Universidade Federal do Maranhão

Centro de Ciências Biológicas e da Saúde

Programa de Pós-Graduação em Ciências da Saúde

Tese de Doutorado

EFEITOS DO EXTRATO RICO EM POLIFENOIS DA FOLHA DE *SYZYGIUM CUMINI* (L.) SKEELS SOBRE O DIABETES INDUZIDO POR ESTRESSE OXIDATIVO

VINICYUS TELES CHAGAS

São Luís

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal do Maranhão, para a obtenção do título de doutor em Ciências da Saúde.

Orientador: Prof. Dr. Antonio Marcus de Andrade Paes

Co-orientador: Prof. Dr. Andres Trostchansky

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Teles Chagas, Vinicyus.
Efeitos do Extrato Rico em Polifenois da folha de Syzygium cumini L. Skeels sobre o diabetes induzido por estresse oxidativo / Vinicyus Teles Chagas. - 2018.
130 p.

Orientador(a): Antonio Marcus de Andrade Paes Andres Trostchansky.
Tese (Doutorado) - Programa de Pós-graduação em Ciências da Saúde/ccbs, Universidade Federal do Maranhão, São Luís, 2018.

1. Diabetes. 2. Flavonoides. 3. Polifenóis. 4. Proteína microsomal transferidora de triacilgliceróis. 5. Syzygium cumini. I. Andres Trostchansky, Antonio Marcus de Andrade Paes. II. Título.

“A ciência serve para nos dar uma ideia
de quão extensa é a nossa ignorância”.

(Félicité Robert de Lamennais)

Dedico esta tese a Deus, pela saúde e encorajamento, principalmente nos momentos de desânimo, e a todos os colaboradores

AGRADECIMENTOS

Ao Senhor Deus por possibilitar os meios para a conclusão de mais esta etapa da jornada da vida.

Ao meu orientador, Prof. Dr. Antonio Marcus de Andrade Paes pela sabedoria na orientação desta tese e incentivo no âmbito acadêmico desde a iniciação científica. Minha admiração ao pesquisador e professor Antonio Marcus por ser um formador de novos docentes amantes da ciência.

Ao co-orientador, Prof. Dr. Andres Trostchansky por receber-me em seu laboratório (Laboratório de Oncologia Básica e Biologia Molecular da Universidad de la República, Montevideo, Uruguai) como estagiário e pela contribuição em diferentes análises químicas presentes neste caderno.

Às alunas de iniciação que participaram ativamente em diversos experimentos que constam nesta tese, Rafaella Coelho e Ivana Letícia. Vocês foram fundamentais para alcance dos objetivos deste trabalho. Muito obrigado! Também, à equipe do Laboratório de Ensino e Pesquisa em Fisiologia (LEFISIO) pelo companheirismo, auxílio nos experimentos e sugestões para melhoria deste trabalho. Obrigado Lucas França, Samira Abdalla, Bruno Serra e aos demais amigos do Lefisio.

À minha família pelo apoio e carinho prestados ao longo de toda a vida estundantil. Jamais teria chegado até aqui sem este fundamento essencial.

Ao programa de Pós-Graduação em Ciências da Saúde/UFMA e à profª Dra. Flávia Raquel Fernandes do Nascimento (coordenadora) por contribui com meu aperfeiçoamento científico. À Universidade Federal do Maranhão (UFMA) pela infra-estrutura disponibilizada ao Programa de Pós-graduação em Ciências da Saúde (PPGCS) e instalações do laboratório de pesquisa, LEFISIO.

Agradeço à Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Maranhão (FAPEMA) pelo apoio financeiro ao projeto vinculado a esta tese "Efeitos do extrato rico em polifenois das folhas de *Syzygium cumini* (L.) Skeels sobre a síntese e exportação de triacilglicerois em hepatócitos".

RESUMO

O estresse oxidativo contribui para o desenvolvimento e progressão das complicações do diabetes. Antioxidantes naturais como os polifenóis podem reduzir ou prevenir os danos oxidativos causados às ilhotas pancreáticas através da atenuação do estresse oxidativo. *Syzygium cumini* (L.) Skeels (Myrtacea) é uma espécie nativa do subcontinente indiano cultivada em todo o território brasileiro que possui elevada concentração de polifenóis em suas diferentes partes. Dentre suas propriedades medicinais estão: atividade anti-inflamatória, cardioprotetora, anti-neoplásica, anti-hiperglicemiante, dentre outras, descritas principalmente para suas sementes. Por seu potencial terapêutico, *S. cumini* vem sendo utilizado popularmente para o controle do diabetes, no entanto, há controvérsias sua eficácia de *S. cumini* em ensaios *in-vivo*. Assim, este trabalho teve como objetivo avaliar os efeitos antidiabeticos e antioxidante do Extrato Rico em Polifenois (ERP) das folhas de *S. cumini*. Inicialmente, reportamos que diferentes mecanismos responsáveis pelas propriedades cardiometabólicas de *S.cumini* como inibição da HMG-CoA, estimulo à secreção insulínica, maior expressão de GLUT-4 são descritos para os polifenóis.A seguir, preparamos o ERP a partir das folhas de *S.cumini*. Estas foram submetidas à maceração empregando álcool:água (7:3) resultando no extrato hidroalcoolico bruto (EHB). O EHB foi particionado com solventes de polaridade crescente (clorofórmio e acetato de etila). A fase acetato foi concentrada e liofilizada gerando o ERP. *Rattus norvegicus* adultos (60 dias) receberam aloxana (150 mg/kg, i.p) para indução do diabetes tipo 1. Estes foram radomizados e divididos em 3 grupos: controle (ALX), tratados antes e após a injeção de aloxana (ALX-PP) tratados após a aloxana (ALX-P). Os animais tratados com ERP (50 mg/kg) apresentaram acentuada redução nos níveis glicêmicos e lipidêmicos, além de apresentarem redução da resistência à insulina, especialmente no grupo previamente tratado. Em seguida realizamos a quantificação de fenóis totais pelo método Azul da Prússia e de flavonoides pelo método do cloreto de alumínio. O teor de polifenóis foi 71.78 ± 8.57 GAE/100 g e a concentração de flavonoides 8.21 ± 0.42 QE/100 g. Para verificar quais polifenois estavam associados aos efeitos, anti-diabeticos observados com o tratamento usando *S.cumini* determinamos o perfil químico do ERP por HPLC-MS empregando eluição por gradiente. Cinco compostos fenólicos foram identificados no ERP: ácido gálico, miricetina 3-α-arabinopiranosídeo, miricetina desoxihexosídeo, miricetina e quercetina. O ERP demonstrou importante atividade antioxidante nos ensaios de inibição do $DPPH^{\bullet}$, $ABTS^{\bullet+}$ e lipoxigenase (LOX) (valores de IC_{50}: 3.88 ± 1.09; 5.98 ± 1.19; 27.63 ± 8.47 µg/mL, respectivamente), sendo que nos ensaios do DPPH e lipoxigenase, a atividade antioxidante do ERP foi equivalente à exibida pelos padrões testados (ácido gálico, miricetina e quercetina). A literatura apresenta que os polifenóis (componentes mais abundantes em *S.cumini*) são promissores agentes hipolipemiantes por inibir parcialmente proteína microsomal transferidora de triacilgliceróis (MTP) hepática, uma chaperona residente no retículo endoplasmático que tem papel essencial na montagem e secreção de VLDL. Desse modo, sugerimos neste trabalho que a via de inibição da MTP hepática pode ser responsável pelo efeito ati-hipertrigliceridêmico de *S.cumini*. Em conjunto, os dados obtidos neste trabalho reforçam o potencial anti-diabetico, antioxidante e hipolipemiante de *S.cumini*.

Palavras-chaves: *Syzygium cumini*; polifenóis; flavonoides; atividade antioxidante; diabetes; proteína microsomal transferidora de triacilgliceróis.

ABSTRACT

Oxidative stress contributes to the development and progression of diabetes complications. Natural antioxidants such as polyphenols can reduce or prevent the oxidative damage caused to the pancreatic islets through the attenuation of oxidative stress. *Syzygium cumini* (L.) Skeels (Myrtacea) is a imported specie from the Indian subcontinent and cultivated throughout the Brazilian territory that has high concentration of polyphenols in its different parts. Among its medicinal properties are: anti-inflammatory, cardioprotective, anti-neoplastic, anti-hyperglycemic activity, among others, mainly described for its seeds. For your terapeutical potential, *S. cumini* has been used over about 130 years for the control of diabetes, however, there are controversies its efficacy of *S. cumini* in in-vivo trials. Thus, the objective of this work was to evaluate the antidiabetic and antioxidant effects of the Polyphenol-Rich extract (PESc) from *S. cumini* leaves. Initially, we report different mechanisms responsible for the cardiometabolic properties of *S.cumini* as inhibition of HMG-CoA, stimulation to insulin secretion, increase of the expression of GLUT-4 are described for polyphenols. Next, we prepare the ERP from the leaves of *S .cumini*. These were submitted to maceration using alcohol: water (7: 3) resulting in the crude hydroalcoholic extract (EHB). The EHB was partitioned with increasing polarity solvents (chloroform and ethyl acetate). The acetate phase was concentrated and lyophilized generating the PESc. Rattus norvegicus adults (60 days) received alloxan (150 mg / kg, ip) for induction of type 1 diabetes. These were radominated and divided into 3 groups (control, treated prior to alloxan injection and treated before and after treatment alloxan). After the treatment with PESc (50 mg / kg), the animals presented a marked reduction in glycemic and lipidemic levels, as well as a reduction in insulin resistance, especially in the previously treated group. Then we quantified total phenols by Prussian Blue method and flavonoids by the aluminum chloride method. The polyphenol content was 71.78 ± 8.57 GAE / 100 g and the flavonoid concentration was 8.21 ± 0.42 QE / 100 g. To verify which polyphenols were associated with the anti-diabetic effects observed with the treatment using *S.cumini* we determined the chemical profile of the PESc by HPLC-MS employing gradient elution. Five phenolic compounds were identified in PESc: gallic acid, myricetin 3-α-arabinopyranoside, myoxycetin deoxyhexoside, myricetin and quercetin. PESc showed significant antioxidant activity in the inhibition assays of $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and lipoxygenase (LOX) (IC_{50} values: 3.88 ± 1.09, 5.98 ± 1.19, 27.63 ± 8.47 μg / mL, respectively). $DPPH^{\bullet}$ and lipoxygenase, the antioxidant activity of ERP was equivalent to that exhibited by the standards tested (gallic acid, myricetin and quercetin). PESc also stimulated insulin secretion in β-cells (INS-1E) non-toxic. We have also collected literature results showing that polyphenols (most abundant components in *S.cumini*) are promising lipid-lowering agents by partially inhibiting hepatic microsomal triglyceride transfer protein (MTP), a chaperone resident in the endoplasmic reticulum that plays a key role in the assembly and secretion of VLDL. Thus, we suggest in this work that the route of inhibition of hepatic MTP may be responsible for the ati-hypertriglyceridemic effect of *S.cumini*. Together, the data obtained in this study reinforce the anti-diabetic, antioxidant and hypolipidemic potential of *S.cumini*.

Keywords: *Syzygium cumini*, polyphenols, flavonoids, antioxidant activity, diabetes, microsomal triglyceride transfer protein.

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LISTA DE ABREVIATURAS E SÍMBOLOS

ABTS$^{\bullet+}$: radical 2,2´- azinobis (3-etilbenzotiazolina-6-ácido sulfônico)

AGEs: “advanced glycation end products”/produtos finais de glicação avançada

PKB/Akt: protein quinase B ou Akt

ApoB: apolipoproteina B

CAT: Catalase

DPPH$^{\bullet}$: radical 2,2-difenil-1-picril-hidrazil

DT1: diabetes tipo 1

eNOS: óxido nítrico-sintase endotelial

ER: espécies reativas

ERP: Extrato Rico em Polifenois

FAS: “first apoptosis signal receptor”/ receptor do primeiro sinal de apoptose

FAS-L: ligante FAS

FOXO1: “forkhead box O1”

GAPDH: gliceraldeído-3-fosfato desidrogenase

G6Pase: glicose-6-fosfatase

iNOS: óxido nítrico-sintase induzida

IRE: elemento responsivo à insulina

Keap-1: “Kelch-like ECH-associated protein 1”

LDL: lipoproteína de baixa densidade

LOX: lipoxigenase

MTP: “Microsomal triglyceride transfer protein”/

MSG: L-glutamato monossódico

m/z: Relação massa-carga

Nrf-2: do inglês “nuclear factor E2-related factor 2”

PESc: “Polyphenol-Rich Extract”

PKC: proteína quinase C

PI3K: fosfoinositol 3-cinase

SOD: Superóxido dismutase

Ref-1: fator redox 1

TG: triacilglicerol

VLDL: lipoproteína de muito baixa densidade

SUMÁRIO

1 INTRODUÇÃO

As espécies reativas (ER), classificadas como espécies reativas do oxigênio, nitrogênio e enxofre (ERO/ERN/ERS), compreendem estruturas radicalares ou não-radicalares formadas em alguns sistemas celulares localizados na membrana plasmática (NADPH oxidases), no citosol (peroxissomos), membranas do retículo endoplasmático (enzimas do citocromo P450) e mitocôndria (cadeia respiratória); a partir da redução parcial do oxigênio durante o metabolismo celular ou em resposta à xenobióticos (RAY et al., 2012; DI MEO et al., 2016).

As ER atuam como mediadores da transferência de elétrons e, através de mecanismos redox, promovem regulação de diferentes processos fisiológicos incluindo apoptose, autofagia, transporte iônico, expressão gênica, fertilização e geração de respostas de defesa em processos infeciosos (LÜ et al., 2010; ARAÚJO et al., 2016). Dentre as principais ER estão o radical hidroxila ($^{\bullet}OH$), espécie que reage rapidamente no sítio de formação devido sua elevada reatividade; ânion radical superóxido ($O_2^{\bullet-}$), predominante no meio celular, importante para o combate à infecções pelos fagócitos e participa de processos de peroxidação; óxido nítrico ($NO^{\bullet}$), molécula sinalizadora no processo de vasosodilatação; radical tiila ($RS^{\bullet}$) que pode oxidar rapidamente proteínas e enzimas com grupos tiólicos; peróxido de hidrogênio (H_2O_2) que modula a atividade de fatores pró-inflamatórios como o NF-κB e participa da oxidação de resíduos de cisteínas em proteínas (GILES e JACOB, 2002; DI MEO et al., 2016; SIES, 2017).

As concentrações basais de ER são importantes para regulação de vias intracelulares, no entanto, a desregularização da sinalização oxidativa pode contribuir para o desenvolvimento de diversas doenças crônicas, desse modo, a sinalização redox é crucial para determinar condições fisológicas ou fisiopatológicas (FINKEL, 2011). Quantidades excessivas de ER são deletérias porque podem provocar oxidação de biomoléculas como ácidos nucleicos (DNA e RNA), proteínas e lipídios (LUSHCHAK, 2014). O conjunto de danos oxidativos causados às estruturas celulares é reconhecido como estresse oxidativo, condição onde há um desbalanço a favor da produção de ER ou deficiência na taxa de remoção (ARAÚJO et al., 2016). O estresse oxidativo contribui para o envelhecimento, desenvolvimento de câncer,

Alzheimer, hipertensão, aterosclerose e diabetes (FINKEL e HOLBROOK, 2000; RAHMAN, 2007; ROBERTS e SINDHU, 2009).

A fisiopatologia do diabetes, bem como, suas complicações neurológicas e vasculares, estão fortemente associadas ao estresse oxidativo (TURK, 2010). Elevados níveis de ER produzem danos a organelas e enzimas celulares reduzindo os mecanismos de proteção antioxidante, aumentando a peroxidação lipídica, levando ao desenvolvimento de resistência à insulina e perda de células β-pancreáticas, sinais peculiares do diabetes (CERIELLO, 2006; DELMASTRO e PIGANELLI, 2011). O diabetes mellitus (DM) é considerado a doença crônica mais comum em todo o mundo (MCGROWDER et al., 2013; Diagnosis and Classification of Diabetes Mellitus, 2014). Esta doença metabólica possui prevalência global de 415 milhões de portadores (2015) e expectativa de atingir cerca de 642 milhões de pessoas até 2040 e apresenta como característica central a hiperglicemia resultante de defeitos na ação da insulina, secreção da insulina ou ambos (OGURTSOVA et al., 2017).

O diabetes tipo 1 (DT1) ou diabetes insulino-dependente é uma desordem auto-imune que representa cerca de 5-10% dos diagnósticos de diabetes mellitus (MCGROWDER et al., 2013). O DT1 foi denominado diabetes juvenil por ocorrer majoritariamente durante a infância ou vida adulta-jovem quando comparado ao diabetes tipo 2, que afeta principalmente os adultos e está associado à resistência à insulina e obesidade (WOMEN'S e HEALTH, 2015). Prejuízos na síntese e secreção da insulina ocorrem no DT1 decorrentes da destruição das células β pancreáticas (DELMASTRO e PIGANELLI, 2011). Desse modo, a insulina exógena é empregada para o tratamento do DT1 (até o presente sem cura), no entanto, apesar da administração do fármaco (insulina) possibilitar a manutenção dos níveis basais de glicose, seu uso não previne as complicações do DT1 que incluem danos aos vasos, rins, retina e coração, além dos eventos de hipoglicemia (LIU et al., 2016).

As células β pancreáticas possuem reduzidas defesas antioxidantes, por isso, são consideradas vulneráveis ao estresse oxidativo em comparação a outras células do organismo (LENZEN et al., 1996). O estresse oxidativo contribui para a disfunção e morte das células β durante o desenvolvimento do DT1 por ativar o ataque auto-imune às ilhotas pancreáticas em indivíduos geneticamente susceptíveis (DELMASTRO e PIGANELLI, 2011). Inicialmente, as espécies reativas causam danos ao tecido pacreático ativando células apresentadoras, principalmente macrófagos, que liberam citocinas como TNFα, IL-6, IL-1β e ER. As ER e

citocinas amplificam as lesões teciduais e desencadeiam a destruição auto-imune das células β (MURATA et al., 2002).

Moléculas capazes de atenuar o estresse oxidativo podem apresentar efeitos protetivos contra o desenvolvimento e complicações do DT1 (BAJAJ e KHAN, 2012; BABU et al., 2013). Nesse contexto, os polifenóis, metabólitos amplamente distribuídos no reino vegetal, tem grande destaque devido sua elevada capacidade antioxidante proporcionada por suas características estruturais que possibilitam reação direta com espécies reativas (transformando-as em moléculas menos deletérias) ou inibição da geração destas por meio do sequestro de metais de transição e inibição da atividade de enzimas oxidativas (PEREIRA et al., 2009).

Syzygium cumini (L.) Skeels (Myrtaceae) é uma espécie nativa do subcontinente indiano bastante cultivada no Brasil apresentando rica composição fenólica (CHAGAS et al., 2015) (Figura 1). Apresenta sinonímia variada: *Syzygium jambolanum* (Lam.) DC, *Eugenia jambolana*, Lam., *Eugenia cumini* (L.) Druce e *Myrtus cumini* L., sendo conhecida popularmen-

Figura 1. *Syzygium cumini* cultivada no campus da UFMA, São Luís-MA.
(Fonte: Chagas, 2018)

te como jambolão, java plum, black plum, Indian blackberry, jaman, jambu e jambul. Dentre as atividades biológicas de *S.cumini* estão atividade hipoglicemicante, antimicrobiana, hepatoprotetora, anti-inflamtória, quimiopreventiva, antigenotóxica, hipolipemiante, descritas principalmente para suas sementes (AYYANAR e SUBASH-BABU, 2012), sendo escassos na literatura informações sobre tais efeitos em tratamentos empregando suas folhas.

A suplementação com antioxidantes naturais como polifenóis pode prevenir a apoptose das células β, contribuir para a homeostase glicídica e elevar as defesas antioxidantes (BABU et al., 2013). Dessa forma, buscamos neste trabalho avaliar a atividade antioxidante *in-vitro* através dos ensaios do $DPPH^{\bullet}$ e $ABTS^{\bullet+}$ e lipoxigenase (LOX), como também *in-vivo*, utilizando o modelo de DT1 induzido por aloxana. Os ensaios utilizando o DPPH e ABTS são úteis na análise da capacidade antioxidante de componentes hidrofílicos como os polifenois, metabólitos que apresentam atividade antioxidante relacionada à reatividade de seus numerosos grupos hidroxilados (FLOEGEL et al., 2011). O ensaio de inibição da lipoxigenase é útil para demonstração da atividade antioxidante (a LOX participa da peroxidação lipídica) e anti-inflamatória (modulação da geração de mediadores inflamatórios)(GERONIKAKI e GAVALAS, 2006). A aloxana promove destruição das células β pancreáticas através da indução do estresse oxidativo. A administração de aloxana leva à geração de ERO, principalemente dos radicais superóxido ($O_2^{\bullet-}$), hidroxila ($OH^{\bullet}$) e também peróxido de hidrogênio (H_2O_2) que danificam e desencadeam o processo de apoptose das células β (ALUWONG et al., 2016).

Dados prévios do nosso laboratório demonstraram que a administração do extrato hidroalcoólico das folhas de *Syzygium cumini* (L.) Skeels a ratos MSG (modelo que apresenta sinais clássicos da síndrome metabólica: resistência à insulina, obesidade, dislipidemia e diabetes) promove efeitos anti–obesidade, anti-hiperlipidêmico e anti-hiperglicêmico. Estes efeitos foram observados após a administração tanto aguda quanto crônica do extrato bruto das folhas (dados não publicados). Sanchez et al. (2016) verificaram que o extrato bruto de *S.cumini* melhora a resistência à insulina, reduz a intolerância à glicose em ratos MSG, além de estimular a secreção insulínica em ensaios *ex-vivo* com ilhotas isoladas destes animais (SANCHES et al., 2016). Outros resultados mostraram que o animal MSG apresenta maior expressão e atividade da MTP hepática, sendo esta a provável causa da elevada concentração de VLDL plasmático (FRANÇA et al., 2013).

Considerando que a literatura descreve os efeitos anti-diabeticos, anti-hipertrigliceridêmico e antioxidante dos polifenois, há fortes indícios que *S. cumini,* devido à sua rica composição fenólica, pode reduzir os níveis glicêmicos e modular a biossíntese de triacilgliceróis via reversão do estresse oxidativo e modulação da MTP hepática.

2 FUNDAMENTAÇÃO TEÓRICA

Durante o desenvolvimento do DT1, as células β são atacadas por citocinas pró-inflamatórias e ER liberadas por linfócitos T autorreativos. A auto-imunidade mediada pelos linfócitos T é precedida pela invasão de células do sistema imune inato, macrófagos e células dendríticas, à ilhota pancreática (DELMASTRO e PIGANELLI, 2011). Os macrófagos reconhecem as células β como antígenos e tornam-se ativados secretando ER e diferentes citocinas (TNFα, IL-6, IL-1β). IL-1β induz ao aumento da atividade da iNOS levando à citólise das células β através da geração de $NO^{\bullet}$; TNFα promove danos as células β por meio do estímulo à secreção de IL-1β, além de ativar células apresentadoras (APCs) e linfócitos (THOMAS e KAY, 2000; DELMASTRO e PIGANELLI, 2011).

Evidências mostram que os linfócitos T autorreativos, em particular os linfócitos T $CD8^+$ e T $CD4^+$, detem papel fundamental na patogênese do DT1 (WONG et al., 1996). Linfócitos T $CD8^+$ são reportados como os principais agentes das lesões pancreáticas através da liberação de granzima B e perforina ou através da via FAS-L/ FAS (receptores da família do TNF) (THOMAS et al., 2010), além disso, camundongos diabéticos não-obesos com deficiência destes linfócitos não desenvolveram auto-imunidade mostrando a sua capital participação nas lesões às ilhotas de Langerhans (SUMIDA et al., 1994). Para que os linfócitos T $CD8^+$ possam entrar no pâncreas, é necessário a atuação de células auxiliadoras, os linfócitos T $CD4^+$, que também levam à morte de células β por liberação de citocinas e recrutamento de macrófagos para o ambiente pancreático (CANTOR e HASKINS, 2007). Resultados mostram que a produção e migração de linfócitos T $CD4^+$ (PIGANELLI et al., 2002) e T $CD8^+$ autorreativos é reduzida com atenuação do estresse oxidativo (SKLAVOS et al., 2008). Assim, o estresse oxidativo possui participação na modulação do sistema imune durante o desenvolvimento do DT1 (LIU et al., 2016) (Figura 2).

O estresse oxidativo leva à prejuízos na função das células β, portanto, as ER possuem impacto crucial na patogênese do DT1 (MCGROWDER et al., 2013). As ilhotas pancreáticas possuem reduzidos níveis de antioxidantes endógenos e, por isso, são altamente sensíveis ao estresse oxidativo. Espécies oxidantes podem causar danos ao metabolismo celular e aos canais de potássio, promovendo inclusive, inativação destes canais que são importantes mediadores da secreção insulínica (AZEVEDO-MARTINS et al., 2003).

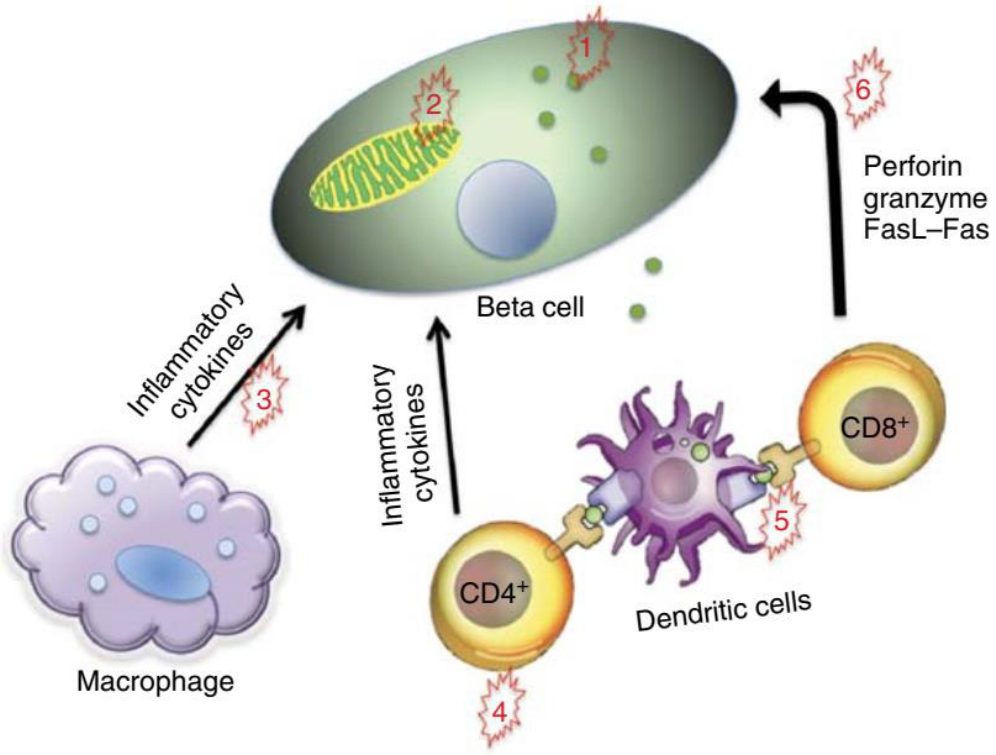

Figura 2. Participação das espécies reativas (ER) em múltiplos estágios do desenvolvimento do diabetes tipo 1 (DT1). (1) ER podem induzir diretamente à disfunções nas células β (2) As ER facilitam a morte programada das células β; (3) As ER produzidas por macrófagos induzem à destruição das células β. (4) ER promovem a proliferação de linfócitos T $CD4^+$ e secreção de citocinas inflamatórias intensificando os dano às células β. (5) As ER participam na apresentação cruzada de antígenos das células dendríticas para linfócitos T CD8+. (6) Os linfócitos T $CD8^+$ destroem células β via ação da perforina, granzima e FASL-FAS. Adaptado de Delmaro et al. (2013).

O estresse oxidativo contribui para a ação citotóxica de linfócitos T autorreativos associados a outras células do sistema imune (macrófagos e células dendríticas) promovendo disfunção e morte das células β e consequentemente levando à hiperglicemia no DT1 (DELMASTRO e PIGANELLI, 2011). A hiperglicemia representa o fator primário para

geração de elevados níveis de ER no DM por quatro vias principais: o aumento do fluxo da via poliol, hiperatividade da via hexosamina, ativação da proteína quinase C (PKC), aumento da formação de produtos finais de glicação avançada (AGEs), elevação na expressão de receptores para AGEs (RAGE) e ativação de seus ligantes (Figura 3) (GIACCO e BROWNLEE, 2010; VANESSA FIORENTINO et al., 2013).

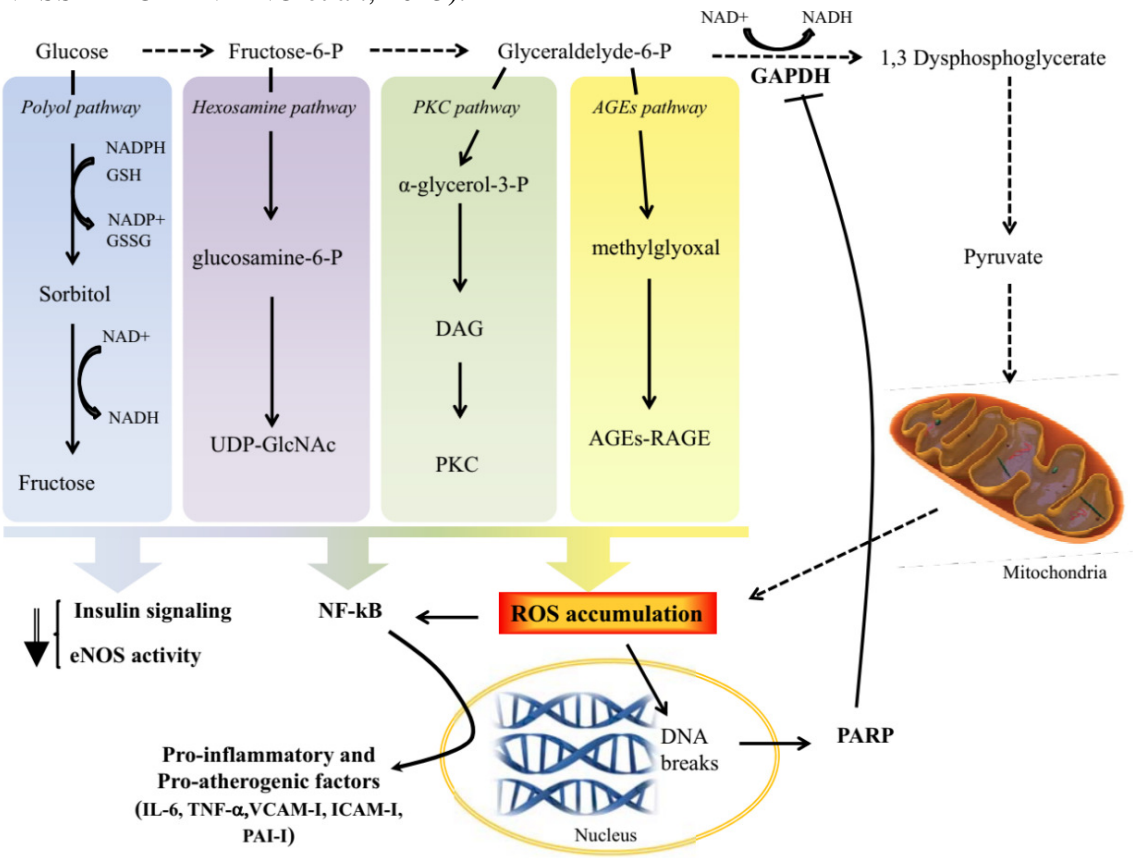

Figure 3. Estresse oxidativo induzido pela hiperglicemia. A hiperglicemia pode ativar as vias poliol, hexosamina, proteína quinase C (PKC) e de geração de produtos finais de glicação avançada (AGEs), bem como, seus ligantes e receptores (RAGE). Os elevados níveis de glicose causam prejuízos na sinalização insulínica e atividade da óxido nítrico sintase endotelial (eNOS) promovendo aumento na expressão de fatores pró-inflamatórios e pró-coagulantes, além de acúmulo de espécies reativas (ER). A hiperglicemia promove disfunção mitocondrial induzindo também ao acúmulo de ER. Elevados níveis de ER levam ao aumento da expressão do fator nuclear-Kappa B (NF-κβ) e estimula a produção de moléculas pró-inflamatórias e pró-coagulantes. ER e NF-kB provocam danos ao DNA. Quebras no DNA podem ativar a poli (ADP-ribose) polimerase (PARP) que inibe a gliceraldeído-3-fosfato desidrogenase (GAPDH) aumentando todos os intermediários glicolíticos e amplificando o estresse oxidativo. Adaptado de Fiorentino et al. (2013).

Através da via poliol, a glicose é convertida a sorbitol por redução da NADPH, reação catalisada pela enzima aldose redutase (AR) e subsequentemente, o sorbitol é oxidado à frutose pela enzima sorbitol desidrogenase (SDH) com concomitante geração de NADH (aumento da taxa NADH/NAD^+). Desse modo, o decréscimo de NADPH (co-fator requerido na redução de glicose a sorbitol via AR e fundamental para a regeneração da enzima antioxidante glutationa reduzida, GSH) e o aumento da taxa NADH/NAD^+ (as moléculas de NADH produzidas durante a conversão de sorbitol à frutose são oxidadas na cadeia respiratória gerando superóxido e outras ER) podem exacerbar o estresse oxidativo (CHIKEZIE et al., 2015).

A via de biossíntese da hexosamina é o menor ramo da glicólise respondendo pela metabolização de cerca de 3% da glicose. Nesta via ativada pela hiperglicemia, a glicose é convertida, por ação da glutamina:frutose-6-fosfato amidotransferase (GFAT), em glucosamina-6-fosfato (BUSE, 2006). Esta molécula é metabolizada a uridina difosfato Nacetilglicosamina (UDP-GlcNAc) que sob a ação da enzima O-GlcNAc transferase (OGT) leva à modificação pos-translacional de proteínas como fatores de transcrição reguladores da expressão de genes inflamatórios e pró-trombóticos como VCAM-1, PAI-I, ICAM-I, além de causar prejuízos na fosforilação de substratos do receptor de insulina (resistência insulínica) na vasculatura via inibição da eNOS desencadeando o estresse oxidativo (RAJAPAKSE et al., 2009) (Figura 3).

As concentrações do metabólito glicolítico, gliceraldeído-3-fosfato, são elevadas sob hiperglicemia induzindo, através do α-glycerol-3-fosfato e diacilglicerol (DAG), a uma maior atividade da PKC (YAO e BROWNLEE, 2010). O aumento na atividade da PKC promove, entre outros efeitos, a produção de ER por ativação de enzimas peroxidases e cicloxigenase. A atividade aumentada da PKC também contribui para promoção de dandos vasculares estimulando a produção de ER via maior expressão da enzima NADPH oxidase (NOX), inibição da óxido nítrico sintase induzida (eNOS), aumento da expressão de PAI-I e NF-κβ (LEE et al., 1989; CHIKEZIE et al., 2015).

Sob elevadas concentrações de glicose, ocorre espontaneamente, reação entre glicose e grupos amino de proteínas gerando resíduos glicados estáveis chamados produtos Amadori (THORNALLEY, 2002; MCGROWDER et al., 2013). A transformação dos produtos Amadori (gerando 3-desoxiglicosona), associada à glicoxiais (produtos da auto-oxidação da

glicose) e metilglicoxiais (substâncias derivadas da fragmentação de gliceraldeído-3-fosfato e dihidroxiacetona fosfato) são responsáveis pela geração dos AGEs, componentes tóxicos encontrados em elevadas quantidade no diabetes (CHIKEZIE et al., 2015). Os AGEs podem causar modificações estruturais e funcionais em proteínas intracelulares. Secundariamente, os AGEs podem formar reações cruzadas com proteínas da matriz extracelular (ex: integrinas expressas na superfície celular) provocando redução da flexibilidade e anormalidade na interação destas com outros componentes da matriz. Proteínas plasmáticas também sofrem modificações causadas pelos AGEs (Ex: albumina) e posteriormente, podem se ligar ao receptor RAGE induzindo à produção de ER por diferentes mecanismos como ativação da via dos poliois, ativação da PKC e fator de (TGF-β) e ativação do fator nuclear (NF-κβ) (CREAGER et al., 2006).

O estresse oxidativo amplifica a ativação do NF-κβ estimulando a produção de diversos genes associados à processos inflamatórios como interleucinas (IL-1, 6, 8), fator de necrose tumoral (TNF-α), interferon-γ (IFN-γ), molécula de adesão (VACAM-I), antígeno inibidor de plasminogênio (PAI-I), proteína quimiotáctica de monocitos 1 (MCP-I) e causando danos ao DNA. As lesões causadas ao DNA podem ativar a poli(ADP-ribose) polimerase (PARP) que inibe a gliceraldeido-3-fosfato desidrogenase (GAPDH)(VANESSA FIORENTINO et al., 2013). Os AGEs podem estimular a atividade da PKC via ativação do NFκβ e NOX o que pode aumentar a geração mitocondrial de ER e citocinas pro-inflamatórias agravando o estresse oxidativo (SIMM et al., 1997).

A hiperglicemia está associada ao aumento do risco cardiovascular em pacientes diabéticos (COLLABORATION, 2010; 2011). De fato, as doenças cardiovasculares são consideradas a maior causa de mortalidade de pessoas com DT1 (LIBBY et al., 2005). Dados sobre a mortalidade por eventos cardiovasculares em portadores do DT1 tem mostrado que o risco aumenta com a nefropatia (pacientes com proteinúria apresentam 37 vezes mais chances de óbito) culminando em hipertensão e desordens do perfil lipídico (BORCH-JOHNSEN e KREINER, 1987). Estudos de meta-análise também têm mostrado que pacientes com DT1 possuem risco de elevação de lipídios devido a fatores como circunferência abdominal, sexo, idade e níveis de hemoglobina glicada (Hbg), sendo que, o tratamento com estatinas foi ineficaz para controle da dislipidemia associada às concentrações aumentadas de Hbg sugerindo que o

controle glicêmico é fundamental para controle lipídico no DT1 (SEIDAH et al., 2003; STEIN et al., 2012).

A fisopatologia da dislipidemia no DT1 não é completamente compreendida, entretanto, está estabelecido a importante relação entre os níveis séricos de lipídios aumentados, a hiperglicemia e resistência à insulina, provocados pelo uso da insulina subcutânea (VERGÈS, 2001). A insulina é fundamental para regulação do metabolismo lipídico, pois, possui ação anti-lipolítica reduzindo o fluxo de ácidos graxos do tecido adiposo (por inibição da lipase hormônio-sensível) para a circulação, contudo, a terapia crônica empregando insulina por via subcutânea induz à hipersinsulinemia periférica modificando o metabolismo das lipoproteínas (VERGÈS, 2009).

A dislipidemia, característica frequente no diabetes, está relacionada com o desenvolvimento da doença aterosclerótica (CHAPMAN et al., 2011). Pacientes com DT1 apresentam 2 a 4 vezes mais chances de desenvolver aterosclerose quando comparados a pacientes não-diabéticos e os eventos cardíacos no DT1 representam 44% do total de mortes (HOMMA et al., 2015). Estudo realizado por Homma e colaboradores (2015) utilizando pacientes brasileiros jovens (5-19 anos) com DT1 mostrou que 81,7 % destes apresentaram dislipidemia com elevação de colesterol total e lipoproteína LDL.

Evidências mostram que há diferenças entre o perfil de lipoproteínas presentes no diabetes tipo 2 (DT2) e tipo 1 devido às suas distintas fisiopatologias. A dislipidemia no DT2 é caracterizada por hipertrigliceridemia (associada à elevação de LDL) e redução dos níveis de HDL, enquanto no DT1, pode ocorrer hipertrigliceridemia e frequentemente os valores de HDL estão normais, salvo se o paciente apresenta nefropatia ou taxas de glicose sérias não controladas (DEAN e DURRINGTON, 1996). No entanto, trabalho consuzindo por Vergès (2009) especifica que pacientes com DT1 não-controlado apresentam hipertrigliceridemia e níveis elevados de LDL (VERGÈS, 2009). Estes resultados concordam com os encontrados por Mona e colaboradores (2015) que verificaram que pacientes com DT1 apresentam dislipidemia caracterizada principalmente por altos níveis de LDL e também por redução nas concentrações de HDL (MONA et al., 2015). Do mesmo modo, Hassan e colaboradores (2015) destacaram com base em seus dados que a principal anormalidade lipídica no DT1 é a elevação nas concentrações de LDL, afetando metade dos pacientes (HASSAN et al., 2015).

As partículas de LDL, remanescentes de VLDL, são consideradas as principais lipoproteínas aterogênicas devido a grande capacidade de penetração e deposição nas paredes das artérias causando redução da luz do vaso e inflamação (DEEDWANIA et al., 2006). As LDL são partículas heterogêneas pertencentes a 4 subespécies maiores: LDL-I (grandes), LDL-II (médias), LDL-III (pequenas) e LDL-IV (muito pequenas) (GRIFFIN et al., 1990). Estas diferem em composição química, densidade, tamanho, aterogênicidade e comportamento metabólico e oxidativo. Dados sugerem que quanto menor e mais densas são as lipoproteínas LDL, mais susceptíveis serão à oxidação levando ao desenvolvimento do fenótipo lipoproteico aterogênico. Estas características permitem que as lipoproteínas LDL infiltrem no espaço endotelial, onde não há antioxidantes plasmáticos, ficando sujeitas à oxidação (RIZZO e BERNEIS, 2006). Os macrófagos interagem com as LDLs oxidadas (LDL-ox) por meio de seus receptores como CD36 e as fagocita, o que aumenta o conteúdo de colesterol no interior dos macrófagos (KETELHUTH e HANSSON, 2011). A elevação no teor interno de colesterol e gotículas de lipídios fazem com que os macrófagos assumam a forma de células espumosas amplificando a quimiotaxia de linfócitos e outros mediadores da inflamação que podem levar à ruptura da placas de gordura e, consequentemente, causando trombose e isquemia (PELUSO et al., 2012). Assim, a LDL-ox é um importante preditor de doenças relacionadas ao estresse oxidativo como a aterosclerose e possui correlação positiva com a hipertrigliceridemia (KONDO et al., 2001; ITABE, 2012).

Os elevados níveis de LDL estão associados à hipertrigliceridemia, maior fator de risco para doenças cardiovasculares (TENENBAUM et al., 2014). Os mecanismos fisiopatológicos da hipertrigliceridemia no diabetes envolve o controle glicêmico exercido pela insulina, de modo que, a deficiência ou resistência à insulina ativa a lipase-hormônio sensível intracelular promovendo maior fluxo de ácidos graxos livres não-esterificados (AGNEs) dos triacilgicerois estocados principalmente no tecido adiposo. (VERGÈS, 2015). Os AGNEs em elevadas concentrações na circulação promovem superprodução hepática de triacilglicerois secretados na forma de VLDL (ADELI et al., 2001).

A montagem de VLDL nos hepatócitos é dependente da proteína microssomal de transferência de triglicerídeos (MTP), uma chaperona heterodímera residente no lúmen do retículo endoplasmático essencial para a incorporação de lipídeos neutros às partículas de apolipoproteína B 100 (apoB-100), estrutura sintetizada no fígado que é componente

fundamental do VLDL e serve como parâmetro para mensuração do número de lipoproteínas aterogênicas) sendo esta uma etapa limitante na formação e secreção de VLDL (SEGREST et al., 2001; DHOTE et al., 2011). Embora a atividade exacerbada da MTP esteja associada à hipertrigliceridemia via produção de VLDL, a literatura mostra que aberrações no gene que codifica a MTP provocando perda de sua atividade e causa uma rara doença autossômica, a abetalipoproteinemia, caracterizada por defeitos na montagem e secreção de partículas ricas em lipídeos e deficiência de vitaminas (BERRIOT-VAROQUEAUX et al., 2000).

O processo de síntese de VLDL é contra-regulado pela insulina e depende da disponibilidade de substrato, pois, na presença de lipídios, a glicoproteína apoB nascente é lipidada pela MTP, enquanto na ausência de lipídios, a apoB não sofre lipidação sendo direcionada para a degradação proteassomal (KAMAGATE e DONG, 2008) (Figure 4).

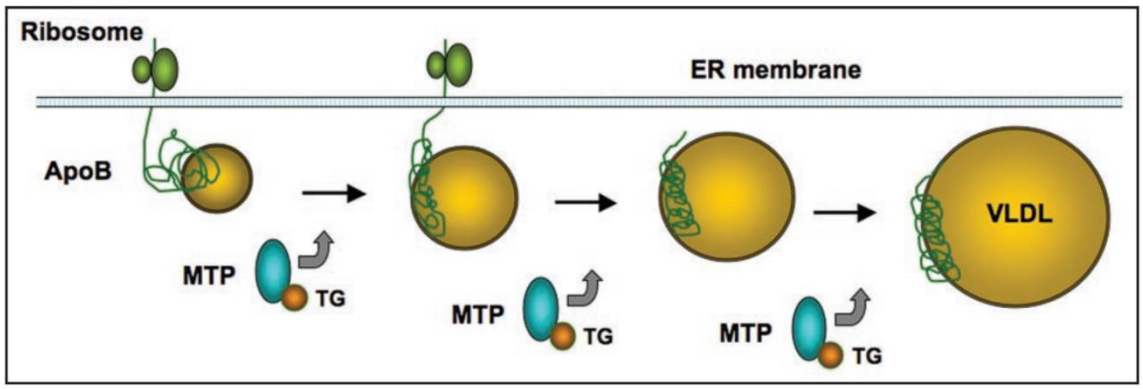

Figure 4. Participação da proteína microssomal transferidora de triacilgliceróis (MTP) na montagem de VLDL nos hepatócitos. A montagem de VLDL inicia com a lipidação de polipeptídeos apoB nascentes em um processo mecanicamente acoplado com tradução da apoB e translocação para o lúmen do retículo endosplamático. A MTP atua como uma chaperona no transporte de lipídios para moléculas de apoB nascentes, resultando na produção de partículas VLDL ricas em triacilglicerois. Há controvérsias se a MTP é necessária para o estágio final da montagem VLDL, considerando que, os lipídios são incorporados na região central da apoB mal lipidada para a maturação de partículas de VLDL. Adaptado de Kamagate & Dong. (2008).

A síntese de VLDL ocorre nos hepatócitos em duas principais etapas: a primeira dá-se no retículo endoplasmático rugoso e a segunda no retículo endoplasmático liso (OLOFSSON et al., 2000). Na primeira etapa, a apoB-100 é co-translacionadamente e pós-transladacionalmente lipidada pela MTP levando à formação de pre-VLDL. A segunda etapa é conduzida pelo ARF-1 (O fator de ribosilação do ADP) e ativação da fosfolipase D, proteínas

responsáveis pela conversão da pre-VLDL em VLDL no interior de compartimentos do retículo endoplasmático liso (OLOFSSON et al., 2000; VERGÈS, 2009).

A expressão da MTP hepática é regulada pelo FoxO1 (forkhead box O1), fator de transcrição com importante papel na sinalização à insulina via regulação das proteínas PKB/Akt (proteína quinase B). O FoxO1 reside no núcleo e exerce sua função de aumentar a expressão de MTP ao ligar-se ao elemento de resposta à insulina (IRE), porém, quando a insulina interage com seu receptor, o FoxO1 é fosforilada via PI3K e exportada para o citosol levando a inibição da expressão gênica. A supressão do FoxO1 inibe a gliconeogênese por ativação da G6Pase (glicose-6-fosfatase) e PEPCK (fosfoenolpiruvato carboxiquinase) e reduz a síntese de Apo B e VLDL por inibir a expressão da MTP (ALTOMONTE et al., 2003). Desse modo, FoxO1 é um promissor alvo terapêutico, pois, sua inibição culmina em reversão do quadro de hiperglicemia por ativação da G6Pase (glicose-6-fosfatase) e PEPCK (fosfoenolpiruvato carboxiquinase) e hipertrigliceridemia via maior ativação e expressão da MTP (KAMAGATE et al., 2008).

Trabalhos tem mostrado que a inibição farmacológica da atividade da MTP reduz a produção de VLDL e os níveis plasmáticos de colesterol em indivíduos com hipercolesterolemia hereditária (CUCHEL, MARINA et al., 2007) e reduz o risco de desenvolvimento da aterosclerose (PHILLIPS et al., 2012). Indivíduos tratados com antagonistas da MTP hepática apresentaram elevadas concentrações plasmáticas de transaminases devido o grande acúmulo de lipídeos nos hepatócitos (CUCHEL, MARINA et al., 2007). Assim, são necessários novos métodos para inibição da MTP sem que ocorra o desenvolvimento de esteatose hepática. Uma alternativa para evitar a injúria hepática pelo acúmulo de lipídeos é a inibição específica da MTP intestinal. Contudo, a deficiência da atividade da MTP intestinal causa esteatorréia, naúseas, flatuência e outros distúrbios gastrointestinais (AGGARWAL et al., 2005). Outra estratégia para evitar a toxicidade hepática é o emprego de compostos antioxidantes que reduzem o acúmulo de lipídios nos hepatócitos e os danos oxidativos (PISONERO-VAQUERO et al., 2015). Flavonoides como a naringenina e hesperidina (extraídos de *Citrus maxima*) e genesteína e daidzeína (isolados de Glycine max) foram reportados como inibidores da atividade da MTP hepática (CHANDLER et al., 2003), efeito associado à importante atividade antioxidante destes metabólitos (WILCOX, L. J. et al., 2001).

De acordo com Halliwell (2007), antioxidante é qualquer substância que quando presente em baixa concentração comparada à do substrato oxidável, regenera o substrato ou previne significativamente sua oxidação (HALLIWELL, 2007). De maneira mais específica, antioxidantes são moléculas que neutralizam radicais livres por doação ou recepção de elétrons. Podem ainda inibir a sua formação ou transformá-las em espécies menos reativas. Esses agentes capazes de proteger as células contra danos oxidativos podem ser classificados em antioxidantes enzimáticos e não-enzimáticos (HELMUT, 1993). Os antioxidantes enzimáticos compreendem os sistemas de defesa endógenos como as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GHPx) e outras (Figura 5). Enquanto, os antioxidantes não-enzi-

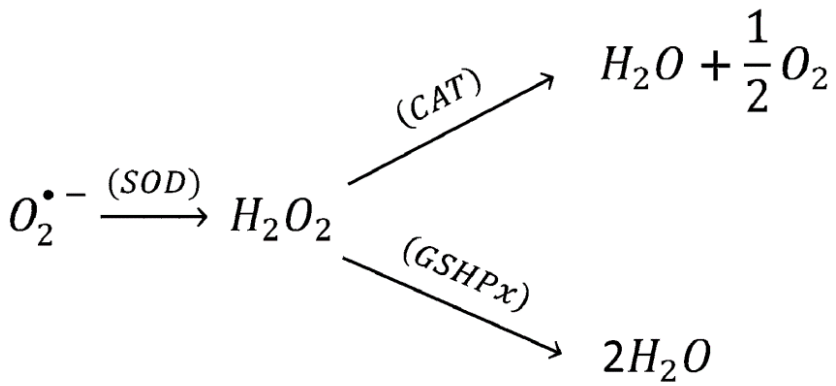

Figura 5. Sequestro radicalar promovido por antioxidantes enzimáticos: superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GSHPx). A SOD (citosólica ou mitocondrial) converte o ânion radical superóxido ($O_2^{\bullet -}$) em peróxido de hidrogênio (H_2O_2) na presença dos cofatores zinco, manganês ou cobre. A CAT, presente no peroxissomo, converte em H_2O_2 em O_2 e a GSHPx, presente no meio extracelular e no citoplasma, trasnformar H_2O_2 em H_2O. Adaptado de Nimse & Pal (2015).

máticos são representados pelo ácido ascórbico (vitamina C), α-tocopherol (vitamina E), glutathiona (GSH), carotenoides, polifenois, e outras moléculas antioxidantes obtidos de fontes exógenas (VALKO et al., 2007) (Figura 6).

As plantas possuem a capacidade intrínseca de sintetizar antioxidantes naturais não-enzimáticos capazes de sequestrar ERO e reduzir os efeitos deletérios do estresse oxidativo (KASOTE et al., 2015). As moléculas antioxidantes extraídas de plantas receberam grande atenção desde a descoberta do ácido ascórbico e, após este marco, as pesquisas envolvendo antioxidantes naturais de plantas tem crescido substancialmente, pois, há evidências que o

estresse oxidativo é o maior fator para desencadeamento e progressão de doenças cardiovasculares e neurodegenerativas (KASOTE et al., 2013). Desse modo, a suplementação com antioxidantes naturais possui propriedades terapêuticas, ao passo que, os antioxidantes sintéticos como BHA (2,3-terc-butil-4-hidroxianisol) e BHT (2,6-diterc-butil-p-creso), largamente utilizados na indústria alimentícia, podem ser carcinogênicos em elevadas concentrações (SHAHIDI e ZHONG, 2010).

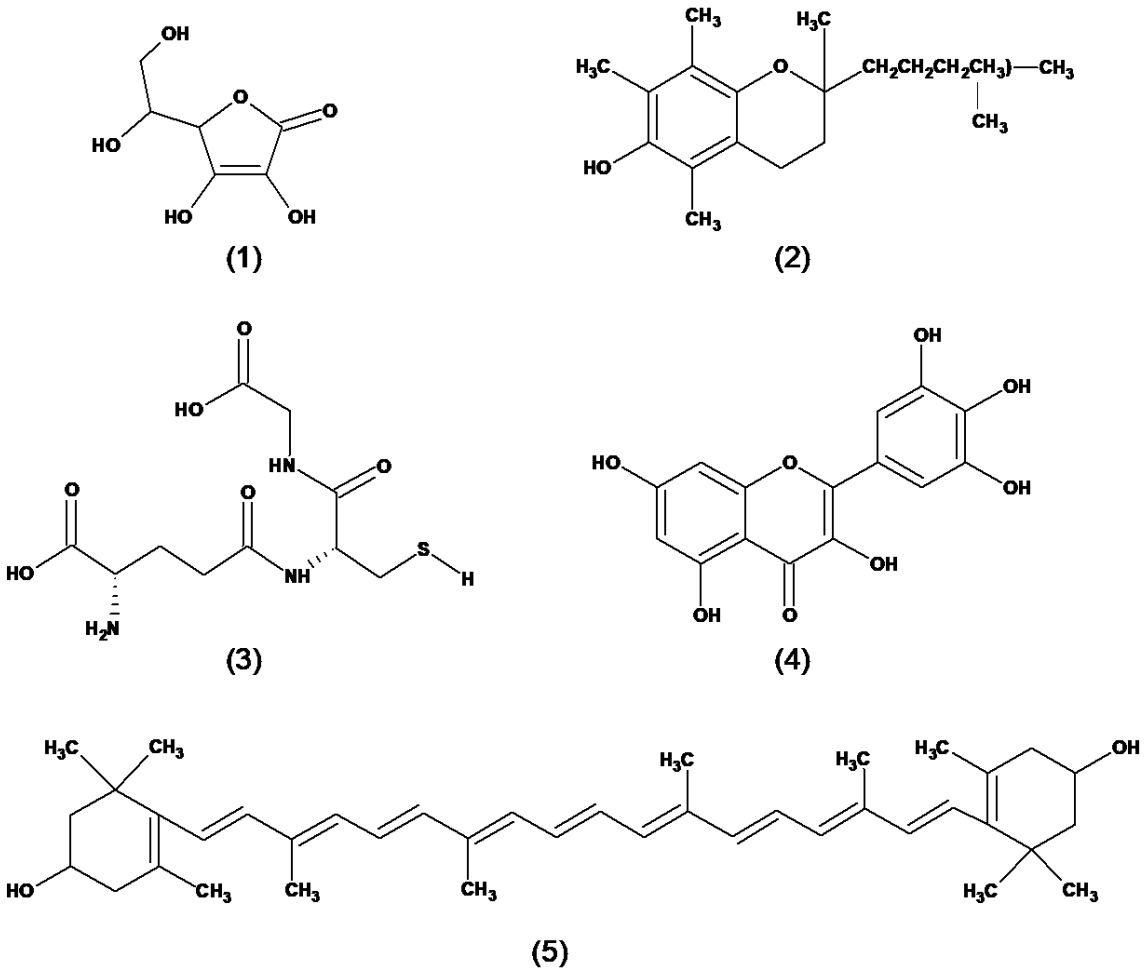

Figura 6. Representantes de antioxidantes não-enzimáticos. Estes antioxidantes naturais atuam interrompendo as reações das espécies reativas. (1) ácido ascórbico, (2) α-tocoferol, (3) glutationa reduzida, (4) miricetina, (5) β-caroteno.

O reino vegetal é uma fonte riquíssima de moléculas antioxidantes naturais, dentre estas, se destacam os polifenois, pois, apresentam uma varidade de atividades biológicas

associados à sua capacidade antioxidante como atividade cardioprotetora, anti-inflamatoria, antimicrobiana, anti-envelhecimento, anticâncer e outras, sendo portanto, úteis na prevenção e manejo de doenças crônicas associadas ao estresse oxidativo como câncer, doenças neurodegenerativas, cardiovasculares e diabetes (LI et al., 2014).

A dieta rica em compostos fenólicos possui propriedades terapêuticas semelhantes à apresentada por fármacos clinicamente empregados no controle do diabetes, sendo, estes benefícios exercidos por diferente vias de sinalização promovendo: redução da resistência à insulina, inflamação e estresse oxidativo no músculo e gordura; aumento da captação de glicose no músculo esquelético e tecido adiposo; regulação do metabolismo glicídico nos hepatócitos; melhora na sercreção de insulina, redução da apoptose e aumento na proliferação de células β-pancreáticas (BABU et al., 2013). Informações epidemiológicas também suportam que a dieta rica em polifenois reduzem os níveis de lipoproteínas VLDL e triacilglicerois em pacientes dislipidêmicos tanto em jejum quanto no pós-pradialmente (ANNUZZI et al., 2014).

Os compostos fenólicos são produto do metabolismo secundário derivados das rotas chiquimato e acetato malonil e nos vegetais podem estar em formas livres ou complexadas a açúcares e proteínas (LÜ et al., 2010). Detém um importante papel antioxidante devido suas características estruturais: nucleofílicidade do anel benzênico e caráter ácido dos grupos fenólicos hábeis na deslocalização de elétrons desemparelhados presentes nos radicais livres (PAIXÃO et al., 2007) (Figura 7). Estes metabólitos secundários podem inibir a geração de espécies reativas por quelar metais de transição ou inibir a atividade de enzimas oxidativas como as cicloxigenases, xantina oxidase, neutralizar espécies reativas ou convertê-las em espécies menos reativas por doação de elétrons ou hidrogênio, (RICE-EVANS et al., 1996; HAVSTEEN, 2002) - e co-antioxidante – regeneração de vitaminas essencias (ZHOU et al., 2005) protegendo macromoléculas celulares contra danos oxidativos (LOTITO e FREI, 2006).

Os flavonoides são polifenóis reportados por exibir múltiplos efeitos biológicos como antividade antibacteriana, antiviral, anti-inflamatória e anti-câncer, associadas às suas propriedades antioxidantes (KUMAR, SHASHANK e PANDEY, ABHAY K, 2013). A atividade antioxidante dos flavonoides é dependente de suas estruturas nucleares e arranjo dos seus grupos funcionais. As substituições no anel B e o aumento no número de hidroxilas são cruciais para as propriedades antioxidantes. Dentre as principais características estruturais responsáveis pela capacidade sequestradora de ERO dos flavonoides estão:

1- a presença do grupo catecol (estrutura orto-dihidroxi) no anel B, determinante para a deslocalização eletrônica (Figura 6);

2-dupla ligação na posição 2,3 conjugada à função cetônica no C-4 no anel C, importante para reforço da deslocalização de elétrons no anel B (Figura 6);

3-grupos hidroxilas na posição 3 e 5 do anel A e C, respectivamente, proporcionando a formação de pontes de hidrogênio com o grupo cetônico (Figura 6).

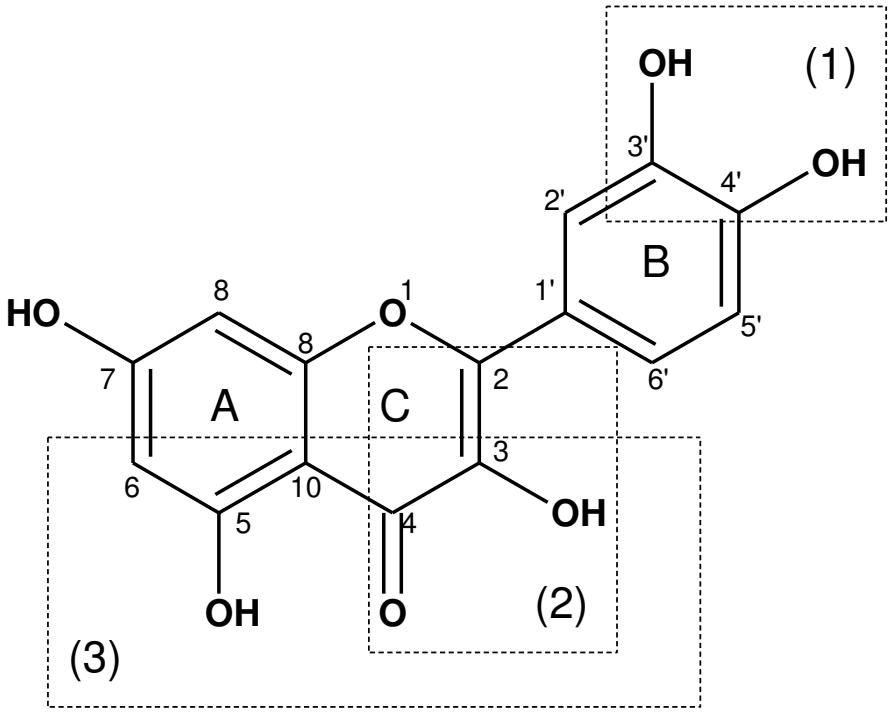

Figura 7. Principais características estruturais dos flavonoides requeridas para sequestro de espécies reativas. (1) Presença do grupo orto-dihidroxi (catecol) no anel B; (2) dupla ligação em C-2,3 e carbonila no anel C; (3) hidroxila em C-3 (anel A) e C-5 (anel C).

A capacidade sequestradora das ER pelos flavonoides ocorre devido a habilidade destes metabólitos na doação de átomos de hidrogênio (Figura 8) (HEIM, KELLY E et al., 2002). Flavonoides podem prevenir os danos causados pelas espécies reativas por diversos mecanismos que incluem sequestro direto de ER, quelação de metais, ativação de enzimas antioxidantes, aumento das propriedades antioxidantes de pequenas moléculas com atividade antioxidante, redução de radicais α-tocoferol, atenuação de estresse oxidativo causado por

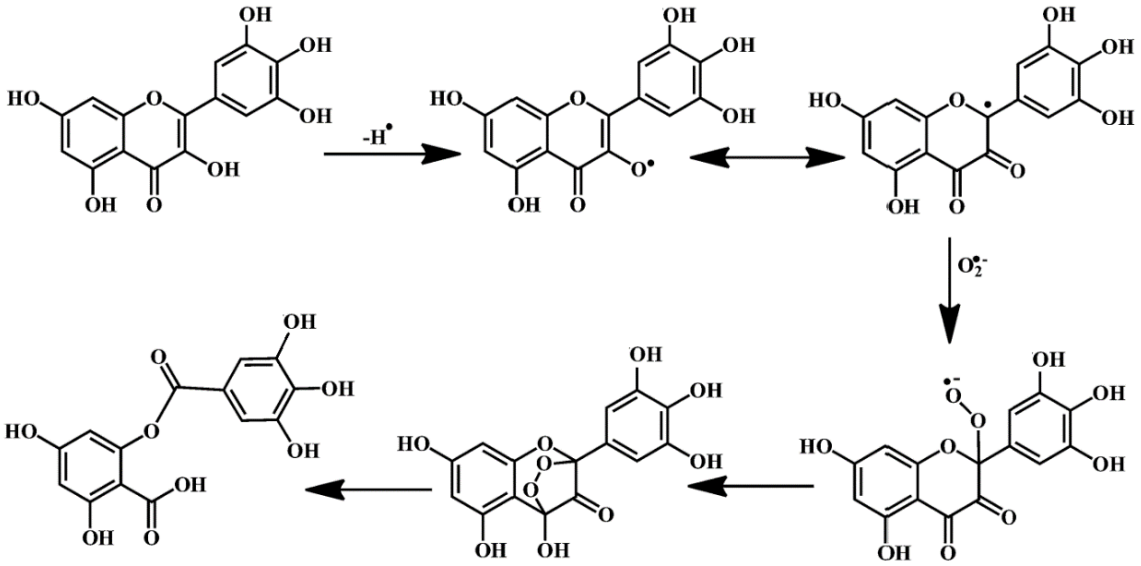

Figura 8. Mecanismo da atividade da miricetina no sequestro do ânion radical superóxido

óxido nítrico, aumento nos níveis de ácido úrico e inibição de oxidases (PROCHÁZKOVÁ et al., 2011). Além de agirem por diferentes mecanismos de ação, os flavonoides possuem capacidade antioxidante superior à apresentada pelas vitaminas C e E (PRIOR e CAO, 2000), por isso, apesar de não serem considerados nutrientes, os flavonoides são importantes para promoção da saúde humana (PROCHÁZKOVÁ et al., 2011).

A potente atividade antioxidante advinda das estruturas dos flavonoides tornam estes metabólitos uteis na prevenção e atenuação dos danos causados pelas ER, e, ademais, estes metabólitos são ubíquos no reino vegetal com mais de 10 000 estruturas identificadas (AGATI et al., 2012).

Syzygium cumini (L.) Skeels, também conhecido como *Eugenia jambolana*, *Syzygium jambolanum, Eugenia cumini*, é uma espécie medicinal pertencente à família Myrtaceae que possui elevado teor de flavonoides (ESHWARAPPA et al., 2014; CHAGAS et al., 2015; PRIYA et al., 2017).

Diferentes partes de *S.cumini* são utilizadas na medicina popular devido suas atividades anti-carminativa, anti-helmíntica, hepatoprotetora, anti-hiperlipidêmica, anti-ulcerogênica, anti-alérgica e anti-inflamatória (AYYANAR e SUBASH-BABU, 2012). No

entanto, a espécie tem sido principalmente empregada contra o diabetes mellitus e co-morbidades (SRIVASTAVA e CHANDRA, 2013).

Os efeitos anti-diabéticos de *S.cumini* podem ocorrer devido sua rica composição flavonoídica, visto que, os flavonoides podem melhorar a sinalização insulínica através da modulação de transportadores de glicose, promover proliferação e redução da apoptose das células β pancreáticas, reduzir à resistência insulínica, reduzir a inflamação e o estresse oxidativo (VINAYAGAM e XU, 2015). Contudo, trabalhos que apresentam a atividade moduladora de *S.cumini* sobre o metabolismo de carboidratos e lipídios no diabetes concentram-se em suas sementes (parte mais estudada do vegetal), assim, há necessidade de estudos *in-vivo* que demonstrem os efeitos farmacológicos das folhas de *S.cumini* no diabetes e suas complicações, bem como, possibilitem a determinação de flavonoides específicos como biomarcadores.

3 OBJETIVOS

3.1 Objetivo Geral

Caracterizar os os efeitos anti-diabéticos do Extrato Rico em Polifenois (ERP) das folhas de *Syzygium cumini* (L.) Skeels em ratos com diabetes induzida por estresse oxidativo.

3.2 Objetivos Específicos

Caracterizar a composição química do ERP da folha de *S. cumini*.

Investigar os efeitos do emprego do ERP sobre o perfil glicídico e lipídico de ratos diabeticos.

Avaliar a capacidade antioxidante do ERP em ensaios *in-vitro* e em modelo *in-vivo* de estresse oxidativo.

Investigar o efeito do ERP sobre a secreção de insulina.

Destacar os principais mecanismos descritos para as propriedades cardiometabólicas descritas para *S. cumini.*

Propor mecanismo para os efeitos do ERP sobre a hipertrigliceridemia presente no diabetes.

4 RESULTADOS

4.1 Capítulo I - *Syzygium cumini* (L.) Skeels: A prominent source of bioactive molecules against cardiometabolic diseases

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Artigo publicado no Frontiers in Pharmacology (ISSN: 1663-9812)

Fator de impacto: 4.400; Qualis Medicina I: A2

Mini-Review

Syzygium cumini (L.) Skeels: A prominent source of bioactive molecules against cardiometabolic diseases

Running title:

Cardiometabolic properties of *Syzygium cumini*.

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Conflict of Interest Statement: Authors declare the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abstract

Syzygium cumini (Myrtaceae) is a worldwide medicinal plant traditionally used in herbal medicines due to its vaunted properties against cardiometabolic disorders, which include: anti-hyperglycemic, hypolipemiant, anti-inflammatory, cardioprotective and antioxidant activities. These properties have been attributed to the presence of bioactive compounds such as phenols, flavonoids and tannins in different parts of the plant, albeit the knowledge on their mechanisms of action is scarce. This mini-review highlights the cardiometabolic properties of *S. cumini* by correlating its already identified phytochemicals with their described mechanisms of action. Data herein compiled show that some compounds target multiple metabolic pathways; thereby, becoming potential pharmacological tools. Moreover, the lack of clinical trials on *S. cumini* usage makes it a fruitful field of interest for both scientific community and pharmaceutical industry.

Keywords: black plum, jamun, myrtaceae, phenolic compounds, metabolic syndrome, ethnopharmacology, medicinal plants, complementary and alternative medicine.

1 Introduction

Cardiometabolic syndrome is associated with multiple risk factors including insulin resistance, dyslipidemia, hypertension and obesity (Alberti et al., 2009). According to World Health Organization, every year about 2.8 million people die worldwide due to overweight or obesity (Lim et al., 2013). Prevalence of diabetes appears with projections to affect about 439 million adults by 2030, whereas cardiovascular diseases account for 30% of deaths annually, including both developed and developing countries (Shaw et al., 2010). Because of their chronic degenerative nature, cardiometabolic-related disorders have long-lasting treatments, costly for both the patient and the health services, in addition to potentially harmful side effects caused by politherapeutic regimens (Yanovski and Yanovski, 2014). In this context, herbal medicines have become the major source of bioactive molecules and emerged as potential therapeutic tools to fulfill a multiple-target strategy, especially because of their inherented large-scale structural diversity as compared with synthetic compounds (Prabhakar and Doble, 2008;Huang et al., 2009).

Myrtaceae family comprises about 140 genera with 4620 species distributed mainly in tropical and subtropical areas (Lago et al., 2011). The genus *Syzygium*, a leading member of this family, embrace 1100 species with deserving attention to *Syzygium cumini* (L.) Skeels (syn.: *Eugenia jambolana*, *Syzygium jambolanum*), which has been used in the treatment of numerous diseases, especially diabetes (Ayyanar and Subash-Babu, 2012). The use of *S. cumini* was introduced in western medicine in the mid-nineteenth century, when the first reports on the investigation of its antidiabetic properties were published (Helmstadter, 2008). *S. cumini* is a large tree native

from Indian subcontinent, but widely cultivated in many countries in Asia, Africa and South America (Srivastava and Chandra, 2013). It is popularly known as jamun in India, black plum in Europe, jambolan in Spanish-spoken countries and jambolão in Brazil (Corrêa, 1984;Vizzotto and Pereira, 2008).

S. cumini is known to possess wide range of medicinal properties, which have been attributed to the presence of bioactive compounds in different parts of the plant .The leaves are used in dermopathies, gastropathies, constipation, leucorrhea and diabetes (Warrier et al., 1996); fruits are used in the treatment of pharyngitis and splenic diseases (Warrier et al., 1996); whereas barks are used as astringents (Rekha et al., 2008), anthelmintic and carminative (Warrier et al., 1996;Jagetia and Baliga, 2002). Furthermore, seeds are used as astringents, diuretic, and especially in the treatment of diabetes (Helmstadter, 2008;Baliga et al., 2011). Pharmacological studies have expanded the biological activities of *S. cumini*, which include anti-hyperglycemic (Kumar et al., 2008a;Sharma et al., 2008b;Sharma et al., 2011), anti-inflammatory (Muruganandan et al., 2001;Kumar et al., 2008b), antibacterial (Oliveira et al., 2007;Bag et al., 2012), cardioprotective (Mastan et al., 2009;Tanwar et al., 2011;Jadeja et al., 2012) and antioxidant (Rekha et al., 2008;Arun et al., 2011). Likewise, some remarkable and well-conducted literature surveys on pharmacological properties, phytochemical constituents as well as nutritive value of *S. cumini* have been published along the last decade (Helmstadter, 2008;Baliga et al., 2011;Ayyanar and Subash-Babu, 2012;Ayyanar et al., 2013;Baliga et al., 2013;Srivastava and Chandra, 2013).

Notwithstanding those findings, however, there is scarcity of data on the relationship of its secondary metabolities with the described biological effects, especially on the mechanisms of action of these compounds. Therefore, this mini-review is aimed to specifically study the cardiometabolic properties of secondary metabolites already identified in this plant species,

trying to correlate them with their potential mechanisms of action. For this purpose, a detailed literature survey has been carried out using both preclinical and clinical studies as an attempt to identify the molecular mechanisms of action for the compounds identified in the various parts of *S. cumini*, even considering they may not have been necessarily isolated from this plant species, but had the same chemical identity of those found in it.

2 Phytochemical constituents

Various secondary metabolites viz., terpenes, phenols, and especially phenolic acids, flavonoids and tannins have been reported in different parts of *S. cumini* (Table 1). For instance, leaves of this plant species contain high levels of flavonoids, especially quercetin, myricetin, myricitrin, kaempferol, kaempferol 3-O-acetyl-α-L 4-rhamnopyranoside, kaempferol-3-O-β-D-glucuronopyranoside in addition to simple phenols: ellagic acid, ferulic acid, chlorogenic acid and gallic acid (Mahmoud et al., 2001;Timbola et al., 2002;Ruan et al., 2008). The essential oil present leaves is high in terpenes such as α-pinene, β-pinene, α-limonene, α-cadinol, pinocarvone, pinocarveol (Shafi et al., 2002;Mohamed et al., 2013). The seeds are the most studied part of the plant, being especially high in hydrolyzable tannins terpenes and phenolic acids, which are abundant throughout the plant (Ramya et al., 2012). The flowers and seeds are found to have similar chemical composition, although studies on identification of compounds in flowers are scarce (Baliga et al., 2011;Gordon et al., 2011). In addition to the compounds mentioned above, the fruits also contain anthocynins as cyanidin, delphinidin and petudinine which impart bright violet color (Ramya et al., 2012). Stem bark has essentially the same phenolic acids, flavonoids and terpenes, as described already for other parts (Bhatia and Bajaj, 1975;Baliga et al., 2011).

Table 1. Phytochemical compounds identified in different parts of *Syzygium cumini*.

Part of plant	Secondary Metabolite Class	Identified Compounds	Reference
Leaves	Flavonoids	catequin, kaempferol, myricetin, myricetin 3-O-β-D-glucuronopyranoside, myricetin-4'-metil ether 3-O-α-rhamnopyranoside, myricetrin 4''-O-acetate, myricetrin 4''-O-acetyl-2-O-gallate, myricitrin, quercetin -3-O-α-rhamno_ pyranoside.	Mahmoud et al., 2001
	Phenolic acids	ellagic acid, gallic acid, ferulic acid	Mahmoud et al., 2001; Ruan et al., 2008b
	Tannins	nilocetin.	(Mahmoud et al., 2001)
	Terpenes	α-pinene, α-cadinol, pinocarvone, pinocarveol, α-terpeneol, myrtenol, eucarvone, muurolol, myrtenal, cineole, geranylacetone.	(Shafi et al., 2002;Mohamed et al., 2013)
Seeds	Flavonoids	Rutin, quercetin, 3,5,7,4'-tetrahydroxy flavanone	(Bhatia and Bajaj, 1975;Karthic et al., 2008;Srivastava and Chandra, 2013)
	Phenolic acids	gallic acid , elagallic acid, ferulic acid, caffeic acid	(Bhatia and Bajaj, 1975;Ramya et al., 2012)
	Tannins	corilagin, 3,6-HHDP glucose, 4,6-HHDP glucose, 1-galloyl glucose, 3–galloyl glucose	Bhatia and Bajaj, 1975
	Terpenes	β-pinene, β-terpinene, ,α-terpineol, eugenol.	Ramya et al., 2012
Fruits	Flavonoids	myricetin, myricetin deoxyhexoside	Gordon et al., 2011
	Phenolic acids	gallic acid	Gordon et al., 2011
	Tannins	trigalloylglucose, HHDP-galloyl glucose	Gordon et al., 2011
	Terpenes	citronellol, geraniol, , hotrienol, nerol, β-phenilethanol , phenylpropanal	Ramya et al., 2012
	Anthocyanins	cyanidin, delfinidin, petudinin	Ramya et al., 2012
Flowers	Flavonoids	isoquercetin, quercetin, kaempferol	Ramya et al., 2012
	Phenolic acids	ellagic acid	Baliga et al., 2011
	Terpenes	eugenol, oleanolic acid	Ramya et al., 2012
Stem barks	Flavonoids	myricetin, quercetin, kaempferol	Baliga et al., 2011
	Phenolic acids	3,3'-di-O-methyl ellagic acid, 3,3', 4-tri-O-methyl ellagic acid, gallic acid	Bhatia and Bajaj, 1975; Ramya et al., 2012
	Terpenes	β-siterol, friedelin, betulinic acid	Baliga et al., 2011

Abbreviations: HHDP, hexahydroxydiphenic acid.

3 Cardiometabolic properties and potential mechanisms of action

3.1 Antihyperglycemic activity

Use of *S. cumini* in the fight against diabetes has been studied by western medicine since more than 130 years (Helmstadter, 2008). In recent years, numerous pre-clinical studies have evaluated extracts of various parts, especially seeds, of this plant species in maintaining anti-hyperglycemic activity (Ravi et al., 2003;Schossler et al., 2004;Anandharajan et al., 2006;Sharma et al., 2008a;Sharma et al., 2008b;Ramya et al., 2012;Sharma et al., 2012). The ethanolic extract of seeds, when administered for 30 days at the dosis of 100 mg/kg/day to rats with streptozotocin-induced diabetes, was found to decrease both blood and urine glucose levels (Ravi et al., 2003). In the same experimental model, administration of a flavonoid-rich extract from seeds reduced blood glucose levels and restored peripheral glucose tolerance in rats (Sharma et al., 2008a) as well as mice (Sharma et al., 2008b). These effects were also observed in cultured 3T3-L1 and attributed to increased activity of PPARs receptors (receptors activated by peroxisome proliferator human, precisely the PPARγ and PPARα) (Sharma et al., 2008a). Furthermore, it increases insulin sensitivity by inducing the production of the adipocyte-derived factors adiponectin and leptin (Yang et al., 2002;Valero-Muñoz et al., 2013), an effect already described for quercetin (Wein et al., 2010).

Rutin, a flavonoid present in the seed, showed anti-hyperglycemic effect, which was attributed to inhibition of hexokinase and glucose-6-phosphatase (Kamalakkannan and Prince, 2006;Sharma et al., 2008a). In addition, studies with extracts of seeds and husks from *S. cumini* have been reported to possess a major insulin secretagogue activity (Schossler et al., 2004;Sharma et al., 2008a). Among the various compounds identified in these parts of the plant (Table 1), it has also been shown that apigenin and rutin increased insulin concentrations in rats with streptozotocin-induced diabetes (Esmaeili et al., 2009), whereas quercetin stimulated

insulin secretion by increasing calcium influx through interaction with L-type calcium channels in isolated rat beta-cells (Bardy et al., 2013).

S. cumini has also been described to promote other insulin-related effects. Methanolic extract of leaves was found to increase mRNA expression of GLUT-4 glucose transporter and phosphatidylinositol-3 kinase (PI3 kinase), both important mediators of insulin action in adipocytes and skeletal muscle (Anandharajan et al., 2006). Myricetin, one of the most prevalent compound in the leaves, also promotes these same actions (Liu et al., 2007). *S. cumini* reduced by 50% the expression of aldose reductase in renal tissue of diabetic rats (Sharma et al., 2008b). Phenolic compounds such as myricetin (Haraguchi et al., 1998), quercetin, kampferol and ellagic acid have been reported as potent aldose reductase inhibitors (Haraguchi et al., 1998). The use of *S. cumini* elevated the concentrations of glycogen in liver and muscle, suggesting a stimulatory effect of glycogen synthase or glycogenolysis inhibition (Sharma et al., 2008b). These effects have been described for oleanolic acid (Ha et al., 2009) and caffeic acid (Jung et al., 2006), compounds found in flowers and seeds, respectively. Moreover, in vitro studies showed that betulinic acid and 3,5,7,4'-tetrahydroxy flavanone, isolated from *S. cumini* seeds, inhibited pancreatic α-amylase, what may cause diminished intestinal absorption of carbohydrates (Karthic et al., 2008).

3.2 Anti-hyperlipidemic activity

Flavonoid-rich extract from *S. cumini* seeds was described to possess anti-dyslipidemic activity by reducing total cholesterol, LDL-cholesterol and triacylglycerols as well as raising HDL-cholesterol levels in rats with streptozotocin-induced diabetes (Sharma et al., 2008b). Similar results were found when the aqueous extract of the fruits was applied (Rekha et al., 2008). Ravi and colleagues showed that hydroalcoholic extract from *S. cumini* seed kernel has anti-hyperlipidemic effects similar to glyburide and restores serum levels of free fatty acids,

cholesterol and triacylglycerols in streptozotocin diabetic rats (Ravi et al., 2005). In this same animal model, administration of Dihar, an Indian mixture of herbs containing *S. cumini* also reduced lipid serum levels (Patel et al., 2009)

Anti-hyperlipidemic properties of *S. cumini* have been ascribed mainly to the inhibition of HMG-CoA reductase, the enzyme responsible for cholesterol synthesis (Ravi et al., 2005;Sharma et al., 2008b;Sharma et al., 2011). The flavonoids present in *S. cumini* might account for this activity, as it was described that this class of compounds increases the expression of cAMP-dependent phosphokinase, enzyme responsible for HMG-CoA inhibition (Havsteen, 2002). Furthermore, quercetin promoted downregulation of adipogenic enzymes, particularly lipoprotein lipase, in OP9 preadipocytes (Seo et al., 2015). Nevertheless, such effects might also come from the reduction of intestinal absorption of cholesterol or increased free fatty acids and triacylglycerols clearances as a result of insulin actions improvement. (Ravi et al., 2005;Birari and Bhutani, 2007;Sharma et al., 2012). Preliminary data from our group has pointed out that anti-hyperlipidemic properties of *S. cumini* leaf may be due inhibition of both activity and expression of the hepatic isoform of microsomal triglyceride-transfer protein.

3.3 Antioxidant activity

Polyphenolic and related antioxidant compounds are recognized as important cardiometabolic agents since they scavenge reactive oxygen/nitrogen species (ROS, RNS) and stimulate antioxidant defenses (Valko et al., 2007). The oral administration of aqueous extract of *S. cumini* seeds to mice treated with urethane 7,12-dimethyl benzanthracene resulted in reduced chromosomal damage, significantly inhibited hepatic lipid peroxidation and reduced GSH levels that was associated with significantly increased activity of glutathione S-transferase, superoxide dismutase and catalase (Arun et al., 2011). Pretreatment of cyclophosphamide-administered rats with methanolic extract of *S. cumini* fruits reduced the formation of hepatic

malondialdehyde, inhibited the frequency of aberrant metaphases, the formation of micronuclei, cytotoxicity against marrow cells and increased the activity of antioxidant enzymes superoxide dismutase and catalase, besides increased GSH levels (Tripathi et al., 2013).

Considering in vitro studies, methanolic extract from leaves and branches has been shown to strongly act against $OH^{\bullet}$ and $DPPH^{\bullet}$ radicals, and decreased Fe^{3+} to Fe^{+2} reduction in the FRAP assay. These activities showed a strong correlation with the high content of polyphenols and flavonoids present in the extract (Eshwarappa et al., 2014). It has also been reported that the significant antioxidant activity of ethyl acetate fraction from the methanolic extract of *S. cumini* leaves is related to its polyphenolic composition, especially to ferulic acid and catechin present in the extract (Ruan et al., 2008). Aqil et al. (2012) reported that ethanolic extracts of fruits and seeds possess potent antioxidant activity in different assays, such as $ABTS^{\bullet+}$, $DPPH^{\bullet}$, FRAP and ORAC. These extracts also showed anti-proliferative activity on lung cancer cells, making them potential sources of anticarcinogenic agents. Anthocyanins (delphinidin, cyanidin, petunidin, peonidin and malvidin), besides ellagic acid and its derivatives (ellagitannins) identified in both extracts were suggested to be responsible for the antioxidant and anti-proliferative activities (Aqil et al., 2012). Antioxidant activity of *S. cumini* fruits was shown to be promoted by gallic acid, and derivatives of tetragalloyl glucose and myricetin (Gordon et al., 2011).

3.4 Cardioprotective activity

S. cumini has also been reported to promote hypotensive and antihypertensive effects. Administration of hydroalcoholic extract of S cumini leaves, as well as its chloroform and aqueous fractions, to normotensive rats reduced the blood pressure due to decreased reactivity of vascular smooth muscle through a suggestive presence of any noncompetitive antagonist of calcium channels (Ribeiro, 2007). Recently, the same group showed a dose-dependent

reduction of blood pressure and heart rate of spontaneously hypertensive rats (SHR) submitted to 8-weeks treatment with the same extract (Ribeiro et al., 2014). Those antihypertensive properties might be attributed to flavonoids such as myricetin, which was reported to promote potent vasodilation by impairment of calcium influx (Herrera et al., 1996). Notwithstanding, it was also verified that quercetin promotes vasospasmolytic effect probably by blockage of calcium influx through L-type calcium channels (Hou et al., 2014).

Besides its effects on vasomotricity, *S. cumini* seems to improve hemodynamics as well. In a recent work, platelets collected from diabetic patients were incubated with *S. cumini* extract resulting in lower platelet aggregation and decreased oxidative damage, as assessed by measument of lipoperoxide and nitric oxide levels and superoxide dismutase activity. The extract was still found to increase platelet cell membrane fluidity and to stimulate Na^+/K^+-ATPase activity. (Raffaelli et al., 2014). In earlier work, De Bona et al had already reported that aqueous extract of *S. cumini* leaf was able to decrease the activities of adenosine deaminase and 5'-nucleotidase, both important enzymes in the thrombogenic process, when incubated with platelets of diabetic patients. Phenolic compounds identified in the extract, such as gallic acid, chlorogenic acid and rutin, were suggested as possible candidates for the above mentioned activities (De Bona et al., 2010).

Administration of α-hydroxy cinnamic acid and other phenolic compounds isolated from the aqueous extract of *S. cumini* fruits to diabetic rats previously fed an atherosclerotic diet promoted antiatherosclerotic effects mainly characterized by decreased oxidized LDL levels, modulation of endothelial nitric oxide synthase (eNOS), lower expression of soluble vascular cell adhesion molecule-1 (sVCAM-1) and significant reduction of atherogenic lipoprotein Apolipoprotein B100 (Apo B100) along with an increase of Apolipoprotein A1 (Apo A1) (Tanwar et al., 2011). Moreover, Mastan et al evaluated the cardioprotective effects of *S. cumini*

on acute myocardial infarction induced by isoproterenol in rats. Treatment with methanolic extract of seed reduced the serum levels of myocardial necrosis biomarkers, specifically aspartate aminotransferase, alanine aminotransferase, uric acid, creatine phosphokinase and lactate dehydrogenase (Mastan et al., 2009).

3.5 Anti-inflammatory activity

Inflammatory processes are directly involved in the development of cardiometabolic diseases including atherosclerosis, type 2 diabetes and cancer (Hotamisligil, 2006;Okada, 2014;Angelovich et al., 2015). Methanolic and ethyl acetate extracts of *S. cumini* leaf reduced carrageenan-induced paw edema in rats (Jain et al., 2011). Methanolic extract of the seeds also reduced paw edema volume and leukocyte migration in rats with adjuvant-induced arthritis (Kumar et al., 2008c). On a deeper characterization, ethanolic extract of the bark was found to reduce the production of prostaglandin E2, serotonin and histamine (Muruganandan et al., 2002). Similar results were found for ethanolic extract of leaf, which reduced the systemic production of pro-inflammatory factors such as TNF-α and $NO^{\bullet}$ (Maciel et al., 2008) and interleukin-5 (Brito et al., 2007).

4 Toxicity

Several studies have shown that *S. cumini* do not produce acute or chronic toxicity when given by oral route. General toxicological screenings including behavioral, histomorphological as well as blood hematological and biochemical parameters have been conducted for extracts from seeds (Chaturvedi et al., 2007), fruit (Kumar et al., 2008a) and leaves (Silva et al., 2012) of *S. cumini*, with no toxic effect noticed. Silva and colleagues demonstrated that acute administration of hydroalcoholic extract of leaf at dosis as high as 2 g/kg produced no toxic effects in rodents. The same study demonstrated that chronic treatment for up to 180 days at doses of 50, 100 and 250 mg/kg did not cause hematological, biochemical or histological alterations in target organs (Silva et al., 2012). These results are corroborated by Bilal et al., whom demonstrated the potential hepatoprotective effect of ethanolic extract of *S. cumini* fruit without any side effects in animals (Bilal et al., 2011).

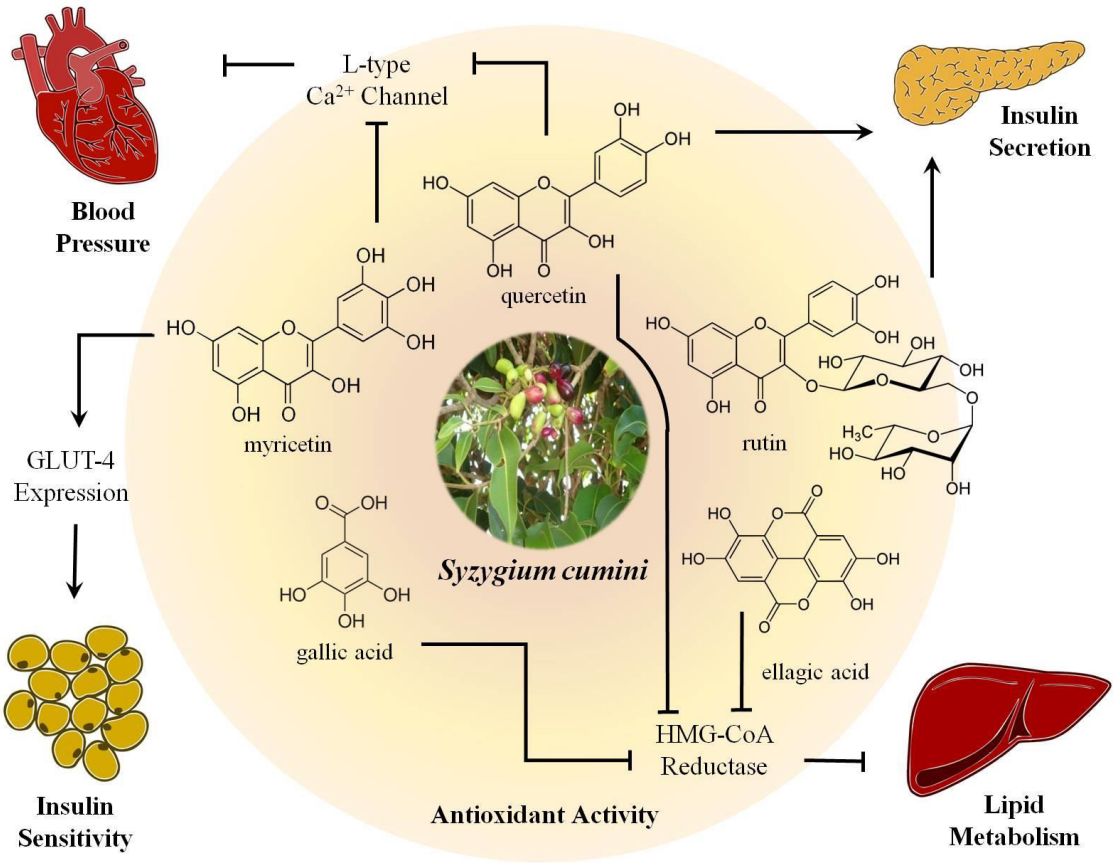

Figure 1. Pharmacological activities and possible mechanisms described for phytochemicals of *Syzygium cumini*. Antioxidant activity: phenolic compounds (e.g: ellagic, gallic and ferulic acids) scavenge reactive oxygen/nitrogen species (ROS and RNS) and stimulate antioxidant defenses like superoxide dismutase (SOD) and catalase (CAT) in plasma and multiple tissues. Cardioprotective activity: the phenolic compound quercetin increase endothelial nitric oxide synthase (eNOS) expression and decrease LDL oxidation (LDL-ox), apolipoproteins B100 (Apo-B100) and soluble vascular cell adhesion molecule-1 (sVCAM-1) expression. Anti-hyperlipidemic activity: the quercetin inhibits the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) in the liver. Moreover, it downregulates lipase lipoprotein (LPL) by decreasing adipogenesis at white adipose tissue. Anti-hyperglycemiant activity: myricetin inhibits the aldose reductase that might avoid renal complications of the hyperglycemia. It also increases the GLUT-4 glucose transporter expression at white adipose tissue. Other flavonoids like rutin, apigenin and quercetin induce regeneration of pancreatic tissue and stimulation of insulin secretion. The betulinic acid and 3,5,7,4'-tetrahydroxy flavanone inhibit pancreatic α-amylase activity. Caffeic and oleanolic acids increase the liver glycogen content by reduction and increase of activity of glucose-6-phosphatase (G6Pase) and glycogen synthase kinase-3β (GSK-3β), respectively. The quercetin enhances the secretion of adiponectin at white adipose tissue.

5 Clinical studies

Notwithstanding all the accumulated knowledge on the pharmacological properties of *S. cumini*, very limited data has came from clinical trials (Helmstadter, 2008). A study conducted by Srivastava and collegues showed that administration of capsuled seed powder to patients with severe diabetes notably reduced blood glucose levels in both fasting and post-prandial condictions (Srivastava et al., 1983). Similarly, Kohli and Singh reinforced the effects of seed powder on type 2 diabetes mellitus patients by showing that besides a 30% reduction of serum glucose levels, a 3-months treatment also improved other classical symptoms of diabetes, like polyphagia, polyuria and polidisia (Kohli and Singh, 1993). On the other hand, a single administration of a decoction from dried powdered leaves of *S. cumini* (2g in 250 mL water) to young normoglycemic patients had no effect on serum glucose levels (Teixeira et al., 2000). In a second approach, Teixeira and colleagues administered the same decoction to type 2 diabetic patients for 28 days observing no effect on glucose levels (Teixeira et al., 2006). Albeit contradictory results showed above, the plethora of pre-clinical studies on the cardiometabolic properties of *S. cumini* supports the necessity of well-designed trials that allow an efficient assessment of therapeutic potentials of *S. cumini* in humans. In addition, the standardization of the extraction method and characterization of the phytochemicals present in the extracts are fundamental to the success of such trials.

6 Conclusion

The present work enlightens cardiometabolic properties described for *S. cumini*, which have been attributed to a limited amount of phytochemicals, particularly flavonoids, phenolic acids and tannins. As summarized in Figure 1, some compounds like quercetin, myricetin, ellagic acid and other phenolic compounds seem to be able to act on distinct pathways of cardiometabolic disorders, thus emerging as potencial multi-targeted drugs. Nevertheless, the knowledge on their precise mechanisms of action is scanty and still deserves in-depth scientific research, especially concerning their jointed action as a phytocomplex. Finally, though toxicological studies have shown the species is safe, clinical trials are barely inexistent pointing out a golden Eldorado for pharmaceutical companies.

7 Acknowlegments

Authors are thankful to Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Estado do Maranhão – FAPEMA, which has importantly funded their research on pharmacological properties of *Syzygium cumini* through the grants #APP01128/10 and #APP00280/12.

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4.2 Capítulo II - Protective effects of a polyphenol-rich extract from *Syzygium cumini* (L.) Skeels leaf on oxidative stress-induced diabetic rats

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Artigo publicado no Oxidative Medicine and Cellular Longevity

(ISSN: 1942-0900)

Fator de impacto: 4.440; Qualis Medicina I: A2

Protective effects of a polyphenol-rich extract from *Syzygium cumini* (L.) Skeels leaf on oxidative stress-induced diabetic rats.

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Conflict of Interest Statement: Authors declare the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Running title: Protective effect of *S. cumini* on diabetic rats

Abstract

Syzygium cumini (L.) Skeels has been reported to exert anti-inflammatory and cardiometabolic activities due to its high content of polyphenols. We characterized the chemical composition and assessed the antidiabetic effects of a novel polyphenol-rich extract (PESc) obtained from *S. cumini* leaf. Rats were injected with alloxan (150 mg/kg, i.p., ALX group) and followed up for 7 days. Some were orally treated with PESc (50 mg/kg/day) for 7 days before and after diabetes induction (ALX-PP) or only for 7 days after alloxan injection (ALX-P). ALX-P and ALX-PP decreased fasting glycemia in 37 and 43%, respectively, as compared to ALX. Triglycerides and total cholesterol serum levels were also significantly reduced in comparison to ALX. PESc presented high polyphenols concentration (71.78 ± 8.57 GAE/100 g), with flavonoid content of 8.21 ± 0.42 QE/100 g. Upon HPLC-MS/MS and MS/MS studies, five main

polyphenols – gallic acid, quercetin, myricetin and its derivatives – were identified. Myricetin was predominant (192.70 ± 16.50 µg/mg PESc), followed by measurable amounts of gallic acid (11.15 ± 0.90 µg/mg PESc) and quercetin (4.72 ± 0.06 µg/mg PESc). Kinetic assessment of total antioxidant capacity revealed PESc high potency, since maximum response was reached within 5-min reaction time in a concentration-dependent manner. Specific antioxidant activity of PESc was assessed against both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, showing strong activity (IC_{50}: 3.88 ± 1.09 and 5.98 ± 1.19 µg/mL, respectively). PESc also inhibited lipoxygenase activity (IC_{50}: 27.63 ± 8.47), confirming its antioxidant activity also on biologically relevant radicals. Finally, PESc induced insulin secretion by directly stimulating INS-1E β cells in absence of any cytotoxic effect. Overall, our results support that PESc is a potent antioxidant phytocomplex with potential pharmacological use as a preventive anti-diabetic natural product.

Keywords: *Syzygium cumini*, polyphenols, mass spectrometry, antioxidants, diabetes.

1 Introduction

Hyperglycemia induces oxidative stress by producing mitochondrial dysfunction and stress of the endoplasmic reticulum. Oxidative stress, in turn, is involved in the development and progression of type 1 diabetes [1]. Reactive oxygen species cause endothelial lesions that are of fundamental importance for macro and micro vesicular complications of diabetes. In addition, oxidative stress also causes damage to pancreatic β cells by activating the autoimmune response in genetically susceptible individuals [2]. Exogenous antioxidants obtained from medicinal plants can neutralize such reactive species and, consequently, their deleterious effects evidenced in diabetes [3].

Syzygium cumini (L.) Skeels (*S. cumini*) is a tree from the Indian subcontinent [4] that was brought to Western countries in the mid-nineteenth century, where it started to be used as anti-diabetic agent even before the discovery of insulin [5]. This plant species is denominated with a variety of names: *Syzygium jambolanum* (Lam.) DC, *Eugenia jambolana Lam.*, *Eugenia cumini* (L.) Druce e *Myrtus cumini* L., and widely spread in several countries, including Brazil. It is popularly known as jambolão, jambolan, java plum, black plum, Indian blackberry, jaman, jambu and jambul [6].

Polyphenols are phytochemicals that have been reported to exert healthy benefits [7]. Epidemiological studies show that consuming polyphenol-rich diets is inversely associated with the development of cardiometabolic and neurodegenerative diseases, as well as cancer [8]. *S. cumini* is phytochemically composed of compounds such as hydrolysable tannins, flavonoids, anthocyanins, terpenes and aliphatic acids [6]. Different parts of *S. cumini* have different composition but all share a high content of polyphenols [9]. Both fruit and flowers are enriched in anthocyanins as cyanidin, delphinidin, peonidin, pelargonidin, petunidin and malvidin [10]. The seed contains rutin and quercetin, and leaf have important secondary metabolites such as kaempferol, quercetin, myricetin and their glycosides[3]

Many important biological activities have been described for *S. cumini* such as hypoglycemic, hypolipidemic, anti-inflammatory, cardioprotective, antibacterial, hepatoprotective, anti-neoplastic and anti-allergic activities [6, 9]. In fact, it has been previously reported a significant anti-hyperglycemic activity in patients administered with *S. cumini* leaf extract, being this effect ascribed to the inhibition of the adenosine deaminase protein [11]. Our group reported that *S. cumini* has a variety of polyphenols, including flavonoids, phenolic acids and tannins widely distributed in different parts of the plant being potentially protective against cardiometabolic diseases [3]. More recently, we showed that the hydroethanolic extract of *S. cumini* leaf improved the metabolic profile of monosodium L-glutamate (MSG)-induced obese rats, especially by reverting triglyceride accumulation in both liver and serum [12].

Evidence in the literature suggests that the anti-hyperglycemic activity of *S. cumini* can be attributed to the antioxidant properties of flavonoids such as quercetin and rutin, which have been identified in *S. cumini* leaf [9]. Thus, in the present report we took advantage of alloxan-induced diabetic rats, a well-known oxidative stress animal model [13], to characterize the anti-diabetic effects of a novel polyphenol-rich extract from *S. cumini* leaf (PESc). Furthermore, main compounds within the enriched extract were identified and chemically characterized as an attempt to correlate them with PESc antioxidant activity in vitro and its insulinagogue actions.

2 Materials and Methods

Chemical Reagents. All reagents and solvents were of analytical grade. The $DPPH^{\bullet}$ (2,2-diphenyl-1-picryl-hydrazyl-1), $ABTS^{\bullet+}$ (2,2'-azinobis- (3-ethylbenzthiazoline sulfonic acid-6)), quercetin, gallic acid, potassium ferricyanide, ferric chloride, sodium dodecyl sulfate, and hydrochloric acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Formic acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Any other solvent used was HPLC grade.

Botanical material. Leaves from *S. cumini* plants were collected during September 2012 from specimens located at the campus of the Federal University of Maranhão (São Luís, Maranhão, Brazil). An exsiccated sample of the plant species was identified and stored at the Herbarium of Maranhão (MAR) under the register number 4574.

Sample preparation. Preparation of the polyphenolic-rich extract (PESc) was performed as previously described with modifications [14]. Briefly, 300 g of powdered dried leaves were extracted by maceration with ethanol: water (70:30, v/v) under constant stirring at 25ºC and filtered after 24 hours. This procedure was repeated twice, totaling three extractions. Extracts were then combined and centrifuged at 3500 rpm for 10 minutes at room temperature. The supernatant was concentrated in a rotary evaporator at 38°C to obtain the hydroethanolic extract (HESc). HESc was partitioned with chloroform (1:1 v/v; 3x), with subsequent partition of the aqueous phase with ethyl acetate (1: 1 v/v; 3x). The ethyl acetate fraction was concentrated under vacuum and lyophilized, yielding polyphenol-enriched extract (PESc).

In vivo model of Alloxan-induced diabetes. To perform in vivo studies using the PESc, male Wistar Hannover rats (Rattus novergicus) of 60 days were used. Rats were hosted in polypropylene boxes, in a maximum of 5 animals per box, maintained at 21 ± 2 °C, 12 h light/dark cycles with food and water ad libitum. After adaptation to these conditions for a week, rats were randomly divided into three groups and at day 7 all of them received a single i.p. injection of Alloxan (150 mg/kg; Sigma Chem. CO., St Louis, USA) after 12 hours of fast to induce diabetes [13, 15]. To prevent a fatal hypoglycemic state due to a fast release of insulin, the animals received 10% glucose in water for 12 h and food included an hour after Alloxan injection. The three groups at day 7 were divided as, i) ALX: rats who received Alloxan at day 7 and then did not receive any other treatment than NaCl 0.9 g/L (1 mL/kg/d, n=9); ii) ALX-P: rats who received Alloxan at day 7 and then 50 mg/kg/d PESc by gavage (n=14) until the animals were euthanized; and iii) ALX–PP: rats who received from day 0 50 mg/kg/day PESc

by gavage (n=14) and continued receiving the extract after the injection of Alloxan. All the protocols used in this paper were approved by the institutional Ethical Committee on Animal Use (CEUA-UFMA), under ruling number 016/13. During the 14 days of treatment, the rats were weighted every day. Blood glucose levels were determined at days 1, 4, 7, 10 and 14 after overnight fasting, and periphery blood collected by puncture of caudal vein to determine glycemic levels using Blood Glucose Test Strip (Accu-Check Active, Roche Diagnostics, Mannheim, Germany).

Analysis of lipid profile and triglyceride/glucose ratio. At day 14, the animals were overnight fasted, anesthetized and blood was collected from aorta puncture and used for triglycerides (TG) and total cholesterol (TC) determination. The serum levels of these lipids were determined by enzymatic commercial kits [16]. Insulin sensitivity was inferred by determining the Triglyceride/Glucose index (TyG) in accordance to the following calculation: TyG = Ln ([TG (mg/dL)] x [glucose (mg/dL)] / 2) [17].

Quantification of total phenols and flavonoids. Total phenolic content was determined as described elsewhere [18]. Briefly, 100 µL of PESc in 96% ethanol was mixed with 630 µL of deionized water, 20 µL of HCl 1 M, 150 µL of 1% K3[Fe(CN)6], 50 µL of 1% sodium dodecyl sulfate (SDS) and 50 µL of 0.2% FeCl3·6H2O. Samples were vortexed and allowed to stand at room temperature for 30 minutes. The polyphenol content of PESc was determined by absorbance at 750 nm using a UV-VIS spectrophotometer (SP-2000 UV Spectrum); quantitation was performed using a gallic acid calibration curve. Results were expressed as gallic acid equivalents in grams per 100 g of dry extract (GAE/100 g).

In addition, flavonoids concentration was determined according to previous method with modifications [19]. 200 µL of 1 mg/mL samples in methanol were incubated with 5% aluminum chloride (1/1, v/v) and reached a final volume of 2 mL with methanol. The reaction mixture was incubated for 30 minutes at room temperature and protected from light. Flavonoids were determined spectrophotometrically at 425 nm in an UV-VIS spectrophotometer (SP-1105, Spectrum), and quantified using a calibration curve with quercetin. Results are expressed as quercetin equivalents per 100 g of dry extract (QE/100 g). Both procedures were repeated at least four times in separate experiments.

Isolation and identification of polyphenols by HPLC-MS/MS. Chemical characterization of polyphenolic compounds present in PESc was performed by HPLC-MS/MS analysis. Polyphenols were separated using a quaternary pump HPLC (Agilent 1260) and separation was performed in an analytical Phenomenex Luna C-18 column (250 x 4.6 mm, 5 µm). Before the run, extracts were filtered (0.45 µM, Millipore) and dissolved in the same solvent used for extraction. The solvent system consisted of H2O/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B) according to the following elution gradient at: 5% B (0-1 min); 5-30% B (1-30 min), and re-equilibrated to initial conditions. Flow rate was 1 mL/min and column maintained at 35°C [20]. From PESc, different fractions were collected, solvent evaporated and resuspended in methanol for MS/MS characterization. Mass spectrometry chemical characterization of polyphenols presents in PESc was done by using a triple quadrupole ion trap QTRAP4500 (AbSciex, Framingham, MA). Samples were characterized in the negative mode with electrospray ionization (ESI) of the samples. Using purified standards, parameters for each compound were obtained by injecting samples at a flow rate of 10 µL/min, a declustering potential (DP) of -70 eV and desolvation temperature of 350 °C. For fragmentation analysis, collision energy (CE) used was between -25 to -37 eV. HPLC-ESI-MS/MS of PESc was performed in the negative ion mode using the multiple reaction monitoring (MRM) scan mode. For LC-MS/MS experiments, the following parameters were used at the mass spectrometer: DP of -100 V, CE of -25 to -37 eV and a desolvation temperature of 550 ºC. The MRM transitions used were *m/z* 169/125 for gallic acid; *m/z* 317/271, *m/z* 317/151 and *m/z* 317/179, for myricetin; *m/z* 463/316, *m/z* 463/151, *m/z* 463/179, *m/z* 449/316, *m/z* 449/271 and *m/z* 449/179, for glycosylated derivatives of myrecitin; and 301/151 and *m/z* 301/179, for quercetin [21, 22]. Analysis and processing of the obtained data was performed using the Analyst 1.6.1 software (Applied Biosystems, Framingham, MA).

Quantification of main polyphenols identified in PESc. Using standards of gallic acid, myricetin and quercetin, calibration curves by HPLC-UV were made. Standards and PESc separation and analysis were performed using a quaternary pump HPLC (Dionex Ultimate 3000) with autosampler and a Diode Array detector. Detection of the different standards and the polyphenols present in PESc was done at 254 nm, 292 nm, 354 nm and 375 nm while chromatographic conditions were the same for LC-MS/MS studies. For quantification purposes, gallic acid and myrecitin calibration curves were done at 254 nm while quercetin quantified at 375 nm [23, 24].

Analysis of antioxidant activity. A) Scavenging of DPPH•: The antioxidant activity was evaluated by incubating 100 µL of PESc with 1900 µL of a methanolic solution of DPPH• (25 µg/mL) leading to a final extract concentration of 0.25-20 µg/mL. The antioxidant activity was measured spectrophotometrically at 515 nm [25]. The DPPH• was prepared just before the beginning of experiment and kept in an amber bottle. The percentage of inhibition was calculated according to the formula:

$$Inhibition = \left[\frac{\text{A control} - \text{A sample}}{\text{A control}}\right] \times 100$$

A = absorbance

IC_{50} (effective concentration for 50% inhibition of pre-formed radical) was determined by nonlinear regression. B) Scavenging of $ABTS^{\bullet+}$: The ABTS solution was prepared in water and potassium persulfate, kept in dark for 16 hours before testing for the complete oxidation of ABTS and the generation of the highly stable chromophore cation radical 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) [26]. The $ABTS^{\bullet+}$ solution was diluted with absolute ethanol until the absorbance at 734 nm reached 0.7 ± 0.02. Readings were performed by reacting 1-20 µg/mL of PESc with the $ABTS^{\bullet+}$ solution. All studies were performed at least in triplicate monitoring the decrease of absorbance for 10 min; results reported corresponded to the % of remaining chromophore compared to conditions in the absence of antioxidants. The IC_{50} values were determined as previously.

Analysis of lipoxygenase activity. Lipoxygenase (LOX) activity was measured spectrophotometrically at 234 nm [27]. The reaction was performed in 100 mM borate buffer pH 9 at 25°C in the presence of 100 µM arachidonic acid (AA) prepared in 0.4% deoxycholate. The reaction was initiated with the addition of AA to the enzyme and the increase in absorbance at 234 nm was monitored for 5 minutes under continuous stirring against a blank sample. Enzyme activity was determined from the initial linear slope. To analyze effects of PESc on LOX, either the extract or the pure standards were added 2 minutes before enzyme activity was initiated. In all cases, specific LOX activity was determined by previously quantifying the amount of protein used in each experiment. Protein content was determined spectrophotometrically at 595 nm using the Bradford method, with BSA as standard [28]. The results shown were reported as the remaining specific LOX activity compared to the condition in the absence of extract or standard.

INS-1E β cells culture and cell proliferation assay. INS-1E β cells were cultured in humidified atmosphere (95%) containing 5% CO2 in complete RPMI 1640 medium (Sigma-Aldrich, Canada). These were supplemented with 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol, 1mM L-glutamine, 5% inactivated fetal calf serum (Hyclone, Logan, UT), 1 U/mL and 1 mg/mL of penicillin and streptomycin respectively (Sigma-Aldrich, Canada). To determine cell proliferation, INS-1E β cells were seeded at 2 x 105 cells/well density and incubated with PESc at increasing concentrations (1 – 1000 µg/mL) for 48 hours at 37˚C. After incubation period, BrdU solution (10 µM) was added for another 4 hours. DNA incorporation by BrdU was measured through colorimetric assay and carried out as per manufacturer (Roche Diagnostics, Mannheim, Germany).

Glucose stimulated insulin secretion in INS-1E β cells. INS-1E β cells were cultured as above mentioned. To examine PESc effects on insulin secretion in vitro, freshly diluted and filtered PESc solution was incubated at 100 and 1000 µg/mL for 4 hours in a 96-wells plate seeded with 2 x 105 cells/well INS-1E β cells at 37˚C. Medium was then washed twice with PBS and replaced by glucose-free Krebs-Ringer Bicarbonate HEPES buffer (KRBH: 135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES and 0.1% BSA, pH 7.4) for 1 hour. Then, 2.8mM glucose was added to KRBH for 30 min, washed with PBS and incubated for 2 hours with 3.3 or 16.5 mM glucose KRBH. Finally, supernatants were collected and stored at –20˚C until analysis. Insulin was measured with an ultra-sensitive rat insulin ELISA kit and all procedures carried out as per manufacturer (Crystal Chem Inc, USA).

Statistical analysis. The results were expressed as mean ± standard error of the means with values of at least three independent experiments and analyzed with GraphPad Prism® (version 5.0). Statistical analyzes were performed by Student t test or analysis of variance (ANOVA) followed by Tukey post-test. Differences were significant when $p \leq 0.05$.

3 Results

PESc reduction of Alloxan-induced hyperglycemia. We evaluated the protective effects of PESc in an in vivo oxidative stress-induced diabetic rat model (Figure 1). Diabetic state was induced by the oxidative environment exerted by the i.p. injection of Alloxan at day 7 [13].

When animals were weighted, rats who received PESc from day 0 (ALX-PP) showed body weight loss from 350.10 ± 7.48g to 325 ± 7.69g before Alloxan injection at day 7 (Figure 1A). Neither ALX nor ALX-P groups showed decrease in body weight during the first 7 days (Figure 1A). Importantly, glycemia was lower at days 4 and 7 in ALX-PP as compared to ALX and ALX-P, suggesting a hypoglycemic activity of the extract (Figure 1B). As expected, alloxan promoted body weight loss on all groups, though ALX-PP data suggest a lower slope of body weight decrease when compared to the other two groups (Figure 1A). Upon alloxan administration, fasting glucose levels measured in ALX rats at day 14 were 3.5-fold higher than those at day 7 (346.33 ± 27.89 mg/dL vs. 98.22 ± 4.66 mg/dL, Figure 1B). For groups receiving PESc, fasting glucose levels verified at day 14 were strongly decreased in 37% for ALX-P and 43% for the ALX–PP group, as compared to ALX group (221.64 ± 38.30 mg/dL for ALX-P and 197.92 ± 38.54 mg/dL for ALX-PP, Figure 1B).

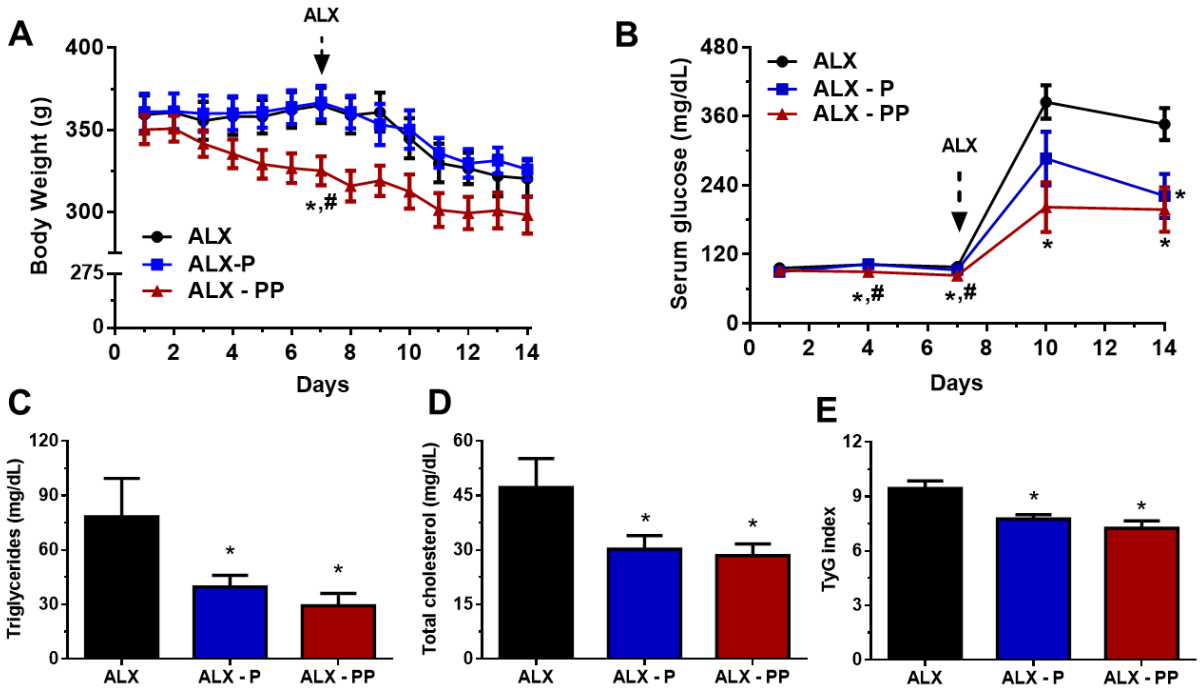


Figure 1. Effects of PESc in an in vivo diabetic model. Male Wistar rats (black squares, ALX, n=9) were treated with PESc (50 mg/kg/d) from day 7 (blue squares, ALX-P, n=14) or from day 0 (red triangles, ALX-PP, n=14). At day 7, all animals received an injection of Alloxan (150 mg/kg, i.p) to induce diabetes as explained in Materials and Methods section. During the experimental procedures changes in body weight (A), serum blood glucose (B), Triglycerides (C) and Total Cholesterol (D) levels were determined. In addition the TyG index (E) was obtained from data in (C) and (D). Results shown correspond to the mean ± SEM; * p<0,05 vs ALX; & p<0,05 vs ALX-P.

PESc prevents insulin resistance on Alloxan-treated rats. Administration of PESc lowered serum TG levels, reaching maximum decrease of 63% on ALX-PP and 49% in ALX-P when compared to ALX (Figure 1C). Similar effects were observed for TC determinations which were reduced from 47.18 ± 7.97 mg/dL on ALX rats to 29.10 ± 3.09 mg/dL and 29.96 ± 3.53 mg/dL on ALX-P and ALX-PP, respectively (Figure 1D). Using fasting glucose and TC levels at day 14, we calculated the TyG index, which suggested decreased hepatic insulin resistance in ALX-P and ALX-PP, since TyG values were significantly reduced by 18% and 23%, respectively.

Total antioxidant capacity of PESc. Levels of polyphenols and flavonoids in PESc were determined spectrophotometrically reaching values of 71.78 ± 8.57 GAE/100 g, and 8.21 ± 0.42 QE/100 g, respectively. Due to the observed protection in an oxidative stress-mediated model of pancreatic damage, we next analyzed the total antioxidant potency of PESc by using DPPH$^{\bullet}$ method (Figure 2). Since PESc reached maximum scavenging effect before 5 minutes, it can be considered as having high antioxidant potency, with a concentration-dependent effect (Figure 2).

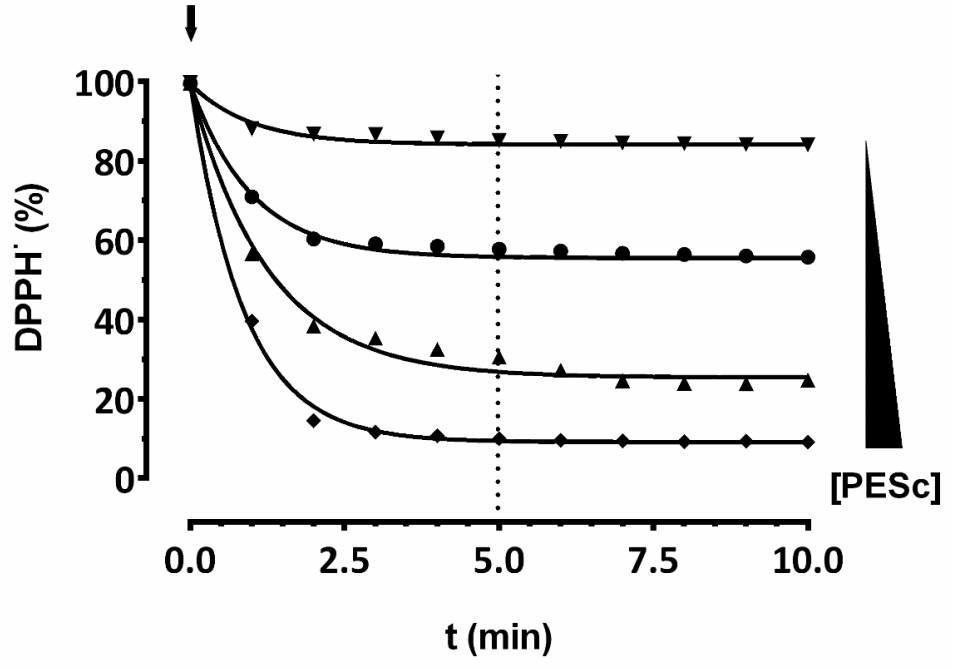


Figure 2. **Kinetics of DPPH. radical consumption after addition of Polyphenol-Rich Extract (PESc)**. PESc (0.62-5 µg/mL) was incubated with DPPH. and the reaction monitored for 10 minutes to determine the maximum antioxidant activity. After 5 minutes maximal effect was reached at all the concentrations tested as shown by the vertical dashed line.

Identification of polyphenols by HPLC-MS/MS. Once antioxidant capacity was verified, we moved to the chemical identification and characterization of compounds present in PESc that could be responsible for such activity (Figure 3). Figure 3A shows the chromatographic elution profile of PESc with MS/MS detection. We were able to isolate, collect and identify five peaks (named 1 to 5) whose structure and identity were obtained by mass spectrometry studies (Figure 3B-F). MS/MS spectra of peak 1 showed the presence of the molecular negative ion ([M – H]- ; *m/z* 169) which fragmented into an ion of *m/z* 125 (Figure 3B), characteristic of gallic acid [29]. The spectrum obtained was identical to the one obtained using a pure standard of gallic acid (not shown). Peaks 2 and 3, in accordance to their MS/MS spectra, were suggested to be myricetin derivatives (Figure 3C and 3D). Peak 2 exhibited a [M – H]- of *m/z* 449 with a fragmentation pattern accordingly to myricetin-3-α-arabinopyranoside mainly formed by the presence of *m/z* 317, *m/z* 271 and *m/z* 179 ions (Figure 3C). The MS/MS spectrum presented in Figure 3D exhibited a [M – H]- of *m/z* 463 which suggested the presence of myricetin deoxyhexoside whose aglycone has been detected from the fragment of *m/z* 317 [21]. Comparing with the available standard, peak 4 corresponded to myricetin due to its chromatographic retention time in addition to the MS/MS analysis (*m/z* 317 and fragments with *m/z* 179 and *m/z* 151; Figure 3E). The final compound characterized was quercetin, whose spectra presented a [M – H]- of *m/z* 301 and fragments of *m/z* 179 and *m/z* 151 (Figure 3F). Finally, we quantified gallic acid, myricetin and quercetin in PESc (Table 1). Myricetin was the most abundant flavonoid, accounting for nearly 20% of total PESc mass. Altogether, these data characterize the polyphenol content of PESc with abudancy of myricetin, gallic acid and quercetin.

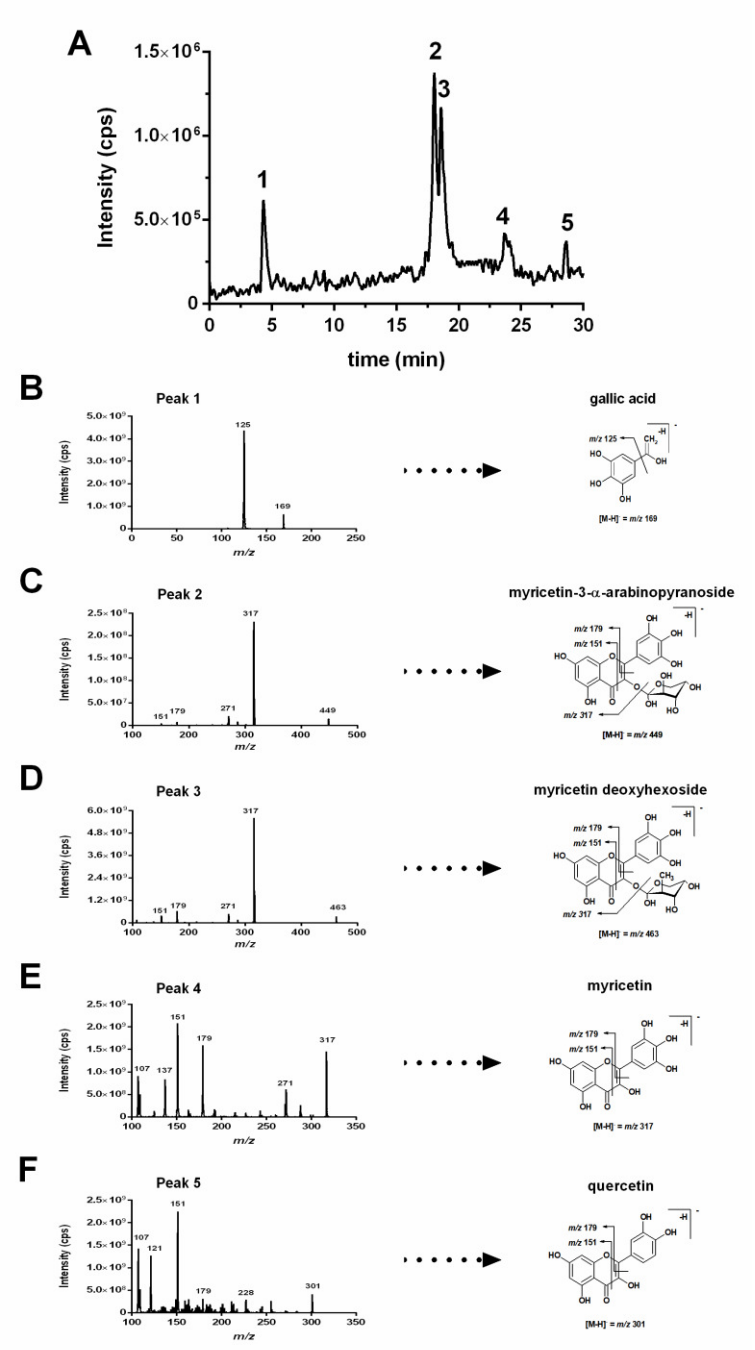
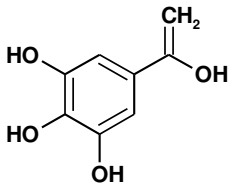
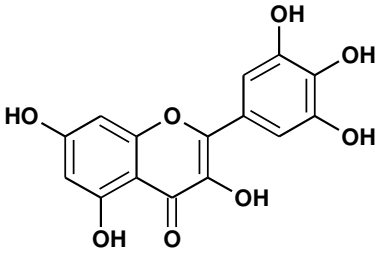
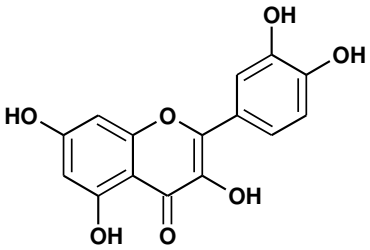


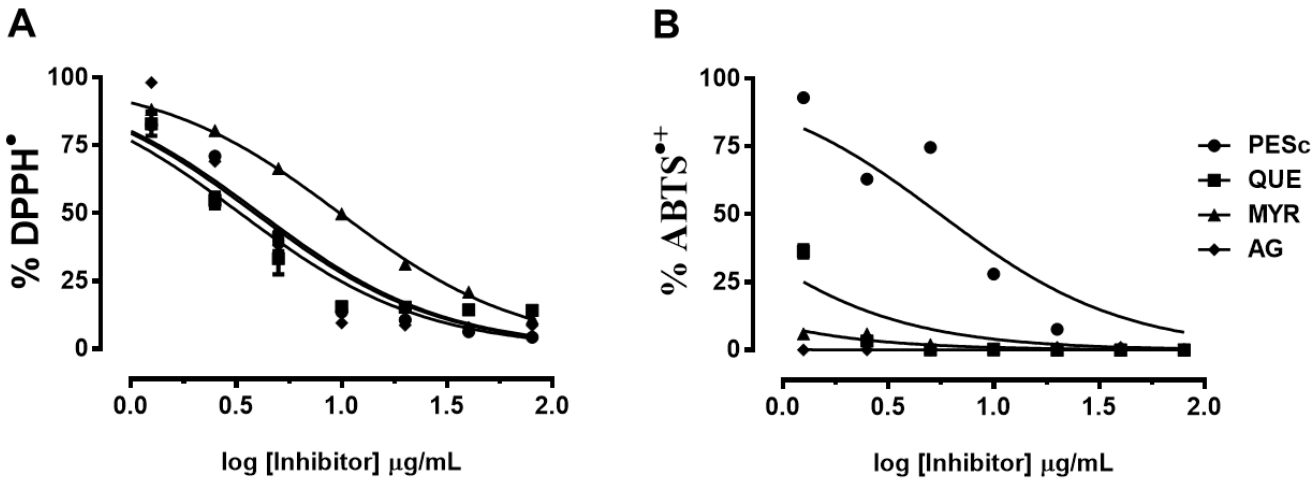
Figure 3. LC-MS/MS analysis of PESc polyphenol content. RP-HPLC-MS/MS chromatographic profile of PESc (A) was obtained. PESc was analyzed by MS/MS using the MRM method. Gallic acid was detected by using *m/z* 169/125, *m/z* 169/97 and *m/z* 169/79. For myricetin, MRM transitions corresponded to *m/z* 317/271, *m/z* 317/151 and *m/z* 317/179 while for glycosylated forms of myricetin *m/z* 449/316, *m/z* 449/271 and *m/z* 449/179. Finally, quercetin was followed by the *m/z* 301/151 and *m/z* 301/179. Chemically identification of the structure of the compounds present in PESc was analyzed by mass spectrometry. Representative structures and fragmentation patters of gallic acid (B); myricetin-3-α-arabinopyranoside (C), myricetin deoxyhexoside (D), myricetin (E), and quercetin (F) are shown. Data is representative of at least three independent experiments.

Table 1. ***Quantification of main polyphenols identified in PESc***

Compounds	Structure	µg/mg of PESc
Gallic acid		**11.15 ± 0.90**
Myricetin		**192.70 ± 16.50**
Quercetin		**4.72 ± 0.06**

Gallic acid (AUC_{254nm}= 624.40 · [GA] + 17.25), myricetin (AUC_{254nm}= 132.90 · [MYR] + 26.34) and quercitin (AUC_{375nm}= 1110.10 · [QUER] - 36.42) were quantified by HPLC-UV. The retention times for those peaks used for quantitation purposes for each compound were previously identified by MS/MS as shown in Figure 3. Results are reported per mg of PESc and correspond to the mean ± SD from three different batches.

Antioxidant activity of the polyphenolic compounds of PESc. Since PESc is rich in myricetin and other flavonoids, we next analyzed the antioxidant activity of PESc in comparison to gallic acid, myricetin and quercetin standards by using $DPPH^{\bullet}$ (Figure 4A) and $ABTS^{\bullet+}$ (Figure 4B) assays. As previously determined, antioxidant reactions were followed for up to five minutes. For the $DPPH^{\bullet}$ method, PESc showed an IC_{50} similar to that measured for quercetin and gallic acid and two times lower than myricetin (Figure 4C). Using $ABTS^{\bullet+}$ method (Figure 4B), PESc presented an antioxidant activity at least ten times lower than pure standards (Figure 4C).

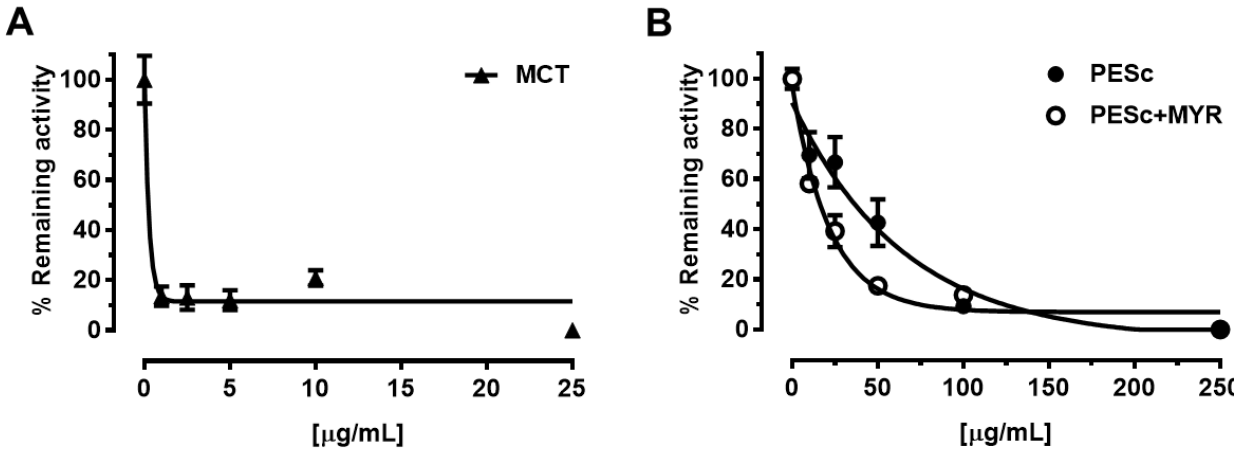


C

SAMPLE	DPPH· (IC_{50}, µg/mL)	ABTS·+ (IC_{50}, µg/mL)
PESc	3.878 ± 0.058	5.591 ± 0.330
MYR	9.810 ± 0.748	0.095 ± 0.003
QUE	3.324 ± 0.107	0.420 ± 0.040
AG	4.084 ± 0.084	0.001 ± 0.0004

Figure 4. Antioxidant activity of PESc. The antioxidant capacity of PESc as well as the pure standards was determined by using DPPH (A) and ABTS (B) assays. In both cases the concentration of extract or fractions leading to a decrease of 50% of initial activity (IC_{50}) was determined (C). Data shown are representative of at least three independent experiments, performed by triplicate for each condition and expressed as the mean ± SEM. *p<0,005

Inhibition of 12/15 lipoxygenase activity. LOX is a biologically-relevant enzyme widely present in plants and mammals that catalyzes the oxidation of unsaturated fatty acids, i.e. AA and linoleic acid. By following changes in absorbance at 234 nm, we analyzed the capacity of either PESc and/or the standards to inhibit 12/15 LOX activity (Figure 5). Both myricetin (Figure 5A) and PESc (Figure 5B) were able to inhibit 12/15 LOX activity in a concentration-dependent manner in a process that was enhanced by the supplementation of PESc with myricetin (Figure 5B and Figure 5C). Neither gallic acid nor quercetin inhibited 12/15 LOX activity (data not shown).



C

SAMPLE	(IC_{50} ± SEM, μg/mL)
PESc	27.63 ± 8.47
MYR	0.34 ± 0.03
PESc + MYR (0,5 μg/mL)	17.76 ± 2.68

Figure 5. Inhibition of LOX by PESc. Lipoxygenase activity was followed by formation of conjugated dienes at 234 nm (ε= 2.5 x 10^{-4} $M^{-1}{\cdot}cm^{-1}$). Activity was determined from the initial linear slope of changes in absorbance. Enzyme protein concentration was determined and specific activity in the absence or presence of the extract or the purified fractions reported. Results are expressed as the remaining initial activity in the presence of the different inhibitors concentrations (A) Dose dependent action of myricetin (MYR) on LOX activity. (B) LOX activity was determined at different PESc concentrations in the absence (closed squares) or presence (open circles) of MYR, to detect if an additive or cooperative action was observed. (C) The obtained IC_{50} values in each condition are reported. In all cases, data are presented as mean ± SD of three independent experiments at least in triplicate for each condition.

Cell proliferation and insulin secretion. Due to the beneficial effects of PESc on alloxan-induced diabetes and its potent antioxidant activity, we determined whether this extract would have any effect on in vitro INS-1E β cell culture proliferation and insulin secretion. After 48 hours incubation, PESc did not compromise cell proliferation up to 100 μg/mL, as measured by BrdU incorporation to DNA (Figure 6A). At 1000 μg/mL PESc incubation abolished cell proliferation probably due to an extremely high concentration. Importantly, acute incubation of 100 μg/mL PESc induced a 4-fold increase in basal insulin secretion and a 2-fold increase in 16.7 mM glucose-stimulated insulin secretion (Figure 6B).

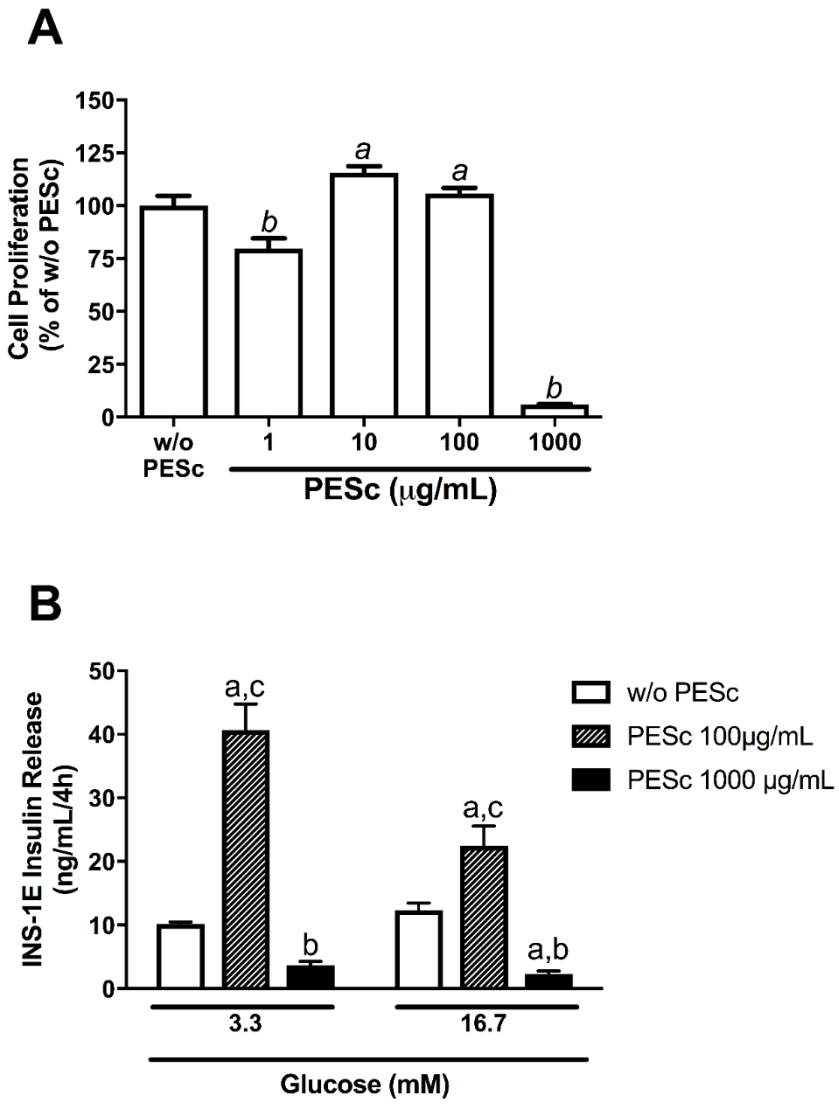


Figure 6. Cell proliferation and insulin release induced by PESc. Cell proliferation was determined by BrdU incorporation to DNA, whereas insulin levels were determined by commercial ELISA kit – both described in Methods. (A) Cell proliferation after 48 hours incubation with or w/o PESc. Results expressed as relative to w/o PESc condition. *a*: $p<0.05$ vs w/o PESc; *b*: $p<0.05$ vs All. n=9 per group. (B) Glucose stimulated insulin secretion (GSIS) in INS-1E insulinoma cells incubated with 100 or 1000 µg/mL PESc for 4 hours at 37˚C. *a*: $p<0.05$ vs w/o PESc; *b*: $p<0.05$ vs PESc 100 µg/mL; *c*: $p<0.05$ vs PESc 1000 µg/mL. n=8 per group. All data were analyzed by one-way ANOVA followed by Tukey post-test and presented as mean ± SEM.

Discussion

The present study strengthens the chemical and pharmacological knowledge of the phytochemical composition of *S. cumini* leaf as well as its applicability in complementary

management of cardiometabolic disorders. Data herein described show that PESc, a novel polyphenol-rich extract prepared from *S. cumini* leaf, presented a protective effect on a model of oxidative stress-induced diabetes. These effects were possibly due to PESc potent antioxidant capacity, demonstrated by a prominent ability to scavenge DPPH• and more importantly to inhibit the biologically-relevant 12/15 LOX enzyme. Chemical characterization evidenced abundance of gallic acid, quercetin and myricetin derivatives. Moreover, PESc induced insulin secretion by INS-1E β cells, without affecting cell proliferation at potentially therapeutic concentrations.

Alloxan produces a diabetic state through the accumulation of reactive oxygen species on pancreatic β cells causing detrimental effects since β cells have incipient antioxidant defense [13]. Upon alloxan injection, all rats presented a marked increase of fasting serum glucose levels consequent to the pancreatic oxidative damage. However, rats from ALX-P and ALX-PP groups had significantly lower increases than those from ALX group, depicting the ability of PESc to attenuate alloxan-induced diabetes regardless of when PESc was administered. Accordingly, PESc administration reduced fasting serum levels of TG and TC in both ALX-P and ALX-PP groups, an effect further assessed by the improved hepatic insulin sensitivity inferred from TyG index calculation. These findings are in line with our previous report of the dual effect of a hydroethanolic extract from *S. cumini* leaf on insulin secretion and peripheral insulin sensitivity of MSG-obese rats [12]. Studies carried out with *S. cumini* seed extract in HepG2 cells [14] and liver from streptozotocin-induced diabetic rats [30] showed hypolipidemic effects, which were attributed to increased PPARγ activity and expression. Importantly, myricetin, a flavonoid widely distributed through *S. cumini* tree, has been shown to improve insulin sensitivity [31] and promote hepatic lipid oxidation by increasing PPARα expression in the liver [32]. Nevertheless, the protective effect of PESc could also come from a direct improvement of cellular redox balance, similar to the islet-regenerative potential showed for a purified fraction of *S. cumini* seeds in streptozotocin-induced diabetic mice [33]. Therefore, here we showed that acute PESc administration before or after diabetes induction was sufficient to attenuate diabetic state.

To investigate whether PESc in vivo effects were due to its antioxidant activities, we first characterized PESc polyphenolic content. PESc presented a polyphenol content 3-fold higher than HESc [12] and virtually even higher than those reported for different extracts prepared

from *S. cumini* seeds [34]. In fact, values reported by Arun et al. [34] may be overestimated, since they were obtained using the Folin-Ciocalteau method, which also quantifies non-phenolic compounds such as aromatic amino acids, sugars, ascorbic acid, and organic acids, reason why it is no longer advised for total phenols quantification [18]. LC-MS/MS studies of PESc allowed us to identify and quantitate the compounds – gallic acid, myricetin and quercetin, showing that approximately 30% of PESc total mass was made of these three compounds. As expected for an RP-chromatography, gallic acid eluted first whereas quercetin was the last compound to elute from the column, explained by different polarities between compounds [35].

Antioxidant capacity of *S. cumini* leaf has been formerly shown for hydroethanolic [12] and aqueous [36] extracts, whereas PESc apparently present higher capacity. Both quercetin and gallic acid showed similar scavenging capacity of $DPPH^{\bullet}$ compared to the extract, while myricetin presented higher IC_{50}. The low IC_{50} found for PESc on $DPPH^{\bullet}$ assay corroborates the novelty of PESc, since there is no data on literature describing such results for a *S. cumini* extract. In fact, PESc scavenging capacity is at least three times higher than those described in different reports [37, 38]. For $ABTS^{\bullet+}$ assay, the standards showed a stronger antioxidant activity when compared to PESc. Despite this, as far as we know, PESc showed the lowest IC_{50} against $ABTS^{\bullet+}$ for a *S. cumini* extract described thus far [39, 40]. $ABTS^{\bullet+}$ is reactive through both hydrophilic and hydrophobic radicals while $DPPH^{\bullet}$ assay is performed in organic solvents where hydrophobic radicals reduce the radical [41]. As expressed before for their chromatographic behavior, polarity of the tested polyphenols is different, supporting the distinct lipophilicity and reactivity of the compounds present in PESc [36]. In addition, it has been reported that flavonoids exert different antioxidant properties due to the localization of hydroxyl groups at C-3 and C-3' on flavonoid B ring [42]. Steric impediments were also suggested to impact the reactivity of gallic acid, quercetin and myricetin with different oxidant models [42] which can also explain the different activities seen against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$.

Since our antioxidant capacity studies used non-biologically relevant oxidants, we next decided to assess PESc action on the enzymatic lipid-derived oxidation of AA by 12/15 LOX, whose signaling pathway encompasses a variety of cellular pro- and anti-inflammatory mediators, such as hydroperoxy- or hydroxy-eicosatetraenoic acids, leukotrienes, and lipoxins [43]. Moreover, catalytic mechanism of AA oxidation by LOX involves the formation of a lipid-

derived radical, which can be sequestered or reduced by the biologically-active polyphenols present in PESc. PESc could concentration-dependently inhibit LOX activity, an effect further enhanced upon addition of 0.5 μg/mL myricetin, leading to a 35% reduction in IC50. Similar additive effect for quercetin supplementation was obtained only at concentrations 20-fold higher than that used for myricetin (data not shown). Moreover, gallic acid was unable to exert inhibition of enzymatic activity (data not shown). Thus, PESc inhibition of 12/15 LOX improves our understanding on the mechanisms of action of both extract and its constituent polyphenolic compounds.

Given the beneficial effects of PESc on an oxidative stress-induced diabetes model, coupled with its antioxidant activities in vitro, we sought to further understand the extract's effect on β cell culture. Importantly, PESc did not interfere with cell proliferation up to 100 μg/mL, while promoting a potent insulinagogue effect, at both basal and glucose-stimulated conditions. These effects support the restoration of islet architecture, as well as glucose-insulin axis seen on ALX-P and most evidently on ALX-PP animals, comparable to effects described for *S. cumini* seed extract on streptozotocin-induced diabetic mice [33]. In addition, it is known that 12/15 LOX is highly relevant in β cell insulin secretion, both at physiologic [44] and diabetic conditions [45]. Thus, by inhibiting 12/15 LOX, PESc might improve insulin secretion directly on β cells, even though other mechanisms cannot be ruled out. Notwithstanding, 12/15 LOX is also relevant in a broader setting of cardiovascular disease, for instance by promoting thrombus formation in vivo [46], which enlarges PESc potentiality as an interesting new tool to treat and prevent thromboembolic outcomes associated to metabolic disorders.

5 Conclusions

In conclusion, our study showed the in vitro and in vivo antioxidant activities of PESc obtained from *S. cumini* leaf in addition to the chemical characterization and identification of its main polyphenolic compounds, which could be responsible for the observed actions. Our findings support that myricetin, quercetin and gallic acid compose a phytocomplex, with poorly understood synergistic mechanisms of action. Nevertheless, the strong attenuation of oxidative stress-derived metabolic outcomes observed in vivo using such a low dose of PESc definitely reinforces the potential use of this novel polyphenol-enriched extract from *S. cumini* leaf as a source of anti-diabetic natural products.

Abbrevations

$DPPH^{\bullet}$	2,2-diphenyl-1-picryl-hydrazyl-1
$ABTS^{\bullet+}$	2,2'-azinobis-(3-ethylbenzthiazoline sulfonic acid-6)
PESc	polyphenolic-rich extract
HESc	hydroethanolic extract
MRM	multiple reaction monitoring
IC_{50}	effective concentration for 50% inhibition
LOX	lipoxygenase
AA	arachidonic acid
TG	triglicerides
TC	total cholesterol
TyG	Triglyceride/Glucose index
BrdU	5-bromo-2'-deoxyuridine

Acknowledgment

We would like to thank Prof Alison Holloway and Prof Deborah Sloboda (McMaster University, Hamilton, Canada) for the support given with regards to INS-1E β cells culture. This work was funded by the Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Estado do Maranhão – FAPEMA (Universal 00280/12 and APCINTER-02698/14), the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (PIBIC fellowships to R.M.R.C.) and Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior – CAPES (Master fellowship to V.T.C). A.T. was supported by Comisión Sectorial de Investigación Científica (CSIC, Grupo 536)-Uruguay and FAPEMA (PVI-05558/15).

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4.3 Capítulo III - Inhibition of hepatic microsomal triglyceride transfer protein by polyphenols

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Artigo a ser submetido ao Journal of Foods and Function

(ISSN: 2042-6496)

Fator de impacto: 3.247; Qualis Medicina I: B1

Mini-review article

Inhibition of hepatic microsomal triglyceride transfer protein by polyphenols

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Conflict of Interest Statement: Authors declare the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abstract

Epidemiological data demonstrate that hypertriglyceridemia is strongly associated with increased deaths from cardiovascular disease. Plasma levels of triacylglycerols directly reflect increased concentrations of triglyceride-rich lipoprotein synthesized by hepatocytes: VLDL and its remnants. The microsomal triglyceride transfer protein (MTP) is essential for the production and secretion of hepatic VLDL particles containing apoB-100. Inhibition of MTP using synthetic inhibitors such as lomitapide is an important pharmacological strategy by reducing plasma TGs and apoB levels, however, this mechanism induces hepatotoxicity. Polyphenols are reported as inhibitors of hepatic MTP through different mechanisms that involve the regulation of lipogenic transcriptional factors and prevent liver damage by reducing the accumulation of cellular lipids and attenuation of endoplasmic reticulum stress and oxidative stress.

Keywords: Microsomal triglyceride transfer protein, hypertriglyceridemia, insulin resistance, obesity, metabolic syndrome, dyslipidaemia, polyphenols, flavonoids.

1 Introduction

Hypertriglyceridemia, defined as the plasma concentration of triacylglycerols (TGs) ≥ 200 mg /mL, is an independent risk factor for the development of metabolic syndrome, type 2 diabetes and atherosclerotic cardiovascular disease (MILLER et al., 2011; VOLPE et al., 2017). Epidemiological information supports that hypertriglyceridemia is the major risk factor for coronary heart disease (SARWAR et al., 2007; TENENBAUM et al., 2014) evidencing the causal relationship between hypertriglyceridemia and cardiac events (JØRGENSEN et al., 2014). Thus, the control of hypertriglyceridemia is crucial for the prevention of deaths due to cardiovascular diseases (TENENBAUM et al., 2014).

The treatment of hypertriglyceridemia is still limited, despite its relevance as a predictor of cardiovascular risk (VOLPE et al., 2017). Drugs such as fibrates, statins, niacin, n-3 polyunsaturated fatty acids are partially effective in reducing plasma levels of TGs (SAHEBKAR et al., 2014) and this is evident from clinical studies showing that there are residual risks for cardiovascular events even after the reduction of triglyceride-rich lipoprotein (TRLs) such as LDL, considered the lipoprotein with the greatest atherogenic potential (CHAPMAN et al., 2011; BANACH et al., 2016).

Elevated TGs levels are a common phenotype in central obesity, metabolic syndrome, type 2 diabetes, among other metabolic disorders, and are commonly associated with increased TRLs such as VLDL, LDL, and reduced HDL. (GUÉRIN et al., 2001; TENENBAUM et al., 2014). Although the reduction of HDL levels is frequent in hypertriglyceridemia, randomized studies have shown that these low levels are not capable of generating cardiovascular diseases (CD), while epidemiological evidence points to TRLs such as LDL, end product of the VLDL particle, as the main marker which predisposes to CD (PACKARD, 2003; NORDESTGAARD e VARBO, 2014). This is because these TRLs, due to their small size, can penetrate and accumulate in the arterial wall leading to the formation of the atherosclerotic plaque (CHAPMAN et al., 2011), furthermore, LDL is less rapidly eliminated by hepatic LDL

receptors, whereas HDL particles are hypercatabolized by the liver and kidneys (PACKARD, 2003).

The increased secretion TRLs such as VLDL, chylomicrons and remnants that induce insulin resistance (WRAY, 1994). This condition reduces the degradation of apolipoprotein B (apoB), increases visceral lipolysis and promotes a greater flow of free fatty acids to the liver favoring the overproduction of VLDL (BERGMAN et al., 2006). This condition reduces the degradation of apoB, increases visceral lipolysis and promotes a greater flow of free fatty acids to the liver favoring the overproduction of VLDL (HUSSAIN et al., 2011). The apoB-100 biosynthesis occurs in the liver and is an essential component for the formation of the VLDL particle and its intermediates, IDL and LDL, as well as being a ligand for the LDL receptor (LDLR) (HOOPER et al., 2015). ApoB-100 presents 5 superdomains in its structure formed by alternation of afiphatic chains α and β (βα1-β1-α2-β2-α3), with strong evidence that the βα1 domain is important for the lipoprotein assembly and stabilization by interacting physically with the triacylglycerol transferring microsomal protein (MTP), chaperone responsible for lipidation of apoB preventing aberrant formations and proteasomal degradation of nascent lipoprotein (HUSSAIN et al., 2003; HUSSAIN et al., 2011).

2 Microsomal Triglyceride Transfer Protein: structure and functions

The MTP consists of a heterodimeric complex residing in the lumen of the endoplasmic reticulum formed by the M (functional subunit, MTP, 97 kDa) and P (disulfide isomerase protein, PDI, 58 kDa) (HUSSAIN et al., 2011), essential for the incorporation of neutral lipids into apoB-containing lipoproteins (DHOTE et al., 2011; HUSSAIN et al., 2011). The M subunit belongs to a broad family of proteins that share homologous sequences and similarity in their secondary and tertiary structures. These proteins act on lipid transfer and include as members apoB, lipoforin and vitellogenin (HUSSAIN et al., 2003; HOOPER et al., 2015). The P subunit itself does not promote the transfer of lipids to nascent proteins because its ability to generate disulfide bonds via its isomerase and reductase activity is inhibited when associated with the M subunit. However, the dissociation of the heteromimer caused by different agents leads to the loss of the catalytic function of MTP, highlighting the crucial role of PDI in the stabilization, solubilization and activity of the MTP complex (WETTERAU et al., 1991).

MTP is expressed primarily in hepatocytes and in small intestine epithelial cells, major producers of TRLs. Different tissues also express MTP to a lesser extent as myocardium

(avoiding accumulation of lipids in the heart) (LI et al., 2005), retina (preventing the onset of maculopathy, a disease characterized by deposition of cholesterol crystals), neurons, cells of the immune system, renal tubule epithelial cells, placenta, among others, sites where MTP can perform the same activity described for the heart and retina (HOOPER et al., 2015).

MTP, MTTP gene product, is essential for the assembly and secretion of apoB-containing particles, however, mutations in the MTTP gene cause abetalipoproteinemia, a rare autosomal recessive disease characterized by the absence of circulating apoB due to the inability to generate VLDL and chylomicrons (HOOPER et al., 2015). Among the clinical manifestations of abetalipoproteinemia are diarrhea, nocturnal blindness, muscular weakness and anemia, signs associated with malabsorption and transport of lipids and fat soluble vitamins (A, D, K and E) (BERRIOT-VAROQUEAUX et al., 2000). Knowledge about the molecular mechanisms involved in the pathophysiology of abetalipoproteinemia has led to the development of new treatments (LIN et al., 1995) for hypertriglyceridemia via partial inhibition of MTP activity (HOOPER et al., 2015).

3 Regulatory mechanisms of hepatic MTP

The regulation of MTP activity and expression occurs through different mechanisms involving transcriptional and post-transcriptional and post-translational levels events capable of altering apoB synthesis. The MTTP promoter region contains several cis-elements that bind to negative regulatory elements such as insulin and sterol, in addition to binding to transcriptional factors such as nuclear hepatic factor (eg, HNF-1α, -1β and HNF-4α), and factors "Forkheads" (eg FoxO1 and FoxA2), elements that regulate positively the expression of the MTP gene. Other factors such as leptin, macronutrients, cyccadian cycle, bile acids are also able to regulate the expression / activity of MTP (HUSSAIN et al., 2011), besides these, the reduction of hepatic oxidative stress can interfere in lipogenic pathways suppressing MTP expression (TANAKA, YUJI et al., 2008).

Insulin promotes decrease in VLDL production and secretion by reducing MTTP gene transcription (HAGAN et al., 1994; LIN et al., 1995). In fact, therapies that improve insulin signaling reduce MTP expression and hypertriglyceridemia (CARPENTIER et al., 2002; CHONG et al., 2006). Insulin inhibits MTP through cascade activation involving phosphorylation of insulin receptor substrate (IRS), PIK3-kinase, Akt/PKB and inhibition of FoxO1 (BIGGS et al., 1999). Au et al. (2003) demonstrated that insulin regulates MTP

expression by activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) cascade ($MAPK^{erk}$) (AU et al., 2003), however, Allister et al. (2005) have shown that inhibition of MTP by insulin may occur by other mechanisms after observing that blocking of $MAPK^{erk}$ activity was able to partially inhibit the generation of hepatic VLDL (ALLISTER et al., 2005). Study using HepG2 cells has highlighted that MTP is also negatively regulated by the sterol and insulin response element (SRE/IRE) when it binds to sterol regulatory element-binding proteins (SREBPs), reducing the expression of MTP (SATO et al., 1999). However, there is controversy over the effectiveness of this mechanism in vivo after finding by microarray analysis that MTP is not a direct target of SREBPs (HORTON et al., 2003).

Elements such as HNF-1 and HNF-4 present in the promoter region of the MTTP gene interact with factors HNF-1α/1β and HNF-4α, respectively, important transcription factors for the regulation of lipid hoemostasis (AKIYAMA et al., 2000). HNF-1α regulates the expression of multiple genes in the liver such as fatty acid synthase (FAS), acyl-CoA carboxylase (ACC1), liver fatty acid-binding protein (L-FABP), among others (AKIYAMA et al., 2000) and mutations in the HNF-1α gene are associated with diseases related to lipid metabolism such as juvenile onset diabetes, a form of non-insulin dependent diabetes that affects individuals before 25 years of age (YAMAGATA et al., 1996). O fator HNF-4α controla a expressão de genes ligados ao metabolismo de carboidratos e lipídios incluindo glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), ApoB e outros. Both HNF-1α and HNF-4α are involved in the regulation of MTP expression in hepatocytes (GONZALEZ, 2008; NAUJOK et al., 2008; HUSSAIN et al., 2011), being that, HNF-4α promotes MTP gene transactivation, while HNF-1α synergistically complements this activity (SHEENA et al., 2005). Results from Hayhurst et al. (2001) emphasize that HNF-4α is critical for the expression of MTP, since HNF-4α knockout mice do not express MTP, however, further studies, especially in humans, are needed to demonstrate the real impact of this mechanism on hypertriglyceridemia (HAYHURST et al., 2001; KAMAGATE e DONG, 2008).

Another pathway of insulin signaling capable of inhibiting MTP involves the transcription factors forkhead box O1 (FoxO1) and forkhead box A2 (FoxA2) (WOLFRUM e STOFFEL, 2006; KAMAGATE et al., 2008). Results of Kamagate et al. (2008) demonstrated that the deletion or mutation of forkhead box O1 (FoxO1) renders it unfit for binding to the promoter

region of the MTTP gene by suppressing hepatic MTP expression and exporting of VLDL particles (KAMAGATE et al., 2008). Wolfrum and Stoffel (2006) showed that FoxA2 contributes to the regulation of hepatic MTP mediated by insulin (WOLFRUM e STOFFEL, 2006).

FoxO1 is a transcription factor belonging to the family of forkhead domain, Akt/PKB substrates that play an important role in insulin signaling. FoxO1 resides in the nucleus and increases MTP expression by binding to the insulin response element (IRE), however, when insulin interacts with its receptor, FoxO1 is phosphorylated via PI3K and exported to the cytosol leading to inhibition of gene expression. Suppression of FoxO1 inhibits reduces the synthesis of apoB and VLDL by inhibiting the expression of MTP (ALTOMONTE et al., 2003), Thus, FoxO1 is a promising therapeutic target, since modulation of this factor prevents excessive hepatic VLDL production, a frequent condition in insulin resistant individuals (KAMAGATE e DONG, 2008).

FoxA2 is also phosphorylated in the presence of insulin by reducing the expression of MTP, however, the overexpression of FoxA2 decreases the content of hepatic TGs and increases the export of TGs to the circulation. The FoxA2 factor is co-activated by the PPARgamma coactivator beta factor (Pgc-1β) inducing the activation of genes involved in mitochondrial oxidation, in addition to increasing the production of VLDL containing apoB, but when phosphorylated, Foxa2 dissociates from its co-reducing the expression of hepatic MTP. These data show that inhibition of insulin-regulated FoxA2/Pgc-1β reduces hepatic lipid secretion and pharmacological interventions affecting FoxA2 or Pgc-1β may be useful in the control of hypertriglyceridemia (WETTERAU et al., 1991).

Oxidative stress is associated with the evolution of insulin resistance in obesity, however, there are indications that oxidative stress is an independent factor for the installation of insulin resistance (PARK et al., 2009). The high production of reactive species (ROS) and reduction of the activity of antioxidant enzymes (present in oxidative stress) also induce the decrease of genes involved in the proper functioning of the β cells causing dinoperations in insulin secretion and cell death (TANGVARASITTICHAI, 2015). The decrease in insulin production added to the insulin resistance state induces hypertriglyceridemia due to increased production and exportation of VLDL by the liver and a decrease in the concentration of HDL (TANGVARASITTICHAI et al., 2010). In contrast, oxidative stress induces hepatic insulin resistance and reduced VLDL secretion leading to hepatocyte lipid accumulation, star cell

activation, necro-inflammation, and is crucially involved in the pathogenesis and progression of non-alcoholic fatty liver disease (NAFLD) (DAY, 2002; LETTERON et al., 2003; GAMBINO et al., 2011; POLIMENI et al., 2015).

4 Main adverse effects caused by hepatic MTP inhibitors

The main adverse effects from the use of MTP inhibitors include lipid accumulation and elevation of hepatic transaminases (LIN et al., 2014). The increase in plasma levels of transaminases, mainly aspartate aminotransferase (AST) and alanine aminotransferase (ALT), occurs in about 10% to 30% of patients treated with MTP inhibitors and is strongly associated with accumulation of lipids in hepatocytes (CUCHEL, MARINA et al., 2007; SAMAHA et al., 2008). However, there are points to be clarified about the toxicity caused by the accumulation of hepatic lipids, since the MTP performs transfer of neutral lipids, TGs and cholesterol esters, avoiding the toxicity caused by the excess of free fatty acids and free cholesterol, therefore, TGs appear to be the major cause of liver damage and development of NAFLD (HUSSAIN et al., 2008). Inhibition of MTP results in oxidative stress and en - tence of the endoplasmic reticulum resulting from the accumulation of free TGs / cholesterol in the reticulum and in the mitochondria. In response to reticulum stress, inhibition of MTP promotes elevation of GPT/GOT1 gene transcription by regulating the IRE1α/cJun pathway by inducing the synthesis and secretion of transaminases (ALT1/AST1). These results demonstrate that the increase of the hepatic transaminases occurs secondarily to the stress of the endoplasmic reticulum (JOSEKUTTY et al., 2013).

5 Antioxidant compounds and inhibition of hepatic MTP: involvement of polyphenols

Factors that reduce cell accumulation of lipids such as insulin resistance and oxidative stress are useful in attenuating the damage caused by the inhibition of MTP such as those present in NAFLD (Lin, Zhao et al. 2014). The literature suggests that consumption of antioxidant compounds such as vitamin E, n-3 polyunsaturated Fas (PUFAs), probucol and polyphenols are effective in therapy against NALFD and may be useful in reducing liver damage caused by inhibition of MTP (AL-BUSAFI et al., 2012; LIN et al., 2014; RODRIGUEZ-RAMIRO et al., 2016).

Polyphenols, compounds recognized for their high antioxidant capacity, are also reported as important inhibitors of MTP by diferente pathways (Figure 1) (WILCOX, LISA J et al., 2001; BORRADAILE, NICA M. et al., 2003; CASASCHI, ADELE et al., 2004; ALLISTER et al.,

2005). These metabolites are the product of the secondary metabolism derived from the chiquimate/acetate routes and can be isolated from the vegetables in their free or complex forms to sugars and proteins (LU et al., 2010). It has an important antioxidant role due to its structural characteristics: nucleophilicity of the benzene ring and acid character of the phenolic groups skilled in the delocalization of the unpaired electrons present in the free radicals (PAIXÃO et al., 2007). Due to these characteristics, these compounds are potent inhibitors of the generation of reactive species by chelating transition metals or inhibiting the activity of oxidative enzymes such as cycloxigenases, xanthine oxidase, neutralizing reactive species or converting them into less reactive species by electron or hydrogen donation (RICE-EVANS et al., 1996) and co-antioxidant - regeneration of essential vitamins (ZHOU et al., 2005) protecting cell macromolecules from oxidative damage (LOTITO e FREI, 2006). Associated with the high antioxidant capacity of polyphenols are their hepatoprotective properties (SALOMONE et al., 2017), as opposed to those caused by therapy with lomitapid, a systemic MTP inhibitor available on the market, which presents considerable hepatotoxicity (CUCHEL, M. et al., 2007; CUCHEL, M. et al., 2013). Sharma et al. (2016) noted that the polyphenol-rich aqueous extract of apple pulp reduced liver damage markers alanine transaminase, aspartate transaminase and alkaline phosphatase, inhibited lipid peroxidation and apoptosis via elevated antioxidant enzyme activity such as superoxide dismutase (SOD) and reduced glutathione (GSH) and increased expression of nuclear erythroid 2-related factor 2 (Nrf2), key transcription factor for the regulation of expression of genes encoding antioxidant proteins (SHARMA et al., 2016).

Nrf2 is a transcription factor crucial for the reduction of lipid accumulation and oxidative stress in hepatocytes by modulating lipogenic pathways, among them the MTP inhibition pathway (Figure 1) (TANAKA, Y. et al., 2008). Under physiological conditions, the activity of Nrf2 is suppressed in the cytoplasm by the Kelch-like ECH-associated protein 1 (Keap1) which leads to the ubiquitination and proteosal degradation of Nrf2. However, under oxidative or electrophilic stress, Nrf2 dissociates from its suppressor (Keap1) and is translocated to the nucleus promoting the transcriptional activation of different genes encoding enzymes involved in the antioxidant response such as perirredoxins, catalase (CAT), SOD, GSH, etc (ITOH et al., 2004; DE VRIES et al., 2008). Nrf2 is a molecular target of polyphenols, among them quercetin (GRANADO-SERRANO et al., 2012), resveratrol (PALSAMY e SUBRAMANIAN, 2011), epigallocatechin-3-gallate (NA e SURH, 2008), ellagic acid (DING et al., 2014) curcumin (DING et al., 2014), among others. In general, electrophilic groups present in α, β unsaturated

carbons of the polyphenols react with Keap1 cysteine residues, preventing its association with Nrf2 or modulating Nrf2 through protein kinases, and although other studies are necessary for a better understanding of the activation of antioxidant responses, polyphenols represent promising agents for the attenuation of oxidative stress targeting Nrf2 (NABAVI et al., 2016).

Among the classes of polyphenols, flavonoids stand out for their antioxidant activity demonstrated *in vitro* and *in vivo* systems (KORKINA e AFANAS' EV, 1996; JIN e YIN, 2012; BANJARNAHOR e ARTANTI, 2014; RAMAN et al., 2016). Flavonoids consist of a large group of phenolic compounds ubiquitously present in the plant kingdom, which contains as a fundamental structure the benzo-γ-pyran nucleus (KUMAR, SHASHANK e PANDEY, ABHAY K., 2013). Multiple hydroxyl groups present in these metabolites confer substantial antioxidant capacity, amplified by the double bond, carbonyl function in the heterocycle or polymerization of the fundamental nucleus making the radical more stable through the electronic delocalization and conjugation (HEIM, K. E. et al., 2002).

A variety of pharmacological activities are associated with the antioxidant capacity of flavonoids, including cardioprotective, antibacterial, antiviral, anticancer, anti-inflammatory and hepatoprotective activity (KUMAR, SHASHANK e PANDEY, ABHAY K., 2013). In addition to these activities, flavonoids have antihypertriglycyridemic activity involving inhibition of MTP (CASASCHI, ADELE et al., 2004; ALLISTER et al., 2005). Casaschi et al. (2004) demonstrated *in vitro* using a hepatoma culture that the flavonoid taxofolin has an antihypertriglyceridemic effect reducing apoB secretion via inhibition of MTP and diacylglycerol acyltransferase (DGAT), an enzyme that participates in the accumulation of TGs in the lumen of the reticulum, without raising lipid levels cell phone (CASASCHI, A. et al., 2004). Kurowska et al. (2004) found similar effects using tangeretin in HepG2 cell culture. This flavonoid reduced the secretion of triglyceride-rich particles-containing apoB by inhibiting the activity of MTP and another enzyme that participates in the accumulation of TGs in the lumen (diacylglycerol acyltransferase, DGAT), as well as activate the peroxisome proliferator-activated receptor), transcription factor that regulates the fatty acid oxidation and the availability of TGs (KUROWSKA et al., 2004). These results suggest that inhibition of lipogenic enzymes such as MTP by flavonoids does not cause hepatic steatosis due to, among other mechanisms, induction of β-oxidation (PPAR activation) and inhibition of synthesis and accumulation of TGs (DGAT inhibition) (HUSSAIN et al., 2008).

Results obtained by Borradaile et al. (2003) show that narigenin decreased secretion and elevated proteasomal degradation of apoB via inhibition of MTP In this work, narigenin directly inhibited MTP activity, contributing to a rapid decrease in apoB100 secretion (Figure 1). It is important to note that narigenin reduced apoB100 serum concentration and the accumulation of newly formed TGs in the lumen in a manner similar to BMS-197636, a specific inhibitor of MTP, in addition to flavonoid showed a two-fold inhibition in ester accumulation of cholesterol compared to BMS-197636.. These data show that narigenin may be useful as a supplement for reducing levels of both TGs and cholesterol (BORRADAILE, N. M. et al., 2003). Allister et al. (2005) investigated the mechanisms involved in the inhibition of hepatic MTP by narigenin and demonstrated that the decrease in MTP expression and inhibition of apoB secretion occurs through the activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) ($MAPK^{erk}$) which is independent of substrate-1/2 activation of the insulin receptor (Figure 1) (ALLISTER et al., 2005). Insulin also reduces MTP expression by activation of MAPK pathway and Choi et al. (1991) have shown that a narigenin derivative isolated from the methanolic stem extract of Prunus davidiana, 7-O-α-D-glucoside, has similar insulin properties by reducing glucose, cholesterol and TGs in rats with diabetes induced by streptozotocin (CHOI et al., 1991).

Although narigenin modulates MTP expression independently of phosphorylation of the insulin receptor substrate (CHOI et al., 1991), Bumke-Vogt et al. (2016) demonstrated that the apigenin and luteolin flavonoids reduce lipogenic capacity by the nuclear translocation of FOXO1 via PKB/AKT-dependent (Figure 1) (BUMKE-VOGT et al., 2014), indicating that the expression of the MTP is orchestrated by polyphenols also through the event cascade including the substrate of the insulin receptor. Research conducted by Li et al. (2015) showed that the treatment with flavonoid, luteolin, showed a reduction in the serum levels of VLDL, LDL and apoB without an increase in the accumulation of lipids in the liver through the inactivation of HNF-4α fator (LI et al., 2015). Recently, Inoue et al. (2017) demonstrated that the flavonoid derivative 4'-nitro-6-hydroxyflavone suppresses the activity of HNF-4α (Figure 1), transcription factor that positively regulates MTP gene expression, and accelerates the degradation of HNF-4α protein via activation of AMP-activated protein kinase (AMPK), a kinase sensitive to glucose deprivation conditions, improving insulin sensitivity and maintenance of energy metabolism (ZHANG et al., 2009). Activation of AMPK also inhibits the activity of SREBP-1c and-2c proteins (Figure 1), lipogenic transcription factors that

regulate genes involved in fatty acid synthesis and TGs (SREBP-1c), and to control cholesterol synthesis and consumption (SREBP-2c). This mechanism allowed the attenuation of hepatic steatosis and atherosclerosis in mice with insulin resistance induced by high fat and sugar diet (LI et al., 2011).

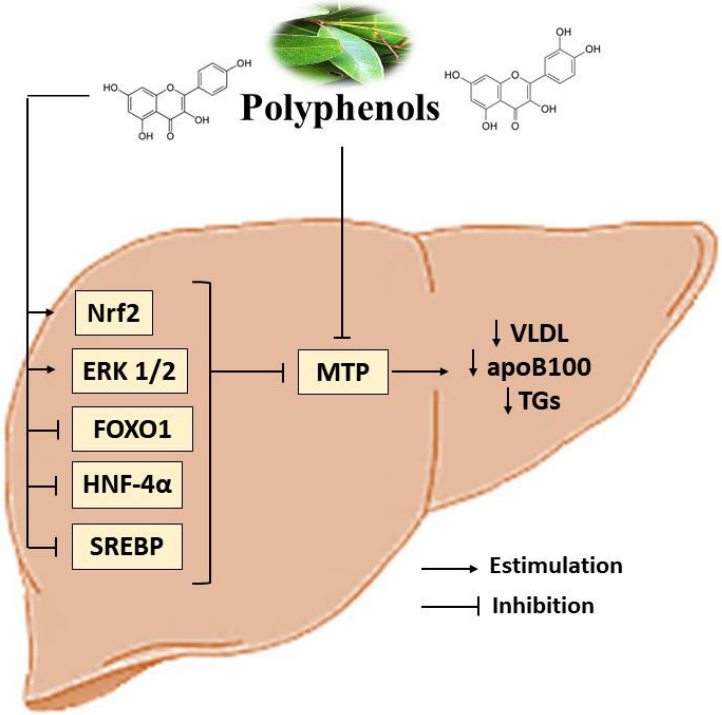

Figure 1. Proposition of mechanisms of inhibition of hepatic microsomal triglyceride transfer protein (MTP) by polyphenols. Phenolic compounds reduce the reticulum stress and oxidative stress by increasing the expression of transcriptional factor Nrf2. They promote phosphorylation of IRK 1/2 withing $MAPK^{erk}$ pathway (cascade independent on the activation of the substrate of the insulin receptor) or inhibition of the activity of the lipogenic fator, FOXO1 (mechanism involving phosphorylation of substrate of the insulin receptor). Furthermore, these metabolities suppress the activity of HNF-4α e SREBP 1c, -2c. Modulation of these pathways culminates in the inhibition of hepatic MTP inducing the reduction in the synthesis and export of VLDL, apoB and TGs.

6 Conclusions

Hypertriglyceridemia is an independent predictor for the development of DCs and causal relationship between elevated levels of TGs and DCs has been reaffirmed (LAAKSO et al., 1993; SARWAR et al., 2007). The high plasma concentration of TGs is associated with obesity,

insulin resistance and dysregulation of the metabolism of TRLs. The plasma accumulation of TRLs with a predominance of VLDL lipoproteins and their remnants results in the atherogenic lipoprotein profile, whereas the reduction in TRLs levels is determinant for the prevention of DCs (SAHEBKAR et al., 2014; TENENBAUM et al., 2014).

MTP is an insulin-regulated key protein required for assembly and hepatic secretion of VLDL. In insulin-resistant individuals, there is an increase in MTP activity inducing the overproduction of VLDL particles and, consequently, elevation of plasma TG levels (KAMAGATE e DONG, 2008). However, pharmacological inhibition of MTP reduces the synthesis of VLDL and apoB (CUCHEL, MARINA et al., 2013).

Decreased MTP activity attenuates hypertriglyceridemia, but is a factor for the development of NFLD due to accumulation of lipids in hepatocytes, in addition to being associated with elevation of transaminases and oxidative stress and reticulum stress (JOSEKUTTY et al., 2013). Antioxidant molecules such as polyphenols are reported as inhibitors of MTP and, in contrast to synthetic inhibitors, these metabolites reduce liver toxicity by preventing the accumulation of cellular lipids (CASASCHI, A. et al., 2004). In this work we suggest that multiple mechanisms are involved in the inhibition of MTP by polyphenols, which include inhibition of oxidative stress and improvement of insulin resistance through increased expression of Nrf2 factor and induction of the activity of antioxidant enzymes, inhibition of the activity of lipogenic factors such as FOXO1 and factors HNF-4α and SREBP via activation of AMPK. In addition, polyphenols stimulate lipid oxidation mediated by PPARs and reduced expression of lipogenic enzymes such as DGAT. The gathering of these data reinforces the therapeutic potential of polyphenols in the treatment of hypertriglyceridemia via inhibition of hepatic MTP expression and activity.

7 Funding

This work was funded by Foundation for the Support of Research, Scientific and Technological Development of the State of Maranhão – FAPEMA through the grants #APP01128/10 and #APP00280/12; and fellowships to JS and RG. VC and KS received fellowships from National Council for Scientific and Technological Development – CNPq.

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5 CONSIDERAÇÕES FINAIS

Syzygium cumini (L.) Skeels é uma espécie medicinal estudada ao longo de cerca de 130 anos devido suas múltiplas propriedades cardiometabólicas, sendo a principal delas, a atividade anti-hiperglicemiante, descrita antes mesmo da descoberta da insulina. Dentre suas atividades biológicas estão: atividade anti-inflamatória, antibacteriana, anti-hiperlipemiante, anti-hipertensiva, hepatoprotetora, dentre outras, reportadas às diferentes partes. Além disso, estudos mostraram que *S.cumini* não apresenta toxicidade em tratamentos agudos ou crônicos preservando valores normais de parâmetros bioquímicos e histológicos.

Os efeitos farmacológicos observados de *S.cumini* são atribuídos à sua composição fenólica que incluem ácidos fenólicos, flavonoides e taninos, moléculas antioxidantes que possuem especial relevância terapêutica devido sua capacidade de inibir a biosíntese ou reagir diretamente com espécies reativas do metabolismo do oxigênio. Assim, neste trabalho padronizamos a obtenção do extrato rico em polifenois (ERP) e o administramos a ratos com diabetes induzida por aloxana.

O tratamento com ERP reduziu as concentração séricas de glicose, colesterol total e TGs. Estes efeitos foram observados tanto nos animais que receberam tratamento anterior e posterior ao diabetes, quanto naqueles tratados somente após à indução do diabetes. É importante ressaltar que os animais que receberam o ERP previamente apresentaram maior controle nos níveis glicêmicos quando comparados àqueles tratados somente após ao diabetes. Isto sugere que os polifenois presentes no ERP podem, além de minimizar os efeitos indesejáveis do diabetes tipo 1, contribuir para sua prevenção através do sequestro de espécies reativas e modulação de mediadores pró-inflamatórios nas ilhotas pâncreáticas. O ERP também estimulou a secreção de insulina em células INS-1E indicando que *S.cumini*, além de proteger o tecido pancreático, pode controlar a hiperglicemia por meio de seu efeito secretagogo.

Os polifenois foram identificados como os metabólitos majoritários no ERP da folha de *S. cumini*. Neste trabalho, nós demonstramos através da análise cromatógrafica associada à espectrometria de massas que o ERP continha ácido gálico, miricetina 3-α-arabinopiranosídeo, miricetina desoxi-hexosíde, miricetina e quercetina. Desse modo, os flavonoides derivados de miricetina foram os fitonutrientes mais abundantes caracterizados no ERP.

O ERP apresentou elevada atividade antioxidante verificada em ensaios *in-vitro* ($DPPH^{\bullet}$, $ABTS^{\bullet+}$ e LOX), sendo esta atividade, similar à demonstrada pelos padrões ácido

gálico, quercetina e miricetina no teste do DPPH•. O ERP também apresentou atividade equivalente à miricetina no ensaio de inibição da LOX.

Os flavonoides presentes no ERP, especialmente os derivados de miricetina, parecem ser os responsáveis pelos efeitos antioxidantes, anti-inflamatórios, anti-hiperglicêmicos e anti-hipertrigliceridêmico. O efeito anti-hipertrigliceridêmico dos polifenois do ERP podem ocorrer por diferentes vias metabólicas incluindo a inibição parcial da MTP. Estes metabólitos, além de reduzirem a concentração plasmática de TGs, possuem efeitos hepatoprotetores diminuindo danos oxidativos e níveis de transaminases. Estes resultados suportam o ERP como potencial agente anti-diabetico e hipolipemiante.

Estudos adicionais voltados para investigação da modulação da expressão e atividade de proteínas pertencentes à expressão e atividade da MTP hepatica encontram-se em andamento. Camundongos matrizes da linhagem *Swis musculus* foram adquiridos do Bioterio Central, acasalados e seus filhotes receberam administração subcutânea de MSG durante os 10 primeiros dias de vida para instalação do diabetes e outras co-morbidades típicas da sindrome metabólica. Estes animais estão sob acompanhamento diário com mensuração peso e consumo energético, além determinação mensal de índices de adiposidade, glicemia, triacilglicerois e colesterol. Os animais serão tratados por 45 dias com o ERP e miricetina (ambos, 50 mg/kg) e após este período serão eutanasiados para coleta de tecido hepático e soro. Estas amostras serão submetidas à análises bioquímicas para avaliação de parâmentros da função hepatica e perfil lipídico, e ainda, serão submetidas à ensaios de expressão gênica e proteica para determinação de marcadores do eixo insulina-MTP para proposição desta via como rota associada ao seu efeito redutor de TGs de *S.cumini*.

Este trabalho reforça o conteúdo literário sobre elevado potencial terapêutico de *S.cumini* na prevenção e atenuação das complicações de doenças metabólicas associadas ao estresse oxidativo como diabetes.

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ANEXO A - Artigo I

frontiers in Pharmacology

MINI REVIEW
published: 03 November 2015
doi: 10.3389/fphar.2015.00259

Syzygium cumini (L.) skeels: a prominent source of bioactive molecules against cardiometabolic diseases

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Syzygium cumini (Myrtaceae) is a worldwide medicinal plant traditionally used in herbal medicines due to its vaunted properties against cardiometabolic disorders, which include: antihyperglycemic, hypolipemiant, antiinflammatory, cardioprotective, and antioxidant activities. These properties have been attributed to the presence of bioactive compounds such as phenols, flavonoids, and tannins in different parts of the plant, albeit the knowledge on their mechanisms of action is scarce. This mini-review highlights the cardiometabolic properties of *S. cumini* by correlating its already identified phytochemicals with their described mechanisms of action. Data herein compiled show that some compounds target multiple metabolic pathways; thereby, becoming potential pharmacological tools. Moreover, the lack of clinical trials on *S. cumini* usage makes it a fruitful field of interest for both scientific community and pharmaceutical industry.

Keywords: black plum, jamun, myrtaceae, phenolic compounds, metabolic syndrome, ethnopharmacology, medicinal plants, complementary and alternative medicine

OPEN ACCESS

Edited by:
Akio Inui,
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Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *10 August 2015*
Accepted: *20 October 2015*
Published: *03 November 2015*

Citation:
Chagas VT, França LM, Malik S and Paes AMA (2015) Syzygium cumini (L.) skeels: a prominent source of bioactive molecules against cardiometabolic diseases. Front. Pharmacol. 6:259. doi: 10.3389/fphar.2015.00259

INTRODUCTION

Cardiometabolic syndrome is associated with multiple risk factors including insulin resistance, dyslipidemia, hypertension, and obesity (Alberti et al., 2009). According to World Health Organization, every year about 2.8 million people die worldwide due to overweight or obesity (Lim et al., 2012). Prevalence of diabetes appears with projections to affect about 439 million adults by 2030, whereas cardiovascular diseases account for 30% of deaths annually, including both developed and developing countries (Shaw et al., 2010). Because of their chronic degenerative nature, cardiometabolic-related disorders have long-lasting treatments, costly for both the patient and the health services, in addition to potentially harmful side effects caused by polytherapeutic regimens (Yanovski and Yanovski, 2014). In this context, herbal medicines have become the major source of bioactive molecules and emerged as potential therapeutic tools to fulfill a multiple-target strategy, especially because of their inherented large-scale structural diversity as compared with synthetic compounds (Prabhakar and Doble, 2008; Vasudeva et al., 2012).

Myrtaceae family comprises about 121 genera with 3800–5800 species of shrubs and trees distributed mainly in tropical and subtropical areas of the world (Stefanello et al., 2011). The genus *Syzygium*, a leading member of this family, embrace 1100 species with deserving attention to *Syzygium cumini* (L.) Skeels (syn.: *Eugenia jambolana, Syzygium jambolanum*), which has been

ANEXO B- Carta de aceite do artigo II

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Dear Dr. Trostchansky,

The review process of Research Article 5386079 titled "Protective effects of a polyphenol-rich extract from Syzygium cumini (L.) Skeels leaf on oxidative stress-induced diabetic rats." by Vinicyus Teles Chagas, Rafaella Moraes Rego de Sousa Coelho, Renato Simões Gaspar, Samira Abdalla da Silva, Mauricio Mastrogiovanni, Cáritas de Jesus Mendonça, Maria Ribeiro, Antonio Marcus de Andrade Paes and Andrés Trostchansky submitted to Oxidative Medicine and Cellular Longevity has been completed. I am pleased to inform you that your manuscript has now been accepted for publication in the journal.

The special issue for which the paper is being processed is
"Therapeutic Potential of Natural Antioxidants"

The publication process of your manuscript will be initiated upon the receipt of electronic files. Please log in to the Manuscript Tracking System at the link below using your username and password, and upload the electronic files of your final accepted version within the next 2-3 days.

http://mts.hindawi.com/author/5386079/upload.files/

The electronic files should include the following:

1- Source file of the final accepted manuscript (Word or TeX/LaTeX).
2- PDF file of the final accepted manuscript.
3- Editable figure files (each figure in a separate EPS/PostScript/Word file) if any, taking into consideration that TIFF, JPG, JPEG, BMP formats are not editable.

Thank you again for submitting your manuscript to Oxidative Medicine and Cellular Longevity.

Best regards,

Kota V. Ramana
kvramana@utmb.edu

ANEXO C-Aprovação no Comitê de Ética de Uso Animal

UNIVERSIDADE FEDERAL DO MARANHÃO **COMISSÃO DE ÉTICA NO USO DE ANIMAIS**	
PARECER CONSUBSTANCIADO INICIAL	**Nº do parecer: 16**
PROJETO DE PESQUISA	**Registro da CEUA: 16/13** **Nº do Protocolo: 23115.01983/2013-41** **Data de entrada no CEUA: 20/12/2013** **Parecer: APROVADO**

I – Identificação

Título do projeto: Avaliação pré-clínica de espécies vegetais utilizadas no tratamento de patologias associadas à síndrome metabólica		
Identificação da equipe executora: Antonio Marcus de Andrade Paes, Lucas Martins França, Vinicyus Teles Chagas, Karla Frida Torres Flister, Caio Fernando F Coelho		
Instituição onde será realizado: UFMA		
Área temática: Não se aplica	**Multicêntrico:** Não	**Data de recebimento:** **20/01/2014**
Cooperação estrangeira:		**Data de devolução:** **25/04/2014**

II – Objetivo:

Investigar mecanismos moleculares envolvidos nas atividades biológicas de plantas medicinais da flora maranhense que sejam popularmente utilizadas para o tratamento de patologias associadas à síndrome metabólica.

III – Sumário do projeto:

O atual projeto trata-se de projeto de pesquisa coordenado pelo Prof Dr. Antonio Marcus de Andrade Paes (UFMA). O projeto será realizado no Laboratório Ensino e Pesquisa em Fisiologia (LeFisio) da Universidade Federal do Maranhão (UFMA) e terá a colaboração de diversos pesquisadores. O objetivo maior do projeto é realizar a avaliação pré-clínica de espécies vegetais utilizadas no tratamento de patologias associadas à síndrome metabólica. O projeto é subdivido em três subprojetos intitulados "Investigação do mecanismo da ação hipolipemiante das folhas de *Syzygium cumini* (L.) Skeels em animais dislipidêmicos", "Investigação do mecanismo da ação hipoglicemiante das folhas de *Averhoa carambola* L. em animais hiperglicêmicos" e "Purificação, isolamento e identificação biomonitorada de

metabólitos secundários em extratos vegetais ricos em polifenóis". Para a realização da pesquisa, serão utilizados vinte e quatro ratos da linhagem Wistar, distribuídos igualmente e aleatoriamente em quatro grupos de seis animais. Os animais serão utilizados com 2 meses de idade e 250 gramas, serão provenientes do Biotério Central da UFMA e mantidos no Biotério Setorial do Laboratório de Fisiologia. A vigência do projeto é de 24 meses a iniciar após a aprovação desta comissão. O orçamento foi apresentado, mas não houve menção sobre a fonte financiadora. A equipe executora tem experiência no tema propostos, o que pode ser comprovado pelos currículos.

IV – Comentário do relator frente à resolução 779 de 26 de agosto de 2010 e complementares em particular sobre:

O projeto **Avaliação pré-clínica de espécies vegetais utilizadas no tratamento de patologias associadas à síndrome metabólica** é relevante e será executado por equipe experiente sob a coordenação do Prof. Dr. Antonio Marcus de Andrade Paes,. O projeto está conciso, bem delineado e coerente quanto aos seus objetivos. Os métodos estão claros e estão sustentados suste por artigos científicos já publicados em revistas conceituadas, entretanto, foi mencionado no formulário (Anexo II da Resolução 779-CONSEPE) que serão usados apenas 24 animais, número considerado inadequado para a realização de todos os testes dos três subprojetos. É necessário que seja feita a correção no Anexo II, acrescentando o número total necessário, e que seja detalhado o número de animais para cada procedimento. Os animais serão anestesiados corretamente para realização dos procedimentos, por meio de cloridrato de quetamina e cloridrato de xilazina. Foi informado que os animais serão oriundos do Biotério Central da UFMA, mas é recomendável que seja acrescentado um ofício do Diretor do Biotério garantido a viabilidade do fornecimento ou então, outra fonte onde os animais possam ser adquiridos. Não há informações sobre o destino das carcaças dos animais coletados, o que seria recomendado ser acrescentado. Outra recomendação é que seja anexado o CD referente ao projeto. É importante que a equipe inserida no corpo do projeto seja a mesma colocada no formulário (Anexo II), é importante esclarecer no projeto se trata-se de estudo multicêntrico, pois caso assim seja, é necessária anuência dos demais centros.

V – Pendências

O projeto não apresenta pendências

VI – Recomendações:

O projeto não apresenta recomendações

VII – Parecer consubstanciado da CEUA

O projeto foi considerado APROVADO.

São Luís, _25__/03__/_2015_

Profa Dra Lucilene Amorim Silva
Presidente da CEUA / UFMA