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VIRGINIA DEMARCHI KAPPEL

ESTUDO DA ATIVIDADE BIOLÓGICA DE *Baccharis articulata*, *Musa x paradisiaca* E RUTINA NA HOMEOSTASIA DA GLICOSE EM MODELOS EXPERIMENTAIS *IN VIVO* E IN *VITRO*

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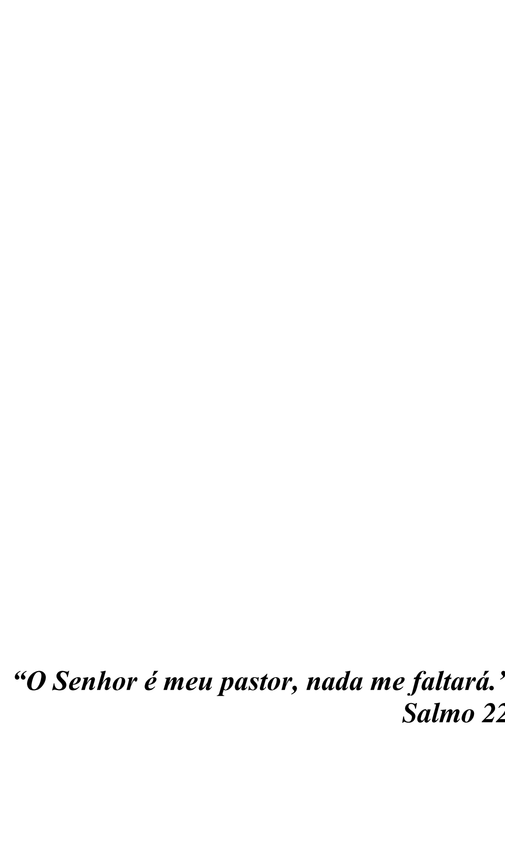
Florianópolis, **17 de agosto de 2012.**

Dedico este trabalho aos meus pais,
Gilberto e Doraci, pelo amor e apoio
constante, que sempre estiveram presentes
em todos os momentos de minha vida.



"O Senhor é meu pastor, nada me faltará."
Salmo 22

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RESUMO

A insulina é o principal hormônio anabólico responsável pelo controle da captação, utilização e armazenamento dos nutrientes celulares como carboidratos, proteínas e lipídios, sendo essencial para a manutenção da homeostasia da glicose, o crescimento e diferenciação celular. Defeitos na ação e/ou na secreção de insulina podem levar à hiperglicemia, característica da diabetes melito. A diabetes melito é uma patologia complexa e multifatorial de elevada morbidade e mortalidade e, por esse motivo, é considerada uma epidemia, caracterizando um problema de saúde pública mundial. Muitas plantas são conhecidas na medicina popular de diferentes culturas pelas propriedades hipoglicemiantes e tem um uso crescente no tratamento da diabetes. Os compostos fenólicos derivados de plantas, especialmente os flavonóides, apresentam diversas propriedades e tem um potencial terapêutico muito investigado. O presente trabalho teve como objetivo caracterizar o efeito de extratos e frações de *Bacharis articulata* (carqueja) e de *Musa x paradisiaca* (banana), assim como do flavonóide rutina, na homeostasia da glicose em modelos experimentais *in vivo* e *in vitro*. As duas espécies foram avaliadas quanto às atividades anti-hiperglicêmica e/ou hipoglicemiante na curva de tolerância à glicose e em modelos de diabetes induzidos experimentalmente, como a secreção de insulina, o conteúdo de glicogênio hepático e muscular, a inibição das enzimas α-glicosidases e a propriedade anti-glicação, assim como o conteúdo de flavonóides. Além disso, foi estudado o mecanismo de ação da rutina na captação de glicose e de cálcio em músculo sóleo, e também a secreção de insulina *in vivo* e o mecanismo de ação da rutina na captação de cálcio em ilhotas pancreáticas isoladas. Para tanto, foram utilizados ratos Wistar machos entre 50-55 dias de idade. Para a realização da curva de tolerância à glicose as coletas de sangue, para determinação da glicose e insulina sérica, foram realizadas nos tempos zero, 15, 30, 60, 120 e 180 minutos. Nos ensaios para a determinação do conteúdo de glicogênio os tecidos foram retirados dos animais 3 h após os tratamentos. As atividades das dissacaridases intestinais e a propriedade anti-glicação foram realizadas *in vitro*. A captação de ^{14}C-glicose e de ^{45}-cálcio ($^{45}Ca^{2+}$) foi estudada após a incubação do músculo sóleo com a rutina. As ilhotas pancreáticas foram isoladas e incubadas com $^{45}Ca^{2+}$ e rutina, na presença ou não de diferentes inibidores e ativadores. Os extratos brutos, as frações *n*-butanol e residual aquosa de *B. articulata* e de *M. x paradisiaca* reduziram significativamente a glicemia de ratos normais hiperglicêmicos e potencializaram a secreção de insulina induzida por

glicose. Além disso, observou-se um aumento no conteúdo de glicogênio no músculo sóleo e fígado após os tratamentos, principalmente com as frações *n*-butanol das duas espécies vegetais. Os extratos e as frações reduziram a atividade da maltase e preveniram a glicação. A rutina estimulou a captação de glicose e cálcio no músculo, estimulando a captação de glicose através da ativação de uma via insulinomimética e uma via independente da sinalização clássica da insulina. Além disso, a rutina estimulou a secreção de insulina *in vivo* e a captação de cálcio em ilhotas pancreática isoladas, atuando como um potencial agente secretagogo de insulina. Desta forma, apoiado nos resultados obtidos neste trabalho, propõe-se que as espécies vegetais, *Baccharis articulata* e *Musa x paradisiaca,* e o flavonóide rutina aqui estudados possam regular a homeostasia da glicose. Os mecanismos envolvem a inibição da enzima que permite a absorção intestinal da glicose, a inibição da glicação, o estímulo da secreção de insulina e o aumento na utilização de glicose pelos tecidos periféricos, evidenciando que estas duas espécies e a rutina podem atuar por múltiplos mecanismos de ação para regular a homeostasia da glicose e colaborar na prevenção das complicações da diabetes.

Palavras-chave: diabetes, hiperglicemia; *Baccharis articulata*; *Musa x paradisiaca*; flavonóides, rutina; glicogênio; insulina; AGEs; dissacaridases; captação de glicose; captação de cálcio; ilhotas pancreáticas.

ABSTRACT

Insulin is the main anabolic hormone responsible for controlling the uptake, use and storage of cellular nutrients such as carbohydrates, proteins and lipids. It is essential for the maintenance of glucose homeostasis, growth and cellular differentiation. Defects in action and / or secretion of insulin may lead to hyperglycemia, which characterizes *diabetes mellitus*. *Diabetes mellitus* is a complex and multifactorial disease with high morbidity and mortality, therefore is considered epidemic causing a public health problem worldwide. Many plants are known in folk medicine of different cultures for their hypoglycemic properties showing an increasing use in the treatment of diabetes. The plant-derived phenolic compounds, especially flavonoids, have several properties and their therapeutic potential has been investigated. The aim of this study was to characterize the effect of extracts and fractions of *Baccharis articulata* ("carqueja") and *Musa x paradisiaca* ("banana"), as well as the flavonoid rutin, in glucose homeostasis using *in vivo* and *in vitro* experimental models. Antihyperglycemic and / or hypoglycemic activity in the curve of glucose tolerance and in models of experimentally induced diabetes, insulin secretion, the hepatic glycogen content and muscle, inhibition of the enzymes α-glucosidases and anti-glycation property were investigated, as well as the content of flavonoids. In addition, was also studied the mechanism of action of rutin in glucose and calcium uptake in soleus muscle, and also the *in vivo* insulin secretion and the mechanism of action of rutin on calcium uptake in rat isolated pancreatic islets. For *in vivo* experiments, Wistar male rats with 50-55 days of age were used. To glycemia and serum insulin determination blood samples were collected at zero, 15, 30, 60, 120 and 180 min in glucose tolerance curve. Tissues were removed from animals 3 h after oral administration of treatments to determine glycogen contents. The intestinal disaccharidases activities and anti-glycation property were performed *in vitro*. The glucose and calcium uptake was studied after incubation of the soleus muscle with rutin, in the presence or not of different inhibitors and of ^{14}C-glucose or calcium ($^{45}Ca^{2+}$). The pancreatic islets were isolated and incubated with $^{45}Ca^{2+}$ and rutin in the presence or absence of various inhibitors or activators. The crude extracts and *n*-butanol and residual aqueous fractions of *B. articulata* and of *M. x paradisiaca* showed potential anti-hyperglycemic activity in hyperglycemic normal rats and potentiated glucose-induced insulin

secretion. Additionaly, it was observed an increase on glycogen content in muscle and liver after treatments, mainly with the *n*-butanol fractions of two species. The extracts and fractions reduced the activity of maltase and prevented glycation. Also, rutin stimulated glucose and calcium uptake in soleus muscle, stimulating glucose uptake via activation of an insulin-mimetic and an insulin-independent signaling pathways. Additionally, rutin stimulated insulin secretion *in vivo* and calcium uptake in isolated pancreatic islets, as a potential insulin secretagogue agent. Thus, these results suggest that *Baccharis articulata*, *M. x paradisiaca* and rutin are able to regulate glucose homeostasis. The mechanisms involve the inhibition of the enzyme that allows the intestinal absorption of glucose, the inhibition of glycation, the stimulation of insulin secretion and increase in glucose utilization by peripheral tissues showed that these plant species and rutin may act by multiple mechanisms of action to regulate glucose homeostasis, thereby contributing to the prevention of diabetes-related complications.

Key-words: diabetes, hyperglycemia, *Baccharis articulata*, *Musa x paradisiaca*, flavonoids, rutin, glycogen, insulin, AGEs, disaccharidases; glucose uptake, calcium uptake; pancreatic islets

LISTA DE ABREVIATURAS E SIGLAS

ADP	Adenosina bifosfato
AGE	Produto final de glicação avançada
AMPK	Proteína cinase ativada por 5’-AMP
AMP	Adenosina monofosfato
APS	Substrato da proteína associada
AS160	Proteína substrato da Akt de 160 kDa
ATP	Adenosina trifosfato
cAMP-PKA	Proteína cinase A dependente de AMP cíclico
C3G	Proteína trocadora de nucleotídeo
Ca^{2+}	Íon cálcio
CAP	Proteína associada à Cbl
CAPE	Ácido caféico éster fenetil
Cbl	Protooncogene
CCDV	Canais de Ca^{2+} dependentes de voltagem
CEUA	Comitê de ética do uso de animais
CLAE	Cromatografia líquida de alta eficiência
CML	Carboximetillisina
CrkII	Proteína adaptadora
DM	Diabetes melito
DPP-IV	Dipeptidilpeptidase 4
ERRO	Espécies reativas de oxigênio
ERK	Cinase reguladora de sinal extracelular
Fyn	Tirosina cinase citoplasmática
G-1-P	Glicose 1-fosfato
G-6-P	Glicose 6-fosfato
GAP	Proteína ativadora de GTPases
GDP	Guanosina bifosfato
GIP	Polipeptídeo insulinotrópico dependente de glicose
GLP-1	Peptídeo semelhante ao glucagon 1
GLUT	Transportador de glicose
Grb2	Proteína adaptadora ligada ao receptor de fator de crescimento
GS	Glicogênio sintase

GSK-3	Glicogênio sintase cinase 3
GTP	Guanosina trifosfato
HbA1c	Hemoglobina glicada
HMIT	Transportador H^+ ligado ao mio-inositol
Ig	Imunoglobulina
IL-6	Interleucina – 6
IRS	Substrato receptor de insulina
JAK2	Janus cinase 2
JNK	Cinase c-jun NH2 terminal
KIF	Proteína cinesina
K_{ATP}	Canais de potássio dependentes de ATP
K_m	Constante cinética de Michaelis Menten
LDL	Lipoproteína de baixa densidade
MAPK	Proteína cinase ativada por mitógeno
MEK	Proteína tirosina/treonina cinase
Mg^{2+}	Íon magnésio
MMP	Matriz metaloproteinases
mTOR	Proteína alvo da rapamicina em mamíferos
n-BuOH	*n*-butanol
NF-κβ	Fator nuclear κβ
PBS	Tampão fosfato salina
PDK	Proteína cinase dependente de 3-fosfoinositídeos
PH	Plekstrina
PI3K	Fosfatidilinositol 3 cinase
PIP3	Fosfatidilinositol-3,4,5-trifosfato
PKB/ Akt	Proteína cinase B
PKC	Proteína cinase C
PKCaM	Proteína cinase dependente de cálcio-calmodulina
PP	Polipeptídeo pancreático
PP1	Proteína fosfatase 1
PP1G	Isoforma da proteína fosfatase 1 ligada ao glicogênio
PPAR	Receptor ativado por proliferador de peroxissomo
PTB	Fosfotirosina
PTG	Proteína regulatória da PP1

PTP	Proteína tirosina fosfatase
Ras	Proteína GTPase
Raf	Proteína serina/treonina cinase
RAGE	Receptor específico de AGE
SGLT	Transportador de glicose dependente de sódio
SHP2	Proteína tirosina fosfatase homóloga de Src 2
SLC5A	Família dos transportadores de glicose dependentes de sódio
SNAP23	Proteína de 23 kD associada à sinaptossoma
SNARE	Proteína do receptor ligada a NSF solúvel
SOCS	Proteína supressora da sinalização de citocina
SOS	Fator trocador de nucleotídeo de guanina
TC10	Proteína G
TGF-β	Fator de crescimento-β
TNF-α	Fator de necrose tumoral- α
TOTG	Teste oral de tolerância à glicose
UDP-G	Uridina-difosfato glicose
V_{max}	Velocidade máxima
VAMP2	Proteína 2 da vesícula associada à membrana
VEGF	Fator de crescimento das células do endotélio vascular

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1 INTRODUÇÃO

1.1 INSULINA E MECANISMOS DE TRANSDUÇÃO DE SINAIS

A insulina é um hormônio polipeptídico sintetizado e armazenado nas células β das ilhotas de Langherans do pâncreas. É composta por 51 aminoácidos dispostos em duas cadeias polipeptídicas, A e B, com 21 e 30 resíduos de aminoácidos, respectivamente, unidas através de ligações dissulfeto (Figura 1). A síntese ocorre a partir de um precursor de 110 aminoácidos, a pré-pró-insulina no retículo endoplasmático rugoso é clivada a pró-insulina. Esta é convertida à insulina e armazenada no complexo de Golgi (NORMAN, LITWACK, 1997; SMITH et al., 2005).

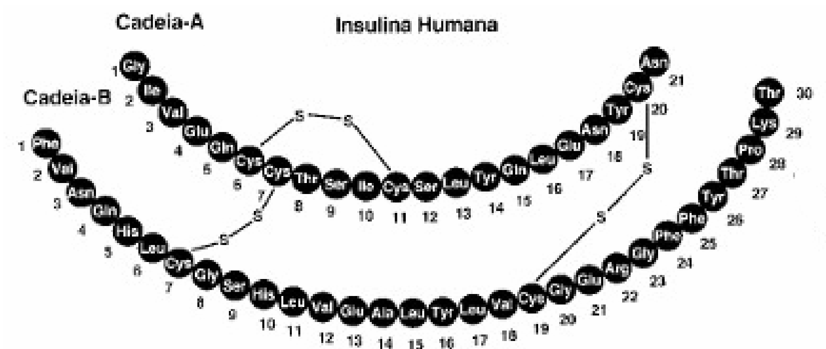


Figura 1. Estrutura básica da insulina: seqüência de aminoácidos.

A insulina é o principal hormônio anabólico responsável pelo controle da captação, utilização e armazenamento dos nutrientes celulares como carboidratos, proteínas e lipídios. Além disso, é essencial para a manutenção da homeostasia da glicose, do crescimento e diferenciação celular (TAHA; KLIP, 1999).

A exocitose das vesículas que estocam insulina e a consequente secreção é estimulada, principalmente, em função da hiperglicemia. A glicose entra na célula β via transportador de glicose específico, GLUT2. No interior da célula a glicose é fosforilada à glicose 6-fosfato pela glicocinase e então, metabolizada. O metabolismo da glicose

aumenta a relação ATP/ADP citoplasmática, gerando o fechamento de canais de potássio dependentes de ATP (K_{ATP}), causando uma despolarização da membrana das células β e abertura de canais de Ca^{2+} dependentes de voltagem, com consequente aumento do influxo de cálcio que, por sua vez, desencadeia o processo de exocitose dos grânulos de insulina, resultando na secreção de insulina (Figura 2) (MACDONALD et al., 2005; OHARA-IMAIZUMI; NAGAMATSU, 2006; SMITH et al., 2005).

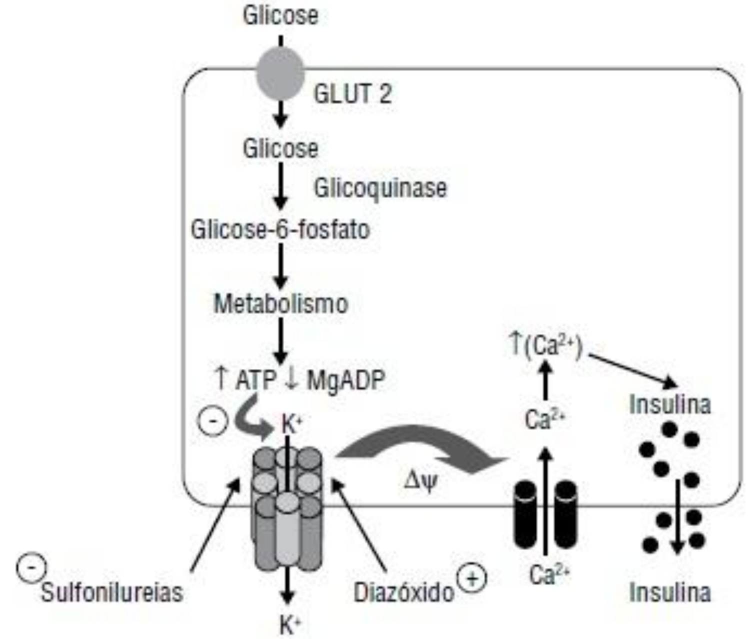


Figura 2. Representação esquemática da célula β pancreática e mecanismo de secreção de insulina (JUNIOR; JUNIOR, 2011).

Quando estimulada pela glicose, a secreção de insulina é bifásica. Primeiramente, ocorre, uma fase rápida (4 – 10 minutos) e transitória, que resulta na liberação da insulina estocada de grânulos localizados próximos à membrana plasmática (estoque de liberação rápida). Em seguida, inicia-se uma segunda fase de maior duração, que ocorre através do deslocamento e ativação de grânulos citoplasmáticos (estoque de reserva) em direção à membrana, bem como através da síntese de

insulina (Figura 3) (HENQUIN et al., 2006; MACDONALD; JOSEPH; RORSMAN, 2005; OHARA-IMAIZUMI; NAGAMATSU, 2006). A meia-vida da insulina circulante é de 3 a 5 minutos e o metabolismo ocorre principalmente no fígado e rins por ação de insulinases (NORMAN; LITWACK, 1997).

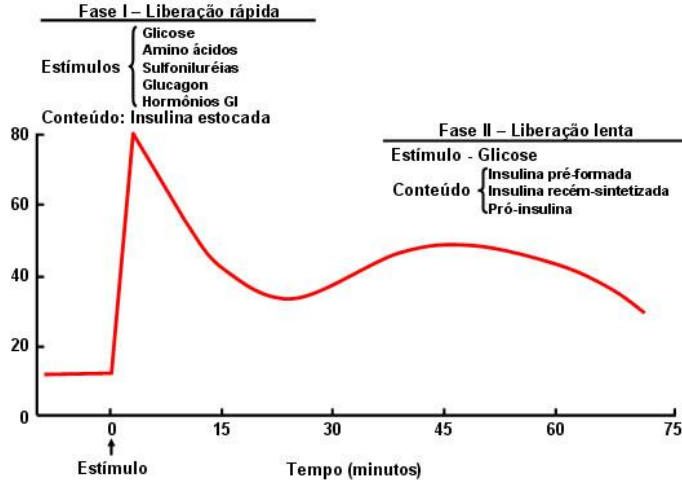


Figura 3. Secreção bifásica de insulina estimulada por glicose (Fonte:http://www.emv.fmb.unesp.br/aulas_on_line/Endocrinologia/diabetes_mellitus/fisiopatologia.asp).

Além da concentração de glicose no sangue, outros fatores podem modular a liberação de insulina. Alguns aminoácidos também podem estimular a secreção de insulina, assim como os hormônios gastrintestinais. Além disso, as ilhotas pancreáticas são inervadas pelo sistema nervoso autônomo, incluindo uma via do nervo vago. Esses sinais neuronais ajudam a coordenar a liberação de insulina com sinais secretórios iniciados pela ingestão de alimentos. Por outro lado, a epinefrina, liberada em resposta ao jejum, estresse, trauma e exercício vigoroso, diminui a liberação de insulina e gera sinais para utilização de energia (SMITH et al., 2005).

A insulina exerce um papel central na regulação da homeostase da glicose e atua de maneira coordenada em eventos celulares que regulam os efeitos metabólicos e de crescimento. Para exercer os efeitos

biológicos, a insulina liga-se a um receptor específico da membrana das células. O receptor de insulina é uma glicoproteína heterotetramérica pertencente à família de receptores tirosina cinases. Consiste de duas subunidades α extracelulares que contém o sítio de ligação à insulina e duas subunidades β trans-membrana com atividade de tirosina cinase (CHANG et al, 2004; SALTIEL; KAHN, 2001; TAHA; KLIP, 1999). O receptor de insulina funciona como uma enzima alostérica, onde a subunidade α inibe a atividade tirosina cinase da subunidade β. A ligação da insulina ao receptor promove autofosforilação da subunidade β em resíduos de tirosina específicos. Além disso, o receptor também sofre mudanças conformacionais que resultam no aumento ainda maior da atividade tirosina cinase do receptor (SALTIEL; KAHN, 2001; CHANG et al., 2004).

A autofosforilação induzida por insulina torna o receptor ativado, aumentando a capacidade de fosforilar um ou mais substratos protéicos intracelulares. Desta forma, a fosforilação de substratos inicia uma série de eventos, incluindo a cascata de reações de fosforilação e desfosforilação que regula os efeitos metabólicos e de crescimento (Figura 4). Além da família de substratos do receptor de insulina (IRS 1-4), diversos outros substratos podem ser fosforilados como, por exemplo, Shc, Gab-1, $p60^{dok}$, Cbl, JAK2 e APS (CHANG et al., 2004; TAHA; KLIP, 1999).

As proteínas IRS-1/2 desempenham função essencial na transmissão do sinal insulínico e a fosforilação desses substratos permite a interação com diversas proteínas adaptadoras ou com atividade enzimática, caracterizando o efeito pleiotrópico da insulina. IRS-1 parece ser a isoforma predominante envolvida na transdução de sinal da insulina no músculo. Já a IRS-2 parece estar envolvida no desenvolvimento das células β pancreáticas e ambas as isoformas são importantes para o metabolismo da glicose no fígado. As funções das IRS-3 e IRS-4 são menos entendidas, mas parecem envolver a regulação das proteínas IRS-1 e IRS-2 (KROOK et al., 2004; SALTIEL; KAHN, 2001)

A ligação das proteínas IRS com o receptor de insulina se dá através dos domínios de ligação a fosfotirosina (PTB) que permitem a fosforilação de diversos resíduos tirosina. Esta fosforilação em tirosina das proteínas IRS cria sítios de reconhecimento para moléculas contendo domínios com homologia a Src 2 (SH2). Muitas dessas moléculas são proteínas adaptadoras como a subunidade regulatória p85 da PI3K, a Grb2 e a CrkII ou enzimas como a fosfotirosina fosfatase,

SHP2, e a tirosina cinase citoplasmática, Fyn (KAHN; PESSIN, 2002; SALTIEL; KAHN, 2001; TAHA; KLIP, 1999; TANIGUCHI et al., 2006).

Além da fosforilação em resíduos de tirosina, tanto o receptor de insulina quanto as proteínas IRS podem ser fosforiladas em resíduos de serina em resposta à insulina e outros estímulos. Este efeito parece ser mediado por diversas cinases como fosfatidilinositol 3-cinase (PI3K), proteína cinase B (PKB/Akt), glicogênio sintase cinase 3 (GSK-3), cinase reguladora de sinal extracelular (ERK), cinase c-jun NH_2 terminal (JNK), proteínas supressoras da sinalização de citocinas (SOCS 1 e 3) e proteína alvo da rapamicina em mamíferos (mTOR) bem como pela interação entre sistemas de sinalização. Estas fosforilações parecem regular negativamente a sinalização insulínica e podem provocar resistência a insulina (SALTIEL; KAHN, 2001; TANIGUCHI et al., 2006). Além disso, a ação da insulina também é atenuada por proteínas tirosina fosfatases (PTPases) as quais catalisam a desfosforilação rápida do receptor de insulina e de substratos, destacando a PTP1B e a SHP2 (CARVALHEIRA, ZECCHIN, SAAD, 2001; SALTIEL; KAHN, 2001; TAHA; KLIP, 1999; TANIGUCHI et al., 2006).

A PI3K é uma enzima importante na regulação da mitogênese e das ações metabólicas da insulina, apresentando uma estreita associação com IRS-1/2. A PI3K é uma serina/treonina cinase formada por uma subunidade catalítica (p110) e uma subunidade regulatória (p85). A ligação dos sítios fosforilados das proteínas IRS 1 e IRS 2 ao domínio SH2 da subunidade p85 da PI3K ativa o domínio catalítico associado. Essa enzima catalisa a fosforilação de fosfoinositídeos na posição 3 do anel inositol, produzindo fosfatidilinositol-3-fosfato, fosfatidilinositol-3,4-fosfato e fosfatidilinositol-3,4,5-trifosfato (PIP3) (SALTIEL; KAHN, 2001; TAHA; KLIP, 1999; TANIGUCHI et al., 2006). O PIP3 funciona como segundo mensageiro intracelular e regula a localização e atividade de diversas proteínas intracelulares, como a proteína cinase 1 dependente de 3-fosfoinositídeos (PDK-1 e PDK-2) e a proteína cinase B (PKB/Akt) através da interação com domínios de homologia de plekstrina (PH). A seguir, a PDK-1 fosforila e ativa a PKB e isoformas de proteína cinase C (PKC) atípica (ξ, λ) (KAHN; PESSIN, 2002; SALTIEL; KAHN, 2001).

A proteina cinase B (PKB), ou Akt, é uma serina treonina cinase que possui três isoformas (Akt1, Akt2 e Akt3), todas expressas em tecido muscular e hepático (DATTA et al., 1999). A PKB tem a habilidade de fosforilar e ativar vários alvos metabólicos, sendo que o mecanismo de ativação da PKB por insulina ocorre em duas etapas. A

primeira etapa envolve a fosforilação em dois sítios, treonina 308 e serina 473 pelas PDK-1 e PDK-2, respectivamente, e uma mudança conformacional da enzima. Já a segunda etapa relaciona-se com a translocação da PKB citosólica para as proximidades da membrana plasmática onde ocorre a fosforilação (HAJDUCH et al., 2001; TAHA; KLIP, 1999).

A PKB estimula a captação de glicose através do aumento da translocação dos transportadores de glicose GLUT4 para a membrana plasmática (KROOK et al., 2004; TANIGUCHI et al., 2006). Além disso, um dos principais alvos da PKB é a enzima glicogênio sintase cinase-3 (GSK-3), estimulando a síntese de glicogênio. Adicionalmente, a PKB possui outras funções não metabólicas, tais como a inibição de apoptose e degradação protéica em músculo esquelético através da fosforilação e inativação da Bad e fatores de transcrição como a FoxO, respectivamente (OGG et al., 1997).

A insulina estimula o transporte de glicose através da ativação de diferentes cascatas de sinalização intracelulares, além da ativação da PI3K, a via da CAP/Cbl é outra via alternativa. Esta cascata envolve a ligação da proteína adaptadora APS ao receptor de insulina com consequente recrutamento e fosforilação de Cbl a qual, em geral, encontra-se associada à proteína adaptadora CAP. Após a fosforilação, o complexo CAP/Cbl migra para a membrana plasmática e interage com a proteína CrkII, que também está constitutivamente associada à proteína C3G. A C3G é uma proteína trocadora de íons que catalisa a troca de guanosina difosfato (GDP) por guanosina trifosfato (GTP) da proteína TC10, ativando-a. A TC10 causa um sinal subsequente para a translocação do GLUT4, em paralelo à via da PI3K (CHANG et al., 2004; KAHN; PESSIN, 2002).

A insulina, semelhante a outros fatores de crescimento, estimula a proteína cinase ativada por mitógeno (MAPK). Essa via inicia com a fosforilação das proteínas IRS e/ou Shc, que interagem com a proteína Grb2 (PAEZ-ESPINOSA et al., 1999). A Grb2 está constitutivamente associada à SOS, proteína que troca GDP por GTP na proteína Ras (codificada pelo proto-oncogene *ras*). SHP2 participa da ativação da Ras, que promove o recrutamento para a membrana da Raf que resulta na ativação da MEK1 e MEK2, as quais fosforilam MAPK/ERK1 e ERK2 em resíduos de tirosina e treonina. A ativação da cascata da MAPK leva à proliferação e diferenciação celulares (BOULTON et al., 1991). Outros alvos também são fosforilados pela MAPK, entre eles a p90S6 cinase ($p90^{rsk}$), fosfolipase A_2 e fatores de transcrição como o ELK1 e $p62^{TCF}$ (SALTIEL; KAHN, 2001; TANIGUCHI et al., 2006). O

bloqueio farmacológico desta via previne a ação da insulina no crescimento celular, mas não tem efeito nas ações metabólicas do hormônio (LAZAR et al., 1995).

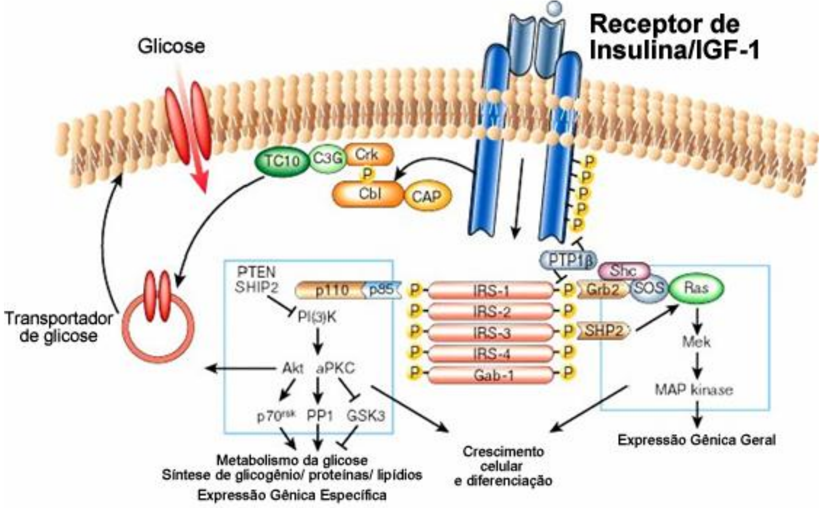


Figura 4. Vias de sinalização da insulina (adaptado de SALTIEL; KAHN, 2001).

1.2 HOMEOSTASIA DA GLICOSE

1.2.1 Absorção e transportadores de glicose

A absorção dos carboidratos da dieta ocorre no intestino. O epitélio intestinal, desde o duodeno até o cólon, é constituído por células denominadas enterócitos que são ligados entre si por junções. Como os carboidratos ingeridos são essencialmente polissacarídeos, para que ocorra a absorção no intestino delgado em nível dos enterócitos das microvilosidades intestinais, os carboidratos sofrem um processo digestivo enzimático para formar unidades mais simples, tais como os monossacarídeos (glicose, galactose e frutose), que são desta forma absorvidos (SMITH et al., 2005).

As glicosidases são enzimas presentes nos enterócitos que participam do processamento de oligossacarídeos provenientes da alimentação, tornando disponíveis os monossacarídeos (KRASIKOV et

al., 2001). Entre as glicosidases estão as α e β-glicosidases localizadas nos enterócitos que revestem a borda em escova das microvilosidades intestinais (GUYTON; HALL, 2002). As α-glicosidades são representadas por enzimas intestinais α-amilase, trealose-6-fosfato hidrolase, sacarase e maltase, enquanto as β-glicosidases compreendem a β-glicosidase, β-galactosidase, β-glicuronidase e β-D-acetil-hexosaminidase (MELO; CARVALHO, 2006).

As α-glicosidades são exoenzimas que hidrolizam a ligação glicosídica do final da cadeia, liberando α-glicoses da porção não redutora da molécula. Estas enzimas estão amplamente distribuídas em microorganismos, plantas e tecidos animais. Várias α-glicosidases podem hidrolizar, não somente oligossacarídeos e α-glicosídeos sintéticos contendo ligações α-glicosídicas, mas também α-glicanos solúveis em água como o amido e glicogênio. Podem também ser denominadas de transglicosidases em razão das atividades hidrolítica e de transglicosilação, catalisando, respectivamente, dois tipos de reações, uma envolvendo a transferência de resíduo de D-glicose do lado não-redutor de oligossacarídeo para molécula de água (hidrólise), ou a transferência deste resíduo para uma unidade aceptora (transglicosilação). Nestas reações, D-glicose é liberada como anômero α, retendo a configuração do substrato no carbono anomérico C-1 no produto de transglicosilação (KRASIKOV et al., 2001).

Após a clivagem dos polissacarídeos em monossacarídeos pela ação das glicosidases, os açúcares são transportados através das células epiteliais do intestino para o sangue para a distribuição a todos os tecidos. A glicose é absorvida através de transportadores específicos dependente de sódio ou difusão facilitada. Desta forma, os transportadores de glicose podem ser classificados em dois grandes grupos: SGLTs e GLUTs (DYER et al., 2003, 2007; KAHN; PESSIN, 2002).

A absorção clássica é mediada pelo co-transporte de sódio e glicose. A família dos transportadores de glicose dependentes de sódio é chamada de SLC5A, (ULDRY; THORENS, 2004). Dentre estes membros, seis deles são denominados SGLT tendo, três deles, funções de transportadores bem estabelecidas. São eles o SGLT1 (SLC5A1), SGLT2 (SLC5A2) e SGLT3 (SLC5A4) (HEDIGER et al., 1987, 1989).

O SGLT1 é encontrado no intestino e participa do transporte de hexoses (D-glicose e D-galactose) do lúmen intestinal para os enterócitos intestinais (HEDIGER et al., 1987, 1989). Este transporta glicose e galactose com afinidades semelhantes e elevadas (constante de afinidade ou K_m =0,1 – 0,6 mM para a glicose), mas com capacidade de

transporte (atividade para elevadas concentrações de substrato ou V_{max}) baixa, sendo esta atividade inibida por florizina (um inibidor competitivo com uma constante de inibição ou $K_i = 0,1$ mM (HEDIGER et al., 1987; TURK et al., 1994).

SGLT2 é predominantemente expresso na membrana apical dos segmentos S1 e S2 do túbulo proximal dos néfrons (KANAI et al., 1994), sendo este transportador o principal responsável pela reabsorção da glicose renal (CALADO et al., 2004; VAN DEN HEUVEL et al., 2002). O SGLT-3 está envolvido na detecção de monossacarídeos na mucosa intestinal (FREEMAN et al., 2006). O SGLT-3 humano (hSGLT-3), que tem 70% de homologia com a SGLT1, é expresso na musculatura lisa e esquelética e, possivelmente, localizado nos neurônios do sistema nervoso entérico. Estudos evidenciam que o SGLT3 pode agir como um sensor da glicose, mas não como um transportador da mesma. O SGLT3 não é expresso nos enterócitos e a existência nas células enteroendrócrinas e enterocromafins ainda não está clara (RAYBOULD, 2007).

A regulação dos SGLTs é essencial para a homeostasia da glicose e a sua expressão é regulada diretamente por concentrações de açúcares na dieta. Essa regulação parece envolver vias de transdução de sinais de receptor acoplado à proteína G e cAMP-PKA (DYER et al., 2003, 2007). SGLT1 possui um local de ligação para sódio que induz uma alteração conformacional no transportador, tornando-o acessível à glicose, que é transportada ativamente contra gradiente de concentração (THOMSON; WILD, 1997a, b). Desse modo, para cada molécula de glicose transportada, dois íons sódio, cujo gradiente transmembranar é gerado pela ATPase-Na^+/K^+ localizada na membrana basolateral, são transportados na mesma direção (WRIGHT et al., 2003).

O transporte facilitado de glicose nos tecido periféricos é mediado através de carreadores solúveis pertencentes à família dos transportadores de glicose, GLUTs. Atualmente, existem 14 membros desta família (GLUTs 1-14) cuja distribuição tecidual, propriedades cinéticas e especificidade de açúcares são variáveis. Essas proteínas são divididas em 3 classes principais, sendo a classe I, a melhor caracterizada, que compreende os GLUTs 1-4 e o GLUT 14 (KAHN; PESSIN, 2002; MANOLESCU et al., 2007).

O GLUT 1 é amplamente expresso, principalmente nos eritrócitos e células endoteliais, e é responsável pela captação basal de glicose. Por outro lado, a isoforma GLUT2 é expressa principalmente nas células β e no fígado além dos rins e intestino. O GLUT2 possui baixa afinidade para a glicose (K_m elevado), e por esse motivo, juntamente com a

hexocinase e/ou glicocinase, funciona como parte do sensor de glicose nestas células. O GLUT3 tem a maior afinidade (menor K_m) para glicose e está presente em tecidos como o cérebro, tecido muscular e coração. O GLUT4 é a isoforma predominante nos tecidos sensíveis à insulina, como músculo e tecido adiposo e é responsável pelo transporte de glicose estimulado por insulina (KAHN; PESSIN, 2002; MANOLESCU et al., 2007; WATSON; PESSIN, 2001).

Na classe II estão incluídos os GLUT 5, GLUT 7, GLUT 9 e GLUT 11. O GLUT 5 é o transportador da frutose presente no intestino, mas também é encontrado nos testículos e rins. O GLUT 7 é encontrado no intestino, colon, testículos e próstata, enquanto que o GLUT 9 é altamente expresso nos rins e fígado. O GLUT 11 apresenta duas formas (uma curta e outra longa), as quais são expressas em diferentes tecidos e apresenta baixa afinidade por glicose além de transportar frutose. A classe III é composta pelas isoformas GLUT 6, GLUT 8, GLUT 10, GLUT 12 e pelo transportador de H^+ ligado ao mio inositol (HMIT). O GLUT 6 apresenta baixa afinidade por glicose, sendo expresso no cérebro, leucócitos e vesícula biliar enquanto que o GLUT 8 apresenta alta afinidade por glicose e é expresso principalmente nos testículos. O GLUT 10 é predominantemente expresso no fígado e pâncreas e está associado com o diabetes do tipo 2. GLUT 12 é expresso predominantemente no coração e próstata e o HMIT no cérebro (SCHEEPERS et al., 2004; WOOD; TRAYHURN, 2003).

O açúcar sai dos enterócitos passivamente através do transportador GLUT2 que, a princípio, existe somente na membrana basolateral. O GLUT2 apresenta baixa afinidade (K_m para a glicose >50 mM) e alta capacidade para a glicose e, além dela, transporta também a frutose, galactose e manose (DROZDOWSKI; THOMSON, 2006; HEDIGER et al., 1987).

Estudos evidenciam que o GLUT2 pode difundir transitoriamente para a membrana apical dos enterócitos, em resposta a altas concentrações de glicose no lúmen intestinal. O GLUT 2 é translocado do interior do enterócito para a superfície da borda em escova por ação da SGLT1 envolvendo proteínas cinases C (PKC), mais especificamente as isoformas PKC βII e proteínas cinases ativadas por mitógenos (MAP), entre outras (Figura 5). A presença deste transportador na membrana apical dos enterócitos explicaria, ao menos em parte, a absorção de glicose quando a mesma se encontra em altas concentrações, uma vez que o transportador SGLT1 apresenta um V_{max} baixo, ou seja, rapidamente saturável, sendo assim, a absorção linear da glicose, após a ingesta alimentar, seria garantida pela ação do GLUT2

(DROZDOWSKI; THOMSON, 2006; HELLIWELL et al., 2003; KELLETT et al., 2008; MIYAMOTO et al., 1993; TURK et al., 1994; WRIGHT et al., 2003).

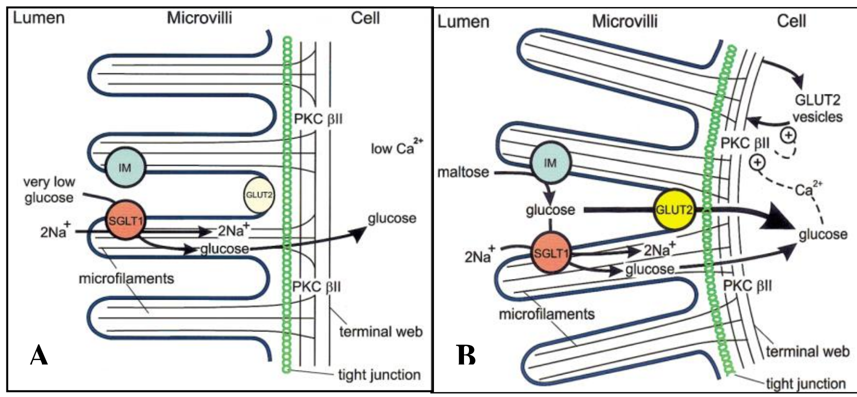


Figura 5. Transporte de glicose no período de jejum via SGLT1 (A) e alimentado (B) via SGLT1 e GLUT2 (Adaptado de KELLETT, 2001).

1.2.2 Captação de glicose em tecidos periféricos

A captação de glicose no músculo e tecido adiposo ocorre através do estímulo e vias de sinalização da insulina. O passo limitante do processo de captação de glicose nesses tecidos sensíveis à insulina é a translocação dos transportadores GLUT4 dos estoques intracelulares para a membrana. Sob condições basais, o GLUT4 está localizado em compartimentos intracelulares responsivos à insulina conhecidos como estruturas tubulovesiculares. A translocação do GLUT4 dos compartimentos de armazenamento para a membrana ocorre após o estímulo da insulina, e quando cessado este estímulo, ocorre a internalização dos tranportadores através de endocitose com fragmentos de membrana plasmática na forma de vesículas (HOU; PESSIN, 2007; KAHN; PESSIN, 2002; WATSON; PESSIN, 2007).

O mecanismo de captação de glicose estimulado por insulina inicia-se com a ligação da insulina ao receptor de membrana e consequente ativação da atividade tirosina cinase do receptor (KAHN; PESSIN, 2002; TAHA; KLIP, 1999). Uma vez ativado, o receptor de insulina promove a fosforilação das proteínas IRS-1 e IRS-2, as quais promovem a subsequente fosforilação de proteínas com domínios SH2 como a subunidade regulatória p85 da PI3K. Essa enzima catalisa a

formação de PIP3 que funciona como segundo mensageiro intracelular e regula a localização e atividade da PDK-1 e PDK-2. A seguir, as PDK-1 e PDK2 fosforilam e ativam a PKB e isoformas de proteína cinase C (PKC) atípica (ξ, λ) (CHANG et al., 2004; KAHN; PESSIN, 2002; TAHA; KLIP, 1999). A PKB (ou Akt) regula a captação de glicose através do aumento da translocação dos transportadores de glicose GLUT4 para a membrana. Esse efeito parece ser mediado pela fosforilação da proteína AS 160, que possui domínios de proteína ativadora de GTPases (GAP). A AS160 tem ações específicas para proteínas da família Rab nos diversos aspectos do tráfego de vesículas intracelulares além de estar envolvida na reorganização do citoesqueleto (HAJDUCH et al., 2001; HOU; PESSIN, 2007; WATSON; PESSIN, 2007). Assim como a PKB, as isoformas atípicas de PKC (ξ, λ) regulam a translocação do GLUT4 induzida por insulina para a membrana plasmática (LIU et al., 2006; TANIGUCHI et al., 2006).

Além da ativação da PI3K, outra via estimulada por insulina para a captação de glicose é a via da CAP/Cbl/T10. Esta cascata envolve a ligação da proteína adaptadora APS ao receptor de insulina com conseqüente recrutamento e fosforilação de Cbl que, em geral, encontra-se associada à proteína adaptadora CAP. Após a fosforilação, o complexo CAP/Cbl migra para a membrana plasmática e interage com a proteína CrkII que também está constitutivamente associada à proteína C3G, esta é uma proteína trocadora de íons que catalisa a troca de GDP por GTP da proteína TC10, ativando-a. Desta forma, a TC10 induz um sinal subsequente para a translocação do GLUT4 em paralelo à ativação da via da PI3K (CHANG et al., 2004; KAHN; PESSIN, 2002).

No processo de ligação e fusão das vesículas contendo GLUT4 com a membrana plasmática dois grupos de proteínas sinalizadoras estão presentes, v-SNAREs e t-SNAREs. Algumas dessas proteínas (VAMP2, sintaxina 4, SNAP23, Munc18 e SYNIP) regulam a fusão das vesículas com a membrana plasmática. A insulina recruta as vesículas de GLUT4 em direção à membrana através da fosforilação e ativação de VAMP2 na superfície das vesículas. Essa proteína interage com SNAP23 e sintaxina 4 na membrana da célula, formando um complexo ternário. Concomitantemente à formação desse complexo, ocorre a dissociação da proteína SYNIP da sintaxina 4 e a mudança conformacional de Munc18, expondo o domínio de ligação do complexo ternário à VAMP2, promovendo a fusão das vesículas com a membrana (CHANG et al., 2004; HOU; PESSIN, 2007; WATSON; PESSIN, 2007).

Adicionalmente, os microtúbulos e os filamentos de actina apresentam funções importantes no tráfego do GLUT4, principalmente pelo direcionamento do movimento das vesículas da região perinuclear para a membrana em resposta à insulina. A regulação da actina cortical pela TC10, bem como a ação de proteínas quinesinas motoras dos microtúbulos KIF5b e KIF3, facilitam o trânsito dos GLUT4, através do remodelamento dinâmico do citoesqueleto e dos microtúbulos (CHANG et al., 2004; HOU; PESSIN, 2007).

Por outro lado, no músculo esquelético, o transporte de glicose pode ser estimulado, independentemente da insulina, através do exercício ou hipóxia. Nesta via de regulação do transporte de glicose não-insulino dependente, um dos reguladores é a proteína cinase ativada por 5'-AMP (AMPK) (KROOK et al., 2004; MUSI; GOODYEAR, 2003).

1.2.3 Síntese de glicogênio

Nos tecidos de mamíferos, os carboidratos são estocados principalmente na forma de glicogênio, sendo que os principais locais de depósito de glicogênio são o fígado e o músculo esquelético. Além destes, tecidos como músculo liso e cardíaco, rins, cérebro e tecido adiposo também são capazes de sintetizar e armazenar glicogênio (ROACH, 2002; SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999).

A insulina regula a síntese de glicogênio em duas etapas: a primeira através do controle da captação de glicose e a segunda pela regulação dos estados de fosforilação e ativação das enzimas envolvidas na síntese e degradação do glicogênio (ROACH, 2002; SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999; VILLAR-PALASÍ; GUINOVART, 1997). Os mecanismos moleculares pelos quais a insulina regula o metabolismo do glicogênio são complexos e podem variar entre diferentes tipos celulares.

A glicose, após entrar na célula, é fosforilada à glicose 6-fosfato pela hexocinase muscular e/ou pela glicocinase/hexocinase hepáticas. A glicose 6-fosfato (G-6-P) é convertida a glicose 1-fosfato (G-1-P) pela enzima fosfoglicomutase e, a seguir, convertida em uridina-difosfato glicose (UDP-G) pela enzima uridina-difosfato glicose pirofosforilase. A UDP-G formada serve como doador de unidades glicosil para a cadeia de glicogênio nascente. Essa reação é catalisada pela enzima glicogênio sintase (GS), ponto-chave na síntese de glicogênio. Além da GS, uma proteína iniciadora chamada glicogenina e uma enzima ramificadora

também contribuem para o processo de síntese e armazenamento de glicogênio (FERRER et al., 2003; ROACH, 2002; SRIVASTAVA; PANDEY, 1998).

A GS é uma proteína multimérica e sua atividade é regulada por mecanismos alostéricos e de fosforilação/desfosforilação. Duas isoformas desta enzima são descritas em mamíferos, uma expressa no fígado e a outra no músculo e em diversos outros tecidos (ROACH, 2002; SRIVASTAVA; PANDEY, 1998).

A insulina modula a atividade da GS através de modificação covalente, translocação e regulação alostérica. Uma das vias que modulam a atividade da GS é a da PI3K. A via da PI3K estimulada pela insulina resulta na ativação das PDK1 e PDK2 que, por sua vez, fosforilam a PKB. A PKB promove a fosforilação e inativação da GSK-3 em resíduos de serina (S21 e S9). Além da PKB, a $p90^{rsk}$ e a p70S6 cinase também parecem fosforilar e inibir a GSK-3. Esta enzima existe sob duas isoformas, GSK-3α e β, constitutivamente ativas, no estado basal, e que através da fosforilação nos sítios 3a, 3b, 3c e 4 inativam a glicogênio sintase. A inibição da GSK-3 reduz a taxa de fosforilação da GS, resultando em ativação da enzima e conseqüente aumento da síntese de glicogênio (BRADY; SALTIEL, 2001; FERRER et al., 2003; FORDE; DALE, 2007; MORA et al., 2005; SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999; VILLAR-PALASÍ; GUINOVART, 1997). Por outro lado, além da GSK-3, a inibição da PKA por insulina, cinases dependentes de calmodulina e AMPK, e ainda a ativação da via da mTOR, também parecem estar envolvidas na ativação da glicogênio sintase (Figura 6) (BRADY; SALTIEL, 2001; ROACH, 2002).

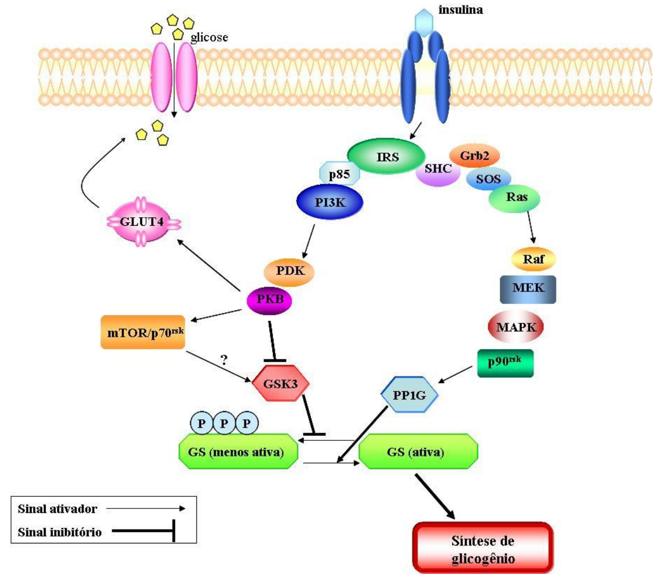


Figura 6. Cascata de fosforilação e ativação da glicogênio sintase.

A inativação da GSK-3 não é suficiente para causar a ativação completa da glicogênio sintase, uma vez que ela não fosforila vários dos resíduos na GS que são desfosforilados pela insulina (1a, 1b, 2, 2a, 5). Mecanismos adicionais podem envolver a desfosforilação da enzima por fosfatases como a proteína fosfatase do tipo 1 (PP1) (BRADY; SALTIEL, 2001; SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999;).

A PP1 é constituída de uma subunidade catalítica (PP1-C) e diferentes subunidades regulatórias. Essas subunidades regulatórias são constituídas de quatro proteínas diferentes, chamadas fosfatases associadas ao glicogênio (PP-1Gs) que estão relacionadas com a ligação da PP1 com as partículas de glicogênio. G_M/R3 e G_L/R4 (PP-1G) são expressas no músculo esquelético e no fígado, respectivamente, enquanto as subunidades alvo do glicogênio PTG (*protein targeting to glycogen*) R5 e R6 são distribuídas no músculo, fígado e tecido adiposo. Essas subunidades regulatórias formam complexos com a subunidade catalítica e ligam a enzima a partículas de glicogênio e a outras estruturas celulares. As PTGs ainda podem interagir e complexar diretamente com a glicogênio-sintase (BRADY; SALTIEL, 2001;

ROACH, 2002; SRIVASTAVA; PANDEY, 1998). A insulina promove a fosforilação e ativação da PP1. O mecanismo inicial proposto é através da cascata da MAPK, leva à ativação da $p90^{rsk}$, e esta fosforila e aumenta a atividade da PP1 (BRADY; SALTIEL, 2001; SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999).

Além da regulação por modificação covalente, a GS também é regulada por efetores alostéricos. Um dos efetores alostéricos envolvidos na ativação da GS é a glicose 6-fosfato (G-6-P). O mecanismo de ativação da enzima parece envolver a ligação da G-6-P à GS leva a uma mudança conformacional que converte a enzima em um substrato melhor para a ação das fosfatases. A ligação da G-6-P na glicogênio fosforilase reduz o efeito inibitório dessa enzima sobre a glicogênio sintase, o que também facilita a ativação da GS (FERRER et al., 2003; SRIVASTAVA; PANDEY, 1998;VILLAR-PALASÍ; GUINOVART, 1997).

O processo de degradação das moléculas de glicogênio é feito pela enzima glicogênio fosforilase. Esta produz glicose 1-fosfato que é convertida em glicose 6-fosfato e, no fígado através da glicose 6-fosfatase, enzima que só está presente no fígado e rins, irá originar moléculas de glicose livre. A glicogênio fosforilase é regulada por fatores alostéricos e por modificações covalentes (fosforilação). Essa regulação se relaciona com a necessidade de energia nas células, de modo que altas concentrações de substratos energéticos (ATP, G-6-P, glicose) inibem a glicose fosforilase enquanto baixos níveis de substratos energéticos a ativam (AMP e outros). A regulação covalente da glicose fosforilase é feita pela fosforilação da fosforilase cinase e desfosforilação pela PP1. A ativação da fosforilase cinase é mediada por concentrações intracelulares aumentadas de Ca^{2+}, fosforilação pela AMPK, e por Mg^{2+}, ADP e pH (FERRER et al., 2003; ROACH, 2002).

1.2.4 Cálcio (Ca^{2+})

O Ca^{2+} é um mensageiro intracelular que pode regular muitos processos biológicos em diferentes células, como secreção, contração, metabolismo, transcrição gênica, apoptose, entre outros (BERRIDGE; BOOTMAN; RODERICK, 2003; CARAFOLI, 2005). Os níveis de Ca^{2+} são 10.000 vezes maiores no meio extracelular do que a concentração intracelular de Ca^{2+} livre ($[Ca^{2+}]_i$), a qual é determinada por um equilíbrio entre influxo e efluxo do íon que envolve canais, bombas e trocadores de Ca^{2+} na membrana plasmática. O Ca^{2+} intracelular livre é

a forma fisiologicamente ativa (BERRIDGE; BOOTMAN; RODERICK, 2003; CARAFOLI, 2005).

Existem diversos canais de membrana plasmática que controlam a entrada de Ca^{2+}, em resposta a estímulos que incluem a despolarização da membrana, agonistas extracelulares, deformação mecânica, mensageiros intracelulares e depleção dos estoques intracelulares. Com base na maneira pela qual eles são ativados, estes canais são classificados como canais Ca^{2+} dependentes de voltagem (CCDV), de receptores de membrana, de segundos mensageiros, entre outros. Os canais Ca^{2+} dependentes de voltagem (CCDV) abrem e fecham em reposta a mudanças no potencial transmembrana, e são subdivididos em muitos subtipos com base na sensibilidade a voltagem e a inibidores, entre os quais canais de Ca^{2+} do tipo T, L, N, P/Q e R. Canais do tipo T são ativados por uma baixa voltagem e controlam o influxo de Ca^{2+} em células excitáveis durante uma pequena despolarização em torno do potencial de repouso. Os canais do tipo L são ativados por alta voltagem e são encontrados principalmente no músculo e células endócrinas, onde iniciam o processo da contração e da secreção (BERRIDGE; BOOTMAN; RODERICK, 2003; CARAFOLI, 2005).

A abertura de canais de Ca^{2+} e o consequente aumento dos níveis do íon levam à formação de sinais intracelulares que podem induzir tanto processos celulares localizados nas proximidades dos canais como estimular processos em nível global, como a ativação de outros canais e proteínas (BERRIDGE; BOOTMAN; RODERICK, 2003).

O Ca^{2+} está envolvido em multiplos processos biológicos e também participa da homeostasia da glicose. Como previamente descrito, a absorção de glicose, no estado alimentado, é realizada através dos transportadores SGLT1 e GLUT2. O transporte de glicose através de SGLT1 induz a um aumento da concentração intracelular de Ca^{2+} que, por sua vez, ativa uma proteína cinase dependente de Ca^{2+} (PKC) que ativa e recruta o GLUT2 para a membrana apical, matendo a absorção da glicose (KELLET, 2001).

O cálcio tem papel essencial na secreção de insulina estimulada pela glicose nas células β. O metabolismo da glicose induz o fechamento de canais K_{ATP} com consequente despolarização da membrana e influxo de Ca^{2+} através de canais de Ca^{2+} dependentes de voltagem. Esse aumento da concentração de Ca^{2+} citosólico desencadeia a exocitose dos grânulos contendo insulina e sua secreção (HENQUIN, 2011).

Estudos recentes demonstram a participação do Ca^{2+} também na captação de glicose mediada pela insulina em tecidos periféricos. O Ca^{2+}

parece atuar sobre os passos finais na cascata de sinalização de insulina, isto é, na ancoragem e fusão das vesículas do GLUT4 com a membrana plasmática. Além disso, o Ca^{2+} participa na ativação de proteínas, como a proteína cinase dependente de cálcio-calmodulina (PKCaM) e AMPK, as quais estimulam a translocação do GLUT4 e captação de glicose através de vias independentes de insulina (LANNER et al., 2008).

1.2.5 Manutenção da homeostasia da glicose

A glicose é o primeiro e principal substrato metabólico para a atividade normal do ser humano. Desta forma, é fundamental a manutenção da homeostasia glicêmica, tanto no estado absortivo e pós-absortivo, quanto no jejum. No estado fisiológico normal esta homeostasia da glicose é mantida através da regulação hormonal da captação periférica e produção endógena de glicose, primariamente pelo músculo, tecido adiposo e fígado, além da secreção de insulina pelo pâncreas (BEARDSALL et al., 2003; MOORE et al., 2003; SALTIEL; KAHN, 2001; TAHA; KLIP, 1999).

A insulina é um hormônio anabólico com papel essencial na regulação do metabolismo, crescimento e diferenciação celular e atua em diversos tecidos. De maneira geral, as ações da insulina incluem o estímulo da captação, utilização e armazenamento intracelular de glicose, aminoácidos e ácidos graxos e a inibição de processos catabólicos como a glicogenólise, lipólise e proteólise (BEARDSALL et al., 2003; MOORE et al., 2003; SALTIEL; KAHN, 2001).

No estado pós-prandial, quando as concentrações de glicose sangüínea estão elevadas, a hiperglicemia sinaliza às células β do pâncreas para produzir e liberar insulina e suprimir a produção de glucagon pelas células α das ilhotas pancreáticas (BEARDSALL et al., 2003; TAHA; KLIP, 1999). Uma vez liberada, a insulina estimula a captação de glicose no músculo através do aumento da translocação dos GLUT4 para a membrana. Além disso, as concentrações aumentadas de glicose no interior das células musculares estimulam a síntese de glicogênio através da ativação da GS, bem como a glicólise para produção de energia. A glicose que não é imediatamente utilizada é captada pelo fígado onde a insulina estimula a produção de glicogênio através da estimulação da GS, inibição da glicogênio fosforilase e inibição da gliconeogênese e da glicogenólise. No tecido adiposo, a insulina estimula a captação de glicose semelhante ao músculo e promove a lipogênese, aumenta a atividade da lipoproteína lipase, que

libera ácidos graxos para a síntese de triglicerídeos e inibe a lipase hormônio-sensível, enzima responsável pela quebra dos estoques de gordura. Com relação ao metabolismo protéico, a insulina também possui um efeito anabólico, promovendo a entrada de aminoácidos nas células e estimulando a síntese protéica (Figura 5) (BEARDSALL et al., 2003; MOORE et al., 2003; TAHA; KLIP, 1999).

Adicionalmente, no período pós-prandial ocorre a liberação de inúmeros peptídeos e neurotransmissores, incluindo os hormônios incretinas, como o peptídeo insulinotrópico dependente de glicose (GIP) e o peptídeo semelhante ao glucagon (GLP-1). Estes hormônios participam do controle da homeostasia da glicose, promovem a liberação de insulina dependente de glicose, e são classificados como hormônios insulinotrópicos (DUBE; BRUBAKER, 2004; PERFETTI et al., 1999).

Durante o jejum ou entre as refeições, as concentrações de insulina diminuem e as de glucagon e outros hormônios contra-regulatórios (epinefrina, cortisol e outros) aumentam. O glucagon atua primariamente no fígado, com o objetivo de ativar vias que levem ao aumento das concentrações plasmáticas de glicose como a gliconeogênese e glicogenólise. Embora as concentrações de glicose sangüínea sejam mantidas inicialmente pela glicogenólise hepática, os estoques de glicogênio são limitados, e após um jejum prolongado, a contribuição da gliconeogênese hepática, bem como renal, aumenta progressivamente, a partir de glicerol, lactato e aminoácidos (BEARDSALL et al., 2003; SALTIEL; KAHN, 2001; ZIERATH; KAWANO, 2003).

Durante o jejum, a captação de glicose no músculo é reduzida e este torna-se altamente dependente da oxidação de ácidos graxos para obtenção de energia. Além disso, ocorre aumento da glicogenólise e proteólise muscular. No tecido adiposo ocorre ativação da lipólise com elevação da liberação de ácidos graxos e glicerol, que servem como precursores gliconeogênicos e cetogênicos no fígado (Figura 7) (BEARDSALL et al., 2003; MOORE et al., 2003).

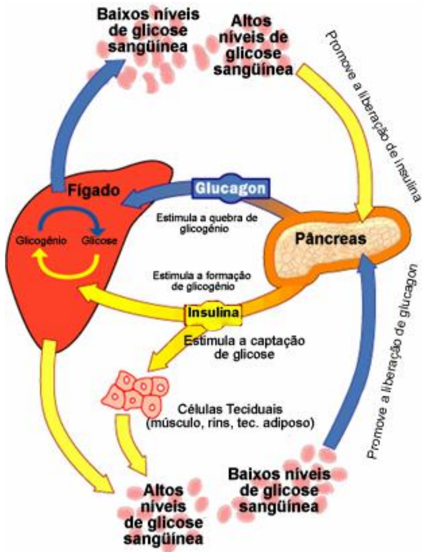


Figura 7. Regulação da homeostasia da glicose
(adaptado de: http://health.howstuffworks.com/diabetes1.htm).

A insulina é o hormônio mais importante que regula o metabolismo energético. Uma deficiência relativa ou absoluta, como no caso da diabetes, leva a severas disfunções nos principais órgãos-alvos da insulina, isto é, fígado, tecido adiposo e músculo esquelético (HEI, 1998). A falta de insulina pode levar ao aumento das concentrações glicêmicas, redução da captação de glicose pelos tecidos periféricos e redução da lipogênese e da síntese proteica, com os aminoácidos sendo utilizados como substrato para a gliconeogênese. Além disso, ocorre ativação da produção hepática de glicose e aumento da lipólise no tecido adiposo, com conseqüente elevação de ácidos graxos na circulação (MOORE et al., 2003).

1.3 DIABETES MELITO

1.3.1 Aspectos Gerais

A diabetes melito (DM) é um grupo heterogêneo de distúrbios metabólicos de etiologia múltipla caracterizados pela hiperglicemia, que é resultante de defeitos na ação e/ou na secreção de insulina. O estado hiperglicêmico crônico está associado a muitas complicações

observadas nos pacientes diabéticos, como disfunções e insuficiência de vários órgãos, especialmente olhos, rins, nervos, cérebro, coração e vasos sangüíneos (BRASIL, 2006; JAMES et al., 2003; SOCIEDADE BRASILEIRA DE DIABETES, 2009).

O envelhecimento da população e a adoção de estilos de vida pouco saudáveis, como sedentarismo, dieta inadequada e obesidade, são os grandes responsáveis pelo aumento da incidência e prevalência da diabetes em todo o mundo. Atualmente, caracterizada como uma epidemia mundial, traduz-se em um grande desafio para os sistemas de saúde de todo o mundo, pois é uma doença que apresenta alta morbi-mortalidade, com perda importante na qualidade de vida (BRASIL, 2006).

Segundo estimativas da Organização Mundial de Saúde (OMS), atualmente mais de 220 milhões de pessoas da população mundial tem diabetes, com expectativa de alcançar 366 milhões de pessoas em 2030. No Brasil, é estimado que atualmente sejam cerca de seis milhões de portadores, devendo alcançar 10 milhões de pessoas em 2025 (BRASIL, 2006; WHO, 2008; WILD et al., 2004).

A classificação da DM utilizada atualmente aceita pela Sociedade Brasileira de Diabetes (SBD) e pela OMS foi proposta em 1997 pela Associação Americana de Diabetes (AMERICAN DIABETES ASSOCIATION - ADA) (AMERICAN DIABETES ASSOCIATION, 2011). A diabetes melito é classificada de acordo com a etiologia da doença e não mais pelo tipo de tratamento. Assim, os termos DM insulino-dependentes e DM não insulino-dependentes, devem ser evitados. Desta forma, a DM é classificada em quatro categorias, que são: DM tipo 1, DM tipo 2, outros tipos específicos de DM e DM gestacional (AMERICAN DIABETES ASSOCIATION, 2011; SOCIEDADE BRASILEIRA DE DIABETES, 2009).

A DM tipo 1 compreende 5-10% dos casos, sendo resultante da destruição das células beta (β)-pancreáticas que leva à deficiência absoluta de insulina, destruição essa que pode ser de causa auto-imune ou idiopática. Por outro lado, a DM tipo 2 abrange 90-95% dos casos e caracteriza-se pela resistência em graus variados à insulina e deficiência relativa de secreção de insulina. Este último tipo, a DM tipo 2, pode ocorrer em qualquer idade, porém geralmente é diagnosticado após os 40 anos, sendo que os pacientes na maioria apresentam sobrepeso ou obesidade (AMERICAN DIABETES ASSOCIATION, 2011; SOCIEDADE BRASILEIRA DE DIABETES, 2009).

Outros tipos de diabetes podem ser causados por defeitos genéticos funcionais das células beta, defeitos genéticos da ação da

insulina, doenças do pâncreas exócrino, endocrinopatias associadas com a produção excessiva de antagonistas da insulina, indução por fármacos ou agentes químicos, ou ainda por infecções associadas à destruição das células beta (AMERICAN DIABETES ASSOCIATION, 2011; SOCIEDADE BRASILEIRA DE DIABETES, 2009).

A DM gestacional é caracterizada pela diminuição da tolerância à glicose, que é diagnosticada pela primeira vez na gestação, podendo ou não persistir após o parto. Desta forma, a paciente deve ser reavaliada 6 semanas após o parto e ser reclassificada como apresentando DM, glicemia de jejum alterada, tolerância à glicose diminuída ou normoglicemia (AMERICAN DIABETES ASSOCIATION, 2011; SOCIEDADE BRASILEIRA DE DIABETES, 2009).

Ainda outras duas categorias são propostas, que são a glicemia de jejum alterada e a tolerância à glicose diminuída, as quais são referidas como pré-diabetes e podem ser fatores de risco para o desenvolvimento de DM e doenças cardiovasculares (AMERICAN DIABETES ASSOCIATION, 2011; SOCIEDADE BRASILEIRA DE DIABETES, 2009)

O diagnóstico clínico da DM em humanos está relacionado aos sintomas clássicos desta patologia decorrentes da hiperglicemia, tais como poliúria, polidipsia, polifagia e emagrecimento, estes principalmente relacionados ao DM tipo 1. Já no tipo 2 cerca de 50% dos pacientes são assintomáticos, ou apresentam sintomas inespecíficos como tonturas, dificuldade visual, astenia e/ou câimbras (FORTI et al., 2006; MILECH; PEIXOTO, 2004).

Os critérios de diagnóstico laboratorial para DM preconizados pela OMS e pela SBD atualizados pela ADA (AMERICAN DIABETES ASSOCIATION, 2011) são:

- Hemoglobina glicada (HA1C) ≥ 6,5 %
- Glicemia de jejum de oito horas ≥ 126 mg/dL (duas ocasiões);
- Glicemia casual (em qualquer hora do dia, independente do horário da última refeição) > 200 mg/dL, em um paciente com sintomas (polidipsia, poliúria, emagrecimento, distúrbios visuais e outros) somado a glicemia de jejum ≥ 126 mg/dL;
- Valor de 2 horas da glicemia ≥ 200 mg/dL durante o teste oral de tolerância à glicose (TOTG) preconizado pela OMS;
- <u>Glicemia de jejum alterada</u> = 100 a 125 mg/dL;
- <u>Tolerância à glicose diminuída</u> = valor de 2 horas da glicemia entre 140 e 199 mg/dL no TOTG.

1.3.2 Tratamento

A DM tipo 2 é uma desordem metabólica que resulta de interações complexas de diversos fatores ambientais e genéticos. Pacientes diabéticos frequentemente apresentam um modelo complexo de anormalidades metabólicas e fisiológicas, incluindo hiperglicemia, hipertensão, obesidade e hiperinsulinemia. Em geral, o tratamento inicial da diabetes envolve mudanças no estilo de vida, especialmente relacionadas à dieta, exercício físico e controle de peso. Porém, quando o paciente com DM tipo 2 não responde às medidas não medicamentosas, é indicada a terapia com fármacos antidiabéticos, com o objetivo de controlar a glicemia e prevenir e/ou reduzir a severidade das complicações oriundas desta patologia (FOWLER, 2007; KOSKI, 2006; SOCIEDADE BRASILEIRA DE DIABETES, 2009).

A escolha do tratamento, monoterapia ou associação entre medicamentos, segue critérios que devem ser avaliados, tais como o peso e idade do paciente, os valores das glicemias de jejum e pós-prandial, valores da hemoglobina glicada (HbA1c), presença de complicações, outros distúrbios metabólicos e doenças associadas, assim como possíveis interações com outros medicamentos, reações adversas e contra-indicações (SOCIEDADE BRASILEIRA DE DIABETES, 2009).

1.3.2.1 Antidiabéticos orais

Antidiabéticos orais são fármacos que tem como finalidade reduzir a glicemia e mantê-la em níveis normais (inferior a 99 mg/dL em jejum). De acordo com o mecanismo de ação, esses medicamentos podem ser divididos entre: os que incrementam a secreção de insulina (sulfoniluréias e glinidas); os que diminuem a produção hepática de glicose (biguanidas); os que aumentam a utilização periférica de glicose (glitazonas); e os que reduzem a velocidade de absorção de glicídios (inibidores das alfa-glicosidases) (SHARIF, 2011).

As sulfoniluréias são medicamentos secretagogos de insulina, ou seja, estimulam a secreção pancreática de insulina. O mecanismo de ação envolve a inibição dos canais da K_{ATP} nas células β no pâncreas, e, em resposta à despolarização das células, os canais de Ca^{2+} da membrana plasmática abrem, propiciando a entrada deste íon no citosol, resultando na geração de um sinal à liberação da insulina estocada para a circulação (BIJLSTRA et al., 1996; DEL et al., 2007). As sulfoniluréias de primeira geração são representadas pela clorpropamida, tolbudamida

e tolazamida. Por outro lado, os agentes de segunda geração das sulfoniluréias, que são mais potentes, seguros e eficazes, são representados pela glibenclamida, glipizida, glimepirida e gliburida. Esta classe tem ação hipoglicemiante prolongada e promove queda de até 2 % na HbA1c. Além disso, esses fármacos são também sensibilizadores da ação de insulina em tecidos periféricos, melhorando a tolerância à glicose e restaurando a função das células β (FOWLER, 2007; SOCIEDADE BRASILEIRA DE DIABETES, 2009).

As metilglinidas ou glinidas (repaglinida e nateglinida) também são fármacos secretagogos de insulina, porém, diferentemente das sulfoniluréias, apresentam um tempo de ação mais curto, abrangendo principalmente o período pós-prandial. A hipoglicemia, como efeito adverso, é menos frequente com estes fármacos do que com as sulfoniluréias, se tomadas antes da ingestão alimentar (FOWLER, 2007; SOCIEDADE BRASILEIRA DE DIABETES, 2009).

A metformina, que é a única representante de biguanidas disponível comercialmente, apresenta maior ação anti-hiperglicêmica, mas não tem o mecanismo de ação totalmente definido, embora sua ação esteja relacionada com a diminuição da produção hepática de glicose acompanhada da ação sensibilizadora da insulina em tecidos periféricos (SOTO et al., 2008).

Rosiglitazona e pioglitazona, representantes da classe das glitazonas (tiazolidinadionas), são conhecidas como sensibilizadores da insulina e promovem o aumento da ação insulínica no tecido adiposo, músculo esquelético e fígado. Além disso, as glitazonas são agonistas de PPAR-γ, que é predominantemente expresso no tecido adiposo. A ativação de PPAR-γ leva ao aumento da expressão de diferentes genes que controlam a homeostasia glicêmica, lipídica e inflamatória, reduzindo, assim, a resistência à insulina e, indiretamente, promovendo a secreção de insulina (JUNCÀ; RICART, 2008).

Os inibidores das α-glicosidades, como a acarbose, agem inibindo as enzimas intestinais responsáveis por hidrolizar os polissacarídeos em monossacarídeos, desta forma reduzem a velocidade de absorção de glicose, atuando numa fase mais precoce, ainda no tubo digestivo, predominantemente controlando a hiperglicemia pós-prandial (GOMIS, 2008).

Contudo, o conhecimento emergente dos mecanismos que envolvem a fisiopatologia da DM permite que novos alvos moleculares para o desenvolvimento de fármacos sejam investigados. O foco de investigação está voltado para a nova classe de substâncias cuja ação baseia-se no efeito incretina, o qual é mediado pelos hormônios GLP-1 e

GIP, que são considerados peptídeos insulinotrópicos glicose-dependente, capazes de aumentar a secreção de insulina apenas quando a glicemia se eleva. Pertencem a esta classe, os medicamentos com ação parecida com o GLP-1 (miméticos e análogos), e os inibidores da enzima dipeptidilpeptidase 4 (DPP-IV) (SOCIEDADE BRASILEIRA DE DIABETES, 2009).

Os agonistas dos receptores do GLP-1 representam uma terapia atrativa para a DM tipo 2 porque atuam no sentido de restaurar a secreção de insulina dependente de glicose, preservam e protegem as células β e, provavelmente, melhoram os fatores de risco como o excesso de peso. Como já foi referido, a secreção de GLP-1 pós-prandial está diminuída em indivíduos com DM tipo 2 (VILSBOLL et al., 2001), e as evidências sugerem que os doentes permanecem relativamente sensíveis ao GLP-1, podendo ser considerado um tratamento muito adequado para esta condição. Estes efeitos ocorreram concomitantes às melhorias na função das células β, desaceleração do esvaziamento gástrico, supressão do apetite, redução do peso corporal e dos ácidos graxos livres (ZANDER et al., 2002). Para esta classe de terapias existe o exenatida, que é um agonista do receptor da GLP-1, altamente resistente à degradação enzimática pela DPP-4, e a liraglutida, que é um análogo da GLP-1, com grande capacidade de se ligar à albumina, o que prolonga a ação no organismo (NIELSEN et al., 2004).

Os inibidores da DPP-IV (ex: sitagliptina, vildagliptina) foram introduzidos na prática clínica com sucesso (BAGGIO et al., 2007). Tipicamente, os inibidores da DPP-IV resultam numa duplicação dos níveis de GLP-1 (HERMAN et al., 2006), em relação às variações fisiológicas. Porém, como existem outros substratos alvos dessa enzima, como neuropeptídeos, citocinas, e outros hormônios gastrointestinais, o uso destes inibidores ainda requer cautela (BAGGIO et al., 2007; BOONACKER et al., 2003).

Existem ainda moléculas ativadoras do receptor de insulina, inibidores da ação da resistina, das fosfatases PTP-1B e SHP2, e da GSK-3. Estão sendo desenvolvidas pesquisas envolvendo estratégias para redução da produção de glicose hepática como antagonistas do receptor de glucagon, inibidores da glicogênio-fosforilase, glicose 6-fosfatase e frutose 1,6-bisfosfatase e ativadores da glicocinase, objetivando a redução da gliconeogênese e glicogenólise, e aumento da glicólise e síntese de glicogênio hepático. Além disso, alternativas que alterem o metabolismo lipídico reduzindo os ácidos graxos livres circulantes, também estão em estudo como a elevação das concentrações de adiponectina, a utilização de agonistas de AMPK, e a diminuição da

expressão da enzima 11β-hidroxi-esteróide desidrogenase tipo 1 no tecido adiposo (FOWLER, 2007, 2008; MORRAL, 2003; VATS et al., 2005).

1.3.2.2 Insulinoterapia

A terapia preconizada para DM tipo 1 consiste na administração diária de doses ajustadas de insulina, uma vez que este tipo de diabetes caracteriza-se justamente pela ausência deste hormônio. A DM tipo 2 pode progredir da resistência à insulina para uma insuficiência da mesma. Dessa forma, pacientes portadores desse tipo de diabetes também podem desenvolver a necessidade da terapia insulínica (BETHEL et al., 2005; SOCIEDADE BRASILEIRA DE DIABETES, 2009). A terapia com insulina é capaz de promover novamente a sensibilidade à insulina e, em alguns casos, reverter a resistência, possivelmente pela eliminação da toxicidade causada pela glicose. Perfis farmacocinéticos mais favoráveis das formulações de insulina reduzem a preocupação com a hipoglicemia e com o ganho de peso, facilitando o uso da insulina por pacientes com DM tipo 2 (RIDDLE, 2002; SCARLETT et al., 1982).

Como a insulina é degradada no trato gastrointestinal é preciso administrá-la pela via subcutânea e, em alguns casos, de severa descompensação metabólica, ela pode ser administrada por via intravenosa ou muscular (SIMÓ; HERNÁNDEZ, 2002). Os riscos e os efeitos adversos mais relevantes da terapia insulínica são a hipoglicemia e o ganho de peso (ELDOR et al., 2005).

A insulina é disponibilizada em diferentes análogos e formas que podem reduzir o número de injeções diárias, garantindo uma excelente eficácia em pacientes diabéticos podendo, assim, evitar as complicações inerentes à doença (STOTLAND, 2006).

Entre as formas de insulina comercializáveis estão as insulinas de ação rápida (lispro, aspart) que apresentam início de ação aproximadamente em 15 minutos, pico máximo de atividade entre 30 minutos e 2 horas, e duração de, no máximo, 4 horas. As de ação curta (regular) iniciam o efeito entre 30 minutos e 1 hora, pico máximo de atividade entre 2 e 3 horas e duração total de efeito entre 6 e 8 horas e as de ação intermediária, incluindo nesse grupo a NPH (insulina com protamina de Hagedorn), tem efeito durante um período mais longo, variando de 16 a 24 horas. As insulinas lentas apresentam perfis de ação bastante prolongados quando comparadas às preparações de ação rápida e intermediária, cujas representantes são a insulina glargina e detemir

(BETHEL; FEINGLOS, 2005; FOWLER, 2008; OIKNINE; MOORADIAN, 2003).

1.3.3 Produtos finais de glicação avançada (AGEs)

As complicações da DM são descritas por apresentarem origens multifatoriais, pois a hiperglicemia crônica está envolvida no desenvolvimento e progressão de muitas doenças, inclusive alterações microvasculares e macrovasculares. Entre as hipóteses que explicam os danos celulares e teciduais induzidos pela hiperglicemia destaca-se o processo bioquímico da formação dos produtos finais de glicação avançada, também chamados AGEs (*Advanced Glycated End-Products*) (GOH; COOPER, 2008; PEPPA et al., 2003).

Os AGEs são resultado da reação de glicação não enzimática entre açúcares redutores ou lipídeos oxidados e proteínas, aminofosfolipídeos ou ácidos nucleicosamino das proteínas (WAUTIER; GUILLAUSSEASU, 2001). A reação de formação de AGEs pode ser separada em três fases. Na primeira ocorre a glicação não enzimática, onde o grupamento carbonila do açúcar redutor condensa-se como o grupo amina livre da proteína formando a base de Schiff que, após sofrer rearranjo, origina os produtos de Amadori, estruturalmente mais estáveis. A HbA1c e a frutosamina são conhecidos exemplos de produtos de Amadori. Na fase intermediária, os produtos de Amadori são transformados em uma série de compostos dicarbonilicos como glioxal, metilglioxal e glicoaldeído, através de oxidações e desidratações. A última fase compreende a reação do dicarbonil novamente com os grupamentos amina primárias acessíveis, formando um composto irreversível chamado AGE. A inibição de qualquer uma destas três fases pode reduzir a formação dos AGEs e beneficiar o tratamento de doenças como câncer, aterosclerose e diabetes (SHUAN, L., et al., 2010).

Carboximetilisina (CML) é um dos produtos mais importantes da fragmentação dos compostos de Amadori junto com a pentosidina. A CML está presente no plasma, tecido renal, retinas e colágenos de pacientes diabéticos e a concentração em humanos é proporcional à idade (PEYROUX; STERNBERG, 2006). A presença de CML também é reportada aos depósitos neurofibrilares em pacientes afetados com a doença de Alzheimer (CERVANTES-LAUREN et al., 2006). Em pacientes diabéticos, a quantificação da HbA1c, a qual carrega um produto de Amadori em sua cadeia β, é usada como indicativo de

hiperglicemia dos últimos três meses, sendo considerada um importante marcador da progressão da diabetes (RAHBAR, 2005).

Alguns AGEs como a pentosidina tem uma fluorescência intrínseca, desta forma a determinação da fluorescência pode ser usada como um marcador de formação dos AGEs. Por outro lado, AGEs não fluorescente, como a CML, podem ser detectados por técnicas como ELISA (GOH; COOPER, 2008).

O AGEs podem atuar via receptores específicos (RAGEs) e o processo de glicação avançada ocorre durante um período prolongado de hiperglicemia, contribuindo para o desenvolvimento das complicações da diabetes. Os AGEs são responsáveis pelas microangiopatias, onde ocorrem alterações no endotélio e células associadas, culminando em oclusão capilar, isquemia e falência de órgãos, e pelas macroangiopatias diabéticas que induzem a complicações cardiovasculares, sendo responsável por altas taxas de morbimortalidade de pacientes diabéticos (BASTA et al., 2004; VLASSARA; PALACE, 2003).

Na diabetes, as células endoteliais dos capilares da retina, mesangiais do glomérulo renal, neurônios e células de Schwann nos nervos periféricos, são vulneráveis à ocorrência de AGEs, pelo fato de não apresentarem grande capacidade de regular a captação de glicose ficando, assim, mais expostas a grandes quantidades de glicose durante as fases de hiperglicemia (BROWNLEE, 2001, 2005).

O desenvolvimento da retinopatia diabética ocorre pela presença dos AGEs nos vasos sanguíneos que, ao se ligarem em receptores específicos (RAGE), desencadeiam um sinal, promovendo a perda de pericitos, que são células mesenquimais que participam no processo de reparação de tecidos dos vasos sanguíneos, além das funções contráteis auxiliadoras da regulação do fluxo sanguíneo (JUNQUEIRA; CARNEIRO, 2008). A interação AGEs-RAGE aumenta a produção do fator de crescimento das células do endotélio vascular (VEGF), o qual estimula a angiogênese e a neovascularização, também envolvidos na retinopatia (AHMED, 2005).

Os rins são o principal alvo dos danos mediados pelos AGEs na diabetes, pois o rim é o principal local de depuração dos AGEs. Nos rins, o complexo AGE-RAGE promove a liberação do fator de crescimento β (TGF-β), estimulando a síntese de componentes da matriz do colágeno, causando o espessamento da membrana basal, alteração na filtração e perda de função glomerular, resultando na nefropatia diabética (VLASSARA; PALACE, 2002).

No sistema nervoso, a mielina glicada torna-se susceptível à fagocitose por macrófagos, que também podem ser estimulados a

secretarem proteases, proporcionando a desmielinização do nervo. Este processo é acompanhado pela degeneração axonal dos neurônios periféricos, anormalidades funcionais reduzindo a condução nervosa e fluxo sanguíneo (AHMED, 2005; GUVEN et al., 2004). Os AGEs também podem estimular reações imunes através da ligação a IgG e IgM, potencializando a desmielinização, ativar NF-κβ e promover a expressão de citocinas pró-inflamatórias com IL-6 e TNF-α (AHMED, 2005). O espessamento dos vasos sanguíneos também pode gerar isquemia nos nervos, agravando ainda mais o quadro de neuropatia diabética (BIERHAUS et al., 2004)

Quando no paciente diabético ocorre lesão do tecido epitelial, é observado um retardo na migração de células inflamatórias ao local lesionado, e essa demora faz com que o processo de recomposição da matriz e fechamento da ferida seja prejudicado. A severidade do caso é agravada com a liberação de moléculas pró-inflamatórias, como o TNF-α e as metaloproteinases (MMPs), devido à ligação AGE-RAGE, que promovem a destruição da matriz e, nos fibroblastos, reduzem a deposição de colágeno necessário no processo de cicatrização (AHMED, 2005).

As alterações macrovasculares dos AGEs são resultado do excesso de ácido graxo circulante proveniente dos adipócitos. A oxidação dos ácidos graxos na mitocôndria aumenta a concentração de espécies reativas de oxigênio (EROS) na cadeia de transporte de elétrons mitocondrial, ativando as vias do poliol, da hexosamina, da PKC e de formação dos AGEs. Os AGEs se complexam com as proteínas plasmáticas, enrijecem as paredes dos vasos, inibem a atividade do óxido nítrico e prostaciclinas, interagem com receptores responsáveis por modular propriedades celulares, aumentam a produção de vasoconstritores como endotelina-1 pela ativação do NF-κβ, oxidam o LDL, aumentam a resposta inflamatória nas células aderidas as placas aterioscleróticas (fagócitos mononucleares e células musculares lisas), aumentam a permeabilidade vascular, a expressão de moléculas de adesão leucocitárias endoteliais, a atividade de processos pró-coagulantes, e a produção de EROS, entre outras ações (BASTA et al., 2004; BIERHAUS et al., 1998; GOLDIN et al., 2006).

Enzimas como a oxaldeído redutase e a aldose redutase atuam na remoção dos intermediários dicarbonílicos, assim como os sistemas enzimáticos glioxilase I e II, a frutosamina-3-cinase e a frutosamina oxidase (amadoriase), que também atuam na interrupção de diferentes etapas da formação dos AGEs (THORNALLEY, 2003). Contudo estes sistemas tornam-se ineficazes frente a excessos de AGEs (VLASSARA;

PALACE, 2002). A remoção tecidual dos AGEs é feita, extracelularmente, por proteólise ou, intracelularmente, por macrófagos, que formam produtos solúveis e de baixo peso molecular, os chamados AGEs-peptídeos, ou então segunda geração de AGEs, que são excretados pelos rins (BIERHAUS et al., 1998; GUGLIUCCII; BENDAYAN, 1996).

Além disso, substâncias com capacidade anti-AGE estão sendo estudadas. Aminoguanidina foi o primeiro inibidor da formação de AGE a ser estudado. Hipoglicemiantes orais como metformina e pioglitazona diminuem a formação de AGEs por minimizar a hiperglicemia, porém evidência tem mostrado capacidade de prevenir a formação dos AGEs e conseqüente glicação, independente do efeito na glicemia (GOH; COOPER, 2008; PEPPA et al., 2003).

1.3.4 Estudo da diabetes em modelos experimentais em animais de laboratório

Os estudos experimentais são muito utilizados e necessários para a busca de esclarecimentos dos mecanismos fisiopatológicos da diabetes, das complicações e de novos tratamentos para as alterações endócrino-metabólicas causadas por esta doença,. Assim, o estudo *in vivo* da diabetes pode ser realizado através de modelos experimentais em animais de laboratórios pelo emprego de substâncias diabetogênicas (aloxana ou estreptozotocina), em animais com diabetes geneticamente modificados (ex. ratos da linhagem Sabra), pelo artifício da pancreatectomia total ou subtotal, ou ainda, através da extração da hipófise anterior (MAIA; CAMPOS, 2005).

Um dos modelos mais empregado para o estudo da diabetes é aquele que utiliza animais diabéticos induzidos com aloxana, que é um derivado da pirimidina com citotoxicidade seletiva pelas células β-pancreáticas. Desta forma, esta substância causa insuficiência insulínica primária do pâncreas, provocando alterações clínicas e laboratoriais características de diabetes grave, as quais possibilitam estudos com parâmetros bioquímicos controlados da diabetes (LERCO et al., 2003).

Estudos indicam que a glicemia normal em jejum de ratos é diferente da observada em humanos, variando de 100 a 130 mg/dL, enquanto que em humanos normoglicêmicos está entre 70 e 99 mg/dL. Desta forma, pesquisas que utilizam o modelo de diabetes experimental quimicamente induzido consideram ratos diabéticos aqueles que apresentam glicemia em jejum acima de 300 mg/dL (JORGE, et al.,

2004; SILVA et al., 2002; SOCIEDADE BRASILEIRA DE DIABETES, 2009; ZANATTA et al., 2007).

O teste oral de tolerância à glicose (TOTG) é um teste simples que se baseia na avaliação da glicemia ao longo do tempo, após uma sobrecarga oral de glicose. É um teste amplamente utilizado na prática clínica para o diagnóstico de intolerância à glicose e DM tipo 2 (ANTUNA-PUENTE et al., 2011). Porém, este ensaio também é muito difundindo na pesquisa em modelos animais para avaliação de potenciais substâncias antidiabéticas. Considerando que além da glicemia, também seja determinada a insulinemia, este teste pode estimar a sensibilidade à insulina e / ou a secreção, inclusive permitindo o cálculo de índices para melhor avaliar esses parâmetros (DAMAZIO et al., 2009; FOLADOR et al., 2010).

1.4 PLANTAS MEDICINAIS E COMPOSTOS NATURAIS

1.4.1 Espécies vegetais e o tratamento da diabetes

A utilização de plantas é uma alternativa para o tratamento da diabetes. Muitas plantas são conhecidas na medicina popular de diferentes culturas pelas propriedades hipoglicemiantes ou anti-hiperglicêmicas. São listadas mais de 1200 espécies vegetais usadas como anti-diabéticas em todo o mundo. Nesse contexto, o Brasil com a sua enorme biodiversidade, pode contribuir no desenvolvimento de novos medicamentos produzidos a partir de plantas medicinais para o tratamento da diabetes. No entanto, apesar da riqueza da flora e da ampla utilização de plantas medicinais pela população, existe ainda uma insuficiência de estudos científicos na investigação das propriedades terapêuticas de espécies vegetais nativas do Brasil (DORNAS et al., 2009; GROVER et al., 2002; IVORRA et al., 1989; LI et al., 2004; TROJAN-RODRIGUES et al., 2012).

Apoiado no conhecimento etnofarmacológico, algumas espécies vegetais, estão sendo experimentalmente analisadas com relação aos efeitos na DM. Muitos estudos confirmam o uso popular antidiabético de algumas espécies, e inclusive relacionam esta atividade com os variados constituintes químicos nelas presentes, os quais podem ser utilizados como modelos para novos agentes hipoglicemiantes (CHAN et al., 2012; NEGRI, 2005).

Espécies vegetais de diversas famílias apresentam atividade hipoglicemiante, principalmente pertencentes ás famílias Fabaceae, Asteraceae e Lamiaceae, entre as quais a título representativo pode-se

citar *Bauhinia forficata* (pata-de-vaca; Fabaceae) (SILVA et al., 2002); *Baccharis trimera* (carqueja; Asteraceae) (OLIVEIRA, A. et al., 2005), *Momordica charantia* (melão-de-são-caetano; Cucurbitaceae) (VOLPATO et al., 2002), *Eugenia jambolana* (jambolão; Myrtaceae) (SAGRAWAT; MANN; KHARYA, 2006), *Allium sativum* (alho; Liliaceae) (KISS et al., 2006) e *Vitex megapotamica* (tarumã; Lamiaceae) (ZANATTA et al., 2007). Entre as plantas mais utilizadas na medicina popular para tratamento do diabetes estão as espécies pertencentes ao gênero *Bauhinia* (Fabaceae), popularmente conhecidas no Brasil como “pata-de-vaca”, devido ao formato das folhas. *B. forficata* é a espécie que apresenta maior número de estudos quanto à atividade hipoglicemiante, sendo considerada pela população como “pata-de-vaca verdadeira”, e muito usada na forma de chás e outras preparações fitoterápicas (SILVA; CECHINEL FILHO, 2002).

Silva e colaboradores (2002) demonstraram que a fração *n*-BuOH de folhas de *B. forficata* apresenta efeito hipoglicemiante, quando administrada via oral em ratos normais e ratos diabéticos induzidos por aloxana. O efeito hipoglicêmico em ratos normoglicêmicos foi observado até 2 horas após o tratamento nas doses de 500 e 600 mg/kg. Por outro lado, nos ratos diabéticos após 1 hora, a dose de 800 mg/kg foi a mais efetiva em reduzir a glicemia sérica, sendo que este resultado manteve-se por até 3 horas após o tratamento. Outras espécies de *Bauhinia* também são descritas em diversos estudos na literatura por apresentaram potencial antidiabético, entre as quais se destacam a *B. sacra*, *B. variegata*, *B. purpurea* e *B. candicans* (NEGRI, 2005; OLIVEIRA,T. et al., 2005; SILVA; CECHINEL FILHO, 2002).

Os metabólitos isolados de espécies vegetais que possuem atividade hipoglicêmica podem pertencer a diversas classes químicas, incluindo alcalóides, terpenóides, flavonóides e cumarinas. Os estudos indicam que esta grande variedade química de compostos ativos poderia estar relacionada com os diversos mecanismos de ação observados pelos extratos de espécies vegetais com potencial antidiabético (CAZAROLLI et al., 2008b; NEGRI, 2005; TROJAN-RODRIGUES et al., 2012).

Dentre os mecanismos de ação hipoglicêmicos ou anti-hiperglicêmicos descritos na literatura para produtos de origem natural podem-se citar: o aumento da liberação de insulina através da estimulação das células β-pancreáticas; a resistência aos hormônios que aumentam a taxa de glicose; o aumento do número e da sensibilidade do sítio receptor de insulina; a diminuição da perda de glicogênio; o aumento do consumo de glicose nos tecidos e orgãos; a diminuição da absoção de glicose; a eliminação de radicais livres; a resistência à

peroxidação de lipídeos; a correção da desordem metabólica causada em lipídeos e proteínas, e o estímulo ao aumento da microcirculação do sangue no organismo (CAZAROLLI et al., 2008b; CHAN et al., 2012; NEGRI, 2005; VOLPATO et al., 2002).

1.4.2 *Baccharis articulata*

O gênero *Baccharis* pertence à família Asteraceae e compreende mais de 500 espécies distribuídas exclusivamente no continente americano, sendo que a maior concentração destas encontra-se no Brasil, Argentina, Colômbia, Chile e México (VERDI et al., 2005). No Brasil estão descritas 120 espécies, conhecidas popularmente como carquejas, sendo que, no Rio Grande do Sul, há relatos de 70 espécies distribuídas desde a região serrana até o litoral (ABAD; BERMEJO, 2007 BARROSO; BUENO, 2002;GIULIANO, 2001).

As espécies do gênero *Baccharis* apresentam elevado valor sócio-econômico, com ampla dispersão nos estados de Santa Catarina, Paraná, São Paulo e Rio Grande do Sul. São utilizadas na medicina popular para controle ou tratamento de várias doenças, consumidas principalmente na forma de chás com indicações para males do estômago, fígado, anemias, inflamações, diabetes, doenças da próstata, sendo também descritas como remédio para o processo de desintoxicação do organismo (CORRÊA, 1984; FRANCO, 1995).

Baccharis articulata (Lam.) Pers. (Figura 8), também conhecida como carqueja-doce, carquejinha ou carqueja-branca, é um subarbusto com ramos lenhosos, articulados, bialados, sendo as alas rígidas, planas, e, às vezes, viscosas e glabras. As folhas são rudimentares, quase nulas. Os capítulos de flores brancas (amareladas nos indivíduos masculinos) são dispostos em espigas densas. O fruto aquênio é linear, pequeno e glabro. Infusões ou decocções das partes aéreas de *B. articulata* são tradicionalmente usadas como diurético, digestivo e anti-diabético na medicina popular local do sul do Brasil (ABAD; BERMEJO, 2007; CORRÊA, 1984; LORENZI; MATOS, 2002).

Figura 8. Espécie vegetal *Baccharis articulata*
(Fonte: http://www6.ufrgs.br/fitoecologia/florars/open_sp.php?img=558)

Entre os compostos de maior ocorrência nas espécies do gênero *Baccharis* destacam-se os flavonóides, diterpenos e triperpenos (DE OLIVEIRA et al., 2006; VERDI et al., 2005). Nos estudos de atividades biológicas são destacados os efeitos alelopáticos, antimicrobianos, citotóxicos, antiinflamatórios e antioxidantes, sendo que algumas atividades relatadas para as espécies de *Baccharis* estão relacionadas com a presença dos flavonóides (BORGO et al., 2010; DE OLIVEIRA et al., 2004; VERDI et al., 2005).

De Oliveira e colaboradores (2002) isolaram o composto BaII (4´-*O*-β-D-glucopyranosyl-3´,5´-dimethoxybenzyl-caffeate) da fração *n*-butanol do extrato aquoso das partes aéreas de *B. articulata* e mostraram que este composto fenólico apresentam significativa atividade antioxidante. Além disso, Borgo e colaboradores (2010) determinaram teores de quercetina em extratos de partes aéreas de *B. articulata*, assim como demonstraram a atividade antioxidante *in vitro* para esses extratos.

Recentemente, *B. trimera* foi relatada como uma das espécies mais utilizadas como anti-diabéticas pela medicina popular do sul do Brasil (TROJAN-RODRIGUES et al., 2012). Com relação à atividade anti-diabética de espécies de *Baccharis*, a revisão da literatura indica somente o estudo de Oliveira, A. e colaboradores (2005). Os autores investigaram o efeito de extrato e frações de partes aéreas de *B. trimera* na glicemia de camundongos normais e diabéticos induzidos com estreptozotocina. Os autores verificaram que após 7 dias de tratamento a

fração aquosa (2000 mg/kg, 2 vezes ao dia) reduziram a glicemia de animais diabéticos.

1.4.3 *Musa x paradisiaca*

O gênero *Musa* pertence à família Musaceae e apresenta uma grande diversidade de variedades conhecidas como bananas. São plantas herbáceas, com caule subterrâneo (rizoma), e a parte aérea é chamada de pseudocaule, que pode alcançar 4-5 metros de altura. O pseudocaule é formado por bainhas foliares, terminando com uma copa de folhas compridas e largas. Uma planta pode emitir de 30 a 70 folhas, com o aparecimento de uma nova folha a cada 7 a 11 dias. A inflorescência sai do centro da copa, em cujas axilas nascem as flores. De cada conjunto de flores são formadas as pencas com número variável de frutos. O fruto é do tipo baga, partenocárpico, isto é, desenvolvido sem polinização, e assim as sementes não se desenvolvem ou são abortivas (BORGES; SOUZA, 2004, CORRÊA, 1984).

Este gênero é originário do continente Asiático, mas atualmente é cultivado na maioria dos países tropicais. A banana é uma das frutas mais consumidas no mundo e constitui uma importante fonte alimentar com alto valor nutricional, podendo ser utilizada verde ou madura, crua ou processada. O Brasil é o quarto maior produtor mundial, o cultivo ocorre em todos os estados, desde a faixa litorânea até os planaltos do interior do Brasil (AMORIM et al., 2011; JESUS et al., 2004).

As espécies de *Musa* são cultivadas principalmente por seus frutos, porém, além do valor nutricional, os frutos e outras partes da planta são utilizados na medicina popular para tratamento de diversas doenças (CORRÊA, 1984). Estudos relatam inúmeras atividades biológicas para diferentes espécies de *Musa*, como, antiulcerogênica, antidiarréica, antitumoral, antimutagênica, antioxidante e antimicrobiana, inclusive a propriedade antidiabética (ELEAZU; OKAFOR; AHAMEFUNA, 2010; KARADI, 2011; OLORUNFEMI et al., 2011; ORHAN, 2001; PANNANGPETCH, 2001; PARI; MAHESWARI, 1999).

A composição química entre as espécies de *Musa* é muito variável, sendo relatada a presença de vitamina C e carotenóides. Algumas espécies, como a *M. sapientum* e a *M. acuminata*, são relatadas por mostrarem altos teores de compostos fenólicos, principalmente flavonóides (AMORIM et al., 2011; JESUS et al., 2004; VIJAYAKUMAR; PRESANNAKUMAR; VIJAYALAKSHMI, 2008).

Recentemente, Aziz e colaboradores (2011) verificaram um significativo conteúdo de compostos fenólicos e flavonóides para o extrato do psedo-caule da espécie *Musa acuminata x balbisiana* Colla cv. Awak.

A parte aérea deste gênero é cortada após a colheita (BORGES; SOUZA, 2004). Desta forma as folhas da *Musa* podem servir como alvo de pesquisas, buscando novas finalidades, inclusive terapêuticas, aplicando o conceito do uso sustentável dos recursos naturais. Um dos focos de estudo deste trabalho incindiu sobre as folhas da espécie *Musa x paradisiaca* L. (*M. x paradisiaca*) (Figura 9), que é assim denominada por ser um híbrido de *M. acuminata* e *M. balbisiana* (VALMAYOR, et al., 2000).

Figura 9. Espécie vegetal *Musa x paradisiaca*
(Fonte: http://musaceaefamily.blogspot.com/)

1.4.4 Compostos Fenólicos

Compostos fenólicos são compostos que, no mínimo, apresentam na estrutura um anel aromático substituído por um ou mais grupamentos hidroxila. No entanto, é um grupo químico que apresenta grande diversidade de estruturas. A origem biogenética determina o padrão de substituição do composto fenólico resultante. Desta forma, os compostos fenólicos podem ser formados através de duas rotas biogenéticas: pela via do ácido chiquímico ou pela via do acetato-polimalato (BRAVO, 1998; CARVALHO et al., 1999).

Flavonóides são compostos fenólicos que tem um ou mais núcleos aromáticos contendo substituintes hidroxilados e/ou os derivados funcionais (ésteres, glicosídeos). O grupo dos flavonóides inclui as subclasses das antocianidinas, flavonóis, flavanóis, flavanonas e flavonas. Entre os metabólitos secundários de espécies vegetais estes compostos são descritos com relativa abundância (CAZAROLLI et al., 2008a; HARBONE; BAXTER, 1999; ZUANAZZI; MONTANHA, 2004).

A maioria dos representantes dos flavonóides possui 15 átomos de carbono no núcleo fundamental (Figura 10), o qual se constitui de duas fenilas ligadas por uma cadeia de três carbonos entre elas. Quando o composto está na forma conjugada com açúcares é conhecido como heterosídeo (ou glicosídeo), e sem o açúcar (forma livre) é chamado de aglicona ou genina. A ligação entre a genina e o açúcar pode ocorrer por intermédio de uma hidroxila (*O*-heterosídeos) ou por um átomo de carbono (*C*-heterosídeos) (ANDERSEN; MARKHAM, 2006; ZUANAZZI; MONTANHA, 2004).

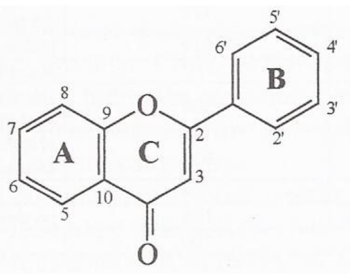


Figura 10. Estrutura básica dos flavonóides (ZUANAZZI; MONTANHA, 2004).

Algumas espécies são relatadas por apresentarem alto teor de flavonóides, entre as quais as espécies de *Baccharis*, que se destacam pela ocorrência de flavonas e flavonóis como componentes predominantes (VERDI, BRIGHENTE, PIZZOLATTI, 2005; ZUANAZZI; MONTANHA, 2004).

Muitas propriedades farmacológicas são descritas para os flavonóides como atividade antioxidante, antimicrobiana, antiinflamatória, antitrombótica, anti-alérgica entre outras (COOK; SAMMAN, 1996; COWAN, 1999; GRYGLEWSK et al.,1987; HARBONE; WILLIAMS, 2000; MIDDLETON; KANDASWAMI, 1992; PIETTA, 2000). Atualmente, essa classe de compostos também

está sendo amplamente pesquisada como hipoglicemiante, tornando os flavonóides potenciais substâncias para auxiliar no tratamento da DM (CAZAROLLI et al., 2008a; DE SOUZA et al., 2004; JORGE et al., 2004; NEGRI, 2005; OLIVEIRA, T. et al., 2005; ZANATTA et al., 2008).

O nosso grupo de pesquisa do Laboratório de Hormônios & Transdução de Sinais já relatou o efeito de diferentes flavonóides sobre a homeostasia da glicose. De Souza e colaboradores (2004) verificaram que o flavonóide presente nas folhas de *B. forficata*, a canferitrina (canferol-3,7-O-(α)-L-diramnosídeo), apresenta significante efeito hipoglicêmico em ratos normais e diabéticos induzidos por aloxana. A redução da glicemia nos ratos diabéticos pela canferitrina, quando administrada via oral, foi observada nas doses de 50, 100 e 200 mg/kg e nos ratos normais somente na maior dose testada após uma hora do tratamento. Corroborando com este estudo, Jorge e colaboradores (2004) também verificaram que a canferitrina, apresenta efeito hipoglicêmico em ratos diabéticos induzidos por aloxano quando administrado via oral na dose de 100 mg/kg. Adicionalmente, os autores também constataram que esse composto estimula *in vitro* a captação de glicose em músculo sóleo de ratos normais, comparando ao efeito da insulina, sugerindo um efeito insulinomimético da canferitrina.

Figura 11 – Estrutura da canferitrina (DE SOUZA et al., 2004)

O estudo de Cazarolli e colaboradores (2006) mostrou que o flavonóide *O*-glicosilado canferol 3-neoesperidosídeo, isolado de *Cyathea phalerata* (Cyatheaceae), conhecida popularmente como xaxim-espinhento, apresentou atividade hipoglicêmica, quando

administrado intraperitonealmente nas doses de 50 e 100 mg/kg em ratos diabéticos induzidos por aloxana. Em outro estudo, de Zanatta e colaboradores (2008), demonstrou-se o efeito insulinomimético *in vitro* para este composto. Os autores verificaram que nas doses de 1 e 110 nM o flavonóide estimula significativamente a captação de glicose em músculo sóleo de ratos em 35 e 21 %, respectivamente.

Figura 12 – Estrutura da canferol 3-neoesperidosídeo (ZANATTA et al., 2008)

Recentemente, Folador e colaboradores (2010) demonstraram que as flavonas *C*-glicosiladas isovitexina e swertisina, isoladas do taiuiá (*Wilbrandia ebracteata*; família Cucurbitaceae), quando administradas via oral na dose de 15 mg/kg, apresentaram um significativo efeito antihiperglicêmico e também potencializaram a secreção de insulina em ratos hiperglicêmicos, demonstrando um potencial efeito secretagogo de insulina para estes flavonóides.

Figura 13 – Estruturas de isovitexina (1) e swertisina (2) (FOLADOR et al., 2010)

1.4.5 Rutina

A rutina (quercetin-3-*O*-rutinosídeo) é um flavonóide pertencente à subclasse dos flavonóis que apresenta em sua estrutura a quercetina como aglicona e o dissacarídeo rutinosídeo (raminose + glicose) ligado a posição 3 do anel pirano (Figura 14). Este flavonóide é encontrado em várias fontes alimentares como cebola, uva, trigo serraceno, feijão vermelho, maçãs, tomates e bebidas como vinho tinto e chá preto (PEDRIALI, 2005; BECHO et al., 2009).

Figura 14 – Estrutura da rutina (BECHO et al., 2009)

Rutina tem sido intensamente pesquisada e muitas atividades farmacológicas são descritas para este flavonóide, tais como a atividade antioxidante (LA CASA et al., 2000), citoprotetora (JANBAZ et al. 2002), antiplaquetária (SHEU et al., 2004), neuroprotetora (GUPTA et al., 2003) e cardioprotetora (ANNAPURNA et al., 2009). Também recentemente a atividade antidiabética foi relatada. Fernandes e colaboradores (2010) verificaram que o tratamento com rutina (50 mg/kg) por 45 dias reduziu a hiperglicemia de ratos diabéticos induzidos com streptozotocina. Além disso, Pereira e colaboradores (2011) demonstraram que o tratamento com rutina via oral (50 mg/kg) reduziu a glicemia em ratos normais hiperglicêmicos e inibiu significativamente a atividade da α-glicosidade maltase.

2 OBJETIVOS

2.1 Objetivo geral

Caracterizar o efeito de extratos e frações de partes aéreas de *Baccharis articulata* (carqueja), de folhas de *Musa x paradisiaca* (banana) e do flavonóide rutina na homeostasia da glicose em modelos experimentais *in vivo* e *in vitro*.

2.2 Objetivos específicos

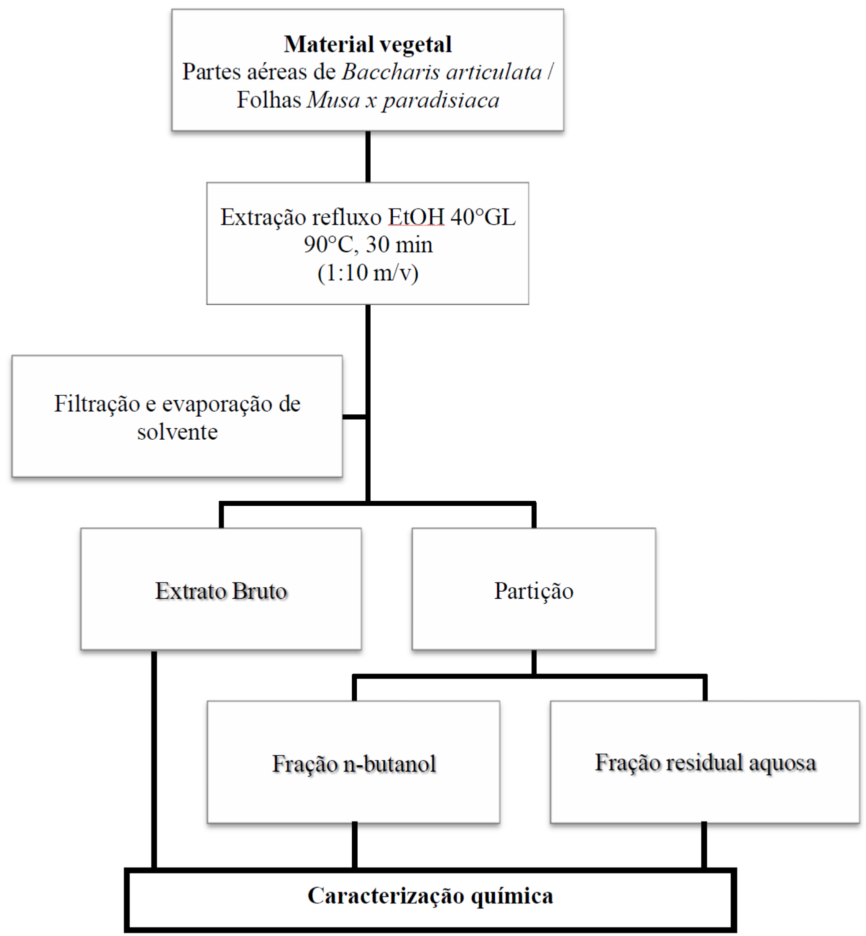
- Quantificar o conteúdo total de fenólicos e flavonóides totais no extrato e nas frações da *B. articulata.*
- Caracterizar o perfil fitoquímico e identificar o composto majoritário do extrato e das frações da *M. x paradisiaca* por CLAE.
- Avaliar o efeito hipoglicemiante e/ou antihiperglicêmico dos extratos e das frações de partes aéreas de *Baccharis articulata* e das folhas de *Musa x paradisiaca* em ratos normais hiperglicêmicos e diabéticos por um período agudo de tratamento por via oral.
- Determinar o efeito *in vivo* dos extratos e das frações de *B. articulata* e de *M. x paradisiaca* no conteúdo de glicogênio muscular e hepático, após o tratamento agudo em ratos normais hiperglicêmicos.
- Determinar o efeito *in vivo* dos extratos e das frações de *B. articulata* e de *M. x paradisiaca* na secreção de insulina após o tratamento agudo em ratos normais hiperglicêmicos.
- Estudar o efeito *in vitro* dos extratos e das frações de *B. articulata* e de *M. x paradisiaca* na atividade das dissacaridases intestinais e na glicação de proteínas.
- Estudar o efeito *in vitro* e o mecanismo de ação da rutina na captação de ^{14}C-deoxi-D-glicose no músculo sóleo de ratos normoglicêmicos, comparando com o efeito estimulatório da insulina na captação de glicose.
- Estudar o efeito *in vitro* e o mecanismo de ação da rutina na captação de $^{45}Ca^{2+}$ no músculo sóleo de ratos normoglicêmicos, verificando o envolvimento do Ca^{2+} com a captação de ^{14}C-deoxi-D-glicose no músculo sóleo.
- Avaliar o efeito da rutina na secreção de insulina após tratamento agudo em ratos hiperglicêmicos.

- Verificar o efeito *in vitro* e o mecanismo de ação da rutina na captação de $^{45}Ca^{2+}$ em ilhotas pancreáticas isoladas.

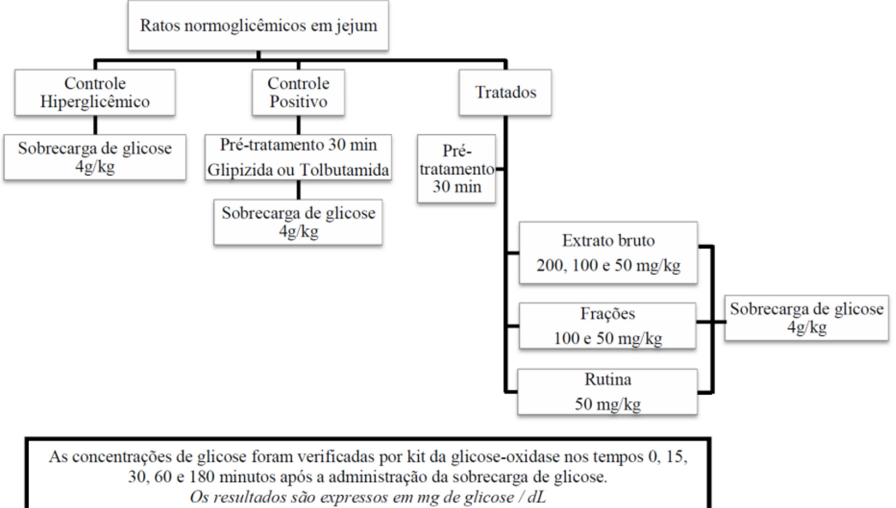
3 METODOLOGIA

A metodologia utilizada neste trabalho está apresentada sob a forma fluxogramas.

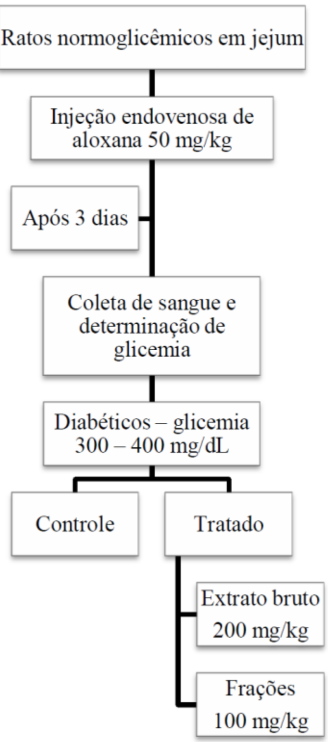
Fluxograma 1: Preparação e caracterização química dos extratos e frações.



Fluxograma 2: Tratamento de ratos normais hiperglicêmicos - Teste oral de tolerância à glicose (TOTG).

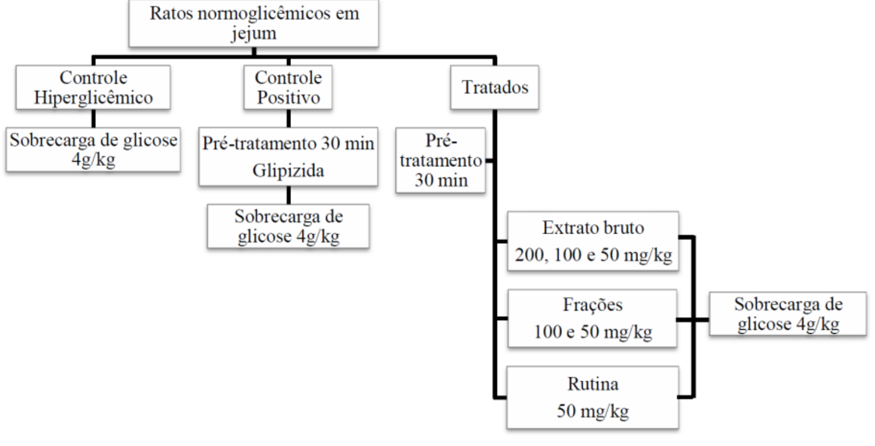


Fluxograma 3: Indução do modelo de diabetes experimental e tratamento de ratos diabéticos.



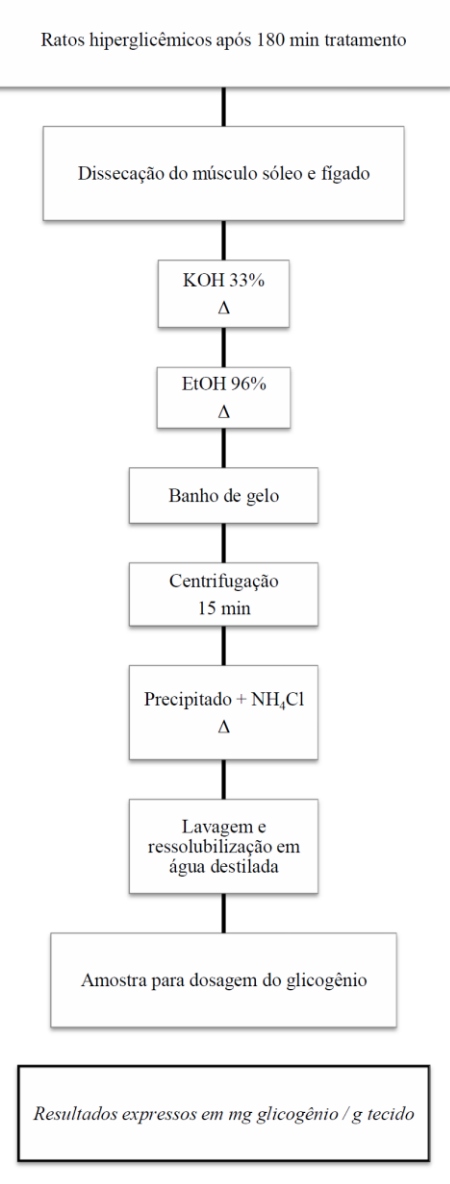
As concentrações de glicose foram verificadas por kit da glicose-oxidase nos tempos 0, 1, 2 e 3 horas após a administração do tratamento.
Os resultados são expressos em mg de glicose / dL

Fluxograma 4: Determinação da insulina sérica.

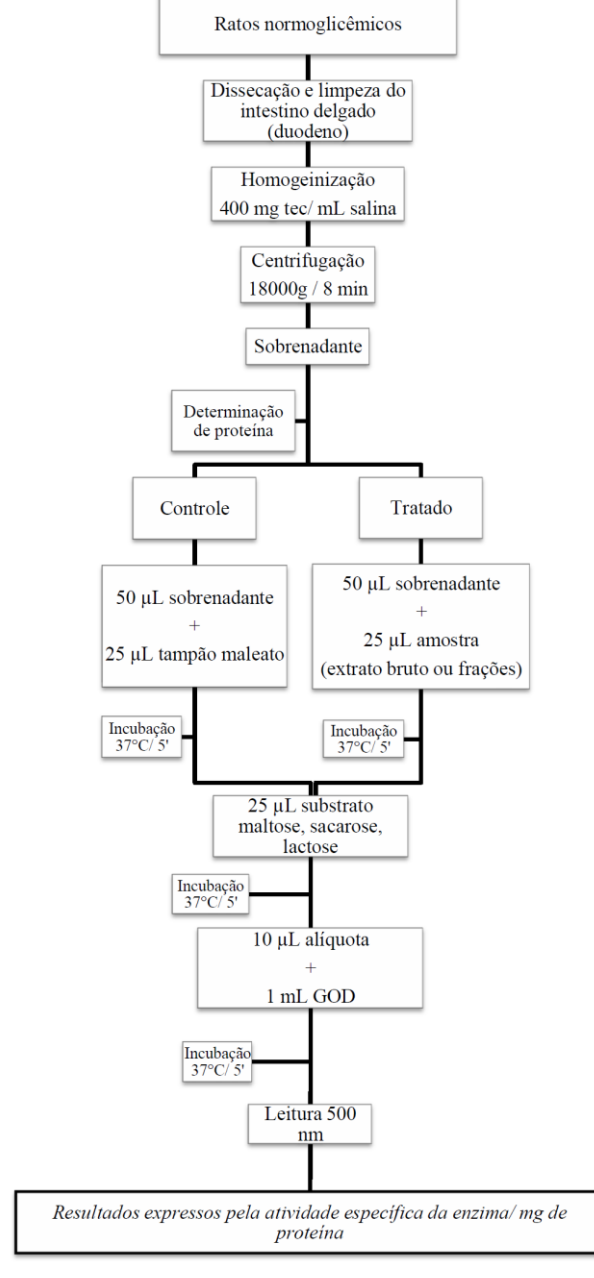


As concentrações de insulina foram verificadas por kit de ELISA nos tempos 0, 15, 30 e 60 após a administração da sobrecarga de glicose.
Os resultados são expressos em ng/mL

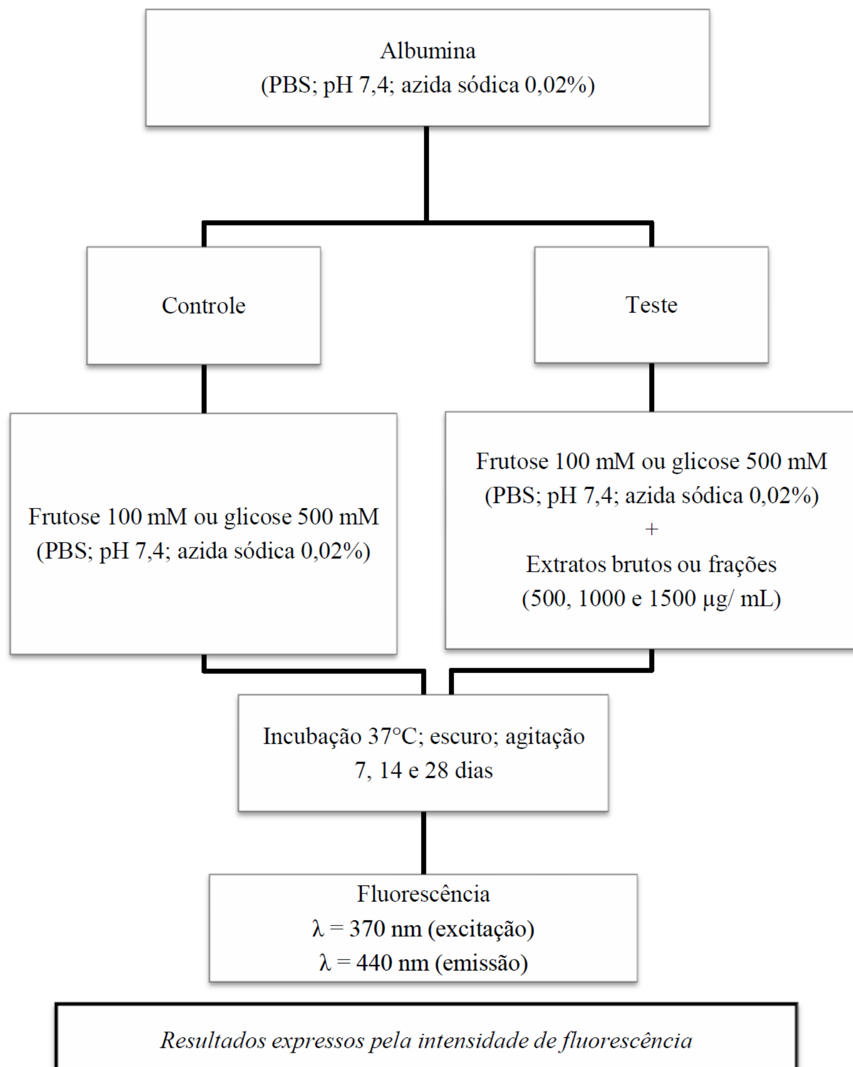
Fluxograma 5: Determinação do conteúdo de glicogênio muscular e hepático.



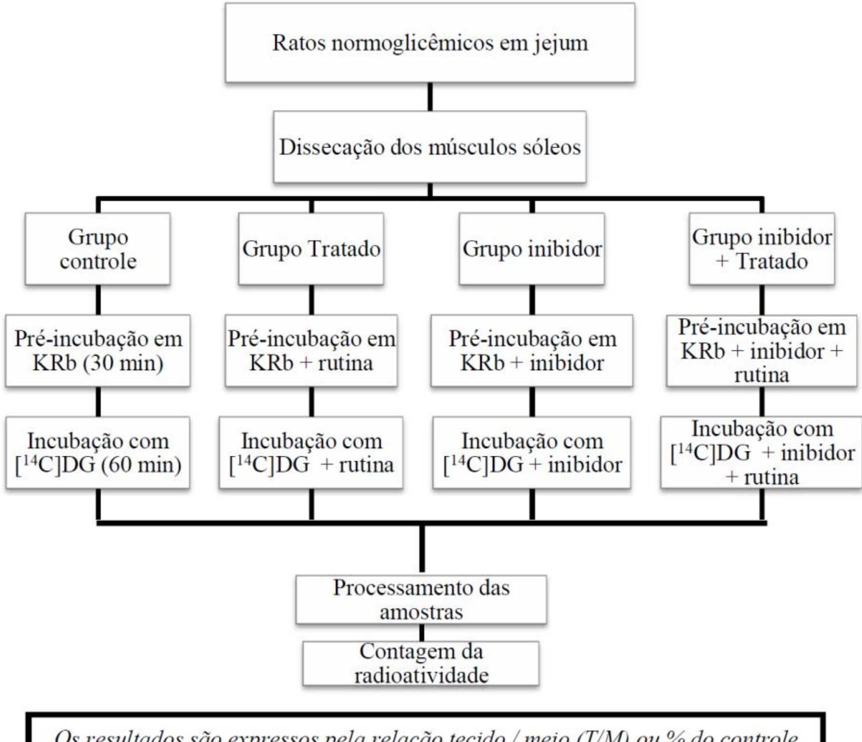
Fluxograma 6: Determinação da atividade das dissacaridases intestinais.



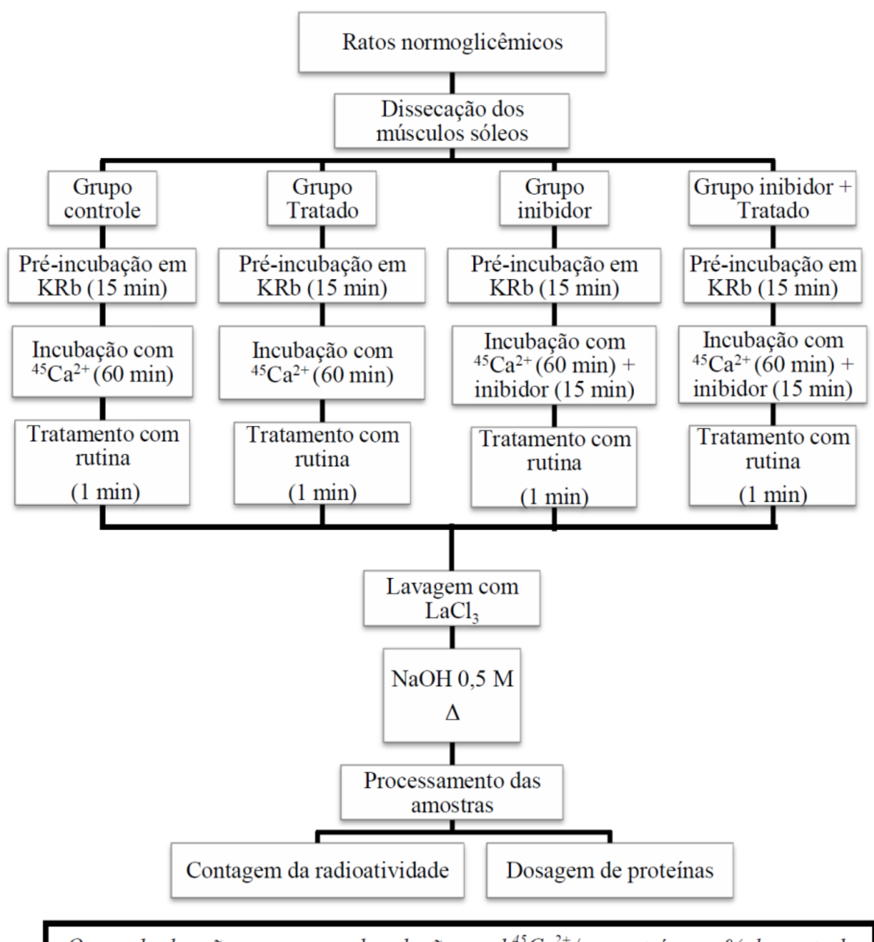
Fluxograma 7: Determinação da atividade anti-glicação de albumina.



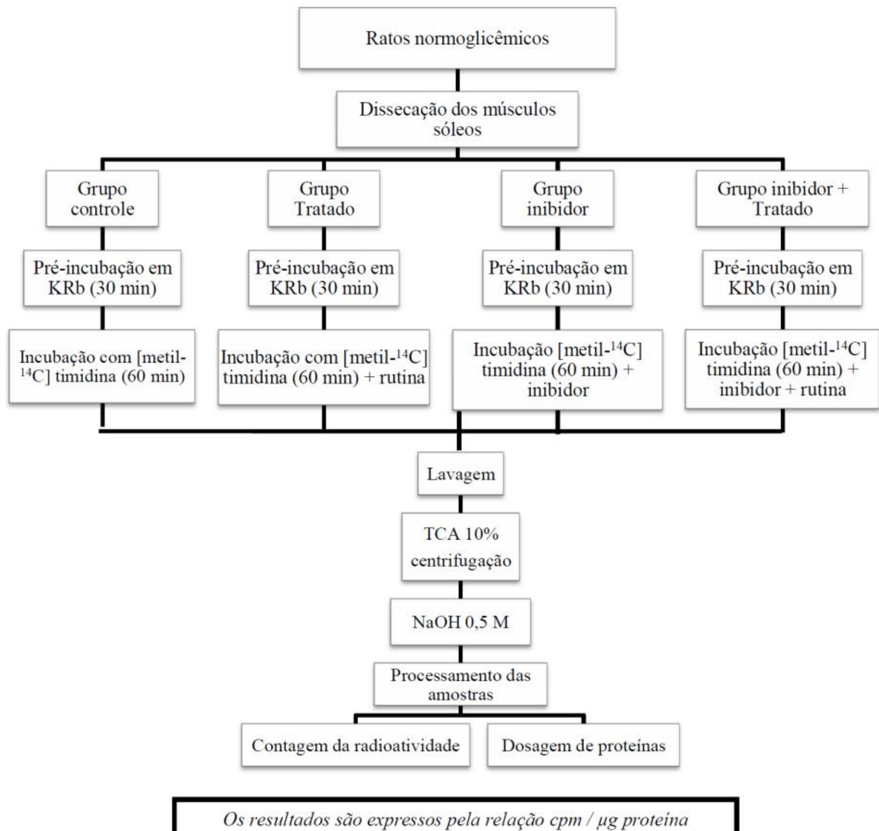
Fluxograma 8: Captação de [U-^{14}C]DG no músculo sóleo.



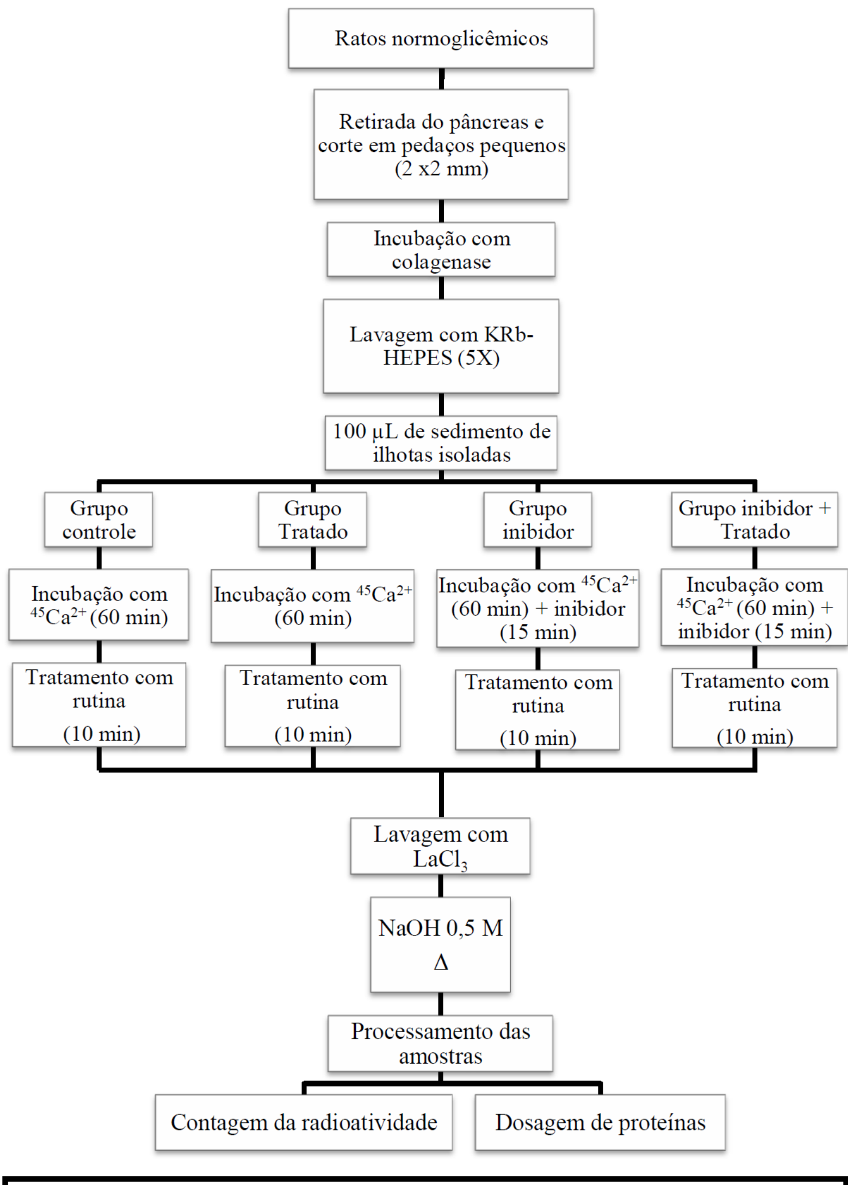
Fluxograma 9: Captação de Cálcio ($^{45}Ca^{2+}$) no músculo sóleo.



Fluxograma 10: Incorporação de timidina no músculo sóleo.



Fluxograma 11: Isolamento das ilhotas pancreáticas e captação de cálcio ($^{45}Ca^{2+}$).



Os resultados são expressos pela relação pmol $^{45}Ca^{2+}$/µg proteína ou % do controle

4 RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas estão detalhadamente descritos nos artigos.

4.1 *Baccharis articulata*: efeitos na homeostasia da glicose

A DM é uma doença crônica de ocorrência mundial que resulta em distúrbios gerais do organismo, alterando o metabolismo dos carboidratos, lipídeos e proteínas e que gera importantes conseqüências como danos, disfunção e falência de vários órgãos (SOCIEDADE BRASILEIRA DE DIABETES, 2009). Desta forma, é oportuno buscar novas formas de tratamento para essa patologia. Além disso, como previamente relatado, os compostos naturais são importantes fontes na pesquisa por novas substâncias com potencial terapêutico. A espécie vegetal *Baccharis articulata* destaca-se na medicina tradicional e possui elevado potencial terapêutico. Porém, os relatos na literatura sobre as propriedades biológicas e de metabólitos isolados desta espécie, especialmente com relação à atividade hipoglicemiante e/ou anti-hiperglicêmica, são escassos. Desta forma, o presente trabalho teve por objetivo estudar a atividade anti-hiperglicêmica de extratos e frações de *B. articulata*, assim como os efeitos gerais de curto e longo prazo na homeostasia da glicose. O efeitos biológicos do extrato bruto e frações foram estudados através de modelos experimentais *in vivo* e *in vitro*, avaliando os níveis séricos de glicose, a secreção de insulina, o conteúdo de glicogênio muscular e hepático, bem como a atividade das dissacaridases intestinais e a atividade anti-glicação de proteínas.

4.1.1 Artigo publicado

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Folha	Linha	Onde se lê	Leia-se
6756	13	B. Articulata	*B. articulata*
6757	16	B. Articulata	*B. articulata*
6760	9	B. Articulata	*B. articulata*
6761	9	B. Articulata	*B. articulata*
6763	27	B. Articulata	*B. articulata*

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Article

Short and Long-Term Effects of *Baccharis articulata* on Glucose Homeostasis

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Abstract: In this study, the *in vivo* effect of the crude extract and *n*-butanol and aqueous residual fractions of *Baccharis articulata* (Lam.) Pers. on serum glucose levels, insulin secretion and liver and muscle glycogen content, as well as *in vitro* action on serum intestinal disaccharidase activity and albumin glycation were investigated. Oral administration of the extract and fractions reduced glycemia in hyperglycemic rats. Additionally, the *n*-butanol fraction, which has high flavonoids content, stimulated insulin secretion, exhibiting an insulinogenic index similar to that of glipizide. Also, the *n*-butanol fraction treatment significantly increased glycogen content in both liver and muscle tissue. *In vitro* incubation with the crude extract and *n*-butanol and aqueous residual fractions inhibited maltase activity and the formation of advanced glycation end-products (AGEs). Thus, the results demonstrated that *B. articulata* exhibits a significant antihyperglycemic and insulin-secretagogue role. These effects on the regulation of glucose homeostasis observed for *B. articulata* indicate potential anti-diabetic properties.

Keywords: *Baccharis articulata*; diabetes; insulin; disaccharidase; glycation

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by a high blood glucose concentration (hyperglycemia) which is due to insulin deficiency and/or insulin resistance. As a consequence, liver and skeletal muscle is unable to take up or utilize glucose or to store it as glycogen. The chronic hyperglycemia of diabetes is associated with long-term damage to many systems of the body, in particular the eyes, kidneys, nerves, heart, and blood vessels [1].

Type 2 diabetes mellitus (T2DM) is the most common metabolic disorder worldwide, and with epidemic proportions the increase in its prevalence is unprecedented, both in developed and developing countries. The primary aim in the management of T2DM is to delay, or even prevent, the complications of the disease by achieving euglycemic levels. In addition to drug therapy, with oral hypoglycemic agents and/or insulin, the glycemic control often involves changes in lifestyle such as diet and amount of exercise. However, the treatment of T2DM is complicated by several factors inherent to the disease process, such as insulin resistance, hyperinsulinemia, impaired insulin secretion and reduced insulin-mediated glucose uptake and utilization [2].

A variety of plant extracts have been used for centuries in folk medicine to treat diabetes. Medicinal plants are particularly interesting since not only can they be used as complementary and alternative remedies to prevent metabolic diseases, but they also serve as an interesting source of compounds which are potential drug candidates [3]. Several plant species have demonstrated anti-diabetic properties and a large number of compounds from plant extracts have been reported to have beneficial effects in the treatment of diabetes [4].

The *Baccharis* genus (Asteraceae) is distributed mainly in Brazil, Argentina, Colombia, Chile and Mexico. The aerial parts of *Baccharis* species, commonly known as "carqueja", have many traditional uses in folk medicine, especially for anti-inflammatory, diuretic, and digestive purposes. In addition, infusions or decoctions of *Baccharis articulata* (Lam.) Pers. (*B. articulata*) are also traditionally used as antidiabetic remedies in local folk medicine in southern Brazil [5]. However, as far as we are aware, there are no reports in the literature concerning the hypoglycemic and/or antihyperglycemic properties of this plant.

Therefore, the aim of this study was to investigate the short- and long-term effects of *Baccharis articulata* on glucose homeostasis. The biological effects of the crude extract and the *n*-butanol and aqueous residual fractions were also studied through *in vivo* and *in vitro* approaches. To this end the serum glucose levels, insulin secretion, and muscle and liver glycogen content as well as the intestinal disaccharidase activity and anti-glycation properties were determined.

2. Results and Discussion

2.1. Phytochemical Characterization

In our investigation the TLC analysis showed a predominance of phenolic compounds and flavonoids in the crude extract (CE) and the *n*-butanol fraction (BF) of *B. articulata*. This was verified

using the Natural Reagent A/UV356 that showed yellow spots corresponding to phenolic compounds (data no shown) as previously described [6]. In addition, it can be observed (Table 1) that the BF showed significantly higher total flavonoids content (44.9 ± 0.71 mg of RE/g of DW, $p < 0.05$) when compared to the CE (38.9 ± 0.66 mg of RE/g of DW) and ARF (25.3 ± 0.42 mg of RE/g of DW). On the other hand the total phenolic content of CE and their related fractions did not shown differences.

Some of the different biological activities of *Baccharis* species are related to the presence of phenolic compounds and flavonoids [7,8]. A large number of studies have demonstrated that phenolic compounds as flavonoids and phenolic acids derivatives have different biological activities, such as antioxidant, anticancer, anti-inflammation and cardioprotective properties, and they can also prevent lipoperoxidation, induce favorable changes in the lipid profile, improve endothelial function, and disclose antithrombotic properties [9]. In addition, the hypoglycemic and/or antihyperglycemic activity of flavonoids has been previously reported [10].

Table 1. Total phenolic [a] and total flavonoids [b] content in crude extract (CE), *n*-butanol fraction (BF) and aqueous residual fraction (ARF) of *B. articulata*.

Extract/fraction	Total phenolic	Total flavonoids
CE	151.8 ± 0.93 [a]	38.9 ± 0.66 [a]
BF	154.1 ± 1.08 [a]	44.9 ± 0.71 [b]
ARF	135.6 ± 0.95 [b]	25.3 ± 0.42 [c]

[a] Data are mean ± S.E.M. values, expressed as mg of acid gallic equivalents/g of dry weight (n = 6); [b] Data are mean ± S.E.M. values, expressed as mg of rutin equivalents/g of dry weight (n = 6); Values in column with the same letters indicate no significant differences ($p < 0.05$).

2.2. Effect of Crude Extract and n-Butanol and Aqueous Residual Fractions of B. Articulata *on Oral Glucose Tolerance Curve*

As expected, in the oral glucose tolerance test, after 15 min of glucose loading the glycemia was significantly increased when compared with zero time. Glipizide (100 mg/kg) an oral hypoglycemic agent of the sulfonylurea class was used as a positive control and produced a typical serum glucose lowering at all periods analyzed (15 to 180 min) compared to the hyperglycemic group (Table 2). At all doses tested (50, 100 and 200 mg/kg) the CE of *B. articulata* leaves was effective in reducing the glycemia at different times after oral treatment compared with the respective hyperglycemic control group (Table 2). The dose of 100 mg/kg of the CE produced the best antihyperglycemic profile at 15 to 60 min and the maximum reduction observed was 26% at 30 min. Administration of 50 mg/kg of the BF of *B. articulata* decreased serum glucose levels significantly at 15, 30 and 60 min, and the glycemic reduction was around 23, 23 and 18%, respectively, whereas the dose of 100 mg/kg was not effective in reducing the serum glucose levels during the times studied (Table 2). In addition, oral administration of the ARF of *B. articulata* also reduced the serum glucose levels in hyperglycemic rats at both doses tested (50 and 100 mg/kg) and the antihyperglycemic effect was better with the lower dose. The reduction was around 15 and 20% at 15 and 30 min after treatment, respectively. At 180 min, glycemic levels were similar to the respective results for the hyperglycemic control groups.

On the other hand, the CE, BF and ARF (100 mg/kg) of *B. articulata* were studied in rats with induced diabetes and no significant changes in the serum glucose levels in an acute treatment were

observed (data not shown). These results point a potential insulin secretagogue effect for *B. articulata* compounds.

Another *Baccharis* species, *Baccharis trimera* reportedly has potential antidiabetic activity. Oliveira *et al.* [11] investigated the effect of its extracts and fractions on glycemia in non-diabetic mice and mice with streptozotocin-induced diabetes. After 7 days of treatment, the aqueous fraction (2,000 mg/kg, twice daily) reduced the glycemia of diabetic mice. However, in contrast to our results for *B. articulata*, none of the extracts or fractions (200 or 2,000 mg/kg) of *B. trimera* induced any effect on glycemia after acute administration on hyperglycemic mice. Thus, to the best of our knowledge, our results represent the first report of the potential antihyperglycemic effect of *B. articulata.*

Diterpenoids, flavonoids and other phenolic compounds have been reported as the major phyto-constituents of the *Baccharis* species and this diverse chemical composition is related to a variety of biological activities described for these species [8]. The results reported herein demonstrate that *B. articulata* has a significant content of flavonoids and other phenolic compounds (Table 1) and the presence of these constituents may be associated with the antihyperglycemic effect observed, since the hypoglycemic activity of phenolics compounds has been previously reported [10].

2.3. Effect of Crude Extract and n-Butanol and Aqueous Residual Fractions of B. Articulata *on Insulin Secretion and Glycogen Content*

In order to evaluate the possible mechanism of action of the extract (CE) and fractions (BF and ARF) of *B. articulata*, their effects on glycogen content and on insulin secretion were investigated. Serum insulin levels were determinated in fasted rats after an oral glucose loading (4 g/kg) as shown in Table 3. As expected a sulfonylurea agent, glipizide, stimulated the insulin secretion by 295, 149 and 191% at 15, 30 and 60 min, respectively, compared to the hyperglycemic control group. The CE potentiated insulin secretion induced by glucose at 15 (167%), 30 (141%) and 60 min (268%), after oral treatment. In addition, the BF increased significantly the insulin secretion by 162, 189 and 244% at 15, 30 and 60 min, respectively. However, the ARF was not able to increase serum insulin levels. The treatments with the CE and BF resulted in around a 2.3-fold increase in the insulinogenic index (II) compared with the hyperglycemic control group (hyperglycemic control 0.44 ng/mg; CE 0.96 ng/mg and BF 1.03 ng/mg), achieving values similar to that calculated for glipizide (1.09 ng/mL). These results indicate, for the first time, the powerful effect of *B. articulata* on insulin secretion.

The glycogen content in the soleus muscle and liver samples of hyperglycemic rats and those which received acute treatments with the CE (100 mg/kg), BF (50 mg/kg) and ARF (50 mg/kg) was determinate as shown in Figure 1A and B. After 3 h of oral treatment with the BF and ARF the glycogen content in the soleus muscle increased significantly, by around 443 and 212%, respectively, compared to the hyperglycemic control group (Figure 1A).

Table 2. Acute effect of crude extract (CE), *n*-butanol fraction (BF) and aqueous residual fraction (ARF) of *Baccharis articulata* on serum glucose levels (mg/dL) in oral glucose tolerance curve [a].

Time (min)	Group I Hyper Glucose (4 g/kg)	Group II Hyper + glipizide (10 mg/kg)	Group III Hyper + CE			Group IV Hyper + BF		Group V Hyper + ARF	
			50 mg/kg	100 mg/kg	200 mg/kg	50 mg/kg	100 mg/kg	50 mg/kg	100 mg/kg
0	112 ± 4 [#]	104 ± 3	110 ± 6	116 ± 5	103 ± 3	116 ± 2	113 ± 3	116 ± 3	103 ± 2
15	162 ± 8	121 ± 4 ***	128 ± 5 **	123 ± 2 ***	134 ± 6 **	124 ± 6 ***	187 ± 4	138 ± 4 **	136 ± 10 *
30	185 ± 6	148 ± 7 ***	144 ± 4 ***	137 ± 1 ***	146 ± 3 ***	143 ± 2 ***	178 ± 6	148 ± 3 ***	159 ± 6 *
60	164 ± 4	122 ± 4 ***	145 ± 5	130 ± 8 **	140 ± 6 *	134 ± 9 **	186 ± 8	154 ± 4	161 ± 10
180	135 ± 4	116 ± 5 **	130 ± 3	129 ± 3	136 ± 4	145 ± 5	126 ± 4	142 ± 5	133 ± 5

[a] Values are expressed as mean ± S.E.M; n= 6 in duplicate for each treatment; Statistically significant difference compared to the corresponding hyperglycemic group; Statistically significant at [#] $p < 0.001$ in relation to 15 min * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

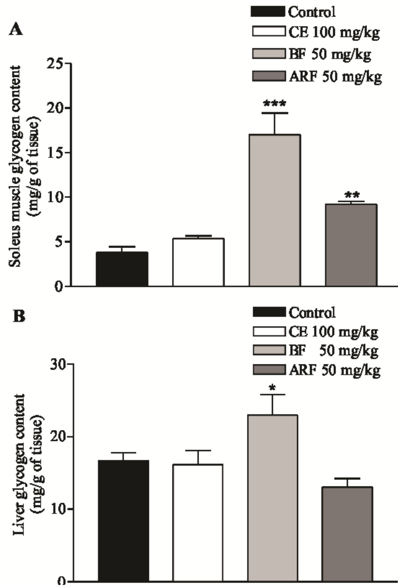
Table 3. Acute effect of crude extract (CE), *n*-butanol fraction (BF), aqueous residual fraction (ARF) of *Baccharis articulata* on serum insulin levels (ng/mL) and insulinogenic index (II; ng/mg) [a].

Time (min)	Hyper Glucose (4 g/kg)	Hyper + glipizide (10 mg/kg)	Hyper + *B. articulata*		
			CE 100 mg/kg	BF 50 mg/kg	ARF 50 mg/kg
0	0.57 ± 0.03	-	-	-	-
15	0.77 ± 0.06 [#]	2.27 ± 0.20 ***	1.29 ± 0.27 **	1.25 ± 0.10 *	0.82 ± 0.04
30	0.90 ± 0.10	1.37 ± 0.02 *	1.30 ± 0.14 *	1.74 ± 0.06 ***	0.79 ± 0.09
60	0.54 ± 0.04	1.03 ± 0.15 *	1.45 ± 0.24 ***	1.32 ± 0.20 ***	0.63 ± 0.10
II	0.44	1.09	0.96	1.03	0.49

[a] Values are expressed as mean ± S.E.M; n = 4 in duplicate for each treatment; Statistically significant at [#] $p < 0.01$ in relation to euglycemic group; Statistically significant difference compared to the corresponding hyperglycemic group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In addition, only the BF treatment was able to significantly increase the glycogen content in the liver when compared with the hyperglycemic control group 3 h after treatment. In percentage terms, this change was 137% (Figure 1B). Taking it in account, it seems that the serum glucose lowering could be related with the effect on increased glycogen content similar to that known to insulin, points to an insulin-mimetic effect.

Figure 1. Effect of crude extract (CE), *n*-butanol fraction (BF) and aqueous residual fraction (ARF) of *B. articulata* on glycogen content in comparison to hyperglycemic rats (control). (**A**) soleus muscle and (**B**) liver 3 h after treatment by oral gavage. Values are expressed as mean ± S.E.M; n = 6 in duplicate for each group. Significantly different to the corresponding hyperglycemic group; * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.



Insulin is secreted into the bloodstream by β-cells of the endocrine pancreas and glucose is the main insulin secretagogue. Insulin is the most important hormone that regulates energy metabolism and has hypoglycemic effect. An absolute or relative lack of insulin, as in the case of diabetes, leads to severe dysfunction in the major insulin target organs such as muscle, liver and adipose tissue [12].

The stimulation of β-cells and subsequent release of insulin and activation of the insulin receptors is a possible mechanism of natural products with potential antidiabetic activity. Folador *et al.* [13] showed that the crude extract, the *n*-butanol fraction and two isolated *C*-glycosylflavones, isovitexin and swertisin, of *Wilbrandia ebracteata* can have an antihyperglycemic action, which was related to the stimulation of *in vivo* insulin secretion.

Glucose homeostasis is maintained by the balance of liver glucose production and glucose utilization by peripheral tissues. In mammals, glucose is stored as glycogen in the liver and muscle,

which are the major sites for glycogen synthesis and storage. It is well known that glycogen deposition from glucose is regulated by insulin. However, it is well reported that flavonoids and plant extracts with proven antihyperglycemic activity can also influence glycogen deposition in different tissues as well as interact with key enzymes of the glycolytic route in rats [14,15].

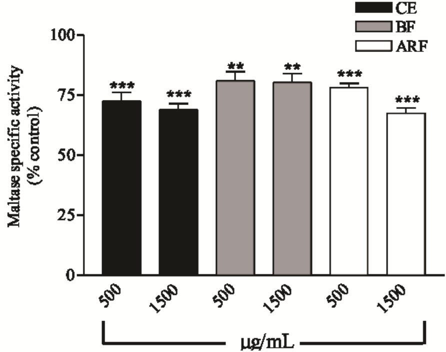
In addition, it is worth noting that plants and phytochemicals that have a hypoglycemic effect may act by different mechanisms of action to regulate glucose homeostasis, including an increase in insulin secretion from the pancreatic islets (insulin-secretagogue) and/or enhancing or reproducing the effect of insulin (insulin-mimetic) as we demonstrated for *B. articulata*.

2.4. Effect of Crude Extract and n-Butanol and Aqueous Residual Fractions of B. Articulata *on the Disaccharidases*

The intestine plays an important role in glucose homeostasis. A therapeutic approach to decreasing postprandial hyperglycemia is to retard the absorption of glucose via inhibition of carbohydrate-hydrolyzing enzymes, such as α-glucosidase, in the intestine. These disaccharidase enzymes are located in the brush border of the small intestine and are required for the breakdown of carbohydrates before monosaccharide absorption. The α-glucosidase inhibitors delay the absorption of ingested carbohydrates, reducing the postprandial glycemia and insulin peaks [16]. Some plants which exhibit properties similar to those of known classes of anti-diabetic drugs, for instance, inhibitors of α-glucosidase such as acarbose, have been identified [17].

The effect of the extract and fractions of *B. articulata* in disaccharidase assays were determined (Figure 2). A significant effect on maltase inhibition was observed after 5 min of incubation of the intestine homogenate in the presence of a maltose substrate. The CE, BF and ARF were effective at inhibiting the enzyme maltase at both doses tested (500 and 1,500 μg/mL) after 5 min of incubation.

Figure 2. *In vitro* effect of crude extract (CE), *n*-butanol fraction (BF) and aqueous residual fraction (ARF) of *B. articulata* on specific activity of maltase, in the duodenal portion of rat intestine. Incubation = 5 min. Values are expressed as mean ± S.E.M.; n = 6 for each group. Significant at ** $p < 0.001$; *** $p < 0.0001$ compared to control group.



The inhibitory effect observed ranged from 15 and 32% and the maximum inhibitory effect on maltase activity, above 30%, was observed for the CE and ARF at the higher dose studied, compared with the respective controls. On the other hand, none of the treatments affected the sucrase and lactase activity at any concentration tested (data not shown).

A number of plants are known to exert antihyperglycemic activity through the inhibition of disaccharidase enzymes in the small intestine, impeding the absorption of carbohydrates. Recently, De Souza *et al.* [18] reported that aqueous and methanolic extracts of *B. trimera* efficiently inhibited β and α-glycosidase activity.

2.5. Effect of Crude Extract and n-Butanol and Aqueous Residual Fractions of B. Articulata *on* in vitro *Albumin Glycation*

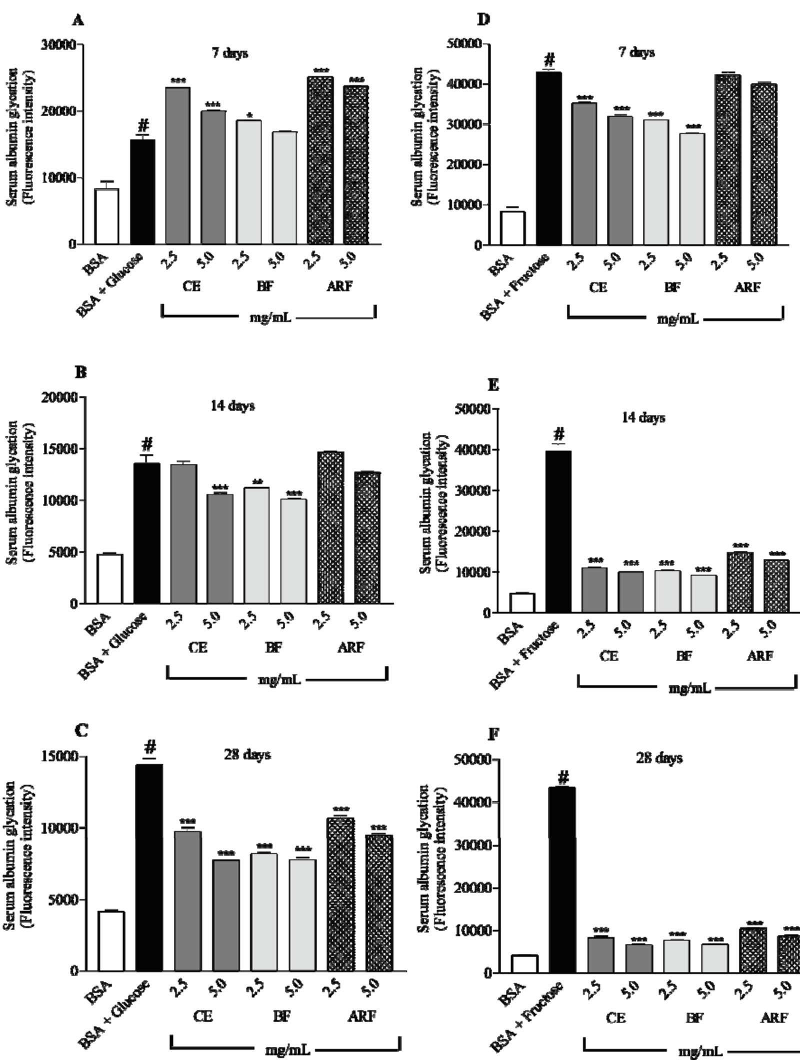
Chronic hyperglycemia and increased oxidative stress during diabetes results in the irreversible formation of advanced glycation endproducts (AGEs), which are a heterogeneous group of molecules formed from non-enzymatic glycation of reducing sugars with free amino groups of proteins, lipids, and nucleic acids. The Schiff's bases formed by glycation rearrange further through stable reactions to form Amadori products which later, by isomerization, condensation, and rearrangement reactions, form AGEs. The AGEs are known to have a wide range of chemical, cellular, and tissue effects implicated in the development and progression of diabetic complications, like nephropathy, neuropathy, retinopathy, and cardiovascular diseases [19].

In the method adopted in this study, BSA was chosen as the model protein and glucose or fructose was used as the glycated agent. This BSA-reducing sugar system is an *in vitro* model widely used in non-enzymatic glycation studies. Proteins can be modified when exposed to reducing sugars through the spontaneous glycation process. The sugar-mediated fluorescence intensity, which is a characteristic of AGEs, increases during incubation at 37 °C for a long period. Figure 3 shows the fluorescence intensity of the products (AGEs) formed in the BSA-glycation model. After incubation at all periods analyzed (7, 14 and 28 days) it was clearly observed that the formation of AGEs was significantly increased in the BSA/glucose (Figure 3A–C) and BSA/fructose (Figure 3D–F) systems when compared with the basal control.

Figure 3B and C show the efficiency of the CE, BF and ARF in the inhibition of albumin glycation with glucose after 14 and 28 days. After 28 days of *in vitro* incubation all treatments caused a glycation reduction of over 30% when compared with positive glycation group (albumin plus glucose). However, a slight increase in glycation was observed for the CE, BF and ARF treatments after 7 days in the BSA/glucose system (Figure 3A). These results show the significant capacity of the extract and fractions of *B. articulata* to reduce the AGE formation after a long period of incubation, the fluorescence intensity being stronger.

The capacity of the CE, BF and ARF to inhibit albumin glycation with fructose for different periods is shown in Figure 3D–F. The reduction in the glycation of albumin by glucose in the presence of the extract and fractions of *B. articulata* increased from 7 to 28 days of treatment. With 7 days of incubation only the CE and BF, at both doses tested, were able to inhibit significantly the albumin glycation. At the maximum period evaluated, more that 75% of glycation reduction was observed when compared with the positive glycation group (albumin plus fructose).

Figure 3. Inhibitory effect of crude extract (CE), *n*-butanol fraction (BF) and aqueous residual fraction (ARF) of *B. articulata* on the formation of fluorescent AGEs in a BSA/glucose or BSA/fructose system. (**A, B and C**) 7, 14 and 28 days BSA/glucose; (**D, E and F**) 7, 14 and 28 days BSA/fructose. Values are expressed as mean ± S.E.M; n = 6 in duplicate for each group. Significantly different to the corresponding control group (BSA/glucose or BSA/fructose); * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.



Several natural compounds have been proposed and tested as inhibitors of glycation and AGE formation, providing additional therapeutic options for the treatment of the various complications associated with diabetes [20]. As the incidence of diabetes continues to rise worldwide, the study of natural products for the treatment and prevention of diabetes, and its associated complications, offers an important opportunity for the development of complementary interventions that may be more acceptable to high-risk populations in the search for non-pharmaceutical alternatives. Our results demonstrate that the extract and fractions of *B. articulata* are potent inhibitors of *in vitro* AGE formation in chronic treatments and this mechanism may help to provide a protective effect against hyperglycemia-mediated protein damage. Recent reports have shown that flavonoids inhibit the formation of AGEs, which is related to their well-known antioxidative effects [21]. In this regard, we verified the high total flavonoid content of *B. articulata*, which may explain the significant AGE inhibition observed for this species.

3. Experimental

3.1. Materials

Glipizide, glycogen and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Glucose, fructose and maltose were purchased from Vetec® AG (Rio de Janeiro, Brazil). All reagents were of analytical grade. Enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of rat insulin (catalogue no. EZRMI-13K) was purchased from Millipore (St Charles, MO, USA). Salts and solvents were purchased from Merck AG (Darmstadt, Germany).

3.2. Plant Material

Aerial parts of *Baccharis articulata* (Lam.) Person were collected in Chapecó, State of Santa Catarina, Brazil, in March 2008. The plant material was identified by Prof. Dr. Geraldo Ceni Coelho (Universidade Federal da Fronteira Sul) and a voucher specimen of the plant (ICN 9057) was deposited at the Herbarium of the Botany Department of Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

3.3. Preparation of Extract and Fractions of B. Articulata

Aerial parts (50 g) of *B. articulata* were crushed and extracted under reflux (90 °C) with 500 mL of ethanol 40 °GL for 30 min. After cooling, each extract was filtered separately, the volume was adjusted to 500 mL with water, and the preparation was separated into two fractions of 250 mL. One fraction was evaporated under reduced pressure to dryness to obtain the crude extract (CE). The ethanol content of the second fraction was removed under reduced pressure, its volume was adjusted to 250 mL with water, and this aqueous suspension was partitioned (3 × 50 mL of *n*-BuOH) yielding the *n*-BuOH (BF) and aqueous residual (ARF) fractions.

3.4. Thin-Layer Chromatographic Analysis

Phytochemical profile of *B. articulata* was performed by thin-layer chromatography (TLC) on silica gel plates (Merck F_{254}, 20 × 20 cm) using as mobile phase chloroform-ethanol-acetic acid ($CHCl_3$:EtOH:HOAc, 60:40:6, v/v) and Diphenylboryloxyethylamine 1% in methanol, (Natural Reagent) as colour reagent [6].

3.5. Determination of Total Phenolic Content

Total phenolic content of the extract/fractions were determined by the Folin-Ciocalteu assay [22]. Briefly, a 125 µL aliquot of the extract/fractions were assayed with 125 µL of Folin-Ciocalteu reagent. After six min, 1.25 mL of sodium carbonate (20%, wt/vol) was added and the mixture vortex-mixed and diluted with distilled water to a final volume of 2 mL. After 90 min, the absorption was measured at 760 nm, and the total phenolic content was expressed as milligrams of gallic acid equivalents in relation to grams of dry weight (mg of GAE/g DW). All analyses were performed in triplicate.

3.6. Determination of Total Flavonoid Content

The total flavonoids content was determined according to Miliauskas *et al.* [23] with minor modifications. Briefly, 1 mL of the extract/fractions at 4 mg/mL was mixed with 1 mL of aluminum trichloride. After 40 min the absorption of each sample (CE, BF and ARF) was measured at 415 nm. The results were expressed as milligrams of rutin equivalents in relation of grams of dry weight (mg of RE/g DW). All analyses were performed in triplicate.

3.7. Animals

The male Wistar rats (180–200 g) used in this study were bred in our animal facility and housed in an air-conditioned room (approximately 22 °C) with controlled lighting on a 12:12 h light/dark cycle (lights on from 06:00 to 18:00 h). The animals were maintained with pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil), while tap water was available *ad libitum*. Fasted animals were deprived of food for at least 16 h but allowed free access to water. All the animals were monitored and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council (CMV) and the Brazilian College of Animal Experimentation (COBEA; Protocol PP00398/CEUA/UFSC).

3.8. Oral Glucose Tolerance Curve (OGTC)

Fasted rats were divided into different groups of six animals for each treatment. Group I, hyperglycemic rats that received glucose (4 g/kg; 8.9 M); Group II, rats that received glipizide at a dose of 10 mg/kg; Group III, rats that received the CE at the doses 50, 100 and 200 mg/kg; Group IV, rats the received the BF at doses of 50 and 100 mg/kg; Group V, rats that received the ARF at doses of 50 and 100 mg/kg. The glycemia was measured before the rats received the treatment (zero time). The rats were treated with extract or fraction and loaded with glucose after 30 min and after the glycemia was measured at 15, 30, 60 and 180 min. All treatments were administrated by oral gavage.

3.9. Determination of the Plasma Glucose Concentration

Blood samples were collected and centrifuged, and the blood glucose levels were determined by the glucose oxidase method [24].

3.10. Insulin Serum Measurements

The insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The range of values detected by this assay was 0.2 ng/mL to 10 ng/mL. The intra- and inter-assay coefficients of variation for insulin were 3.22 and 6.95, respectively, with a sensitivity of 0.2 ng/mL. All insulin levels were estimated by means of colorimetric measurements at 450 nm with an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) by interpolation from a standard curve. Samples were analyzed in duplicate and results were expressed as ng of insulin serum mL^{-1} [14]. The incremental areas under the response curves (AUCs) were calculated. The insulinogenic index (II) was calculated as the ratio between the $AUC_{insulin}$ and $AUC_{glucose}$ (from zero to 60 min) [25].

3.11. Glycogen Content Measurements

The soleus muscles and livers were harvested from untreated hyperglycemic rats and from those treated with the CE (100 mg/kg), BF (50 mg/kg) and ARF (50 mg/kg) used for the assay of glycogen content immediately after 3 h of treatment. Glycogen was isolated from these tissues as described by Krisman [26], with minor modifications [27]. The tissues were weighed, homogenized in 33% KOH and boiled at 100 °C for 20 min, with occasional stirring. After cooling, 96% ethanol was added to the samples which were then heated to boiling followed by cooling in an ice bath to aid the precipitation of glycogen. The homogenates were centrifuged at 1,300 × g for 15 min, the supernatant was discarded and the pellets were neutralized with saturated NH_4Cl before being maintained at 100 °C for 5 min, washed and resolubilized in water. Glycogen content was determined by treatment with iodine reagent and the absorbance was measured at 460 nm. The results are expressed as mg of glycogen/g of tissue.

3.12. Disaccharidase Extraction and Assays

A segment of the small intestine was removed, washed in 0.9% NaCl solution, dried on filter paper, weighed, trimmed and homogenized (300 rpm) with 0.9% NaCl (400 mg of duodenum per mL) for 1 min at 4 °C. The resulting extract was centrifuged at 8,000 rpm for 8 min. The supernatant was used for the measurement of *in vitro* maltase, sucrase and lactase activities and for protein determination.

Maltase (EC 3.2.1.20), lactase (EC 3.2.1.23) and sucrase (EC 3.2.1.48) activities were determined using a glucose diagnosis kit based on the glucose oxidase reagent. For the determination of disaccharidase activity, 50 µL of homogenate were pre-incubated at 37 °C for 5 min, in the absence (control) or in the presence of the CE, BF or ARF of *B. articulata* (treated). The concentrations 500 and 1,500 µg/mL were used. The duodenum homogenates were then incubated at 37 °C for 5 min with 25 µL of the substrate (corresponding to 0.056 µM of maltose, sucrose or lactose) [28].

One enzyme unit (U) was defined as the amount of enzyme that catalyzed the release of 1 µmol of glucose per min under the assay conditions. The specific activity was defined as enzyme activity (U)

per mg of protein. Protein concentration was determined by the method described by Lowry method [29] using bovine serum albumin as the standard. The assays were performed in duplicate and conducted along with appropriate controls.

3.13. Formation of Advanced Glycation End-Products (AGEs) in Bovine Serum Albumin/Glucose and Fructose Systems

AGE was formed in *in vitro* systems using a previously described method [30]. In brief, BSA (10 mg/mL) in phosphate buffered-saline (PBS, pH 7.4) containing 0.02% sodium azide was incubated with glucose (500 mM) or fructose (100 mM) at 37 °C for 14 and 28 days in the absence (control) and presence of the CE, BF or ARF of *B. articulata* (2.5 and 5.0 µg/mL). The protein, glucose or fructose, and the prospective inhibitor were simultaneously introduced into the incubation mixture. Each solution was kept in the dark in a capped vial, and incubation was allowed to proceed in triplicate vials. In the time-course experiments on AGE formation, we measured characteristic fluorescence (excitation wavelength of 370 nm and emission wavelength of 440 nm) with an Infiniti M200 (TECAN).

3.14. Data and Statistical Analysis

Data were expressed as means ± S.E.M. One-way analysis of variance (ANOVA) followed by the Bonferroni *post-hoc* test or unpaired Student's *t*-test to determine significant differences between the groups. Differences were considered to be significant at $p < 0.05$.

4. Conclusions

In conclusion, the results reported herein indicate that *B. articulata* possesses antihyperglycemic potential. The *n*-butanol fraction, with the highest flavonoids content, showed an important insulin secretagogue effect, exhibiting an insulinogenic index similar to that of glipizide, and also contributed significantly to the storage of glucose as glycogen in muscle and liver tissue. Additionally, the extract and fractions of *B. articulata* demonstrated an inhibitory *in vitro* effect on maltase activity and AGE formation, resulting in short and long-term influence on the decrease in glucose absorption and the prevention of protein glycation. Overall, the results of this study suggests that *B. articulata* has a beneficial *in vivo* and *in vitro* biological effect on glucose homeostasis, which may ameliorate significantly diabetes mellitus status.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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Sample Availability: Samples of the extracts are available from the authors.

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4.1.2 Conclusões parciais

A partir dos resultados obtidos neste trabalho, podemos concluir que:

O extrato bruto e as frações *n*-butanol e residual aquosa do extrato de partes aéreas de *Baccharis articulata* reduziram a glicemia de ratos normais hiperglicêmicos após período agudo de tratamento.

O extrato bruto e a fração *n*-butanol de *Baccharis articulata* potencializaram a secreção de insulina estimulada por glicose após tratamento agudo por via oral, apresentando um índice insulinogênico similar à glipizida.

As frações *n*-butanol e residual aquosa de *Baccharis articulata* aumentaram significativamente o conteúdo de glicogênio em músculo sóleo de ratos normais hiperglicêmicos após o tratamento.

A fração *n*-butanol de *Baccharis articulata* aumentou significativamente o conteúdo de glicogênio em fígado de ratos normais hiperglicêmicos após o tratamento.

O extrato bruto, frações *n*-butanol e residual aquosa de *Baccharis articulata* inibiram a atividade da enzima maltase.

O extrato bruto, frações *n*-butanol e residual aquosa de *Baccharis articulata* inibiram o processo de glicação, prevenindo a formação dos AGEs.

O extrato bruto e a fração *n*-butanol de *Baccharis articulata* apresentaram teores significativos de fenólicos e flavonóides totais, e estes compostos podem estar relacionados com as atividades biológicas aqui observadas.

4.2 *Musa x paradisiaca*: efeitos na homeostasia da glicose

As espécies do gênero *Musa* são conhecidas como bananas. Várias partes dessas espécies, como frutos, folhas, flores, raízes, entre outras, tem sido usadas como remédio na medicina tradicional para variados fins, inclusive como anti-diabético (CORRÊA, 1984; OLORUNFEMI et al., 2011; PARI; MAHESWARI, 1999). No entanto, uma ampla revisão de literatura mostrou que não há relatos sobre a caracterização química e as propriedades farmacológicas das folhas de *Musa x paradisiaca*. Assim, o objetivo deste trabalho foi estudar o efeito *in vivo* e *in vitro* na homeostasia da glicose do extrato bruto e das frações (*n*-butanol e residual aquosa) das folhas de *Musa x paradisiaca*. Nesse sentido, realizou-se uma caracterização fitoquímica do extrato e das frações, e também avaliou-se em ratos hiperglicêmicos os níveis séricos de glicose, a secreção de insulina, o conteúdo de glicogênio muscular e hepático, e *in vitro* a glicação de albumina sérica e a atividade das dissacaridases intestinais.

4.2.1 Artigo submetido para publicação

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Nota: A formatação dos artigos submetidos não corresponde exatamente à forma submetida ao periódico, uma vez que as figuras foram inseridas no corpo do texto a fim de facilitar a leitura e compreensão.

Beneficial effects of banana leaves (*Musa x paradisiaca*) on glucose homeostasis in rats: multiple sites of action

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ABSTRACT

The acute effect of crude extract, *n*-butanol and aqueous residual fractions of *Musa* x *paradisiaca* leaves on glycemia, serum insulin secretion and glycogen content in an *in vivo* approach was evaluated. In addition, the *in vitro* effect on disaccharidases activity and albumin glycation was studied. The crude extract and fractions, *n*-butanol and aqueous residual, reduced glycemia and increased liver glycogen content in hyperglycemic rats, inhibited maltase activity and the formation of advanced glycation end-products *in vitro*. Also, a significant increase in insulin secretion and muscle glycogen content in hyperglycemic rats was observed with oral administration of the *n*-butanol fraction. Phytochemical analysis demonstrated the presence of rutin in crude extract and fractions of *M.* x *paradisiaca* leaves as the major compound. These beneficial effects on the regulation of glucose homeostasis observed for *M.* x *paradisiaca* leaves indicate potential anti-diabetic properties.

Keywords: *Musa* x *paradisiaca* Lineu; Banana; Glycemia; Insulin; Glycation; Glycogen.

1. Introduction

Diabetes mellitus is the most common metabolic disorder and is a major cause of ill health all over the world. It is caused by defects in insulin secretion or action and, consequently, is characterized by hyperglycemia. Several pathogenic processes are involved in the development of diabetes and the chronic hyperglycemia is associated with long-term damage, dysfunction and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Treatment of hyperglycemia in diabetes involves diet control, exercise and the use of oral anti-diabetic drugs, insulin therapy or combination of both [1]. Nonetheless, they fail to alter the course of complications and tend to result in undesirable side effects.

Plants continue to be an important source of bioactive compounds and involve a multidisciplinary approach combining ethnobotanical, phytochemical and biological techniques to provide new chemical compounds. For many years people have used plants to treat diabetes. In this context, the hypoglycemic effect of several plants used as antidiabetic remedies has been confirmed, and the mechanisms of hypoglycemic activity of these plants and their major compounds are being investigated [2].

Musa x *paradisiaca* L. (Musaceae), popularly known as ‘banana’, is a perennial tree-like herb cultivated in many tropical and subtropical regions around the world. Banana, eaten as a fruit or a vegetable, is one of the most important crops in several countries due to its enriched food and versatile medicinal value. Various parts of the *Musa* plants have been used orally or topically as remedies in folk medicine and some studies have demonstrated this medicinal potential. The fruits, peel, leaves, roots and pseudostem of *Musa* plants have shown antiulcerogenic, antioxidant and antimicrobial activity, among others activities [3-5]. In addition, studies have shown that some species of *Musa* possess anti-diabetic, antihyperglycemic and hypoglycemic activity [6-8].

However, as far as we aware, there is no reports concerning chemical characterization and pharmacological properties of their leaves. Thus, the aim of the present study was to investigate the *in vivo* and *in vitro* effect of crude extract, *n*-butanol and aqueous residual fractions of leaves of *Musa* x *paradisiaca* L. (*M.* x *paradisiaca*) on serum glucose levels, insulin secretion, liver and muscle glycogen content, serum albumin glycation and intestinal disaccharidase activity. Also, a

phytochemical characterization of crude extract and fractions was carried out.

2. Methods and materials

2.1. Chemicals

Rutin (≥98%), glycogen, bovine serum albumin (BSA), tolbutamide were purchased from Sigma Chemical Company® (St. Louis, MO, USA). Glucose, fructose, maltose, sucrose and all other solvents were purchased from Vetec® AG (Rio de Janeiro, Brazil). All reagents were of analytical grade. The solvents used for HPLC analysis were purchased from Tedia® (HPLC grade; Fairfield, OH, USA). Enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of rat insulin (catalogue n°. EZRMI-13K) was purchased from Millipore (St Charles, MO, USA).

2.2. Plant material

Leaves of *Musa* x *paradisiaca* L. were collected in Florianópolis, State of Santa Catarina, Brazil, in April 2008. The voucher specimen was identified by Dr. Geraldo Ceni Coelho and is deposited in the herbarium at Universidade Federal de Santa Catarina (FLOR 3832).

2.3. Preparation of the extracts and fractions of Musa x paradisaca

The crude extract (CE) and fractions were prepared according to Costa et al.[9] with minor modifications. Briefly, dried leaves (50 g) of *M.* x *paradisiaca* were crushed and extracted under reflux (90ºC) with 500 mL of ethanol 40% for 30 min. After cooling, the extract was filtered, the volume was adjusted to 500 mL with water, and the extract was separated into two fractions of 250 mL. One fraction was evaporated under reduced pressure to dryness to obtain CE. The ethanol content of the second fraction was removed under reduced pressure, its volume was adjusted to 250 mL with water, and this aqueous suspension was partitioned (3 x 100 mL) with *n*-BuOH, yielding the *n*-BuOH (BF) and aqueous residual fractions (ARF).

2.4. Chemical characterization of crude extract and fractions

The presence of different constituents in crude extract and fractions from *M. x paradisiaca* was established by thin-layer chromatography (TLC) on silica gel plates (Merck 60 F_{254} 20x20 cm) using several mobile phases. Detection was performed, respectively, with chlorosulfonic acid–glacial acetic acid reagent spraying and heating for terpenoids, and for phenolic compounds, fluorescence at 365 nm after spraying with 1% diphenylboryloxyethylamine in MeOH. The high performance liquid chromatography (HPLC) analyses were performed in a PerkinElmer Series 200 HPLC, composed of a Photo Diode Array Detector (PDA), quaternary pump and autosampler. The data acquisition system was TotalChrom Workstation software. All samples were dissolved in MeOH: H_2O (1:1 v/v), filtered using a 0.45 µm syringe filter (PVDF, Millipore®) and 10 µL aliquots were injected for HPLC analysis. The extracts and fractions were analyzed at 1,000 mg/mL while the rutin standard solution was analyzed at 100 µg/mL. The separation was performed on a Perkin Elmer Brownlee Choice C_{18} column (250 x 4.6 mm i.d.; 5µm) and the mobile phase used was a gradient of solvent A (acetonitrile) and solvent B (acetic acid 1%, adjusted to pH 3.0) as follow: 10-20% A (0 - 40 min) and isocratic 20% A (40-45 min). The flow rate was kept at 1.0 mL/min. The chromatograms were recorded at 340 nm while the UV spectra were monitored over the range of 200 - 450 nm. The flavonoids in the CE, BF and ARF of *M.* x *paradisiaca* were characterized by comparing the retention time and UV spectra with the reference standards, and by the co-injection of the sample and authentic samples [9].

2.5. Animals

The male Wistar rats (190 – 220 g) used in this study were bred in animal facility and housed in an air-conditioned room (approximately 22 ºC) with controlled lighting on a 12:12 h light/dark cycle (lights on from 06:00 to 18:00 h). The animals were maintained with pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil), while tap water was available *ad libitum*. Fasted rats were deprived of food for at least 16 h but allowed free access to water. All the animals were monitored and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation. This study was approved by the Committee for Ethics in Animal Research of UFSC (Protocol CEUA PP00398).

2.6. Oral glucose tolerance curve (OGTC)

Fasted rats were divided into different groups of six animals for each treatment. Group I, hyperglycemic rats that received glucose (4 g/kg; 8.9 M); Group II, rats that received tolbutamide at a dose of 100 mg/ kg; Group III, rats that received the CE at doses of 50, 100 and 200 mg/ kg; Group IV, rats that received BF at doses of 50 and 100 mg/ kg; Group V, rats that received ARF at doses of 50 and 100 mg/ kg. The glycemia was measured before the rats received the treatment (zero time). The rats were treated with extract or fraction and loaded with glucose after 30 min and after the glycemia was measured at 15, 30 60 and 180 min. All treatments were administrated by oral gavage.

2.7. Determination of the serum glucose concentration

Blood samples were collected, centrifuged and the serum was used to determine the glycemia by the glucose oxidase method.

2.8. Disaccharidase extraction and assays

A segment of the small intestine was removed, washed in 0.9% NaCl solution, dried on filter paper, weighed, trimmed and homogenized (300 rpm) with 0.9% NaCl (400 mg of duodenum per mL) for 1 min at 4 ºC. The resulting extract was centrifuged at 8,000*g* for 8 min. The supernatant was used for the measurement of *in vitro* maltase, sucrase and lactase activities and for total protein determination. Maltase (EC 3.2.1.20), lactase (EC 3.2.1.23) and sucrase (EC 3.2.1.48) activities were determined using a glucose diagnosis kit based on the glucose oxidase reagent. For determination of disaccharidase activity 50 µL of homogenate were pre-incubated at 37 °C for 5 min, in the absence (control) or in the presence of the CE, BF or ARF of *M.* x *paradisiaca* (treated groups). The concentrations used were 500, 1000 and 1500 µg/ mL. The duodenum homogenates were then incubated at 37 °C for 5 min with 25 µL of the substrate corresponding to 0.056 µM of maltose, sucrose or lactose [10]. One enzyme unit (U) was defined as the amount of enzyme that catalyzed the release of 1 µM of glucose per min under the assay conditions. The specific activity was defined as enzyme activity (U) per mg of protein. Protein concentration was determined by the method described by Lowry method using bovine serum albumin (BSA) as the standard [11]. The assays were performed in duplicate and conducted along with appropriate controls.

2.9. Formation of advanced glycation end-products in the bovine serum albumin/glucose and fructose systems

Advanced glycation end-products (AGEs) were formed in the in vitro system using a previously described method [12]. In brief, BSA (10 mg/ mL) in phosphate buffered-saline (PBS, pH 7.4) containing 0.02% sodium azide was incubated with glucose (500 mM) or fructose (100 mM) at 37°C for 14 and 28 days in the absence (control) and presence of the CE, BF or ARF of *M.* x *paradisiaca* (2.5 and 5.0 µg/ mL). The protein, glucose or fructose, and the prospective inhibitor were simultaneously introduced into the incubation mixture. Each solution was kept in the dark in a capped vial, and incubation was allowed to proceed in triplicate vials. To the time-course experiments on AGE formation it was measured the characteristic fluorescence (excitation wavelength of 370 nm and emission wavelength of 440 nm) with Infiniti M200 (TECAN).

2.10. Glycogen content measurements

The soleus muscle and liver were harvested from untreated hyperglycemic rats, treated with CE (200 mg/ kg), BF (50 mg/ kg) and ARF (100 mg/ kg) and used for the assay of glycogen content immediately after 3 h of treatment. Glycogen was isolated from tissues as described by Krisman [13]. The tissues were weighed, homogenized in 33% KOH and boiled at 100 °C for 20 min, with occasional stirring. After cooling, 96% ethanol was added to the samples which were then heated to boiling followed by cooling in an ice bath to aid the precipitation of glycogen. The homogenates were centrifuged at 1,300*g* for 15 min, the supernatant was discarded and the pellets were neutralized with saturated NH_4Cl before being maintained at 100 °C for 5 min, washed and resolubilized in water. Glycogen content was determined by treatment with iodine reagent and the absorbance was measured at 460 nm. The results are expressed as mg of glycogen/g of tissue.

2.11. Insulin serum measurements

The insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. The range of values detected by this assay was 0.2 ng/ mL to 10 ng/ mL. The intra-

and inter-assay coefficients of variation (CV) for insulin were 3.22 and 6.95, respectively, with a sensitivity of 0.2 ng/ mL. All insulin levels were estimated by means of colorimetric measurements at 450 nm with an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) by interpolation from a standard curve. Samples were analyzed in duplicate and results were expressed as ng of insulin serum mL^{-1}.

2.12. Data and statistical analysis

Data were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by the Bonferroni *post-hoc* test or unpaired Student's *t*-test to identify significant differences between groups. Differences were considered to be significant at $p \leq 0.05$.

3. Results

3.1. Phytochemical characterization

The chemical composition of CE and BF and ARF fractions of *Musa x paradisiaca* were determined by TLC and by RP-HPLC. The TLC analyses showed a predominance of flavonoids and the major spot detected in all analyses showed Rf and color similar to rutin (data not shown). Figure 1 shows the HPLC profiles for CE, BF and ARF of *M.* x *paradisiaca*. These analyzes identified the presence of rutin (rt=30.5 min) on crude extracts and fractions of *M.* x *paradisiaca* leaves as the major compound.

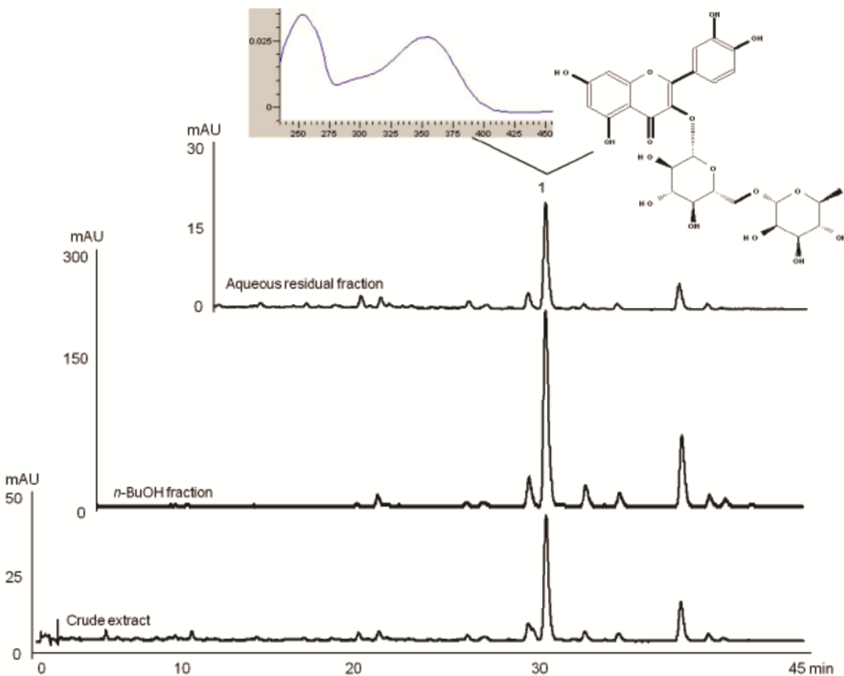

Figure 1 HPLC chromatograms of crude extract, aqueous residual fraction and *n*-butanol fraction of leaves of *M.* x *paradisiaca* with photo-diode array (PDA) detection at 340 nm. (1) = Rutin (rt = 30.6 min).

3.2. Effect of crude extract and n-BuOH and aqueous residual fractions of M. x paradisiaca on the oral glucose tolerance curve

As expected, after starting the glucose tolerance test the serum glucose concentration was significantly increased when compared with zero time. Tolbutamide (100 mg/kg) an oral hypoglycemic agent of sulfonylurea class was used as a positive control and produced a typical serum glucose lowering at 15, 30 and 60 min compared to the hyperglycemic group (Table 1). The CE at all doses tested (50, 100 and 200 mg/kg) was effective in reducing the glycemia at different times after oral treatment when compared with the respective hyperglycemic control group (Table 1). The CE (200 mg/kg) produced the best antihyperglycemic profile from 15 to 60 min and the maximum

reduction observed was 19% at 30 min, compared to tolbutamide. Table 1 also shows the effect of the BF at 50 and 100 mg/kg on oral glucose tolerance curve (OGTC). Although both doses showed an antihyperglycemic effect, the 50 mg/kg dose was more effective in terms of serum glucose lowering than 100 mg/kg, since it reduced significantly the glycemia at 15, 30 and 60 min. In addition, oral administration of ARF of also reduced the serum glucose levels in hyperglycemic rats at the two doses tested (50 and 100 mg/kg). The antihyperglycemic effect observed was around 22% and 25% at 30 and 60 min, respectively, after treatment with 100 mg/kg. At 180 min, glycemic levels were similar to the respective data for the hyperglycemic control groups.

On the other hand, acute treatments with CE, BF and ARF tested were ineffective to change serum glucose levels in alloxan-induced diabetic rats (data not shown).

Table 1. Acute effect of crude extract (CE), *n*-butanol fraction (BF) and aqueous residual fraction (ARF) of *M.* x *paradisiaca* on serum glucose levels (mg/dL) in oral glucose tolerance curve.[a]

Time (min)	Group I Hyper Glucose (4 g/kg)	Group II Hyper+ tolbutamide (100 mg/kg)	Group III Hyper+ CE			Group IV Hyper + BF		Group V Hyper + ARF	
			50 mg/kg	100 mg/kg	200 mg/kg	50 mg/kg	100 mg/kg	50 mg/kg	100 mg/kg
0	112 ± 4	111 ± 3	120 ± 3	110 ± 2	110 ± 3	121 ± 4	123 ± 3	103 ± 2	110 ± 5
15	162 ± 8	134 ± 9**	137 ± 8*	145 ± 8	138 ± 3*	128 ± 6**	157 ± 5	148 ± 6	147 ± 4
30	185 ± 6	140 ± 9***	151 ± 5**	147 ± 7**	150 ± 5***	152 ± 4**	155 ± 4**	149 ± 4**	144 ± 4***
60	164 ± 4	128 ± 7***	171 ± 8	139 ± 8*	136 ± 1*	141 ± 6*	143 ± 5	141 ± 5*	123 ± 5***
180	135 ± 4	123 ± 2	143 ± 5	137 ± 3	144 ± 5	142 ± 3	130 ± 3	134 ± 5	129 ± 2

[a] Values are expressed as mean ± S.E.M; n= 6 in duplicate for each treatment. Statistically significant difference compared to the corresponding hyperglycemic group. **p* ≤0.05; ***p* ≤0.01; ****p* ≤0.001.

3.3. Effect of crude extract and n-BuOH and aqueous residual fractions on in vitro albumin glycation

In the method adopted in this study, BSA was chosen as the model protein and glucose or fructose was used as the glycated agent. The BSA-reducing sugar system is an *in vitro* model widely used in non-enzymatic glycation studies. Proteins can be modified when exposed to reducing sugars through the spontaneous glycation process. The sugar-mediated fluorescence intensity, which is a characteristic of AGEs, increases during incubation at 37 ^{o}C for a long period. In Figs. 2 -A, B, C and D- the fluorescence intensity of the products (AGEs) formed in the BSA-glycation model can be observed. After incubation for both periods analyzed (14 and 28 days) it was clear that the formation of AGEs was significantly increased in the BSA/glucose (Figures 2 A and B) and BSA/fructose (Figures 2 C and D) systems when compared to the basal control group.

Figure 2A shows the *in vitro* ability of CE and BF to suppress the AGE formation in the BSA/glucose system after 14 days of incubation. All concentrations of CE, BF and ARF inhibited significantly the formation of AGEs in the BSA/fructose system after 14 days of incubation (Figure 2C).

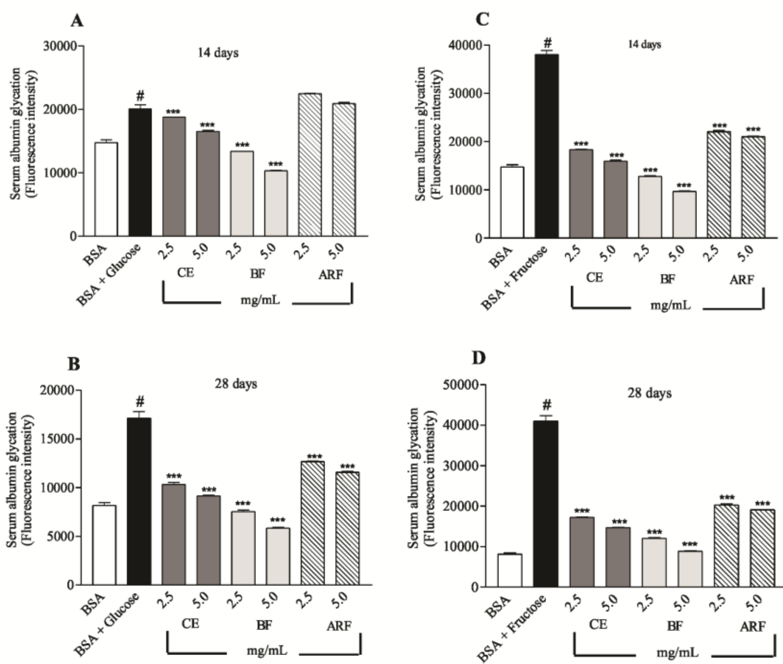

Figure 2 Inhibitory effect of crude extract (CE), *n*-butanol fraction (BF) and aqueous residual fraction (ARF) of leaves of *M.* x *paradisiaca* on the fluorescent AGEs formation in a BSA/glucose or BSA/fructose system. (A and B) 14 and 28-days BSA/glucose; (C and D) 14 and 28-days BSA/fructose. Values are expressed as mean ± S.E.M; n=6 in duplicate for each group. Significantly different to the corresponding control group (BSA/glucose or BSA/fructose); ***$p \leq 0.001$

3.4. Effect of crude extract and n-BuOH and aqueous residual fractions on the disaccharidases

We observed that CE, BF and ARF were able to reduce slightly maltase specific activity at the maximal dose tested (1500 μg/mL) after 5 min of *in vitro* incubation when compared with the basal group (Figure 3). The maximum effect was observed for BF which inhibited 20% of maltase activity, while this inhibitory effect was around 13% and 14% for the CE and ARF, respectively. On the other hand, none of

the treatments affected the sucrase and lactase activity at any concentration tested (data not shown).

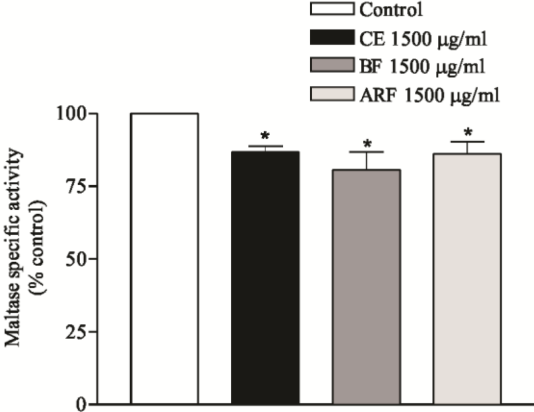

Figure 3 *In vitro* effect of crude extract (CE), *n*-butanol fraction (BF) and aqueous residual fraction (ARF) of leaves of *M.* x *paradisiaca* on specific activity of maltase, in the duodenal portion of rat intestine. Incubation = 5 min. Values are expressed as mean ± S.E.M.; n = 6 for each group. Significant at $*p \leq 0.05$ compared to control group.

3.5. Effect of crude extract and n-BuOH and aqueous residual fractions on the glycogen content in soleus muscle and liver

The glycogen content in the soleus muscle and liver of hyperglycemic normal rats was determined after acute treatment with CE (200 mg/kg), BF (50 mg/kg) and ARF (100 mg/kg). Figure 4A shows that only BF were able to significantly increase the glycogen content in soleus muscle (183%) when compared with hyperglycemic control rats at 3 h after treatment.

Additionally, the CE, BF and ARF showed a stimulatory effect on hepatic glycogen storage (Figure 4B). When compared with hyperglycemic rats CE and ARF groups significantly increased glycogen content in the liver around 123 and 136%, respectively. In this experimental condition, the maximum effect observed was around 142% for the BF.

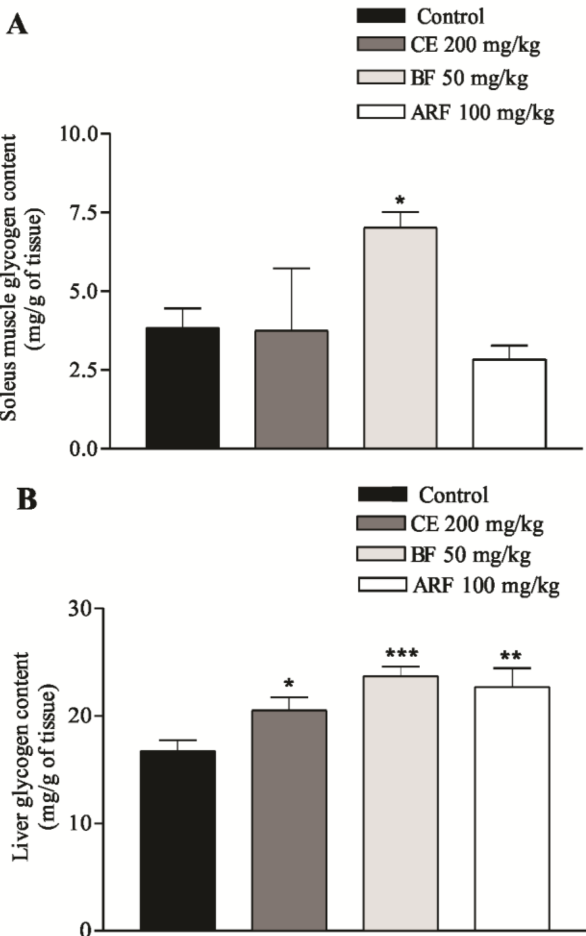

Figure 4 Effect of crude extract (CE), *n*-butanol fraction (BF) and aqueous residual fraction (ARF) and of leaves of *M.* x *paradisiaca* on the glycogen content in normal hyperglycemic rats. (A) soleus muscle and (B) liver 3h after treatment by oral gavage. Values are expressed as mean ± S.E.M; n=6 in duplicate for each group. Significantly different to the corresponding hyperglycemic group; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

3.6. Effect of crude extract and n-BuOH and aqueous residual fractions on insulin secretion

The insulin secretion after *in vivo* treatment with the extract and fractions of *M.* x *paradisiaca* was measured. Serum insulin levels were determined in fasted rats after an oral glucose loading (4 g/kg), as shown in Table 2. BF (50 mg/kg) stimulated significantly the insulin secretion at 15 min by around 154% when compared to the hyperglycemic control group (Table 2). So, the increase in glycogen content in the soleus muscle may be due to insulin release from β-cells stimulated by BF treatment. In addition, the high serum insulin secretion is according with the antihyperglycemic effect of the BF at a dose of 50 mg/kg seen on the glucose tolerance curve (Table 1).

Table 2. Acute effect of crude extract (CE), *n*-butanol fraction (BF), and aqueous residual fraction (ARF) of *M.* x *paradisiaca* on serum insulin levels (ng/mL).[a]

Serum insulin levels in hyperglycemic rats (ng/ml)				
Time (min)		*M.* x *paradisiaca*		
	Control Glucose (4 g/kg)	CE 200 mg/kg	BF 50 mg/kg	ARF 100 mg/kg
0	0.57 ±0.03	-	-	-
15	0.77 ±0.06[#]	0.90 ± 0.20	1.29 ± 0.08**	0.88 ±0.15
30	0.90 ± 0.10	0.90 ± 0.10	0.92 ± 0.10	0.66 ± 0.01
60	0.54 ±0.04	0.71 ±0.08	0.72 ± 0.15	0.73± 0.10

[a]Values are expressed as mean ± S.E.M; n= 4 in duplicate for each treatment. Statistically significant at $^{\#}p \leq 0.01$ in relation to euglycemic group. Statistically significant difference compared to the corresponding hyperglycemic group. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

4. Discussion

In the present study, we demonstrated the beneficial effects of *M.* x *paradisiaca* leaves on the regulation of glucose homeostasis. Antihyperglycemic potency of extract and fractions, *n*-butanol and aqueous residual, was demonstrated here by glucose levels decreased, glycogen and plasma insulin levels increased after *M.* x *paradisiac*a

administration in hyperglycemic rats. We have also observed that *M. x paradisiaca* inhibited maltase activity and the formation of AGEs *in vitro*. The present study highlights the ability of *Musa x paradisiaca* leaves to improve carbohydrate metabolism.

The phytochemical analysis demonstrated the presence of flavonoids in crude extract and fractions of *M.* x *paradisiaca* leaves and rutin as the major compound. The presence of a variety of phenolic compounds has been reported in many parts of *Musa* species and these phytochemicals have been associated with the observed biological activity of these plants. The presence of bioactive compounds like apigenin glycosides, myricetin glycoside, myricetin-3-*O*-rutinoside, naringenin glycosides, kaempferol-3-*O*-rutinoside, dopamine, N-acetyl serotonin, and rutin, has been reported in different species of *Musa* [14]. However, to the best of our knowledge, this is the first report of the presence of rutin in the leaves of *M.* x *paradisiaca*.

Several medicinal plants show potential hypoglycemic and/or anti-hyperglycemic activity, including *Musa*. Intra-gastric administration of a fresh flower decoction (4 mL/kg) to hyperglycemic rabbits significantly decreased the hyperglycemic peak and/or the area under the glucose tolerance curve [15]. Furthermore, it has been reported in the literature that the extracts from different parts of *Musa* species showed a hypoglycemic effect in diabetic animal models [6-8, 16, 17]. Pari and Maheswari observed that in a chronic daily treatment the oral administration of various doses of the chloroform extract of *M. sapientum* flowers reduced blood glucose levels in alloxanized rats [16]. Similarly, it has been shown that pectin isolated from the juice of the inflorescence of *M. sapientum* showed significant hypoglycemic effect in alloxan-induced diabetic rats [17]. More recently, Adewoye *et al.* demonstrated an anti-diabetic activity for aqueous and methanolic extracts of *M. sapientum* roots in alloxan-induced diabetic rats [8].

Also, a hypoglycemic effect of a methanolic extract of *M. x paradisiaca* fruit in streptozotocin-induced diabetic mice was demonstrated [6]. Additionally, a composite extract of seeds of *Eugenia jambolana* and roots of *M. x paradisiaca* showed antihyperglycemic and anti-hyperlipidemic effect in streptozotocin-induced diabetic albino rats [7]. However all these works did not investigated the pharmacological activity as well as did not identify the bioactive compound present in the extracts of the leaves of *M. x paradisiaca*. In the present study, we demonstrated the significant antihyperglycemic effect of extract and fractions of *M. x paradisiaca* leaves.

Chronic hyperglycemia and increased oxidative stress during diabetes results in the irreversible formation of AGEs, which are a heterogeneous group of molecules formed from the non-enzymatic glycation of reducing sugars with free amino groups of proteins, lipids, and nucleic acids. The Schiff's bases formed by glycation rearrange further, through stable reactions, to form Amadori products which later, by isomerization, condensation, and rearrangement reactions, form AGEs. The AGEs are known to have a wide range of chemical, cellular, and tissue effects implicated in the development and progression of diabetic complications, like nephropathy, neuropathy, retinopathy, and cardiovascular diseases [18].

Our results indicated that extract and fractions of *M. x paradisiaca* leaves exerted stronger inhibitory effect on AGEs formation. In line with these results, Bhaskar *et al.* also showed that the flower and pseudostem of *Musa* sp. var. elakki bale inhibited the formation of AGEs in rats with streptozotocin-induced diabetes, showing the anti-AGE properties of *Musa* sp [19].

Also, the presence of flavonoids such as rutin may be related to inhibit the formation of AGEs [20], as we have demonstrated for the extract and fractions of *M.* x *paradisiaca*, particularly for the BF which presented the highest content of rutin and showed the best anti-AGEs effect. In agreement with these results, anti-AGEs effect was also previously reported for other flavonoids [21].

Some tissues, like those of the intestine, play an important role in glucose homeostasis. Thus, one therapeutic approach to decrease postprandial hyperglycemia is to retard the absorption of glucose via inhibition of carbohydrate-hydrolyzing enzymes, such as α-glucosidase, in the intestine. These disaccharidase enzymes are located in the brush border of the small intestine and are required for the breakdown of carbohydrates before monosaccharide absorption. The α-glucosidase inhibitors delay the absorption of ingested carbohydrates, reducing the postprandial glycemia and insulin peaks [22].

In the present study, we demonstrated that extract and fractions of *M. x paradisiaca* leaves reduced the maltase activity. A number of plants are known to exert antihyperglycemic activity through the inhibition of carbohydrate-hydrolyzing enzymes in the small intestine. The polyphenols in plants play an important role in the mechanism for regulating these disaccharidases. Andrade-Cetto *et al.* reported that flavonoid-enriched extracts from some Mexican plants efficiently

inhibited α-glucosidase activity and significantly reduced serum-glucose levels in diabetic rats [23].

In mammals, carbohydrate is stored mainly in the form of glycogen, with skeletal muscle and liver being the major storage sites. Glycogen metabolism is regulated by insulin/glucagon through activation and/or inhibition of several enzymes and proteins [24]. The present results demonstrated that BF of *M. x paradisiaca* leaves significantly increased the glycogen content in soleus muscle and liver. The high amount of rutin into BF can be involved on increased muscle glycogen content after acute treatment, since Fernandes *et al.* reported that treatment with rutin was associated with a marked elevation on glycogen in rats in streptozotocin-induced diabetes [25]. It was observed that the hepatic glycogen content and cardiac tissue glycogen content increased significantly in treated diabetic rats when compared to untreated diabetic rats.

Additionally, CE and ARF also showed a stimulatory effect on hepatic glycogen storage. In line with these results, it has been reported that rats fed with dietary fiber from *Musa*, showed significant lower levels of fasting blood glucose and higher concentrations of liver glycogen. Also, the activities of some enzymes of glycolysis were inhibited [26].

Glucose metabolism is regulated by several hormones but only insulin possesses hypoglycemic properties. Insulin is secreted into the blood stream by β-cells of endocrine pancreas and glucose is the main insulin secretagogue. An absolute or relative lack of insulin, as in the case of diabetes type I, leads to severe dysfunction in the major insulin target organs such as muscle, liver and adipose tissue [27].

The stimulation of β-cells, subsequent release of insulin and activation of the insulin receptors is a possible mechanism of natural products with potential antidiabetic activity. In the present study, it was demonstrated that BF potentiated significantly the glucose-induced insulin secretion. In this line, Folador *et al.* showed that the crude extract, *n*-butanol fraction and two isolated *C*-glycosylflavones, isovitexin and swertisin, of *Wilbrandia ebracteata* produced antihyperglycemic action, related to *in vivo* insulin secretion [28].

Additionally, flavonoids are reportedly insulin secretagogues. Genistein and daidzein have been found to increase insulin secretion stimulated by glucose *in vivo* and *in vitro* [29]. Also, rutin has been reported to enhance insulin release and decrease blood glucose levels [30]. Thus, rutin, a potential insulin-secretagogue agent, which is found

in quite high amounts in the BF, is thought to be related to the antihyperglycemic activity observed in the present study for *M.* x *paradisiaca*.

In conclusion, we showed that the crude extract, *n*-butanol and aqueous residual fractions of *M.* x *paradisiaca* leaves exhibit potential antihyperglycemic action. The reduction on serum glucose levels, stimulation of insulin secretion, stimulation of glycogen storage, and inhibition of enzyme activity related to glucose absorption and AGE formation corroborates the beneficial effects on the regulation of glucose homeostasis observed for *M.* x *paradisiac*a leaves. In addition, *M.* x *paradisiac*a leaves that in general there is no commercial interest can provide an excellent source of rutin, which is a candidate for the development of anti-diabetic drugs in the near future.

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4.2.2 Conclusões parciais

A partir dos resultados obtidos neste trabalho, podemos concluir que:

O extrato bruto e as frações *n*-butanol e residual aquosa do extrato das folhas de *M. x paradisiaca* reduziram a glicemia de ratos normais hiperglicêmicos após período agudo de tratamento.

A fração *n*-butanol do extrato das folhas de *M. x paradisiaca* estimulou a secreção de insulina estimulada por glicose após tratamento agudo por via oral.

A fração *n*-butanol do extrato das folhas de *M. x paradisiaca* aumentou significativamente o conteúdo de glicogênio em músculo sóleo de ratos normais hiperglicêmicos após o tratamento.

O extrato bruto e frações *n*-butanol e residual aquosa das folhas de *M. x paradisiaca* aumentaram significativamente o conteúdo de glicogênio em fígado de ratos normais hiperglicêmicos após o tratamento.

O extrato bruto e as frações *n*-butanol e residual aquosa do extrato das folhas de *M. x paradisiaca* inibiram a atividade da enzima maltase.

O extrato bruto e as frações *n*-butanol e residual aquosa do extrato das folhas de *M. x paradisiaca* inibiram o processo de glicação, prevenindo a formação dos AGEs.

O flavonóide rutina foi identificado como o composto majoritário do extrato e das frações *n*-butanol e residual aquosa do extrato das folhas de *M. x paradisiaca*.

4.3 Rutina: efeitos na homeostasia da glicose

Estudos comprovam o efeito hipoglicemiante de flavonóides e descrevem alguns dos possíveis mecanismos de ação envolvidos. Entre os mecanismos propostos podem ser citados: o estímulo da captação de glicose pelos tecidos periféricos, a regulação da atividade e/ou da expressão das enzimas limitantes das reações do metabolismo dos carboidratos, e o efeito como secretagogo ou protetor da função das células β pancreáticas. Ainda, podem atuar como miméticos de insulina, influenciando o mecanismo pleiotrópico da sinalização de insulina (CAZAROLLI et al. 2008b).

A rutina é um flavonóide glicosilado comumente encontrado nas plantas e está presente em grandes quantidades na dieta (HUSSAIN et al., 2009). Diversas atividades farmacológicas são descritas para a rutina (ANNAPURNA et al., 2009; FERNANDES et al., 2010; GUPTA et al., 2003; JANBAZ et al. 2002; LA CASA et al., 2000, SHEU et al., 2004). Recentemente, a rutina foi foco de investigação em estudo de nosso grupo do Laboratório de Hormônios & Transdução de Sinais, que demonstrou um efeito anti-hiperglicêmico no TOTG em ratos e, ainda, os autores verificaram que este flavonóide inibiu significativamente a atividade da maltase *in vitro* (PEREIRA et al., 2011).

Como previamente descrito, a rutina foi identificada como composto majoritário no extrato e frações das folhas *M. x paradisiaca*, um dos objetos de estudo desta tese, o que nos permitiu sugerir que a presença da rutina possa contribuir com os efeitos benéficos das folhas da banana na homeostasia da glicose. Considerando que há evidências que a rutina pode regular a homeostasia da glicose, que os mecanismos exatos desse efeito são escassos na literatura e buscando dar continuidade aos estudos prévios do grupo, o objetivo deste trabalho foi estudar o efeito da rutina na manutenção da homeostasia da glicose através da elucidação de possíveis mecanismos de ação em tecidos que contribuem com a manutenção da homeostasia da glicose, como o músculo sóleo e o pâncreas.

4.3.1 Artigo submetido para publicação

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Involvement of GLUT-4 in the Stimulatory Effect of Rutin on Glucose Uptake in Soleus Muscle

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Abstract

Objectives The aim of the study reported herein was to investigate the in vitro effect of rutin on glucose uptake in an insulin target (soleus muscle) and the mechanism of action involved.
Methods Isolated soleus muscles from rats were treated with rutin (500 microM) with/without the following inhibitors; HNMPA(AM)$_3$, an insulin receptor tyrosine kinase activity inhibitor, wortmannin, an inhibitor of PI3K, RO318220, an inhibitor of PKC, colchicine, a microtubule-depolymerizing agent, PD98059, an inhibitor of MEK, and cycloheximide, an inhibitor of protein synthesis on fresh Krebs Ringer-bicarbonate plus [U-^{14}C]-2-deoxi-D-glucose (0.1 microCi/mL). Aliquots of tissue medium were used for the radioactivity measurements.
Key findings Rutin increased the glucose uptake in rat soleus muscle. In addition, the effect of rutin on glucose uptake was completely inhibited by pretreatment with HNMPA(AM)$_3$, wortmannin, RO318220, colchicine, PD98059, and cycloheximide. These results suggest that rutin stimulates glucose uptake in the rat soleus muscle via the PI3K, aPKC and MAPK pathways. Also, rutin may influence glucose transporter translocation and directly activate the synthesis of the transporter GLUT-4
Conclusion The similarities of rutin action on glucose uptake compared with the signaling pathways of insulin constitute strong evidence for the insulin-mimetic role of rutin in glucose homeostasis.

Keywords: Rutin; Flavonoid; Insulin; Glucose uptake; GLUT-4; diabetes.

Introduction

Type 2 diabetes mellitus (T2DM), the most common metabolic disorder worldwide, is characterized by a high blood glucose concentration (hyperglycemia) and the main metabolic feature of T2DM is insulin resistance.[1] As a consequence, type 2 diabetic patients are characterized by a marked decrease in insulin-stimulated glucose utilization in muscle mainly due to reduced glucose uptake and storage. Skeletal muscle composes a large percentage of total body mass and is the major site for insulin-dependent glucose disposal, making it critically important in glucose homeostasis.[2]

Glucose transport into skeletal muscle is primarily mediated by a membrane-associated glucose transport protein known as GLUT-4. Under normal resting conditions, most of the GLUT-4 molecules reside in membrane vesicles inside the muscle cell. In response to insulin or muscle contraction, GLUT-4 is translocated to the cell membrane where it increases glucose transport.[3]

Insulin stimulates glucose uptake by increasing the translocation of GLUT-4-containing vesicles to the plasma membrane and by modifying the activity of enzymes involved in glucose metabolism. Insulin action is initiated by binding of the hormone to cell membranes and activation of the insulin receptor tyrosine kinase, which results in the stimulation of multiple intracellular signaling pathways. [4] Among these cascades, the phosphatidylinositol 3-kinase (PI3K) pathway is thought to play a crucial role in the insulin intracellular transduction glucose homeostasis. PI3K catalyzes the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), an allosteric activator of phosphoinositide-dependent kinase (PDK). Targets of PDK include protein kinase B (PKB) and the atypical protein kinase C (aPKC) isoforms, which, when activated via phosphorylation, stimulate the translocation of GLUT-4-containing vesicles to the plasma membrane Also, insulin mediates a wide spectrum of biological responses including activation of the transcription of specific genes, and modulation of cellular growth and differentiation.[4]

Flavonoids are a class of secondary metabolites widely distributed in plants and are the largest group of natural products known. They are important components in the human diet and are found in fruits, vegetables, seeds, nuts, grains, spices and beverages (wine, tea and beer). There is considerable evidence indicating that adequate fruit and vegetable consumption has a role in maintaining health and preventing disease. Several investigations have demonstrated a broad range of biological activities for these compounds and numerous studies have

been carried out on their potential role in the prevention and treatment of diabetes.[5,6] Therefore, the investigation of such agents obtained from medicinal plants has recently been gaining importance.

Previously, many studies have demonstrated the hypoglycemic effects of flavonoids as well as their action in glucose uptake and glycogen metabolism.[6] Recently, our group reported that kaempferitrin and kaempferol 3-neohesperidoside were able to reduce serum glucose levels and stimulate glucose uptake in rat soleus muscle.[7,8]

Rutin (quercetin-3-*O*-rutinoside) is a flavonol glycoside composed of quercetin and the disaccharide rutinose. Rutin is present in large amounts in the diet and is present in many plants, for example, onion, black tea, apple and red wine. Many studies have demonstrated that rutin is a pharmacologically active phytochemical which exhibits multiple biological activities.[9,10] Fernandes et al. have shown that rutin can improve the metabolic status of rats with experimentally-induced diabetes.[11] Although the ability of rutin to ameliorate diabetic status has been reported, studies to determine the exact mechanism of action involved in the regulation of glucose homeostasis are scarce. Furthermore, our group has also demonstrated that rutin improves the glucose tolerance curve and inhibits α-glucosidase activity.[12] Thus, the aim of the study reported herein was to investigate the *in vitro* effect of rutin on glucose uptake in an insulin target (soleus muscle) and the mechanism of action involved.

Materials and methods

Chemicals

Rutin, bovine serum albumin (BSA), hydroxy-2-naphthalenylmethylphosphonic acid trisacetoxymethyl ester (HNMPA$(AM)_3$), bisindoylmaleimidine IX, 2-{1-[3-(amidinothio) propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl) maleimide methanesulfonate salt (RO 31–8220), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 98059), colchicine, wortmannin, cycloheximide, acrylamide and bis-acrylamide were purchased from Sigma Chemical Company® (St. Louis, MO, USA). D - [^{14}C (U)] – glucose (^{14}C-G), specific activity 9.25 GBq/mmol and biodegradable liquid scintillation fluid were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA). In relation to the antibodies, GLUT-4 (sc-53566) was obtained from Santa Cruz Biotechnology (California, USA) and peroxidase conjugated goat anti-mouse IgG from Millipore (Temecula, California, USA). The Immobilon™ Western

chemiluminescent horseradish peroxidase (HRP) substrate was obtained from Millipore. All other chemicals were of analytical grade.

Animals

The male Wistar rats (190 – 220 g) used in this study were bred in our animal facility and housed in an air-conditioned room (approximately 22 ºC) with controlled lighting on a 12:12 h light/dark cycle (lights on from 06:00 to 18:00 h). The animals were maintained with pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil), while tap water was available ad libitum. Fasted rats were deprived of food for at least 16 h but allowed free access to water. All the animals were monitored and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation. This study was approved by the Committee for Ethics in Animal Research of UFSC (approved in 03/06/2011; Protocol CEUA PP00398).

Studies on ^{14}C-glucose uptake in rat soleus muscle

For the [U-^{14}C]-2-deoxy-D-glucose (^{14}C-DG) uptake experiments, the soleus muscles of euglycemic fasted rats were used. Slices of soleus muscle were distributed (alternately left and right) between basal and treated groups. The muscles were dissected, weighed, and preincubated and incubated at 37 °C in Krebs Ringer-bicarbonate (KRb) buffer with a composition of 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, and 25 mM $NaHCO_3$ and bubbled with O_2/CO_2 (95%:5%, v/v) until pH 7.4. Rutin (10 nM, 10 and 500 µM) and insulin (10 nM) were added to the preincubation (30 min) and incubation (60 min) medium in the presence or absence of 100 µM HNMPA(AM)$_3$, 100 nM wortmannin, 40 μM RO318220, 50 μM PD98059, 0.35 mM cycloheximide or 1 µM colchicine. ^{14}C-DG (0.1 μCi/mL) was added to each sample during the incubation period. After incubation, the muscle samples were homogenized in 0.5 N NaOH, and boiled at 100 ^{o}C for 10 min, with occasional stirring. Aliquots (25 µL) of tissue homogenate and external medium were placed in scintillation liquid on an LKB rack beta liquid scintillation spectrometer (model 1215; EG and G-Wallac, Turku, Finland), for the radioactivity measurements. The results were expressed as the tissue/medium (T/M) ratio: cpm/mL tissue fluid per cpm/mL incubation medium.[7]

Total tissue homogenate
Rat soleus muscles were preincubated (30 min) and incubated (60 min) at 37 °C in KRb with/without rutin (500 μM). The muscle samples were then rapidly homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, 4% (w/v) and the protein concentration was determined.

Total protein determination
The protein concentration was determined by the Lowry method[13] using serum bovine albumin as the standard.

Polyacrylamide gel electrophoresis (SDS-PAGE)
Total tissue homogenates were prepared as described above. For the electrophoresis analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris-HCl, pH 6.8 and boiled for 3 min. Equal protein concentrations were loaded onto 10 % polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli[14] and transferred to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS).

Western blot analysis
The nitrocellulose membranes were incubated for 2 h in blocking solution (TBS; 0.5 M NaCl, 20 mM Trizma, plus 5% defatted dried milk) and then incubated overnight at 4 °C with anti-GLUT-4 diluted to a ratio of 1:500. Membranes were incubated for 120 min with anti-mouse IgG (1:1000) and immunoreactive bands were visualized using the Immobilon™ Western chemiluminescence HRP substrate kit.[15] Autoradiograms were quantified by scanning the films with a Hewlett–Packard Scanjet 6100C scanner and determining optical densities with an OptiQuant version 02.00 software (Packard Instrument Company).

Data and statistical analysis
Data were expressed as mean ± S.E.M. When multiple comparisons were performed, evaluation was carried out using one-way ANOVA followed by the Bonferroni multiple comparison test. Differences were considered to be significant when $p < 0.05$.

Results

Effect of rutin on ^{14}C-glucose uptake in rat soleus muscle

Figure 1 shows the in vitro effect of rutin on glucose uptake in soleus muscle after 60 min of incubation. The stimulatory effect of rutin on ^{14}C-DG uptake was significant at 500 µM compared to the basal group. This stimulatory effect represents 27% of the glucose uptake compared to the basal level at 60 min. Also, rutin presented similar action when compared to stimulatory action of insulin on glucose uptake.

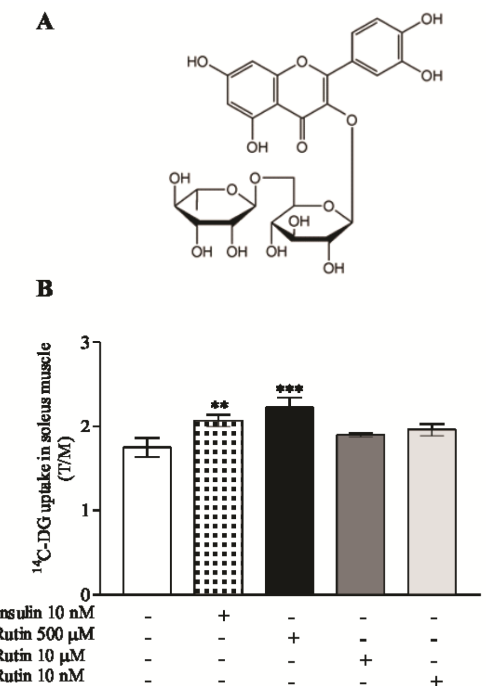

Fig. 1. Rutin structure (A) and concentration–response curve showing the effect of rutin on ^{14}C-glucose uptake in rat soleus muscle (B). Basal group = no treatment. Signal (+) and (-) indicates presence and absence, respectively, of each substance in the incubation medium. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; n = 6 in duplicate for each group. Significant at $***p \leq 0.001$ and $**p \leq 0.01$ in relation to basal group.

Studies on the mechanism of action of rutin on ^{14}C-glucose uptake in rat soleus muscle

In order to study the mechanism of action of the effect of rutin on glucose uptake in soleus muscle, we performed the glucose uptake assay with 100 nM wortmannin, an inhibitor of PI3K[16], 40 μM RO318220, a specific inhibitor of PKC isoforms[8], 50 μM PD98059, an inhibitor of MEK[8], 0.35 mM cycloheximide, an inhibitor of protein synthesis[8], 100 μM HNMPA(AM)$_3$, an insulin receptor tyrosine kinase activity inhibitor[17,18], or 1 μM colchicine, a microtubule-depolymerizing agent[16]. Figure 2 shows that the stimulation of glucose uptake by rutin was completely inhibited by HNMPA(AM)$_3$ pretreatment. Additionally, the stimulatory effect of rutin was completely inhibited by the wortmannin, RO318220 and colchicine pretreatments (Figure 3). However, when only these inhibitors were added to the muscle samples, no significant change resulted compared with the glucose uptake in the basal group.

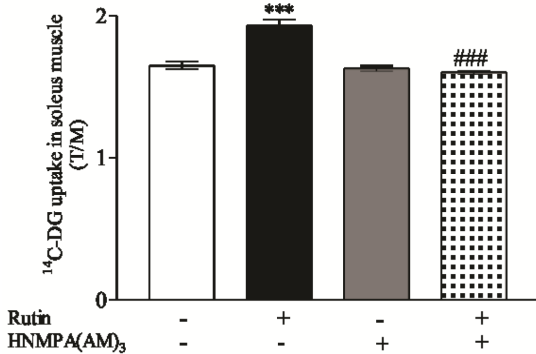

Fig. 2. Effect of 100 μM HNMPA(AM)$_3$ on the stimulatory action of 500 μM rutin on ^{14}C-glucose uptake in rat soleus muscle. Basal group = no treatment. Signal (+) and (-) indicates presence and absence, respectively, of each substance in the incubation medium. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; n = 6 in duplicate for each group. Significant at $^{***}p \leq 0.001$ in relation to basal group. Significant to $^{\#\#\#}p \leq 0.001$ in relation to rutin group.

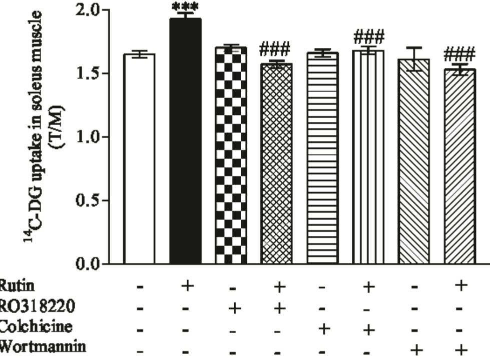

Fig. 3. Effect of enzyme inhibitors, 100 nM Wortmannin, 40 μM RO318220, 1 µM colchicine on the stimulatory action of 500 µM rutin on ^{14}C-glucose uptake in rat soleus muscle. Basal group = no treatment. Signal (+) and (-) indicates presence and absence, respectively, of each substance in the incubation medium. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; $n = 6$ in duplicate for each group. Significant to $^{***}p \leq 0.001$ in relation to basal group. Significant at $^{###}p \leq 0.001$ in relation to rutin group.

To address the possibility that MEK and/or protein synthesis inhibitors influence the glucose uptake activity of rutin, we examined the effect of PD-98059 or cycloheximide on rutin-stimulated glucose uptake (Figure 4). Both inhibitors completely blocked the increase in glucose uptake compared with the control group.

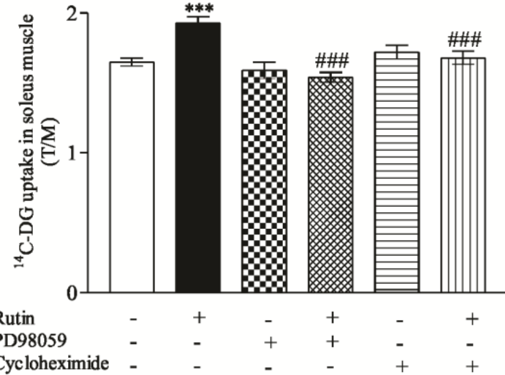

Fig. 4. Effect of 50 μM PD98059 and 0.35 mM cycloheximide on the stimulatory action of 500 µM rutin on ^{14}C-glucose uptake in rat soleus muscle. Basal group = no treatment. Signal (+) and (-) indicates presence and absence, respectively, of each substance in the incubation medium. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; n = 6 in duplicate for each group. Significant to $^{***}p \leq 0.001$ in relation to basal group. Significant at $^{\#\#\#}p \leq 0.001$ in relation to rutin (**1**) group.

Effect of rutin on GLUT-4 immunocontent in skeletal muscle

As shown in Figure 5 rutin significantly increased the content of muscle GLUT-4 compared with the basal group, the results being similarly to those obtained for the insulin group.

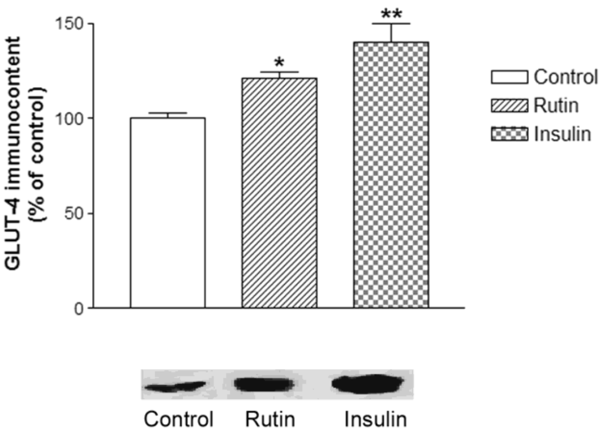

Fig. 5. Effect of rutin on total GLUT-4 immunocontent in rat soleus muscle. The total GLUT-4 levels were measured, as described in Section 2. Values are expressed as mean ± S.E.M.; for three independent experiments carried out in quadruplicate. Significant to $^{**}p \leq 0.01$ and $^{*}p \leq 0.05$ compared with control group. Representative immunoblots are shown.

Discussion

Many biological activities are described for the flavonoid rutin.[9,10] In this study we demonstrated that rutin increases the glucose uptake in rat

soleus muscle and the intracellular pathways of this stimulatory effect of rutin are similar to mechanism of action of insulin on glucose uptake. There are many reports on the effects of flavonoids on glucose uptake in skeletal muscle.[7,8,16] As far as we know, this is the first time that it is shown the mechanism of action of rutin on glucose uptake in skeletal muscle.

The stimulatory effect of rutin on glucose uptake observed herein are in line with those previously published concerning the hypoglycemic effect of rutin in hyperglycemic normal rats and on glucose uptake in a human choriocarcinoma cell line.[12,19] Other flavonoids, both *C* and *O*-glycosides, have been shown to stimulate glucose uptake. Kaempferitrin and kaempferol 3-neohesperidoside increased glucose utilization by stimulation of glucose uptake in soleus muscle.[7,8] Additionally, naringenin and apigenin-6-C-(2"-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside have shown a stimulatory dose-dependent action on glucose uptake in muscle.[16,20] In this approach, our results demonstrate a potential insulin-mimetic effect of rutin on glucose uptake in one of the insulin target tissues, soleus muscle.

The first step by which insulin increases energy storage or utilization involves the regulated transport of glucose into the cell through the activation of the insulin-signaling pathway. In skeletal muscle, the insulin signal transduction is mediated by a series of phosphorylation cascades linking the initial activation of the insulin receptor (IR) tyrosine kinase activity to downstream substrates.[21,22] The insulin receptor is composed of two extra-cellular α-subunits and two transmembrane β-subunits. Insulin binding to the α-subunits induces a conformational change that consequently activates the intrinsic tyrosine kinase of the β-subunits through the autophosphorylation of tyrosine residues in several regions of the intracellular β-subunit resulting in increased catalytic activity and tyrosine phosphorylation of intracellular substrates such as the IRS-proteins.[21,22] It has been demonstrated that the presence of HNMPA$(AM)_3$ totally blocked the stimulatory effect of insulin on glucose uptake and on glucose oxidation in CHO cells.[17] Also, studies have shown that the inhibition of fatty acid release in adipocytes by insulin as well as by zinc (II) complexes and vanadium complexes is reversed by the addition of HNMPA-$(AM)_3$ in a concentration-dependent manner.[23,24] Since the rutin effect completely disappeared in the presence of HNMPA$(AM)_3$, it seems that rutin influences the insulin receptor tyrosine kinase activity culminating in rutin stimulating glucose uptake.

It has been demonstrated that the increased glucose uptake in muscle in response to insulin is driven through signaling via the insulin receptor and PI3K.[22,25] Also, downstream PI3K signals activate several proteins, particularly PKB, AS160 and atypical PKCs, which have been demonstrated to elicit the translocation of GLUT-4 from an intracellular pool to the plasma membrane.[22,25-27] Additionally, the cellular cytoskeleton structure is engaged in the efficient transport of vesicles through intracellular membrane-sorting pathways. The presence of intact microtubules and actin filaments is particularly important for insulin-stimulated GLUT-4 translocation since the disruption of microtubules caused by colchicine and other microtubule-depolymerizing agents inhibits insulin-induced GLUT-4 translocation and glucose uptake .[22,28,29] The stimulatory effect of rutin on glucose uptake is mediated, at least in part, through the PI3K and PKC pathways together with the involvement of the intact microtubules and actin filaments of the cytoskeleton.

Additionally, rutin can promote an anti-apoptotic effect and improve myocardial contractile functions through the activation of the PI3K pathway, suppressing apoptosis and promoting cell survival.[30] Thus, the results reported herein are in agreement with those from the literature concerning the effect of other flavonoids. Another example is that epigallocatechin gallate and kaempferitrin reportedly stimulated glucose uptake in muscle and adipocytes by PI3K and PKB activation, the classical insulin transduction pathway.[31,32] Similarly, the stimulatory effect of kaempferol 3-neohesperidoside seems to be mediated through insulin signal transduction involving the PI3K and PKC pathways.[8] Additionally, the presence of PI3K, PKC inhibitors and the microtubule-depolymerizing agent totally inhibited the effect of apigenin -6-C-(2”-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside on muscle glucose uptake.[16]

Besides the effect of insulin on the metabolism of glucose and lipids, the binding of this hormone to its receptor triggers multiple signaling pathways that participate broadly in cellular growth and differentiation, such as that followed by MAPK pathways.[33] It is worth noting that there is cross-talk between the PI3K and MAPK pathways, raising the possibility that MAPK does indeed contribute to insulin-stimulated glucose uptake.[34,35] Inhibition of MEK with PD98059 or U0126 markedly reduced insulin-stimulated glucose uptake without affecting either the PI3K pathway or GLUT-4 translocation, suggesting that MEK plays a role in insulin-stimulated glucose uptake, possibly through the activation of GLUT-4.[35,36]

The stimulatory effect of rutin was inhibited by PD-98059 and cycloheximide. In accordance with our results, it was reported that apigenin-6-C-(2”-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside extracts of *Aegles marmelos* and *Syzygium cumini*, plants rich in flavonoids, stimulated glucose uptake and this effect was totally inhibited in the presence of cycloheximide, suggesting that active protein synthesis is important in terms of maintaining glucose transport at suitable levels.[37] Thus, these data indicate that active protein synthesis is necessary for rutin-stimulated glucose transport and, coupled with the involvement of PI3K and PKC pathways, reinforces the insulin-mimetic effect of this flavonoid in an insulin-sensitive tissue, soleus muscle.

Rutin may act through the MAPK pathway, stimulating the expression of glucose transporters or proteins from the insulin phosphorylation cascades, since protein synthesis was completely inhibited in the presence of cycloheximide. Thus, in order to evaluate this possible effect on the synthesis of glucose transporters, we assessed the effect of rutin on the GLUT-4 immunocontent in soleus muscle.

Skeletal muscle plays a pivotal role in maintaining glucose homeostasis and in this tissue the glucose transport capacity and GLUT-4 content are closely correlated. Therefore, the amount of GLUT-4 protein is a primary factor in determining the maximal rate of glucose transport into skeletal muscle. The regulation of GLUT-4 expression is not entirely understood, but insulin has been shown to upregulate the expression of GLUT-4 and levels of this transporter in skeletal muscle are a good indicator of whole-body insulin sensitivity.[38] Thus, the significant increase on muscle GLUT-4 content by rutin points a potential target for pharmacological intervention strategies to control glucose homeostasis.

Conclusion

In conclusion, we have shown that rutin stimulates glucose uptake in soleus muscle. As proposed and shown in Fig. 6, the data support the role of PI3K, aPKC and MAPK in the signaling pathways of rutin leading to a stimulatory affect on glucose uptake. Additionally, rutin may influence glucose transporter translocation and directly activate the synthesis of the transporter GLUT-4. These similarities in the involvement of protein kinases and phosphatases in the stimulatory effect of rutin and insulin on glucose uptake constitute strong evidence for the insulin-mimetic role of rutin in glucose homeostasis, effectively, through GLUT-4 translocation.

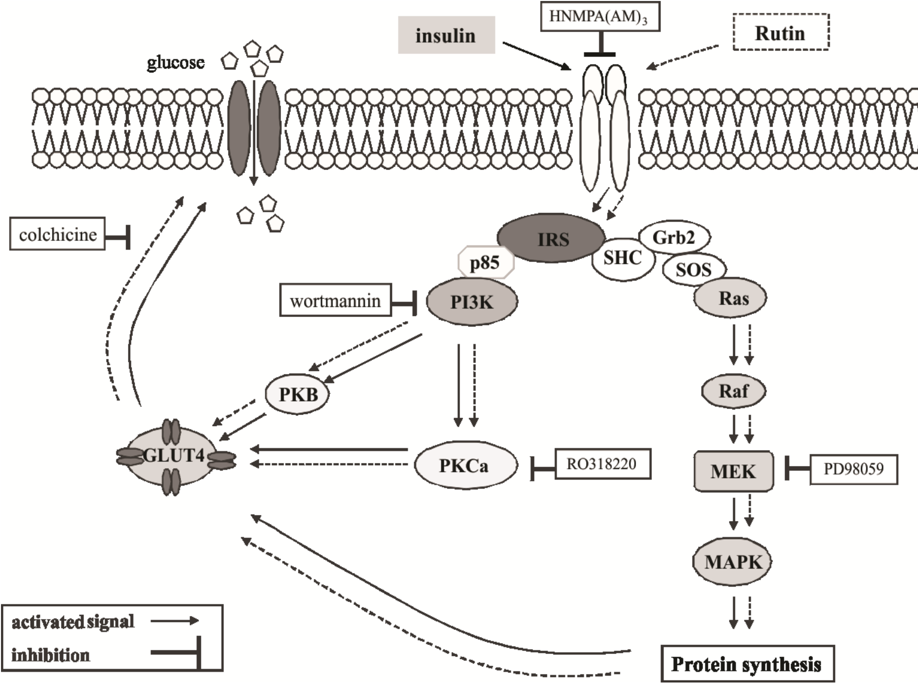

Fig. 6. Proposed signaling pathways of rutin on glucose uptake in rat soleus muscle

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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4.3.2 Conclusão Parcial

A partir dos resultados obtidos neste trabalho, podemos concluir que:

A rutina estimulou *in vitro* a captação de glicose em músculo sóleo.

A rutina ativa diretamente a síntese do tranportador de glicose GLUT4 no músculo sóleo.

O mecanismo de captação de glicose em músculo sóleo estimulado pela rutina é similar ao mecanismo de transdução de sinais da insulina, sugerindo um efeito insulino-mimético.

4.3.3 Artigo submetido para publicação

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KAPPEL, V. D.; ZANATTA, L.; POSTAL, B. G.; SILVA, F. R. M. B.
Rutin potentiates calcium uptake via L-type calcium channel associated with stimulation of glucose uptake in skeletal muscle.

Rutin potentiates calcium uptake via L-type calcium channel associated with stimulation of glucose uptake in skeletal muscle

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ABSTRACT

Rutin is a flavonoid with several pharmacological properties and it has been demonstrated that rutin can also modulate glucose homeostasis. In skeletal muscle, an increase in intracellular calcium concentration may induce GLUT-4 translocation with consequent glucose uptake. The aim of this study was to investigate the effect of rutin and intracellular pathways on calcium uptake as well as the involvement of Ca^{2+} in glucose uptake in skeletal muscle. The results show that rutin significantly stimulated calcium uptake and L-type voltage-dependent calcium channels, CaMKII, MEK and PKA signaling pathways are involved in the stimulatory action of rutin on calcium uptake. Also, rutin stimulated glucose uptake in the soleus muscle and this effect was mediated by extracellular calcium and CaMKII activation. In conclusion, rutin significantly stimulates calcium uptake in rat soleus muscles. Furthermore, an increase in intracellular calcium concentration is involved in rutin DNA activation. Also, rutin induced-glucose uptake by CaMKII may result in GLUT-4 translocation to the plasma membrane, characterizing an insulin-independent pathway. These findings indicate that rutin is a potential drug candidate for diabetes therapy.

Keywords: Rutin; soleus muscle; calcium uptake; glucose uptake; CaMKII; diabetes.

Introduction

The maintenance of glucose homeostasis, under physiological conditions, is due to hormonal regulation of glucose uptake and production by the skeletal muscle and liver, respectively [1]. Insulin stimulates glucose uptake in striated muscle and both resistance to insulin in this tissue and type-2 diabetes constitute important and increasing health problems worldwide. In skeletal muscle, the insulin-mediated glucose transport in the post-prandial state plays a major role in maintaining glucose homeostasis. Insulin increases glucose uptake through the activation of different signaling pathways culminating in glucose transporter-4 (GLUT-4) translocation to the plasma membrane [2,3]. However, some reports have shown insulin-independent mechanisms regulating glucose uptake in striated muscle in which calcium plays a central role [3,4].

In skeletal muscle, the increase in intracellular calcium concentration ($[Ca^{2+}]_i$) is due to influx from the extracellular medium or release from the sarcoplasmic reticulum and can be induced by muscle contraction or other stimuli, such as plasma membrane depolarization, insulin and diacylglycerol (DAG), resulting in GLUT-4 translocation with consequent glucose uptake. The signals involved in mediating glucose uptake in response to $[Ca^{2+}]_i$ increase described so far are protein kinase C (PKC), calcium-calmodulin-dependent protein kinase II (CaMKII) and 5’-AMP-activated protein kinase (AMPK) [3,5].

Flavonoids are polyphenolic compounds that are widespread in the plant kingdom, and they are important components of various fruits and vegetables [6]. Several flavonoids exert an effect on glucose metabolism, interfering with the transport and insulin receptor function, on disaccharidase activity and on insulin secretion and consequently they can affect diabetes (for review Cazarolli et al. [3]). We have previously reported the acute hypoglycemic effect of isolated flavonoids (kaempferitin, kaempferol-3-neohepheridoside, apigenin, isovitexin and swertisin) in diabetic and in hyperglycemic rats [7-9]. Besides the hypoglycemic effect, we have also demonstrated the mechanism of action of these flavonoids in relation to glucose uptake in skeletal muscle, glycogen synthesis and insulin secretion [10-14]. More recently, the effect of different flavonoids on rat intestinal disaccharidase inhibition with a consequent reduction in the intestinal glucose absorption has been described [15].

Rutin (3, 3’, 4’, 5, 7-pentahydrohyflavone-3-rhamnoglucoside), a glycoside of the flavonol quercetin, is widely found in fruits, including tomatoes, vegetables, and beverages such as tea and wine [16]. The

pharmacological properties of rutin have been demonstrated including antioxidant, cytoprotective, antiplatelet, neuroprotective and cardioprotective activity [17-21]. It has also been demonstrated that rutin modulates glucose and lipid homeostasis. Furthermore, rutin can decrease lipogenesis in hepatocytes, plasma total cholesterol and LDL and glycemia, increase HDL cholesterol and inhibit α-glucosidase [15, 16, 22-24]. Recent research indicates an important role of Ca^{2+} in glucose uptake through activation in skeletal muscle via an insulin-independent pathway [25]. Furthermore, alterations in the glucose uptake of insulin-resistant tissue can lead to diabetes. In this context, the characterization of new effectors as well as a comprehension of the mechanism of action involved in glucose uptake by insulin-independent pathways can highlight new molecular targets for drugs aimed at diabetes therapy. The aim of this study was to investigate the effect of rutin and intracellular pathways on calcium uptake as well as the involvement of Ca^{2+} in glucose uptake in skeletal muscle.

Materials and Methods

Chemicals

1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), ethylene glycol-bis(2-aminoethyether)-N,N,N',N'-tetraacetic (EGTA), N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), bisindoylmaleimidine IX, 2-{1-[3-(amidinothio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl) maleimide methanesulfonate salt (RO-318220), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD-98059), dibutyryl cAMP, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), N-[2-[[[3-(4′-chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]- N-(2-hydroxyethyl)-4′ methoxybenzenesulfonamide phosphate salt (KN93), rutin, theophylline, nifedipine, flunarizine and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thymidine [methyl-^{14}C] (sp. act. 1.7464 GBq/mmol), [^{45}Ca]$CaCl_2$ (sp. act. 321 KBq/mg Ca^{2+}), [U-^{14}C]-2-deoxy-D-glucose (sp. act. 10.6 GBq/mmol) and Optiphase Hisafe III biodegradable scintillation liquid were purchased from Perkin-Elmer (Boston, USA). All other chemicals were of analytical grade.

Animals

50-day-old male Wistar rats were bred in our animal facility and housed in an air conditioned room (around 21°C) with controlled lighting (12 h/12 h light/dark cycle). All animals were maintained with pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water available *ad libitum*. All the animals were carefully monitored and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (CEUA/PP00398).

Calcium uptake in the rat soleus muscle

Animals were killed by decapitation and the soleus muscle was removed; one muscle (alternately left and right) from each rat was used as the experimental sample and the contralateral one was used as the control. The muscles were pre-incubated in Krebs Ringer-bicarbonate (KRb) buffer (122 mM NaCl; 3 mM KCl; 1.2 mM $MgSO_4$; 1.3 mM $CaCl_2$; 0.4 mM KH_2PO_4; 25 mM $NaHCO_3$) for 15 min in a Dubnoff metabolic incubator at 37 °C, pH 7.4 and gassed with O_2:CO_2 (95:5; v/v). The medium was replaced with fresh KRb with 0.1 μCi/mL $^{45}Ca^{2+}$ and the sample incubated for 60 min. Finally, the tissue was incubated for 0.5; 1; 5 and 10 min with $^{45}Ca^{2+}$ either in the absence (control) or the presence of rutin (10^{-18}; 10^{-14}; 10^{-10} and 10^{-7} M) or insulin (10^{-8} M). In some experiments channel blockers or kinase inhibitors were added 15 min before the flavonoid addition and maintained during the entire incubation period. The following drugs were prepared in KRb buffer for use in the experiments: BAPTA-AM (50 μM), U-73122 (1 μM), KN93 (10 μM) [26]; nifedipine (1 μM), flunarizine (1 μM) [27]; RO-318220 (1 μM), PD-98059 (10 μM), H-89 (10 μM) and theophylline (50 μM) [28]; dibutyryl cAMP (500 μM) [29]; and EGTA (2 mM) [30].

At the end of the incubation period, extracellular $^{45}Ca^{2+}$ from muscles was thoroughly washed off in 127.5 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 10 mM HEPES, 11 mM glucose, and 10 mM $LaCl_3$, at pH 7.4 (30 min in washing solution). The presence of La^{3+} during the washing stage was found to be essential to prevent release of the intracellular $^{45}Ca^{2+}$ [31]. After La^{3+} tissue washing, muscles were homogenized with 0.5 M NaOH solution; 50 □L aliquots of tissue medium were placed in scintillation fluid for counting in a Beckman coulter beta liquid scintillation spectrometer (model LS 6500; Fullerton, California, USA), and 5 μL aliquots were used for total protein quantification by the Lowry method (1951). The results are expressed as

pmol $^{45}Ca^{2+}$/µg protein or % of control, which represents an average of 25.08 ± 2.7 pmol $^{45}Ca^{2+}$/ µg protein [28].

Glucose Uptake in the rat soleus muscle

For the ^{14}C-Deoxy-D-Glucose (^{14}C-DG) uptake experiments muscles were dissected, weighed, pre-incubated and incubated at 37 °C in Krebs Ringer-bicarbonate (KRb) buffer bubbled with O_2/CO_2 (95%:5%, v/v) up to pH 7.4. Rutin (10^{-14} M) was added to the pre-incubation (30 min) and incubation (60 min) medium in the presence or absence of 1 µM nifedipine, 10 µM KN93 or 2 mM EGTA. ^{14}C-DG (0.1 µCi/mL) was added to each sample during the incubation period. After incubation, the muscles were transferred to screw cap tubes containing 300 µL NaOH 0.5 M. These were then boiled for 10 min; 25 µL aliquots of the tissue medium were placed in scintillation fluid for counting in a Beckman coulter beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman Coulter, Boston, USA), and 5 µL aliquots were used for total protein quantification [14]. The results are expressed as % of control.

Thymidine incorporation into DNA in the rat soleus muscle

For experiments on ^{14}C-thymidine incorporation into DNA, muscles were incubated in KRb buffer with [methyl-^{14}C] thymidine (1 µCi/mL) in the absence (control) or presence of rutin (10^{-14} M) with/without EGTA (2 mM), nifedipine (1 µM) or KN93 (10 µM) for 60 min at 37 °C, pH 7.4. At the end of the incubation, the muscles were rinsed twice with cold buffer to remove the unincorporated [^{14}C]-thymidine. Ice-cold trichloroacetic acid (10%) was added and the acid-insoluble material was dissolved with 0.5 M NaOH. Radioactivity was measured by liquid scintillation using a LKB rack beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman Coulter, Boston, USA). The protein concentrations were determined by the Lowry method [32] and the results expressed as cpm/µg of protein [33].

Statistical Analysis

The results are reported as mean values ± S.E.M. When multiple comparisons were performed, evaluation was carried out using one-way ANOVA followed by the Bonferroni multiple comparison test. Differences were considered to be significant when $p < 0.05$.

Results

Mechanism involved in rutin action on $^{45}Ca^{2+}$ uptake

In this study we determined the time-course and dose-response curve for the effect of rutin on $^{45}Ca^{2+}$ uptake in rat soleus muscle. After the calcium equilibrium obtained through 60 min of incubation with $^{45}Ca^{2+}$, calcium uptake was additionally monitored for 0.5, 1, 5 and 10 min without stimuli (control) and in the presence of rutin (10^{-18}; 10^{-14}; 10^{-10} and 10^{-7} M).

The measurement of calcium uptake in soleus muscle using the radioisotope $^{45}Ca^{2+}$ showed that rutin increased the calcium uptake after exposures of 1 min (58 %) and 10 min (38 %) and no effect was observed after 0.5 and 5 min in the presence of the flavonoid (Fig. 1A). Fig. 1B shows that 10^{-14} M and 10^{-10} M of rutin increased $^{45}Ca^{2+}$ uptake (by 75 % and 63 %, respectively) after 1 min of incubation compared with the respective control groups. Therefore, we selected a concentration of 10^{-14} M rutin and an exposure time of 1 min for subsequent experiments. Insulin was used in these experiments as a positive control and was found to increase the calcium uptake to the same extent as 10^{-10} M rutin.

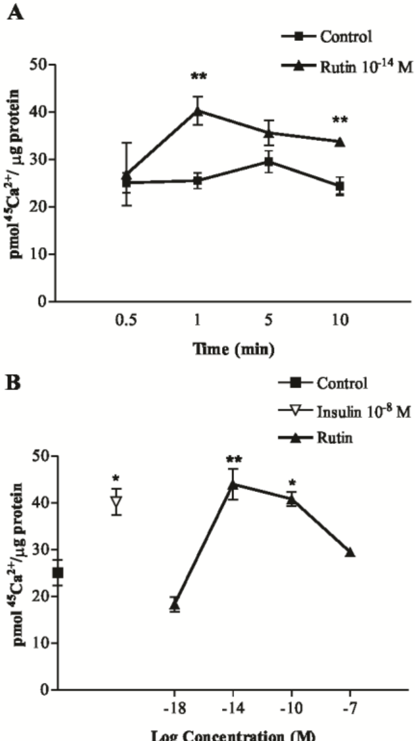

Fig. 1. Time-course (A) and dose-response curve (B) showing the effect of rutin on $^{45}Ca^{2+}$ uptake in rat soleus muscle. The muscles were pre-incubated for 60 min in the presence of 0.1 µCi/mL of $^{45}Ca^{2+}$. The muscles were incubated with/without rutin at different concentrations (10^{-18}, 10^{-14}, 10^{-10}, and 10^{-7} M) for 1 min. Values are means ± S.E.M. of 4 animals in each group. $^{*}p < 0.05$ and $^{**}p < 0.01$ and compared with control group.

In order to verify the participation of T- and L-type voltage-dependent calcium channels (L-VDCC) in the mechanism involved in the rutin action on $^{45}Ca^{2+}$ uptake, flunarizine and nifedipine were used to block these channels, respectively. The results showed that nifedipine but not flunarizine inhibited the effect of rutin, indicating the involvement of L-VDCC in the rutin action in the soleus muscle (Fig. 2A).

Since the results indicated that calcium influx occurs through L-VDCC in soleus muscle, we also sought to determine whether intracellular calcium levels and CaMKII play a role in this effect, using BAPTA-AM (an intracellular calcium chelator) and KN93 (a CaMKII inhibitor). It was demonstrated that in the presence of BAPTA-AM the stimulatory effect of rutin was not changed. The use of KN93 allowed us to verify the participation of CaMKII, a calcium-dependent kinase, in the mechanism of action of rutin (Fig. 2B).

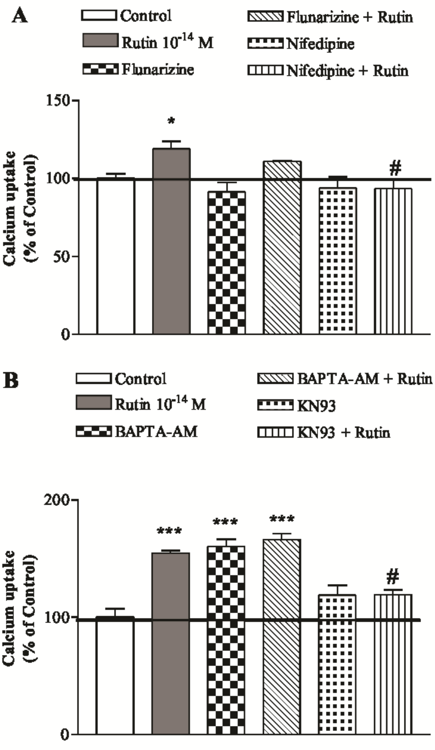

Fig. 2. Influence of voltage-dependent calcium channels (VDCCs) (A) and influence of intracellular calcium and PKCaM II (B) on stimulatory effect of rutin on $^{45}Ca^{2+}$ uptake in rat soleus muscle. The muscles were pre-incubated for 60 min with 0.1 µCi/mL of $^{45}Ca^{2+}$. In the last 15 min of pre-incubation 1 µM nifedipine (L-VDCC blocker), 1 µM flunarizine (T-VDCC blocker), 50 µM BAPTA-AM (intracellular calcium chelator) or 10 µM KN93 (PKCaM II inhibitor) was added to the incubation

medium. The tissue was then treated with or without 10^{-14} rutin for 1 min (incubation). Values are means ± S.E.M. of 4 animals in each group. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to control group. $^{\#}p < 0.05$ compared to rutin group.

Considering that calcium channel activity can be regulated by phosphorylation [34,35], the involvement of protein kinase C (PKC), phospholipase C (PLC) and mitogen-activated kinase (MEK) in rutin-induced calcium uptake was investigated. To this aim, soleus muscle was incubated in the presence or absence of rutin with/without specific inhibitors - RO-318220, U-73122 and PD-98059. The results showed that the inhibitors used did not change the basal calcium uptake. PD-98059 completely prevented the rutin action. However, RO-318220 and U-73122 were not able to significantly alter the flavonoid effect, suggesting that PLC and PKC are not associated with the stimulatory effect of rutin on calcium uptake (Fig.3A).

Also studied was the participation of the PKA signaling pathway in the stimulatory effect of rutin on calcium uptake. A localized elevation of cAMP, produced on using di-BucAMP (cAMP analog) or theophylline (phosphodiesterase inhibitor), did not alter significantly the basal calcium uptake. However, both potentiated the effect of rutin on calcium uptake. As expected, PKA is involved in calcium uptake, since H-89 blocked the calcium uptake induced by rutin (Fig. 3B).

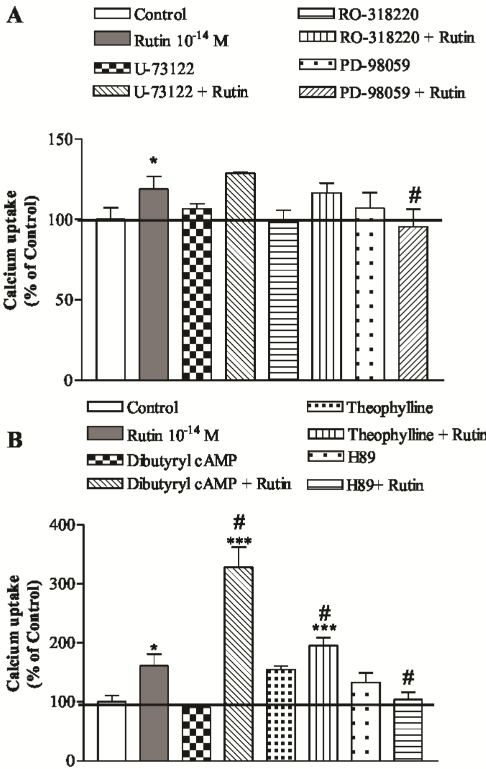

Fig. 3. Influence of PLC, PKC and MEK (A) and of PKA signaling pathway (B) on stimulatory effect of rutin on $^{45}Ca^{2+}$ uptake in rat soleus muscle. The muscles were pre-incubated for 60 min with 0.1 µCi/mL of $^{45}Ca^{2+}$. In the last 15 min of pre-incubation 1 µM U-73122 (PLC inhibitor), 1 µM RO-318220 (PKC inhibitor), 10 µM PD-98059 (MEK inhibitor), 500 µM dibutyryl cAMP (cAMP analog), 50 µM theophylline or 10 µM H-89 (PKA inhibitor) was added to the incubation medium. The tissue was then treated with or without 10^{-14} M rutin for 1 min (incubation). Values are means ± S.E.M. of 4 animals in each group. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to control group. $^{\#}p < 0.05$ compared to rutin group.

Effect of rutin on thymidine incorporation into DNA

To determine whether rutin stimulates nuclear activity, thymidine incorporation into the DNA of soleus muscle was analyzed. Fig. 4 shows that 10^{-14} M rutin increased the thymidine incorporation. In addition, the EGTA extracellular calcium chelator alone did not alter the thymidine incorporation, but in the presence of rutin it completely inhibited the stimulatory effect of the flavonoid. This result demonstrates the role of extracellular Ca^{2+} in rutin–induced thymidine incorporation into DNA.

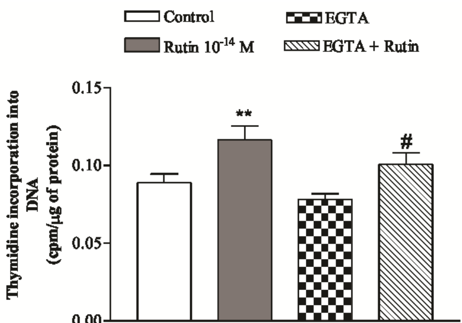

Fig. 4. Influence of EGTA on stimulatory effect of rutin (10^{-14} M) in thymidine incorporation into DNA in rat soleus muscle. The muscles were incubated for 60 min with [methyl-^{14}C] thymidine (1 µCi/mL) in the absence (control) or presence of rutin (10^{-14} M) with/without EGTA (2 mM). Values are means ± S.E.M. of 4 animals in each group. $^{**}p < 0.01$ compared to control group. $^{\#}p < 0.05$ compared to rutin group.

Effect of rutin on ^{14}C-Deoxy-D-Glucose

Fig. 5 shows a significant stimulatory effect (around 40%) of 10^{-14} M rutin on glucose uptake on skeletal muscle. Moreover, in the presence of EGTA the stimulatory action of rutin was abrogated. Also, when nifedipine was used the rutin-stimulated glucose uptake was blunted. Furthermore, in the presence of KN93, a CaMKII inhibitor, the glucose uptake induced by rutin was totally blocked.

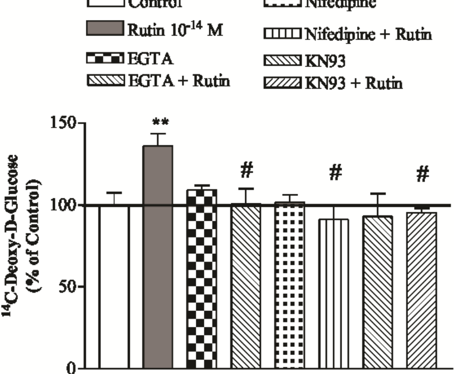

Fig. 5. Influence of extracellular calcium, L-type VDCC and PKCaM II on the stimulatory action of rutin on ^{14}C-glucose uptake in the rat soleus muscle. The muscles were pre-incubated for 30 min either in the absence (control) or in the presence of rutin (10^{-14} M) with/without EGTA (2 mM), nifedipine (1 μM) or KN93 (10 μM). The muscles were then incubated for 60 min with ^{14}C-deoxy-D-glucose (0.1 μCi/mL) in the absence (control) or presence of rutin (10^{-14} M) with/without EGTA (2 mM), nifedipine (1 μM) or KN93 (10 μM). Values are means ± S.E.M. of 4 animals in each group. **$p < 0.01$ compared to control group. $^{\#}p < 0.05$ compared to rutin group.

Discussion

Rutin is a flavonoid with a wide range of biological activities [16-24]. In this study we demonstrated that rutin quickly increases the calcium uptake in rat soleus muscle and that these changes in calcium influx have a significant involvement in glucose uptake and thymidine incorporation into DNA. There are many reports on the effects of flavonoids on glucose uptake in skeletal muscle [9-13]. As far as we know, this is the first time that it is shown that rutin is able to stimulate calcium uptake leading to glucose uptake in skeletal muscle.

Calcium is necessary for muscle contraction and acts as a second messenger in insulin-dependent and independent pathways that lead to glucose uptake [2,5]. The free cytosolic calcium level in skeletal muscle is tightly controlled and can be altered both by chemical and electrical signaling [36]. An increase of cytosolic calcium can occur through release from the sarcoplasmic reticulum, which is induced through depolarization of the adjacent transverse tubules and by inositol 3-

phosphate (IP3) formation or from the extracellular medium through plasma membrane calcium channel opening [36]. The present study has demonstrated that rutin-induced calcium influx in soleus muscle is mediated, at least in part, by L-VDCC, as also reported for insulin [2, 37]. In addition, our findings indicated that calcium uptake can also result from cytosolic calcium depletion since calcium chelation with BAPTA-AM increased the cation influx. Two forms of calcium entry have been characterized in skeletal muscle: triggered by depolarization or by depletion of intracellular calcium stores, which requires functioning L-type VDCC and ryanodine receptor (RYR1) channels [38].

In order to evaluate the mechanisms that could lead to calcium influx in skeletal muscle we investigated the participation of different kinases using pharmacological tools which allowed us to determine that the effect of rutin on calcium uptake is dependent on CaMKII, MEK and PKA but not on the PLC-PKC signaling pathway. In this context, the calcium influx through L-VDCCs could be modulated by complex mechanisms involving the activity of these protein kinases through direct phosphorylation of the channel [27,34,39].

The data reported herein suggest that rutin-induced calcium uptake is triggered by the influx of this ion through L-VDDC and that CaMKII, PKA and MEK play an important role in mediating this effect. Previous studies have shown that CaMKII can modulate L-VDCC and consequently induce it to enter into a gating mode [40]. Similarly, Hao et al. [41] suggested that the activity of both CaMKII and PKA is required to maintain basal L-type calcium activity in mammalian cardiac ventricular myocytes. Moreover, it has been demonstrated that L-VDCC phosphorylation by PKA in skeletal muscle resulted in activation and opening of the channels [42-44]. Vela et al. [45] also showed that PKA activation leads to depolarization-induced calcium entry through L-VDCC in GH3 cells. Surprisingly, the addition of a cAMP analog and a phosphodiesterase inhibitor potentiated the effect of rutin on calcium uptake suggesting that rutin can activate PKA through an alternative pathway, which is not dependent on 5' cAMP, as proposed by other research groups [46,47].

The participation of MEK in calcium influx demonstrated in this study is in agreement with previous reports from our group for the effect of $1,25(OH)_2$-vitamin D_3 on testis [28], suggesting that this kinase can phosphorylate and induce L-VDCC to enter into a gating mode. Also, Fitzgerald [48] reported that an MEK inhibitor reduced the calcium current in rat sensory neurons and Jeong et al. [49] recently described

the phosphorylation of ERK1/2 by rutin supporting the occurrence of cross-talk between rutin-induced calcium uptake and MEK.

Calcium is a highly versatile intracellular signal that can regulate many different cellular functions, among them, nuclear events. Calcium that enters the cell from the outside is a principal source of calcium signaling during the events [36]. Based on these findings we aimed to clarify whether the rutin-induced calcium influx could lead to thymidine incorporation into DNA in soleus muscle. Interestingly, our findings indicated that besides the rapid and plasma-membrane localized effect, rutin also produces a nuclear effect inducing thymidine incorporation into DNA. Furthermore, our results provide evidence that thymidine incorporation induced by rutin is associated with calcium influx since EGTA attenuated the flavonoid-mediated nuclear action.

It is well-known that extracellular calcium is a link able to connect external information to a specific site of action in the cell. Accordingly, the calcium influx can result in a range of biological effects including DNA transcription and protein expression [36]. The nuclear effect triggered by rutin and mediated by calcium in soleus muscle could lead to GLUT-4 biogenesis and consequent glucose uptake as previously reported by Ojuka et al. [50] and more recently by Wright [51]. Among the signaling pathways activated by insulin is MEK/ERK which generates transcriptional-dependent events such as GLUT-4 translation [52]. Considering these findings together, we suggest that rutin can also produce nuclear effects through ERK activation, since this was demonstrated to be involved in the effect of the flavonoid on soleus muscle.

Several studies have shown that glucose transport is increased in mammalian muscle, when cytoplasmic calcium concentrations are elevated via an insulin-independent pathway [53, 54]. Taking into account that the increase on calcium influx in skeletal muscle can lead to glucose transport [2] and that rutin plays a role in glucose homeostasis [15, 23] and calcium metabolism, we studied the effect of rutin on glucose uptake. As evidenced herein, rutin stimulated the glucose uptake in soleus muscle and its effect was mediated by extracellular calcium since EGTA and nifedipine blocked the glucose transport.

Considering that the increase in calcium concentration in the cells can lead to calcium-calmodulin complex formation with subsequent CaMKII activation, the involvement of kinase was also investigated applying this approach. Our findings demonstrated the participation of CaMKII in glucose uptake induced by rutin.

CaMKII, the predominant isoform expressed in skeletal muscle, can be activated and autophosphorylated by a calcium-calmodulin complex (Wijesekara et al., 2006). Also, the skeletal muscle contains voltage-dependent, dihydropyridine-sensitive calcium channels located in the transverse tubules, which is also where GLUT-4 is concentrated [55]. Therefore, the results presented suggest that calcium influx induced by rutin through L-VDCC can activate different downstream effectors leading to GLUT-4 translocation and glucose uptake. Two proteins have been identified in this context: PKC isoforms and CaMKII [5], and in the study reported herein the involvement of CaMKII was verified.

Conclusions

Rutin significantly stimulates calcium uptake in rat soleus muscles by L-VDCC opening through PKA, MEK and CaMKII activation. Furthermore, the increase in intracellular calcium concentration is involved in rutin induced-glucose uptake through CaMKII, which may result in GLUT-4 translocation to the plasma membrane, characterizing an insulin-independent pathway. Also, calcium influences the stimulatory effect of rutin on DNA activity, as schematically represented in Fig. 6. Studies are underway in order to clarify the genomic effect of rutin. The modulation of calcium entry into skeletal muscle via rutin might represent an alternative to glucose uptake regulation, mainly in insulin-resistant tissues. These findings highlight rutin as a potential candidate for use, at least as an adjuvant, in pharmacological therapies for diabetes.

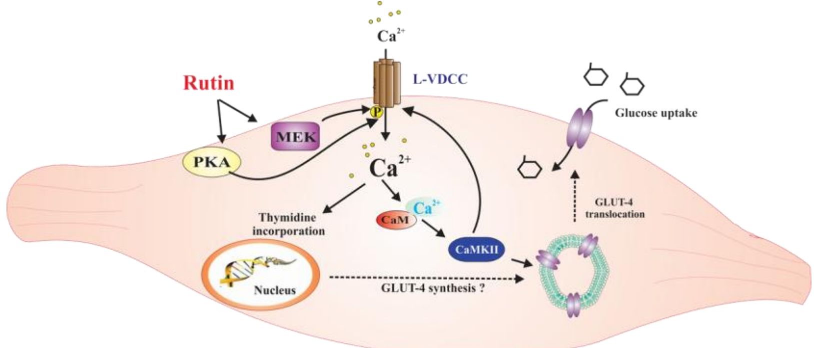

Fig. 6. Schematic representation of rutin-induced signaling pathways mediating **rat soleus muscle** calcium and glucose uptake. Rutin stimulates a variety of signaling pathways such as PKA, MEK and CaMKII. These kinases phosphorylate L-VDCC which promotes the

entry of extracellular calcium into the cells. Calcium influx can lead to thymidine incorporation into DNA and glucose uptake. Also, rutin-activated CaMKII could result in GLUT-4 translocation and consequent glucose uptake.

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4.3.4 Conclusões parciais

A partir dos resultados obtidos neste trabalho, podemos concluir que:

A rutina estimulou *in vitro* a captação de cálcio em músculo sóleo.

O mecanismo de captação de cálcio em músculo sóleo estimulado pela rutina envolve canais de cálcio dependentes de voltagem do tipo L e vias de ativação da PKA, MEK e PKCaMII.

O aumento de cálcio intracelular estimulado pela rutina está envolvido na incorporação de timidina e na captação de glicose também estimulados pela rutina.

A captação de glicose estimulada pela rutina em músculo sóleo envolve a PKCaMII, caracterizando uma via independente da sinalização clássica da insulina.

4.3.5 Artigo submetido para publicação

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The role of calcium in intracellular pathways of rutin in rat pancreatic islets: potential insulin secretagogue effect

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ABSTRACT

Rutin is a flavonol glycoside with multiple biological activities and it has been demonstrated that rutin modulates glucose homeostasis. In pancreatic β-cell, an increase in intracellular calcium concentration triggers exocytosis and thus insulin secretion. The aim of the study reported herein was to investigate the effect of rutin and associated intracellular pathways on calcium uptake in isolated rat pancreatic islets. We focused on the acute effects of rutin on *in vivo* insulin secretion and the *in vitro* cellular signaling of pancreatic islets related to this effect. The results show that rutin significantly increased glucose-induced insulin secretion in an *in vivo* treatment. Moreover, it was demonstrated that rutin stimulated calcium uptake after 10 min of incubation compared with the respective control group. The involvement of L-type voltage-dependent calcium channels was evidenced using nifedipine, while the use of glibenclamide and diazoxide demonstrated that the ATP-sensitive potassium channels are not involved in the rutin action in pancreatic islets. In conclusion, rutin potentiated insulin secretion in hyperglycemic rats and significantly stimulated calcium uptake in rat pancreatic islets. A novel cellular mechanism of action of rutin in Ca^{2+} fluxes on pancreatic β-cells was elucidated. Rutin modulates calcium uptake in pancreatic islets by opening L-VDCC which involves intracellular calcium, PLC and PKC signaling pathways, characterizing K_{ATP} channel-independent pathways. This represents a potential insulinotropic action of rutin which may potentiate glucose-induced insulin secretion. These findings highlight rutin, a dietary adjuvant, as a potential insulin secretagogue contributing to glucose homeostasis.

Keywords: Rutin; rat pancreatic islets; calcium uptake; insulin; PLC; PKC.

1. Introduction

Diabetes mellitus (DM) is the most common metabolic disorder and is currently one of the most serious health problems worldwide. DM is characterized by deranged metabolism and inappropriate hyperglycemia, resulting from defects in the secretion and cellular action of insulin. Treatments aimed at enhancing β-cell function and reducing insulin resistance are therefore key to improving metabolic control and retarding the development of diabetic complications (American Diabetes Association, 2011).

Glucose homeostasis is maintained by the hormonal regulation of glucose uptake and endogenous glucose production, primarily by muscle and liver, respectively. Insulin is the key regulator of glucose uptake in the fed state (Beardsall et al., 2006). After a meal, the peptide hormone insulin is secreted from β-cells located in the islets of Langerhans in the pancreas. Insulin inhibits glucose output from the liver and promotes the uptake of glucose from the blood stream into skeletal muscle and adipose tissue (Rowland et al., 2011).

Glucose is the primary stimulus for insulin secretion, although there are many other metabolic, endocrine, and neural control mechanisms (Beardsall et al., 2006). Exposure of the pancreatic β-cell to stimulatory glucose concentrations leads to the activation of a cascade of reactions, which ends in the release of stored insulin. Metabolism of glucose in glycolysis and the Krebs cycle results in the generation of ATP. The resulting increase in the cytosolic ATP/ADP-ratio closes ATP-sensitive potassium (K_{ATP}) channels in the plasma membrane, which leads to depolarization of the cell and influx of Ca^{2+} through L-type voltage-dependent Ca^{2+} channels (L-VDCCs). The increase in Ca^{2+} concentration triggers exocytosis and thus insulin secretion (Barg, 2003).

Different mechanisms of action have been reported for therapeutic drugs used in the treatment of diabetes (Sharif, 2011). Some pharmacological agents with insulinotropic properties can directly and positively modulate insulin release (Doyle and Egan, 2003). Recent studies have explored the insulinotropic effects of natural products (Pinent et al., 2008). Flavonoids are a class of such bioactive compounds, usually found in fruits and other plant organs and therefore widely consumed. Several beneficial effects have been reported for flavonoids and published data suggest that there might be direct effects of flavonoids on insulin secretion (Cazarolli et al., 2008ab; Folador et al., 2011).

Rutin (quercetin-3-*O*-rutinoside) is a flavonol glycoside composed of quercetin and the disaccharide rutinose. Many studies have demonstrated that rutin is a pharmacologically active phytochemical which exhibits multiple biological activities (Hertog et al., 1993; Janbaz et al., 2002; Rotelli et al., 2003). It has also been demonstrated that rutin modulates glucose homeostasis. Rutin can decrease glycemia, increase insulin secretion and inhibit α-glucosidase (Fernandes et al., 2010; Pereira et al., 201; Prince and Kamalakkannan, 2006;). Although the ability of rutin to improve diabetic status has been reported, studies to determine the exact mechanism of action involved in the regulation of glucose homeostasis are scarce. Thus, the aim of this study was to investigate the effect of rutin and intracellular pathways on calcium uptake in isolated pancreatic islets. We focused on the acute effects of rutin on *in vivo* insulin secretion, and the *in vitro* cellular signaling of pancreatic islets related to this effect.

2. Materials and Methods

2.1 Chemicals

1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), (bisindoylmaleimidine IX, 2-{1-[3-(amidinothio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl) maleimide methanesulfonate salt) RO-318220, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), rutin, nifedipine, diazoxide, glibenclamide and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{45}Ca]$CaCl_2$ (sp. act. 321 KBq/mg Ca^{2+}) and Optiphase Hisafe III biodegradable scintillation liquid were purchased from Perkin-Elmer (Massachusetts, USA). All other chemicals were of analytical grade. Enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of rat insulin (catalogue n^{o}. EZRMI-13K) was purchased from Millipore (St Charles, MO, USA).

2.2 *Animals*

The male Wistar rats (190 – 220 g) used in this study were bred in our animal facility and housed in an air-conditioned room (approximately 22 ºC) with controlled lighting on a 12:12 h light/dark cycle (lights on from 06:00 to 18:00 h). The animals were maintained with pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil), while

tap water was available *ad libitum*. In the *in vivo* treatments rats were deprived of food for at least 16 h but allowed free access to water. In the *in vitro* experiments the animals were not left in fasting. All the animals were monitored and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation. This study was approved by the Committee for Ethics in Animal Research of UFSC (Protocol CEUA PP00398).

2.3 Oral glucose tolerance curve (OGTC)

Fasted rats were divided into two groups of four animals for each treatment. Group I, normal hyperglycemic rats that received glucose (4 g/kg; 8.9 M); Group II, rats that received glipizide at a dose of 10 mg/ kg (corresponding to 0.01 M); Group III, rats that received rutin at a dose of 50 mg/ kg (corresponding to 0.04 M). All treatments were administrated by oral gavage. The glucose and insulin levels were measured before the administration of treatment (zero time). The glucose loading was applied 30 min after the treatment and, subsequently, the glycemia and insulin levels were determined at 15, 30, 60 min (Folador et al., 2011).

2.4 Insulin serum measurements

The insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The range of values detected by this assay was 0.2 ng/ mL to 10 ng/ mL. The intra- and inter-assay coefficients of variation (CV) for insulin were 3.22 and 6.95, respectively, with a sensitivity of 0.2 ng/ mL. All insulin levels were estimated by means of colorimetric measurements at 450 nm with an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) by interpolation from a standard curve. Samples were analyzed in duplicate and results were expressed as ng per mL of insulin serum (Folador et al., 2011). The incremental areas under the response curves (AUCs) were calculated. The insulinogenic index (II) was calculated as the ratio between the $AUC_{insulin}$ and $AUC_{glucose}$ (from zero to 60 min) (Damazio et al., 2009).

2.5 Rat islet isolation

The rat pancreas was visualized by way of a central abdominal incision. The bile duct was clamped at the tip of the duodenum and cannulated at a point sufficiently proximal to the liver. Krebs Ringer buffer (KRb) supplemented with HEPES (8 mM) and glucose (3 mM) (KRb-HEPES) medium was introduced slowly into the bile duct by syringe until the pancreas was clearly distended. The pancreas was then gently removed and kept in a Petri dish with KRb-HEPES medium. The pancreatic tissue was cut into small pieces (2 x 2 mm), and incubated in plastic tubes in KRb-HEPES medium supplemented with collagenase (3 mg/mL). After incubation, the mixture was transferred to a conical tube (110 x 15 mm), resuspended in 10 mL with collagenase-free medium, and centrifuged at room temperature for 3 min at 4,500*g* in an Excelsa Baby centrifuge (model 206), FANEM, São Paulo, SP, Brazil. The supernatant was discarded and the sediment resuspended in fresh KRb-HEPES medium. This washing procedure was repeated five times and in the last two washings the islets were allowed to settle without centrifugation. Aliquots (100 µL) of the final sediment with the isolated islets were transferred to eppendorff tubes with the incubation medium KRb-HEPES (Lacy and Kostianovsky, 1967).

2.6 Calcium uptake experiments

The isolated islets were pre-incubated for 60 min in a Dubnoff metabolic incubator to equilibrate in KRb-HEPES buffer containing 0.1 µCi/mL $^{45}Ca^{2+}$ at 37°C, pH 7.4 and gassed with O_2:CO_2 (95:5; v/v). The islets were then incubated for 10 min in KRb-HEPES without (control) or with rutin. In some experiments channel blockers or kinase inhibitors were added during the last 15 min before the treatment and maintained during the entire incubation period (see figure legends). The following drugs were used: diazoxide (100 µM), glibenclamide (60 µM), nifedipine (1 µM) (Zamoner et al., 2007), BAPTA-AM (50 µM), RO 31-8240 (20 µM), U-73122 (1 µM) (Zanatta et al., 2011). One mL of cold buffer with lanthanum choride (10 mM) at 2°C was added to the samples to stop calcium fluxes. The tubes were centrifuged for 1 min at 1,500*g*. The supernatant was preserved and the islets were washed twice in cold lanthanum chloride solution. The presence of La^{3+} during the washing stage was found to be essential to prevent release of the intracellular $^{45}Ca^{2+}$ (Batra and Sjögren, 1983). After La^{3+} tissue washing, islets were homogenized with 300 µL of 0.5 M NaOH solution and boiled at 100°C for 5 min. Aliquots of 50 µL were taken from each sample for radioactivity measurement in scintillation liquid in an LKB

rack beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman Coulter, Boston, USA) and 5 μL aliquots were used for protein quantification by the Lowry method (1976).

2.7 Data and statistical analysis

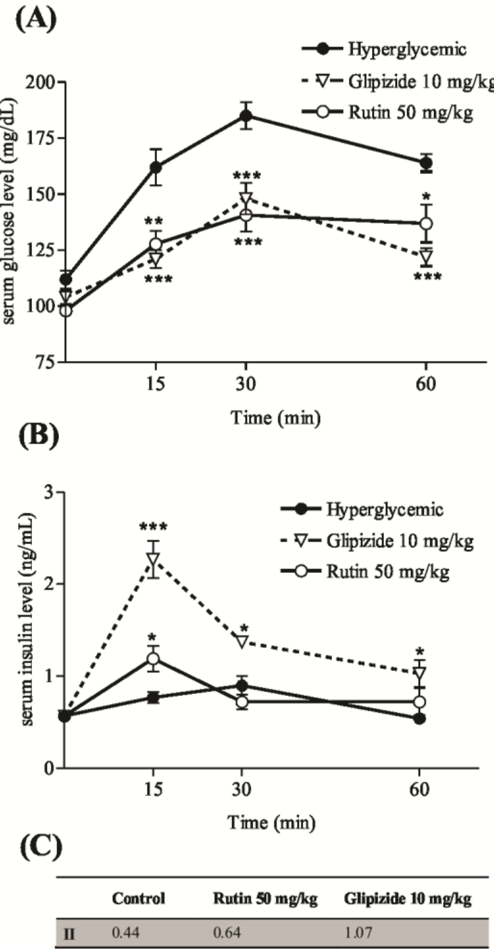
Data were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) was carried out followed by the Bonferroni *post hoc* or unpaired Student's *t*-test to determine the significance of differences between groups. Differences were considered to be significant at $p < 0.05$.

3. Results

3.1 Effect of rutin on oral glucose tolerance curve

Figure 1A shows the acute effect of rutin on serum glucose levels by way of the OGTC. Fifteen minutes after the glucose loading the glycemia was significantly increased when compared with zero time on the OGTC. The sulfonylurea glipizide (10 mg/kg), an oral hypoglycemic agent, was used as a positive control and produced a typical serum glucose lowering for all periods analyzed (15 to 60 min) compared to the hyperglycemic group. With the oral administration of rutin (50 mg/kg) the serum glucose levels were also significantly reduced at 15, 30 and 60 min after the glucose loading in hyperglycemic rats, the glycemic reduction being around 21, 24 and 17 %, respectively.

We studied the effect of *in vivo* rutin treatment by oral gavage on the insulin secretion at 15, 30 and 60 min after glucose loading on an OGTC. Serum insulin levels in fasted rats were determined after an oral glucose loading (4 g/kg) as shown in **Figure 1B**. As expected, a sulfonylurea agent, glipizide, stimulated insulin secretion by 295, 152 and 191% at 15, 30 and 60 min after glucose loading, respectively, compared to the hyperglycemic control group. The rutin (50 mg/kg) potentiated insulin secretion induced by glucose by around 155 % 15 min after glucose loading. The treatment with rutin resulted in around a 1.5-fold increase in II (0.64 mg/mg) compared with the hyperglycemic control group (0.44 ng/mg) (**Figure 1C**).



	Control	Rutin 50 mg/kg	Glipizide 10 mg/kg
II	0.44	0.64	1.07

Figure 1. Acute effect of rutin on serum (A) glucose and (B) insulin levels and (C) insulinogenic index (II; ng/mg). Values are expressed as mean±S.E.M.; n=4 in duplicate for each treatment. Statistically significant difference compared to the corresponding hyperglycemic group, ***p <0.001; **p <0.01 and *p <0.05.

3.2 Mechanism of action of rutin associated with $^{45}Ca^{2+}$ uptake in isolated rat pancreatic islets

Calcium is well known to be involved in insulin secretion by β-cells. Thus, in order to study the mechanism of action of rutin on insulin secretion, *in vitro* studies were carried out. Firstly, the time-course for the effect of rutin on 0.1 μCi/mL $^{45}Ca^{2+}$ uptake in isolated pancreatic islets was determined. The calcium equilibrium obtained through 60 min of islet incubation with $^{45}Ca^{2+}$, calcium uptake was then monitored for 1, 5 and 10 min without stimuli (control) and in the presence of rutin (10^{-14} M). The treatment with rutin significantly increased the calcium uptake in isolated pancreatic islets after 10 min (58 %). However, no effect was observed after 1 and 5 min of incubation with rutin (**Figure 2**).

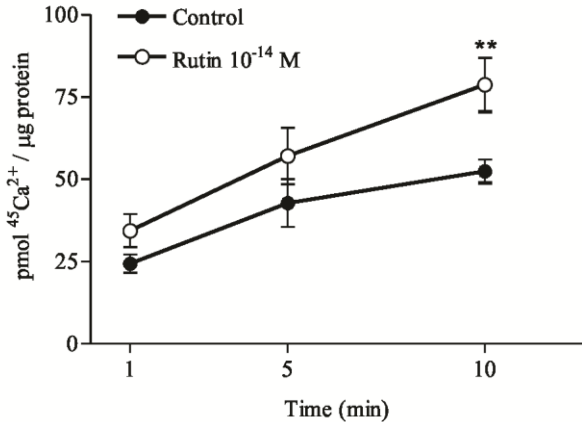


Figure 2. Time-course of rutin effect on $^{45}Ca^{2+}$ uptake in isolated rat pancreatic islets. The pancreatic islets were pre-incubated for 60 min in the presence of 0.1 μCi/mL of $^{45}Ca^{2+}$. The pancreatic islets were incubated with/without 10^{-14} M rutin for 1, 5 and 10 min. Values are means ± S.E.M. (quadruplicate analysis for each group). **$p < 0.01$ compared with control group.

To study the involvement of K_{ATP} channels in the stimulatory effect of rutin on calcium uptake glibenclamide, a potassium channel blocker, and diazoxide, a potassium channel activator, were used. As expected, the treatment with glibenclamide significantly increased the calcium

uptake in isolated pancreatic islets after 10 min. Also, in the presence of glibenclamide an additional effect on calcium uptake stimulated by rutin was observed (**Figure 3A**). **Figure 3B** shows that diazoxide significantly decreased the calcium uptake. On the other hand, the presence of diazoxide did not influence the stimulatory effect of rutin on calcium uptake in isolated pancreatic islets (**Figure 3B**).

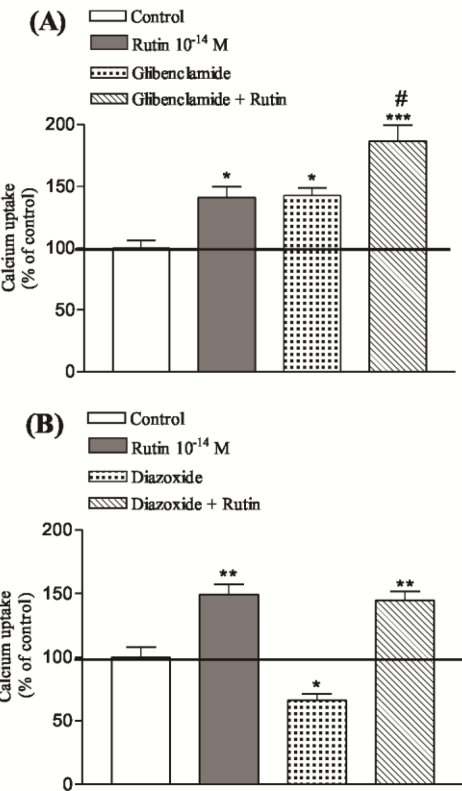


Figure 3. Influence of K^+ channel on stimulatory effect of rutin in $^{45}Ca^{2+}$ uptake in isolated rat pancreatic islets. The pancreatic islets were pre-incubated for 60 min with 0.1 µCi/mL of $^{45}Ca^{2+}$. In the last 15 min of pre-incubation (A) 60 µM glibenclamide (blocker) and (B) 100 µM diazoxide (activator) was added to the incubation medium. The tissue was then treated with or without 10^{-14} M rutin for 10 min (incubation). Values are means ± S.E.M. (quadruplicate analysis for each group). *p <

0.05, **p <0.01 and ***p <0.001 compared to control group. $^{\#}p < 0.05$ compared to rutin group.

In order to verify the participation of L-type voltage-dependent calcium channels (L-VDCCs) in the mechanism involved in the stimulatory effect of rutin action on $^{45}Ca^{2+}$ uptake, nifedipine was used. **Figure 4A** shows that nifedipine did not change the basal level of calcium uptake. However, nifedipine inhibited the effect of rutin.

The participation of intracellular calcium in the stimulatory effect of rutin on calcium uptake was investigated using BAPTA-AM, an intracellular calcium chelator. **Figure 4B** shows that BAPTA-AM did not alter the basal level of calcium uptake. However, BAPTA-AM totally inhibited the stimulatory effect of rutin on $^{45}Ca^{2+}$ uptake.

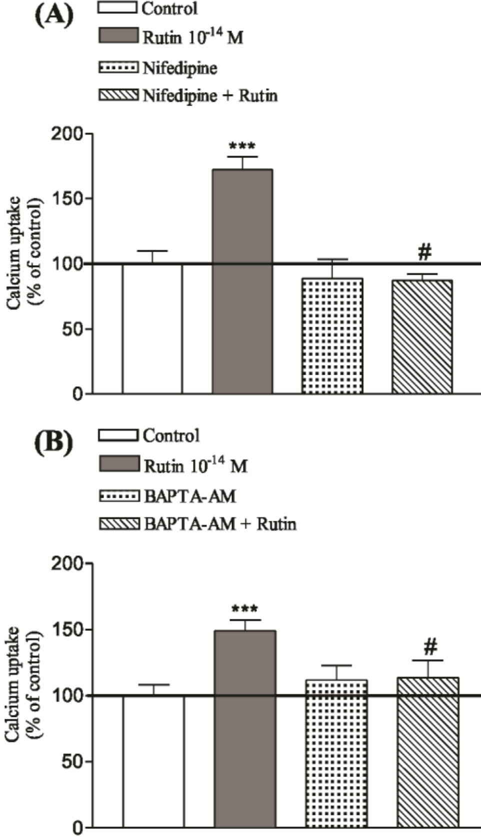


Figure 4. Influence of L-type voltage-dependent calcium channel (L-VDCC) and intracellular Ca^{2+}on stimulatory effect of rutin in $^{45}Ca^{2+}$ uptake in isolated rat pancreatic islets. The pancreatics islets were pre-incubated for 60 min with 0.1 µCi/mL of $^{45}Ca^{2+}$. In the last 15 min of pre-incubation (A) 1 µM nifedipine (L-VDCC blocker) and (B) 50 µM BAPTA-AM (intracellular calcium chelator) were added to the incubation medium. The tissue was then treated with or without 10^{-14} M rutin for 10 min (incubation). Values are means ± S.E.M. (quadruplicate analysis for each group). ***$p < 0.001$ compared to control group. $^{\#}p < 0.05$ compared to rutin group.

Considering that calcium channel activity can be regulated by phosphorylation (Kamp and Hell, 2000; Keef et al., 2001), the involvement of phospholipase C (PLC) and protein kinase C (PKC) in rutin-induced calcium uptake was investigated. To this aim, isolated pancreatic islets were incubated in the presence or absence of rutin with/without specific inhibitors - U-73122 and RO-318220. These results showed that the inhibitors used did not change the basal level of calcium uptake. On the other hand, U-73122 and RO-318220 completely prevented the rutin stimulatory effect (**Figure 5A and B**).

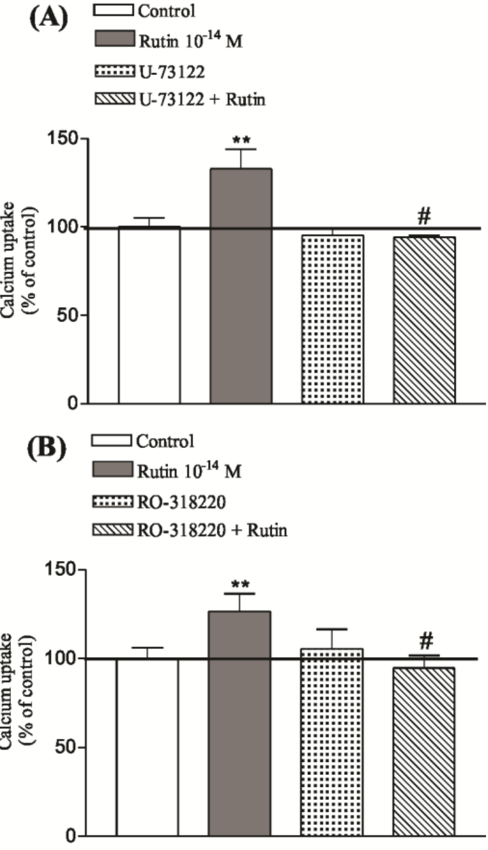


Figure 5. Influence of PLC and PKC on stimulatory effect of rutin in $^{45}Ca^{2+}$ uptake in isolated rat pancreatic islets. The pancreatic islets were pre-incubated for 60 min with 0.1 µCi/mL of $^{45}Ca^{2+}$. In the last 15 min of pre-incubation, (B) 1 µM U-73122 (PLC inhibitor) and (C) 1 µM RO-318220 (PKC inhibitor) was added to the incubation medium. The tissue was then treated with or without 10^{-14} M rutin for 10 min (incubation). Values are means ± S.E.M. (quadruplicate analysis for each group). **$p < 0.01$ compared to control group. $^{\#}p < 0.05$ compared to rutin group.

4. Discussion

Rutin is a flavonoid present in large amounts in the diet and is found in many plants, for example black tea and apple peel. Different types of biological activity have been described for this flavonoid (Gupta et al., 2003; Janbaz et al. 2002; La Casa et al., 2000; Sheu et al., 2004). In the study reported herein, we observed that rutin was effective in reducing glycemia at different times on an OGTC after oral treatment in hyperglycemic rats. Recently, Pereira et al. (2011) also evidenced the antihyperglycemic effect of rutin. and demonstrated that rutin (50 mg/kg) reduced serum glucose levels at 15, 30, and 60 min when administered by oral gavage 30 min before glucose overload in rats.

In addition, we studied the effect of rutin on insulin secretion in an *in vivo* approach. Our results demonstrated that rutin significantly increased glucose-induced insulin secretion *in vivo* with a marked increase in the insulinogenic index (II). In this way, we obtained evidence that rutin is an insulinotropic agent, since it was observed that rutin potentiated insulin secretion *in vivo*. In agreement with our result, Kamalakkannan and Prince (2006) demonstrated trough an *in vivo* treatment that rutin increase insulin levels. They suggest that the increased insulin levels could be due to the stimulatory effect of rutin, thereby potentiating insulin secretion from the existing β-cells of the islets of Langerhans in diabetic treated rats. However, no mechanism of action for the effect of rutin on β-cells was described.

Glucose-stimulated insulin secretion by β-cell is characterized by a biphasic pattern, a rapid first phase of insulin release for about 10 min followed by a gradual increase in a second phase reaching a plateau after 25 to 30 min (Yang and Berggren, 2006). Our results clearly demonstrated that rutin potentiates the first phase of insulin secretion. On the other hand, glipizide, a sulphonylurea, significantly increased the insulin levels during all periods studied, indicating that it potentiated the first and second phase of insulin release.

Sulphonylureas (e.g. glibenclamide, glipizide, tolbutamide) are pharmacological agents that close the K_{ATP} channels, depolarize β-cells, stimulate Ca^{2+} influx, raise $[Ca^{2+}]_i$ and trigger insulin secretion. The insulin secretagogue mechanism of sulphonylureas is independent of changes in glucose metabolism in β-cell, and act in both phases of insulin secretion. This represents a limitation to the use of sulphonyureas because this class of insulin secretagogues has a greater propensity to induce hypoglycemia as an adverse effect (Doyle and Egan 2003; Sharif, 2011). The search for more effective and safer

antidiabetic drugs based on natural products is still attractive, because they have demonstrated alternative mechanisms of action (Patel et al., 2012). In this regard, the biological effect of rutin on insulin secretion demonstrated herein highlights this natural product, as a nutraceutical as well as a pharmaceutical agent, with a significant role in glucose homeostasis.

Calcium signals regulate the first and second phases of glucose-induced insulin secretion (Henquin, 2011). Thus, in order to elucidate the possible mechanism of action of the insulin secretagogue effect of rutin, we studied the effect of rutin on calcium uptake in isolated pancreatic islets and the intracellular pathways related to this effect. Our results demonstrated that rutin quickly increases the calcium uptake in isolated rat pancreatic islets. These changes in calcium influx could explain the increase in insulin secretion stimulated by acute treatment with rutin *in vivo*. Numerous reports have demonstrated that flavonoids can act as insulin secretagogues (Brahmachari, 2011). To the best of our knowledge, this is the first time that it is shown that rutin is able to alter calcium fluxes in isolated pancreatic islets and the mechanism of its potential secretagogue activity is proposed.

Calcium acts as a second messenger on insulin secretion on pancreatic β-cells. Maintaining serum glucose homeostasis is a complex process which is dependents on pancreatic islet hormone secretion. Insulin secretion from islets is coupled to calcium entry which results from the electrical activity regenerative islet cells. Therefore, the mechanisms that regulate calcium entry into islet cells are crucial for maintaining normal glucose homeostasis (Denton and Jacobson, 2012).

Some studies suggest that flavonoids may act on islet function, at least in part, *via* the alteration of Ca^{2+} fluxes. Hii and Howell (1985) showed that exposure of rat isolated islets to certain flavonoids such as epicatechin or quercetin, aglycone of rutin, enhanced insulin release and increased the intracellular Ca^{2+} level in rat islets of Langerhans. They suggested that such flavonoids may act on islet function via changes in Ca^{2+} metabolism. More recently, it was also demonstrated that quercetin increases intracellular Ca^{2+} and potentiates glucose-induced insulin secretion which is correlated with ERK1/2 activation (Youl et al., 2010). In the study reported herein, we demonstrated that a glycosylated flavonoid stimulates calcium uptake and also regulates the intracellular calcium pool. This suggests that both intra and extracellular calcium are involved in the mechanism of action of rutin.

The pancreatic β-cell functions are regulated by glucose metabolism. K_{ATP} channels are metabolic sensors that couple glucose metabolism to

cell excitability. Under conditions were high levels of glucose are present an increase in the ATP/ADP ratio inhibits K_{ATP} channels. This inhibition influences the islet plasma membrane potential, causing islet membrane depolarization which activates several ion channels, including VDCCs, voltage-gated sodium (NaV) channels, and potassium channels. Islet cell calcium entry and hormone secretion are precisely modulated by changes in the membrane potential, thus, K_{ATP} channels are important for the maintenance of glucose homeostasis and glucose-stimulated insulin secretion from pancreatic β-cells (Denton and Jacobson, 2012; McTaggart et al., 2010).

Glibenclamide is a antihyperglycemic sulfonylurea that stimulates insulin secretion by directly blocking K_{ATP} channels of the β-cell membrane and increasing $[Ca^{2+}]i$. On the other hand, diazoxide inhibits insulin secretion by opening the K_{ATP} channels in the membrane, with repolarization, closure of the VDCCs and lowering of $[Ca^{2+}]i$ (Mariot et al., 1998). In our study, as expected, we demonstrated an increase in calcium uptake of isolated pancreatic islet with glibenclamide and a decrease with diazoxide. However, the co-treatment with this blocker and this activator of K_{ATP} channels did not alter the stimulatory effect of rutin on calcium uptake, suggesting that this effect is independent of islet membrane K_{ATP} channels.

Our results indicate that rutin stimulates extracellular calcium influx and the L-VDCCs were involved in this rutin action in pancreatic islets. The glucose metabolism in pancreatic β-cells activates several intracellular signaling pathways including intracellular Ca^{2+}-signaling that targets various proteins such as VDCC. The metabolism of glucose leads to closure of the K_{ATP} channels, depolarization of the plasma membrane and subsequently an influx of Ca^{2+} through VDCCs followed by insulin granule exocytosis. Thus, the L-VDCCs play an important role in the spatio-temporal regulation of intracellular calcium concentration ([Ca2+]i) and glucose-stimulated insulin secretion by β-cells. Dysfunctional β-cell VDCCs have been observed in diabetic patients and also in diabetic animal models (Yang and Berggren, 2005).

Phenolic compounds have been demonstrated to interfere with the insulin signaling cascade in the pancreas. They may modulate beta-cell function and insulin secretion in part via this interaction with the insulin signaling cascade (Pinent et al., 2008). Recently, Adisakwattana et al. (2011) demonstrated that a cinnamic acid derivative, *p*-methoxycinnamic acid, stimulated insulin secretion from pancreatic β-cells by increasing Ca^{2+} influx via the L-type Ca^{2+} channels, but not through the closure of ATP-sensitive K^+ channels. In agreement with

these data, our results obtained in a model which closely represents the physiological state, i.e., rat pancreatic islets, indicate that rutin induces intracellular Ca^{2+} increase through L-type Ca^{2+} channels, but without the involvement of the K_{ATP} channels.

The K_{ATP} channel-dependent pathway plays a central role in the β-cell stimulus-secretion coupling. However, it was demonstrated that the elimination of this pathway does not entirely block glucose-stimulated insulin secretion. In this context, K_{ATP} channel-independent pathways which mediate Ca^{2+} influx have been reported to participate in glucose-stimulated insulin secretion (Yang and Berggren, 2006). Herein, we described a novel mechanism of calcium uptake stimulated by rutin that is a K_{ATP} channel-independent pathway. In this way, rutin may act as a potentiator of insulin secretion by amplifying glucose-stimulated action potentials, enhancing the activity of VDCCs and the entry of Ca^{2+}, since we demonstrated that rutin potentiated glucose-induced insulin secretion.

Glucose-induced calcium entry activates various kinases in β-cells, including protein kinase C (PKC) (Nesher et al., 2002). Insulinotropic agents may act either by direct stimulation of insulin secretion or by amplifying insulin secretion induced by other means. Potentiators may activate phospholipase C (PLC) which culminates in PKC activation (Doyle and Egan, 2003). In the study reported herein, it was demonstrated that activation of the PLC / PKC system associated with a higher Ca^{2+} mobilization from both external and internal pools is involved in the stimulatory effect of rutin on calcium uptake in rat pancreatic islets. The physiologic regulation of glucose-induced insulin secretion is dependent upon the activation of information flow in the PLC/ PKC signal transduction system. The enzyme PLC regulates various cellular processes by catalyzing the formation of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol-4,5- bisphosphate (PIP2). In pancreatic β-cells, PLC mediates the potentiation of glucose-induced insulin secretion by many hormones and neurotransmitters (Zawalich et al. 1997; Zawalich and Zawalich 2001). IP3 mediates rapid mobilization of Ca^{2+} from the endoplasmic reticulum (ER), whereas DAG stimulates PKC (Berridge et al., 2003). PKC plays multiple roles in the β-cell signal-transduction pathway. It has been demonstrated that PKC modulates L-type VDCC in β-cells, enabling appropriate functioning of this channel in the insulin secretion process (Arkhammar et al., 1994).

5. Conclusions

In conclusion, rutin potentiated insulin secretion in hyperglycemic rats and significantly stimulated calcium uptake in rat pancreatic islets. A novel cellular mechanism of action of rutin on Ca^{2+} fluxes in pancreatic β-cells was elucidated (**Figure 6**). Rutin modulates calcium uptake in pancreatic islets by opening L-VDCC with the involvement of stored calcium, PLC and PKC signaling pathways, characterizing K_{ATP} channel-independent pathways. This represents a potential insulinotropic action for rutin that may potentiate glucose-induced insulin secretion. These findings highlight rutin, a dietary adjuvant, as a potential insulin secretagogue contributing to glucose homeostasis.

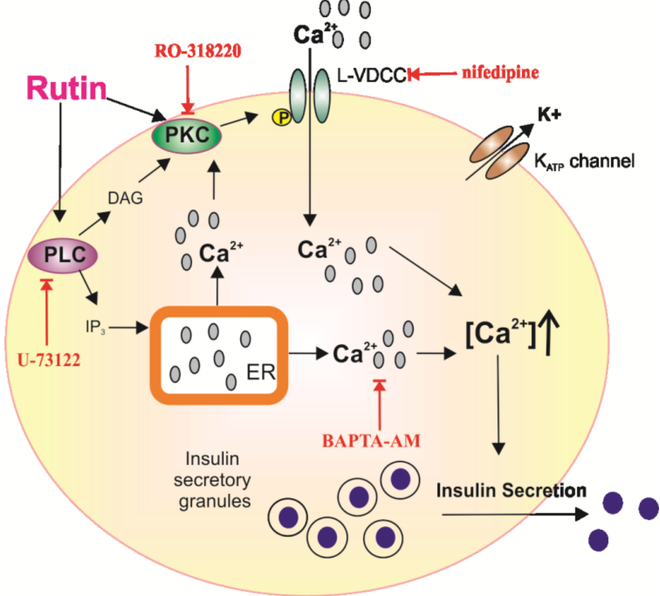


Figure 6. Schematic representation of the main rutin-induced signaling pathways mediating calcium uptake in pancreatic β-cells. Rutin stimulates a variety of signaling pathways such as the PLC / PKC system which phosphorylates L-VDCCs promoting a rapid influx of extracellular calcium into the cells. PLC produces inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol-4,5- bisphosphate (PIP2).IP3, in turn, can also increase the intracellular calcium concentration ($[Ca^{2+}]$) by mobilizing endoplasmic reticulum (ER) calcium stores. DAG activates PKC, at

least partly, by sensitizing it to Ca^{2+}. The increase in intracellular calcium concentration [Ca^{2+}] triggers the fusion of insulin-containing secretory vesicles to the plasma membrane, and exocytosis of insulin follows rapidly resulting in insulin secretion.

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4.3.6 Conclusões parciais

A partir dos resultados obtidos neste trabalho, podemos concluir que:

O tratamento com rutina estimulou a secreção de insulina em ratos hiperglicêmicos, sugerindo um efeito potencial secretagogo de insulina para este flavonoide.

A rutina estimulou *in vitro* a captação de cálcio em ilhotas pancreáticas isoladas.

O mecanismo de estímulo de captação de cálcio pela rutina em ilhotas pancreáticas isoladas envolve canais de cálcio dependentes de voltagem do tipo L e vias de ativação da PLC, PKC e cálcio do estoque, caracterizando uma via indepentente de canais de potássio sensíveis a ATP.

O aumento de cálcio intracelular estimulado pela rutina pode mediar a secreção de insulina pelas células β.

5 DISCUSSÃO GERAL

Os resultados no TOTG demonstraram que, tanto o extrato bruto quanto as frações *n*-butanol e residual aquosa de ambas as espécies, *B. articulata* e *M. x paradisiaca*, reduziram significativamente a glicemia de ratos normais hiperglicêmicos. O efeito da fração *n*-butanol do extrato das folhas de *M. x paradisiaca* foi comparável ao promovido pela tolbutamida, um agente antidiabético da classe das sulfoniluréias. Por outro lado, o extrato bruto e as frações das duas espécies vegetais não apresentaram efeito em ratos diabéticos induzidos por aloxana. Esses resultados indicam um potencial efeito anti-hiperglicêmico para estas plantas.

Corroborando estes resultados, Folador e colaboradores (2010) verificaram que frações da espécie *Wilbrandia ebracteata* também apresentaram melhor efeito na redução da glicemia de ratos hiperglicêmicos após o tratamento com a fração *n*-butanol e ineficácia de ação, quando testados em ratos diabéticos. Portanto, os autores sugeriram que o efeito das frações poderia estar relacionado com a secreção de insulina pelas células β pancreáticas.

Outros estudos também relataram que extratos de plantas com potencial antidiabético, como *Eugenia jambolana* e *Tinospora cordifolia,* só apresentaram efeito em diabetes leve e moderado induzido por estreptozotocina, sem alterar a glicemia de ratos diabéticos severos. Os efeitos observados na glicemia com estas plantas parecem envolver o estímulo da liberação de insulina das células β, bem como a atividade insulino-mimética de algum componente dos extratos ou ainda uma combinação de ambos, uma vez que os extratos não apresentaram efeito em ratos diabéticos severos (GROVER et al., 2000).

Diferentes mecanismos são evidenciados em plantas para reduzir os níveis de glicose. Estudos indicam que algumas plantas exibem propriedades similares aos fármacos pertencentes à classe das sulfoniluréias, como glibenclamida, tolbutamida e glipizida, em que o efeito hipoglicêmico ocorre pelo estímulo da secreção de insulina pelas células β pancreáticas (FOLADOR et al., 2010; MAROO et al., 2002).

O tratamento com o extrato bruto e fração *n*-butanol da *B. articulata* aumentou as concentrações plasmáticas de insulina, apresentando um índice insulinogênico similar à glipizida. A fração *n*-butanol da *M. x paradisiaca* também estimulou a secreção de insulina. Desta forma, os resultados permitem inferir que o efeito anti-hiperglicêmico observado para estas espécies pode, em parte, ocorrer através de mecanismos que envolvam a liberação de insulina das células

β pancreáticas, demonstrando um potencial secretagogo de insulina para os extratos e frações de *B. articulata* e *M. x paradisiaca*.

A insulina é o hormônio mais importante na regulação do metabolismo energético. As ações na homeostasia da glicose incluem, basicamente, síntese e armazenamento de carboidratos, proteínas e lipídeos, modulação do crescimento e diferenciação celulares, bem como a inibição do catabolismo (SALTIEL; KHAN, 2001; TAHA; KLIP, 1999). A insulina regula a síntese de glicogênio através do controle da captação de glicose e pela regulação dos estados de fosforilação e ativação das diversas enzimas envolvidas na síntese e degradação do glicogênio, principalmente a glicogênio sintase e a glicogênio fosforilase. O fígado e o músculo esquelético são os principais depósitos de glicogênio no organismo no estado alimentado. (FERRER et al., 2003; ROACH, 2002; SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999).

O nível de glicogênio pode ser considerado como um bom marcador para assegurar a atividade hipoglicemiante de qualquer fármaco, pois indica que a glicose está sendo estocada no fígado e no músculo na forma de glicogênio através do aumento da glicogênese. Muitas plantas são descritas por apresentarem efeito hipoglicemiante através do aumento da glicogênese, estimulando o consumo de glicose, principalmente, pelo fígado e músculo esquelético para a formação do glicogênio (NEGRI, 2005). Os resultados do presente trabalho demonstraram que, simultaneamente ao estímulo da secreção de insulina, o tratamento, principalmente com a fração *n*-butanol das duas espécies, resultou também em um aumento do conteúdo de glicogênio muscular e hepático, indicando também um efeito significativo em tecidos periféricos responsivos à insulina.

A alta atividade das enzimas no intestino aumenta a digestão dos açúcares e a absorção de glicose, colaborando para a hiperglicemia pós-prandial. Desta forma, uma das maneiras mais efetivas para controlar esse aumento do nível de glicose sanguíneo é inibir as enzimas α-amilase e α-glicosidase. A acarbose é um inibidor das α-glicosidases utilizado na prática clínica para melhorar o controle glicêmico em pacientes diabéticos, reduzindo a absorção dos carboidratos (FOWLER, 2007). Muitas plantas são relatadas por apresentarem efeito anti-hiperglicêmico, através da inibição da atividade das α-glicosidases (GAD et al. 2006; HEACOCK et al., 2006; KIM et al., 2004). Desta forma, foram realizados também experimentos *in vitro* para verificar o efeito inibitório dos extratos de *B. articulata* e *M. x paradisiaca* e suas frações frente a atividade das dissacaridades. Como resultado, foi

verificado que os extratos e as frações de ambas as espécies inibiram significativamente a atividade da maltase, apontando desta forma, para mais um alvo no controle da glicemia.

Durante situações de hiperglicemia prolongada altas concentrações dos AGEs são formadas, que por sua vez, podem ligar-se a proteínas. A glicação de proteínas altera a função celular e causa alterações em vários tecidos. Adicionalmente, a ligação dos AGEs em receptores específicos (RAGEs) pode levar à modificação na sinalização celular e aumentar a produção de radicais livres. Deste modo, o acúmulo de AGEs e o aumento do estresse oxidativo contribuiem com a patogênese das condições associadas às complicações decorrentes da diabetes (GOH; COOPER, 2008).

Evidências sugerem que a prevenção da formação e acúmulo dos AGEs podem, minimizar o desenvolvimento e progressão das complicações da diabetes (GOH; COOPER, 2008). Vários agentes terapêuticos que inibem ou revertem o progresso da glicação, estão sendo investigados e há um interesse crescente em produtos naturais com essas propriedades anti-glicação. De fato, algumas plantas e os fitoconstituintes inibem a formação dos AGEs e a glicação (CHOMPOO et al., 2011; URIOS; BORSOS; STERNBERG, 2007; WU; YEN, 2005).

Neste trabalho também estudou-se o efeito dos extratos e frações de *B. articulata* e *M. x paradisiaca* na formação dos AGEs *in vitro*. Os resultados mostraram um potente efeito inibitório na formação dos AGEs, indicando uma propriedade anti-glicação para estas plantas.

Com relação à caracterização química dos extratos e frações de *B. articulata* e *M. x paradisiaca* foi observado que ambas espécies possuem compostos fenólicos como constituintes majoritários. Especificamente para as espécies do gênero de *Baccharis*, os relatos da literatura destacam a presença de compostos fenólicos (VERDI et al., 2005; DE OLIVEIRA et al., 2006), e os resultados aqui apresentados demonstraram que *B. articulata* possui teores significativos destes compostos, especialmente a fração *n*-butanol. Já para a espécie *M. x paradisiaca* são escassos os trabalhos que descrevam a composição química das folhas desta espécie. No presente trabalho, a análise por cromatografia líquida de alta eficiência (CLAE) permitiu identificar a presença de rutina como composto polifenólico majoritário no extrato e frações das folhas, destacando a presença marcante deste flavonóide na fração *n*-butanol.

A presença de compostos fenólicos, particularmente dos flavonóides, pode estar relacionada com as atividades biológicas observadas *in vivo* e *in vitro* para as espécies estudadas, visto que

existem estudos que relatam a ação destes compostos na homeostasia da glicose. Os flavonóides podem atuar através de diferentes maneiras na manutenção da homeostasia da glicose, incluindo mecanismos relacionados à liberação de insulina das células β pancreáticas, ou através da modulação da atividade de enzimas regulatórias do metabolismo de carboidratos e/ou ações insulino-miméticas ou, ainda, interferindo na absorção de glicose no intestino. Adicionalmente, os flavonóides são conhecidos pela atividade antioxidante e propriedade anti-glicação (CAZAROLLI et al., 2008a, b; WU; YEN, 2005).

Desta forma, identificando a rutina como composto majoritário das folhas de *M. x paradisiaca*, e considerando os relatos prévios na literatura sobre os efeitos deste flavonóide na homestasia da glicose (FERNANDES et al., 2010; PEREIRA et al., 2011), objetivou-se estudar a influência *in vitro* e *in vivo* da rutina na manutenção da homeostasia da glicose, elucidando possíveis mecanismos de ação. Avaliou-se o efeito *in vitro* da rutina na captação de glicose em um tecido-alvo da insulina, o músculo sóleo. Assim como a insulina, a rutina estimulou a captação de glicose e o mecanismo de ação estudado parece envolver as vias clássicas de transdução de sinal do hormônio. Esses resultados indicam que este flavonóide pode atuar como agente insulino-mimético em tecidos responsivos ao hormônio.

Corroborando estes resultados, estudos anteriores já haviam demonstrado que flavonóides podem atuar como agentes insulino-miméticos em um tecido-alvo da ação da insulina, o músculo, aumentando a captação de glicose e o conteúdo de glicogênio (CAZAROLLI et al., 2009 a, b; JORGE et al., 2004; ZANATTA et al., 2008). O canferol 3-neohesperidosídeo, isolado do xaxim-espinhento (*Cyathea phalerata*, Cyatheaceae), foi estudado quanto ao mecanismo de ação na regulação da glicemia. Este flavonóide aumentou a captação de glicose em músculo sóleo através de ações mediadas via transdução de sinal da insulina (ZANATTA et al., 2008), similarmente ao mecanismo de ação proposto para a rutina.

Além da captação de glicose mediada pela insulina, são descritos mecanismos indepententes de insulina que também regulam a captação de glicose no músculo esquelético, nos quais o aumento de cálcio intracelular parece estar envolvido (WITCZAK et al., 2007). Deste modo, também avaliou-se o efeito da rutina na captação de cálcio em músculo sóleo, relacionando-o com a captação de glicose. Os resultados mostraram que a rutina aumentou significativamente os níveis intracelulares de cálcio. Ainda, a captação de glicose estimulada pela rutina em músculo sóleo estaria relacionada com o Ca^{2+} e a via de

ativação da PKCaMII, caracterizando uma via indepentente da sinalização clássica da insulina para captação de glicose estimulada pela rutina.

Estudos demonstraram que compostos naturais, além de ativar vias similares à insulina, caracterizando uma ação insulino-mimética, podem ativar diferentes vias intracelulares independente de insulina para regular a captação de glicose no músculo. Lee e colaboradores (2007) verificaram que o composto ácido caféico fenetil éster (CAPE) estimulou a captação de glicose em células musculares, através da ativação da via da Akt e da AMPK, caracterizando, respectivamente, uma via insulino-mimética e uma via insulina-independente para captação de glicose.

Adicionalmente, no presente trabalho foi demonstrado que o tratamento por via oral com a rutina estimulou a secreção de insulina em ratos normais hiperglicêmicos, indicando que este flavonóide poderia atuar também como secretagogo de insulina. Tendo em vista que o cálcio tem papel importante na secreção de insulina pelas células β, avaliou-se o efeito da rutina na captação de cálcio em ilhotas pancreáticas isoladas. A rutina estimulou a captação de cálcio em ilhotas pancreáticas isoladas, e este aumento intracelular de cálcio pode mediar a secreção de insulina estimulada pela rutina. Recentemente, um estudo demonstrou atividade secretagoga de insulina do extrato das folhas de lótus (*Nelumbo nucifera*) e de seu composto ativo, o flavonóide catequina. Os autores relacionaram essa ação secretagoga ao aumento dos níveis de cálcio intracelular estimulado pelo extrato e à ativação de vias intracelulares envolvidas no processo de secreção de insulina nas células β (HUANG et al., 2011).

Relatos da literatura descrevem a atividade de plantas ricas em flavonóides e de compostos isolados na homeostasia da glicose, seja atuando como insulino-miméticos, secretagogos de insulina ou ambos (CAZAROLLI et al., 2008b; TROJAN-RODRIGUES et al., 2012). O extrato bruto e as frações da *Gentiana Olivieri* (Gentianaceae) e o flavonóide isolado, isoorientina, reduziram a glicemia de ratos normais hiperglicêmicos e diabéticos, após tratamento agudo por via oral. O mecanismo de ação proposto para a isoorientina envolveria a proteção das células β do dano oxidativo e o aumento da secreção da insulina. Além disso, esse flavonóide aumentaria a sensibilidade dos tecidos periféricos em resposta à insulina (SEZIK et al., 2005). Ainda, estudos com luteolina, apigenina e seus glicosídeos demonstraram que estes compostos protegem as células β e aumentam as concentrações

plasmáticas de insulina. Ainda, a redução da glicemia em ratos diabéticos observada após tratamentos crônicos sugere que estes compostos atuam aumentando a utilização da glicose por tecidos periféricos responsivos à insulina, além de estimularem a liberação deste hormônio das células β pancreáticas (PANDA; KAR, 2007; LI et al., 2007).

Os resultados aqui apresentados indicam que a rutina pode atuar de maneira dualística na homeostasia de glicose, tanto como secretagogo de insulina como também aumentando a captação da glicose em tecidos periféricos, através da ativação de diferentes vias intracelulares.

6 CONCLUSÃO GERAL

Com base nos resultados obtidos neste trabalho propõe-se que as espécies vegetais *Baccharis articulata* e *Musa x paradisiaca,* e o flavonóide rutina, aqui estudados, possam regular a homeostasia da glicose. Os mecanismos envolvem a inibição da enzima que permite a absorção intestinal da glicose, a inibição da glicação, o estímulo da secreção de insulina e o aumento na utilização da glicose pelos tecidos periféricos, evidenciando que estas duas espécies, bem como a rutina, podem atuar por múltiplos mecanismos de ação para regular a homeostasia da glicose e colaborar para a prevenção das complicações da diabetes.

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APÊNDICE A – Colaboração em outros trabalhos: Capítulo de livro aceito para publicação

Studies in Natural Products Chemistry, in press, v.37

NATURAL AND SYNTHETIC CHALCONES: TOOLS FOR THE STUDY OF TARGETS OF ACTION - INSULIN SECRETAGOGUE OR INSULIN MIMETIC?

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ABSTRACT: Chalcones are a group of plant-derived polyphenolic compounds that possess a wide variety of biological activities. Several lead compounds with various pharmacological properties have been developed based on the chalcone skeleton. The beneficial effect of these substances has been studied in relation to diabetes mellitus. Several studies have demonstrated that chalcones either from natural or synthetic sources can influence carbohydrate pathways, especially glucose metabolism. These studies verified the effectiveness of chalcones as antihyperglycemic and/or hypoglycemic agents through *in vitro* and *in vivo* experimental responses. In this context, these molecules are attractive substances which can enrich the current therapy

options and they have become the subject of considerable interest in both academia and industry. The purpose of this review is to discuss the recent developments related to the chemistry and medicinal properties of chalcones, especially concerning their role in glucose homeostasis and carbohydrate metabolism. Also, taking into account the relevant structure-activity relationships of these compounds, the development of new approaches to study the interactions of chalcones in specific targets combining *in silico* (computational modeling) and *in vitro* pharmacological studies on β-cells represents a challenge for future perspectives aimed at characterizing molecular targets for diabetes therapy.

APÊNDICE B – Colaboração em outros trabalhos: artigos completos publicados

1. PEREIRA, D.F.; KAPPEL, V.D.; CAZAROLLI, L.H.; BOLIGON, A.A.; ATHAYDE, M.L.; GUESSER, S.M.; DA SILVA, E.L.; SILVA, F.R.M.B. Influence of the traditional Brazilian drink *Ilex paraguariensis* tea on glucose homeostasis. **Phytomedicine**, v. 19,p. 868-877, 2012.

2. CAZAROLLI, L.H.; KAPPEL, V.D.; PEREIRA, D.F.; MORESCO, H.H.; BRIGHENTE, I.M.C.; PIZZOLATTI, M.G.; SILVA, F.R.M.B. Anti-hyperglycemic action of apigenin-6-C-β-fucopyranoside from Averrhoa carambola. **Fitoterapia**, *in press*, 2012.