha irmã e amiga Marta pelo carinho, apoio incondicional e por sempre demonstrarem orgulho desta jornada.

À minha querida prima e amiga Camila e ao meu irmão Roberto, mesmo que distantes fisicamente, por me tranquilizarem nos momentos difíceis e por me inspiraram como pesquisadores.

Aos meus colegas de laboratório e amigos queridos: Alice, Carlos, Ciça, Daniel, Diully, Flavinha, Gabi Cunha, Gabi Pedrosa, Helô, Luana, Marcela, Marcio, Renata e Taty pela companhia, amizade e troca de conhecimentos, obrigada a todos vocês!

À Fernanda Santana, por sua amizade e ajuda com as análises de lipídeos.

À Beatriz Mendonça, “minha filhinha” querida, pela participação na parte experimental, sempre com comprometimento e alegria.

À Joana, por nos ajudar a manter o “antigo” laboratório em ordem.

Aos funcionários do Biotério Fátima, Flávia, Livia e Roseni pelos ensinamentos durante os experimentos com os animais.

Aos técnicos do Departamento de Alimentos e Nutrição Experimental Alexandre Pimentel, Ivanir, Luciene e Rosa Maria pelos equipamentos utilizados e auxílio em algumas análises.

À técnica Rosana e ao prof. Dr. Areas do Departamento de Nutrição da Faculdade de Saúde Pública, pelo uso do liofilizador.

À Fernanda Stefani, da empresa 100% Amazônia e à Bela Iaçã, pelo fornecimento das polpas de camu camu. Ao Sítio do Bello, pelo fornecimento da polpa de cupuaçu.

Ao pessoal do laboratório do prof. Dr. William, sempre fui muito bem acolhida!

Aos professores que de alguma forma participaram da minha formação durante esses 4 anos.

Aos secretários do Departamento Edilson, Monica, Roberta e Cleo.

À CAPES pela bolsa-sanduíche, ao CNPQ pela bolsa durante o doutorado e a FAPESP pelo suporte financeiro ao laboratório.

RESUMO

BARROS, H. R. M. **Efeito dos compostos fenólicos do camu camu e do cupuaçu no desenvolvimento da obesidade e diabetes mellitus tipo 2.** 2015. Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo.

A incidência da obesidade e do diabetes do tipo 2 tomou proporções epidêmicas nos últimos anos, atingindo bilhões de indivíduos em todo o mundo. A descoberta de formas inovadoras capazes de reduzir as alterações metabólicas associadas a estas doenças é fundamental para minimizar o seu impacto na qualidade de vida da população e na economia dos países. Muitos estudos têm demonstrado que os compostos bioativos de alimentos possuem efeitos benéficos à saúde. O camu-camu e o cupuaçu são frutas nativas da região amazônica com potencial agroeconômico ainda inexplorado, que contêm um grande número de compostos fitoquímicos que podem atuar sobre o metabolismo corporal. Desta forma, o objetivo deste estudo foi verificar os efeitos dos compostos fenólicos de extratos do camu-camu e do cupuaçu no desenvolvimento da obesidade e do diabetes tipo 2 em ensaios *in vivo* e *in vitro*, e identificar os possíveis metabólitos envolvidos nestes efeitos. Os extratos ricos em compostos fenólicos da polpa comercial destes frutos foram extraídos em fase sólida, caracterizados por cromatografia líquida de alta eficiência (CLAE/DAD) e avaliados quanto à inibição da atividade de enzimas digestivas *in vitro*. Os extratos obtidos foram então testados em duas diferentes concentrações (2,25 e 4,5 mg de equivalentes de catequina/Kg de peso corporal para o cupuaçu; 7 e 14 mg de equivalentes de ácido gálico/Kg de peso corporal para o camu camu) em um modelo animal de obesidade e resistência insulínica induzida por dieta com alto teor de lipídios e sacarose em camundongos C57BL/6J. Foram investigados os efeitos destes compostos sobre as homeostases glicídica e lipídica através de análises séricas, testes de tolerância à insulina e à glicose e conteúdo de lipídios hepáticos e fecais. O extrato do camu camu apresentou flavonóis, ácido elágico e elagitaninos em sua composição. A suplementação com o extrato de compostos fenólicos do camu camu reduziu o ganho de peso corporal e diminuiu a intolerância à glicose e à insulina, independente da dose administrada. No entanto, a administração destes extratos não apresentou efeitos sobre o metabolismo lipídico. Estes resultados foram associados a um possível efeito de saciedade, com consequente redução da ingestão da dieta e da glicolipotoxicidade, e com um efeito anti-inflamatório devido à diminuição dos níveis de proteína C reativa. Já o extrato de cupuaçu apresentou flavanóis, flavonas e proantocianidinas em sua composição. A suplementação com o extrato de cupuaçu

na maior dose testada melhorou a homeostase da glicose e principalmente dos lipídios, protegendo o tecido hepático dos danos causados pela dieta com alto teor de lipídios e sacarose. Estes efeitos foram associados à inibição de enzimas digestivas, com consequente menor absorção de lipídios provenientes da dieta, reduzindo assim a resistência à insulina no fígado, a hiperglicemia e a dislipidemia. Ainda, foi avaliada a distribuição de metabólitos no trato gastrointestinal de camundongos após a administração aguda do extrato de cupuaçu. Foi possível identificar a complexa mistura de polifenóis presentes no extrato de cupuaçu ao longo do trato gastrointestinal, que posteriormente foi metabolizada pela microbiota. Entre os metabólitos encontrados estão as agliconas hipolaetina e isoscutelareína, e os metabólitos microbianos da epicatequina como o 3,4-diHPP-2-ol e a 5-(3,4-dihidroxifenil)- γ -valerolactona. De acordo com estes resultados, as diferenças na composição de compostos fenólicos encontradas entre os extratos das duas frutas foram responsáveis pelos diferentes resultados nos protocolos *in vivo* e a identificação dos metabólitos microbianos possibilita o conhecimento dos compostos possivelmente implicados nos efeitos benéficos. Novos estudos podem contribuir para um melhor entendimento dos mecanismos, bem como quais metabólitos estão associados aos efeitos benéficos que os compostos presentes nestas duas frutas apresentaram neste estudo.

Palavras chave polifenóis, dislipidemia, homeostase da glicose, *theobroma grandiflorum*, *myrciaria dúbia*, metabólitos.

ABSTRACT

BARROS, H. R. M. **Effects of camu camu and cupuassu phenolic compounds on obesity and type 2 diabetes mellitus development.** 2015. Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo.

The incidence of obesity and type 2 diabetes reached epidemic proportions in recent years, arriving to billions of people around the world. The discovery of innovative ways that can reduce the metabolic abnormalities associated with these diseases is essential to minimize its impact on the population's quality of life and the economy of the countries. Many studies have demonstrated that food bioactive compounds have beneficial health effects. Camu-camu and cupuassu are native fruits of the Amazon region with unexplored agroeconomic potential, which contain a large number of phytochemical compounds that can act on body metabolism. Thus, the objective of this study was verify the effects of phenolic compounds of camu-camu and cupuassu extracts on the development of obesity and type 2 diabetes *in vivo* and *in vitro*, and identify the possible metabolites involved in these effects. The phenolic compound-rich extracts were obtained from commercial frozen fruit pulps by solid phase extraction, characterized by high-performance liquid chromatography (HPLC/DAD) and evaluated for inhibition of digestive enzymes activities *in vitro*. Then, the extracts were tested at two different doses (2.25 and 4.5 mg catechin equivalents/kg body weight for cupuassu; 7 and 14 mg of gallic acid equivalents/kg body weight for camu camu) in an animal model (C57BL/6J mice) of obesity and insulin resistance induced by high fat high sucrose diet. The effects of extract supplementation on glucose and lipid homeostasis were assessed by serum analysis, insulin and glucose tolerance tests in mice, and contents of fat in liver and fecal samples. Camu camu extract presented flavonols, ellagic acid and ellagitannins in its composition. Supplementation with camu camu phenolic extract reduced weight gain and decreased glucose and insulin intolerance independent of the dose administered. However, no effects on lipid metabolism were found. These findings were associated with a possible effect of satiety with a consequent reduction in energy intake and glicolipotoxicity, and anti-inflammatory properties. Cupuassu extract presented flavanols, flavones and proanthocyanidins in its composition. Supplementation with cupuacu extract at the highest dose improved glucose homeostasis and plasmatic lipid levels, protecting the liver tissue from damage caused by diet rich in lipids and sucrose. These effects were associated with inhibition of digestive enzymes, with consequent lower absorption of lipids from the diet, thereby reducing the insulin

resistance in the liver, the hyperglycemia and dyslipidemia. Furthermore, the distribution of metabolites in the gastrointestinal tract of mice was evaluated after acute administration of cupuassu extract by HPLC-ESI-QTOF. We identified the complex mixture of polyphenols present in cupuassu extract along the gastrointestinal tract, which was subsequently metabolized by the intestinal microbiota. Among detected metabolites are hypolaetin and isoscutellarein aglycones and microbial metabolites of epicatechin as 3,4-diHPP-2-ol and 5-(3,4-dihydroxyphenyl)- γ -valerolactone. According to these results, the differences in the composition of phenolic compounds found between the two fruit extracts were responsible for the different effects *in vivo* and identification of microbial metabolites enables the knowledge of the compounds potentially implicated in the beneficial effects. New studies can contribute to a better understanding of the metabolism and mechanisms of action.

Keywords: polyphenols, dyslipidemia, glucose homeostasis, *theobroma grandiflorum*, *myrciaria dúbia*, metabolites.

LISTA DE ABREVIATURAS E SIGLAS

°C	Graus Celsius/ <i>Celsius degree</i>
ADA	<i>American Diabetes Association</i>
Akt	Proteína quinase B/ <i>protein kinase B</i>
ALT	Alanina aminotransferase/ <i>alanine aminotransferase</i>
AMPK	<i>AMP-activated kinase</i>
ANOVA	Análise de variância/ <i>analysis of variance</i>
ASP	Proteína estimuladora de ascilação
AST	Aspartato aminotransferase/ <i>aspartate aminotransferase</i>
BHT	Hidroxitolueno butilado
CBG	β -glicosidase citosólica
CCPE	<i>Camu camu phenolic extract</i>
CE	<i>Catechin equivalent</i>
CEBAS	<i>Centro de Edafología y Biología Aplicada del Segura</i>
CEUA	Comissão de Ética no Uso de Animais
CLAE	Cromatografia Líquida de Alta Eficiência
CLUE	Cromatografia Líquida de Ultra Eficiência
CPE	<i>Cupuassu phenolic extract</i>
CRP	<i>C -reactive protein</i>
CSIC	<i>Consejo Superior de Investigaciones Científicas</i>
CT	Colesterol total
DAD	Detector com arranjo de diodos/ <i>diode array detector</i>
DCNT	Doenças crônicas não transmissíveis
EAG	Equivalente de ácido gálico
EC	Equivalente de catequina
EGC	<i>Epigallocatechin</i>
EGCG	<i>Epigallocatechin gallate</i>
ESI	Ionização por eletrospray/ <i>Electrospray ionization</i>
EUA	Estados Unidos da América
FCF	Faculdade de Ciências Farmacêuticas
FFA	<i>Free fatty acids</i>

<i>g</i>	Constante gravitacional
<i>g</i>	Grama/ <i>gram</i>
GAE	Gallic acid equivalents
GAPDH	Desidrogenase de gliceroaldeído-3-fosfato/ <i>glyceraldehyde 3-phosphate dehydrogenase</i>
GLP	<i>Glucagon like peptide</i>
GLUT	Transportador de glicose
h	Horas/ <i>hours</i>
HCl	Ácido clorídrico
HDL	Lipoproteína de alta densidade/ <i>high-density lipoprotein</i>
HF/HS	<i>High fat high sucrose</i> /alto teor de lipídios e sacarose
HOMA-IR	<i>Homeostatic model assessment of insulin resistance</i>
HPLC	<i>High performance liquid chromatography</i>
IBGE	Instituto Brasileiro de Geografia e Estatística
IC 50	<i>Half maximal inhibitory concentration</i>
IGF	<i>Insulin-like growth factor</i>
IKK	Quinase do inibidor do fator de transcrição NF-κb
IL	Interleucina
IMC	Índice de massa corporal
ipGTT	Teste intraperitoneal de tolerância à glicose/ <i>Intraperitoneal glucose tolerance test</i>
ipITT	Teste intraperitoneal de tolerância à insulina/ <i>Intraperitoneal Insulin tolerance test</i>
IR	Receptor de insulina
IRS	Substrato do Receptor de Insulina
IT	<i>Íon trap</i>
JNK	Proteína Jun N-terminal quinase
Kcal	Quilocalorias/ <i>kilocalories</i>
Kg	Quilogramas/ <i>kilograms</i>
L	Litro/ <i>liter</i>
LDL	Lipoproteína de baixa densidade/ <i>low-density lipoprotein</i>
LOX	Lipoxigenase
LPH	Hidrolase floridzina lactase

LPS	Lipopolissacarídeos
m	Massa/ <i>mass</i>
M1	Macrófagos classicamente ativados
M2	Macrófagos alternativamente ativados
MCP	Proteínas quimiotáticas para monócitos
MDA	Molondialdeído/ <i>malondialdehyde</i>
µg	Micrograma/ <i>microgram</i>
mg	Miligrama/ <i>miligram</i>
µL	Microlitro/ <i>microliter</i>
mL	Mililitro/ <i>mililiter</i>
µM	Micromol
mM	Milimol
MS	Espectrometria de massas
mTORC1	Complexo 1 da quinase alvo da rapamicina em mamíferos
MU	Metilumbeliferil/ <i>methylumbelliferyl</i>
NAFLD	<i>Non-alcoholic fatty liver disease</i>
NPY	<i>Neuropeptide Y</i>
nM	Nanomol
OMS	Organização Mundial da Saúde
PAI	Sistema vascular angiotensinogênio
PBS	Solução salina fosfato tamponada
pH	Potencial de hidrogênio/ <i>hidrogen potencial</i>
PI3q	Fosfatidilinositol 3-quinase
PKC	Proteína quinase C
POF	Pesquisa de Orçamentos Familiares
PTFE	<i>Polytetrafluoroethylene</i>
QUICKI	<i>Quantitative insulin sensitivity check index</i>
rpm	Rotações por minuto
sd	<i>Standard deviation</i>
SD	<i>Standard diet</i>
SEM	<i>Standard error</i>
SGLT	Transportador da glicose sódio dependente
STZ	Estreptozotocina

TAG	Triacilglicerol
TBA	Ácido tiobarbitúrico
TC	<i>Total cholesterol</i>
TCA	Ácido clorofluroacético
TLR	<i>Toll like receptors</i>
TNF	Fator de Necrose Tumoral
UK	<i>United Kingdon</i>
USA	<i>United States of America</i>
USP	Universidade de São Paulo
v	Volume
VLDL	Lipoproteína de muito baixa densidade/ <i>very-low-density lipoprotein</i>
WHO	<i>World Health Organization</i>

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1. INTRODUÇÃO

A obesidade é uma doença definida pela Organização Mundial da Saúde (OMS) como acúmulo excessivo de gordura corporal prejudicial à saúde de um indivíduo. O índice de massa corporal (IMC) é utilizado para avaliar esse excesso de gordura corporal, sendo indivíduos com IMC entre 25 e 30 considerados com sobrepeso e IMC maior que 30 como obesos (OMS, 2015b). Acredita-se que o desequilíbrio no balanço energético, resultante de fatores ambientais, comportamentais como também de fatores genéticos, é um fator determinante no aumento da incidência da obesidade a qual tomou proporções epidêmicas nos últimos anos atingindo bilhões de indivíduos mundialmente (MANZEL et al., 2014; WANG et al., 2014).

Em 2014, aproximadamente 1,9 bilhão de adultos apresentavam sobrepeso e pelo menos 600 milhões estavam obesos, indicando que a prevalência mundial da obesidade mais do que duplicou entre os anos de 1980 e 2014 (OMS, 2015b). Corroborando com estes dados, a Pesquisa de Orçamentos Familiares- POF 2008-2009 (IBGE, 2010) demonstrou que no Brasil a obesidade cresceu mais de quatro vezes entre os homens, e mais de duas vezes entre as mulheres ao longo de 35 anos. Atualmente, indivíduos adultos com sobrepeso e obesos correspondem a 52,5% e 17,9% da população, respectivamente (BRASIL, 2015a). Ainda, o excesso de peso foi observado em 33,5% das crianças entre cinco e nove anos e em 20,5% dos adolescentes, o que pode repercutir na vida adulta, aumentando ainda mais a prevalência de sobrepeso nos próximos anos (BRASIL, 2015b).

A obesidade está frequentemente associada a alterações metabólicas que levam ao desenvolvimento do diabetes mellitus tipo 2 (KAHN; COOPER; DEL PRATO, 2014; OSBORN; OLEFSKY, 2012). O diabetes mellitus tipo 2 representa cerca de 90 a 95% dos casos de diabetes, e caracteriza-se por defeitos na secreção e/ou ação da insulina (ADA, 2014). Dados sobre a prevalência de diabetes tipo 2 no Brasil são escassos, no entanto um estudo recente sobre associação entre excesso de peso e maior prevalência de diabetes tipo 2 demonstrou que 65,5% dos casos de diabetes mellitus foram atribuíveis ao sobrepeso (FLOR et al., 2015). Essa associação torna-se preocupante, uma vez que mais da metade da população do Brasil está acima do peso ideal.

Tanto a obesidade como o diabetes estão associados a hiperglicemia e resistência à insulina; altas concentrações de lipídios nos tecidos; produção endotelial de espécies reativas

de oxigênio; defesas antioxidantes inadequadas e inflamação crônica (LAY et al., 2014; MCARDLE et al., 2013; VAN GREEVENBROEK; SCHALKWIJK; STEHOUWER, 2013). Estas alterações levam ao desenvolvimento de outras doenças crônicas não transmissíveis (DCNT) como hipertensão arterial, doenças cardiovasculares, doença hepática gordurosa não alcoólica e vários tipos de câncer (ROVIRA-LLOPIS et al., 2014; MEYDANI; HASAN, 2010; TCHERNOF; DESPRÉS, 2013), o que determina a elevação da morbimortalidade e a diminuição da qualidade de vida das populações, e conseqüentemente, gera maiores custos dos sistemas de saúde, sendo assim consideradas como os maiores problemas de saúde pública no mundo (DEE et al., 2014; IMES; BURKE, 2014).

Por outro lado, ensaios clínicos e epidemiológicos têm estabelecido uma correlação inversa entre a ingestão de frutas, legumes e verduras e a ocorrência de DCNT (BOHN, 2014; LIU et al., 2014; VON RUESTEN et al., 2013). As frutas, legumes e verduras fornecem compostos bioativos como fibras, vitaminas, minerais, carotenoides e compostos fenólicos que promovem benefícios à saúde e reduzem o risco de desenvolvimento dessas doenças (SLAVIN; LLOYD, 2012; TORRES-FUENTES et al., 2014). Entre estes diferentes compostos bioativos presentes nos vegetais, os compostos fenólicos destacam-se por suas propriedades antioxidante, antiinflamatória, e mais recentemente por seus efeitos sobre a sinalização celular, a expressão gênica e a microbiota intestinal, mecanismos importantes na prevenção do desenvolvimento de diabetes tipo 2 e da obesidade (ANHÊ et al. 2013, GARCÍA-CONESA, 2015; HANHINEVA et al., 2010; MEYDANI; HASSAN, 2010; XIAO; HÖGGER, 2015; WILLIAMSON, 2013).

1.1 OBESIDADE ASSOCIADA AO DIABETES TIPO 2

Como descrito anteriormente, a obesidade está associada a uma variedade de resultados adversos à saúde, tais como a resistência à insulina, principal preditor do desenvolvimento de diabetes tipo 2. A resistência à insulina é definida como uma resposta inadequada aos efeitos fisiológicos da insulina circulante por tecidos-alvo, tais como músculo esquelético, fígado e tecido adiposo (KALUPAHANA; MOUSTAID-MOUSSA; CLAYCOMBE, 2012; SCHENK; SABERI; OLEFSKY, 2008). O mecanismo envolvido na falha da ação da insulina ainda não é totalmente conhecido. Contudo, alterações moleculares

na via de sinalização da insulina são determinantes no estado de resistência à insulina em tecidos periféricos (PAULI et al., 2009).

A insulina é um hormônio polipeptídico anabólico produzido pelas células β pancreáticas, cuja síntese é ativada pelo aumento dos níveis circulantes de glicose e aminoácidos após as refeições. Seus efeitos metabólicos imediatos incluem: aumento da absorção de glicose, principalmente em tecido muscular e adiposo; aumento da síntese de proteínas, ácidos graxos e glicogênio; bem como inibição da produção hepática de glicose, da lipólise no tecido adiposo e da proteólise no tecido muscular (FREITAS; CESCHINI; RAMALLO, 2014; HANHINEVA et al., 2010).

A sinalização intracelular da insulina inicia com sua ligação a um receptor específico de membrana, denominado receptor de insulina (IR). A ativação do IR resulta em fosforilação em tirosina de diversos substratos, incluindo substratos do receptor de insulina 1 e 2 (IRS-1 e IRS-2). A fosforilação das proteínas IRS promovem a ativação de outra proteína citosólica, denominada fosfatidilinositol 3-quinase (PI3q). A ativação da PI3q aumenta a fosforilação em serina da proteína quinase B (Akt) e isso permite o transporte de glicose no músculo e no tecido adiposo, através da translocação da proteína de transporte da glicose -4 (GLUT-4) para a membrana celular. A translocação do GLUT-4 para a membrana permite então a entrada de glicose por difusão facilitada (CHOI; KIM, 2010; SCHENK; SABERI; OLEFSKY, 2008).

Figura 1. Via de sinalização da insulina (Fonte: FREITAS; CESCHINI; RAMALLO, 2014).

O tecido adiposo humano é um tecido bem vascularizado e innervado, que além dos adipócitos (células mais abundantes), é composto por uma fração de células estromais, constituída por pré-adipócitos, células endoteliais e numerosas células do sistema imunológico incluindo macrófagos, células T e células dendríticas (ITOH et al., 2011; MCARDLE et al., 2013). Antes considerado apenas como órgão de armazenamento de energia, atualmente é reconhecido como órgão multifuncional, produtor e secretor de inúmeros peptídeos e proteínas bioativas (adipocinas), e mantém intensa comunicação com os demais órgãos e sistemas orgânicos. As adipocinas (leptina, adiponectina, resistina, proteína estimuladora de oscilação - ASP, fator de necrose tumoral - TNF- α , interleucina-6 - IL-6 e o sistema vascular angiotensinogênio - PAI-1) influenciam uma variedade de processos fisiológicos, entre eles, o controle da ingestão alimentar, a homeostase energética, a sensibilidade à insulina, a proteção vascular, a regulação da pressão, a coagulação sanguínea e a inflamação. Alterações na secreção dessas adipocinas, consequentes à hipertrofia e/ou hiperplasia dos adipócitos, podem estar relacionadas à gênese do processo fisiopatológico da obesidade e suas complicações (OUCHI et al., 2011; SHOELSON; HERRERO; NAAZ, 2007).

Em condições de excesso de nutrientes, o tecido adiposo se expande e os adipócitos se tornam hipoperfundidos, o que leva a uma diminuição do fluxo sanguíneo local e hipóxia em algumas regiões do tecido (EMANUELA et al., 2012). Isso promove a secreção de proteínas quimiotáticas para monócitos (MCP-1) e de citocinas pró-inflamatórias, ocasionando então a infiltração de células do sistema imune como monócitos, que em seguida diferenciam-se em macrófagos. A expansão dos adipócitos também diminui o número de células T regulatórias e aumenta o número de células T_H1, que por sua vez levam à polarização de macrófagos do tipo M2 “alternativamente ativados” para um fenótipo pró-inflamatório chamado de “classicamente ativados” ou M1, aumentando assim a secreção de citocinas inflamatórias como TNF- α , IL-1b e IL-6 (PATEL, BURAS, BALASUBRAMANYAM, 2013; MCARDLE et al., 2013).

Figura 2. Expansão do tecido adiposo e infiltração de células imunitárias em condições de excesso de energia (Adaptado de McArdle et al., 2013).

Com o remodelamento do tecido, a capacidade de estocar lipídios se torna prejudicada resultando no extravasamento de ácidos graxos livres para a circulação. O excesso de ácidos graxos circulantes leva ao acúmulo de triacilglicerídeos e de metabólitos dos ácidos graxos (diacilglicerol, acetil-CoA e ceramidas) ectopicamente em tecidos como fígado, músculo esquelético e pâncreas (SAMUEL; SHULMAN, 2012; STINKENS et al., 2015). Esta condição de lipotoxicidade, inflamação crônica e liberação de citocinas inflamatórias ativam quinases como a quinase IKB (IkK), a proteína quinase C (PKC), a Jun N-terminal quinase (JNK) e a *mammalian target of rapamycin complex 1* (mTORC1/S6K), que por sua vez promovem a fosforilação em serina dos IRS, fazendo com que a ativação de proteínas-chave da via da insulina seja prejudicada, contribuindo assim para o desenvolvimento da resistência à insulina nos tecidos periféricos (EMANUELA et al., 2012; FESTUCCIA et al., 2014; FREITAS; CESCHINI; RAMALLO, 2014; OSBORN; OLEFSKY, 2012).

Portanto, em indivíduos obesos, o tecido adiposo se torna disfuncional principalmente pela hipertrofia dos adipócitos, infiltração de macrófagos e distúrbios na secreção de adipocinas, que levam ao desenvolvimento de um estado de inflamação crônica e a um

acúmulo excessivo de gordura em tecidos periféricos e que juntos podem resultar em resistência à insulina e, finalmente, no aumento de glicose plasmática.

Sob concentrações elevadas de glicose plasmática, as células β pancreáticas respondem à demanda aumentada da insulina por vários mecanismos que incluem o aumento da secreção da insulina, hipertrofia de células β existentes e formação de novas células. A hiperglicemia e hiperlipidemia por períodos prolongados (glicolipotoxicidade), comuns na obesidade, conduzem à deficiência orgânica das células β pancreáticas, refletida na resistência à insulina autócrina, na expressão diminuída dos genes envolvidos na produção da insulina, na diminuição de células β causada por apoptose e finalmente na secreção inadequada de insulina. Consequentemente, a deficiência de insulina produzida no pâncreas relativa à obesidade é consequência do dano celular e da ineficiência na síntese de insulina, acarretando o desenvolvimento do diabetes tipo 2 (ALEJANDRO et al, 2015; BENSELLAM; LAYBUTT; JONAS, 2012).

1.2 DIETA, MICROBIOTA E INFLAMAÇÃO

A importância da nutrição para a prevenção e gestão de doenças crônicas é bem conhecida (OMS, 2015a). A dieta ocidental, caracterizada pelo excesso de alguns nutrientes específicos como gordura saturada ou carboidratos de rápida absorção e pela alta densidade energética, tem sido implicada no risco de desenvolvimento da resistência à insulina, doenças cardiovasculares e doenças do sistema imune. Tanto nutrientes específicos como o excesso de energia tem sido associados com estresse oxidativo e com o aumento de biomarcadores inflamatórios, mesmo em indivíduos saudáveis (EGGER DIXON, 2014; MANZEL et al., 2014; LÓPEZ-ALARCÓN et al., 2014).

A ingestão de carboidratos de rápida absorção, como a sacarose e o xarope de milho com alto teor de frutose, causa um rápido aumento dos níveis de glicose e insulina plasmáticos, e estão associados a alterações na produção de hormônios da saciedade (leptina e grelina) e nos níveis de ácidos graxos livres circulantes, bem como ao estresse oxidativo e ao aumento de citocinas pró inflamatórias como IL-6, IL-18, e TNF- α (MYLES, 2014; JAMMEEL et al., 2014).

Entre os efeitos potencialmente prejudiciais do consumo de gorduras em excesso estão as alterações nos lípidos das membranas de células do sistema imunológico, a ativação dos

sistemas de prostaglandinas, e a sua capacidade para desencadear diretamente um processo inflamatório (MYLES, 2014). Altas concentrações de gorduras saturadas interagem com receptores do sistema imunológico chamados *toll like receptors* (TLR), que também funcionam como mediadores da via inflamatória, interferindo na ação da insulina e em sinais mediados por hormônios controladores da fome e do gasto energético, resultando em obesidade (JOHNSON; OLEFSKY, 2013; HIRAI et al., 2010).

Atualmente, sabe-se que a microbiota intestinal influencia diversos processos imunitários e metabólicos, e mudanças em seu ecossistema podem contribuir para o desenvolvimento de obesidade e resistência à insulina (FALLUCA et al., 2014; MANZEL et al., 2014). Estudos tem demonstrado que dietas ricas em gorduras e açúcares são capazes de modular a microbiota, aumentando a quantidade de microorganismos patogênicos, alteração conhecida como disbiose (MYLES, 2014; TREMAROLI; BACKHED, 2012). Com isso ocorre o aumento da produção de lipopolissacarídeos (LPS) derivados de bactérias gram negativas, que apresentam efeitos semelhantes aos das gorduras, interagindo com os TLRs e acionando as vias inflamatórias e a endotoxemia (DELZENNE et al., 2015; TORRES-FUENTES et al., 2014; MYLES, 2014).

Adicionalmente, as dietas ricas em gorduras e a própria endotoxemia local podem reduzir a expressão de proteínas epiteliais das *tigh junctions*, aumentando assim a permeabilidade intestinal, o que facilitaria a entrada de lipopolissacarídeos na circulação sistêmica, bem como a entrada de lipídios (BOHN, 2014; CARDONA et al., 2013). Assim, a microbiota intestinal pode influenciar a absorção de nutrientes tanto pelo aumento da permeabilidade intestinal quanto pelo desencadeamento de um mecanismo que leva à secreção do peptídeo YY que está envolvido na inibição da motilidade intestinal, retardando o trânsito intestinal (FALLUCA et al., 2014) .

Portanto, tanto o excesso de nutrientes quanto a qualidade destes estão associados à disbiose, aumento da secreção de citocinas e células imunes e inflamação crônica, podendo assim interferir na sinalização da insulina, resultando em resistência à insulina e obesidade.

1.3 ATIVIDADE BIOLÓGICA DOS COMPOSTOS FENÓLICOS, DIABETES TIPO 2 E OBESIDADE

Entre os compostos bioativos presentes nos vegetais estão os compostos fenólicos.

Estes podem ser definidos como substâncias que possuem um anel aromático com um ou mais grupos hidroxila, e normalmente encontram-se conjugados com mono e polissacarídeos (CARDONA et al., 2013; REIN et al., 2013).

Os compostos fenólicos são produtos do metabolismo secundário dos vegetais e lhes conferem sabor e cor, assim como possuem funções de defesa contra herbívoros e infecções microbianas, contra as radiações ultra-violeta do sol, podendo também atuar como atrativo para insetos polinizadores e como agentes alelopáticos (DEL RIO et al., 2013; MARÍN et al., 2015). Já em animais e humanos, as propriedades benéficas destes compostos podem ser atribuídas à sua capacidade de sequestrar os radicais livres, de modular a sinalização celular e de influenciar a expressão de genes relacionados ao desenvolvimento de DCNT (FRAGA; OTEIZA, 2011; THILAKARATHNA; RUPASINGHE, 2013).

Além das propriedades antioxidantes, estudos mais recentes têm demonstrado que os compostos fenólicos podem, ainda, influenciar a homeostase metabólica, bem como a composição da microbiota intestinal, mecanismos importantes na prevenção e tratamento do diabetes tipo 2 e da obesidade. Entre os possíveis mecanismos relacionados a estes benefícios destacam-se: a inibição da digestão e da absorção dos carboidratos e dos lipídios no intestino; a regulação dos níveis de hormônios intestinais, pancreáticos e das adipocinas; a modulação da produção hepática da glicose; a ativação dos receptores de insulina; a inibição da diferenciação e proliferação dos adipócitos; efeitos prebióticos e redução da inflamação crônica (ANHÊ et al., 2013; GARCÍA-CONESA, 2015; HANHINEVA et al., 2010; MEYDANI; HASAN, 2010; NEYRINCK et al., 2013; HIRAI, 2010; WANG et al., 2014; WILLIAMSON, 2013; YUN, 2010).

Devido a estas propriedades, alimentos de origem vegetal, bem como seus extratos e os próprios compostos fenólicos têm sido investigados como adjuvantes aos tratamentos utilizados para reduzir as alterações metabólicas desencadeadas pela obesidade e diabetes (MUNIR et al., 2013). Um grande número de estudos tem demonstrado que ácidos fenólicos, flavonoides e taninos apresentam efeitos benéficos como a redução do ganho de peso corporal e dos níveis de lipídios e glicose circulantes, a melhora da sensibilidade à insulina, a diminuição da pressão arterial e da inflamação crônica em modelos animais de obesidade e/ou diabetes (GARCÍA-CONESA, 2015).

Entre os alimentos mais estudados e seus possíveis benefícios em humanos estão: as *berries* (ricas em antocianinas e elagitaninos), relacionadas à modificação das concentrações de biomarcadores inflamatórios (JOSEPH; EDIRISINGHE; BURTON-FREEMAN, 2014); o chá verde, relacionado à redução dos níveis de lipídios, glicose e insulina plasmáticos

(JOHNSON; BRYANT; HUNTLEY, 2012; LIU et al., 2013; ZHENG et al., 2013) e o cacau e seus derivados (rico em flavan-3-ols e proantocianidinas), associados à melhora da pressão arterial, da resistência à insulina, da função plaquetária e dos níveis de lipídios circulantes (LATHAM; HENSEN; MINOR.,2014). No entanto, estudos com humanos apresentam resultados divergentes e não conclusivos principalmente devido à utilização de controles inadequados, bem como caracterização deficiente dos alimentos ou fontes de compostos fenólicos estudados.

Em geral, existem significantes evidências que o consumo regular destes compostos pode contribuir para a redução ou prevenção de algumas alterações metabólicas que acompanham o diabetes e a obesidade. Apesar do aumento do número de estudos com animais e humanos, algumas questões fundamentais permanecem sem respostas, como as doses, os mecanismos e quais moléculas são realmente responsáveis por estes efeitos. Portanto, um importante tópico para esclarecer estas questões seria a metabolização destes compostos, ou seja, a biodisponibilidade.

1.4 BIODISPONIBILIDADE DE COMPOSTOS FENÓLICOS

Anteriormente foi demonstrado que dietas ricas em compostos fenólicos estão associadas com a menor incidência de obesidade e diabetes mellitus tipo 2. No entanto, a quantidade de polifenóis presentes nos alimentos não reflete necessariamente a quantidade absorvida e metabolizada pelo organismo. A biodisponibilidade (absorção, metabolismo e excreção) de compostos fenólicos varia largamente de um polifenol para outro, ou seja, os polifenóis mais abundantes de nossa dieta não são necessariamente os que levam às maiores quantidades de metabólitos ativos aos tecidos (MANACH et al., 2004). Com isso, nos últimos anos, tornou-se extremamente importante compreender a biodisponibilidade de compostos fenólicos no organismo humano, sendo um passo essencial para a compreensão de sua atividade biológica em tecidos-alvo (DEL RIO et al., 2013; ZANOTTI et al., 2014).

A maioria dos compostos fenólicos se encontra na forma glicosilada ou como polímeros, e portanto precisam ser hidrolisados através da ação de enzimas digestivas e da microflora bacteriana antes de serem absorvidos (BOHN, 2014; REIN et al., 2013). Apesar de alguns estudos terem demonstrado que alguns compostos fenólicos como as antocianinas e alguns ácidos fenólicos podem ser absorvidos no estômago, o primeiro órgão fortemente

envolvido na digestão e modificação de polifenóis é o intestino delgado (LEWANDOWSKA et al. 2013).

Normalmente, a absorção de polifenóis glicosídeos consiste na clivagem dos compostos através da ação da lactase floridzina hidrolase (LPH) presente na borda em escova das células epiteliais do intestino e posterior liberação da aglicona. A aglicona liberada pode então entrar nas células epiteliais por difusão passiva (CHEN et al., 2014; LEWANDOWSKA et al., 2013). Um mecanismo alternativo a este envolve a absorção dos glicosídeos sem prévia hidrólise, através dos transportadores de glicose sódio dependente (SGLT1) presentes na borda em escova das células epiteliais e posterior desglicosilação pela ação da β -glicosidase citosólica (CBG) (MARÍN et al., 2015; RODRIGUEZ-MATEOS et al., 2014).

Uma vez nos enterócitos, as agliconas passam pela ação enzimática de fase II formando metabólitos sulfatados, glicuronados ou metilados. Subsequentemente, os metabólitos conjugados podem retornar ao lúmen intestinal através do efluxo destes compostos ou entrar na circulação portal alcançando rapidamente o fígado (DEL RIO et al., 2013). No fígado, estes metabólitos estão sujeitos a um novo metabolismo de fase II, e voltam então para o intestino delgado através da excreção biliar ou seguem para a circulação sistêmica (CHEN et al., 2014).

Contudo, estima-se que apenas uma pequena parte dos polifenóis ingeridos (5-10%) é efetivamente absorvida (ZANOTTI et al., 2014). O remanescente de polifenóis não modificados (90-95%), em conjunto com os conjugados excretados para o lúmen intestinal através da bÍlis ou do efluxo dos enterócitos, prossegue então através do trato gastrointestinal e se acumula no intestino grosso, onde são expostos à ação da microbiota intestinal (ANHÊ et al., 2015; CARDONA et al., 2013).

O cólon é um grande ecossistema capaz de modificar estruturalmente compostos polifenólicos, conduzindo à produção de metabólitos com diferentes relevâncias fisiológicas. A microbiota intestinal é responsável pela degradação de compostos fenólicos a compostos de baixo peso molecular, que podem ser mais absorvíveis que suas contrapartes originais encontradas em alimentos de origem vegetal. Esta transformação ocorre através de múltiplos passos como hidrólise de ésteres e glicosídeos, desmetilação, desidroxilação, e descarboxilação por diferentes bactérias (SELMA; ESPÍN; TOMÁS-BARBERAN, 2009). Metabólitos produzidos no intestino grosso, subsequentemente, são submetidos novamente ao metabolismo de fase II localmente e/ou no fígado após a absorção (RODRIGUEZ-MATEOS et al., 2014). Eles, então, entram na corrente sanguínea, atingem tecidos periféricos, e, finalmente, são excretados na urina em quantidades substanciais, ultrapassando largamente a

quantidade de metabólitos excretados pelo trato gastrointestinal (CROZIER; DEL RIO; CLIFFORD, 2010; DEL RIO et al., 2013).

Portanto, os metabólitos fenólicos provenientes de degradação microbiana acrescidos da conjugação de fase II podem representar os verdadeiros responsáveis pelos efeitos sobre a saúde derivados do consumo de alimentos ricos em polifenóis. Além disso, estima-se que após o consumo de uma dieta rica em vegetais (frutas, cereais e legumes) e seus derivados (suco, café e chá), a ingestão de polifenóis chegue a mais de 1 g por dia (LANDETE, 2012), porém as concentrações plasmáticas não ultrapassam de nM (HOLMAN, 2014). Assim, o trato gastrointestinal, bem como a microbiota se tornam alvos importantes dos possíveis efeitos metabólicos desses compostos (ANHÊ et al., 2015; FRAGA; OTEIZA, 2011; WILLIAMSON, 2013).

Figura 3. Rotas de absorção e metabolismo dos polifenóis e seus metabólitos (Adaptado de Marín et al., 2015).

1.5 CAMU-CAMU E CUPUAÇU

O camu-camu (*Myrciaria dubia* H. B. K. Mc Vaugh), pertencente à Myrtaceae, e o cupuaçu (*Theobroma grandiflorum* Willd. Ex Spreng. K. Shum), pertencente à Sterculiaceae, são frutas nativas do Brasil encontradas principalmente na região amazônica, como também, em outros países da América do Sul como Colômbia, Peru e Venezuela (CHIRINOS et al., 2011; VENTURIERI, 2011).

A polpa do fruto do camu-camu é macia e succulenta, e no Brasil seu consumo se limita apenas à região norte onde a comercialização da fruta e da polpa congelada é mais comum. Na Amazônia peruana, o camu-camu é bastante consumido, sendo utilizado para o preparo de refresco, sorvete, picolé, geleia, doce, licor, ou para conferir sabor a tortas e sobremesas (ALVES, 2002). No entanto, o camu-camu tem despertado interesse pelo seu elevado teor de vitamina C (2606 mg por 100 g de fruto), superior ao teor encontrado em outras frutas consideradas ricas nesta vitamina como acerola, caju e frutas cítricas (ALVES et al., 2002; ZANATTA et al., 2005). Atualmente, derivados do camu-camu, tais como o extrato, polpa e suco têm como principais importadores países da União Europeia e o Japão (AKTER et al., 2011).

Figura 4. Frutos de camu camu (Fonte: <http://amazonorigins.com/camu-fruit/>).

Além da vitamina C, estudos mais recentes têm demonstrado que o camu-camu é também uma importante fonte de outros compostos bioativos, como os compostos fenólicos e os carotenoides (CHIRINOS et al., 2010; FRACASSETI et al., 2013; ZANATTA; MERCADANTE, 2007). Gonçalves et al. (2010) e Rufino et al. (2010) compararam o teor de compostos fenólicos totais e a capacidade antioxidante do camu-camu com diversas outras frutas nativas do Brasil, e observaram que o camu-camu apresentou os maiores valores em

ambos estudos. Entre os compostos fenólicos foram identificados ácidos fenólicos como o ácido gálico, flavonoides como catequina; quercetina, kaempferol, naringenina, eriodictiol e antocianinas como a cianidina-3-glicosídeo e a delphinidina-3-glicosídeo, e altos teores de taninos como os elagitaninos (AKTER et al., 2011; FRACASSETI et al., 2013).

Uma vez que o camu camu apresenta alto teor de compostos fenólicos, alguns estudos foram realizados visando verificar os possíveis efeitos benéficos do camu camu e seus derivados. A ingestão do extrato camu camu melhorou o perfil lipídico e reduziu o estresse oxidativo de ratos com diabetes induzida por estreptozotocina (Gonçalves et al., 2014), e a ingestão do suco de camu camu por ratos com obesidade induzida por glutamato foi capaz de reduzir os níveis plasmáticos de glicose, insulina e lipídios (NASCIMENTO et al., 2013). Outros estudos demonstraram propriedades antiinflamatórias (INOUE et al., 2008; YAZAWA et al., 2011) hepatoprotetivas (AKACHI et al., 2010) e antimicrobianas (MYODA et al., 2010), o que sugere que este fruto pode ser utilizado como um ingrediente funcional no controle de doenças crônicas ligadas à obesidade.

Já o cupuaçu apresenta uma polpa branco-amarelada de sabor forte muito apreciado pelas comunidades locais. Dentre os frutos amazônicos, é o fruto que reúne melhores condições de aproveitamento industrial e sua polpa possui grandes possibilidades de utilização na indústria de alimentos. A polpa é utilizada na elaboração de suco, iogurte, sorvete, creme, licor, torta, geleia, compota, biscoito, e outros doces. As sementes podem ser utilizadas para a elaboração do cupulate, produto com características nutritivas e organolépticas similares ao chocolate, e a gordura tem sido utilizada na indústria de cosméticos (GENOVESE; LANNES, 2009; OLIVEIRA; GENOVESE, 2013; PUGLIESE et al., 2013).

Figura 5: Fruto do cupuaçu (Fonte: <http://www.frutosdobrasil.com.br/en/momento-natural/dicas/novidades-sobre-o-cupuacu>).

A busca de novos produtos e sabores exóticos levou o cupuaçu a conquistar mercados internacionais. O interesse comercial nesta fruta expandiu sua produção no Brasil para outros estados como a Bahia, promovendo o desenvolvimento de comunidades locais e indústrias e incentivando novas pesquisas. Além disso, o cultivo do cupuaçu propicia a formação de consórcios com outras plantas, permitindo bons resultados econômicos e ecológicos, despontando como um importante produto agrícola (LANNES; MEDEIROS, 2003; LOPES; PEZOA-GARCIA; AMAYA-FARFÁN, 2008).

Entre os compostos fenólicos identificados no cupuaçu estão os flavonoides e os taninos. Entre os flavonoides foram identificados a epicatequina, e os derivados glicuronados e sulfatados da isoscutelareína e da hipolaetina, sendo que a hypolaetina-3'-metil éster 8-O- β -D-glucuronídeo-3"-sulfato nunca foi descrita em outras frutas na literatura. Entre os taninos foram identificadas as proantocianidinas, que são oligômeros de flavonoides como a catequina, epicatequina, galocatequina e a epigalocatequina (YANG et al, 2003; PUGLIESE et al., 2013).

Quanto aos efeitos biológicos, estudos recentes avaliaram o efeito da ingestão crônica de líquido de cupuaçu sobre o estresse oxidativo e perfil lipídico de ratos com diabetes induzido por estreptozotocina (OLIVEIRA; GENOVESE, 2013); e sobre alterações metabólicas associadas a uma dieta com alto teor de lipídios (OLIVEIRA; ROGERO e GENOVESE, 2015). A suplementação de líquido de cupuaçu melhorou o perfil de lipídios plasmáticos, as defesas antioxidantes, a intolerância à glicose e reduziu danos ao fígado. Estes resultados sugerem que o cupuaçu pode minimizar as alterações metabólicas causadas pelo diabetes e por uma dieta inadequada, representando uma possível alternativa para diminuir o risco de desenvolvimento de DCNT.

1.6 JUSTIFICATIVA

A incidência da obesidade e do diabetes mellitus tipo 2 aumentou drasticamente nos últimos anos, sendo consideradas como epidemia global e um desafio das nossas sociedades. Estas doenças afetam diversos órgãos e tecidos como músculo, fígado, tecido adiposo, pâncreas, células inflamatórias e defesas contra o estresse oxidativo intracelular, aumentando também a incidência de várias outras doenças que reduzem a expectativa de vida e acarretam um grande aumento nos custos dos sistemas de saúde.

Os tratamentos medicamentosos a partir de drogas como orlistat e sibutramina apresentam resultados somente a curto prazo, além de efeitos colaterais e altos custos. Por outro lado, muitos estudos têm demonstrado que os compostos bioativos de alimentos podem influenciar o metabolismo corporal, podendo então auxiliar na diminuição do risco de desenvolvimento da obesidade e do diabetes tipo 2. Embora estes compostos bioativos possam ser encontrados naturalmente em frutas e outros vegetais, a população em geral ainda não consome o número de porções recomendado pelos guias alimentares. Neste contexto, o incentivo ao consumo regular de alimentos de origem vegetal, bem como de seus derivados é particularmente justificado.

O camu-camu e o cupuaçu são frutas nativas da região amazônica com potencial agroeconômico ainda inexplorado, que contêm um grande número de compostos fitoquímicos que podem atuar sobre o metabolismo corporal. O estudo dos efeitos dos extratos purificados de compostos fenólicos desses frutos no desenvolvimento da obesidade e do diabetes tipo 2 pode trazer informações importantes para futuras intervenções contra o desenvolvimento de DCNT.

2. OBJETIVOS

- ✓ Obter extratos ricos em compostos fenólicos a partir de polpas comerciais congeladas de camu e cupuaçu.
- ✓ Determinar o teor de compostos fenólicos totais dos extratos ricos em compostos fenólicos de camu camu e cupuaçu.
- ✓ Identificar os flavonoides presentes nos extratos ricos em compostos fenólicos de camu camu e cupuaçu.
- ✓ Determinar o teor de proantocianidinas dos extratos ricos em compostos fenólicos de camu camu e cupuaçu.
- ✓ Avaliar o potencial dos extratos ricos em compostos fenólicos de camu camu e cupuaçu em inibir a atividade das enzimas α -glicosidade e lipase pancreática *in vitro*.
- ✓ Avaliar os efeitos da suplementação de extratos ricos em compostos fenólicos do camu-camu e do cupuaçu em camundongos alimentados com dieta com alto teor de lipídios e sacarose em relação ao:
 - ganho de peso corporal, ingestão alimentar e adiposidade
 - homeostase glicídica
 - homeostase lipídica e lipidemia
 - função hepática.
- ✓ Identificar os metabólitos produzidos no trato gastrointestinal de camundongos após administração aguda do extrato do cupuaçu.

3. MATERIAL E MÉTODOS

Esta seção foi dividida em três partes de acordo com os estudos realizados.

3.1 EFEITOS DOS COMPOSTOS FENÓLICOS DE CUPUAÇU NO DESENVOLVIMENTO DA OBESIDADE/DIABETES TIPO 2

3.1.1 Material

As polpas comerciais congeladas de cupuaçu da marca “Tentação Amazônica” foram produzidas pela Cooperativa Mista dos Produtores Rurais da Região de Carajás em abril de 2013. Os frutos de cupuaçu utilizados para a produção de polpas foram cultivados em Parauapebas, no Estado do Pará, Brasil.

3.1.2 Extração e purificação de compostos fenólicos do cupuaçu

A extração dos compostos fenólicos foi realizada segundo Arabbi, Genovese e Lajolo (2004). A polpa foi extraída com metanol aquoso 70% na proporção de 1:10 (m/v), através da utilização de Ultra-Turrax (IKA Labortechnik, Wilmington, EUA), por 1 minuto em velocidade 5, em banho de gelo. O extrato obtido foi filtrado em papel-filtro Whatman nº 6 e o resíduo foi re-extraído mais duas vezes, obtendo-se então um extrato bruto. Para a purificação dos compostos fenólicos, o extrato bruto obtido foi concentrado utilizando rotaevaporador (RE 20 Büchi, Flawil, Suíça) com temperatura de banho de 40 °C. O extrato foi purificado por extração em fase sólida em coluna com resina C18 (Phenomenex, Torrance, EUA), preparada em seringa própria (HPLC Technology). A coluna foi pré-condicionada com a passagem de metanol e água. Após a passagem do extrato aquoso, a coluna foi lavada com água e a eluição dos compostos fenólicos foi feita com metanol. Após secagem completa através de rotaevaporação, o extrato foi ressuspenso em água. Este extrato foi posteriormente utilizado para a quantificação de fenólicos totais, nos ensaios *in vitro* e *in vivo*.

3.1.3 Quantificação de compostos fenólicos do extrato de cupuaçu

O teor de compostos fenólicos totais foi determinado por espectrofotometria, por meio do reagente de Folin-Ciocalteu, segundo a metodologia descrita por Singleton, Orthofer e Lamuela-Raventos (1999) com adaptações propostas por Genovese et al. (2003). Uma

alíquota de 0,25 mL do extrato foi misturada com 0,25 mL de reagente de Folin-Ciocalteu e 2 mL de água destilada. Em seguida, adicionou-se 0,25 mL de solução saturada de carbonato de sódio e esta mistura foi colocada em banho-maria a 37 °C por 30 minutos. A absorbância foi determinada a 750 nm em espectrofotômetro (Ultrospec 2000, Pharmacia Biotec, Cambridge, Reino Unido). A catequina (Sigma Chemical Co., St. Louis, EUA) foi utilizada para fazer a curva padrão. Essa análise foi realizada em triplicata e os resultados obtidos foram expressos em mg de catequina equivalentes (CE)/ mL de extrato.

3.1.4 Tentativa de identificação de flavonoides por Cromatografia Líquida de Alta Eficiência acoplada a detector com arranjo de diodos (CLAE-DAD)

O mesmo extrato seco obtido por rotaevaporação no item 3.1.2 foi resuspenso em metanol de grau analítico e filtrado através de filtro de polietileno de 0,22 µm (Millipore Ltd., Bedford, MA, EUA). A tentativa de identificação e a quantificação dos flavonoides foram realizadas em cromatógrafo (Hewlett-Packard 1100) com injetor automático de amostras e bomba quaternária acoplada a um detector com arranjo de diodos de acordo com o método de Arabbi, Genovese e Lajolo (2004). A coluna utilizada foi uma Prodigy 5 µm ODS3 de fase reversa (250-4.6mm -Phenomenex Ltd., Torrance, EUA), e os solventes de eluição foram (A) água/tetra-hidrofurano/ácido trifluoroacético (98: 2: 0,1) e (B) acetonitrila. A proporção do solvente B foi alterado nas seguintes proporções: 15% do início até 2 minutos, 25% em 5 minutos, 35% em 8 minutos, 50% em 5 minutos, 90% em 10 minutos e retorno às condições iniciais em 5 minutos. O fluxo utilizado foi de 1 mL/minuto a 25 °C. Os cromatogramas foram registrados a 270 nm e os flavanóis foram quantificados usando epicatequina (Sigma Chemical Co., St Louis, EUA) e as flavonas foram quantificadas usando apigenina (Sigma Chemical Co., St Louis, EUA) como padrões externos, respectivamente. Os resultados foram expressos em µg de cada um dos compostos por mL de extrato.

3.1.5 Inibição da atividade da enzima α -glicosidase *in vitro*

O ensaio de inibição da atividade da enzima α -glicosidase baseou-se no método descrito por Watanabe et al. (1997), com algumas modificações propostas por Kim et al. (2000). Resumidamente, a α -glucosidase de levedura (EC 3.2.1.20, tipo I, Sigma Chemical Co., St Louis, EUA) foi dissolvida em 100 mM de tampão de fosfato (pH 7,0) contendo 2 g/L de albumina bovina sérica (Sigma Chemical Co., St Louis, EUA) e 0,2g/L de azida sódica. O substrato p-nitrofenil 5 mM foi preparado no mesmo tampão (pH 7,0). Em microplaca, a solução da enzima (100 µL) e o extrato (20 µL) foram misturados, e a absorbância foi lida a

405 nm (tempo zero) no leitor de microplaca Sinergy H1 (Biotek, IL, EUA). Após 5 min de incubação a 37 °C, o substrato (100 uL) foi adicionado e a microplaca incubada durante mais 5 min a 37 °C. Então, foi realizada nova leitura a 405 nm. Os extratos foram diluídos em várias concentrações e comparados com um controle (20 µL de água em vez de extrato). Os resultados foram expressos em mg de catequina equivalentes (CE) por mL de reação necessários para inibir 50% da produção de glicose.

3.1.6 Inibição da atividade da enzima lipase pancreática *in vitro*

A inibição da atividade da lipase foi determinada através da quantidade de 4-metilumbeliferil liberada utilizando leitor de microplaca Sinergy H1 (Biotek, IL, EUA) como descrito por Nakai et al. (2005). A lipase pancreática suína (EC 3.1.1.1.3, tipo II, Sigma Chemical Co., St Louis, EUA) e o oleato de 4-metilumbeliferil (4-MU oleato) foram diluídos em solução tampão Tris-HCl pH 8,0 nas concentrações 16,7 U/mL e 0,1M, respectivamente. A microplaca foi então preparada com 25 uL de extrato em diferentes concentrações ou inibidor de lipase (Orlistat, O4139, Sigma Chemical Co., St Louis, EUA) e 25 uL da solução de lipase. A reação iniciou-se pela adição de 50 uL do substrato 4-MU oleato. Após incubação durante 30 min a 37 °C, a produção do 4-metilumbeliferil foi medida em comprimento de onda de excitação de 355 nm e de emissão de 460 nm. Os resultados foram expressos em mg de catequina equivalentes (CE) por mL de reação necessários para inibir 50% da liberação do produto 4-metilumbeliferil.

3.1.7 Efeito dos compostos fenólicos do cupuaçu no desenvolvimento da obesidade e diabetes tipo 2 *in vivo*

Para verificar os efeitos dos compostos fenólicos de cupuaçu no desenvolvimento da obesidade/diabetes tipo 2, camundongos C57BL/6 receberam dieta padrão ou dieta com alto teor de lipídios e sacarose (HF/HS) para a indução da obesidade e resistência à insulina. O protocolo experimental foi aprovado pela Comissão de Ética no Uso de Animais da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (CEUA/FCF/USP), protocolo CEUA nº 353 (Anexo A). Todos os experimentos foram realizados em conformidade com a legislação vigente sobre o assunto no país. Camundongos com 8 semanas de vida, procedentes do Biotério da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, foram divididos em 4 grupos de 10 animais cada e submetidos a uma adaptação de 7 dias ao ambiente experimental, recebendo dieta padrão de laboratório.

A suplementação com extratos fenólicos foi realizada durante 8 semanas e iniciou-se juntamente com a administração da dieta com alto teor de lipídios e sacarose. Os extratos fenólicos purificados obtidos anteriormente foram administrados por gavagem uma vez ao dia em duas doses diferentes. Os grupos que não receberam suplementação de extratos fenólicos receberam água por gavagem como descrito a seguir:

Grupo controle = dieta padrão + gavagem de água

Grupo HF/HS = dieta com alto teor de lipídios e sacarose + gavagem de água

Grupo HF/HS + cupuaçu 0,5 = dieta com alto teor de lipídios e sacarose + gavagem de extrato de compostos fenólicos de cupuaçu (2,25 mg de catequina equivalentes/kg peso corporal)

Grupo HF/HS + cupuaçu 1 = dieta com alto teor de lipídios e sacarose + gavagem de extrato de compostos fenólicos de cupuaçu (4,5 mg de catequina equivalentes/kg peso corporal)

A dieta padrão Nuvilab CR1 (Nuvital Nutrientes SA, Curitiba, PR, Brasil) utilizada fornece 3,6 Kcal/g, sendo 23,7% da energia proveniente de proteínas, 12% de lipídios e 56% de carboidratos. A dieta com alto teor de lipídios e sacarose foi elaborada como descrito anteriormente por Lemieux et al. (2003) e fornece 4,6 Kcal/g, sendo 20% da energia proveniente de proteínas, 41% proveniente de carboidratos (39% proveniente da sacarose) e 39% proveniente de lipídios (19% óleo de soja, 19% banha de porco).

No período dos experimentos, os animais foram mantidos com água e dieta *ad libitum* e as condições ambientais controladas (temperatura de 22 °C, umidade relativa de 65% e ciclo de claro e escuro de 12 horas).

3.1.7.1 Ingestão alimentar, ingestão hídrica, peso corporal e glicemia

A ingestão alimentar, a ingestão hídrica e o peso dos animais foram medidos em dias alternados. A glicemia foi avaliada semanalmente após jejum de 5 horas com glicosímetro Accu-check® (Roche Diagnostics, Mannheim, Alemanha) a partir de amostras de sangue coletadas através de pequena incisão na ponta da cauda dos camundongos. A eficiência alimentar foi calculada pela razão entre o ganho de peso corporal (g) e a quantidade total de calorias consumidas (Kcal) por camundongo, multiplicado por 100.

3.1.7.2 Teste intraperitoneal de tolerância à insulina (ipITT)

O teste de tolerância à insulina foi realizado através de injeção intraperitoneal de insulina regular (0,75 U/ kg de massa corporal) em animais alimentados, na sétima semana do experimento. A concentração de glicose no sangue foi então determinada através de corte na extremidade da cauda do animal, com o glicosímetro Accu-check® (Roche Diagnostics, Mannheim, Alemanha) nos tempos 0, 15, 30, 45 e 60 minutos após administração da insulina.

3.1.7.3 Teste intraperitoneal de tolerância à glicose (ipGTT)

O teste intraperitoneal de tolerância à glicose (ipGTT) foi realizado após 6 horas de jejum na última semana do experimento. Uma primeira coleta de sangue foi feita através de corte na extremidade da cauda do animal no tempo 0. Em seguida, uma solução de glicose a 10 % (1 g/ kg de massa corporal) foi administrada aos camundongos intraperitonealmente. A concentração de glicose no sangue foi então determinada com o glicosímetro Accu-check® nos tempos 15, 30, 45, 60 e 90 minutos após administração da carga de glicose. Para análise de insulina plasmática durante o ipGTT, amostras de sangue foram coletadas através de pequena incisão na ponta da cauda dos camundongos nos tempos 0, 30 e 90 minutos após a injeção de glicose. As amostras de sangue foram centrifugadas a 2000 g por 10 minutos a 4 °C, e o soro foi recolhido para análise de insulina utilizando kit de insulina para camundongos ELISA (EZRMI-13K) da Millipore (Billerica, MA, EUA). Posteriormente, a sensibilidade à insulina foi determinada pelo índice quantitativo de sensibilidade à insulina (*Quantitative Insulin Sensitivity Check Index* - Quicki) utilizando a fórmula: $1/\log \text{ insulina } (\mu\text{U/mL}) \times \text{glicose log (mg/dL)}$.

3.1.7.4 Análise de triacilglicerídeos e ácidos graxos livres do soro no jejum e 3 horas após alimentação

Os níveis de triacilglicerídeos e ácidos graxos livres foram avaliados após jejum de 12 horas e 3 horas após realimentação, a partir de amostras de sangue coletadas através de pequena incisão na ponta da cauda dos camundongos na 6ª semana do experimento. Posteriormente, as amostras de sangue foram centrifugadas a 2000 g por 10 minutos a 4 °C, e o soro foi recolhido para as análises. Para a quantificação de triacilglicerídeos foi utilizado o teste comercial Labtest (Minas Gerais, Brasil) e o analisador automático LabMax 240 (Labtest Diagnóstica, Minas Gerais, Brasil), e para a quantificação de ácidos graxos livres foi utilizado o teste Waco (Richmond, VA, EUA).

3.1.7.5 Coleta de tecidos e soro

Nos dias 56-58 do experimento, os animais foram anestesiados e foi realizada a punção cardíaca. As amostras de sangue foram centrifugadas a 2000 g durante 10 min a 4 °C. Fígado, músculo (gastrocnemius) e tecido adiposo (epididimal , inguinal, retroperitoneal e o tecido adiposo marrom) foram coletados, pesados, congelados em nitrogênio líquido e armazenados a -80 °C.

3.1.7.6 Avaliação bioquímica do soro

Os níveis de glicose, triacilglicerol (TAG), colesterol total (CT), lipoproteína de alta densidade colesterol (HDLc), lipoproteína de baixa densidade colesterol (LDLc), alanina aminotransferase (ALT) e aspartato aminotransferase (AST) foram quantificados por analisador automático LabMax 240 (Labtest Diagnóstica, Minas Gerais, Brasil). A lipoproteína de muito baixa densidade (VLDL) foi calculada através da seguinte fórmula: $VLDL = CT - (HDLc + LDLc)$.

3.1.7.7 Quantificação de lipídios e análise de peroxidação lipídica em tecido hepático

Os tecidos foram homogeneizados em nitrogênio líquido. Os lipídios totais foram extraídos de acordo com o método de Folch; Lees e Sloane-Stanley (1957). Amostras dos lipídios totais extraídos anteriormente foram resuspensas em isopropanol e utilizadas para a quantificação de triacilgliceróis e colesterol total utilizando o analisador automático LabMax 240 (Labtest Diagnóstica, Minas Gerais, Brasil). A peroxidação lipídica no tecido hepático foi avaliada através da quantificação de malondialdeído livre (MDA) utilizando cromatografia líquida de alta eficiência (CLAE) com detecção de fluorescência como descrito por Hong et al. (2000). Cerca de 50 mg do tecido hepático foram homogeneizados com 500 µL de solução salina fosfato tamponada (PBS) e posteriormente centrifugados a 12000 rpm, a 4 °C por 10 minutos. O sobrenadante foi então retirado e diluído mais três vezes em PBS. A 50 µL deste homogenato, adicionou-se 12,5 µL de hidroxitolueno butilado (BHT) e 6,25 µL de NaOH 10N, e esta mistura permaneceu em banho a 60 °C por 30 minutos. Foram então adicionados 750 µL de ácido tricloroacético (TCA) 7,2% contendo 1 % de iodeto de potássio e após resfriar, foram centrifugados a 12000 rpm, a 4 °C por 10 minutos. A 500 µL do sobrenadante foram adicionados 250 µL de ácido tiobarbitúrico (TBA) 0,6% e esta solução foi levada novamente ao banho a 95 °C por 30 minutos. Após resfriar, foram adicionados 750 µL de butanol e novamente centrifugadas a 12000 rpm, a 4 °C por 10 minutos. O sobrenadante foi então filtrado em filtro Millex e injetado em CLAE. Para a curva padrão foi utilizada uma

solução estoque de MDA 20 μ M. O homogenato do tecido hepático em PBS foi previamente analisado quanto ao teor de proteína. Os resultados foram expressos em mmol de MDA/mg de proteína.

3.1.7.8 Western Blot do tecido hepático

Os tecidos foram homogeneizados em nitrogênio líquido e analisados quanto ao teor proteico. Quantidades equivalentes de proteínas foram carregadas num gel de acrilamida (Invitrogen, Life Technologies, Carlsbad, CA, EUA), sujeitas a SDS-PAGE e em seguida, transferidas electroforéticamente para membranas de nitrocelulose. As membranas foram bloqueadas com anticorpos da proteína quinase B fosforilada em serina 473 (p-Akt S473), da proteína quinase B (AKT) e da desidrogenase de gliceraldeído-3-fosfato (GAPDH) da Cell Signaling Technology (Beverly, MA, EUA), diluídos 1: 1000 em leite a 5%. As membranas foram incubadas com anticorpo secundário (1: 10.000) e reveladas com substrato ECL quimioluminescência aumentada (GE Healthcare, Buckinghamshire, Reino Unido).

3.1.8 Análise estatística

As análises estatísticas foram realizadas com o software GraphPad Prism 6 (San Diego, CA, EUA). Os dados experimentais foram testados para normalidade pelo teste de Shapiro-Wilk. As diferenças entre os grupos foram analisadas por meio de análise de variância (ANOVA), seguido de teste de Tukey ou testes não paramétricos. O ganho de peso corporal e a glicemia de jejum semanal foram comparados estatisticamente através de ANOVA *two way* para medidas repetidas. Os dados foram apresentados como médias e desvio-padrão ou erro padrão da média. Um valor de probabilidade de $p < 0,05$ foi usado como o critério para a significância estatística.

3.2 PROTOCOLO DE BIODISPONIBILIDADE DE COMPOSTOS FENÓLICOS DE CUPUAÇU

Este protocolo foi realizado em duas partes. A primeira foi realizada na Faculdade de Ciências Farmacêuticas da Universidade de São Paulo e a segunda no *Centro de Edafología y*

Biología Aplicada del Seguro (CEBAS) do *Consejo Superior de Investigaciones Científicas* (CSIC) na Espanha.

3.2.1. Primeira parte

3.2.1.1 Material

A polpa comercial congelada de cupuaçu da marca “Tentação Amazônica” foi produzida pela Cooperativa Mista dos Produtores Rurais da Região de Carajás em abril de 2013. Os frutos de cupuaçu utilizados para a produção das polpas foram cultivados em Parauapebas, no Estado do Pará, Brasil.

3.2.1.2 Extração e purificação dos compostos fenólicos do cupuaçu

A polpa de cupuaçu (100g de polpa congelada) foi extraída em metanol aquoso 70% na proporção de 1:10 (m/v), através da utilização de Ultra-Turrax (IKA Labortechnik, Wilmington, EUA), por 1 minuto em velocidade 5, em banho de gelo. O extrato obtido foi filtrado em papel-filtro Whatman nº 1 e o resíduo foi re-extraído mais duas vezes, obtendo-se então um extrato bruto. Para a purificação dos compostos fenólicos, o extrato bruto obtido foi concentrado utilizando rotaevaporador (RE 20 Büchi, Flawil, Suíça) com temperatura de banho de 40 °C. O extrato foi purificado por extração em fase sólida em coluna com resina C18 (Phenomenex, Torrance, EUA), preparada em seringa própria (HPLC Technology). A coluna foi pré-condicionada com a passagem de metanol e água. Após a passagem do extrato aquoso, a coluna foi lavada com água e a eluição dos compostos fenólicos foi feita com metanol. Após secagem completa através de rotaevaporação, o extrato foi ressuspenso em 50 mL de água destilada. Dez (10) mL deste extrato foi posteriormente utilizado para a quantificação de fenólicos totais e no experimento *in vivo*. Os outros 40 mL do extrato foram liofilizados para posterior caracterização extrato. O rendimento do extrato fenólico aquoso de cupuaçu (40 mL) foi de 20 mg de extrato seco.

3.2.1.3 Quantificação de compostos fenólicos no extrato do cupuaçu

O teor de compostos fenólicos totais foi determinado por espectrofotometria, por meio do reagente de Folin-Ciocalteu, segundo a metodologia descrita por Singleton, Orthofer e Lamuela-Raventos (1999), com adaptações propostas por Genovese et al. (2003). Uma alíquota de 0,25 mL do extrato fenólico de cupuaçu foi misturada com 0,25 mL de reagente de Folin-Ciocalteu e 2 mL de água destilada. Em seguida, adicionou-se 0,25 mL de solução

saturada de carbonato de sódio e esta mistura foi colocada em banho-maria a 37 °C por 30 minutos. A absorbância foi determinada a 750 nm em espectrofotômetro (Ultrospec 2000, Pharmacia Biotec, Cambridge, Reino Unido). A catequina (Sigma Chemical Co., St. Louis, EUA) foi utilizada para fazer a curva padrão. Essa análise foi realizada em triplicata e os resultados obtidos foram expressos em mg equivalente de catequina/mL de extrato. A quantidade total de polifenóis medido pelo método de Folin-Ciocalteu (0,45 mg de catequina equivalentes (CE)/mL de extrato) foi utilizada para calcular a dose para cada camundongo, considerando o peso corporal dos animais.

3.2.1.4 Experimento *in vivo*

O estudo foi aprovado pelo Comitê de Ética em Experimentação Animal da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, de acordo com as orientações do Colégio Brasileiro de Experimentação Animal (protocolo número 353- Anexo A).

Foram utilizados vinte e sete camundongos machos C57BL/6 (8 semanas de idade, média de 25 g de peso corporal) alimentados com dieta Nuvilab CR1 (Nuvital Nutrientes, Curitiba, PR, Brasil) e água *ad libitum*. A dose do extrato administrada aos animais foi baseada na quantidade de compostos fenólicos em uma única porção de suco cupuaçu (100 g de polpa fresca comercial para fazer um copo de 300 mL de suco, o que corresponde a aproximadamente 70 mg de compostos fenólicos). Para uma pessoa de 70 Kg, essa dose equivale a 1 mg de equivalente de catequina por Kg de peso corporal.

Para melhorar a capacidade de detectar a distribuição de metabólitos, uma dose cinco vezes maior foi usada no estudo *in vivo* (5 mg de equivalentes de catequina por Kg de peso corporal). Após 6 horas de jejum, os camundongos receberam extrato fenólico de cupuaçu por gavagem e posteriormente foram eutanasiados em diferentes tempos (0, 0,5, 1, 2, 3, 4, 6, 12 e 24 horas). As amostras de sangue foram centrifugadas a 2000 g durante 10 min a 4 °C e os tecidos (fígado, rim, estômago, ceco, cólon e intestino) foram coletados, pesados e armazenados a -80 °C. Amostras de fezes foram coletadas nos tempos 0, 3, 4, 6 e 12 h após gavagem e então armazenadas a -80 °C.

Para a realização da segunda parte deste protocolo, as amostras de soro, tecidos, fezes, bem como amostras do extrato utilizado, foram liofilizadas para posteriormente serem analisadas no CEBAS.

3.2.2. Segunda parte

3.2.2.1 Caracterização do extrato fenólico do cupuaçu

3.2.2.1.1 Quantificação de compostos fenólicos por Cromatografia Líquida de Alta Eficiência acoplada ao detector com arranjo de diodos e detector de massas com analisador *íon trap* (CLAE-DAD-IT MS/MS)

O extrato liofilizado (10 mg) foi ressuspenso em 1 mL de metanol: água (80:20, v/v) contendo 0,1% de ácido fórmico e analisado em cromatógrafo (Agilent 1200 series) acoplado ao detector com arranjo de diodos e detector de massas com analisador *íon trap* (Bruker Daltonics, Bremen, Alemanha). A separação dos compostos fenólicos foi realizada em coluna C18 Pursuit XRs, com 250 × 4 mm; tamanho de partícula 5 µm (Agilent Technologies, Aldbronn, Alemanha). O gradiente de solventes consistiu em água: ácido fórmico (99:1, v/v - solvente A) e acetonitrila grau analítico (solvente B), e fluxo de 0,8 mL/min. A eluição foi realizada com o gradiente iniciando com 5% de B em A, até chegar em de 12% B em A em 10 min, 25% de B em A em 20 min, 35% de B em A em 30 min, 70% de B em A em 35 min, 90% de B em A em 36 min e depois isocrático por 5 min. A eluição foi monitorada nos comprimentos de onda de 280 e 340 nm. O detector de massas realizou o scan entre m/z 100 até m/z 800. Dados da espectrometria de massas foram adquiridos em modo negativo. A quantificação de clovamida e flavonas foi realizada através curvas de calibração obtidas com os padrões de ácido cafeíco (1–1000 mg/L) e apigenina (1–500 mg/L), respectivamente, em seus apropriados comprimentos de onda. Os padrões utilizados foram adquiridos da Sigma-Aldrich (St.Louis, MO, EUA). Todas as amostras e padrões foram injetados em triplicata.

3.2.2.1.2 Análise do extrato de cupuaçu por Cromatografia Líquida de Ultra Eficiência com ionização por eletrospray acoplada a detector de massas de alta resolução quadrupolo-tempo de vó (CLUE-ESI-QTOF)

Para confirmar os compostos fenólicos tentativamente identificados por CLAE, o extrato de cupuaçu foi analisado por CLUE-ESI-QTOF. O extrato foi analisado utilizando um cromatógrafo Agilent 1290 acoplado ao espectrômetro de massas quadrupolo (6550 Accurate Agilent Technologies, Waldbronn, Alemanha), utilizando uma interface de electrospray com tecnologia de jato de fluxo. A separação foi realizada em coluna de fase reversa Poroshell 120 CE-C18 (3 × 100 mm, 2,7 µm; Agilent) operando a 30 °C. As fases móveis utilizadas foram: água: ácido fórmico (99,9: 0,1 v/v; fase A) e acetonitrila: ácido fórmico (99,9: 0,1 v/v; fase

B). O gradiente de eluição dos compostos foi: 0-4 min, 5-18% de B; 4-15 min, 18-30% de B; 15-20 min, 30- 90% de B, 1 min a 90% e, em seguida para as condições iniciais em 1 minuto. O fluxo foi ajustado para 0,4 mL/min e o volume de injeção foi de 3 µL. As condições ótimas de interface de electropulverização foram: temperatura do gás de 280 °C, gás de secagem 9 L/min, nebulizador 45 psi, temperatura do gás de 400 °C, fluxo de gás 12 L/min. Os espectros foram adquiridos em modo de MS único com escala de m/z 100-1100, polaridade negativa, e uma taxa de aquisição de espectros de 1,5/s.

3.2.2.1.2 Análise e quantificação de proantocianidinas por floroglucinólise

As proantocianidinas foram quantificadas com descrito anteriormente por Kennedy e Jones (2001) com modificações propostas por Pugliese et al. (2013). Inicialmente foi preparada uma solução de floroglucinol em metanol acidificado com HCl 0,1 N contendo 50 g/L de floroglucinol e 10 g/L de ácido ascórbico. O extrato liofilizado (10 mg) foi então dissolvido nesta solução (150 µL), homogeneizado em vórtex e colocado em banho a 50 °C por 20 min. Posteriormente, a amostra foi resfriada em gelo e foi adicionado 150 µL de acetato de sódio 40 mM para parar a reação. A amostra foi então centrifugada 3000g por 10 minutos a 4 °C, filtrada com um filtro de 0,22 µm, e injetada em CLAE-DAD-MS/MS. A identificação e quantificação de catequina, epicatequina e seus derivados foi efetuada por cromatógrafo Agilent 1100 equipado com detector de arranjo de diodos (Agilent Technologies, Waldbronn, Alemanha), como anteriormente descrito (Pugliese et al., 2013). A coluna utilizada foi uma C 18 Atlantis (250 mm x 4,6 mm, 5 µm, Milford, MA, EUA), operando em um fluxo de 1 mL/min e volume de injeção foi de 10 µL. As fases móveis utilizadas foram ácido acético 2,5% em água (A) e acetonitrila (B) com um gradiente de eluição iniciando com 3% de B em A em 0 min, 9% em 5 minutos, 16% aos 15 minutos, 50% aos 45 minutos. As proantocianidinas foram quantificadas a 280 nm com uma curva de calibração de catequina (1-2000 mg/L) e epicatequina (1-2000 mg/L). Os padrões utilizados foram adquiridos da Sigma-Aldrich (St.Louis, MO, EUA). Para calcular o grau médio de polimerização, a soma de todas as subunidades (monómeros de flavan-3-ol e aductos de floroglucinol em mols) foi dividido pela soma de todos os monómeros de flavan-3-ol (em mols).

3.2.2.2 Análise das amostras de soro, tecidos e fezes por CLUE-ESI-QTOF

3.2.2.2.1 Extração de compostos fenólicos dos tecidos liofilizados

Amostras liofilizadas (20 - 80 mg) foram homogeneizadas com metanol:água (80:20, v/v) contendo 0,1% de ácido fórmico em vórtex e colocadas em banho de ultrassom por 10 minutos. Posteriormente, as amostras foram centrifugadas a 3000 g durante 10 min a 4 °C, e o sobrenadante foi filtrado através de filtro de 0,22 µm (Millipore, Bedford, MA, EUA) e injetado no CLUE-ESI-QTOF.

3.2.2.2.2 Extração de compostos fenólicos dos soros liofilizados

Amostras de soro liofilizadas foram reconstituídas com 300 µl de água milliQ. Uma aliquota de 200 µl das amostras reconstituídas foi misturada com 600 µl de acetonitrila com 0,2% de ácido fórmico, homogeneizadas em vórtex e colocadas em banho de ultrassom por 10 minutos. As amostras foram então centrifugadas a 3000 g durante 10 min a 4 °C. Os sobrenadantes foram filtrados e, em seguida, evaporados em um concentrador de amostras (Speed –Vac). As amostras foram então reconstituídas com 100 µL de metanol, filtradas através de filtro de 0,22 µm (Millipore, Bedford, MA, EUA) e analisadas por CLUE-ESI-QTOF.

3.2.2.2.3 Identificação de compostos fenólicos em soro e tecidos por CLUE-ESI-QTOF

As amostras de soro e de tecidos foram analisadas por cromatógrafo Agilent 1290 acoplado ao espectrômetro de massa quadrupolo, como descrito no item 3.2.2.1.1. As identidades dos picos dos metabolitos foram obtidas combinando os pesos moleculares dos íons ($M - H^+$) obtidos por LC-MS/MS Q-TOF com os pesos moleculares teóricos esperados. Uma estratégia de rastreio foi aplicada para a pesquisa qualitativa dos possíveis metabólitos, que consiste na busca de uma lista de compostos-alvo. Esta lista foi criada a partir dos compostos identificados no extrato fenólico de cupuaçu e de informações do metabolismo destes compostos disponíveis na literatura (Liang et al., 2014; Marín et al., 2015; Monagas et al., 2010; Stanoeva; Stefova, 2013). Para identificar os possíveis metabólitos dos compostos fenólicos do extrato de cupuaçu, cromatogramas de íons extraídos de amostras de plasma ou tecidos de camundongos eutanasiados no tempo 0 (controle) foram comparados com os cromatogramas de íons extraídos nos outros tempos, com o fim de excluir picos cromatográficos que apareciam no controle.

3.3 EFEITOS DOS COMPOSTOS FENÓLICOS DE CAMU CAMU NO DESENVOLVIMENTO DA OBESIDADE/DIABETES TIPO 2

3.3.1 Material

As polpas comerciais congeladas de camu-camu foram adquiridas da empresa Bela Iaçá Indústria e Comércio de Polpas de Frutas Ltda., em abril de 2012. Os frutos utilizados para a produção das polpas foram adquiridos de árvores localizadas em Castanhal, Estado do Pará, Brasil.

3.3.2 Extração e purificação de compostos fenólicos do camu camu

A extração dos compostos fenólicos foi realizada segundo Arabbi, Genovese e Lajolo (2004). As amostras foram extraídas com metanol aquoso 70% com 0,5% de ácido acético na proporção de 1:20 (m/v), através de agitação com barra magnética por duas horas em temperatura ambiente. Os extratos obtidos foram filtrados em papel-filtro Whatman nº 6 e resíduo foi re-extraído mais duas vezes, obtendo-se então um extrato bruto. Para a purificação dos compostos fenólicos, os extratos brutos obtidos foram concentrados utilizando rotaevaporador com temperatura de banho de 40 °C. O extrato foi purificado em coluna com resina C18 (Phenomenex Ltd., Torrance, EUA), preparada em seringa própria (HPLC Technology). A coluna foi pré-condicionada com a passagem de metanol e água. Após a passagem do extrato aquoso, a coluna foi lavada com água e a eluição dos compostos fenólicos foi feita com metanol. Após secagem completa através de rotaevaporação, o extrato foi ressuspenso em água. Este extrato foi posteriormente utilizado para a quantificação de fenólicos totais, nos experimentos *in vitro* e *in vivo*.

3.3.3 Quantificação de compostos fenólicos do extrato de camu camu

A conteúdo de fenólicos totais dos extratos purificados foi realizada pelo método de Singleton, Orthofer e Lamuela-Raventos (1999) com adaptações propostas por Genovese et al. (2003). Uma alíquota de 0,25 mL do extrato foi misturada com 0,25 mL de reagente de Folin-Ciocalteu e 2 mL de água destilada. Em seguida, adicionou-se 0,25 mL de solução saturada de carbonato de sódio e esta mistura foi colocada em banho-maria a 37 °C por 30 minutos. A absorbância foi determinada a 750 nm em espectrofotômetro (Ultrospec 2000, Pharmacia Biotec, Cambridge, Reino Unido). O ácido gálico (Sigma Chemical Co., St. Louis, EUA) foi utilizado para fazer a curva padrão. Essa análise foi realizada em triplicata e os resultados obtidos foram expressos em mg de ácido gálico equivalentes (AGE)/mL de extrato.

3.3.4 Quantificação de proantocianidinas no extrato de camu camu

As proantocianidinas foram quantificadas pelo método do butanol acidificado descrito por Porter; Hrstich e Chan (1985). Preparou-se uma solução 2:3 de HCl e n-butanol (200 mL de HCl com 300 mL de n-butanol) e adicionou-se 77 mg de $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Uma alíquota de 0,25 mL do extrato previamente diluída foi então misturada a 2,5 mL da solução de n-butanol em tubo de ensaio com tampa. Os tubos foram tampados e aquecidos em banho maria a 95 °C por 15 minutos. Após o tempo da reação, a mostra foi resfriada e realizada a leitura em espectrofotômetro a 540 nm. A cianidina-3-rutnosídeo (Extrasynthèse, Genay, França) foi utilizada para fazer a curva padrão. Essa análise foi realizada com 5 replicatas e os resultados obtidos foram expressos em expressos em mg de cianidina equivalentes/mL de extrato.

3.3.5 Tentativa de identificação de flavonoides por Cromatografia Líquida de Alta Eficiência acoplada a detector com arranjo de diodo (CLAE-DAD)

O mesmo extrato seco obtido por rotaevaporação no item 3.1.2 foi resuspenso em metanol de grau analítico e filtrado através de filtro de polietileno de 0,22 um (Millipore Ltd., Bedford, MA). A identificação e a quantificação dos flavonóides foram obtidas por um cromatógrafo (Hewlett-Packard 1100) com injetor automático de amostras e bomba quaternária acoplada a um detector de arranjo de diodos de acordo com o método de Arabbi, Genovese e Lajolo (2004). A coluna utilizada foi uma Prodigy 5 um ODS3 de fase reversa (250-4.6mm-Phenomenex Ltd., Torrance, CA), e os solventes de eluição foram (A) água/tetra-hidrofurano /ácido trifluoroacético (98: 2: 0,1) e (B) acetonitrila. O gradiente de eluição iniciou-se com 17% de B por 2 min, aumentando pa 25% de após 5 min, 35% de B após mais 8 min e 50% de B apór 5 min. Os cromatogramas foram registados a 270 nm e os flavonoides foram quantificados usando quercetina, miricetina e acido elágico (Sigma, St Louis, EUA) como padrões. Os compostos fenólicos foram tentativamente identificados através da comparação do tempo de retenção e do espectro apresentados pelos padrões. Os resultados foram expressos em µg de cada um dos compostos por ml de extrato.

3.3.6 Quantificação de ácido elágico total por Cromatografia Liquida de Alta Eficiência acoplada a detector com arranjo de diodos (CLAE-DAD)

A determinação de ácido elágico total foi realizada de acordo com Pinto et al. (2008). A amostra (0,5 g polpa base seca) foi extraída com acetona aquosa 80% (20 mL), e uma alíquota (2 ml) foi seca sob nitrogênio (Evaporador Analítico Organomation Berlim, MA). A amostra foi então hidrolisada através da adição 2 mL de ácido trifluoroacético 2N e auto

clavada a 120 °C por 90 minutos. Após a hidrólise, foi adicionado 1 mL de álcool terc-bútilico e, em seguida, a amostra foi novamente seca em nitrogênio. Por fim, a amostra foi ressuspensa em 1 mL de metanol grau analítico e filtrada com filtro de polietileno de 0,22 µm (Millipore Ltd, Bedford, MA) para posterior análise por CLAE-DAD.

3.3.7 Inibição da atividade da enzima lipase pancreática *in vitro*

A inibição da atividade da lipase foi determinada através da quantidade de 4-metilumbeliferil liberada utilizando leitor de microplaca Sinergy H1 (Biotek, IL, EUA) como descrito por Nakai et al. (2005). A lipase pancreática suína (EC 3.1.1.1.3, tipo II, Sigma Chemical Co., St Louis, EUA) e o oleato de 4-metilumbeliferil (4-MU oleato) foram diluídos em solução tampão Tris-HCl pH 8,0 nas concentrações 16,7 U/mL e 0,1M, respectivamente. A microplaca foi então preparada com 25 µL de extrato em diferentes concentrações ou inibidor de lipase (Orlistat, O4139, Sigma Chemical Co., St Louis, EUA) e 25 µL da solução de lipase. A reação iniciou-se pela adição de 50 µL do substrato 4-MU oleato. Após incubação durante 30 min a 37 °C, a produção do 4-metilumbeliferil foi medida em comprimento de onda de excitação de 355 nm e de emissão de 460 nm. Os resultados foram expressos em mg equivalente de ácido gálico (EAG) por mL de reação necessários para inibir 50% da liberação do produto 4-metilumbeliferil.

3.3.8 Efeito dos compostos fenólicos de camu camu no desenvolvimento da obesidade e diabetes tipo 2 *in vivo*

Para verificar os efeitos dos compostos fenólicos de camu camu no desenvolvimento da obesidade e diabetes tipo 2, camundongos C57BL/6 receberam dieta padrão ou dieta com alto teor de lipídios e sacarose (HF/HS) para a indução da obesidade e resistência à insulina. O protocolo experimental foi aprovado pela Comissão de Ética no Uso de Animais da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (CEUA/FCF/USP), protocolo CEUA nº 353 (Anexo A). Todos os experimentos foram realizados em conformidade com a legislação vigente sobre o assunto no país. Camundongos com 8 semanas de vida, procedentes do Biotério do Instituto de Ciências Biomédicas da Universidade de São Paulo, foram divididos em 4 grupos de 10 animais cada e submetidos a uma adaptação de 7 dias ao ambiente experimental, recebendo dieta padrão de laboratório.

A suplementação com extratos fenólicos foi realizada durante 8 semanas e iniciou-se juntamente com a administração da dieta com alto teor de lipídios e sacarose. Os extratos fenólicos purificados obtidos anteriormente foram administrados por gavagem uma vez ao dia

em duas doses diferentes. Os grupos que não receberam suplementação de extratos fenólicos receberam água por gavagem como descrito a seguir:

Grupo controle = dieta padrão + gavagem de água

Grupo HF/HS = dieta com alto teor de lipídios e sacarose + gavagem de água

Grupo HF/HS + camu camu 1 = dieta com alto teor de lipídios e sacarose + gavagem de extrato de compostos fenólicos de camu camu (7 mg de ácido gálico equivalentes/kg peso corporal)

Grupo HF/HS + camu camu 2 = dieta com alto teor de lipídios e sacarose + gavagem de extrato de compostos fenólicos de camu camu (14 mg de ácido gálico equivalentes/kg peso corporal)

A dieta padrão Nuvilab CR1 (Nuvital Nutrientes SA, Curitiba, PR, Brasil) utilizada fornece 3,6 Kcal/g, sendo 23,7% da energia proveniente de proteínas, 12% de lipídios e 56% de carboidratos. A dieta com alto teor de lipídios e sacarose foi elaborada como descrito anteriormente por Lemieux et al. (2003) e fornece 4,6 Kcal/g, sendo 20% da energia proveniente de proteínas, 41% proveniente de carboidratos (39% proveniente da sacarose) e 39% proveniente de lipídios (19% óleo de soja, 19% banha de porco).

No período dos experimentos, os animais foram mantidos com água e dieta *ad libitum* e as condições ambientais controladas (temperatura de 22 °C, umidade relativa de 65% e ciclo de claro e escuro de 12 horas).

3.3.8.1 Ingestão alimentar, ingestão hídrica, peso corporal e glicemia

A ingestão alimentar, a ingestão hídrica e o peso dos animais foram medidos em dias alternados. A glicemia foi avaliada semanalmente após jejum de 5 horas com glicosímetro Accu-check® a partir de amostras de sangue coletadas através de pequena incisão na ponta da cauda dos camundongos. A eficiência alimentar foi calculada pela razão entre o ganho de peso corporal (g) e a quantidade total de calorias consumidas (Kcal) por camundongo, multiplicado por 100.

3.3.8.2 Teste intraperitoneal de tolerância à glicose (ipGTT)

O teste intraperitoneal de tolerância à glicose (ipGTT) foi realizado após 6 horas de jejum na última semana do experimento. Uma primeira coleta de sangue foi feita através de corte na extremidade da cauda do animal no tempo 0. Em seguida, uma solução de glicose a

10 % (1 g/ kg de massa corporal) foi administrada aos camundongos intraperitonealmente. A concentração de glicose no sangue foi então determinada com o glicosímetro Accu-check® nos tempos 15, 30, 45, 60 e 90 minutos após administração da carga de glicose. Para análise de insulina durante o ipGTT, amostras de sangue foram coletadas através de pequena incisão na ponta da cauda dos camundongos nos tempos 0, 30 e 90 minutos após a injeção de glicose. As amostras foram centrifugadas a 2000 g por 10 minutos a 4 °C, e o soro foi recolhido para análise de insulina utilizando kit de insulina para camundongos ELISA (EZRMI-13K) da Millipore (Billerica, MA, EUA). Posteriormente, a sensibilidade à insulina foi determinada pelo índice quantitativo de sensibilidade a insulina (*Quantitative Insulin Sensitivity Check Index* - Quicki) utilizando a fórmula: $1/\log \text{ insulina } (\mu\text{U/mL}) \times \log \text{ glicose } (\text{mg/dL})$.

3.3.8.3 Coleta de tecidos e soro

Nos dias 56-58 do experimento, os animais foram anestesiados e foi realizada a punção cardíaca. As amostras de sangue foram centrifugadas a 2000 g durante 10 min a 4 °C. Fígado, músculo (gastrocnemius) e tecido adiposo (epididimal , inguinal, retroperitoneal e o tecido adiposo marrom) foram coletados, pesados, congelados em nitrogênio líquido e armazenados a -80 °C.

3.3.8.4 Avaliação bioquímica do soro

Os níveis de glicose, triacilglicerol (TAG), colesterol total (CT), lipoproteína de alta densidade colesterol (HDLc), lipoproteína de baixa densidade colesterol (LDLc), proteína C reativa , alanina aminotransferase (ALT) e aspartato aminotransferase (AST) foram quantificados por analisador automático LabMax 240 (Labtest Diagnóstica, Minas Gerais, Brasil).

3.3.8.5 Quantificação de lipídios em tecido hepático

Os tecidos foram homogeneizados em nitrogênio líquido. Os lipídios foram extraídos de acordo com o método de Hara e Radin (1978). Em um eppendorff, 50 mg do fígado homogeneizado foi misturado com 1,125 mL de hexano/isopropanol (3:2). Esta mistura foi então centrifugada a 1000 g por 15 minutos e o sobrenadante foi retirado e misturado com 1,5 mL de sulfato de sódio anidro (6.6%) e decantado por 1 minuto. Uma alíquota do sobrenadante (200 µL) foi então removida, seca em nitrogênio e reconstituída em 500 µL de isopropanol. Esta solução foi utilizada para quantificar as concentrações de triacilgliceróis usando o Labmax 240 (Labtest Diagnóstica, Minas Gerais, Brasil).

3.3.9 Análise estatística

As análises estatísticas foram realizadas com o software GraphPad Prism 6 (San Diego, CA, EUA). Os dados experimentais foram testados para normalidade pelo teste de Shapiro Wilk. As diferenças entre os grupos foram analisadas por meio de análise de variância (ANOVA), seguido de teste de Tukey ou testes não paramétricos. O ganho de peso corporal e a glicemia de jejum semanal foram comparados estatisticamente através de ANOVA *two way* para medidas repetidas. Os dados foram apresentados como médias e desvio-padrão ou erro padrão da média. Um valor de probabilidade de $p < 0,05$ foi utilizado como o critério para a significância estatística.

4. RESULTADOS

Esta seção foi dividida em três partes, de acordo com os resultados obtidos.

4.1 CUPUASSU (*THEOBROMA GRANDIFLORUM*) POLYPHENOLS IMPROVE
HEPATIC INSULIN SENSITIVITY REDUCING STEATOSIS AND DYSLIPIDEMIA IN
HIGH-FAT-HIGH-SUCROSE-FED C57BL/6 MICE

**HELENA RUDGE DE MORAES BARROS^{1*}, THIAGO BELCHIOR DE OLIVEIRA²,
WILLIAM T. FESTUCCIA², MARIA INÉS GENOVESE¹**

* Corresponding author (Tel: 55-11-30911525; Fax: 55-11-38154410; e-mail:
lebarros79@gmail.com)

¹ Laboratório de Compostos Bioativos de Alimentos, Departamento de Alimentos e Nutrição Experimental, FCF, Universidade de São Paulo, Av. Prof. Lineu Prestes 580, Bloco 13A, 05508-900 São Paulo, SP, Brazil. E-mail address: genovese@usp.br (Genovese, M.I.)

² Departamento de Fisiologia, ICB-1, Universidade de São Paulo, Av. Prof. Lineu Prestes 1524, Lab. 125, 05508-900 São Paulo, SP, Brazil. E-mail address: tbelchior12@gmail.com (Oliveira, T. B.); william.festuccia@gmail.com (Festuccia, W.T.)

ABSTRACT

The effect of a Brazilian *Theobroma grandiflorum* fruit extract on the development of obesity, glucose intolerance and dyslipidemia induced by high fat/high sucrose (HF/HS) diet intake was evaluated. C57BL/6J mice fed with HF/HS diet were treated by daily gavage with water or different doses of cupuassu phenolic extract (CPE, 2.25 and 4.5 mg of catechin equivalents/Kg body weight). CPE administration at the higher dose significantly decreased digestive gut enzyme activities, fasting glucose levels, plasma LDL and VLDL cholesterol levels, hepatic lipid accumulation and hepatic aminotransferases activities; and increased the phosphorylation of AKT in liver. Therefore, CPE improved glucose and lipid metabolism protecting liver from steatosis promoted by HF/HS diet, indicating that cupuassu is an attractive fruit with valuable health benefits upon glucose and lipid metabolism, being a potential agent in the reduction of the risk of hepatic steatosis development induced by HF/HS diet.

Keywords: cupuassu, dyslipidemia, glucose homeostasis phenolic compounds.

Chemical compounds studied in this article

(-) Epicatechin (PubChem CID: 72276), Procyanidin B2 (PubChem CID: 122738), Isoscutellarein-8-O- β -D-glucuronine (PubChem CID: 44258568), Hypolaetin-8-O- β -D-glucuronide 3''-sulfate (PubChem CID: 11272944).

1. Introduction

The incidence of obesity and type 2 diabetes has increased dramatically in recent years, being regarded as a global epidemic and a challenge to the modern society (World Health Organization, 2014). These diseases are characterized by a chronic low grade inflammation that affect various organs and tissues such as muscle, liver, adipose tissue, and pancreas impairing their ability to defend against intracellular oxidative stress and to properly respond to insulin (Esser, Legrand-Poels, Piette, Scheen, & Paquot, 2014). Thus, the increased incidence of these two diseases leads to the development of several other co-morbidities that reduce life expectancy and entail an expressive increase in the costs of health systems (Thorpe & Philyaw, 2012).

Several studies have shown that regular intake of vegetables and fruits reduces the risk of obesity and type 2 diabetes development (Xiao & Högger, 2015; Wang et al., 2014). Such beneficial actions are partly attributed to bioactive compounds known as polyphenols (Del Rio et al., 2013) that, through different mechanisms, affect glucose and lipids metabolism. Indeed, polyphenols were shown to inhibit the digestion and absorption of carbohydrates and lipids in the intestine; to stimulate insulin secretion, modulate hepatic glucose production and activate insulin receptors promoting glucose uptake in peripheral insulin-sensitive tissues; and reduce preadipocyte proliferation and differentiation and chronic inflammation (Anhê et al., 2013; Meydani & Hasan, 2010; Williamson, 2013).

Among the several classes of polyphenols, flavanols are the most largely consumed polyphenols in Western populations and have been widely *studied* for their impact on health. Many studies have suggested that these compounds, mainly found in cocoa (*Theobroma cacao* L.), green tea and grapes derivatives, play a beneficial role in prevention of chronic diseases, including type 2 diabetes, obesity and cardiovascular diseases (Park et al., 2013; Pinent, Cedó, Montagut, Blay, & Ardévol, 2012; Stote et al., 2012; Yamashita, Okabe, Natsume, & Ashida, 2012).

Cupuassu (*Theobroma grandiflorum* Willd. Ex Spreng) Shum. is a native fruit from the Amazon region phylogenetically close to cocoa, which contains flavanols and other phenolic compounds that are known by their antioxidant capacity and by their potent inhibition of α -amylase activity *in vitro* (Gonçalves, Lajolo, & Genovese, 2010; Pinent et al., 2015). Moreover, the effects of chronic daily intake of cupuassu liquor upon metabolic alterations associated to type1 diabetes or to a high fat diet were recently evaluated, showing improvement of plasma lipid profile, antioxidant defenses and glucose tolerance (Oliveira;

Rogero, & Genovese, 2013; Oliveira, & Genovese, 2015). Herein we tested the hypothesis that administration of cupuassu polyphenols counteract the metabolic disarrangements associated to the development of obesity and type 2 diabetes induced by the intake of a high fat/high sucrose diet.

2. Material and Methods

2.1 Cupuassu samples. Cupuassu commercial frozen pulps were obtained from *Tentação Amazônica*, a fruit producer. Commercial frozen pulps were produced from fruits cultivated in Paraupabas, Pará State, Brazil, in August 2012.

2.2 C18-Purified Cupuassu Phenolic Extracts (CPE). Extraction was performed according to the method of Arabbi, Genovese, & Lajolo (2004) with some modifications. Samples of commercial frozen pulp were homogenized and extracted three times in a solvent mixture of methanol/water (70:30) at speed 4 for 1 minute (Brinkmann homogenizer, Polytron; Kinematica GmbH) in ice. The homogenate was filtered under reduced pressure through filter paper (Whatman no. 1), evaporated under vacuum at 40 °C in a rotatory evaporator (Rotavapor RE 120; Büchi, Flawil, Switzerland) and suspended in water. The cupuassu extract was added to a C18 column (Applied Separations, New York, USA) preconditioned with methanol and water. The column was washed with water and further eluted with methanol. This fraction was evaporated to dryness under pressure at 40 °C, and then dissolved in distilled water for animal administration and for extract characterization.

2.3 Total Phenolic Contents in CPE. The phenolic content was determined according to the method of Singleton, Orthofer, & Lamuela-Raventós (1999). A 0.25 mL aliquot of CPE was mixed with 0.25 mL of Folin-Ciocalteu reagent and 2 mL of distilled water. After 3 min at room temperature, 0.25 mL of a saturated sodium carbonate solution was added and the mixture was placed at 37 °C in a water bath for 30 min. The absorbance was measured at 750 nm using a model Ultrospec 2000 UV-visible spectrophotometer (Amersham Biosciences, Cambridge, UK). The results were expressed as milligrams of catechin equivalents (CE) per mL of extract.

2.4 Identification of phenolic compounds in CPE. Evaporated fractions obtained above were res-suspended in HPLC grade methanol, filtered through 0.22 μm polytetrafluoroethylene (PTFE) filters (Millipore Ltd., Bedford, MA), and then analyzed in a HPLC-DAD. Identification and quantification of flavonoids were achieved using analytical reversed-phase HPLC in a Hewlett-Packard 1100 system with autosampler and quaternary pump coupled to a diode array detector, according to the method of Arabbi, Genovese, & Lajolo (2004). The column used was 250-4.6 mm, i.d., 5 μm , Prodigy ODS3 reversed phase silica (Phenomenex Ltd., Torrance, CA, USA), and elution solvents were (A) water/tetrahydrofuran/ trifluoroacetic acid (98:2:0.1) and (B) acetonitrile. Chromatograms were recorded at 270 nm. Flavanols were quantified using epicatechin and flavones were quantified using apigenin as external standards, respectively. The results were expressed in μg of each compound per mL of extract.

2.5 α -Glucosidase inhibition assay. The α -glucosidase inhibitory assay was based on the chromogenic method described by Kim, Kwon, & Son (2000). Briefly, yeast α -glucosidase (0.7 U) was dissolved in 100 mM phosphate buffer (pH 7.0) containing 2 g/L bovine serum albumin and 0.2 g/L sodium azide. The substrate solution, 5 mM p-nitrophenyl-R-D-glucopyranoside, was prepared in the same buffer (pH 7.0). Enzyme solution (100 μL) and sample (20 μL) were mixed, and the absorbance was read at 405 nm (time zero) (Sinergy H1 hybrid reader, Biotek, IL, USA). After 5 min of incubation at 37 $^{\circ}\text{C}$, the substrate solution (100 μL) was added and incubated for another 5 min at 37 $^{\circ}\text{C}$, and the absorbance at 405 nm was determined (10 min). The extracts were diluted in several rates and compared to a control (20 μL of water instead of fruit extract). Results were expressed as mg of catechin equivalent per mL of reaction necessary to inhibit 50% of glucose production.

2.6 Pancreatic lipase inhibition assay. The inhibition of lipase activity was determined by measuring the amount of 4-methylumbelliferone product released by lipase using a plate reader (Sinergy H1 hybrid reader, Biotek, IL, USA) following a reported method by Nakai et al. (2005). Pancreatic lipase (EC 3.1.1.1.3, type II, from porcine pancreas, Sigma Aldrich, St. Louis, USA) and 4-methylumbelliferyl oleate (4-MU oleate) were the reaction enzyme and substrate, respectively. The reaction mixture was prepared with 25 μL of the cupuassu extract at different concentrations or lipase inhibitor (Orlistat, O4139, Sigma Aldrich, St. Louis, USA) and 25 μL of 16.7 U/mL lipase in pH 8.0 Tris-HCl buffer solution. The reaction was initiated by adding 50 μL of 0.1 M 4-MU oleate in pH 8.0 Tris-HCl buffer solution. After

incubation at 37 °C for 30 min, the rate of release of 4-methylumbeliferone was measured with a plate reader at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Results were expressed as mg of catechin equivalent (CE) per mL of reaction necessary to inhibit 50% of 4-methylumbeliferone production.

2.7 Animals. The study was approved by the Ethics Committee on Animal Experimentation of the Faculty of Pharmaceutical Sciences, University of São Paulo, according to the guidelines of the Brazilian College on Animal Experimentation (protocol number 353). Mice were kept at 25 ±1 °C and on a 12:12 h light/dark cycle. After one week adaptation period, male C57BL/6J mice (8 weeks of age) were randomly divided into four groups of ten animals each. The groups received: (1) standard chow diet (SD) and water by oral gavage; (2) high-fat high-sucrose diet (HF/HS) and water by oral gavage; (3) HF/HS diet and oral gavage of 2.25 mg catechin equivalents of CPE/ Kg of body weight (HF/HS + CPE 0.5) and (4) HF/HS diet and oral gavage of 4.50 mg catechin equivalents of CPE/ Kg of body weight (HF/HS + CPE 1) for 8 weeks. The SD (Nuvilab CR-1 Nuvital Nutrientes SA, Curitiba, PR, Brazil) provided 3.6 kcal/g, of which 12% kcal were from fat, 25% from proteins and 63% from carbohydrates. The HF/HS diet was previously described by Lemieux, Picard, Labrie, Richard, & Deshaies (2003) and provided 4.6 kcal/g, of which 39% kcal were from fat (18.5% lard, 18.5% soy oil), 20% from proteins and 41% from carbohydrates (39% from sucrose). Food and water were provided *ad libitum*. Body weight, food and water intake were determined in alternate days. Feed efficiency was calculated as the ratio between the body mass gain in grams and the food consumed in kilocalories per animal, multiplied by 100.

2.7.1 Measurement of serum triacylglycerol and free fatty acids. Blood was collected from the tail vein after 12 h-fasting and after 3 h re-feeding. The serum triacylglycerol (TG) levels were measured using an automated analyzer (Labmax 240, Labtest Diagnóstica, Minas Gerais, Brazil). Free fatty acids (FFA) were measured using the Wako NEFA-C assay (Richmond, VA, USA). Hydrogen-peroxide produced from FFAs breakdown were measured at absorbance of 550 nm (blue-pigment).

2.7.2 Intraperitoneal Insulin Tolerance Test (ipITT). Insulin tolerance test was performed after 7 weeks of treatment by injecting intraperitoneally 0.75 U of insulin/Kg of body weight in fed mice (no fasting period). Blood glucose was measured at baseline and at 15, 30, 45, 60

and 90 min after insulin injection using a glucometer (Accu-check® performa, Roche Diagnostics, Mannheim, Germany).

2.7.3 Intraperitoneal Glucose Tolerance Test (ipGTT). Glucose tolerance test was performed after 8 weeks of treatment by injecting intraperitoneally 1 g of glucose/kg of body weight after a 5 h-fasting period. Blood glucose was measured at baseline and at 15, 30, 45, 60 and 90 min after glucose injection using a glucometer (Accu chek® performa, Roche Diagnostics, Mannheim, Germany). Blood was collected from tail vein at baseline and 30 and 90 min after glucose injection for serum insulin measurement with a rat/mouse insulin ELISA kit (EZRMI-13K, Millipore Billerica, MA, USA). Insulin sensitivity was determined by the Quantitative Insulin Sensitivity Check Index (QUICKI) using the formula: $1/\log \text{ insulin (uU/ml)} \times \log \text{ glucose (mg/dl)}$.

2.7.4 Tissues and serum collection. On days 56-58, mice were anesthetized and cardiac puncture was performed followed by euthanasia. Blood was centrifuged at 2000 g for 10 min at 4 °C for serum separation. Liver, muscle (gastrocnemius) and adipose tissues (epididymal, inguinal, retroperitoneal and brown adipose tissue) were collected, weighed, frozen under liquid N₂ and stored at -80 °C.

2.7.5 Biochemical evaluation of serum. The plasma levels of glucose, triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDLc), low-density lipoprotein cholesterol (LDLc), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using an automated analyzer (Labmax 240, Labtest Diagnóstica, Minas Gerais, Brazil). Very-low-density lipoprotein (VLDL) was calculated as the difference between TC and HDLc and LDLc.

2.7.6 Liver tissue analysis. Tissues were grounded to powder under liquid nitrogen and homogenized. Total lipids were extracted according to the Folch method (1957). Aliquots of the extracted liver lipids were used for the measurement of the triacylglycerol and total cholesterol levels using Labmax 240 as described above. Lipid peroxidation was estimated by measuring the production of free malondialdehyde (MDA) in hepatic tissue using high performance liquid chromatography with fluorescence detection as described by Hong, Yeh, Chang, & Hu (2000). Tissues were homogenized in pH 7.4 phosphate-buffered saline solution

(PBS), centrifuged at 12,000 rpm for 10 minutes at 4 °C and the supernatants were then used for the estimation of lipid peroxidation.

2.7.7 Western blot analysis. Liver was homogenized and analyzed for protein content. Equal amounts of proteins were loaded onto an acrylamide gel, subjected to SDS-PAGE, and then transferred onto PVDF membranes. Membranes were blocked, probed with antibodies against phosphorylated protein kinase B serine 473 (p-Akt S473), protein kinase B (Akt) from Cell Signalling Technology (Beverly, MA, USA) diluted 1:1000 in 5% milk, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) diluted 1:5000 in 5% milk. Membranes were incubated with peroxidase-conjugated secondary antibody (1:10000) and revealed with ECL enhanced chemiluminescence substrate (GE Healthcare, Buckinghamshire, UK).

2.8 Statistical analysis. Statistical analyses were performed with GraphPad Prism 6 software (San Diego, CA, USA). Experimental data were tested for normality by Shapiro Wilk test. Differences among groups were tested using analysis of variance (ANOVA) followed by Tukey's post-test or non-parametric tests. Body weight gain and weekly fasting glycemia were statistically compared using two-way repeated measures ANOVA. Data were presented as means and standard error of mean (SEM). A probability value of $p < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1 Chemical characterization of Cupuassu Phenolic Extract (CPE).

3.1.1 Phenolic contents. CPE was examined for its flavonoids composition using HPLC/DAD. CPE presented the flavanols epicatechin and procyanidin B2, and the flavones hypolaetin and isoscutellarein derivatives. The contents of each phenolic compound in the extract were presented in **Table 1**. The total amount of polyphenols measured by the Folin Ciocalteu (0.9 mg catechin equivalents (CE)/mL of the extract) was used to calculate the dose to each mouse per day, considering the body weight of the animals.

3.1.2 *In vitro* α -glucosidase and pancreatic lipase inhibitory activity. The ability of CPE to inhibit yeast α -glucosidase and pancreatic lipase activities was evaluated at different

concentrations of CPE (**Figure 1**). Overall, CPE inhibited α -glucosidase and pancreatic lipase activities in a dose-dependent manner and was more effective than acarbose in inhibiting α -glucosidase. CPE presented an IC₅₀ of 0.113 mg CE/mL of reaction, while acarbose presented an IC₅₀ of 0.322 mg of acarbose/mL of reaction for α -glucosidase inhibitory activity. Regarding pancreatic lipase inhibitory activity, the IC₅₀ value of CPE was 0.131 mg CE/mL of reaction, which was lower than that of orlistat (0.069 mg/mL of reaction).

3.2 Effects of CPE on metabolic alterations associated with obesity/diabetes induced by a high fat/high sucrose diet in C57BL6 mice.

3.2.1 Body weight gain and food intake. Mice cumulative body weight gain is shown in **Figure 2**. As expected, the HF/HS-fed mice had a higher incremental body weight than mice fed with standard chow diet (SD) (30 vs 14%). CPE 1 administration reduced HF/HS induced body weight gain (20%) to levels that were not significantly different from either HF/HS or SD fed mice.

Food intake data are presented in **Table 2**. The HF/HS-fed mice featured a significant reduction in daily food intake when compared to mice fed a SD ($p < 0.05$). However, the total energy intake during the experimental protocol did not differ between groups due to the higher caloric density of the HF/HS diet compared to the SD. As expected, feed efficiency in mice receiving the HF/HS diet was significantly higher compared than that of SD group. CPE administration in both doses presented similar total energy intake and feed efficiency than HF/HS-fed mice group, although CPE 1 administration trend to reduce this efficiency due to the body weight gain reduction.

Intake of a HF/HS diet increased retroperitoneal, inguinal, epididymal white adipose depots e brown fat without significantly affecting liver and muscle masses. These variables were not affected by treatment with CPE, the exception being liver mass that was significantly reduced by CPE 1 (**Table 2**).

3.2.2 Glucose homeostasis. Fasting plasma glucose levels were measured weekly (**Figure 3A**). Plasma glucose levels were significantly higher in HF/HS-fed mice compared with SD group from 4th to 8th weeks. Within the HF/HS diet fed groups, fasting plasma glucose levels in the CPE 1 group were significantly lower than those in CPE 0.5 or HF/HS control groups from 5th to 8th weeks.

To estimate whole-body insulin sensitivity, we performed insulin tolerance test (ipITT) and calculated the constant of plasma glucose disappearance after the injection of

insulin (Kitt). The ipITT was performed on fed mice at 7th week (**Figure 3B**). There were no significant changes on rates of glucose disappearance between groups with the exception of HF/HS-fed mice treated with CPE 1, which presented a higher kITT than mice fed with a HF/HS and treated with CPE 0.5 (**Figure 3C**).

We next investigated the effects of CPE on glucose tolerance through an ipGTT (**Figure 3D**). The area under the plasma glucose curve (AUC) was significantly higher ($p < 0.05$) in mice given the HF/HS diet compared to the SD group (**Figure 3E**), indicating glucose intolerance. Administration of CPE did not alter GTT AUC when compared to HF/HS group. However, supplementation with CPE 1 seems to reduce glucose intolerance induced by HF/HS intake, since their AUC and plasma insulin levels during ipGTT do not differ from those displayed by the SD-fed group (**Figures 3E and F**). In addition, from fasting insulin and glucose data, we calculated QUICKI index (**Figure 3H**). The HF/HS-fed group demonstrated a decrease of insulin sensitivity compared to SD-fed group, while the administration of CPE did not result in differences from SD-fed group neither from HF/HS fed group.

3.2.3 Lipid metabolism. Serum triglycerides were not altered by diet or CPE administration (**Figure 4A**). However, the HF/HS-fed mice group presented an increase in total cholesterol (**Figure 4B**) and VLDL cholesterol (**Figure 3E**) levels compared to SD-fed group. On the other hand, HDL-c levels were significantly higher in CPE 1 group (**Figure 4C**), while LDL-c and VLDL were reduced (**Figure 4D and E**) when compared to HF/HS-fed group. As expected, HF/HS diet led to a higher TG fecal excretion compared to SD-fed group (**Figure 4F**), and CPE 1 group increased TG fecal excretion in 50% when compared to HF/HS-fed group. Although no statistical differences were found in plasma triglycerides or free-fatty acids levels upon fasting and re-feeding conditions between all the groups (**Figure 5**), CPE 1 group demonstrated a trend to improve lipid homeostasis compared to HF/HS-fed group.

3.2.4 Hepatic function. CPE 1 administration reduced liver weight (**Figure 6A**) and hepatic TG accumulation (**Figure 6B**), and maintained liver oxidative stress to SD group levels, as indicated by **Figure 6C**. The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were decreased by CPE supplementation at both doses (**Figures 6D and 6E**). To further investigate the molecular basis of insulin resistance in HF/HS mice, we analyzed insulin-stimulated signaling in liver. Phosphorylation of AKT was significantly

reduced in liver of HF/HS fed mice when compared with SD fed mice (**Figure 6F**), while CPE administration presented similar results than those found in SD-fed mice.

4. Discussion

A variety of naturally occurring polyphenols have been proposed to protect against development of chronic diseases as obesity and type 2 diabetes (Xiao, & Högger, 2015; Wang et al., 2014). Cupuassu, a fruit that belongs to the same genus of cacao and is highly appreciated due to its characteristic flavor, contains a variety of phenolic compounds with promising biologic effects against dyslipidemia and oxidative stress (Oliveira et al., 2013; Oliveira, & Genovese, 2015). Herein, we explored the hypothesis that administration of a cupuassu extract rich in polyphenols counteracts the metabolic disarrangements associated to the intake of a HF/HS diet. As expected, 8 weeks of HF/HS feeding induced obesity, glucose intolerance, insulin resistance and dyslipidemia in mice. Among those deleterious effects, administration of CPE attenuated HF/HS induced body weight gain, reduced fasting glucose levels, improved plasma cholesterol levels and protected liver against steatosis development.

Analysis of the phenolic composition of CPE revealed the presence of flavanols that are widespread in edible plants and highly concentrated in cocoa and green tea (Del Rio et al., 2013). In addition, flavones as glucuronide and sulfo-glucuronide derivatives of hypolaetin and isoscutellarein were also found in CPE as previously described by Pugliese, et al. (2013). These flavones have been described in species of the genus *Sideritis* L., which belongs to the Lamiaceae family and are used to prepare “mountain tea”, a traditional beverage of Mediterranean and Balkan countries widely used in folk medicine to treat respiratory diseases and gastrointestinal disorders (Stanoeva, & Stefova, 2013). Moreover, CPE presents hypolaetin-3'-methyl-ether-8-O- β -D-glucuronide-3''-sulfate, which is a novel conjugate in nature (Pugliese et al., 2013).

These polyphenol classes (flavanols and flavones) have been reported to exert biological activities including improvement of glucose and lipid metabolism, antioxidant and anti-inflammatory activities (Singh, Kaur, & Om, 2014; Xiao, & Hogger, 2015; Zanotti et al., 2014). Therefore, this particular mixture of different phenolic compounds makes CPE an interesting source of phytochemicals with potential health benefits. The higher dose of CPE administered to mice (4.5 mg of catechin equivalents/ Kg bw) is equivalent to the

supplementation of 315 mg of polyphenols to a 70-kg person per day. This dose is close to those derived from the consumption of cocoa products or from berries and wine in some European countries (Zanotti et al., 2014) and is thoroughly feasible by diet or supplementation, since estimate dietary polyphenol intake accounts roughly for 1 g of polyphenols per day (Grosso, Stepaniak, Topor-Mądry, Szafraniec, & Pająk, 2014).

Previous studies have demonstrated that ingestion of a diet high in fat and sucrose that mimics Western-style diets significantly elevated adipose tissue mass, insulin levels and lead to hepatic steatosis in mice (Yang, Miyahara, Takeo, & Katayama, 2012). In spite of consuming similar amounts of energy than SD group, mice fed with HF/HS diet featured obesity, glucose intolerance, hepatic steatosis, hyperglycemia and hyperinsulinemia. Administration of CPE at the higher dose attenuated HF/HS induced body weight gain, which may associated to both a slightly decrease in feed efficiency and an increase of TG fecal excretion due to inhibition of pancreatic lipase activity. Similarly to these findings, Min et al. (2013) found that high fat fed mice supplemented with a cocoa polyphenol extract at dose of 200 mg/kg BW also had a reduced body weight gain. Such anti-obesity effect was partly attributed to a reduced feed efficiency and to an inhibition of pancreatic lipase activity by cocoa polyphenols. In fact, many polyphenols (including flava-3-ols and flavones) and plant extracts from Lamiacea family have shown inhibitory effects on pancreatic lipase activity (De la Garza, Milagro, Boque, Campión, & Martínez, 2011).

Along with body weight, CPE administration at the higher dose reduced fasting glucose levels and promoted a trend to attenuate the insulin and glucose intolerance induced by HF/HS intake. One effective therapeutic approach to decrease postprandial hyperglycemia consists in reducing the absorption of glucose through the inhibition of carbohydrate-hydrolyzing enzymes such as α -glucosidase and α -amylase, and the inhibition of glucose transporters in the gastrointestinal tract (Williamson, 2013; Lacroix, & Lin-Chan, 2013). Our findings that CPE can inhibit α -glucosidase activity *in vitro*, along with those of a previous study (Gonçalves, Lajolo, & Genovese, 2010) showing that cupuassu pulp extract strongly inhibits α -amylase activity *in vitro*, indicate a possible modulation of carbohydrate hydrolysis and absorption in the gastrointestinal tract by cupuassu polyphenols. Further studies, however, are required to test this hypothesis *in vivo*.

A previous study also demonstrated that cupuassu phenolic-rich extract was effective in improving the insulin and glucose tolerance of HFD-fed rats, suggesting that cupuassu phenolic compounds reduce peripheral insulin resistance and improve tissue sensitivity to this hormone (Oliveira et al., 2015). Although not investigated here, many studies have shown

that polyphenols may influence glucose metabolism by stimulating peripheral glucose uptake in insulin-sensitive tissues (Anhê et al., 2013, Williamson, 2013). Procyanidin extracts (one of the phenolic classes found in CPE) can act as an insulinomimetic agent, since it stimulates glucose uptake via the PI3K-Akt pathway (Pinent et al., 2012). Proanthocyanidins were also shown to promote, independently of insulin, the translocation of glucose transporter 4 (GLUT 4) in muscle and adipose tissue by enhancing AMPK α phosphorylation in high fat-fed mice (Yamashita, Okabe, Natsume, & Ashida, 2012). In this sense, our findings indicate that CPE 1 administration protected mice from fasting hyperglycemia, which is determined by the balance between glucose production mainly by the liver and glucose utilization. CPE administration at the higher dose reestablished the phosphorylation of Akt and therefore insulin signaling in the liver, indicating that this phenolic extract may attenuate HF/HS induced disarrangements in glucose homeostasis by reestablishing insulin ability to properly inhibit hepatic glucose production.

The development of hepatic insulin resistance has been linked to the ectopic accumulation of lipids, inflammation and oxidative stress in liver, which in combination may lead to non-alcoholic fatty liver disease (NAFLD), the most common chronic liver disease in Europe and in the USA (Vernon, Baranova, & Younossi, 2011; Yang et al., 2012). Herein we found that the improvement in liver insulin signaling induced by CPE was associated with a reduction in hepatic triacylglycerol accumulation and oxidative stress, as evaluated by the production of MDA in hepatic tissues and plasma transaminase activities.

The altered capacity to store lipids in obesity and type 2 diabetes plus HF/HS feeding may result in excessive lipid accumulation in non-adipose tissues and liver is one of the main targets of this lipotoxicity. Our results suggest that CPE administration reduced lipid absorption by inhibiting pancreatic lipase activity and increasing TG fecal excretion, thus possibly decreasing the lipotoxic condition and hepatic triglyceride accumulation. Furthermore, analysis of plasma TG and free-fatty acids levels in fasting and re-feeding states showed that CPE administration induces a trend for restoration of these variables to levels of SD fed mice. In this context, CPE 1 administration also reduced plasma VLDL levels, which would be a result of improvement of lipid homeostasis, thus reducing plasma FFA levels that are the main substrate for hepatic TG production in the form of VLDL (Karpe, Dickmann, & Frayn, 2011).

In addition, recent studies (Gu, Yu, & Lambert, 2014; Oliveira et al., 2015) demonstrated that administration of cocoa and cupuassu derivatives protected against high fat diet-induced liver oxidative stress by reducing transaminase activities. Abnormal circulating

concentrations of hepatic enzymes and cytokines are a common sign of liver and cardiovascular disease and oxidative stress plays a critical role in hepatic cell damage and dysfunction (Vernon et al., 2011). Since polyphenols levels are too low to provide any relevant direct antioxidant action compared to other compounds in tissues, polyphenols and their metabolites could promote indirect antioxidant effects through the regulation of enzymes that generate oxidants or by preventing protein–receptor coupling that initiates oxidant production and interacting with membranes, modulating cell signaling. Flavones, epicatechin and its metabolites are recognized by their antioxidant and anti-inflammatory actions (Ali, Ismail, & Kersten 2014; Mena et al., 2014; Singh et al., 2014). Therefore, CPE supplementation could result in indirect antioxidant or anti-inflammatory effects, slowing the progression of hepatotoxicity produced by obesity and diabetes.

In addition to insulin resistance and hepatic steatosis, obesity and diabetes are also commonly associated with a dyslipidemia characterized by high plasma triglyceride and LDL-cholesterol and low HDL-cholesterol levels (Mooradian, 2009). In this study, serum triacylglycerides were not altered by HF/HS diet. However, CPE at higher dose promoted an increase in HDL-c and a decrease in LDL-c levels. The mechanisms accounting for the improvement lipid homeostasis induced by CPE are unknown, but they may be related to the inhibition of intestinal lipid absorption with consequent reduced chylomicron secretion by the intestine and VLDL secretion by the liver; and reduction of hepatic *de novo* lipid synthesis associated to apolipoprotein synthesis. Importantly, similar results were observed in STZ-diabetic rats treated with cupuassu and cocoa liquors (Oliveira, & Genovese, 2013). Several studies in animals and humans have also reported a significant reduction in plasma total cholesterol and LDL-cholesterol, and an increase in HDL-cholesterol after supplementation with foods containing polyphenols, mainly proanthocyanidins (Bladé, Arola, & Salvadó, 2010). Mechanistic studies have found that flavanols and their metabolites affect the apolipoprotein synthesis in cultured human hepatic and intestinal cells, increasing ApoA1 and decreasing ApoB levels, indicating a direct action of these molecules on liver (Yasuda, Natsume, Osakabe, Kawahata, & Koga, 2011; Guerrero et al., 2013). Whether those mechanisms are also involved in the improvement in lipid homeostasis induced by CPE remains to be investigated.

5. Conclusion

In conclusion, CPE supplementation at higher dose (4.5 mg EC/Kg b.w.) attenuated body weight gain and glucose intolerance, improved fasting hyperglycemia and lipid metabolism, slowing the progression of liver damage promoted by HF/HS diet. These effects were associated to inhibition of digestive gut enzymes, thus decreasing the glucolipotoxicity and improving insulin resistance and oxidative stress in liver. Further studies are needed to elucidate which mechanisms are associated to CPE effects on glucose and lipid metabolism, including hepatic insulin sensitivity. However, these findings suggest that cupuassu is an attractive fruit with valuable health benefits that could contribute to slow the progression of metabolic changes associated with obesity and diabetes development.

ACKNOWLEDGEMENTS

We thank *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq 162218/2011-7) and *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP 10/52192-2) for financial support.

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Table 1. Content of phenolic compounds in cupuassu phenolic extract (CPE)^a.

	ml of CPE
Flavanols (µg EE)	
Epicatechin	17.57 ± 0.58
Procyanidin B2	10.94 ± 0.30
Flavones (µg AE)	
Hypolaetin 8-O-β-D-glucuronide	16.02 ± 0.55
Hypolaetin 3'-methyl ether 8-O-β-D-glucuronide	7.73 ± 0.24
Isoscutellarein 8-O-β-D-glucuronide	14.14 ± 1.14
Hypolaetin 8-O-β-D-glucuronide-3''-O-sulfate	5.17 ± 0.29
Isoscutellarein 8-O-β-D-glucuronide 3''-O-sulfate	10.82 ± 0.38
Hypolaetin 3'-methyl ether 8-O-β-D-glucuronide 3''-O-sulfate	10.14 ± 0.30
Total polyphenol contents (mg CE)	0.9

^aData are presented as means ± SEM (n= 3). CPE = Cupuassu phenolic extract, EE = Epicatechin equivalents, AE= Apigenin equivalents, CE = Catechin equivalents.

Table 2. Food intake and tissue weights of mice fed a standard diet (SD) or a high fat/ high sucrose diet (HF/HS) receiving or not cupuassu phenolic extracts by gavage for 8 weeks^a.

	SD	HF/HS	HF/HS + CPE 0.5	HF/HS + CPE 1
<i>Intake</i>				
Total Kcal intake (56 days/mice)	787 ± 42 a	737 ± 26 a	691 ± 31 a	658 ± 45 a
Feed efficiency	0.38 ± 0.07 a	0.94 ± 0.04 b	1.02 ± 0.07 b	0.70 ± 0.10 ab
<i>Tissue weights</i>				
Liver	1.15 ± 0.04 a	1.24 ± 0.04 a	1.16 ± 0.05 ab	1.01 ± 0.03 b
Muscle	0.15 ± 0.01 a	0.16 ± 0.01 a	0.16 ± 0.01 a	0.14 ± 0.00 a
Epididymal fat	0.26 ± 0.02 a	0.96 ± 0.06 b	1.10 ± 0.08 b	0.88 ± 0.13 b
Retroperitoneal fat	0.08 ± 0.01 a	0.40 ± 0.02 b	0.43 ± 0.02 b	0.35 ± 0.05 b
Inguinal fat	0.16 ± 0.01 a	0.54 ± 0.05 b	0.62 ± 0.06 b	0.53 ± 0.08 b
Brown adipose tissue	0.08 ± 0.01 a	0.14 ± 0.04 bc	0.18 ± 0.02 b	0.13 ± 0.01 ac
Total white fat (E + R + I)	0.49 ± 0.04 a	1.90 ± 0.11 b	2.15 ± 0.15 b	1.58 ± 0.28 b

^aData are presented as means ± SEM (n= 10). Means within rows with different letters are significantly different (p<0.05). E= Epididymal fat, R= Retroperitoneal fat, I= Inguinal fat.

FIGURE CAPTIONS

Figure 1. *In vitro* inhibitory effects of CPE at different concentrations against α -glucosidase (A) and pancreatic lipase (B) activities. Data are presented as means $\pm$ SEM (n= 4).

Figure 2. Cumulative body weight gain (A) and % weight gain (B) of mice fed a standard diet (SD) or a HF/HS diet receiving or not CPE by gavage for 8 weeks. Values are means $\pm$ SEM (n= 10). *SD vs. HFHS ($p < 0.05$); #SD vs HFHS and CPE 0.5 ($p < 0.05$). Bars with different letters are significantly different ($p < 0.05$).

Figure 3. Effect of CPE supplementation on Glucose metabolism. Weekly fasting glucose levels during the experiment (A). Blood glucose levels during intraperitoneal insulin tolerance test (B). Constant of plasma glucose disappearance (Kitt) after intraperitoneal injection of insulin (C). Blood glucose levels during intraperitoneal glucose tolerance test (D). Area under the curve (AUC) for ipGTT (E). Serum insulin levels during ipGTT (F). Area under the curve (AUC) for insulin (G). QUICKI index (H). n= 10 (A-C, G-H) and n=5 (D-F). Values are means $\pm$ SEM. * SD vs. HFHS ($p < 0.05$); + SD and CPE 1 vs HFHS and CPE 0.5 ($p < 0.05$). Bars with different letters are significantly different ($p < 0.05$).

Figure 4. Effect of CPE supplementation on Lipid profile. Serum triacylglycerol (A), serum total cholesterol (B), serum HDL cholesterol (C), serum LDL cholesterol (D), serum VLDL cholesterol (E) and fecal triacylglycerol (F). Values are means $\pm$ SEM (n= 7-10). Bars with different letters are significantly different ($p < 0.05$).

Figure 5. Lipid homeostasis. Serum triacylglycerol levels (A) and serum FFA levels (B) upon fasting and re-feeding.

Figure 6. Effect of CPE supplementation on hepatic function: Liver weight (A), hepatic triacylglyceride accumulation (B), hepatic malondialdehyde (MDA) levels (C), serum activity of ALT (D) and AST (E); Western blot of liver pAkt . Values are means $\pm$ SEM. n= 10 (A-C) and n=5 (D-E) and n=7 (F). Bars with different letters are significantly different ($p < 0.05$).

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

4.2 EVALUATION OF THE DISTRIBUTION AND METABOLISM OF POLYPHENOLS
DERIVED FROM CUPUASSU (*THEOBROMA GRANDIFLORUM*) IN MICE
GASTROINTESTINAL TRACT BY UPLC-ESI-QTOF.

**HELENA RUDGE DE MORAES BARROS^{1*}, ROCÍO GARCÍA-VILLALBA²,
FRANCISCO A. TOMÁS-BARBERÁN², MARIA INÉS GENOVESE¹**

¹ Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes 580, Bloco 14, 05508-900, São Paulo, SP, Brazil. E-mail adress: genovese@usp.br (M.I. Genovese)

² Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, P.O. Box 164, 30100, Campus de Espinardo, Murcia, Spain. E-mail adress: rgvillalba@cebas.csic.es (R. García-Villalba; fatomas@cebas.csic.es (F.A. Tomás-Barberán)

***Correspondence:** (Tel: 55-11-30911525; Fax: 55-11-38154410; e-mail:
lebarros79@hotmail.com)

ABSTRACT

Cupuassu (*Theobroma grandiflorum* Schum) is a native fruit from northern Brazil, phylogenetically close to cocoa (*Theobroma cacao*), with a great economic and health potential. It possesses a unique flavonoid composition that combines epicatechin-derived proanthocyanidins characteristic of cocoa and glucuronide and sulfate conjugates of the unusual 8-hydroxy-flavones hypolaetin, isoscutellarein and hypolaetin methyl ether. The present experiment was performed to study the fate and metabolism of this combination of flavonoids from cupuassu in the gastrointestinal tract of mice. Fasted male mice C57BL/6 (n=27) were administered by gavage a cupuassu phenolic extract. Tissue samples (stomach, small intestine, cecum, and colon) and feces were collected at different times between 0.5 and 12 h after dosing and then were analyzed by UPLC-ESI-QTOF. Parent compounds were found along the gastro intestinal tract, especially in stomach and small intestine, with residence times around 3 hours that would allow flavonoids to exert their local beneficial effects. Fewer amounts were found in cecum and colon because of their transformation to microbial metabolites by the colonic bacteria. Flavone conjugates were mainly hydrolyzed into their respective aglycones, hypolaetin, isoscutellarein and hypolaethin methyl ether, that were already observed at stomach level. Microbial metabolites of epicatechin (3,4-diHPP-2-ol, 3-HPP-2-ol, 5-(3,4-dihydroxyphenyl)- γ valerolactone and 3,4-dihydroxyphenylvaleric acid) were detected in cecum (max 2-3 h), colon (max 3-4 h) and feces (around 12 h). No extensive metabolism towards smaller phenolic acids was observed in any case. Differences in the qualitative profile of microbial metabolites could be due to interactions of the microbiota with the characteristic mixture of flavonoids present in the cupuassu phenolic extract. These metabolites could also contribute to host health benefits as protection against gastrointestinal disorders and pathogens, modulation of nutrients digestion, intestinal permeability and intestinal immune response.

Keywords: cupuassu; flavone glucuronide; procyanidins; microbial metabolism; gastrointestinal tract; UPLC-ESI-QTOF.

1. Introduction

Cupuassu (*Theobroma grandiflorum* Willd. ex Spreng. K.) Schum. is a tropical fruit widely cultivated in the North of Brazil, with the largest production in Pará, followed by Amazonas, Rondônia and Acre and also in other South American countries, like Colombia, Costa Rica, Ecuador and Venezuela (Lim, 2012). Cupuassu, as cocoa, belongs to *Theobroma* genus, and it is one of the most popular fruits on the Amazonian market and second within this genus in terms of economic importance. The fruit pulp, highly appreciated because of its characteristic acidic taste and intense fragrance is widely used to prepare juices, ice cream, sorbet, sweets, jellies and other desserts (Vriesmann, Silveira, & Petkowicz, 2010). The seeds have received special attention because of their potential for being used in a similar way to that of cocoa seeds in chocolate or analog derivative products (Cucaita, Hernández, & Gutiérrez, 2014).

Phytochemical studies have demonstrated that cupuassu pulp and seeds contain potent antioxidant polyphenols including flavones, flavan-3-ols and proanthocyanidins (**Figure 1**) (Yang et al. 2003; Pugliese, Tomás-Barberán, Truchado, & Genovese, 2013). The 8-O- β -D-glucuronides and the corresponding 3''-sulfates of isoscutellarein (5,7,8,4'-tetrahydroxyflavone), hypolaetin (5,7,8,3',4'-pentahydroxyflavone) and hypolaetin 3-methyl ether (5,7,8,4'-tetrahydroxy-3'-methoxy-flavone) were detected in cupuassu seeds, and also in pulp at much lower concentrations. Cupuassu also contains proanthocyanidins mainly derived from epicatechin, similar to other *Theobroma* species, in particular *Theobroma cacao* (Pugliese et al., 2013).

Health beneficial properties have been proposed for cupuassu, being the antioxidant capacity one of the most studied (Santos et al., 2010; Pinent et al., 2015). Antidiabetic potential was also evaluated and cupuassu fruit showed the most potent inhibition of α -amylase activity among sixteen Brazilian native fruits and six commercial frozen pulps (Gonçalves, Lajolo, & Genovese, 2010). The effect of daily intake of cupuassu liquor, prepared from fermented seeds, on oxidative stress and lipid profile of rats with streptozotocin-induced diabetes was recently evaluated, showing that supplementation with cupuassu liquor resulted in reduced plasma triglycerides levels, increased HDL cholesterol levels and improved plasma antioxidant capacity (Oliveira & Genovese, 2013). In addition, chronic intake of a phenolic-rich extract from cupuassu liquor upon metabolic changes associated with a high fat diet decreased lipid peroxidation, increased plasma antioxidant capacity and improved glucose tolerance (Oliveira, Rogero, & Genovese, 2015).

All these beneficial effects have been attributed to the presence of phenolic compounds. However, to promote the biological effects a chemical compound must be bioaccessible in the gastrointestinal tract and further absorbed to achieve the target tissues (Rein et al., 2013). Flavonoids have shown low bioavailability with levels of plasma and tissue metabolites rarely exceeding nanomolar concentrations (Chen, Zheng, Li, & Jiang, 2014; Lewandowska, Szewczyk, Hrabec, Janecka, & Gorlach, 2013). Higher concentrations of parent compounds and their primary metabolites have been reported throughout the gastrointestinal tract, where they can exert their beneficial effects and establish close contact with the intestinal microbiota (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013).

Although extensive metabolism of different classes of flavonoids by the microflora have been reported (Aura et al., 2008; Marín, Miguélez, Villar, & Lombó, 2015; Selma, Espín, & Tomás-Barberán, 2009), little is known about flavones and particularly about those present in cupuassu (hypolaetin and isoscutellarein). Regarding procyanidins, it is known that the metabolites observed in the colon will depend on the matrix being consumed (Monagas et al. 2010; Van Duynhoven et al. 2013). Therefore, although the microbial metabolism of procyanidins has been extensively studied, their distribution and metabolic conversion in a unique matrix such as cupuassu, which is a combination of different type of flavonoids, deserves to be considered.

In this context, this study aimed to evaluate the gastrointestinal distribution and metabolic conversion of polyphenols derived from cupuassu phenolic extract (CPE) in an *in vivo* assay, using C57BL/6 mice. Gastrointestinal bioavailability of cupuassu phenolic compounds may help to understand the role of specific phenolic compounds in health effects. UPLC-ESI-QTOF MS methodology was used to analyze gastrointestinal tract (stomach, small intestine, colon, and cecum) and feces samples.

2. Material and methods

2.1 Chemicals

Phloroglucinol and standards of (+)-catechin, (-)-epicatechin, procyanidin B2, caffeic acid and apigenin were purchased from Sigma-Aldrich (St.Louis, MO, USA). Stock solutions at concentration of 2000 mg/L for each phenolic were first prepared in methanol and then serially diluted to working concentrations. Methanol and acetonitrile were supplied from J. T.

Baker (Deventer, The Netherlands), formic acid, HCl and sodium acetate from Panreac (Barcelona, Spain) and acetic acid from Scharlau (Barcelona, Spain). Ascorbic acid was from Acros Organics (Geel, Belgium). All other chemicals and reagents were of analytical grade. Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout the study.

2.2 Preparation of Cupuassu Phenolic Extract (CPE)

Cupuassu frozen pulp was obtained from *Tentação Amazônica* (Parauapebas, Pará State, Brazil), a Brazilian producer that manufactures fruits of Amazonian region. Commercial cupuassu pulp (100 g fresh weight) was extracted three times in a solvent mixture (200 mL the first time, 100 mL the next two times) comprising methanol/water/acetic acid (70/29.5/0.5, v/v/v) in an Ultraturrax equipment (Polytron-Kinematica GmbH, Kriens-Luzern, Sweden) at 24000 rpm for 1 min. The extract was filtered under reduced pressure through filter paper (Whatman no. 1), and then concentrated until methanol elimination on a rotatory evaporator (Rotavapor RE 120; Büchi, Flawil, Switzerland) at 40 °C. The aqueous phase was filtered through a reverse phase C-18 cartridge (Phenomenex, Torrance, USA), previously activated with methanol and water, in order to retain phenolic compounds and remove other highly hydrophilic compounds. The phenolic compounds retained in each cartridge were eluted with methanol. The collected methanolic fraction was evaporated to dryness and then dissolved in distilled water (50 mL). Ten mL of the extract were used for determination of phenolic contents by Folin-Ciocalteu method and for the *in vivo* study. The other 40 mL of the extract were then freeze-dried for posterior extract characterization analysis. The yield of cupuassu phenolic extract (40 mL) was 20 mg dried weight.

2.3 Total Phenolic Contents

The phenolic content was determined according to previous method (Singleton, Orthofer, & Lamuela-Raventos, 1999). A 0.25 mL aliquot of CPE was mixed with 0.25 mL of the Folin-Ciocalteu reagent and 2 mL of distilled water. After 3 min at room temperature, 0.25 mL of a saturated sodium carbonate solution was added and the mixture placed at 37 °C in a water bath for 30 min. The absorbance was measured at 750 nm using a model Ultrospec 2000 UV-visible spectrophotometer (Amersham Biosciences, Cambridge, UK). The results were expressed as milligrams of catechin equivalents (CE) per mL of extract. The total amount of polyphenols measured by the Folin Ciocalteu (0.45 mg catechin equivalents

(CE)/mL of the extract) was used to calculate the dose to each mouse, considering the body weight of the animals.

2.4 Characterization of cupuassu phenolic extract by HPLC-DAD-IT (MS/MS)

2.4.1 Analysis of phenolic compounds by HPLC-DAD-IT (MS/MS)

The dried CPE (10 mg) was res-suspended in 1 mL of methanol: water (80:20, v/v) with 0.1% formic acid and after vortexing (1 min) and standing in an ultrasonic bath for 10 min. The solution was centrifuged at 13000 g for 10 min and the supernatant filtered through a 0.22 μ m PVDF filter. Samples were analyzed using an Agilent HPLC 1200 series instrument equipped with a diode array detector (Agilent Technologies, Waldbronn, Germany) and an ion-trap mass spectrometer detector in series (Bruker Daltonics, Bremen, Germany). Separation of phenolic compounds was achieved on a reverse-phase C18 Pursuit XRs column (Agilent Technologies, Aldbronn, Germany) (250 $\times$ 4 mm; 5 μ m particle size). The mobile phase was water/formic acid (99:1, v/v) (solvent A) and HPLC grade acetonitrile (solvent B), at flow rate of 0.8 mL/min. Elution was performed with a gradient starting with 5% B in A, to reach 12% B in A at 10 min, 25% B in A at 20 min, 35% B in A at 30 min, 70% B in A at 35 min, 90% B in A at 36 min and then became isocratic for 5 min. UV chromatograms were recorded at 280 and 340 nm. Nitrogen was used as drying and nebulising gas in the electrospray interface (ESI) with pressure at 65 psi, flow 11 L/min and temperature of 350°C. Mass scan (MS) and daughter (MS– MS) spectra were measured from m/z 100 up to m/z 800. Collision induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative mode. Clovamide was quantified with the calibration curve of caffeic acid (1–1000 mg/L) at 280 nm and flavones (hypolaetin and isoscutellarein derivatives) with the calibration curve of apigenin (1–500 mg/L) at 340 nm. All the samples and standards were injected in triplicate.

2.4.2 Analysis of proanthocyanidins by phloroglucinolysis

Proanthocyanidins were quantified as previously reported by Kennedy & Jones (2001) with modifications proposed by Pugliese et al. (2013). Briefly, a solution of 0.1 N HCl in methanol containing 50 g/L of phloroglucinol and 10 g/L of ascorbic acid was prepared. Nine mg of dried cupuassu phenolic extract were reacted with the phloroglucinolysis reagent (150 μ L) in a water bath for 20 min at 50 °C. The reaction was stopped by placing the vials in ice and adding 150 μ L of 40 mM sodium acetate. The sample was centrifuged and filtered with a

0.22 μ m PVDF filter prior to the injection. For the analysis of these samples, the same HPLC–DAD–IT equipment described with the same instrumental parameters previously reported by Pugliese et al. (2013) were used. For the quantitation of total flavan-3-ols (flavan-3-ol monomers and phloroglucynol adducts) catechin was used as quantitative standard (1–2000 mg/L). To calculate the apparent mean degree of polymerization (mDP), the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, in moles) was divided by the sum of all flavan-3-ol monomers (in moles).

2.5 Animals and experimental design

The study was approved by the Ethics Committee on Animal Experimentation of the Faculty of Pharmaceutical Sciences, University of São Paulo, according to the guidelines of the Brazilian College on Animal Experimentation (protocol number 353). Twenty-seven male C57BL/6 mice (8 weeks old, 22–28 g body weight) were fed Nuvilab CR-1 diets (Nuvital Nutrientes SA, Paraná, Brazil) and water *ad libitum* and were kept under standard laboratory conditions of temperature (23 ± 2 °C), relative humidity ($50 \pm 5\%$) and 12 h light-dark cycle. For the study, mice were later kept under fasting conditions for 6 h prior to aqueous CPE administration by gavage, containing 5 mg of catechin equivalents/kg of body weight (b.w.). Blood and tissue samples (stomach, intestine, colon and cecum) were collected at 0, 0.5, 1, 2, 3, 4, 6, 12 and 24 h after dosing (3 mice per time). The plasma samples were obtained by centrifugation (2,000 g for 10 min at 4 °C) and then stored at -80 °C. The tissues were weighted, immediately frozen under liquid nitrogen and stored at -80 °C. Feces were collected at 0, 3, 4, 6 and 12 h after dosing and stored at -80 °C. All the samples (plasma, tissues and feces) were then freeze-dried for posterior phenolic extraction and chromatographic analysis.

2.5.1 Sample preparation

Lyophilized samples of stomach, intestine, colon, cecum and feces were weighted (between 20 and 100 mg) and extracted with methanol: water (80:20, v/v) plus 0.1% formic acid (at least 1 mL of extraction solvent per 50 mg of tissue sample). The mixture was vortexed for 2 min followed by ultrasonic bath for 10 min and then centrifuged at 3000 g for 10 min. The supernatant was filtered (0.22 μ m PVDF filter) prior to the analysis.

Lyophilized samples of serum were reconstituted with 300 μ L of milliQ water. 200 μ L of the reconstituted plasma was mixture with 600 μ L of acetonitrile: formic acid (99.8:0.2, v/v) by vortexing for 2 minutes followed by ultrasonic bath for 10 min to precipitate proteins.

The mixture was centrifuged at 10000 *g* for 10 min and the supernatant was reduced to dryness in a speed vacuum concentrator. The dried samples were res-suspended in 100 μ L of methanol and filtered (0.22 μ m PVDF filter) prior to analysis.

2.6. UPLC-ESI-QTOF analysis of serum and tissue samples

Tissue samples and feces were analyzed using an Agilent 1290 Infinity UPLC system coupled to the 6550 Accurate-Mass quadrupole time-of-flight mass spectrometer (QTOF) (Agilent Technologies, Waldbronn, Germany) using an electrospray interface with Jet Stream technology. Separation was achieved on a reverse phase Poroshell 120 EC-C18 column (3 $\times$ 100 mm, 2.7 μ m; Agilent) operating at 30 °C. The mobile phases were water: formic acid (99.9:0.1 v/v; phase A) and ACN: formic acid (99.9:0.1 v/v; phase B). Gradient program was as follows: 0–4 min, 5–18% B; 4–15 min, 18–30% B; 15–20 min, 30–90% B, 1 min at 90% and then back to the initial conditions in 1 minute and the column was equilibrated during 5 minutes. The flow rate was set constant at 0.4 mL/min and the injection volume was 3 μ L. The optimal conditions of the electrospray interface were as follows: gas temperature 280 °C, drying gas 9 L/min, nebulizer 45 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min. Spectra were acquired in single MS mode with *m/z* range of 100–1100, negative polarity, and an acquisition rate of 1.5 spectra/s. Continuous internal calibration was performed during analyses using two reference masses (112.9855 and 1033.9881). A target screening strategy was applied to all the samples for the identification of possible derived metabolites. This strategy consists of searching for a list of potential biotransformation products after MS full acquisition to generate a series of extracted ion chromatograms (EICs). Screening was based on mass filtering at the exact mass of the compound investigated using narrow mass extraction windows (0.01 *m/z*). This list was created taking into account the original compounds present in the extracts and the information available in literature about the microbial metabolism of flavonoids (Liang et al., 2014; Marín, Miguélez, Villar, & Lombó, 2015; Monagas et al., 2010; Stanoeva & Stefova, 2013). A theoretical metabolic pathway was proposed for the flavones (hypolaetin and isoscutellarein) and flavan-3-ols (epicatechin) (Supplementary Figure 1 and 2). All the metabolites proposed in these routes along with hydroxylated, methylated, glucuronidated and sulfated conjugates were searched in the targeted analysis. These EICs were compared between control and treated samples to eliminate those chromatographic peaks that also appeared in the control samples.

3. Results

3.1 Analysis of Cupuassu Phenolic Extract (CPE)

Identification and peak assignment of the native phenolic compounds present in CPE were based on comparison of their retention times and UV and mass spectral data with those of standards and published data (Pugliese, Tomás-Barberán, Truchado, & Genovese, 2013; Yang et al., 2003). In total, 7 compounds were detected by HPLC-DAD-ESI-MS/MS chromatogram (**Figure 2**) and their characteristics and content in CPE are shown in **Table 1**. As described by Pugliese et al. (2013), glucuronide and sulfo-glucuronide derivatives of hypolaetin (peaks 2 and 5), hypolaethin-methyl ether (peaks 4 and 7) and isoscutellarein (peaks 3 and 6) were identified with maxima of absorption around 340 nm and the same fragmentation profiles previously reported for these compounds (**Table 1**). Total amount of flavones was 87.48 ± 0.38 mg/g d.w., being hypolaetin 8-O- β -D glucuronide and isoscutellarein 8-O- β -D glucuronide 3''-sulfate the predominant ones. Hypolaetin and isoscutellarein are not common dietary flavones and have only been previously found as different glycosidic conjugates, in different plants (*Sideritis* spp. *Gratiola* spp. and the liverwort *Bucegia*) (Tomás-Barberán, Mañez, & Villar, 1987; Grayer-Barkmeijer & Tomás-Barberán, 1993). *Sideritis* species (Lamiaceae) are widely used in Mediterranean countries to prepare beverages and tisanes with digestive and anti-inflammatory properties (Tomás-Barberán, Mañez, & Villar, 1987; Stanoeva & Stefova, 2013).

Clovamide (Peak 1, λ max = 280 nm), a polyphenolic amide previously reported in cocoa (Arlorio et al., 2008), was identified for the first time in cupuassu. The presence of the parent ion at m/z 358 and the same daughter ions at m/z 222, 178, 161 and 133, previously reported in literature (Arlorio et al., 2008), confirmed its identification. Clovamide was present at concentration of 23.03 ± 0.25 mg/g d.w.

In addition, peaks of epicatechin and procyanidin B2 were detected but only in MS. More accurate quantitation of proanthocyanidins was done using phloroglucynolysis (**Table 2**). Epicatechin and catechin were present as terminal units in the proanthocyanidin oligomers but epicatechin was 13 times more abundant than catechin. As previously reported (Pugliese, Tomás-Barberán, Truchado, & Genovese, 2013), the extension units were only epicatechin. The total content was 63 mg epicatechin equivalents/g of CPE d.w., and the mean degree of polymerization was 2.

3.2 Identification of parent compounds and metabolites in tissues and feces

The list of parent compounds (P) and metabolites (M) detected in the gastro intestinal tissues and feces is presented in **Table 3**. A total of 20 compounds were tentatively identified taking into account the molecular formula generated with high scores (≥ 90) and low errors (≤ 2.5 ppm). In addition, information on the fragmentation pattern was very useful to support the identification. The original compounds characterized in the extract were detected in most of the gastrointestinal tract samples. As can be observed in **Table 3**, the major fragment ion of procyanidin B2 (**P1**) was m/z 289 corresponding to the monomer epicatechin. Epicatechin (**P2**) and clovamide (**P3**) showed characteristic fragmentation patterns in agreement with those found in literature (Arlorio et al., 2008; Cádiz-Gurrea et al., 2014). The MS² of glucuronides (**P4**, **P6**, **P7**) showed fragment ions at m/z 175 and others corresponding to the aglycones (m/z 301, 315 or 285). Sulfoglucuronide derivatives (**P5**, **P8**, **P9**) showed fragments at [M-80] and [M-80-176] indicating the sequential loss of the sulfate and glucuronide to lead to the aglycone molecule. The sulfoglucuronide fragment at m/z 255 was also observed.

Flavone glucuronide and sulfoglucuronide conjugates (**P4-P9**) detected in the gastrointestinal samples showed the same retention time that those found in the extract, indicating that the same ingested compounds were accumulated along the gastrointestinal tract and no transformation to other isomers was produced. Apart from the glucuronide and sulfoglucuronide conjugates present in the extract, we specifically searched for phase I and phase II conjugates (glucuronide, sulfate, sulfoglucuronide, methyl-derivatives and hydroxylated derivatives) of the original compounds: procyanidin B2, epicatechin and clovamide. None of these metabolites were observed along the gastrointestinal tract.

The highest metabolic activity was observed in cecum and colon populated by a large number of bacteria. A theoretical metabolic pathway was proposed for the flavones hypolaetin and isoscutellarein (**Supplementary Figure 1**). After an exhaustive search of all the proposed metabolites and their conjugated, only flavone aglycones (**M9**, **M10**, **M11**) characterized by a fragment ion at [M-168] were identified along the gastrointestinal tract with high intensity in the colon.

Regarding flavan-3-ol and procyanidins, the targeted analysis was focused on looking for metabolites previously described in literature (Monagas et al., 2010; Liang et al., 2014) (**Supplementary Figure 2**). Microbial metabolites derived from epicatechin were detected in cecum, colon and feces. Metabolite **M3**, with m/z 291.0874 (molecular formula C₁₅H₁₆O₆) and characteristic fragments at m/z 247, 205, 167, 151, 135 and 123, was identified as 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (3,4-diHPP-2-ol); and metabolite

M8, with m/z 275.0925 and a molecular formula with one oxygen less than the previous compound, was identified as 1-(3-hydroxyphenyl)-3-(2, 4, 6-trihydroxyphenyl) propan-2-ol (3-HHP-2 ol). Metabolite **M5**, with m/z 207.0282 (molecular formula $C_{11}H_{12}O_4$) and characteristic fragments at m/z 175, 163, 143 and 122, was identified as 5-(3,4-dihydroxyphenyl valerolactone). Traces level of a metabolite **M7** with molecular formula $C_{11}H_{14}O_4$ was tentatively identified as 3,4-dihydroxyphenylvaleric acid. These compounds were previously identified as (epi)catechin catabolic metabolites and the MS/MS fragments were in agreement with those previously reported (Appeldoorn, Vincken, Aura, Hollman, & Gruppen, 2009; Liang et al., 2014; Kutschera, Engst, Blaut, & Braune, 2011). According to the molecular formula and fragmentation pattern, metabolite **M4** was tentatively identified as a sulfate derivative of 5-(3-hydroxyphenyl)- γ -valerolactone.

Other metabolites were detected in cecum, colon and feces samples after the administration of the CPE, although precise identification was not achieved. **M2** and **M6** with molecular formula $C_{17}H_{18}O_6$ and $C_{17}H_{18}O_7$ and fragmentation pattern similar to those found for 3,4-diHPP-2-ol (m/z 151, 135, 123 and 109) could be considered derivatives of epicatechin ($C_{15}H_{14}O_6$) with two extra methyl groups in **M2** and an additional hydroxyl group in **M6**. However, both compounds could be also considered degradation products of flavones originated by hydrogenation, C-ring opening and subsequent demethylation. Proposed metabolite for the molecular formula $C_{17}H_{18}O_7$ is showed in **Supplementary Figure 1**. More knowledge about the identity of these unknown compounds and the position of the extra-groups could not be achieved with the information available. In addition, a sulfate derivative **M1** of the metabolite **M2** was also proposed with molecular formula $C_{17}H_{18}O_9S$ and fragment at m/z 315 [M-82].

Figure 3 illustrates EIC chromatogram of the main parent compounds and metabolites detected in stomach and colon respectively, at different times after the administration of the CPE.

3.3 Distribution of parent compounds and metabolites in the GI along the time

Due to the absence of available standards, the distribution was studied using area under the curve of the different compounds. Peak areas obtained using Extracted Ion Chromatograms (EIC) was corrected with the ratio milligrams of tissue (dry weight)/quantity of solvent used during the extraction of each sample. Although quantitative comparison between compounds was not possible, using corrected area enables to plot the evolution of each compound along the different tissues of the gastrointestinal tract. The distribution of the

parent compounds along the stomach, small intestine, cecum, colon and feces is shown in **Figure 4**. Despite the high variability of the results, a clear tendency could be observed.

Results showed that clovamide decreased in the gastric lumen over time reaching maxima around 0.5 h in stomach, 1 h in small intestine, 2 h in cecum and 3 h in colon (**Figure 4a**). Similar behavior was observed for epicatechin and proanthocyanidin (**Figure 4b and 4c**) although in both cases the amount increased in the small intestine between 0.5 and 2 hours, probably because of the partial hydrolysis of proanthocyanidin oligomers to dimers and monomers. Despite the controversial, hydrolysis of procyanidin polymers in the stomach to facilitate their absorption in the small intestine has been previously reported (Spencer, 2003; Margalef, Pons, Bravo, Muguerza, & Arola-Arnal, 2015). The higher amounts of these three parent compounds were detected in stomach and small intestine, and then decreased along the gastrointestinal tract. This could be due to their absorption at intestinal level and possible metabolism to glucuronide and sulfate conjugates or their transformation to microbial metabolites by the bacteria present in the colon.

Regarding flavones, evolution along the GI tract of the flavones present in the extract, hypolaetin glucuronide and hypolaetin glucuronide-sulfate was showed in **Figures 4d and 4e**, respectively. In addition, the distribution of the aglycone hypolaetin, generated in different parts of the GI tract was also considered (**Figure 4f**). Similar behavior was observed for aglycones and conjugates of the other two flavones (hypolaetin methyl ether and isoscutellarein). Glucuronide derivatives were found in stomach and small intestine (**Figure 4e**) with a residence time of around 2-3 hours. Their absence from cecum and colon could indicate absorption at intestinal level to the blood stream or hydrolysis to generate the aglycones. In the case of sulfo-glucuronides, large amounts were detected in cecum 3 h after supplementation. As indicated above, in all samples glucuronide and sulfoglucuronide conjugates were detected with identical retention times to those detected in the extracts. As can be observed in **Figure 4f**, aglycones that were not originally present in the extracts were first detected in the stomach. This suggests partial hydrolysis of the flavone conjugates at this level by microbial populations that has been described to be higher in the stomach of rodents compared to humans (Day et al., 2000). However, aglycones just appeared in one of the three mice analyzed at the different times, showing a high variability. More homogeneous results were observed in cecum and colon with the highest concentration of aglycones at 2 and 4 hours, respectively, showing the role of microbiota in the transformation of flavone conjugates to their aglycones.

All the rest of microbial metabolites were detected with high intensity in cecum and colon (**Supplementary Figure 3**). Flavan-3-ol C-ring fission products (**M3**, **M5** and **M8**) appeared 2 h after the ingestion and remained until 6 h with maxima around 2–3 h in cecum and 3–4 h in colon. Unknown metabolite **M2** also remained in cecum and colon between 3 and 6 h, and metabolite **M6** appeared in highest concentration at 3 h after the supplementation. Metabolites **M1** and **M4** identified as sulfated conjugates were also detected in small intestine, suggesting that their corresponding aglycones were probably absorbed into the enterocytes where it would undergo extensive metabolism and then return into the gut lumen by efflux (Chen, Zheng, Li, & Jiang, 2014). Small amounts of all microbial metabolites appeared in fecal samples at 12 hours after supplementation.

4. Discussion

Nowadays, it is known that health benefits derived from polyphenol consumption depend on their bioaccessibility in the gastrointestinal tract and the close contact with the gut microbiota (Cardona et al., 2013; Selma et al., 2009). Therefore, the gastrointestinal tract was chosen as the primary target to evaluate the gut bioavailability of polyphenols from CPE. Cupuassu (*Theobroma grandiflorum*), has emerged as another Theobromaceae fruit phylogenetically close to cocoa with high agroeconomic and health potential. Despite their phylogenetic proximity, cocoa and cupuassu show differences in their phenolic composition. Both are known to have high levels of flavan-3-ols and procyanidins (Cucaita et al., 2014; Oliveira & Genovese, 2013) but cupuassu present also a mixture of glucuronide and sulfo-glucuronide derivatives of the flavones hypolaetin, isoscutellarein and hypolaetin methyl ether that are absent in cocoa. Gastrointestinal distribution and microbial metabolic conversion of this unique combination of flavonoids present in cupuassu: flavan-3-ols, procyanidins and 8-hydroxy-flavones was studied. Mice were fed a single acute dose of a CPE with a mean amount of 5 mg of catechin equivalents/ Kg b.w., which is equivalent to 350 mg of polyphenols for a 70-kg person per day or about 4 glasses of juice made with commercial frozen pulp. This intake level of polyphenols is close to those derived from the consumption of cocoa products in some European countries (Zanotti et al., 2015) and is easily reached through diet.

All parent compounds present in CPE (procyanidin B2, clovamide, epicatechin and glucuronide and sulfo-glucuronide derivatives of hypolaetin, isoscutellarein and hypolaetin

methyl ether) were detected in stomach and small intestine (**Figure 4**). Smaller amounts were found in cecum and colon, except for sulfo-glucuronide derivatives that were present at higher concentrations in cecum, probably because of the enterohepatic circulation (Dai et al 2015). The accumulation of these compounds during some hours in the tissues could exert local effects that would contribute to their health benefits. In particular, their presence in small intestine could interfere with key signaling and metabolic pathways with potential benefits to the host (García-Conesa, 2015). The inhibition of gut digestive enzyme activities, as well the modulation of receptors and nutrient transporters, have been described as adjuvants in the treatment against the alterations in glucose and lipids homeostasis (Williamson, 2013; García-Conesa, 2015). Previously, a cupuassu extract showed potent inhibition of α -amylase activity (Gonçalves et al., 2010) and some flavones also showed strong inhibitory activity of α -glucosidase (Xiao, 2015), while flavan-3-ols apparently have stronger lipase inhibitory activity compared to other classes of polyphenols (Gonzales et al., 2015). In addition, flavan-3-ols have also the potential to modulate incretins as glucagon-like peptide-1 (GLP-1) and dipeptidyl-peptidase 4 (DPP4) activity *in vitro* (González-Albuín et al., 2014), and reduce sugar absorption through the inhibition of the main transporters of glucose present in the intestinal epithelial cells (Williamson, 2013). Therefore, previous beneficial effects attributed to the presence of phenolic compounds in cupuassu (Oliveira & Genovese, 2014; Oliveira et al., 2015), as the improvement of glucose tolerance and dyslipidemia, could be partly attributed to the modulation of these described metabolic pathways. However, further studies are necessary to confirm this hypothesis *in vivo*.

The decrease in cecum and colon of the CPE compounds could be due to their transformation to microbial metabolites by the colonic bacteria. As has been widely reported, most of the procyanidins and flavonoids consumed in the diet are unabsorbed and reach the colon where they are transformed by the resident microbiota (Selma, Espín, & Tomás-Barberán, 2009; Marín, et al., 2015). Knowledge of the formation and identity these metabolites is important as they could contribute to the potential health effects of the consumed food products (Celep, Rastmanesh, & Marotta, 2014).

A targeted metabolic analysis was developed looking for all the possible microbial metabolites derived from flavones (hypolaetin and isoscutellarein) and flavan-3-ols (epicatechin) (See **supplementary Figure 1 and 2**). Comparing to other flavonoids, there are few studies considering microbial metabolism of flavones. In particular, information about this group of 5,7,8-OH flavones present in CPE is scarce. In the same way as with other flavonoid sub-classes, deglycosilation is the first microbial action. *In vitro* studies suggested

the transformation of apigenin and luteolin aglycones into naringenin and eriodictyol respectively, as intermediate metabolites, which were further transformed to hydroxyphenyl propionic acids (Schoefer, Mohan, Schwiertz, Braune, & Blaut, 2003). Consistent with *in vitro* data, 3-(4'-hydroxyphenyl) propionic acid was the main metabolite detected in urine after the administration of apigenin-7-O-glucoside to rats (Hanske, Loh, Sczesny, Blaut, & Braune, 2009). Based on these results and information about microbial transformation of other families of flavonoids, a metabolic pathway was proposed for the flavones described in CPE (**Supplementary Figure 1**). After an exhaustive search, only the aglycones (hypolaetin - **M9**, isoscutellarein - **M10** and hypolaetin methylether - **M11**) were identified with high intensity in colon. No extensive metabolism to other smaller phenolic acids was found, showing poor colonic fermentation of this group of flavones. This was in accordance with data previously published for the metabolism of scutellarin (scutellarein-7-O- β -D glucuronide), the main active compound from *Erigeron breviscapus* extract, which was hydrolyzed into scutellarein via β -glucuronidase from the gastrointestinal bacteria (Tang et al., 2014; Wang, Xia, Liu, Qiu, & Di, 2014). The urinary excretion of polyphenols described by Stanoeva & Stefova (2013) after ingestion of a cup of mountain tea (*Sideritis scardia*), in which the dominant flavonoids are glucosides of hypolaetin and isoscutellarein, also support our results. The most abundant metabolites were glucuronide derivatives of flavones, obtained after absorption and glucuronidation of the aglycones that were hydrolyzed from the parent compounds in the intestinal tract.

The same metabolic behavior was observed for baicalein and wogonoside, flavones with similar A-ring structure as those from cupuassu, that are present as glucuronide conjugates in the rhizome of *Scutellaria baicalensis*. These compounds are poorly absorbed from the gastrointestinal tract in its native form and are hydrolyzed by intestinal microflora to their aglycones in human and rats (Kang et al. 2014; Xing, Wang, Peng, Chen, & Li, 2014). Reductions of the double bond, methylations and hydroxylations have been also proposed in the metabolic pathways of the baicalein (Zhang et al. 2015; Du et al. 2015). However, as occurred with the flavones in CPE, degradation into low molecular weight phenolic acids through the cleavage of the C-ring has not been described. These flavones have in common the presence of three hydroxyl groups in the A-ring of their molecules that somehow could affect the fission of the heterocyclic C-ring. Further researches would be needed to test this hypothesis. Two unknown metabolites detected in cecum, colon and feces could be tentatively identified as dihydrochalcones derived from hypolaetin and isoscutellarein. However, more solid evidence would be necessary to support this identification.

Regarding monomeric flavan-3-ols and procyanidins, their microbial degradation pathway has been partially elucidated by numerous *in vitro* and *in vivo* studies using pure standards as well as grapes, tea, cocoa, berries, red wines and their extracts (Liang et al., 2014; Monagas et al. 2010; Van Duynhoven et al. 2013). Based on the literature, a colonic pathway was proposed in **Supplementary Figure 2**. The main metabolites identified in cecum and colon of mice after consumption of CPE were 3,4-diHPP-2-ol (**M3**), obtained by reductive cleavage of the (epi)catechin C-ring, 3-HPP-2-ol (**M8**), 5-(3,4-dihydroxyphenyl)- γ -valerolactone (**M5**), and 3,4-dihydroxyphenylvaleric acid (**M7**). This qualitative profile was slightly different from those found after the intake of other procyanidin-rich foods (Margalef et al., 2015; Goodrich et al. 2014).

The characteristic metabolites of flavan-3-ols consumption, hydroxyphenyl valeric acid and valerolactone derivatives, were previously identified, but in other matrixes, and the colonic degradation reached low molecular weight phenolic acids such as hydroxyphenyl acetic, hydroxyphenylpropionic and hydroxybenzoic acids. Among the microbial phenolic acids identified after cocoa consumption, product with similar procyanidin pattern than cupuassu, 3,4-dihydroxyphenyl propionic acid, 3-hydroxyphenyl acetic acid and phenylvalerolactones (5-(3',4'-dihydroxyphenyl)- γ -valerolactone and 5-(3-methoxy-4-hydroxyphenyl)- γ -valerolactone were found in rat urine (Urpi-Sarda, et al., 2009) and mainly hydroxybenzoic acids were quantified in mice gastrointestinal tissues (Goodrich et al., 2014). These metabolites described previously in literature with lower molecular weight were not found after CPE consumption. In contrast, diarylpropan-2-ol metabolites, the first intermediate compounds formed after the fission of the heterocyclic C-ring, were detected with high intensity. These metabolites have been previously described *in vitro* after incubation with human and rat intestinal bacteria (Takagaki & Nanjo, 2010; Kutschera, Engst, Blaut, & Braune, 2011) but only two studies described their presence *in vivo* after consumption of epicatechin gallate or catechin by rats (Takagaki & Nanjo, 2010; Liang et al 2014). Their absence in other studies was attributed to their fast conversion to other metabolites. It seems that a slower microbial metabolism has been produced in the presence of CPE compared with other similar matrixes as cocoa products. This variation in microbial metabolism could be resulted from qualitative differences in the profile of native flavan-3-ols or because of the presence in the extract of other polyphenols as flavones that could interfere in flavan-3-ols microbial metabolism. In addition, the two-way interaction between microbiota and polyphenols has been recently study indicating the modulatory capacity of these bioactive compounds on gut microbiota composition (Cardona et al., 2013; Marín et al., 2015).

The presence of sulfate metabolite (**M1** and **M4**) in the small intestine, cecum and colon suggests that part of the metabolites originated in the gut could be absorbed conjugated and then back into the small intestine by enterohepatic circulation as occurred with other flavonoids (Dai et al 2015).

Native flavonoids and their microbial metabolites are retained for a period of time (around 3 hours) in the colonic mucosa where they can exert their beneficial effects including anti-inflammatory and anticarcinogenic activity, improved barrier function, reduced permeability to lipopolysaccharide and modulations of the gut microflora populations through selective prebiotic effects and antimicrobial activities (Celep et al., 2014; Marín et al., 2015). Flavan-3-ol monomers and proanthocyanidins may favor beneficial bacteria such as *Bifidobacterium*, *Enterococcus*, *Lactobacillus* spp. as well increase butyrate production; but may also inhibit other groups such *Clostridium* spp. and *Staphylococcus* genera (Cardona et al., 2013; Tzounis et al., 2011). No studies about hypolaetin and isoscutellarein on microbiota modulation were found. However, another flavone, luteolin, showed antibacterial effects against *B. subtilis*, *S. aureus*, *P. fluorescens* and *E. coli*. (Marín et al., 2015).

5. Conclusions

Gastrointestinal distribution and microbial metabolic conversion of a unique combination of flavonoids (flavan-3-ols, procyanidins and flavones) present in cupuassu phenolic extract (CPE) was studied. Original compounds were accumulated in the different gastrointestinal tissues, especially in stomach and small intestine where they could exert local effects that would explain the beneficial effects observed in previous studies. The decrease in cecum and colon of the CPE compounds could be due to their transformation to microbial metabolites by the colonic bacteria. The qualitative profile of microbial metabolites of flavones and procyanidins was showed. Flavones present as glucuronides were mainly hydrolyzed into their respective aglycones, hypolaetin, isoscutellarein and hypolaethin methylether. No extensive metabolism to other smaller phenolic acids was found, showing poor colonic fermentation of this group of flavones. Microbial metabolism of procyanidins from CPE was different compared with other similar matrixes as cocoa products. They were mainly transformed to diarylpropan-2-ol metabolites, the first intermediate metabolites formed after the fission of the heterocyclic C-ring, 5-(3,4-dihydroxyphenyl)- γ -valerolactone and 3,4-dihydroxyphenylvaleric acid and. No further metabolism to smaller phenolic acids was

observed. The qualitative profile of microbial metabolites could be affected by the presence in the matrix of different polyphenols that could modulate the gut microbiota. In addition to systemic activities, native flavonoids and their microbial metabolites are thought to act in the colon. Further studies with higher dose or a longer treatment may provide a better understanding of the metabolism of cupuassu extract.

ACKNOWLEDGEMENTS

We thank *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq 162218/2011-7) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES 99999.003759/2014-08) for financial support.

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Table 1. HPLC-DAD-IT analysis and content (mg/g d.w.) of flavone glucuronides and clovamide in cupuassu phenolic extract (CPE).

No	Compound	tr (min)	[M-H] ⁻	MS/MS fragments	Content* (mg/g d.w.)
1	Clovamide	20.01	358	222, 178, 161	23.03 ± 0.25
2	Hypolaetin 8-O-β-D glucuronide	25.93	477	301	22.13 ± 0.07
3	Isoscutellarein 8-O-β-D glucuronide	28.25	461	285	10.63 ± 0.03
4	Hypolaetin 3'-methyl ether 8-O-β-D glucuronide	28.47	491	315, 301	7.81 ± 0.02
5	Hypolaetin 8-O-β-D glucuronide 3''-sulfate	36.21	557	447, 301, 255	9.07 ± 0.03
6	Isoscutellarein 8-O-β-D glucuronide 3''-sulfate	43.59	541	461, 285, 255	23.06 ± 0.04
7	Hypolaetin 3'-methyl ether 8-O-β-D glucuronide 3''-sulfate	44.30	571	491, 315, 255	14.76 ± 0.25

* Data are presented as means (n=3) and standard deviation.

Table 2. Quantification of procyanidins in cupuassu phenolic extract (CPE) by phloroglucinolysis.

Compound	tr (min)	[M-H]⁻	mg/g d.w.
Epicatechin adduct (extension unit)	8.23	413	31.23
Epicatechin (terminal unit)	14.99	289	29.17
Total Epicatechin			60.40
Catechin (terminal unit)	11.12	289	2.20
Total flavan-3-ols			62.61
mDP*			2.00

*mDP= mean degree of polymerization

Table 3. Parent compounds and main metabolites identified along the gastrointestinal tract after the intake of cupuassu phenolic extract (CPE).

No	Compounds	Retention time (min)	Exact mass	MS/MS fragments	Score	Error	Molecular Formula	Samples ^{a)}				
PARENT COMPOUNDS												
P1	Procyanidin B2	4.95	577.1351	289, 245, 125	92.21	0.27	C ₃₀ H ₂₆ O ₁₂	ST,	SI,	CE,	C*	
P2	Epicatechin	5.42	289.0718	245, 203, 137, 125, 109	99.91	0.19	C ₁₅ H ₁₄ O ₆	ST,	SI,	CE,	C	
P3	Clovamide	6.08	358.0932	178, 161, 135	99.21	0.75	C ₁₈ H ₁₇ NO ₇	ST,	SI,	CE,	C	
P4	Hypolaetin 8-O-β-D glucuronide	9.05	477.0675	301,175	94.70	0.40	C ₂₁ H ₁₈ O ₁₃	ST,	SI,			
P5	Hypolaetin 8-O-β-D glucuronide 3''-sulfate	10.48	557.0243	477, 301, 255	90.84	0.53	C ₂₁ H ₁₈ O ₁₆ S	ST,	SI,	CE,	C, F	
P6	Hypolaetin 3'-methyl ether 8-O-β-D glucuronide	10.74	491.0831	315, 300, 175	94.24	1.91	C ₂₂ H ₂₀ O ₁₃	ST,	SI,			
P7	Isoscutellarein 8-O-β-D glucuronide	10.85	461.0725	285, 175	92.48	2.52	C ₂₁ H ₁₈ O ₁₂	ST,	SI,			
P8	Hypolaetin 3'-methyl ether 8-O-β-D glucuronide 3''-sulfate	12.24	571.0399	491, 315, 255		1.32		ST,	SI,	CE,	C, F	
P9	Isoscutellatellarein 8-O-β-D glucuronide 3''-sulfate	12.52	541.0294	461, 285, 255	97.87	-0.34	C ₂₂ H ₂₀ O ₁₆ S					
					98.34		C ₂₁ H ₁₈ O ₁₅ S	ST,	SI,	CE,	C, F	
METABOLITES												
M1	Sulfate derivative of M2	4.42/6.58	397.0592	315, 271, 205, 163,151, 135, 123, 109	93.55	1.63	C ₁₇ H ₁₈ O ₉ S		SI,	CE,	C, F	
M2	Unknown	5.23/5.76	317.1031	193, 151, 135, 123, 109	92.45	0.21	C ₁₇ H ₁₈ O ₆			CE,	C, F	
M3	3,4-diHPP-2-ol	5.63	291.0874	247, 205, 167, 151, 135, 123, 109	96.77	2.50	C ₁₅ H ₁₆ O ₆			CE,	C, F	
M4	5-(3-Hydroxyphenyl)-γ-valerolactone sulfate	5.81	271.0282	191	96.44	0.73	C ₁₁ H ₁₂ O ₆ S		SI,	CE,	C, F	
M5	5-(3,4-Dihydroxyphenyl)-γ-valerolactone	6.20	207.0663	175, 163, 143, 122	96.13	1.46	C ₁₁ H ₁₂ O ₄			CE,	C,	
M6	Unknown*	6.47/7.53	333.0980	-	99.26	0.81	C ₁₇ H ₁₈ O ₇			CE	C F	
M7	3,4-dihydroxyphenylvaleric acid*	7.22	209.0819	-	92.57	0.18	C ₁₁ H ₁₄ O ₄			CE	C F	
M8	3-HPP-2-ol	7.56	275.0925	191, 167, 147, 125, 107	98.22	2.05	C ₁₅ H ₁₆ O ₅			CE,	C, F	
M9	Hypolaetin	9.49	301.0354	133, 104, 89, 65	98.40	1.83	C ₁₅ H ₁₀ O ₇	ST,	SI*,	CE,	C, F	
M10	Isoscutellarein	11.50	285.0405	117, 65	97.75	2.00	C ₁₅ H ₁₀ O ₆	ST,	SI*,	CE,	C, F	
M11	Hypolaetin 3'-methyl ether	12.23	315.0510	133, 65	90.81	2.10	C ₁₆ H ₁₂ O ₇	ST,		CE,	C, F	

a) Sample identification, ST: stomach; SI: small intestine; CE: cecum; C: colon; F: feces. *Detected at traces level. M6 and M7 were detected at too low intensity to obtain optima MS/MS analysis.

FIGURE CAPTIONS

Figure 1. Structures of the main compounds found in cupuassu phenolic extract (CPE). Numbers correspond to compounds described in Table 1.

Figure 2. HPLC-UV chromatogram at 340 nm of cupuassu phenolic extract (CPE). Number correspond to compounds described in Table 1. (1) clovamide; (2) hypolaetin 8-O- β -D-glucuronide; (3) isoscutellarein 8-O- β -D-glucuronide; (4) hypolaetin 3'-methyl ether 8-O- β -D-glucuronide; (5) hypolaetin 8-O- β -D-glucuronide 3''-O-sulfate; (6) isoscutellarein 8-O- β -D-glucuronide 3''-O-sulfate; (7) hypolaetin 3'-methyl ether 8-O- β -D-glucuronide 3''-O-sulfate.

Figure 3. Extracted Ion Chromatograms (EICs) of the parent compounds (P) and metabolites (M) identified in a) stomach (0.5 h after CPE administration) and b) colon (4 h after CPE administration). Numbers correspond to compounds identified in Table 3.

Figure 4. Distribution of parent compounds from Cupuassu phenolic extract : a) clovamide, b) epicatechin, c) procyanidin B2, d) hypolaetin glucuronide e) Hypolaetin glucuronide-sulfate and the main flavone metabolite f) hypolaetin in different tissues along the gastrointestinal tract: stomach; small intestine; cecum; colon ; feces. Values are means of three mice with SD shown by vertical bars. Results are expressed in Areas corrected by the ratio mg of tissue/mL of extraction solvent.

Flavones

Compound	R ₁	R ₂
2	OH	H
3	OH	SO ₃ H
4	H	H
5	OCH ₃	H
6	H	SO ₃ H
7	OCH ₃	SO ₃ H

Clovamide

Epicatechin and related procyanidins

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Supplementary Figure 1. Proposed metabolic pathways of hypolaetin 8-O-β-D glucuronide by intestinal bacteria. Similar behavior was observed for the other flavones (isoscuteallarein and hypolaetin methyl ether). In blue we have indicated the compounds identified in the microbial metabolism of flavones from CPE and in big arrows the most probable pathway. In red tentative metabolite proposed for the molecular formula C₁₇H₁₈O₇. More information would be necessary for the complete characterization of this molecule.

Supplementary Figure 2. Proposed metabolic pathways of flavan-3-ols (epicatechin) by intestinal bacteria. In blue we have indicated the compounds identified in the microbial metabolism of procyanidins from CPE and in big arrows the most probable pathway.

Supplementary Figure 3. Time-course of the main microbial metabolites detected in small intestine (■), cecum (▲), colon (◊) and feces (×). Numbers correspond to metabolites identified in Table 3.

4.3 POLYPHENOLS FROM CAMU CAMU (*MYRCIARIA DUBIA* (VELL.) BERG)
ATTENUATE WEIGHT GAIN AND IMPROVE GLUCOSE HOMEOSTASIS IN HIGH
FAT HIGH SUCROSE DIET FED MICE

**HELENA RUDGE DE MORAES BARROS ¹, LUANA JORGE SOUSA, MARIA INÉS
GENOVESE ^{1*}**

* corresponding author (Tel: 55-11-30911525; Fax: 55-11-38154410; e-mail:
genovese@usp.br)

¹ Laboratório de Compostos Bioativos de Alimentos, Departamento de Alimentos e Nutrição
Experimental, FCF, Universidade de São Paulo, Av. Prof. Lineu Prestes 580, Bloco 14, 05508-
900 São Paulo, SP, Brazil.

ABSTRACT

Obesity, defined as ‘abnormal or excessive fat accumulation that may impair health’, is characterized by a low-grade systemic inflammation, which associated with elevated fasting blood glucose and reduced insulin sensitivity, increases the risks of developing type 2 diabetes. Herein, the regular consumption of phytochemical-rich foods has been proposed to protect against the development of metabolic diseases as obesity and type 2 diabetes. Camu camu is a fruit from Amazonian region, which is an excellent source of different classes of polyphenols with potential health benefits under to explore. Therefore, the aim of this study was to evaluate the effects of camu-camu phenolic extracts (CCPE) on metabolic disarrangements induced by a high fat high sucrose diet (HF/HS). Male C57BL/6J mice were assigned the following diets for 8 weeks: Standard chow diet (SD); HF/HS diet or HF/HS diet supplemented with CCPE in two doses (7 and 14 mg of gallic acid equivalents/Kg body weight) administered by gavage. Supplementation of CCPE can reduced the weight gain, improved fasting glucose levels, reduced glucose intolerance and insulin resistance and decreases serum C reactive protein levels (CRP). These beneficial effects occurred similarly in both doses, except by fasting plasma glucose and CRP levels, which was more pronounced in higher dose. These effects were possibly associated with reduced in food intake, increase in glucose uptake by tissues and anti-inflammatory activity. Despite the absence of improvements in lipid metabolism, these findings suggest that supplementation of phenolic compounds from camu camu could minimize glucose metabolism disarrangements induced by a HF/HS diet.

Keywords: anti-inflammatory activity, camu camu, glucose homeostasis.

1. Introduction

Obesity is defined by the World Health Organization (WHO) as ‘abnormal or excessive fat accumulation that may impair health’ (WHO, 2015) and is characterized by a low-grade systemic inflammation, primarily resulting from increase of adipocyte size and the recruitment of macrophages into the white adipose tissue (Moreno et al., 2014). Deregulation of energy balance due to high caloric intake and sedentary habits, as well variations in gut microbiota composition and host genetic are the major factors contributing to the increase of metabolic abnormalities from obesity (Moran & Shanahan, 2014; García-Conesa, 2015; Wang et al., 2014). Among these metabolic abnormalities, elevated fasting blood glucose and reduced insulin sensitivity associated to chronic low-grade systemic inflammation increase the risks of developing type 2 diabetes.

Caloric restriction and lifestyle changes, such as increase in physical activity, are generally considered the initial approach against insulin resistance associated to obesity, with variable efficiency (Evert & Riddle, 2015). Despite availability of many pharmacological interventions including the use of inhibitors of gut digestive enzymes, noradrenergic and oral hypoglycemic agents for obesity and diabetes management, the chronic use of these drugs can have reduced effectiveness, secondary adverse effects and high costs (García-Conesa, 2015; Torres Fuentes, Schellekens, Dinan, & Cryan, 2015; Yanovski & Yanovski, 2014).

As an adjuvant and safer intervention, the consumption of phytochemical-rich foods such as fruits, vegetables, spices and some beverages, as red wine and green tea, has been proposed to protect against development of metabolic disorders as obesity and type 2 diabetes (Bozzetto et al., 2015; Xiao, Högger, 2015; Wang, 2014). Among fruits, berries are reported as being a good source of dietary fiber, minerals, vitamin C and polyphenols, which are suggested to play a role in health benefits of fruit and vegetables based diets (Fracassetti, Costa, Moulay, & Tomás-Barberán, 2013; Heyman et al., 2014). In addition, recent clinical evidence indicates that intake of berries and their products may improve plasma lipid profile, reduce chronic inflammation and ameliorate postprandial glycemic response, specially in population with increased risk for metabolic syndrome development (Puupponen-Pimia et al., 2013; Yang & Kortesniemi., 2015). These effects were associate with several classes of polyphenols present in berries such as anthocyanins, ellagitannins, flavan-3-ols, procyanidins, flavonols and hydroxybenzoate derivatives (Del Rio, Borges, & Crozier, 2010).

Despite both production and consumption of berries increased propelled by the positive impact on human health all over the world (McDougall & Stewart, 2012), these well

known berries are mainly produced in temperate climates of Europe, Asia and North America. So, the richest known dietetic sources of ellagitannins such as blackberries, mulberries and raspberries (Zanotti et al., 2014) are not commonly consumed in tropical areas such as in Brazil. Among several Brazilian native fruits, the highest amounts of ellagic acid derivatives were found in the species of the Myrtaceae family, such as jaboticaba, cagaita, cambuci and camu camu (Abe, Lajolo, & Genovese, 2012; Gonçalves, Lajolo, & Genovese, 2010).

Camu camu fruit is a berry from bushes growing naturally in seasonally flooded environments of Amazonian region. Compared to other fruits, camu camu is an excellent source of vitamin C and presents different classes of polyphenols like other berries (Fracassetti et al., 2013). The high antioxidant potential of camu camu has been well established through several studies and previous animal experiments have reported, hypolipidemic, hepatoprotective and anti-inflammatory effects of camu camu (Gonçalves, Lellis-Santos, Curi, Lajolo, & Genovese, 2014; Langley, Pergolizzi, Taylor, & Ridgway, 2015). Therefore, the aim of this study was to evaluate the effects of camu-camu phenolic extracts on metabolic disarrangements induced by a high fat high sucrose diet (HF/HS).

2. Material and methods

2.1 Materials. Camu camu commercial frozen pulps were obtained from a fruit producer (*Bela Iaçá Polpa de Frutas Indústria e Comércio Ltda*) and were produced in Pará State, Brazil in August 2013.

2.2 C18-Purified Camu camu Phenolic Extracts (CCPE). Extraction was performed according to the method of Arabbi, Genovese & Lajolo (2004) with some modifications. Samples of homogenized commercial frozen pulp were extracted in a solvent mixture comprising methanol/water/acetic acid (70:30:0.5, v/v/v) for two hours. The homogenate was filtered under reduced pressure through filter paper (Whatman no. 1), and then it was evaporated under vacuum at 40 °C in a rotator evaporator (Rotavapor RE 120; Büchi, Flawil, Switzerland) and res-suspended with water. An aliquot of the extract was added to a C18 column (Applied Separations, New York, USA) preconditioned with methanol and water. The column was washed with water and further eluted with methanol. This fraction was evaporated to dryness under pressure at 40 °C, and dissolved in distilled water.

2.3 Total Phenolic Contents. The determination was performed according to the method of Singleton, Orthofer & Lamuela-Raventos (1999). A 0.25 mL aliquot of CCPE was mixed with 0.25 mL of the Folin-Ciocalteu reagent and 2 mL of distilled water. After 3 min at room temperature, 0.25 mL of a saturated sodium carbonate solution was added and the mixture placed at 37 °C in a water bath for 30 min. The absorbance was measured at 750 nm using a model Ultrospec 2000 UV-visible spectrophotometer (Hitachi U-1100, Tokyo, Japan). The results were expressed as milligrams of gallic acid equivalents (GAE) per mL of extract.

2.4 Flavonoid Contents. The same evaporated fractions obtained above were redissolved in HPLC grade methanol, filtered through 0.22 µm polytetrafluoroethylene (PTFE) filters (Millipore Ltd., Bedford, MA), and analyzed by HPLC. Identification and quantification of flavonoids were achieved using analytical reversed-phase HPLC in a Hewlett-Packard 1100 system with autosampler and quaternary pump coupled to a diode array detector according to the method of Arabbi, Genovese & Lajolo (2004). The column used was 250-4.6mm, i.d., 5 µm, Prodigy ODS3 reversed phase silica (Phenomenex Ltd., Torrance, CA), and elution solvents were (A) water/tetrahydrofuran/ trifluoroacetic acid (98:2:0.1) and (B) acetonitrile. Ellagic acid, quercetin and myricetin were quantified using the respective standards from Sigma-Aldrich (St.Louis, USA). The results were expressed in µg of each compound per mL of extract.

2.5 Proanthocyanidin content. The total proanthocyanidin contents were determined by UV spectrophotometry method based on acid hydrolysis and color formation according to Porter, Hrstich, & Chan (1986). Total amount was calculated using commercially available cyanidin (Extrasynthèse, Genay, France) as standard, and the calibration curve was in the range of 25-100 µg/mL. The result was expressed in µg of cyanidin equivalents per mL of extract.

2.6 Total ellagic acid content. Total ellagic acid was determined after extraction and acid hydrolysis according to Pinto, Lajolo & Genovese (2008). The extracts were dried under nitrogen, 2 N trifluoroacetic acid was added, and the hydrolysis was performed at 120 °C for 90 min. The hydrolyzed samples were evaporated under nitrogen, redissolved in methanol and filtered for HPLC analysis. The result was expressed in µg of ellagic acid equivalents per mL of extract.

2.7 Pancreatic lipase inhibition assay. The inhibition of lipase activity was determined by measuring the amount of 4-methylumbelliferone product released by lipase using a plate reader (Sinerigy H1 hybrid reader, Bioteck, IL, USA) following a reported method by Nakai et al. (2005). Pancreatic lipase (3.1.1.3, type II, from porcine pancreas, Sigma Aldrich, St. Louis, USA) and 4-methylumbelliferyl oleate (4-MU oleate) served as the reaction enzyme and substrate, respectively. The reaction mixture was prepared with 25 μ L of the cupuassu extract at different concentrations or lipase inhibitor (Orlistat, O4139, Sigma Aldrich, St. Louis, USA) and 25 μ L of 16.7 U/mL lipase in pH 8.0 Tris-HCl buffer solution. The reaction was initiated by adding 50 μ L of 0.1 M 4-MU oleate in pH 8.0 Tris-HCl buffer solution. After incubation at 37° C for 30 min, the rate of release of the 4-methylumbelliferone product was measured with a plate reader at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The readings were compared with the controls, containing buffer in place of sample extract. Results were expressed as mg of gallic acid equivalent (GAE) per mL of reaction necessary to inhibit 50% of 4-methylumbelliferone product.

2.8 Animals and experimental protocol. Eight-week-old male C57BL/6J mice (n = 40) were housed (3-4/cage) in plastic cages in a room with controlled temperature (22°C) and a relative humidity (55%) and kept on a regular light cycle (06:00-18:00 light, 18:00-06:00 dark). All experimental procedures were approved by the Ethics Committee on Animal Experimentation of the Faculty of Pharmaceutical Sciences, University of São Paulo, according to the guidelines of the Brazilian College on Animal Experimentation (protocol number 353). After acclimatization for one week, mice were randomly divided into four groups consisting of 10 mice each as follows: 1) standard chow diet (SD) fed-group that received a daily water administration by gavage; 2) a high fat high sucrose diet (HF/HS) fed group that received a daily water administration by gavage; 3) a HF/HS fed-group that received a daily administration of camu camu phenolic extract by gavage (7 mg GAE/kg body weight, CCPE1); and 4) an HF/HS fed group that received a daily administration of camu camu phenolic extract by gavage (14 mg GAE/kg body weight, CCPE2). Diets and water were available *ad libitum* during the experimental period of 8 weeks. The HF/HS diet contained 10% soy oil, 10% lard, 45% sucrose and 23% proteins and provided 4.6 kcal/g. The SD contained 56% carbohydrates, 5% lipids and 23 % proteins and provided 3.6 kcal/g. The composition of the HF/HS diet was based on a previous study (Lemieux, Picard, Labrie, Richard, & Deshaies, 2003). The fat-calorie percentages of the SD and HF/HS diets were 11.5% and 39%, respectively. HF/HS diet contained the same amounts of minerals and

vitamins per kilocalorie as the SD did. Weights, food and water intake were measured in alternate days. Feed efficiency was calculated as the ratio between the body mass gain in grams and the food consumed in kilocalories per animal, multiplied by 100.

2.8.1 Intraperitoneal Glucose Tolerance Test (ipGTT). Glucose tolerance test was performed at 8th week by injecting intraperitoneally 1 g of glucose/kg of body weight after a 5 h fasting period. The blood glucose was measured at baseline and at 15, 30, 45, 60 and 90 minutes using a glucometer (Accu chek performa®, Roche Diagnostics). Blood was collected in the tail vein at baseline and at 30 and 90 min after glucose injection. The tubes were centrifuged at 2000 g for 10 min at 4 °C, and serum was collected for insulin analysis using a rat/mouse insulin ELISA kit (EZRMI-13K) from Millipore (St. Charles, MO, USA). Insulin sensitivity was determined by the Quantitative Insulin Sensitivity Check Index (QUICKI) using the formula: $1 / \log \text{insulin (uU/mL)} \times \log \text{glucose (mg/dL)}$.

2.8.2 Tissues and blood collection. On days 56-58 of the experiment, the animals were sacrificed after euthanization with isofluorane. Bloody was collected by cardiac puncture, separated by centrifugation at 2000 g for 10 min at 4 °C. Heart, liver, muscle (gastrocnemius) and adipose tissues (epididymal, inguinal, retroperitoneal and brown adipose tissue) were collected, weighed, frozen under liquid N₂ and stored at -80 °C until further analyses.

2.8.3 Biochemical evaluation of serum. The serum levels of glucose, triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), C-reactive protein (CRP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using an automated analyzer (Labmax 240, Labtest Diagnóstica, Minas Gerais, Brazil).

2.8.4 Histological Analysis of White Adipose Tissue (WAT). Freshly isolated epididymal adipose tissue sections were fixed in 10% formalin for 24 h for histopathological analysis. Following a rinse in flowing water, tissues were processed in a paraffin. The paraffin-embedded samples were sliced into 5-µm thick sections and stained with hematoxylin and eosin.

2.8.5 Liver tissue analysis. Tissues were ground to a powder under liquid nitrogen and homogenized, and lipids were extracted according to the method of Hara & Radin (1978).

Briefly, in a test tube, 50 mg of liver tissue was weighted and 1.125 mL of hexane/isopropanol (3:2) were added. This mixture was then centrifuged at 1000 g for 15 min and the supernatant was removed, mixed with 1.5 mL of aqueous sodium sulfate (6.6%) and decanted for 1 min. One aliquot of the extracted liver lipids (200 µL) was then removed, dried, reconstituted in 500 µL of isopropanol and used for the measurement of the triacylglycerol concentrations using Labmax 240 (Labtest Diagnóstica, Minas Gerais, Brazil).

2.9 Statistical analysis. The statistical analyses were performed with GraphPad Prism 6 software (San Diego, CA, USA). Experimental data were tested for normality by Shapiro Wilk test. Differences among groups were analyzed using analysis of variance (ANOVA) followed by Tukey's test or non parametric tests. Body weight gain and weekly fasting glycaemia were statistically compared using two-way repeated measures ANOVA. Data were presented as means and standard deviation (SD) or standard error (SEM). A probability value of $p < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1 Phenolic composition of camu camu phenolic extract (CCPE)

Phenolic compounds tentatively identified in camu-camu extract are presented in **Table 1**. The total phenolic content was 2.53 mg of gallic acid equivalents/mL of extract and the amount of proanthocyanidins was 269 µg of cyaniding equivalents/mL. The content of ellagic acid was 215 µg of ellagic acid equivalents/mL, of which 8 % was free ellagic acid. Quercetin and myricetin derivatives were also found in CCPE.

3.2 *In vitro* pancreatic lipase inhibitory activity

The ability of CCPE to inhibit pancreatic lipase was evaluated at different concentrations of the extract. Overall, CCPE had the capacity to inhibit the pancreatic lipase in a dose-dependent manner. CCPE presented an IC₅₀ of 0.194 mg GAE /mL of reaction, which was 3 times lower than that of orlistat (0.069 mg/mL of reaction), on a weight basis.

3.3 Effect of camu camu phenolic extracts on body composition and food intake

The initial body weights of the four groups of mice were not significantly different. Subsequent to the 8-week treatment, the increase in body weight of the HF/HS-fed group was

significantly greater than that of the SD group (**Figure 1A**). Mice gavaged with extract from camu camu had significantly lower weight (g) than HF/HS-fed group in the 7th to 8th weeks independently the supplemented dose. Although no significant differences between relative body weight gains in all HF/HS fed groups were found, mice supplemented with both doses of camu camu extract decreased about 9 % of weight gain in relation to HF/HS control group (**Figure 1B**). In addition, supplementation with camu camu presented a tendency to reduced white adipose tissue gain (retroperitoneal, epididymal and inguinal) as demonstrated in **Figures 1C, D and E**. Accordingly, the histological microscopic analysis of the epididymal adipose tissue demonstrated that adipocytes in the SD-fed mice were markedly smaller than those of the HF/HS-fed mice, and camu camu supplementation slightly decreased the sizes of the adipocytes compared to the adipocytes in the HF/HS-fed mice (**Figure 2**).

Regarding food intake, HF/HS-fed mice group presented a significant reduction in daily food intake (g) when compared with mice fed SD ($p < 0.05$). However, the daily and total energy intake during the experimental protocol were significantly higher to HF/HS-fed groups due to the higher caloric density of the HF/HS diet when compared to the SD. Therefore, the feed efficiency in mice receiving the HF/HS diet was significantly higher compared to SD group. Mice gavaged with CCPE consumed significantly less kilocalories per day than mice in the HF/HS group without supplementation (**Table 2**). However, the total calorie intake only differed significantly within the group supplemented with the higher dose when compared to HF/HS control group, and this difference corresponded to a reduction of 17 % in total calorie and lipid intake. Moreover, feed efficiency was significantly lower to both groups HF/HS diet fed supplemented with camu camu than group without supplementation ($p < 0.05$).

3.4 Effect of camu camu phenolic extracts on glucose metabolism

Fasting plasma glucose levels were measured weekly during the experiment (**Figure 3A**). Plasma glucose levels were significantly higher in HF/HS-fed group compared to SD-fed group from 3rd to 8th weeks. Within the HF/HS diet fed groups, fasting plasma glucose levels in the CCPE 2 group were significantly lower than those in HF/HS control group from 5th to 8th weeks ($p < 0.005$) and CCPE 1 presented lower level than HF/HS group only at 8th week ($p < 0.05$).

To investigate the effects of CCPE on prevention of postprandial hyperglycemia, ipGTT was performed on fasted mice at 8th week (**Figure 2B**). The glycaemia were not different among the groups at baseline. The HF/HS fed control group showed higher glucose

levels at 15 to 90 min when compared to SD group, while mice supplemented with CCPE in both doses demonstrated higher glucose levels at 30 to 60 min when compared to SD group. Plasma glucose levels of mice supplemented with CCPE 2 were lower when compared within HF/HS at 45 to 90 min. CCPE 1 demonstrated lower level at 60 to 90 min, when compared to HF/HS fed control group. Thus, the area under the plasma glucose curve (AUC) was significantly higher ($p < 0.05$) in the HF/HS-fed compared to the SD group (**Figure 2C**), showing that they are glucose intolerant comparing to SD-mice. Supplementation of CCPE in both doses significantly reduced the glucose AUC when compared to HF/HS-fed group. Along with hyperglycemia during ipGTT, HF/HS-fed group presented higher insulinemia than SD-fed mice during all ipGTT ($p < 0.05$), while both doses of CCPE supplementation group presented no differences in relation to SD-fed group (**Figures 2D**). In addition, from fasting insulin and glucose data, we calculated HOMA-IR index (**Figure 2E**). The HF/HS-fed group demonstrated a significantly decrease of insulin sensitivity in relation to SD-fed group ($p < 0.0005$), while mice supplemented with CCPE 2 did not differ from SD-fed group.

3.5 Effect of camu camu phenolic extracts on lipid metabolism

The plasma lipid profiles are shown in **Figure 3**. Plasma triglycerides were not altered by diet or CCPE supplementation. However, HF/HD-fed group had an increase in the total cholesterol and their fractions (HDLc and LDLc) when compared to SD. CCPE supplementation in both doses attenuated these differences in relation to SD when compared to HF/HS-fed group (**Figures 3 B, C and D**). In addition, HF/HS diet leads to a higher triglyceride fecal excretion but not cholesterol than that in SD (**Figure 3F and G**). CCPE 2 supplemented group increased 21% of TG fecal excretion, while CCPE 1 increased 27% of cholesterol excretion when compared to HF/HS-fed group.

3.6 Effect of camu camu phenolic extracts on liver damage

Although there were no statistical differences in liver weights (g) between SD and HF/HS-fed group, HF/HS presented higher hepatic triacylglycerol contents than SD-fed group (**Figure 4A and B**). The liver masses in mice supplemented with CCPE 2 were significantly lower compared to HF/HS-fed group and the liver triacylglycerol contents presented a tendency to decrease (**Figure 4B**). Whereas no differences in ALT and AST were observed among all the groups (**Figures 4C and D**), C-reactive protein levels were also significantly lower in mice supplemented with CCPE in higher dose compared to HF/HS group without supplementation (**Figure 4E**).

4. Discussion

Recent clinical evidence indicates a great potential of a berry-rich diet in reducing the risk of chronic diseases development. In this study, we aimed to study the efficacy of camu camu polyphenols to attenuate the development of metabolic disarrangements induced by HF/HS diet. Mice were daily supplemented with two different doses of CCPE by 8 weeks. The higher dose fed to mice was 14 mg of gallic acid equivalents/ Kg b.w., which is equivalent to 980 mg of polyphenols for a 70-kg person per day. This intake level is close to the estimated dietary polyphenol intake, which accounts roughly for 1 g of polyphenols per day (Van Dam, Naidoo, & Landberg, 2013).

As in other berries, the main phenolic compounds in camu camu are proanthocyanidins, ellagic acid derivatives, followed by myricetin and quercetin derivatives. Among proanthocyanidins, epigallocatechin (EGC) and epigallocatechin gallate (EGCG), which are also found in green tea, have been previously identified in camu camu powders (Fracassetti, Costa, Moulay, & Tomás-Barberán, 2013). Health benefits of these classes of polyphenols include their antioxidant, anti-inflammatory, anti-obesity and anti-diabetic effects (Nile & Park, 2014; Dragan, Andrica, Serban, & Timar, 2014; Wang et al., 2014; Yang et al., 2015). Here, we demonstrated that supplementation of CCPE can decelerate the weight gain and attenuate metabolic disarrangements induced by HF/HS diet. Among the beneficial effects are the improvement of fasting glucose levels and insulin sensitivity, reduction of glucose intolerance and a decrease in levels of an inflammatory biomarker.

The ability of CCPE to decrease the body weight gain and attenuate white adipose tissue gain compared to their counterpart given water (HF/HS) may be due to the reduction of feed efficiency. High caloric intake from an excess of dietary lipids and carbohydrates in the diet is one of the main factors in determining the development of obesity, insulin resistance, β -cell dysfunction and glucose intolerance. Caloric restriction is generally considered the initial approach to reduce metabolic changes associated to obesity. Previous studies with blueberry and lingonberry supplementation demonstrated satiating effects and decreased food intake in mice ((Eid, Brault, Ouchfoun, & Thong, 2014; Molan, Lila, & Mawson, 2008). In this context, it has been proposed that the consumption of polyphenols has some effects on the production of hormones involved in satiating effects such as leptin; insulin-like growth factor I (IGF-1), amylin, neuropeptide Y (NPY), resistin; glucagon like peptide 1 (GLP-1) and ghrelin (García-Conesa, 2014; Panickar, 2013; Wang et al., 2014).

Beneficial effects on adiposity of other berries, such as bilberries, blackcurrants and raspberries, were previously associated with an increased excretion of triacylglycerol in mice fed a high fat diet (Heyman et al., 2014). The presence of ellagitannins and proanthocyanidins in CCPE could be important for lipase activity inhibition. The most effective berry types in inhibiting lipase activity *in vitro* are strawberry, raspberry, arctic bramble and cloudberry, which are rich in ellagitannins (McDougall & Stewart, 2012). Cagaita, other fruit rich in ellagitannins and that belongs to the same genus of camu camu, presented a high inhibition of pancreatic lipase activity *in vitro* (Donado-Pestana, Oliveira, & Genovese, 2015), practically the same presented by Orlistat, a well-known pancreatic lipase inhibitor used as an adjuvant in treatment of obesity and hyperlipidemia. Compared to cagaita ($IC_{50} = 25.26 \mu\text{g GAE/mL}$ of reaction), camu camu presented a much lower ability to decrease lipase activity ($194 \mu\text{g GAE/mL}$ of reaction).

Nevertheless, in this study, the pancreatic lipase inhibitory activity seems to have a little contribution against weight gain in CCPE-supplemented group, since supplementation did not increase fat excretion significantly in relation to HF/HS fed mice. On the other hand, if the reduced energy intake is taken into account, CCPE 2 supplementation reduced 17% of total food intake and CCPE 1 supplementation resulted in a slightly (but not significantly) reduced total energy intake of about 50 Kcal, when compared to HF/HS-fed group, throughout the 8-week treatment period. Therefore, this small reduction in energy intake and absorption, which accounts for 6% of total energy, could suggest that a regular intake of dietary polyphenols may mimic a small but significant caloric restriction of some of the current inhibitory enzyme activities drugs and could contribute to moderate the energy metabolism regulation.

However, the similar effects of different doses on weight gain, despite the differences in food intake, also point to additional distinct metabolic effects of CCPE supplementation. Among these metabolic effects could be the enhancing fat oxidation in adipose tissue and skeletal muscle (Dragan, Andrica, Serban, & Timar, 2014), the increasing of glucose utilization (Eid, Brault, Ouchfoun, & Thong, 2014; Hajiaghaalipour, Khalilpourfarshbafi, & Arya, 2015), and the attenuation on inflammatory responses and the suppression on oxidative stress (McDougall & Stewart, 2012, Yang & Kortensniemi, 2015). Further analyses are needed to elucidate the involved mechanisms for the prevention of weight gain in this study.

Moreover, the reduced food intake was not able to improve dyslipidemia caused by HF/HS intake. Serum triglycerides were not altered by diet, nevertheless HF/HS diet leads to an increase in all cholesterol fractions and CCPE supplementation groups only presented a

tendency to decrease these levels. Despite CCPE supplementation did not improve dyslipidemia, the levels of hepatic triglycerides demonstrated a tendency to reduce. Hepatic fat accumulation is associated with impaired insulin sensitivity in liver, elevated activities of serum aminotransferases and the production of inflammatory markers. While changes in ALT and AST activities were not found, the significant decrease of C-reactive protein (CRP) level in serum of CCPE 2-supplemented mice may be relevant. Elevated levels of circulating CRP are positively associated with increased adiposity, and increased risk for developing diabetes, cardiovascular diseases and leptin resistance (Ebron et al, 2015, Hribal, Fiorentino, Sesti, 2014, Kahn et al., 2014). This possible anti-inflammatory action could contribute to reduce insulin resistance in other tissues.

Mice supplemented with CCPE presented lower fasting glucose levels and improvement in glucose tolerance and insulin sensitivity as demonstrated by HOMA-IR. Thus, the reduced food intake associated with the reduction of total sucrose intake in camu camu-supplemented animals may be partly responsible for the improvement of glucose homeostasis, thus protecting pancreatic cells against glucose toxicity. Camu camu aqueous extracts were also reported as highly effective in inhibiting α -glucosidase activity (Fujita et al., 2015), and some polyphenols found in camu camu, including quercetin, myricetin, ellagitannins and EGCG, have been demonstrated to inhibit the sodium dependent glucose transporter and GLUT 2 *in vitro* (Hanhineva et al., 2010). However, as we analyzed glucose tolerance intraperitoneally, it is not clear if these mechanisms of decreasing intestinal carbohydrate absorption could be related to the improvement of glucose homeostasis observed.

Other studies have shown that berry extracts significantly increased glucose uptake, induced GLUT4 translocation and inhibited hepatic glucose production through an insulin-independent mechanism involving AMPK (Eid, Brault, Ouchfoun, & Thong, 2014; Heyman-Lindén et al., 2014). In addition, HF-induced hepatic inflammation was counteracted by supplementation with berries by inhibiting pathways related to inflammatory responses and downregulating several inflammatory genes, important mechanisms by which berries prevent HF-induced steatosis, low-grade inflammation and insulin resistance (Heyman-Lindén et al., 2015). In this way, the lower feed efficiency, the increased glucose uptake, the suppression of hepatic glucose production, and the lower CPR plasmatic levels are plausible mechanisms to explain the improvement of glucose homeostasis in this study. However, further studies are essential to elucidate the exact mechanism of this modulatory effect and to evaluate the potential therapeutic CCPE effects on glucose homeostasis.

5. Conclusion

This study demonstrated that the administration of phenolic compounds from camu camu provided beneficial health effects against HF/HS diet-induced metabolic disarrangements by reducing weight gain and plasmatic C reactive protein levels and improving glucose metabolism. These effects were mainly associated with reduced food intake and consequently lower glucolipotoxicity, improvement of insulin resistance and fasting glucose level, as well anti-inflammatory properties of CCPE. The inclusion of foods that may improve satiety may be a practical way to maintain a stable body weight, decreasing the risk of metabolic disorders development. Further experiments are underway to determine the additional mechanisms involved in the effects observed.

ACKNOWLEDGEMENTS

We thank *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq 162218/2011-7) and *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP 10/52192-2) for financial support.

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Table 1. Content of phenolic compounds in camu camu phenolic extract (CCPE).

	CCPE
Myricetin derivatives (µg myricetin equivalents/mL)	15.2 ± 0.4
Quercetin derivatives (µg quercetin equivalentes/mL)	18.7 ± 0.3
Free ellagic acid (µg ellagic acid equivalents/mL)	17.6 ± 0.8
Total ellagic acid (µg ellagic acid equivalentes/mL)	215 ± 7
Proanthocyanidins (µg cyanidin equivalentes/mL)	269 ± 3
Total polyphenol contents (mg GAE/mL)	2.53 ± 0.03

Data are presented as means ± sd (n= 6). CCPE = Camu camu phenolic extract; GAE = galic acid equivalents.

Table 2. Food intake of mice fed a standard diet (SD) or high fat/ high sucrose diet (HF/HS) supplemented with different doses of CCPE.

	SD	HF/HS	CCPE 1	CCPE 2
<i>Intake</i>				
Diet intake (g/ day/ mice)	3.6 ± 0.1 a	3.2 ± 0.1 b	3.0 ± 0.1 b	2.6 ± 0.1 c
Kcal intake (Kcal/ day/ mice)	12.9 ± 0.2 ac	14.4 ± 0.2 b	13.5 ± 0.3 a	11.9 ± 0.2 c
Total Kcal (56 days/mice)	727 ± 15 ac	809 ± 12 b	758 ± 12 ab	669 ± 10 c
Feed efficiency	0.33 ± 0.01 a	0.98 ± 0.02 b	0.71 ± 0.01 c	0.79 ± 0.01 d
Fat intake (mg/day/mice)	166 ± 2 a	623 ± 10 b	584 ± 11 b	515 ± 11 c

Data are presented as means ± SEM (n= 10). Means within rows with different letters are significantly different (p<0.05).

FIGURE CAPTIONS

Figure 1. Effects of camu camu phenolic extract (CCPE) supplementation on body composition. Mice were fed a standard diet (SD) or a high fat high sucrose (HF/HS) diet by 8 weeks. HF/HS-fed mice were supplemented daily with two doses of CCPE (7 or 14 mg galic acid equivalents/Kg body weight) by gavage. SD fed mice and another HF/HS control group were daily gavage with water. Cumulative body weight gain (A); % Weight gain (B); Retroperitoneal (C); Epididymal (D) and Inguinal adipose tissue weights (E). Values are means $\pm$ SEM (n= 10). # CCPP 1 and CCPE 2 vs HF/HS; SD vs. other groups *(p < 0.05), ** (p < 0.005), *** (p < 0.001) and **** (p < 0.0001).

Figure 2. Histology of the epididymal adipose tissue with hematoxylin and eosin staining (400 $\times$ original magnification).

Figure 3. Effects of camu camu phenolic extract (CCPE) supplementation on glucose metabolism. Weekly fasting glucose level during the experiment (A); Blood glucose levels during intraperitoneal glucose tolerance test- ipGTT (B); area under the curve (AUC) for ipGTT (C); serum insulin levels during ipGTT (D); area under the curve (AUC) for insulin (E); Homa-IR index (F). Values are means $\pm$ SEM; n= 10 (A-C) and n=5 (D-F). *SD vs. HF/HS groups (p < 0.05). Bars with different letters are significantly different (p< 0.05).

Figure 4. Effect of camu camu phenolic extract (CCPE) supplementation on lipid profile. Serum triacylglycerol (A), total cholesterol (B), HDL cholesterol (C), LDL cholesterol (D); fecal triacylglycerol (E) and fecal cholesterol. Values are means $\pm$ SEM; n= 7-10 (A-D) and n=3 (E-F). *SD vs. HF/HS groups (p < 0.05). Bars with different letters are significantly different (p< 0.05).

Figure 5. Effect of CPE supplementation on hepatic function and inflammation: Liver weight (A), hepatic triglyceride accumulation (B), serum activity of ALT (C); AST (D) and serum ractive protein C (E). Values are means $\pm$ SEM; n= 7-10 (A-E). *SD vs. HF/HS groups (p < 0.05). Bars with different letters are significantly different (p< 0.05).

Figure1.

SD

HF/HS

CCPE 1

CCPE 2

Figure 2.

Figure 3.

Figure 4.

Figure 5.

5. CONCLUSÕES

Os extratos ricos em compostos fenólicos do camu camu e do cupuaçu foram caracterizados quanto ao teor de compostos fenólicos totais, proantocianidinas e flavonoides. O extrato de cupuaçu apresentou flavanóis como a epicatequina, flavonas como os derivados glicuronados da hypolaetinas e da isoscutelareina, e proantocianidinas como procianidina B2. Já extrato do camu camu apresentou flavonóis como a quercetina e a miricetina, o ácido elágico e as proantocianidinas em sua composição.

O extrato do cupuaçu apresentou efetiva capacidade inibitória da enzima lipase pancreática *in vitro*, o que foi comprovado pelo aumento da excreção de lipídios fecais no experimento biológico. Já o extrato de camu camu apresentou inibição da atividade da lipase pancreática *in vitro*, porém a dose administrada não apresentou efeitos significativos em relação a excreção de lipídios fecais.

A suplementação crônica do extrato de cupuaçu na maior dose testada melhorou a glicemia de jejum e a homeostase dos lipídios, protegendo o tecido hepático dos danos causados pela dieta com alto teor de lipídios e sacarose. Estes efeitos foram associados à inibição de enzimas digestivas comprovado pelo aumento da excreção de lipídios nas fezes, com consequente menor absorção de lipídios provenientes da dieta, reduzindo assim a resistência à insulina e o estresse oxidativo no fígado, a hiperglicemia e a dislipidemia.

A suplementação crônica do extrato de compostos fenólicos do camu camu reduziu o ganho de peso corporal, melhorou a tolerância à glicose e a sensibilidade à insulina independente da dose administrada. No entanto, não apresentou efeitos sobre o metabolismo lipídico. Estes resultados foram associados a um possível efeito de saciedade, com consequente redução da ingestão da dieta, e com a diminuição dos níveis de proteína C reativa.

Portanto, as diferenças da composição de compostos fenólicos encontradas entre os extratos das duas frutas foram responsáveis pelos diferentes resultados nos protocolos *in vivo*.

O modelo de biodisponibilidade *in vivo* permitiu verificar a distribuição e a formação de metabólitos no trato gastrointestinal de camundongos a partir da administração do extrato

de compostos fenólicos do cupuaçu por gavagem. Os compostos “originais” presentes no extrato foram identificados em diferentes tecidos gastrointestinais, principalmente no estômago e intestino delgado, onde poderiam exercer os efeitos benéficos observados em alguns estudos. A diminuição da concentração destes compostos ao longo do trato gastrointestinal (cólon e ceco) se deve possivelmente à transformação destes compostos pela microbiota. As flavonas glicuronadas foram hidrolizadas a agliconas, enquanto que as proantocianidinas e epicatequina foram transformadas em diarilpropanol e 5-(3,4-dihidroxifenil)- γ -valerolactona. A metabolização destes compostos a ácidos fenólicos, como comumente descrito na literatura, não foi observada, o que pode ter ocorrido devido à presença de diferentes polifenóis na matriz que podem ter interferido nessa metabolização. Estudos adicionais com uma dose mais alta ou um tratamento mais longo poderiam proporcionar uma melhor compreensão do metabolismo de extracto de cupuaçu.

O consumo da fruta e/ou dos derivados das frutas estudadas pode auxiliar na redução de alterações metabólicas que propiciam o desenvolvimento de obesidade e diabetes mellitus tipo 2.

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ZANOTTI, I.; DALL’ASTA, M.; MENA, P.; MELE, L.; BRUNI, R.; RAY, S.; DEL RIO, D.. Atheroprotective effects of (poly)phenols: a focus on cell cholesterol metabolism. **Food Function**, v. 6, n. 1, p. 13–31, 2015.

ZANATTA, C. F., MERCADANTE, A. Z. Carotenoid composition from the Brazilian tropical fruit camu-camu (*Myrciaria dubia*). **Food Chemistry**, v. 101, n. 4, p. 1526–1532, 2007.

ZANATTA, C. F., CUEVAS, E., BOBBIO, E. O., WINTERHALTER, P., MERCADANTE, A. Z. Determination of anthocyanins from camu-camu (*Myrciaria dubia*) by HPLC–PDA, HPLC–MS, and NMR. **Journal of Agricultural and Food Chemistry**, v. 53, p. 9531–9535, 2005.

ZHENG, X. X.; XU, Y. L.; LI, S. H., HUI, R.; WU, Y. J.; HUANG, X. H. Effects of green tea catechins with or without caffeine on glycemic control in adults: a meta-analysis of randomized controlled trials. **American Journal of Clinical Nutrition**., v. 97, n. 4, p. 750–62, 2013.

ANEXOS

ANEXO A - PARECER DO COMITÊ DE ÉTICA

UNIVERSIDADE DE SÃO PAULO**FACULDADE DE CIÊNCIAS FARMACÊUTICAS**
Comissão de Ética no Uso de Animais - CEUA

Ofício CEUA/FCF/17 /2012

CERTIFICADO

A Comissão de Ética no Uso de Animais da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo Certifica que o Projeto "Efeito dos compostos fenólicos do camu camu e do cupuaçu no tratamento e prevenção da obesidade e diabetes mellitus tipo 2" (Protocolo CEUA/FCF/353), de responsabilidade da pesquisadora **Helena Rudge de Moraes Barros** sob a orientação da Profa. Dra. Maria Inês Genovese, está de acordo com as normas do Conselho Nacional de Controle de Experimentação Animal - CONCEA e foi **APROVADO** em reunião de 05 de Março de 2012.

São Paulo, 5 de março de 2012.

Prof. Dr. Marco Antonio Stephano
Coordenador da Comissão de Ética no Uso de Animais
CEUA/FCF/USP

ANEXO B - FICHA DO ALUNO

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9131 - 7898300/1 - Helena Rudge de Moraes Barros

Email: helenabarro@usp.br
Cédula de Identidade: RG - 30.003.169-5 - SP
Local de Nascimento: Estado de São Paulo
Nacionalidade: Brasileira
Graduação: Nutricionista - Centro Universitário São Camilo - São Paulo - Brasil - 2001
Mestrado: Mestre em Nutrição e Saúde (1) - Universidade Federal de Goiás - Goiás - Brasil - 2011

Curso: Doutorado
Programa: Ciência dos Alimentos
Área: Bromatologia
Data de Matrícula: 24/11/2011
Início da Contagem de Prazo: 24/11/2011
Data Limite para o Depósito: 24/11/2015
Orientador: Prof(a). Dr(a). Maria Ines Genovese Rodriguez - 24/11/2011 até o presente. Email: genovese@usp.br
Proficiência em Línguas: Inglês, Aprovado em 24/11/2011
Data de Aprovação no Exame de Qualificação: Aprovado em 12/11/2013
Data do Depósito do Trabalho:
Título do Trabalho:
Data Máxima para Aprovação da Banca:
Data de Aprovação da Banca:
Data Máxima para Defesa:
Data da Defesa:
Resultado da Defesa:
Histórico de Ocorrências: Primeira Matrícula em 24/11/2011

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).

Última ocorrência: Matrícula de Acompanhamento em 20/07/2015

Impresso em: 27/10/2015 09:32:09

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

Documento sem validade oficial

FICHA DO ALUNO

9131 - 7898300/1 - Helena Rudge de Moraes Barros

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Disp.	Situação
BMB5816-2/3	Metodologias Básicas de Experimentação Laboratorial no Departamento de Fisiologia e Biofísica (Instituto de Ciências Biomédicas - Universidade de São Paulo)	27/02/2012	04/03/2012	15	1	100	A	N	Concluída
FBA5899-2/2	Biodisponibilidade de Nutrientes e de Substâncias Bioativas em Alimentos e Dietas	05/03/2012	15/04/2012	90	6	90	A	N	Concluída
FBA5712-5/4	Fisiologia da Nutrição I	16/04/2012	27/05/2012	90	6	100	A	N	Concluída
MCM5797-4/3	Obesidade (Faculdade de Medicina - Universidade de São Paulo)	11/06/2012	01/07/2012	60	4	95	A	N	Concluída
MCM5716-5/4	Atualização em Diabetes Mellitus Tipo 1 e 2 e Síndrome Metabólica (Faculdade de Medicina - Universidade de São Paulo)	13/08/2012	16/09/2012	75	5	85	A	N	Concluída
EDM5102-3/1	Preparação Pedagógica PAE (Faculdade de Educação - Universidade de São Paulo)	14/08/2012	24/09/2012	60	4	100	A	N	Concluída
BMB5804-2/5	O Órgão Adiposo como Centro Regulador do Metabolismo (Instituto de Ciências Biomédicas - Universidade de São Paulo)	22/10/2012	03/12/2012	60	4	90	A	N	Concluída
FBA5895-5/1	Carboidratos na Nutrição Humana	08/04/2013	12/05/2013	75	5	100	A	N	Concluída
FBC5780-2/2	Análise de Dados Aplicados às Pesquisas Biológicas	10/03/2014	20/04/2014	90	6	100	A	N	Concluída
BIB5762-6/3	Métodos de Análise de Metabólitos Secundários (Instituto de Biociências - Universidade de São Paulo)	23/04/2014	03/06/2014	120	0	-	-	N	Matrícula cancelada

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	20	41
Estágios:			
Total:	0	20	41

Créditos Atribuídos à Tese: 167

Observações:

1) Curso com validade nacional, de acordo com o disposto na Portaria nº 590, de 18.06.2009..

Conceito a partir de 02/01/1997:
A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.
Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 20/07/2015

Impresso em: 27/10/2015 09:32:09

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

Documento sem validade oficial

FICHA DO ALUNO

9131 - 7898300/1 - Helena Rudge de Moraes Barros

Comissão julgadora da tese de doutorado:			
NUSP	Nome	Vínculo	Função
2112371	Maria Ines Genovese Rodriguez	FCF - USP	Presidente

Última ocorrência: Matrícula de Acompanhamento em 20/07/2015

Impresso em: 27/10/2015 09:32:09

ANEXO C - CURRICULO LATTES

Helena Rudge de Moraes Barros

Bolsista de Doutorado do CNPq

- Endereço para acessar este CV:
<http://lattes.cnpq.br/5163026855878451>
- Última atualização do currículo em 12/10/2015

Doutoranda em Ciência dos Alimentos pela Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (FCF-USP). Mestre em Nutrição e Saúde pela Universidade Federal de Goiás (UFG) na área de qualidade de alimentos e dietas, bromatologia. Graduada em Nutrição pelo Centro Universitário São Camilo- São Paulo- SP (2001). **(Texto informado pelo autor)**

Identificação

Nome

Helena Rudge de Moraes Barros

Nome em citações bibliográficas

Barros, Helena Rudge de Moraes; BARROS, H. R. M.;Barros, H.R.M., Barros, Helena Rudge;de Moraes Barros, Helena Rudge

Endereço

Endereço Profissional

Universidade de São Paulo, Faculdade de Ciências Farmacêuticas.

Av. Lineu Prestes 580, Bloco 14 - Cjto das Químicas

Butantã

05508-000 - São Paulo, SP - Brasil

Telefone: (011) 30914238

Formação acadêmica/titulação

2011 Doutorado em andamento em Ciência dos Alimentos, Faculdade de Ciências Farmacêuticas-USP, com **período sanduíche** em Consejo Superior de Investigaciones Científicas (Orientador: Francisco Tomás-Barberán).

Título: Efeito dos compostos fenólicos de camu-camu e cupuaçu no desenvolvimento da obesidade/diabetes tipo 2,

Orientador: Maria Inés Genovese Rodriguez.

Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico.

2009 - 2011 Mestrado em Nutrição e Saúde (Conceito CAPES 3).

Universidade Federal de Goiás, UFG, Brasil.

Título: Composição mineral e capacidade antioxidante de citros cultivados no Estado de Goiás. Ano de Obtenção: 2011.

Orientador: Tânia Aparecida Pinto de Castro Ferreira.
 Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.
 Palavras-chave: antioxidantes; minerais; compostos fenólicos; ácido ascórbico.

1998 - 2001 Graduação em Nutrição. Centro Universitário São Camilo.

Formação Complementar

2012 - 2012 Workshop de Capacitação para Pesquisadores da USP. (Carga horária: 16h).
 Instituto de Ciências Biomédicas - USP.

2010 - 2010 Extensão universitária em Aplicações de Métodos Instrumentais para Análises. (Carga horária: 25h).
 Universidade Federal de Goiás, UFG, Brasil.

2010 - 2010 Curso de Aperfeiçoamento em SPSS. (Carga horária: 40h).
 Faculdade de Nutrição da Universidade Federal de Goiás.

2009 - 2009 Gestão de Resíduos Sólidos em UANs. (Carga horária: 8h).
 Universidade Federal de Goiás, UFG, Brasil.

2009 - 2009 Nutrição, Atividade Física e Esporte. (Carga horária: 4h).
 Sociedade Brasileira de Alimentação e Nutrição.

2008 - 2008 Curso de Formação do Personal Diet. (Carga horária: 80h).
 NTR Cursos.

2004 - 2004 Treinamento para membros da CIPA. (Carga horária: 20h).
 Pronutri Refeições Coletivas Ltda.

2003 - 2003 Curso Prático de Atendimento Nutricional em Consul. (Carga horária: 16h).
 Centro Universitário Senac.

2000 - 2000 Planejamento Físico de Sistema de Alimentação. (Carga horária: 8h).
 Centro Universitário São Camilo.

Atuação Profissional

Universidade de São Paulo, USP, Brasil.

Vínculo institucional

2010 - 2010 Vínculo: estagiário, Enquadramento Funcional: estagiário, Carga horária: 8

Universidade Federal de Goiás, UFG, Brasil.

Vínculo institucional

2010 - 2010 Vínculo: Estágio docência, Enquadramento Funcional: Estágio, Carga horária: 6

Outras informações Estágio docência na disciplina de Bromatologia, oferecida ao curso de graduação em Nutrição.

Vínculo institucional

2009 - 2009 Vínculo: Estágio docência, Enquadramento Funcional: Estagiário, Carga horária: 6

Outras informações Estágio docência na disciplina Introdução à Prática Profissional, oferecida ao curso de graduação em Nutrição.

Pronutri Refeições Coletivas Ltda, PTN, Brasil.

Vínculo institucional

2002 - 2008 Vínculo: Empregado salariado, Enquadramento Funcional: Administrador de Restaurante Industrial, Carga horária: 40, Regime: Dedicação exclusiva.

Residencial Israelita Albert Einstein Lar Golda Meyer, ALBERT EINSTEIN, Brasil.

Vínculo institucional

2001 - 2001 Vínculo: Estagiária, Enquadramento Funcional: Estagiaria, Carga horária: 30

Hospital do Coração, HCOR, Brasil.

Vínculo institucional

2001 - 2001 Vínculo: Estagiária, Enquadramento Funcional: Estagiária, Carga horária: 30

Banco de Alimentos de São Paulo, ONG BA, Brasil.

Vínculo institucional

2001 - 2001 Vínculo: Estagiária, Enquadramento Funcional: Estagiária, Carga horária: 40

Centro Tecnológico da Marinha em São Paulo (CTMSP) Geraldo J. Coan CIA LTDA, COAN PAMPAS, Brasil.

Vínculo institucional

2001 - 2001 Vínculo: Estagiária em UAN, Enquadramento Funcional: Estagária, Carga horária: 30

Revisor de periódico

2013 - Atual Periódico: African Journal of Agricultural Research
2013 - Atual Periódico: Pharmaceutical Biology (Online)
2012 - Atual Periódico: Journal of Agricultural and Food Chemistry
2015 - Atual Periódico: Current Pharmaceutical Biotechnology
2015 - Atual Periódico: Journal of Functional Foods
2014 - Atual Periódico: Journal of Food Biochemistry
2014 - Atual Periódico: Journal of Nutrition & Food Sciences

Áreas de atuação

1.

Grande área: Ciências da Saúde / Área: Nutrição / Subárea: Bioquímica da Nutrição.

2.

Grande área: Ciências da Saúde / Área: Nutrição / Subárea: Valor nutricional dos alimentos.

Idiomas

Inglês

Compreende Bem, Fala Razoavelmente, Lê Bem, Escreve Razoavelmente.

Francês

Compreende Pouco, Fala Pouco, Lê Pouco, Escreve Pouco.

Espanhol

Compreende Razoavelmente, Fala Razoavelmente Lê bem, Escreve Razoavelmente.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

1. ★ **Barros, Helena Rudge de Moraes** ; Ferreira, Tânia Aparecida Pinto de Castro ; Genovese, Maria Inés . Antioxidant capacity and mineral content of pulp and peel from commercial cultivars of citrus from Brazil. Food Chemistry **JCR**, v. 134, p. 1892-1898, 2012.

Citações: **WEB OF SCIENCE**™ 17 | **SCOPUS** 19

2. ALDIN, M. N. ; **Barros, Helena Rudge de Moraes** ; JAIME, P. C. ; MATTOS, R. C. ; NUNES, M. S. ; OLIVEIRA, P. G. ; RIBEIRO, F. P. ; SAVOY, P. M. . Eficácia dos Flavonóides na dislipdemia. O Mundo da Saúde, v. 25, p. 149-152, 2000.

Resumos publicados em anais de congressos

1. ★ **Barros, H.R.M., Barros, Helena Rudge** ; FESTUCCIA, W. ; Oliveira, T. B. ; Genovese, Maria Inés . Effect of cupuassu phenolic compounds on glycaemic control in diet-induced obesity and type 2 diabetes. In: 8th ISANH Congress on Polyphenols Applicatios, 2014, Lisboa. Abstracts book, 2014. p. 91-91.

2. Pestana, C. M. Donado ; **Barros, H.R.M., Barros, Helena Rudge** ; Genovese, Maria Inés . Phenolics compounds from cagaita fruit reduce body fat accumulation and inflammatory status in mice fed a high-fat high-sucrose diet. In: 8th ISANH Congress on Polyphenols Applicatios, 2014, Lisboa. Abstracts book, 2014. p. 90.

3. BETETO, F. M. ; Barros, H.R.M., Barros, Helena Rudge ; ROCHETTI, A. ; FUKUMASU, H. ; Genovese, Maria Inés .

Antiproliferative activity of fruits in A544 cell line (human lung adenocarcinoma). In: VI International Conference on Polyphenols and Health, 2013, Buenos Aires. VI International Conference on Polyphenols and Health, 2013.

4. Barros, H.R.M., Barros, Helena Rudge ; LAJOLO, F. ; GENOVESE, M. I. . Cupuassu phenolic extract supplementation attenuates the development of glucose intolerance associated with a high fat/ high sucrose diet in C57BL/6J mice. In: VI International Conference on Polyphenols and Health, 2013, Buenos Aires. VI International Conference on Polyphenols and Health, 2013.

5. ARAUJO, R. L. ; COLOMBO, K. ; Barros, H.R.M., Barros, Helena Rudge ; GENOVESE, M. I. . Inhibition of pancreatic lipase in vitro by camu camu (*Myrciaria dubia*). In: VI International Conference on Polyphenols and Health, 2013, Buenos Aires. VI International Conference on Polyphenols and Health, 2013.

6. Barros, H.R.M., Barros, Helena Rudge ; ALEZANDRO, M. ; SEELAENDER, M. ; GENOVESE, M. I. . Tratamento com extrato de jabuticaba reverte alterações metabólicas em ratos wistar com estresse oxidativo relacionado à caquexia. In: XII Congresso Nacional da Sociedade Brasileira de Alimentação e Nutrição - SBAN, 2013, Foz do Iguaçu. Nutrire Revista da Sociedade Brasileira de Alimentação e Nutrição, 2013. v. 38. p. 219.

7. Barros, H.R.M., Barros, Helena Rudge ; CURI, R. ; LAJOLO, F. ; Genovese, Maria Inés . Efeito da suplementação de compostos fenólicos do cupuaçu no desenvolvimento de intolerância à glicose induzida por uma dieta rica em gorduras e sacarose em camundongos. In: XII Congresso Nacional da Sociedade Brasileira de Alimentação e Nutrição - SBAN, 2013, Foz do Iguaçu. Nutrire Revista da Sociedade Brasileira de Alimentação e Nutrição, 2013. v. 38. p. 93-93.

Apresentações de Trabalho

1. MENDONCA, B. G. ; Barros, H.R.M., Barros, Helena Rudge ; Genovese, Maria Inés . Potencial anti-obesidade de compostos bioativos fenólicos de frutas nativas brasileiras camu-camu e cupuaçu. 2015. (Apresentação de Trabalho/Simpósio).

2. ★Barros, H.R.M., Barros, Helena Rudge ; FESTUCCIA, W. ; Oliveira, T. B. ; Genovese, Maria Inés . Effect of cupuassu phenolic compounds on glycaemic control in diet-induced obesity and type 2 diabetes. 2014. (Apresentação de Trabalho/Congresso).

3. Pestana, C. M. Donado ; Barros, H.R.M., Barros, Helena Rudge ; Genovese, Maria Inés . Phenolics compounds from cagaita fruit reduce body fat accumulation and inflammatory status in mice fed a high-fat high-sucrose diet. 2014. (Apresentação de Trabalho/Congresso).

4. BARROS, H. R. M. ; LAJOLO, F. ; CURI, R. ; Genovese, Maria Inés ; Barros, Helena Rudge de Moraes, BARROS, H. R. M. . Efeito da suplementação de compostos fenólicos do cupuaçu no desenvolvimento de intolerância à glicose induzida por uma dieta rica em gorduras e sacarose em camundongos. 2013. (Apresentação de Trabalho/Congresso).

5. ALEZANDRO, M. ; Barros, Helena Rudge de Moraes, BARROS, H. R. M. ; SEELAENDER, M. ; GENOVESE, M. I. . Tratamento com extrato de jabuticaba reverte alterações metabólicas em ratos wistar com estresse oxidativo relacionado à caquexia. 2013. (Apresentação de Trabalho/Congresso).

6. BETETO, F. M. ; Barros, H.R.M., Barros, Helena Rudge ; ROCHETTI, A. ; FUKUMASU, H. ; RODRIGUES, M. I. G. . Antiproliferative activity of fruits in A544 cell line (human lung adenocarcinoma). 2013. (Apresentação de Trabalho/Conferência ou palestra).

7. ARAUJO, R. L. ; COLOMBO, K. ; BARROS, H. R. M. ; RODRIGUES, M. I. G. . Inhibition of pancreatic lipase in vitro from camu camu (*Myrciaria dubia*). 2013. (Apresentação de Trabalho/Conferência ou palestra).

8. BARROS, H. R. M. ; LAJOLO, F. ; RODRIGUES, M. I. G. . Cupuassu phenolic extract supplementation attenuates the development of glucose intolerance associated with a high fat/ high sucrose diet in C57BL/6J mice. 2013. (Apresentação de Trabalho/Conferência ou palestra).

9. BARROS, H. R. M. ; FESTUCCIA, W. ; LAJOLO, F. ; CURI, R. ; GENOVESE, M. I. ; **BARROS, H. R. M.** . CUPUAÇU PHENOLIC COMPOUNDS PREVENTED THE DEVELOPMENT OF GLUCOSE INTOLERANCE ASSOCIATED WITH A HIGH FAT/ HIGH SUCROSE DIET. 2012. (Apresentação de Trabalho/Congresso).

10. BARROS, H. R. M. ; FERREIRA, T. A. P. C. . Teor de compostos fenólicos e capacidade antioxidante de polpas de citros cultivados em Goiás. 2010. (Apresentação de Trabalho/Seminário).

11. BARROS, H. R. M. ; FERREIRA, T. A. P. C. ; GENOVESE, M. I. . Comparação de métodos de extração de compostos fenólicos e atividade antioxidante em casca e polpa de laranja pêra. 2010. (Apresentação de Trabalho/Congresso).

12. ★ BARROS, H. R. M. ; FERREIRA, T. A. P. C. . Teor de compostos fenólicos e capacidade antioxidante de polpas de citros cultivados em Goiás. 2010. (Apresentação de Trabalho/Congresso).

13. Barros, H.R.M., Barros, Helena Rudge ; LOPES, Synara ; FERREIRA, T. A. P. C. . COMPARAÇÃO DA COMPOSIÇÃO QUÍMICA CENTESIMAL DE PARTES DE FRUTAS E HORTALIÇAS USUALMENTE NÃO CONSUMIDAS COM COMUMENTE CONSUMIDAS. 2009. (Apresentação de Trabalho/Congresso).

14. ALDIN, M. N. ; Barros, Helena Rudge de Moraes ; MATTOS, R. C. ; NUNES, M. S. ; OLIVEIRA, P. G. ; SAVOY, P. M. . "Avaliação das Políticas Nacionais de Fortificação de Alimentos e Controle de Deficiências Nutricionais de Vitamina A e Iodo". 2000. (Apresentação de Trabalho/Simpósio).

15. Aldin, Marlene Nunez ; Barros, Helena Rudge de Moraes ; MATTOS, R. C. ; Nunes, Mariana Salton ; Oliveira, Priscila Gil de ; Savoy, Paula Marcela . "Avaliação Nutricional de Adolescentes de 7 e 8 séries em Escola Particular. 2000. (Apresentação de Trabalho/Simpósio).

Produção técnica

Entrevistas, mesas redondas, programas e comentários na mídia

1. Barros, H.R.M., Barros, Helena Rudge . Frutas vermelhas ajudam a combater problemas de memória. 2014. (Programa de rádio ou TV/Comentário).

2. Barros, H.R.M., Barros, Helena Rudge ; Fujita, Alice . Fruta consumida no exterior em falta no Brasil. 2014. (Programa de rádio ou TV/Comentário).

Eventos

Participação em eventos, congressos, exposições e feiras

1. FoRC Symposium: Advances in Food. 2015. (Simpósio).
2. 23º SIICUSP. Potencial anti-obesidade de compostos bioativos fenólicos de frutas nativas brasileiras camu-camu e cupuaçu. 2015. (Simpósio).
3. "Prebiotics, Probiotics and Synbiotics and their Impact on Nutrients, Vitamins and Minerals". 2015. (Outra).
4. Intestinal microbiota in Synthesis and Activation of Vitamin B12. 2015. (Outra).
5. Mechanistic in an in vitro model of the colon to study the role of the gut microbiota in obesity. 2015. (Outra).
6. 8th World Congress on Polyphenols Applications. Effect of cupuassu phenolic compounds on glycaemic control in diet-induced obesity and type 2 diabetes. 2014. (Congresso).
7. VI International Conference on Polyphenols and Health. Cupuassu phenolic extract supplementation attenuates the development of glucose intolerance associated with a high fat/ high sucrose diet in C57BL/6J mice. 2013. (Congresso).
8. XII Congresso Nacional da Sociedade Brasileira de Alimentação e Nutrição - SBAN. Efeito da suplementação de compostos fenólicos do cupuaçu no desenvolvimento de intolerância à glicose induzida por uma dieta rica em gorduras e sacarose em camundongos. 2013. (Congresso).
9. 6th Congress of the International Society of Nutrigenetics/Nutrigenomics (ISNN). CUPUAÇU PHENOLIC COMPOUNDS PREVENTED THE DEVELOPMENT OF GLUCOSE INTOLERANCE ASSOCIATED WITH A HIGH FAT/ HIGH SUCROSE DIET. 2012. (Congresso).
10. Conceitos gerais da Cromatografia Líquida de Alta Performance. 2012. (Seminário).
11. VIII Simpósio de Lipídeos e Saúde. 2012. (Simpósio).
12. Conferencia Vitafoods South America. 2012. (Outra).
13. VII Simpósio de lipídeos e saúde: "Dos lipídeos à prevenção da doença cardiovascular". 2011. (Simpósio).
14. Colunas de HPLC e GC e Cartuchos de SPE: Como Escolher. 2011. (Outra).
15. XXII Congresso Brasileiro de Ciência e Tecnologia de Alimentos. Comparação de métodos de extração de compostos fenólicos e atividade antioxidante em casca e polpa de laranja pêra. 2010. (Congresso).
16. VII Congresso de Pós Graduação da UFG. Teor de compostos fenólicos e capacidade antioxidante de polpas de citros cultivados em Goiás. 2010. (Congresso).
17. V Simpósio de síndrome metabólica do instituto central do Hospital das Clínicas da FMUSP. 2010. (Simpósio).
18. X Congresso Nacional da Sociedade Brasileira de Alimentação e Nutrição-SBAN. 2009. (Congresso).
19. V Congresso Pan-Americano de incentivo ao consumo de frutas e hortaliças para a promoção da saúde. Comparação da composição química centesimal de partes de frutas e hortaliças usualmente não consumidas com comumente consumidas. 2009. (Congresso).
20. IV Seminário Integrado de Segurança Alimentar da PUC GOIÁS. 2009. (Seminário).

- 21.** III Mostra Parceria Ensino-Serviço-Comunidade/UFG - MOPESCO. 2009. (Encontro).
- 22.** I CURSO DE METODOLOGIA CIENTÍFICA BÁSICA DO CA XXI de ABRIL (CAXXIA). 2009. (Outra).
- 23.** Gestão de resíduos sólidos em unidades de alimentação e nutrição2009. 2009. (Outra).
- 24.** IX International Congress on Obesity. 2002. (Congresso).
- 25.** 1 seminário Científico Aspectos Funcionais do Licopeno para a Saúde. 2000. (Seminário).
- 26.** III SIMPONUTRI - Simpósio de Nutrição.Avaliação Nutricional de Adolescentes de 7 e 8 séries em escola particular. 2000. (Simpósio).
- 27.** II SIMPONUTRI- simpósio de Nutrição. 1999. (Simpósio).
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Organização de eventos, congressos, exposições e feiras

- 1. BARROS, H. R. M. .** III Mostra Parceria Ensino-Serviço-Comunidade/UFG. 2009. (Outro).