LUISA HELENA CAZAROLLI

CARACTERIZAÇÃO DE COMPOSTOS NATURAIS E AVALIAÇÃO DA ATIVIDADE INSULINO-MIMÉTICA EM TECIDOS ALVOS DA INSULINA EM ESTUDOS *IN VIVO* E *IN VITRO*

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Tese apresentada ao Programa de Pós-graduação em Farmácia do Centro de Ciências da Saúde da Universidade Federal de Santa Catarina, como requisito parcial para obtenção do título de Doutor em Farmácia.

Orientadora: Profª. Drª. FÁTIMA REGINA MENA BARRETO SILVA

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"Caracterização de compostos naturais e avaliação da atividade insulino-mimética em tecidos alvos da insulina in vivo e in vitro"

POR

Luisa Helena Cazarolli

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RESUMO

A diabetes é considerada uma patologia complexa e multifatorial de elevada morbidade e mortalidade e, por esse motivo, é considerada um problema significativo de saúde pública mundial. É caracterizada por distúrbios no metabolismo de carboidratos, proteínas e lipídios resultantes da absoluta ou relativa insuficiência na secreção e/ou ação da insulina. A classificação da diabetes melito está baseada na etiologia da doença e a divide clinicamente em duas formas básicas: tipo 1 e tipo 2 sendo que, a diabetes melito tipo 2 é a forma prevalente da doença estando presente em 90 a 95% dos casos. Muitas plantas são conhecidas na medicina popular de diferentes culturas pelas propriedades hipoglicemiantes e pelo uso crescente no tratamento da diabetes. Flavonóides são compostos fenólicos, derivados de plantas, que apresentam diversas propriedades e cujo potencial terapêutico é cada vez mais investigado. O presente trabalho teve como objetivos estudar os efeitos do extrato bruto, frações e compostos isolados das folhas da *Averrhoa carambola* na glicemia, na secreção de insulina e no conteúdo de glicogênio em ratos normais hiperglicêmicos. Além disso, estudar o mecanismo de ação do canferol-3-neohesperidosídeo, obtido da *Cyathea phalerata*, bem como da apigenina-6-C-β-L-fucopiranosídeo (composto 1) e da apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo (composto 2) na síntese de glicogênio muscular e na captação de glicose e comparar com o efeito estimulatório da insulina. 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 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 após 3 h da administração dos flavonóides. A síntese de glicogênio muscular e a captação de glicose foram estudadas após a incubação do músculo sóleo com os respectivos flavonóides e/ou insulina, na presença ou não de diferentes inibidores e do radioisótopo no período de 1 h. O extrato bruto, as frações acetato de etila, *n*-butanol e os flavonóides apigenina-6-C-β-L-fucopiranosídeo (composto 1) e apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo (composto 2) reduziram significativamente a glicemia de ratos normais hiperglicêmicos e potencializaram a secreção de insulina induzida por glicose. Além disso, os compostos 1 e 2 aumentaram o conteúdo de glicogênio no músculo sóleo e fígado após os tratamentos. A síntese de glicogênio foi estimulada significativamente pelo canferol-3-neohesperidosídeo e pela apigenina-6-C-β-L-fucopiranosídeo. Este aumento foi mediado através da via da PI3K-PKB-GSK-3 e MAPK-PP1. A apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo (composto 2) estimulou a captação de glicose no músculo sóleo através de uma via, pelo menos parcialmente comum, à via de sinalização da insulina. Os resultados da ação dos flavonóides estudados neste trabalho na regulação da homeostasia da glicose, em estudos *in vivo* e *in vitro*, demonstram o potencial efeito insulino-mimético e/ou anti-hiperglicêmico destes compostos.

Palavras-chave: flavonóides, síntese de glicogênio, captação de glicose, hiperglicemia, *Averrhoa carambola*, diabetes.

ABSTRACT

Diabetes is a complex and multifactorial disease which presents high death rates and morbidity. Because of that, diabetes is considered a World public health problem. It is characterized by hyperglycemia resulting from defects in insulin secretion and/or in insulin action. Basically, this disorder is divided into type 1 and type 2 diabetes and the last one represents the great majority of the cases. Many plant species are known in popular medicine for their hypoglycemic properties and their increasing use for treating diabetes. Flavonoids are phenolic compounds derived from plants, which present diverse properties and whose therapeutic potential is being increasingly investigated. The objectives of the present investigation were to study the effects of crude extract, fractions and isolated compounds, apigenin-6-C-β-L-fucopyranoside (compound 1) and apigenin-6-C-(2”-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (compound 2) from leaves of *Averrhoa carambola* on glycemia, on insulin secretion as well as on glycogen content in muscle and liver from hyperglycemic rats. In addition, we intended to study the mechanism of action of kaempferol-3-neohesperidoside, obtained from *Cyathea phalerata*, and of apigenin-6-C-β-L-fucopyranoside (compound 1) and apigenin-6-C-(2”-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (compound 2) on glycogen synthesis and on glucose uptake and compare them with the stimulatory effect of insulin. Male Wistar rats aged 50-55 days were used. To the glucose tolerance curve, blood samples were collected just prior to and at 15, 30, 60, 120 and 180 min after the glucose loading and serum glucose levels were measured. In the assays for the determination of the glycogen content tissues were removed from animals after 3 h of the administration of the compounds 1 and 2. The glycogen synthesis and the glucose uptake were studied after incubation of the soleus muscle for 1 h with the respective flavonoids and/or insulin, in the presence or not of different inhibitors and of the radioisotope. The oral administration of crude extract, ethyl acetate and *n*-butanol fractions and compounds 1 and 2 of *Averrhoa carambola* leaves exhibited a potential hypoglycemic activity in the hyperglycemic normal rats and potentiated the glucose-induced insulin secretion. Additionaly, compounds 1 and 2 also increased glycogen content in muscle and liver from the hyperglycemic rats after 3 h of the treatments. The glycogen synthesis was significantly stimulated by kaempferol-3-neohesperidoside as well as by apigenin-6-C-β-L-fucopyranoside (compound 1). This effect is mediated through the PI3K-PKB-GSK-3 and MAPK-PP1 pathways. Furthermore, apigenin-6-C-(2”-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (compound 2) stimulated glucose uptake in soleus muscle through the same signaling pathway as insulin. The results observed in this work support the flavonoid’s effect in glucose homeostasis through *in vivo* and *in vitro* studies. This demonstrates the insulinmimetic and/or antihyperglycemic potential of these compounds.

Key-words: flavonoids, glycogen synthesis, glucose uptake, hyperglycemia, *Averrhoa carambola,* diabetes.

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LISTA DE FLUXOGRAMAS

LISTA DE ABREVIATURAS

AcOEt	Acetato de etila
ADP	Adenosina difosfato
AMPK	Proteína cinase ativada por 5'-AMP
APS	Substrato da proteína associada
ATP	Adenosina trifosfato
AS160	Proteína substrato da Akt de 160 kDa
CAP	Proteína associada à cbl
[^{14}C]DG	[U-^{14}C]-2-deoxi-D-glicose
[^{14}C]G ou D[U-^{14}C]G	D - [^{14}C (U)] – glicose
CEUA	Comitê de Ética do Uso de Animais
C3G	Proteína trocadora de nucleotídeos
cpm	Contas por minuto
DPP-IV	Dipeptidilpeptidase 4
ERK	Cinase reguladora de sinal extracelular
EtOH	Etanol
GAP	Proteína ativadora de GTPases
GDP	Guanosina difosfato
GIP	Polipeptídeo insulinotrópico
GLP-1	Peptídeo semelhante ao glucagon 1
GLUT	Transportador de glicose
G-6-P	Glicose 6 fosfato
G-1-P	Glicose 1 fosfato
Grb2	Proteína 2 ligada ao receptor de fator de crescimento
GS	Glicogênio sintase
GSK-3	Glicogênio sintase cinase-3
GTP	Guanosina trifosfato

HMIT	Transportador H^+ ligado ao mio-inositol
IRS	Substrato do receptor de insulina
JAK2	Janus cinase 2
JNK	Cinase c-jun NH_2 terminal
KIF	proteína quinesina
K_m	Constante Cinética de Michaelis Menten
MAPK	Proteína cinase ativada por mitógeno
MEK	Proteína cinase cinase ativada por mitógeno
mTOR	Alvo da rapamicina em mamíferos
n-BuOH	*n*-butanol
NPH	Insulina com protamina neutra de Hagedorn
PDK-1 e 2	Proteína cinase 1 e 2 dependentes de PI-3K
PH	Domínios de homologia de plekstrina
PI-3K	Fosfatidilinositol 3-cinase
PIP3	Fosfatidilinositol-3,4,5-trifosfato
PKA	Proteína Cinase A
PKB/AKt	Proteína Cinase B
PKC	Proteína Cinase C
p38 MAPK	p38 Proteína cinase ativada por mitógeno
PP1	Proteína fosfatase 1
PP1G	Isoforma da proteína fosfatase 1 ligada ao glicogênio
PRG	Proteína regulatória da glicocinase
PTB	Domínios de ligação a fosfotirosina
PTG	Proteína regulatória da PP1
PTPase	Proteína fosfatase
PTP1B	Proteína fosfatase 1B
$p70^{rsk}$	p70 ribossomal S6 cinase
$p90^{rsk}$	p90 ribossomal S6 cinase
RTK	Receptor do tipo tirosina cinase

SH2	Homólogo 2 Src
SHP2	Proteína tirosina fosfatase homóloga de Src 2
SNAP23	Proteína de 23 kD associada à sinaptossoma
SNARE	Proteína do receptor ligada a NSF solúvel
SOCS 1 e 3	Proteínas supressoras da sinalização de citocinas
UDP-G	Uridina difosfato glicose
VAMP2	Proteína 2 da vesícula associada à membrana

SUMÁRIO

1 INTRODUÇÃO

1.1 DIABETES MELITO

Diabetes melito é um grupo heterogêneo de distúrbios metabólicos que apresentam em comum a hiperglicemia. É caracterizada por distúrbios no metabolismo de carboidratos, proteínas e lipídios resultantes da absoluta ou relativa insuficiência na secreção e/ou ação da insulina. (ISLAS-ANDRADE et al., 2000; SOCIEDADE BRASILEIRA DE DIABETES, 2003; SOCIEDADE BRASILEIRA DE DIABETES, 2007; AMERICAN DIABETES ASSOCIATION, 2008; WHO, 2009a). Segundo estudos, no ano de 2000 aproximadamente 171 milhões de adultos com mais de 20 anos tinham diabetes em todo o mundo. Além disso, estima-se que neste mesmo ano, 2,9 milhões de pessoas morreram em conseqüência da doença (WILD et al., 2004; ROGLIC et al., 2005; WHO, 2009b). Em 2007, já eram 246 milhões de pessoas com idade entre 20 e 79 anos com diabetes (7,3% da população adulta) (INTERNATIONAL DIABETES FEDERATION, 2009). Projeções indicam que pode haver 366 milhões de casos até o ano de 2030 (GADSBY, 2002; WILD et al., 2004; WHO, 2009b). No Brasil, a prevalência de pacientes diabéticos em 2000 era de 4,6 milhões de casos, em 2007 6,9 milhões e estima-se que até o ano de 2030 sejam 11,3 milhões de diabéticos (WILD et al., 2004; INTERNATIONAL DIABETES FEDERATION, 2009; WHO, 2009c).

A classificação da diabetes melito atualmente é baseada na etiologia da doença sendo dividida clinicamente em duas formas básicas: tipo 1 e tipo 2 (SOCIEDADE BRASILEIRA DE DIABETES, 2003; SOCIEDADE BRASILEIRA DE DIABETES, 2007; AMERICAN DIABETES ASSOCIATION, 2008). A diabetes melito tipo 1, presente em 5 a 10% dos casos, se caracteriza pela destruição auto-imune das células β pancreáticas mediada por células T CD4+ e CD8+ bem como macrófagos infiltrados nas ilhotas (ROSMALEN et al., 2002; KAWASAKI et al., 2004; GILLESPIE, 2006). Inúmeros fatores genéticos e ambientais contribuem para a ativação imunológica que desencadeia esse processo destrutivo e que se caracteriza pela ausência da secreção endógena de insulina (KAWASAKI et al., 2004; GILLESPIE, 2006; AMERICAN DIABETES ASSOCIATION, 2008). A diabetes melito tipo 2 é a forma prevalente da doença estando presente em 90 a 95% dos casos sendo que a maioria dos pacientes apresenta sobrepeso ou obesidade (SOCIEDADE BRASILEIRA DE DIABETES, 2003; AMERICAN DIABETES ASSOCIATION, 2008). Esta desordem metabólica se caracteriza por dois defeitos fisiopatológicos principais: a redução da secreção de insulina pelas células β pancreáticas e a resistência periférica à ação da insulina em diversos tecidos (músculo, fígado e tecido adiposo) (LEROITH, 2002; CHENG, FANTUS, 2005; KASUGA, 2006). A resposta inicial da célula β à resistência a insulina é aumentar a secreção do hormônio promovendo uma hiperinsulinemia. À medida que a doença progride, a produção bem como a secreção de insulina diminuem gradativamente levando a estágios progressivos de hiperglicemia (LEROITH, 2002; KASUGA, 2006).

A resistência a insulina geralmente precede o início da diabetes e pode ser influenciada por inúmeros fatores genéticos e ambientais como, por exemplo, idade, obesidade e sedentarismo. Indivíduos insulino-resistentes apresentam diversas outras características como dislipidemia, hipertensão, excesso de adiposidade visceral, fibrinólise alterada, disfunção endotelial, inflamação vascular e aterosclerose prematura (CHENG; FANTUS, 2005). Além disso, defeitos na função do receptor e/ou na sinalização da insulina pós-receptor, aumento da lipólise no tecido adiposo com conseqüente elevação das concentrações plasmáticas de ácidos graxos livres e inabilidade de suprimir a produção de glicose hepática são condições que contribuem para a patogênese da diabetes (LEROITH, 2002; KASUGA, 2006).

A diabetes é considerada uma patologia complexa e multifatorial de elevadas morbidade e mortalidade e, por esse motivo, é considerada um problema significativo de saúde pública mundial. O número de casos de diabetes vem aumentando gradativamente entre a população, devido basicamente ao crescimento populacional e aumento da expectativa de vida, urbanização, obesidade

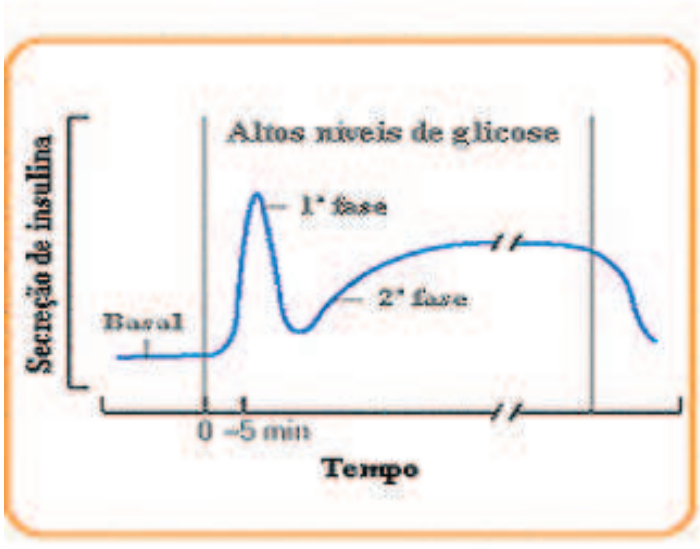
e sedentarismo (NARAYAN et al., 2000; BOYLE et al., 2001; GADSBY, 2002; WILD et al., 2004).

1.2 INSULINA E MECANISMOS DE TRANSDUÇÃO DE SINAL

A insulina é um hormônio polipeptídico produzido pelas células β das ilhotas de Langerhans no pâncreas. É composta por 51 aminoácidos dispostos em duas cadeias polipeptídicas, A e B, unidas através de pontes dissulfeto. A síntese ocorre a partir de um precursor de 110 aminoácidos, a pré-pró-insulina no retículo endoplasmático rugoso onde é clivada a pró-insulina. Esta é convertida à insulina e armazenada no complexo de golgi (KRAUSS, 2008). A glicose é o estímulo primário para a secreção de insulina, embora outros mecanismos de controle possam estar envolvidos como a ação coordenada de vários nutrientes, hormônios gastrintestinais, pancreáticos e neurotransmissores (BEARDSALL et al., 2003).

Quando estimulada por glicose, a secreção de insulina é bifásica. Uma fase inicial rápida (4 – 8 minutos) e transitória resultante da liberação da insulina estocada de grânulos localizados próximos à membrana plasmática (estoque de liberação rápida). Uma segunda fase de maior duração ocorre através do deslocamento e ativação de grânulos citoplasmáticos (estoque de reserva) em direção à membrana bem como através da síntese do hormônio (Figura 1A) (RORSMAN, 1997; MACDONALD et al., 2005; OHARA-IMAIZUMI; NAGAMATSU, 2006; HENQUIN et al., 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 (KRAUSS, 2008).

A secreção de insulina ocorre, principalmente, em função da hiperglicemia. Quando a célula β é exposta à glicose, esta é internalizada através de difusão facilitada pelo GLUT2. No interior da célula a glicose é fosforilada a glicose 6-fosfato pela glicocinase e então, metabolizada. A elevação do metabolismo da glicose aumenta a relação ATP/ADP citoplasmática gerando um fechamento de canais de potássio (K^+) dependentes de ATP. A redução da saída de potássio causa uma despolarização da membrana das células β e abertura de canais de Ca^{2+} dependentes de voltagem com conseqüente aumento do influxo de cálcio que, por sua vez, desencadeia o processo de exocitose dos grânulos de insulina (Figura 1B) (RORSMAN, 1997; MACDONALD et al., 2005; OHARA-IMAIZUMI; NAGAMATSU, 2006).



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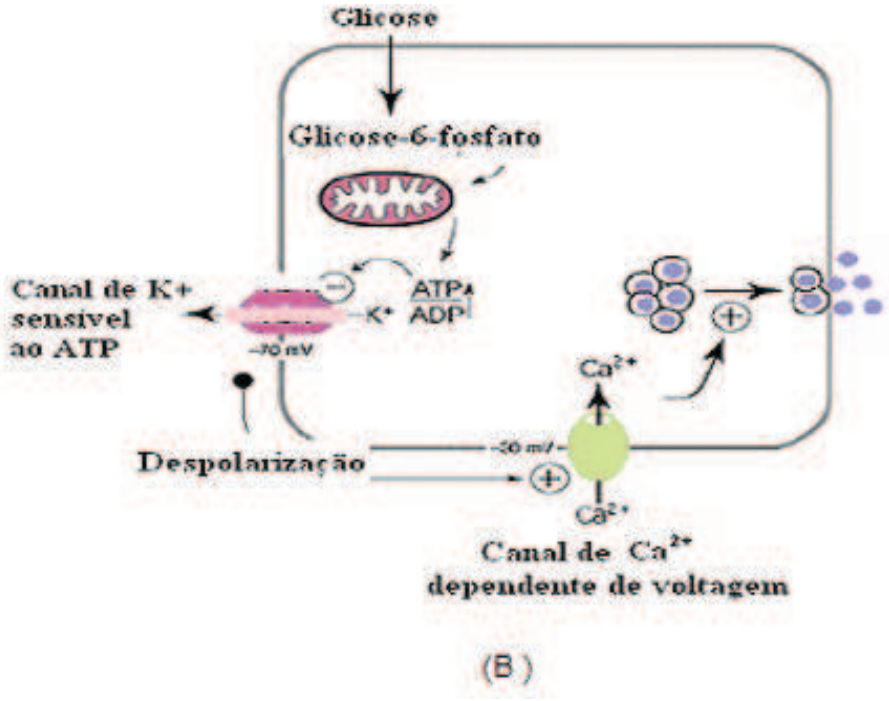


Figura 1. Secreção bifásica de insulina estimulada por glicose (A) (adaptado de http://www.servier.com/imgs/Pro/diabeto/diabetographia/ud/1/1.gif). Processo de secreção de insulina pelo pâncreas (B) (adaptado de http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=diabetes.section.621).

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 (HEI, 1998; TAHA; KLIP, 1999). A insulina exerce os efeitos biológicos através da fosforilação/desfosforilação de proteínas, resultando em aumento do transporte e metabolismo de glicose, mitogênese, bem como, através do controle da atividade/síntese de enzimas regulatórias de vias metabólicas centrais (TAHA; KLIP, 1999).

Para exercer os efeitos metabólicos, a insulina se liga a um receptor específico da membrana das células. O receptor de insulina é uma proteína heterotetramérica pertencente à família de receptores tirosina cinases (RTKs). 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 (TAHA; KLIP, 1999; SALTIEL; KAHN, 2001; CHANG et al., 2004). 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 passa por mudanças conformacionais que resultam no aumento ainda maior da atividade tirosina cinase do receptor (Figura 2) (SALTIEL; KAHN, 2001; CHANG et al., 2004).

Uma vez ativado, o receptor de insulina promove a fosforilação de diversos substratos protéicos em resíduos de tirosina como, por exemplo, a família de substratos do receptor de insulina (IRS 1-4). Outros substratos incluem Shc, Gab-1, $p60^{dok}$, Cbl, JAK2 e APS (TAHA; KLIP, 1999; CHANG et al., 2004). As proteínas IRS-1 e IRS-2 estão amplamente distribuídas nos tecidos de mamíferos e são as mais importantes para as ações 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 e ambas as isoformas são importantes para o metabolismo da glicose no fígado (KROOK et al., 2004). As funções das IRS-3 e IRS-4 são menos entendidas, mas parecem envolver a regulação das proteínas IRS-1 e IRS-2 (HEI, 1998; SALTIEL; KAHN, 2001).

Em geral, as proteínas IRS apresentam regiões em comum: domínios de homologia com plekstrina (PH) que ligam fosfolipídios, domínios de ligação a fosfotirosina (PTB), sítios de ligação a proteínas com domínios com homologia Src 2 (SH2), regiões ricas em prolina e regiões ricas em serina/treonina. 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 nas IRS (TAHA; KLIP, 1999; KAHN; PESSIN, 2002; TANIGUCHI et al., 2006; KRAUSS, 2008).

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-se a PTP1B e a SHP2 (TAHA; KLIP, 1999; SALTIEL; KAHN, 2001; TANIGUCHI et al., 2006; KRAUSS, 2008).

Os resíduos tirosina fosforilados das IRS formam sítios de ligação para proteínas com domínios SH2 (TAHA; KLIP, 1999; KAHN; PESSIN, 2002; TANIGUCHI et al., 2006). Muitas dessas proteínas são moléculas 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 (SALTIEL; KAHN, 2001; KRAUSS, 2008).

A PI3K é uma enzima importante na regulação da mitogênese e das ações metabólicas da insulina como, por exemplo, o estímulo da translocação dos transportadores de glicose GLUT4 para a membrana. Consiste de uma subunidade catalítica (p110) e uma subunidade regulatória (p85). 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) (TAHA; KLIP, 1999; SALTIEL; KAHN, 2001; 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) (HEI, 1998; SALTIEL; KAHN, 2001; KAHN; PESSIN, 2002; KRAUSS, 2008).

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

Um dos principais alvos fisiológicos da PKB é a glicogênio sintase cinase 3 (GSK-3). A fosforilação e inativação da GSK-3 resulta em aumento da síntese de glicogênio. A PKB também 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 está envolvida na reorganização do citoesqueleto (HAJDUCH et al., 2001; KROOK et al., 2004; TANIGUCHI et al., 2006). Além disso, a PKB atua na síntese e bloqueio da degradação de proteínas através da fosforilação do alvo da rapamicina em mamíferos (mTOR) que por sua vez

ativa a p70S6 cinase ($p70^{rsk}$) assim como 4E-BP1 e eIF-4E (TAHA; KLIP, 1999; SALTIEL; KAHN, 2001; TANIGUCHI et al., 2006; KRAUSS, 2008).

Assim como a PKB, as isoformas atípicas de PKC (ξ, λ) estão envolvidas na translocação do GLUT4, induzida pela insulina, para a membrana plasmática. Além disso, as isoformas atípicas de PKC exercem efeitos na síntese de glicogênio, secreção de insulina e no remodelamento do citoesqueleto (TANIGUCHI et al., 2006; LIU et al., 2006).

Além da ativação da PI3K, diferentes cascatas de sinalização intracelulares são necessárias para que a insulina estimule o transporte de glicose. Uma das vias propostas é a da CAP/Cbl. Esta cascata envolve a ligação da proteína adaptadora APS ao receptor de insulina com conseqüente recrutamento e fosforilação de Cbl que, em geral, se encontra 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 GDP por GTP da proteína TC10 ativando-a. Uma vez ativada, a TC10 causa um sinal subseqüente para a translocação do GLUT4 em paralelo à ativação da via da PI3K (KAHN; PESSIN, 2002; CHANG et al., 2004).

Além da via da PI3K e semelhante a outros fatores de crescimento, a insulina estimula a via da proteína cinase ativada por mitógeno (MAPK) relacionada ao crescimento, sobrevivência e diferenciação celular. Essa via se inicia com a fosforilação das proteínas IRS e/ou Shc, que interagem com a proteína Grb2. A Grb2 está constitutivamente ligada a SOS, proteína que troca GDP por GTP da Ras ativando-a. Uma vez ativada, a Ras promove o recrutamento para a membrana e a ativação da Raf que resulta na ativação da MEK1 e MEK2 as quais fosforilam e ativam as MAPK/ERK1 e ERK2 em resíduos tirosina e treonina. As MAPK ativadas fosforilam diversos alvos intracelulares entre eles a p90S6 cinase ($p90^{rsk}$), fosfolipase A_2 e fatores de transcrição como o ELK1 e $p62^{TCF}$ (TAHA; KLIP, 1999; SALTIEL; KAHN, 2001; TANIGUCHI et al., 2006; KRAUSS, 2008).

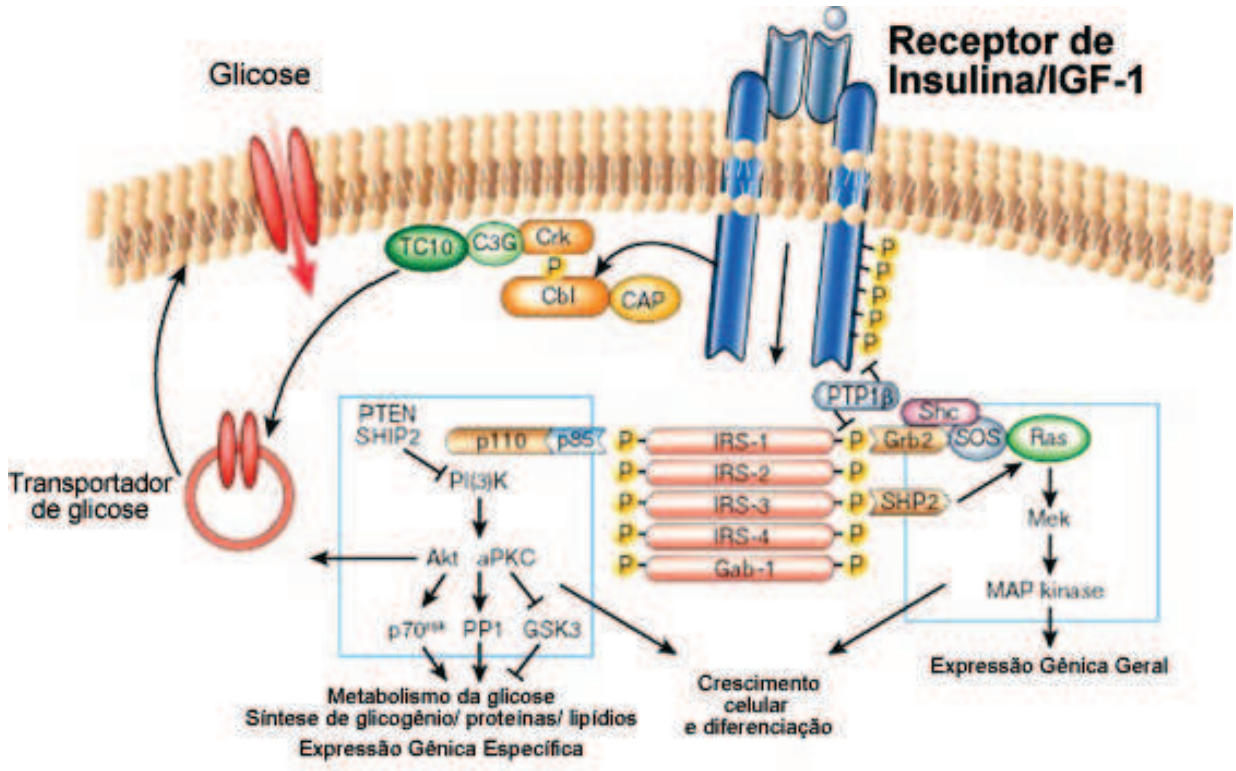


Figura 2. Vias de sinalização da insulina (adaptado de SALTIEL; KAHN, 2001).

1.3 REGULAÇÃO DO METABOLISMO DA GLICOSE

1.3.1 Transportadores de glicose e Captação de Glicose

A captação de glicose nas células ocorre através de difusão facilitada por transportadores específicos (KAHN; PESSIN, 2002). 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 carboidratos é variável. Essas proteínas são divididas em 3 classes principais, sendo a classe I a melhor caracterizada e compreende os GLUTs 1-4 e o GLUT 14 (KAHN; PESSIN, 2002; MANOLESCU et al., 2007).

O GLUT1 é amplamente expresso, principalmente nos eritrócitos e células endoteliais e é responsável pela captação basal de glicose. A isoforma GLUT2 é expressa principalmente nas células β e no fígado além da membrana basal tubular dos rins e no intestino. O GLUT2 possui baixa afinidade (K_m elevado) para a glicose 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 esquelético, coração, placenta e durante o desenvolvimento fetal. O GLUT4 é a isoforma predominante nos tecidos sensíveis à insulina, como músculo esquelético e cardíaco e tecido adiposo e é responsável pelo transporte de glicose estimulado por insulina (WATSON; PESSIN, 2001; KAHN; PESSIN, 2002; MANOLESCU et al., 2007). A classe II é composta pelos GLUTs 5, 7, 9 e 11 e a classe III pelos transportadores GLUT 6, 8, 10, 12 e 13 (HMIT) (MANOLESCU et al., 2007).

A insulina estimula a captação de glicose no músculo e tecido adiposo através de vias de sinalização complexas que iniciam com a ligação da insulina ao receptor de membrana e ativação da atividade tirosina cinase do receptor (TAHA; KLIP, 1999; KAHN; PESSIN, 2002; KRAUSS, 2008). Uma vez ativado, o receptor de insulina promove a fosforilação das proteínas IRS-1 e IRS-2 que ativadas promovem a subseqüente 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) (TAHA; KLIP, 1999; KAHN; PESSIN, 2002; CHANG et al., 2004; KRAUSS, 2008).

A PKB 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; WATSON; PESSIN, 2007; HOU; PESSIN, 2007). Assim como a PKB, as isoformas atípicas de PKC (ξ, λ) regulam a translocação do GLUT4 induzida por insulina para a membrana (TANIGUCHI et al., 2006; LIU et al., 2006).

Além da ativação da PI3K, outras cascatas de sinalização intracelulares são necessárias para que a insulina estimule o transporte de glicose. Uma das vias propostas é a da CAP/Cbl que ocorre nos microdomínios lipídicos da membrana. Esta via resulta na ativação da proteína TC10, membro da família Rho de GTPases que regulam a actina do citoesqueleto. Além disso, após a ativação, a TC10 pode interagir com diversas moléculas efetoras como a proteína CIP4 e com componentes do complexo *exocyst* como o Exo70, ambos envolvidos no processo de ancoragem, ligação e fusão das vesículas contendo GLUT4 com a membrana plasmática (KAHN; PESSIN, 2002; CHANG et al., 2004; HOU; PESSIN, 2007).

A translocação dos transportadores dos estoques intracelulares para a membrana é considerada o passo limitante do processo de captação de glicose (KAHN; PESSIN, 2002). Sob condições basais, o GLUT4 está localizado em compartimentos intracelulares responsivos a insulina

conhecidos como estruturas tubulovesiculares. A translocação do GLUT4 dos compartimentos de armazenamento para a membrana após estímulo da insulina e o retorno para as vesículas de armazenamento envolve um processo complexo de exocitose e endocitose com tráfego, ligação e/ou fusão de vesículas com a membrana plasmática (HEI, 1998; WATSON; PESSIN, 2007; HOU; PESSIN, 2007).

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).

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 munc18c expondo o domínio de ligação do complexo ternário à VAMP2, promovendo a fusão das vesículas com a membrana (HEI, 1998; CHANG et al., 2004; WATSON; PESSIN, 2007; HOU; PESSIN, 2007).

Também vale a pena mencionar que 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), para revisão ver Musi; Goodyear, (2003) e Krook et al., (2004).

1.3.2 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 (SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999; ROACH, 2002).

A insulina regula a síntese de glicogênio em duas etapas: a primeira através do controle da captação e transporte de glicose e a segunda pela regulação dos estados de fosforilação e ativação das enzimas envolvidas na síntese e degradação do glicogênio (VILLAR-PALASÍ; GUINOVART, 1997; SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999; ROACH, 2002). Após entrar na célula, a glicose é fosforilada a glicose 6-fosfato pela hexocinase muscular e/ou pela glicocinase/hexocinase hepáticas. A glicose 6-fosfato (G-6-P) é convertida à 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 (Figura 3) (SRIVASTAVA; PANDEY, 1998; ROACH, 2002; FERRER et al., 2003).

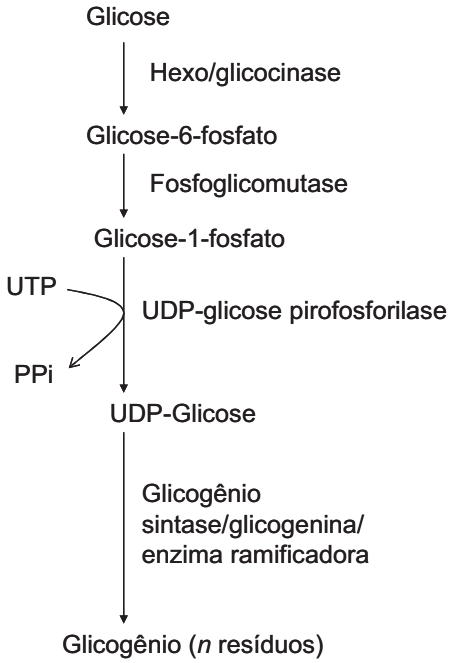


Figura 3. Síntese de glicogênio (Adaptado de SRIVASTAVA; PANDEY, 1998).

A GS é uma proteína multimérica, cuja atividade é regulada por mecanismos alostéricos e de fosforilação/desfosforilação. Ela existe basicamente sob duas isoformas em mamíferos, uma expressa no fígado e outra no músculo e em diversos outros tecidos (SRIVASTAVA; PANDEY, 1998; ROACH, 2002). A insulina modula a atividade da GS através de modificação covalente, translocação e regulação alostérica (VILLAR-PALASÍ; GUINOVART, 1997; BRADY; SALTIEL, 2001; FERRER et al., 2003).

Os mecanismos moleculares pelos quais a insulina regula o metabolismo do glicogênio são complexos e podem variar entre diferentes tipos celulares. Uma das cascatas envolvidas na ativação da GS é a da PI3K. A ativação da PI3K pela insulina resulta na ativação das PDK1 e PDK2 que por sua vez fosforilam e ativam a PKB. A PKB ativada 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. A GSK-3 existe sob duas isoformas, GSK-3α e β, constitutivamente ativas no estado basal que promovem a fosforilação nos sítios 3a, 3b, 3c e 4 da glicogênio sintase inativando-a. A inibição da GSK-3 reduz a taxa de fosforilação da GS resultando em ativação da enzima e conseqüente aumento da síntese de glicogênio (SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999; HAJDUCH et al., 2001; MORA et al., 2005; FORDE; DALE, 2007). 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 4) (BRADY; SALTIEL, 2001; ROACH, 2002).

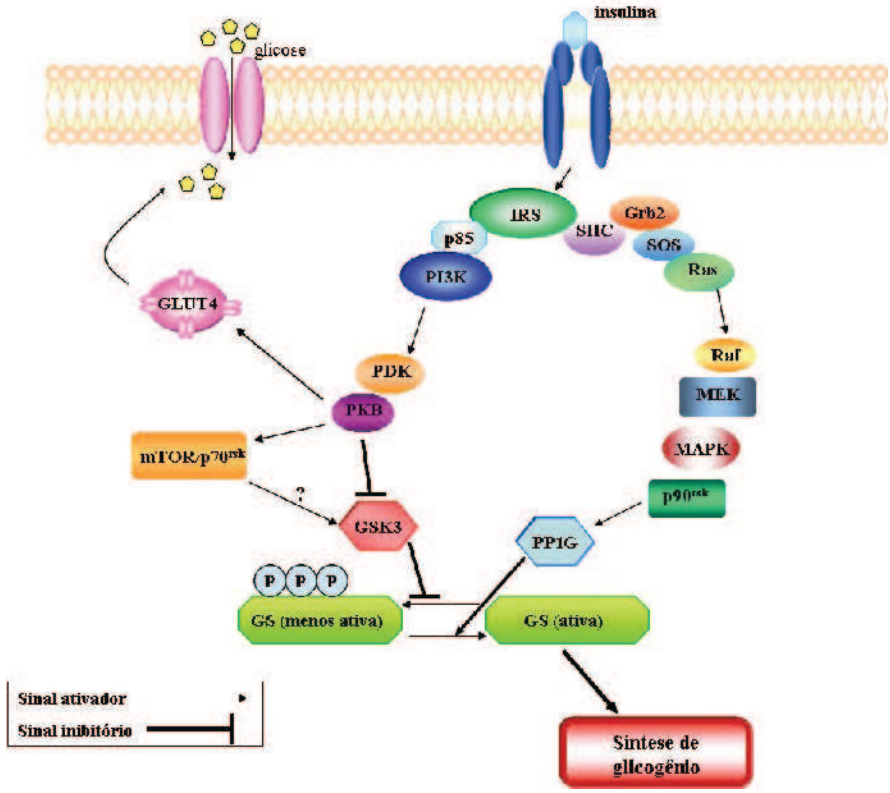


Figura 4. Cascata de fosforilação e ativação da glicogênio sintase (Adaptado de SRIVASTAVA; PANDEY, 1998).

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) (SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999; BRADY; SALTIEL, 2001; KRAUSS, 2008).

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 PTG/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 (SRIVASTAVA; PANDEY, 1998; RAGOLIA; BEGUM, 1998; BRADY; SALTIEL, 2001; ROACH, 2002; KRAUSS, 2008). A insulina promove a fosforilação e ativação da PP1. O mecanismo proposto inicialmente é através da cascata da MAPK, levando a ativação da $p90^{rsk}$ e esta, fosforilando e aumentando a atividade da PP1. No entanto, o envolvimento desta via na ativação da PP1 deve ser melhor estudado (SRIVASTAVA; PANDEY, 1998; TAHA; KLIP, 1999; BRADY; SALTIEL, 2001).

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 levando a uma mudança conformacional que converte a enzima em um substrato melhor para a ação das fosfatases (VILLAR-PALASÍ; GUINOVART, 1997; SRIVASTAVA; PANDEY, 1998; FERRER et al., 2003).

Um dos mecanismos propostos para a regulação alostérica da síntese de glicogênio envolve a ligação entre a ativação da GS e a inativação da glicogênio fosforilase. Isto ocorre devido à inibição

da desfosforilação da GS pela forma fosforilada da glicogênio fosforilase (fosforilase **a**). Desta forma sinais como a G-6-P que ativam a síntese de glicogênio causam inicialmente a desfosforilação e inibição da glicogênio fosforilase seguido da liberação da GS da inibição promovida pela forma ativa da glicogênio fosforilase resultando no aumento da síntese de glicogênio (ROACH, 2002; AISTON et al., 2003a,b).

A ligação da G-6-P à glicogênio fosforilase reduz o efeito inibitório dessa enzima sobre a glicogênio sintase o que facilita a ativação da GS (VILLAR-PALASÍ; GUINOVART, 1997; SRIVASTAVA; PANDEY, 1998; FERRER et al., 2003). Em hepatócitos, quando as concentrações intracelulares de glicose estão reduzidas a glicogênio fosforilase **a** (forma fosforilada, mais ativa) inibe a desfosforilação da glicogênio sintase mantendo-a na forma inativa (fosforilada). Em concentrações aumentadas de glicose, a G-6-P se ligaria à glicogênio fosforilase inibindo-a diretamente e também facilitando a atividade da PP1 na desfosforilação da glicogênio sintase. A conversão da glicogênio fosforilase para a forma menos ativa (desfosforilada) também diminui a inibição que esta enzima exerce na própria PP1, permitindo sua ativação (ROACH, 2002; FERRER et al., 2003; AISTON et al., 2003a).

A potência da G-6-P para a ativação da GS em hepatócitos depende da origem, uma vez que a G-6-P oriunda da ação da glicocinase é mais efetiva em mediar a ativação da enzima do que o mesmo metabólito oriundo da hexocinase. Como resultado, no hepatócito a deposição de glicogênio está sujeita a um sistema de controle onde a GS é regulada pela glicocinase e pelo transporte de glicose através do GLUT2 (capacidade de fosforilar a glicose). Já no músculo, o controle da síntese é mantido entre o transporte de glicose estimulado pela insulina através do GLUT4 e a GS já que a hexocinase I possui alta afinidade para glicose (independente da capacidade de fosforilar a glicose) (VILLAR-PALASÍ; GUINOVART, 1997; FERRER et al., 2003).

Além disso, as enzimas chaves do metabolismo do glicogênio mudam as localizações intracelulares em resposta à glicose o que constitui um mecanismo adicional de controle. Na ausência de carboidratos, a glicocinase está localizada no núcleo dos hepatócitos ligada à proteína regulatória da glicocinase (PRG) e se move para o citosol quando as concentrações de glicose intracelulares aumentam. Já a glicogênio sintase se encontra distribuída no citosol na ausência de glicose e se acumula na periferia da membrana quando as concentrações dessa hexose aumentam (FERRER et al., 2003). No músculo, a hexocinase se encontra associada à membrana mitocondrial quando as concentrações de G-6-P estão baixas, o que estimula a atividade da enzima. Já a GS se encontra no núcleo em concentrações baixas de glicose e transloca para o citosol quando as concentrações aumentam (FERRER et al., 2003).

As mudanças nas distribuições intracelulares das enzimas ativadas pela glicose se correlacionam com o estímulo da síntese de glicogênio. As partículas que dão origem às novas moléculas de glicogênio se encontram próximas à membrana da célula e inicialmente, a síntese se concentra nessa região. À medida que a síntese progride, esta se torna ativa no interior celular em adição à periferia da membrana (FERRER et al., 2003).

A degradação do glicogênio é catalisada pela glicogênio fosforilase resultando em glicose 1 fosfato, substrato para a glicose 6-fosfatase. A queda dos estoques de glicogênio é a primeira resposta dos tecidos, especialmente do fígado, para a manutenção das concentrações normais de glicose sangüínea frente a uma redução das concentrações plasmáticas de insulina e aumento das concentrações de glucagon. A degradação do glicogênio armazenado é regulada pela ação de cinases e fosfatases. Assim como a GS, a glicogênio fosforilase também sofre regulação alostérica por G-6-P, modificação colavente por fosforilação pela fosforilase cinase e desfosforilação pela PP1, além de translocação celular. A ativação da fosforilase cinase é mediada por concentrações intracelulares aumentadas de Ca^{2+} ou por fosforilação pela AMPK. Ainda, a atividade pode ser modulada por Mg^{2+}, ADP e pH (JOHNSON, 1992; BOLLEN et al., 1998; ROACH, 2002; FERRER et al., 2003).

1.3.3 Regulação do metabolismo da glicose

No estado fisiológico, a manutenção da homeostasia da glicose é mantida, principalmente, 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 e da secreção de hormônios contra-regulatórios (TAHA; KLIP, 1999; SALTIEL; KAHN, 2001; BEARDSALL et al., 2003; MOORE et al., 2003).

A insulina é um dos hormônios essenciais que regulam o metabolismo, o crescimento e a diferenciação celular, atuando em diversos tecidos. De maneira geral, as ações anabólicas da insulina incluem o estímulo da captação, da utilização e do armazenamento intracelular de glicose, aminoácidos e ácidos graxos e a inibição de processos catabólicos como a glicogenólise, lipólise e proteólise. Além disso, a insulina também inibe a gliconeogênese hepática (SALTIEL; KAHN, 2001; BEARDSALL et al., 2003; MOORE et al., 2003).

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 (RORSMAN, 1997; TAHA; KLIP, 1999; BEARDSALL et al., 2003). 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 e a presença da insulina 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 pelo músculo e/ou tecido adiposo 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, aumentando 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) (HEI, 1998; TAHA; KLIP, 1999; BEARDSALL et al., 2003; MOORE et al., 2003).

Durante o jejum ou entre as refeições, as concentrações de insulina diminuem e as de glucagon e outros hormônios contra-regulatórios da insulina 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 a partir de glicerol, lactato e aminoácidos aumenta progressivamente (SALTIEL; KAHN, 2001; BEARDSALL et al., 2003; ZIERATH; KAWANO, 2003).

Durante o jejum, a captação de glicose no músculo é reduzida e este se torna 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 5) (BEARDSALL et al., 2003; MOORE et al., 2003).

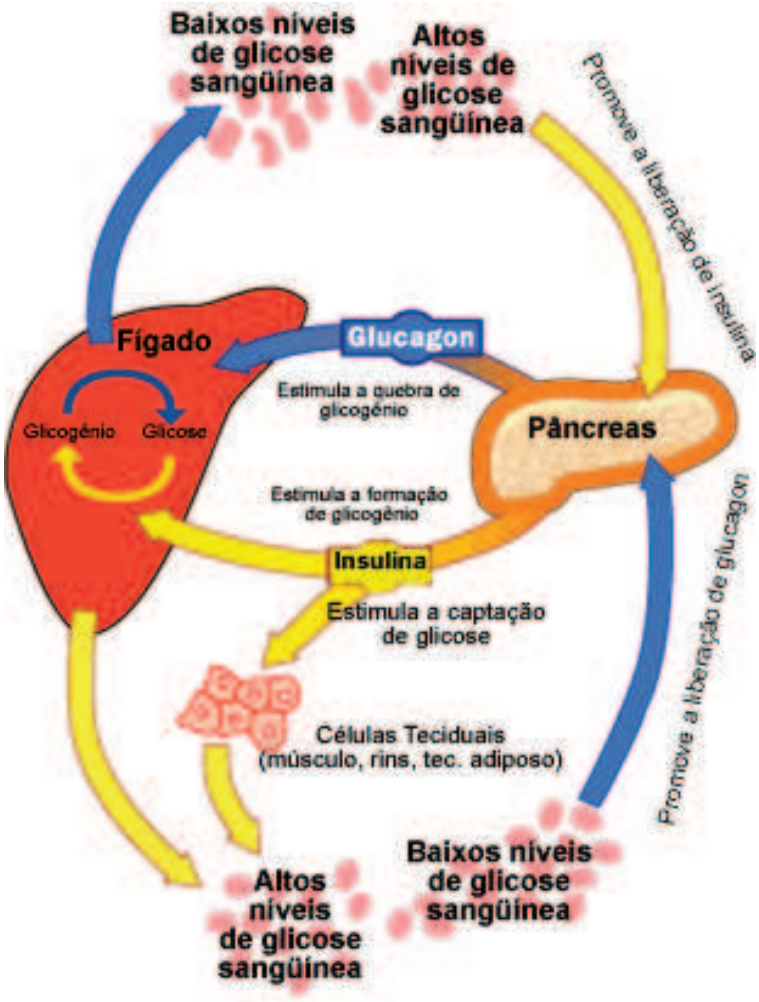


Figura 5. Regulação do metabolismo da glicose (adaptado de http://health.howstuffworks.com/diabetes1.htm).

A insulina é o hormônio anabólico 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 (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 protéica 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). Se não controlada, a hiperglicemia crônica resulta no desenvolvimento de diversas complicações que levam a disfunção, dano ou falência de vários órgãos (SOCIEDADE BRASILEIRA DE DIABETES, 2003; AMERICAN DIABETES ASSOCIATION, 2008; WHO, 2009a).

1.4 HIPOGLICEMIANTES ORAIS

Pacientes diabéticos freqüentemente 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 (SOCIEDADE BRASILEIRA DE DIABETES, 2003; CHENG; FANTUS, 2005; KOSKI, 2006; FOWLER, 2007).

Quando o controle glicêmico adequado não é atingido com essas medidas, os pacientes fazem uso dos medicamentos conhecidos como hipoglicemiantes orais. Esses medicamentos são divididos em classes de acordo com o efeito geral. Os fármacos chamados de "sensibilizadores da insulina" atuam sobre a resistência à insulina, exercendo efeitos terapêuticos através do estímulo à captação de glicose pelos tecidos periféricos e da redução da liberação de glicose pelo fígado. Este grupo inclui duas classes terapêuticas, as biguanidas e as glitazonas ou tiazolidinodionas. A metformina, único representante da classe das biguanidas, atua através da redução da produção hepática de glicose e, em menor extensão da diminuição da resistência à insulina nos tecidos periféricos. As glitazonas ou tiazolidinodionas, representadas pela rosiglitazona e pioglitazona, exercem sua ação através da redução da resistência à insulina (SOCIEDADE BRASILEIRA DE DIABETES, 2003; CHENG; FANTUS, 2005; KOSKI, 2006; FOWLER, 2007).

Outro grupo de fármacos hipoglicemiantes inclui os chamados "secretagogos de insulina" que atuam estimulando a produção de insulina pelas células β pancreáticas, sendo representado pelas classes das glinidas e das sulfoniluréias. As sulfoniluréias atuam estimulando a secreção de insulina e reduzindo as concentrações plasmáticas de glicose. Essa classe de substâncias ocupa um sítio específico (subunidade SUR-1) do receptor de sulfoniluréia nos canais de potássio dependentes de ATP. As sulfoniluréias de primeira geração são representadas pela clorpropamida, tolbutamida, acetohexamida e tolazamida. Os agentes de segunda geração representados pela gliburida, glipizida, glibenclamida e glimepirida são mais potentes, seguros e eficazes que os da primeira geração. As glinidas (repaglinida e nateglinida) atuam pelo mesmo mecanismo de ação, porém ocupam outra subunidade do receptor e possuem duração de ação mais curta comparado às sulfoniluréias. Há ainda fármacos que retardam a absorção intestinal de glicose através da inibição da atividade das α-glicosidases, como a acarbose e o miglitol (SOCIEDADE BRASILEIRA DE DIABETES, 2003; CHENG; FANTUS, 2005; KOSKI, 2006; FOWLER, 2007).

Além da gama de medicamentos atualmente disponíveis para o tratamento da diabetes, o conhecimento emergente dos mecanismos que envolvem a patogenia dessa desordem fornece novos alvos moleculares para o desenvolvimento de fármacos. Dentre os novos fármacos em desenvolvimento estão os que estimulam a secreção de insulina como as incretinas peptídeo semelhante ao glucagon 1 (GLP-1) e polipeptídio insulinotrópico (GIP), os inibidores da dipeptidil peptidase IV (DPP IV) que cliva o GLP-1 e agonistas do receptor de GLP-1 como o exenadina. Esses agentes aumentam a secreção de insulina, promovem a proliferação das ilhotas e suprimem a liberação de glucagon (MORRAL, 2003; VATS et al., 2005; FOWLER, 2007, 2008). Além destes, também são alvos de estudos os antagonistas do adrenoreceptor α_2 como por exemplo, RS79948-197, fentolamina e efaroxano, que estimulam a secreção de insulina nas células β pancreáticas principalmente através do bloqueio dos adrenoreceptor α_2 impedindo a ação das catecolaminas (MORGAN, 1994; ABDEL-ZAHER et al., 2001; FAGERHOLM et al., 2008). Ainda, os que melhoram a ação da insulina, como moléculas ativadoras do receptor de insulina, inibidores da ação da resistina, das fosfatases PTP-1B e SHP2, e da GSK-3. Existem ainda, 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 hepáticas. 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 o aumento da expressão da enzima 11β-hidroxi-esteróide desidrogenase tipo 1 (11βHSD-1) no tecido adiposo (MORRAL, 2003; VATS et al., 2005; FOWLER, 2007, 2008). Ainda, agonistas do adrenoreceptor β_3 apresentam atividade termogênica e anti-obesidade e efeitos sensibilizadores da insulina no tecido adiposo branco e marrom e no músculo esquelético. Estes agentes atuam através do aumento da oxidação dos lipídeos resultando em perda de peso e aumento da sensibilidade à insulina nestes tecidos e também estão entre as alternativas em estudo para o desenvolvimento de novas moléculas para o tratamento da diabetes e obesidade (VATS et al., 2005; ARCH, 2002, 2008).

1.5 TERAPIA INSULÍNICA

A terapia com insulina é a base do tratamento da diabetes tipo 1 e quando um controle glicêmico adequado não é atingido com mudanças no estilo de vida e/ou terapia farmacológica, a insulina se torna a alternativa para os diabéticos do tipo 2 (SOCIEDADE BRASILEIRA DE DIABETES, 2003; BETHEL; FEINGLOS, 2005; YADAV; PARAKH, 2006; FOWLER, 2008).

Desde a descoberta da insulina a indústria farmacêutica vem propondo modificações na molécula nativa do hormônio com o objetivo de melhorar as características farmacocinéticas. Essas alterações podem acelerar os efeitos da insulina na circulação sangüínea ou ainda, prolongar o perfil farmacocinético (SOCIEDADE BRASILEIRA DE DIABETES, 2003; BETHEL; FEINGLOS, 2005; YADAV; PARAKH, 2006; FOWLER, 2008). Atualmente existem diversas preparações de insulina disponíveis para o tratamento da diabetes. Estas incluem as insulinas de ação curta e rápida (regular, lispro, aspart e glusilina). A insulina regular foi a primeira preparação de insulina disponível. Tem início de ação entre 30 a 60 minutos após a injeção, com pico de ação entre 2 a 3 horas e duração total entre 8 a 10 horas. As insulinas lispro, aspart e glusilina contêm alterações estruturais que permitem rápida dissociação e absorção após a injeção. Elas têm início de ação entre 5 a 15 minutos, com pico entre 30 a 90 minutos e duração de ação entre 4 a 6 horas. Essas insulinas de ação rápida exibem melhor controle sobre as concentrações de glicose pós-prandiais e causam menos hipoglicemia que a insulina regular (SOCIEDADE BRASILEIRA DE DIABETES, 2003; BETHEL; FEINGLOS, 2005; YADAV; PARAKH, 2006; FOWLER, 2008).

As insulinas de ação intermediária e longa resultam de modificações na estrutura da insulina regular bem como na solução de administração. A adição da protamina neutra de Hagedorn (NPH) forma a insulina NPH de ação intermediária cujo início de ação é entre 2 a 4 horas, com pico de atividade entre 4 a 10 horas e duração total de 10 a 16 horas. Por causa do perfil de ação, a insulina NPH está bastante associada a episódios de hipoglicemia. Além da protamina, zinco também pode ser associado às preparações de insulina aumentando os perfis de ação (insulinas de ação lenta). Ainda, as insulinas de ação longa representadas pela insulina glargina e detemir apresentam perfis de ação bastante prolongados quando comparadas às preparações de ação rápida e intermediária. A insulina glargina possui modificações na molécula que atrasam a absorção, resultando em ausência de pico de atividade característico e duração de ação de aproximadamente 24 horas após a administração. A insulina detemir consiste de uma molécula de insulina ligada a um ácido graxo de 14 carbonos que facilita a ligação e o transporte dessa insulina na albumina da circulação. Isso resulta em um tempo de ação de 14 a 21 horas. Tanto a insulina glargina quanto a detemir tem início de ação muito lento e os níveis séricos se mantêm durante todo o dia (BETHEL; FEINGLOS, 2005; YADAV; PARAKH, 2006; FOWLER, 2008).

1.6 PLANTAS MEDICINAIS

Os produtos naturais representam uma das principais fontes de estruturas químicas únicas para avaliação e pesquisa de novos fármacos com potencial utilidade na indústria farmacêutica. Muitos produtos naturais e derivados sintéticos e semi-sintéticos foram desenvolvidos para uso clínico em quase todas as áreas terapêuticas com sucesso como, por exemplo, a atropina isolada da *Atropa belladona* L., morfina e codeína isoladas da espécie *Papaver somniferum* L., quinina isolada da *Cinchona* spp., taxol® isolado da espécie *Taxus brevifolia* entre outros (SIMÕES et al., 2001; BUTLER, 2004; BALUNAS; KINGORN, 2005; BAKER et al., 2007).

Existem cerca de 1200 espécies de plantas, pertencentes a 190 famílias que são citadas de uso na diabetes. Embora muitas dessas plantas não possuam uma avaliação científica completa, a grande maioria das espécies estudadas demonstra, experimentalmente, atividade antidiabética e/ou anti-hiperglicêmica (MARLES; FARNSWOTH, 1995; GROVER et al., 2002; SILVA et al., 2002;

ZAREBA et al., 2005; MUKHERJEE et al., 2006; LEDUC et al., 2006). Alguns dos fármacos atualmente utilizados no tratamento da diabetes têm origem em plantas, como a metformina que foi desenvolvida a partir de um protótipo (galegina) que foi identificado nas flores da espécie *Galena officinalis* e acarbose isolada da *Actinoplances* spp., (OUBRÉ et al., 1997; LEDUC et al., 2006; NEWMAN; CRAGG, 2007).

1.6.1 *Averrhoa carambola*

Averrhoa carambola L. pertencente à família das Oxalidaceae é uma árvore de porte médio (6 a 9 metros) conhecida popularmente como caramboleira ou carambola. Possui folhas decíduas e alternas de 15 a 20 cm de comprimento e flores pequenas e variegadas, brancas e púrpuras. Os frutos do tipo baga com formato oblongo anguloso (5 ângulos ou arestas longitudinais) são de coloração amarelo âmbar e quando cortados no sentido transversal lembram o formato de uma estrela (CORRÊA, 1984; MORTON, 1987). Além disso, os frutos se caracterizam pelo elevado conteúdo de ácido oxálico entre outros como ácidos tartárico, cítrico, málico, ascórbico, succínico e fumárico. Esta planta é nativa das regiões tropicais e subtropicais da Ásia e foi introduzida no Brasil em 1817, em Pernambuco de onde se espalhou para todas as regiões do país (Figura 6) (CORRÊA, 1984; MORTON, 1987).

Figura 6. Espécie vegetal *Averrhoa carambola*.

De acordo com a medicina popular, na Índia o suco dos frutos maduros da carambola são utilizados para estancar hemorragias, febres e afecções oculares. No Brasil, as folhas da planta são utilizadas como diurético, estimulante do apetite, antiemético, antidiarréico, e antifebril. Também é utilizada no tratamento de eczemas, diabetes, escorbuto, hipertensão, enxaqueca, angustia, náusea e tosse. A *Averrhoa carambola* também é utilizada topicamente em picadas de insetos (BURKHILL, 1935; CORRÊA, 1984; MOREIRA, 1985; MORTON, 1987; IAMONI 1997). O suco dos frutos da carambola também é utilizado para limpar e polir metais, uma vez que remove ferrugem e manchas e para alvejar roupas brancas, devido ao elevado conteúdo ácido (CORRÊA, 1984; MORTON, 1987).

Os frutos da carambola são ricos em fibras insolúveis além da presença de ácido oxálico, ácido L-ascórbico, epicatequina e ácido gálico na forma de galotaninos e proantocianidinas (dímeros, trímeros, tetrâmeros e pentâmeros da catequina e epicatequina) (SHUI; LEONG, 2004, 2006). Essas fibras apresentam efeitos hipolipemiantes e hipocolesterolemiantes (redutoras de colesterol), ações hipoglicemiantes, atividade antioxidante além da melhora das funções intestinais (CHAU et al., 2004a; CHAU et al., 2004b; CHAU; CHEN, 2006; SHUI; LEONG, 2006).

Estudos *in vitro* com coração ou átrio isolado de cobaias realizados por Vasconcelos e colaboradores (2005, 2006) demonstraram que o extrato aquoso das folhas da *Averrhoa carambola* L., apresentou ação sobre o ritmo cardíaco e a força contráctil do miocárdio. Os resultados sugerem que o efeito redutor sobre o ritmo e a força contráctil cardíaca seria mediado através do bloqueio de canais de Ca^{2+} do tipo L.

Foram identificados taninos, triterpenos e flavonóides a partir do extrato bruto hidroalcoólico das folhas da *Averrhoa carambola* (PROVASI et al., 2001; ARAHO et al., 2005). Recentemente, o extrato bruto liofilizado, extrato aquoso e frações semi-purificadas das folhas da *Averrhoa carambola* apresentaram efeito antihiperglicêmico em ratos normais e normais hiperglicêmicos. (MARTHA et al., 2000; PROVASI et al., 2001; PROVASI et al., 2005).

Outras espécies do gênero Averrhoa, como a *Averrhoa bilimbi* Linn., também são conhecidas popularmente pelas propriedades antiinflamatórias, antiescorbúticas, adstringentes, antibacterianas e antidiabéticas (GOH et al., 1995). Extratos e frações semi-purificadas das folhas de *Averrhoa bilimbi* apresentaram efeitos hipoglicemiantes e hipolipemiantes quando administradas pelas vias oral e intraperitoneal em ratos diabéticos induzidos por estreptozotocina, após tratamentos agudos e crônicos (TAN et al., 1996; PUSHPARAJ et al., 2000; 2001; TAN et al., 2005). Além disso, o tratamento crônico de ratos diabéticos com a fração aquosa do extrato bruto das folhas da *Averrhoa bilimbi* aumentou as concentrações plasmáticas de insulina e reduziu a atividade da enzima glicose 6-fosfatase hepática (PUSHPARAJ et al., 2001).

1.6.2 *Cyathea phalerata*

Com mais de 12000 espécies, as samambaias arborescentes encontradas em algumas famílias tropicais como a *Cyatheaceae* constituem uma importante divisão de representantes do reino vegetal (BRINGMANN et al., 1999). O gênero *Cyathea* consiste de samambaias arborescentes que variam de 4 a 8 m de altura e se encontram amplamente distribuídas pelas áreas tropicais e subtropicais do planeta. Muitas espécies são utilizadas em algumas regiões para o tratamento de doenças sexualmente transmissíveis e como anti-helmíntico (ARAI et al., 1994; ARAI et al., 1995; BRINGMANN et al., 1999). Ameríndios da Bolívia e Equador fazem uso da espécie *Cyathea pungens* como antiinflamatório (MACÍA, 2004) e no México a infusão de raízes e folhas da espécie *Cyathea fulva* é utilizada como hipoglicemiante, porém sem nenhuma comprovação científica desta atividade (ANDRADE-CETTO; HEINRICH, 2005).

Em geral, os flavonóides das samambaias são quatro grupos principais: flavonóis, C-glicosilflavonas, flavonas e flavanonas. Outros tipos conhecidos de forma mais esporádica são as antocianidinas, xantonas, chalconas, flavanonóis e biflavonas (WALLACE, 1989). A primeira investigação sobre os constituintes flavonóides no gênero Cyathea (Cyatheaceae) foi realizada por Harada et al. (1955, 1958) que analisou as folhas de *Cyathea fauriei* e *Cyathea hancockii* durante um levantamento sobre a distribuição de flavonas, flavonóis e flavanonas nas samambaias japonesas. De acordo com eles, as espécies continham canferol, cirtominetina, farrerol e vitexina (in: HIRAOKA; HASEGAWA, 1975). Concordando com a distribuição dos flavonóides heterosídeos neste gênero, Soeder e Bass (1972) demonstraram a ocorrência de flavonas C-heterosídeos (vitexina e isovitexina) nas folhas de três espécies do gênero Cyathea da América Latina (in: HIRAOKA; HASEGAWA, 1975).

Espécies representantes da família Cyatheaceae acumulam flavonóis O-heterosídeos, predominantemente 3-O-glicosídeos do canferol e quercetina e C-glicosilflavonas e flavanonas (HIRAOKA; HASEGAWA, 1975; HIRAOKA; MAEDA, 1979; WALLACE, 1989). Além desses compostos são características a presença de constituintes triterpenóides e esteróides em determinadas espécies do gênero Cyathea (ARAI et al., 1994; ARAI et al., 1995; BRINGMANN et al., 1999). Porém a fitoquímica do gênero Cyathea é pouco conhecida, apresentando até o momento escassa literatura a respeito. Os poucos estudos realizados mostram que o gênero é caracterizado fitoquimicamente pelo acúmulo de triterpenos hopanóides e fernanos e compostos flavonoídicos glicosilados (CUNHA et al., 2003).

No estudo fitoquímico da *Cyathea phalerata* (*C. phalerata*) realizado por Cunha e colaboradores (2003) (Figura 7), foram isolados e caracterizados um flavonóide glicosídeo contendo o canferol como aglicona e um derivado do ácido caféico: canferol 3-O-α-L-ramnopiranosil (1→2) β-D-glicopiranosídeo (canferol-3-neohesperidosídeo) e ácido-4-O-β-D-glicopiranosil caféico. Além disso, mais recentemente, foram isolados e identificados um derivado spiropiranosil do ácido protocatechuico, ciatenosídeo A e um glicosídeo, o ácido 4-O-β-D-glicopiranosil cumárico (PIZZOLATTI et al., 2007).

Figura 7. Espécie vegetal *Cyathea phalerata.*

Popularmente conhecida como xaxim, *Cyathea phalerata* Mart. é utilizada na medicina popular regional para o tratamento de diversas doenças inflamatórias. Na região sul do Brasil, o extrato alcoólico do caule é utilizado para o tratamento de varicosas e hemorróidas. Além disso, é utilizada para o tratamento de afecções renais, amarelão, reumatismo e gripes, porém sem comprovação científica (CUNHA et al., 2003, PIZZOLATTI et al., 2007).

1.6.3 Flavonóides

Os flavonóides constituem um grupo importante de polifenóis de baixo peso molecular amplamente distribuídos em frutas, plantas e vegetais. A síntese dos flavonóides ocorre a partir da combinação de derivados da fenilalanina e ácido acético. A estrutura é baseada no núcleo flavonoídico que consiste de três anéis fenólicos denominados A, B e C. Os flavonóides podem ocorrer como agliconas, heterosídeos ou derivados metilados e as diferentes classes de flavonóides diferem entre si pelo nível de oxidação e modelo de substituição do anel C. Já os compostos individuais de uma mesma classe diferem no modelo de substituição nos anéis A e B. As principais classes de flavonóides incluem as antocianidinas, flavanóis (catequinas), flavonas, flavonóis, flavanonas e isoflavonas entre outras (HAVSTEEN, 1983; PIETTA, 2000; AHERNE; O'BRIEN, 2002; CAZAROLLI et al., 2008a, item 4.5).

Os flavonóides são encontrados na natureza principalmente como heterosídeos e o tipo de ligação do carboidrato à molécula de flavonóide dá origem aos O-heterosídeos ou aos C-heterosídeos. O carboidrato mais comumente encontrado é a D-glicose mas outros carboidratos

como L-ramnose, glicoramnose, galactose, xilose e arabinose ou combinações destes podem estar presentes. O grande número de flavonóides encontrados na natureza é devido às inúmeras possibilidades de combinações entre as agliconas e os diferentes carboidratos (AHERNE; O'BRIEN, 2002; CAZAROLLI et al., 2008a, item 4.5). São amplamente distribuídos em folhas, sementes, caule e flores das plantas, onde conferem proteção contra a radiação ultravioleta, patógenos e herbívoros. Estão presentes em vegetais e frutas, no entanto, a ingestão diária varia conforme a região geográfica e a cultura (Para revisão, ver CAZAROLLI et al., 2008a, item 4.5).

Uma vez ingeridos, os flavonóides são absorvidos no intestino delgado e metabolizados principalmente pela mucosa intestinal e pelo fígado, porém túbulos renais, pele, pulmão e placenta exerçam essa função secundariamente. Os flavonóides intactos e derivados também podem sofrer metabolismo por bactérias do cólon e serem reabsorvidos entrando no ciclo entero-hepático ou serem excretados através das fezes ou da urina (Para revisão, ver CAZAROLLI et al., 2008a, item 4.5). Os flavonóides e derivados são amplamente explorados em função das inúmeras aplicações terapêuticas que apresentam como: ações antialérgica, anti-oxidante, antiinflamatória, anti-carcinogênica, anti-viral, anti-ulcerogênica, antiparasitária e protetora do sistema cardiovascular e do sistema nervoso central (Para revisão, ver CAZAROLLI et al., 2008a, item 4.5).

Diversos trabalhos demonstram o efeito antidiabético dos flavonóides e as ações no metabolismo de carboidratos através da redução da absorção de glicose ou da melhora da tolerância à glicose. Os flavonóides estimulam a captação e metabolismo da glicose, regulam a atividade e/ou a expressão de enzimas chave do metabolismo de carboidratos, atuam como secretagogos de insulina ou protetores da função das células β pancreáticas. Ainda, podem atuar como insulino-miméticos provavelmente influenciando mecanismos pleiotrópicos da sinalização insulínica intracelular contribuindo para melhorar o estado diabético (CAZAROLLI et al., 2008b, item 4.6).

Estudos anteriores deste laboratório, com plantas utilizadas popularmente como hipoglicemiantes e ricas em flavonóides, demonstraram resultados que estimulam a continuidade destas investigações. Dentre as plantas estudadas em relação ao potencial hipoglicemiante, a fração *n*-butanol oriunda das folhas da *Bauhinia forficata*, rica em flavonóides glicosilados, apresentou efeito hipoglicemiante após tratamento agudo em ratos diabéticos sem alterar a curva de tolerância à glicose (SILVA et al., 2002). Desta fração foram isolados diferentes flavonóides, sendo o composto majoritário, a canferitrina (Figura 8) (PIZZOLATTI et al., 2003). A curva de dose-resposta deste composto mostrou um efeito hipoglicemiante significativamente melhor do que o observado para a fração *n*-butanol e reduziu a glicemia em ratos diabéticos em todos os tempos e doses estudados. Também, com doses semelhantes àquelas estudadas *in vivo*, exibiu um efeito anti-oxidante *in vitro* por mostrar alta reatividade com o 1,1-difenil-2-picril hidrazil, inibiu a atividade da mieloperoxidase, diminuiu a peroxidação lipídica induzida pelo radical ascorbil tanto em microssomas de fígado de ratos como em lipossomas de fosfatidilcolina e asolecitina (DE SOUSA et al., 2004).

O estudo deste composto em ratos normoglicêmicos e diabéticos revelou uma ação significativa na captação de glicose no músculo sóleo, não influenciou a síntese protéica *in vitro* e não interferiu na glicosúria após tratamento por via oral. Ainda, a canferitrina promoveu um aumento significativo do conteúdo de glicogênio muscular após tratamento de animais normoglicêmicos e diabéticos. Estes resultados mostraram um efeito agudo da canferitrina percentualmente tão eficaz quanto à insulina embora utilizado em doses mais altas do que o hormônio purificado (JORGE et al., 2004).

Além disso, quando complexado com vanádio, um metal já descrito como um regulador do receptor de insulina, formou uma espécie ativa em pH fisiológico que reduziu significativamente a glicemia em ratos diabéticos após um tratamento agudo (CAZAROLLI et al., 2006). Também demonstramos que a ação hipoglicemiante dos flavonóides depende do tipo, número e da posição dos carboidratos ligados ao núcleo flavonoídico, uma vez que o flavonóide canferol, também identificado das folhas da *B. forficata* não apresentou efeito em ratos diabéticos (Figura 8) (CAZAROLLI, 2004; CAZAROLLI et al., 2006).

Levando em consideração a similaridade estrutural entre os compostos (Figura 8) e os efeitos da canferitrina na glicemia e no metabolismo de carboidratos, foi pesquisado também a atividade hipoglicemiante do canferol-3-neohesperidosídeo, constituinte isolado do caule fresco da *Cyathea phalerata*. Este flavonóide se mostrou eficaz em diminuir a glicemia de animais diabéticos pelas vias oral e intraperitoneal. Além disso, quando complexado com o íon vanádio (IV) demonstrou um pronunciado efeito hipoglicêmico. Este efeito foi maior do que o observado com a canferitrina e com o canferol-3-neohesperidosídeo livre (CAZAROLLI et al., 2006). Com o objetivo de determinar o mecanismo de ação do canferol 3-neohesperidosídeo na glicemia, foi estudado o efeito na captação de glicose e no conteúdo de glicogênio muscular. O canferol 3-neohesperidosídeo estimulou a captação de glicose em músculo sóleo através de ações mediadas via transdução de sinal da insulina e promoveu o aumento do conteúdo de glicogênio muscular após tratamento em ratos diabéticos sugerindo um potencial efeito insulino-mimético (ZANATTA et al., 2008). Após observado o efeito estimulatório do canferol-3-neohesperidosídeo na captação de glicose e no conteúdo de glicogênio, neste trabalho, foi estudado o efeito bem como o mecanismo de ação deste composto na síntese de glicogênio muscular com a finalidade de elucidar a hipótese insulino-mimética de ação deste composto na regulação da glicemia. A ação e o mecanismo de ação de diversos flavonóides, além dos acima citados, foram revisados recentemente, bem como, foi proposto um mecanismo geral de ação do canferol-3-neohesperidosídeo, o flavonóide com melhor efeito hipoglicemiante determinado por este grupo até o momento (CAZAROLLI et al., 2008a, b, itens 4.5 e 4.6).

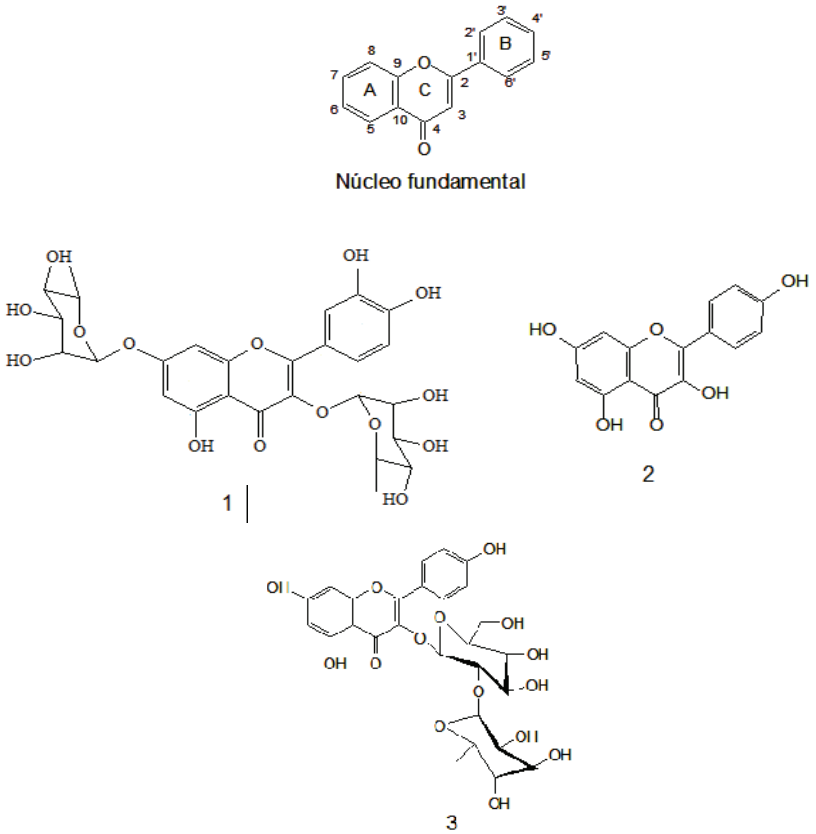


Figura 8. Estruturas do núcleo fundamental dos flavonóides, canferitrina (1), canferol (2) e canferol 3-neohesperidosídeo (3).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Caracterização de compostos naturais como hipoglicemiantes em modelo de diabetes experimental. Estudo do mecanismo de ação destes compostos sobre o metabolismo de carboidratos.

2.2 OBJETIVOS ESPECÍFICOS

Determinar o efeito do extrato bruto, de frações e de compostos isolados das folhas de *Averrhoa carambola* na glicemia de animais hiperglicêmicos e diabéticos após período agudo de tratamento.

Determinar o efeito dos compostos isolados das folhas de *Averrhoa carambola*, apigenina-6-C-β-L-fucopiranosídeo e apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo no conteúdo de glicogênio hepático e muscular.

Estudar o efeito dos compostos isolados das folhas da *Averrhoa carambola*, apigenina-6-C-β-L-fucopiranosídeo e apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo na secreção de insulina após tratamento agudo em ratos normais hiperglicêmicos.

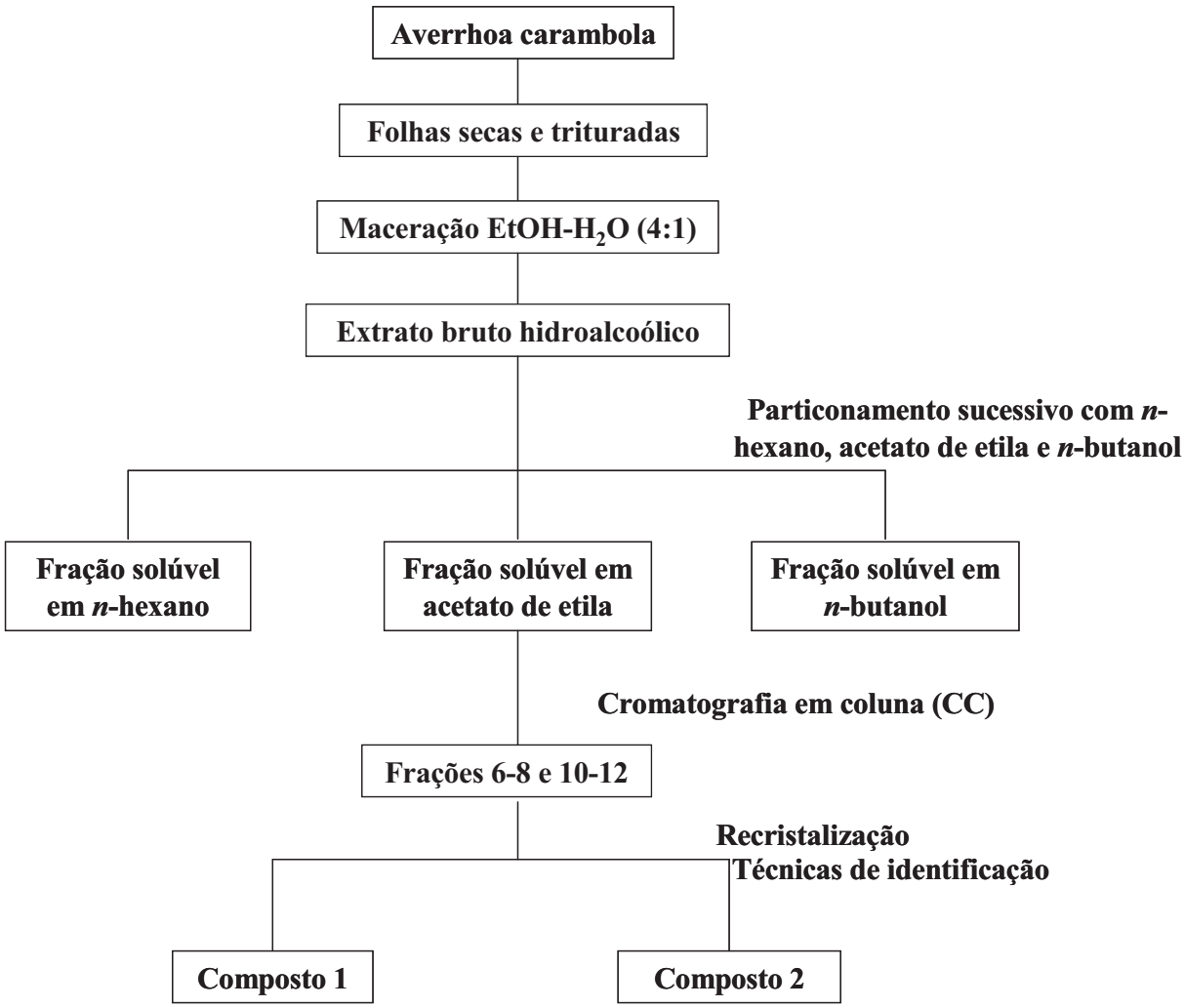
Estudar o efeito, bem como, o mecanismo de ação do canferol-3-neohesperidosídeo, isolado do caule da *Cyathea phalerata*, na síntese de glicogênio muscular *in vitro* via incorporação de D - [^{14}C (U)] – glicose e comparar com o efeito da insulina em ratos normoglicêmicos.

Estudar o efeito, bem como, o mecanismo de ação da apigenina-6-C-β-L-fucopiranosídeo, isolada das folhas da *Averrhoa carambola*, na síntese de glicogênio muscular *in vitro* via incorporação de D - [^{14}C (U)] – glicose e comparar com o efeito da insulina em ratos normoglicêmicos.

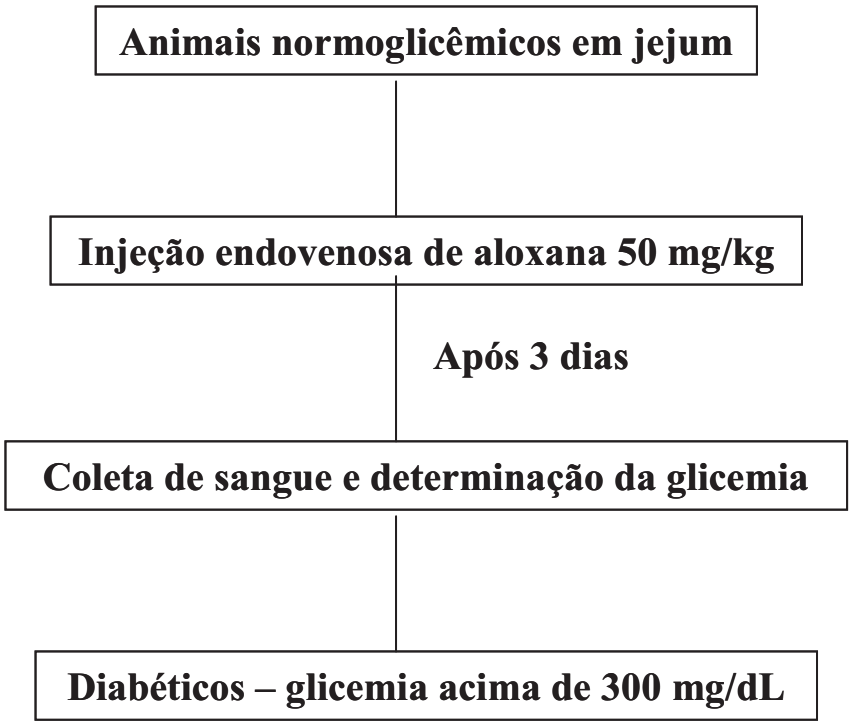
Estudar o efeito da apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo, isolado das folhas da *Averrhoa carambola*, na captação de [U-^{14}C]-2-deoxi-D-glicose em músculo sóleo de ratos normoglicêmicos, bem como o mecanismo de ação deste composto e ainda comparar com a ação da insulina.

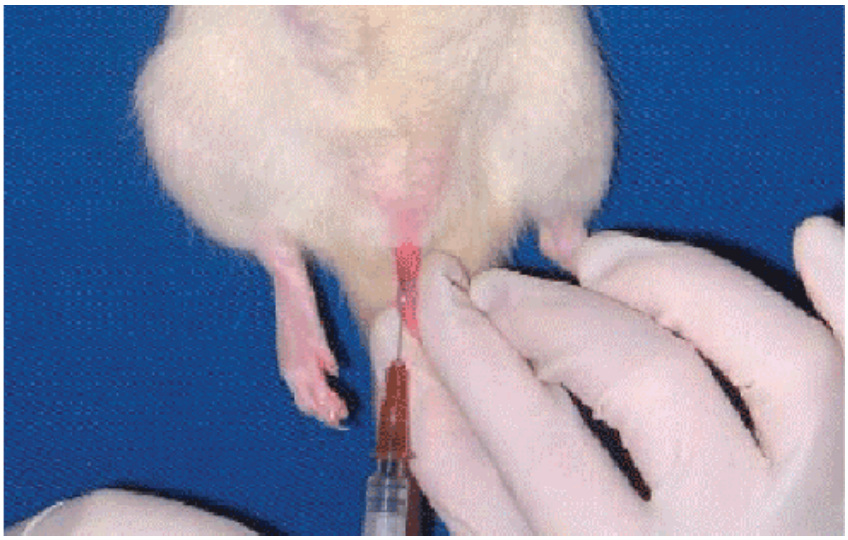
Comparar o efeito e o mecanismo de ação de produtos naturais (extrato bruto, frações e compostos isolados), principalmente flavonóides, com os efeitos da insulina (insulino-miméticos) e com os secretagogos da insulina (anti-hiperglicêmicos) na regulação da captação e metabolização da glicose estudados nesta tese com os relatados dos últimos dez anos da literatura mundial.

3 METODOLOGIA

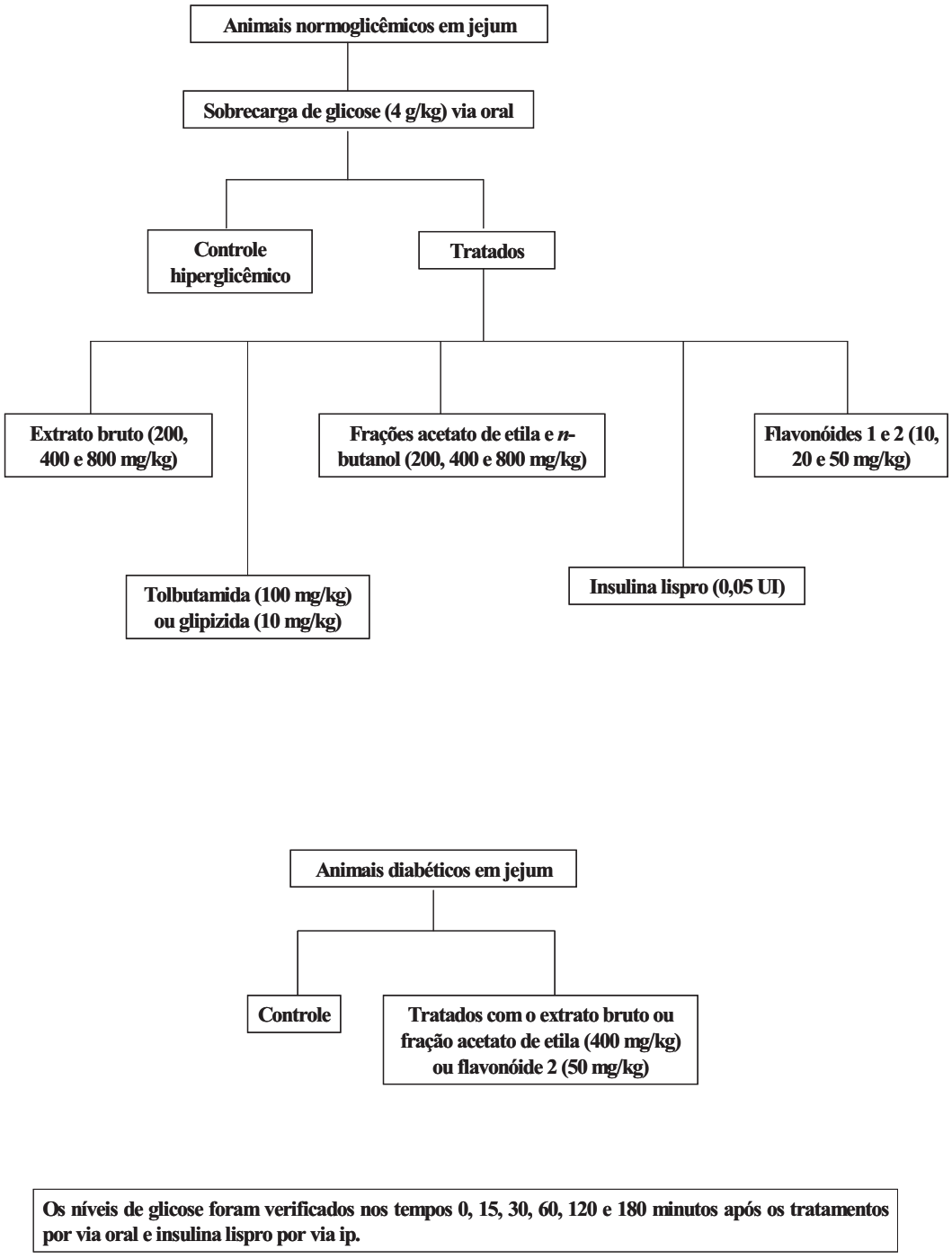


Fluxograma 1. Extração e isolamento dos flavonóides da *Averrhoa carambola*.

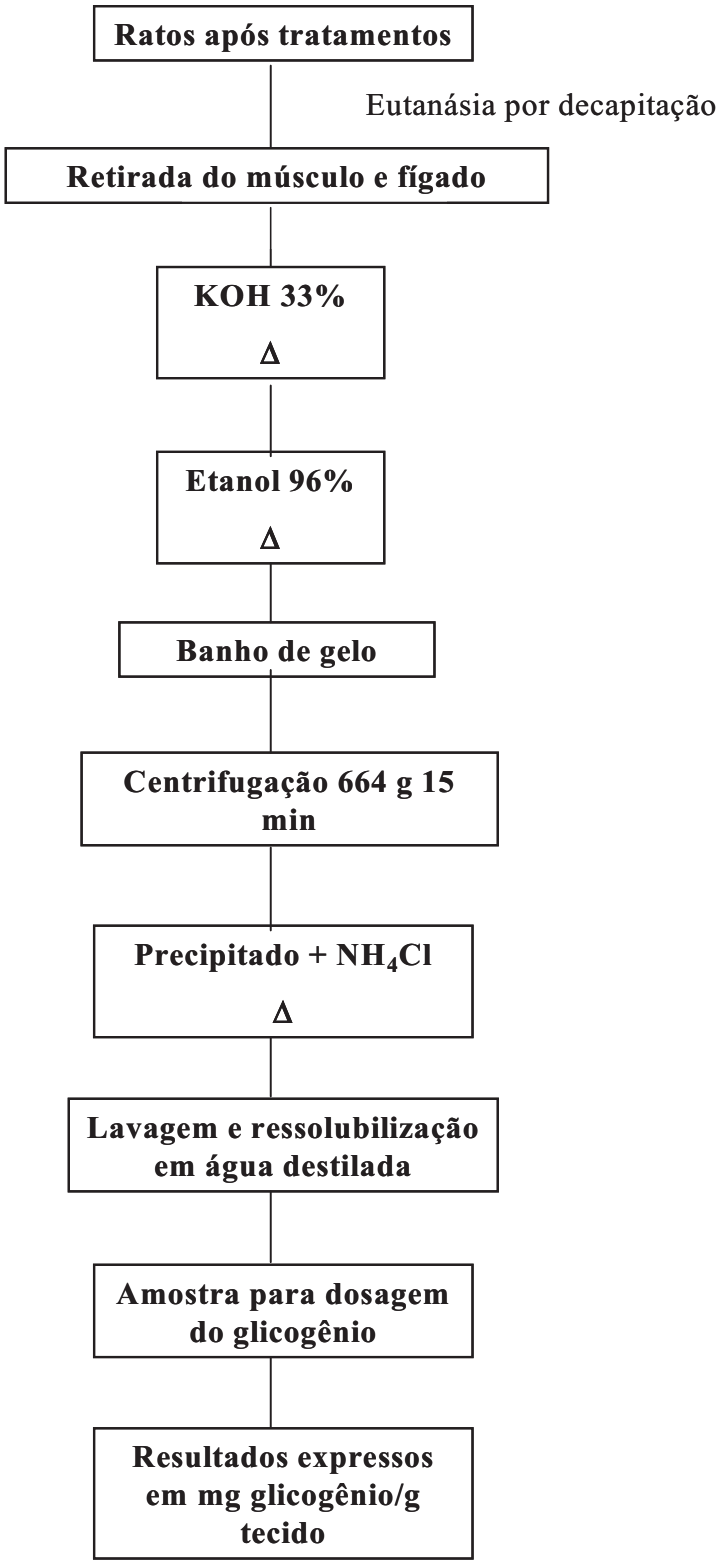




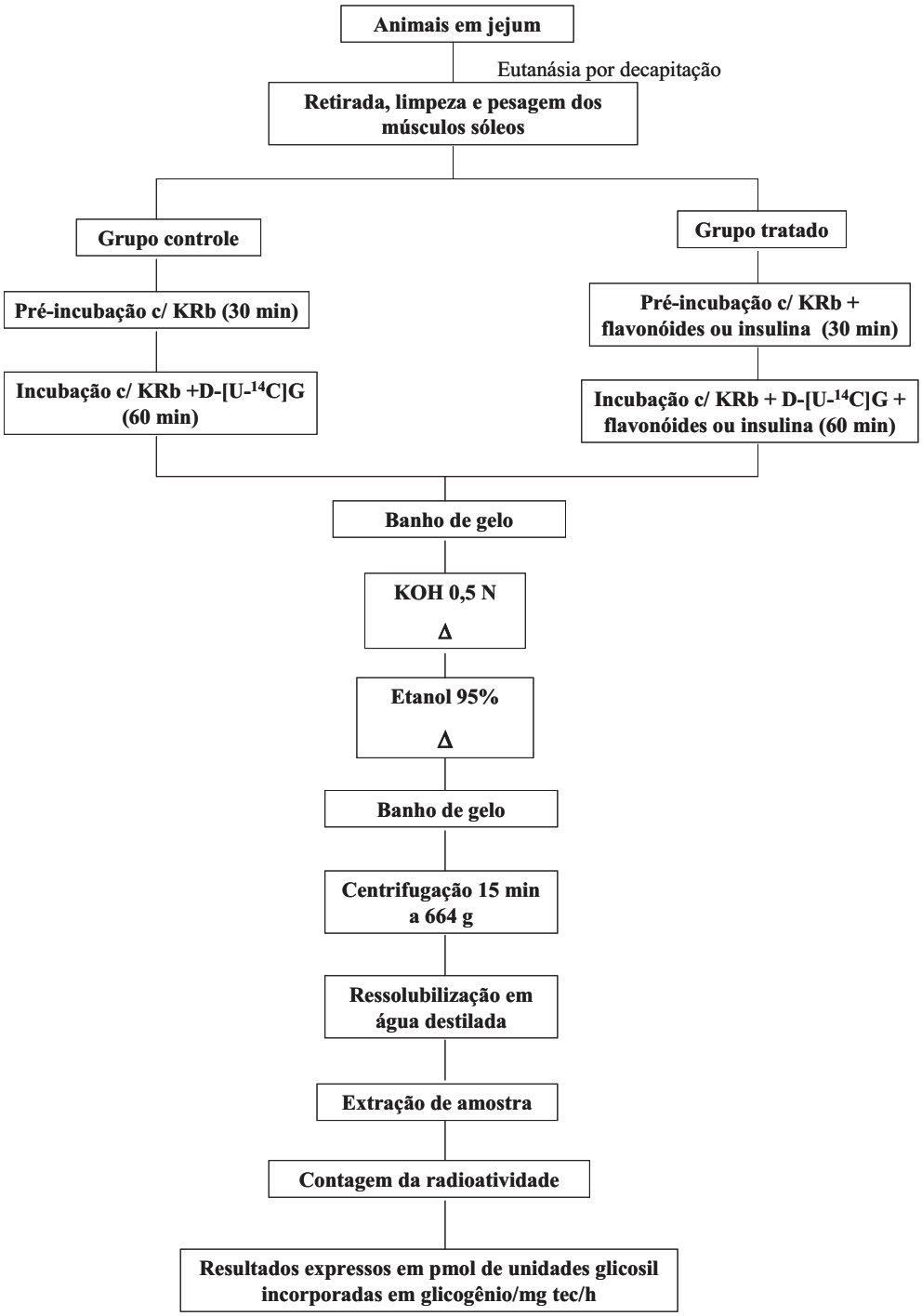
Fluxograma 2. Indução do modelo de diabetes experimental.



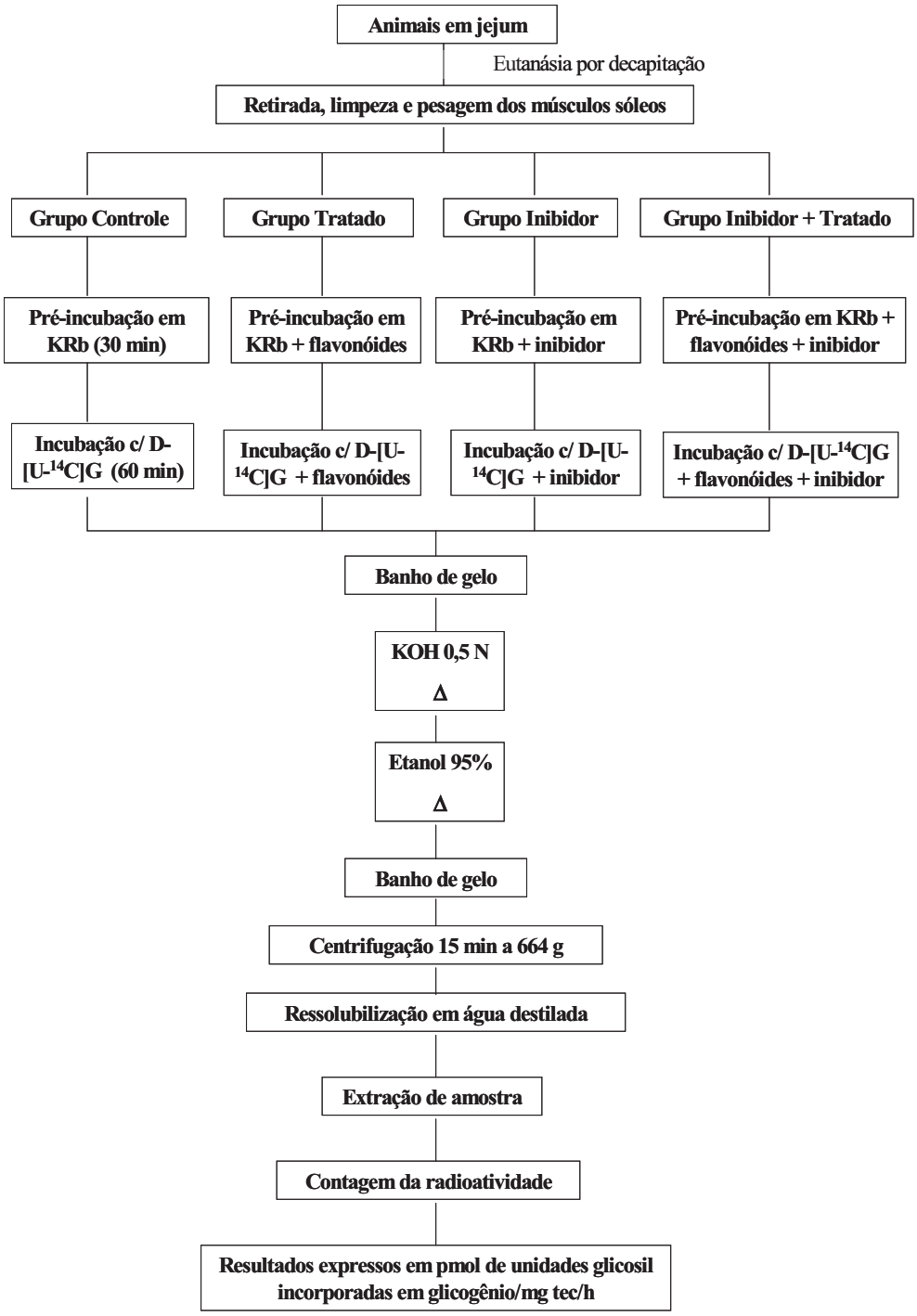
Fluxograma 3. Tratamentos dos animais normais hiperglicêmicos e diabéticos.



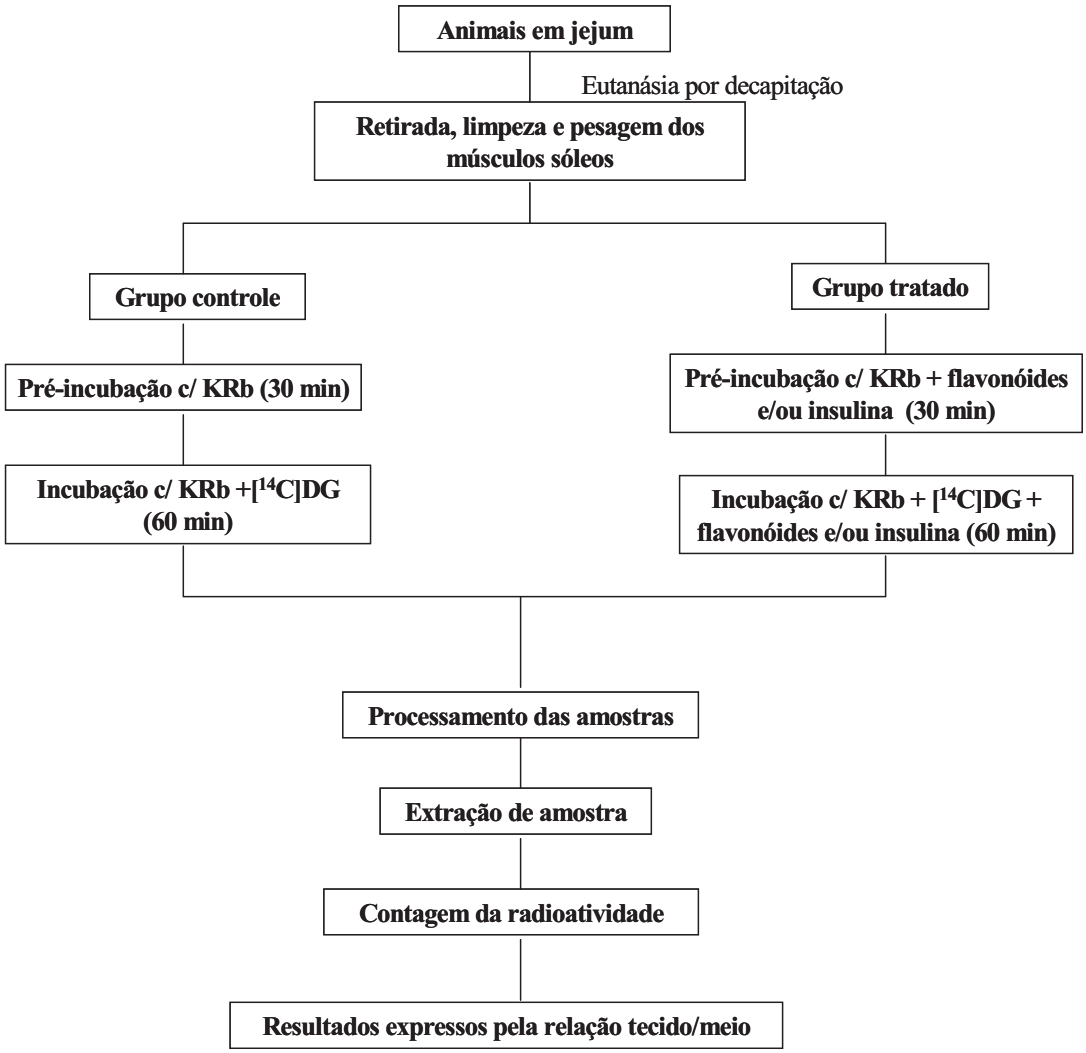
Fluxograma 4. Extração do glicogênio muscular.



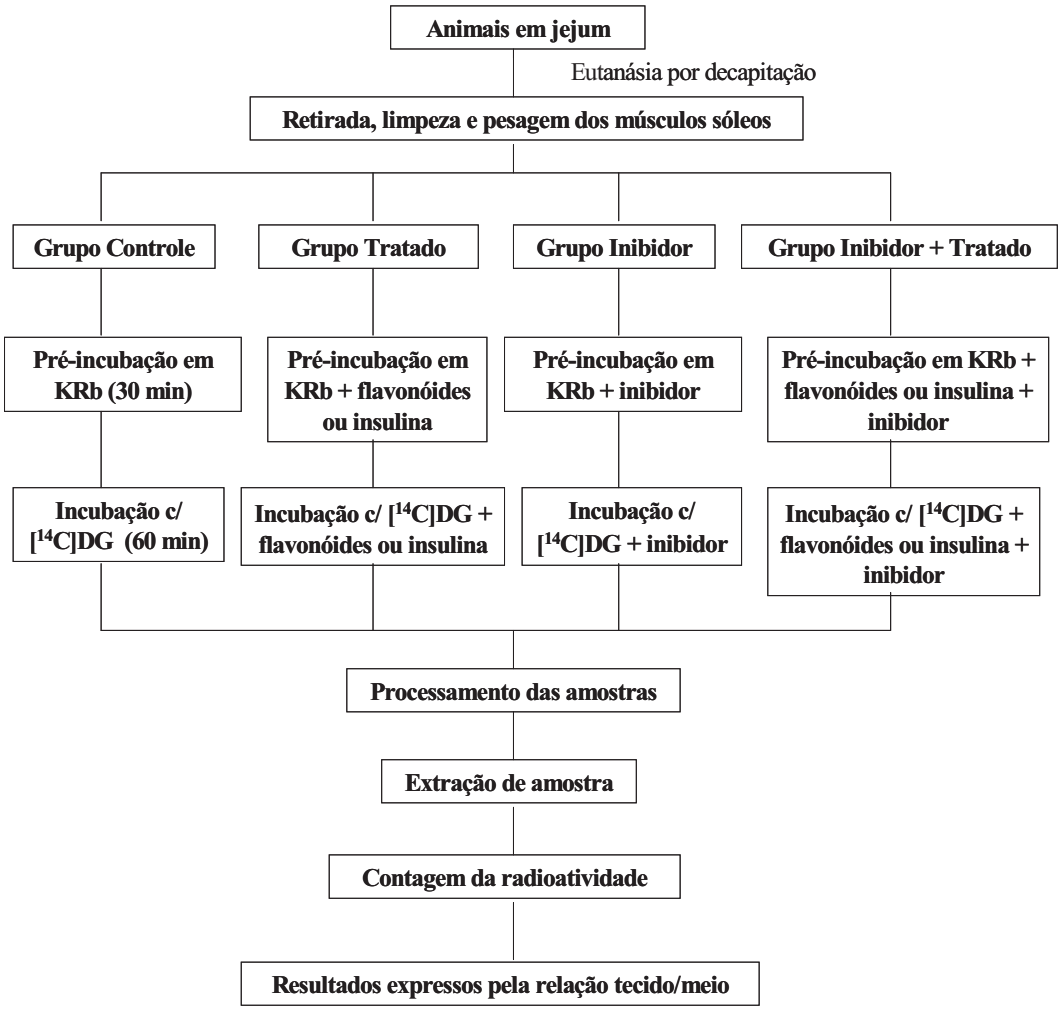
Fluxograma 5. Representação esquemática do ensaio de incorporação de D-[U-^{14}C]G em glicogênio no músculo sóleo incubado *in vitro* para a curva de dose-resposta com os flavonóides ou insulina.



Fluxograma 6. Representação esquemática do ensaio de incorporação de D-[U-^{14}C]G em glicogênio no músculo sóleo incubado com os flavonóides e com inibidores da PI-3K, GSK-3, MEK e PP1.



Fluxograma 7. Representação esquemática do ensaio de captação de [^{14}C]-DG no músculo para a curva de dose-resposta com os flavonóides ou insulina.



Fluxograma 8. Representação esquemática do ensaio de captação de [^{14}C]-DG no músculo sóleo incubado com os flavonóides e com diferentes inibidores.

4 ARTIGOS

4.1 ARTIGO SUBMETIDO PARA PUBLICAÇÃO

Periódico – Journal of Agricultural and Food Chemistry

CAZAROLLI, L.H.; MACHADO, L.M.; FOLADOR, P.; MORESCO, H.H.; BRIGHENTE, I.M.C.; PIZZOLATTI, M.G.; SILVA, F.R.M.B. **Potential antihyperglycemic role of crude extract, fractions and flavonoids from Averrhoa carambola leaves in rats.**

Nota: A formatação deste artigo não corresponde extamente à forma submetida ao periódico uma vez que as figuras foram inseridas no meio do texto a fim de facilitar a leitura e compreensão.

Potential antihyperglycemic role of crude extract, fractions and flavonoids from *Averrhoa carambola* leaves in rats

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Abstract

The effect of the crude extract, ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) fractions and two flavonoids, apigenin-6-C-β-L-fucopyranoside (compound **1**) and apigenin-6-C-(2”-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (compound **2**) isolated from *Averrhoa carambola* L. (Oxalidaceae), were investigated on glycemia and on glycogen content in muscle and liver from hyperglycemic rats. These effects were compared with insulin and/ or tolbutamide in hyperglycemic and diabetic rats. The oral administration of crude extract, ethyl acetate and *n*-butanol fractions of *Averrhoa carambola* leaves exhibited a potential hypoglycemic activity in hyperglycemic normal rats. The maximum hypoglycemic effect detected was with the 400 mg/kg dose of the EtOAc fraction in a very rapid response, 15 min after treatment in hyperglycemic rats. Nevertheless, no alteration in the serum glucose level was detected in diabetic rats with these substances. Also, using this approach, compound **2** (20 mg/kg), a glycosylated flavonoid isolated from the EtOAc fraction, appeared to be a potential natural hypoglycemic agent. Although both compounds exhibited a hypoglycemic effect they appear to act on glucose management through different mechanisms since the compound **1** was less potent as a hypoglycemic substance but more potent in increasing glycogen content of soleus muscle as well as that of the liver.

Keywords: *Averrhoa carambola;* Flavonoids; Diabetes; Hypoglycemia; Antihyperglycemic

Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and/or in insulin action (*1*). Although there has been broad discussion about the etiopathogenesis of diabetes, the vast majority of cases of diabetes fall into type 1 (the cause of which is an absolute deficiency of insulin secretion) or, in a much more prevalent category, type 2 (caused by combination of resistance to insulin action and an inadequate compensatory insulin secretory response) *(1).* The number of people with diabetes is increasing and estimates of diabetes prevalence warn of a worldwide epidemic in the coming years. Along with the increasing prevalence, diabetes care means high costs to the public health service as well as to the individual (*2*).

Medicinal plants used in folk medicine and cited in the scientific literature for the treatment of diabetes may be an alternative to the classical drugs used such as insulin and oral hypoglycemic agents (*3*). Several studies have demonstrated that phytotherapies appear to be effective as hypoglycemic agents being used as adjuvants in the glucose management with no or very few side effects, as well as resulting in a low cost for the maintenance of the entire treatment (*3, 4*). The flavonoids are 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). They have a broad range of biological activities and numerous studies have been carried out on their potential role in the treatment of diabetes (*5, 6*). Therefore, the investigation of such agents from medicinal plants has become more important.

One such plant is *Averrhoa carambola* L**.** (Oxalidaceae), also known as star fruit. This plant is native from tropical and subtropical regions of Asia and it was successfully introduced to Brazil in 1817. *Averrhoa carambola* (*A. carambola*) has been used as an appetite stimulant, a diuretic, an antiemetic, an antidiarrheal, an antifebrile and for the treatment of eczemas (*7*). Ripe carambolas are consumed as fresh fruits, cooked or served in salads. Also, carambola juice is served as a cooling beverage. The fruits are very popular consumed food items (*8*). Recently, the decoction of *A. carambola* leaves has been used in the treatment of diabetes (*9*). Additionally, the fruits of this plant are reported to be rich in insoluble fiber that presents hypoglycemic, antioxidant and hypocholesterolemic effects (*10, 11, 12*).

Other species of the *Averrhoa* genus, such as *Averrhoa bilimbi* Linn. are known for their anti-inflammatory, anti-scorbutic, astringent, anti-bacterial and antidiabetic properties (*13*). In addition, a leaf extract and semi-purified fractions of *A. bilimbi* exhibited hypoglycemic and hypolipidemic effects when administered intraperitoneally (*14*) as well as orally in diabetic rats (*15, 16, 17*). Based on this, the present study investigated the acute effect of crude extract, EtOAc and *n*-BuOH fractions as well as isolated compounds from *A. carambola* leaves on serum glucose levels. Also, the glycogen content in normal hyperglycemic rats was measured in soleus muscle and liver after treatment with glycosylated flavonoids from *A. carambola*.

Materials and methods

Drugs

Alloxan monohydrate, tolbutamide and oyster glycogen type IIwere purchased from Sigma Chemical Company® (St. Louis, MO, USA). Lispro insulin (100 IU/mL; batch FF6D46D; Humalog®—Lilly, São Paulo, SP, Brazil) was purchased from a commercial source. Glucose and all other solvents were purchased from Merck® AG (Darmstadt, Germany). The iodine reagents ($CaCl_2$ + I_2 + KI) were purchased from VETEC, Rio de Janeiro, Brazil. All reagents were of analytical grade.

Plant material

The leaves of *A. carambola* were collected (March, 2003; co-ordinates -27.687799 latitude; -48.777296 longitude) at Santo Amaro da Imperatriz, Santa Catarina, Brazil and identified by Prof.

Daniel de Barcellos Falkenberg. A voucher specimen was deposited at the herbarium of the Botany Department at the Universidade Federal de Santa Catarina, Florianópolis, under number FLOR-24.144.

Extraction and isolation

The powdered, dried leaves (281 g) were extracted with ethanol-water (EtOH-H_2O) (4:1). The extract was concentrated to dryness by rotatory vaporization at 60°C under reduced pressure (41.3 g; crude extract-dry leaves ratio = 14.7%). The concentrated extract was then suspended in EtOH-H_2O (4:1) and successively extracted with *n*-hexane, ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). The ethyl acetate soluble fraction (EtOAc) of 6.9 g was subjected to silica gel (100-200 mesh) CC and eluted with an ethyl acetate/ethanol mixture gradient to afford 36 fractions. Fractions 6-8 (150 mg) and 10-12 (200 mg) were purified by recrystallization from methanol (MeOH) to give pure (HPTLC in ethyl acetate/methanol/acetic acid 80:14:6 and NMR) compounds **1** and **2**, respectively. These compounds were identified by nuclear magnetic resonance spectroscopy (NMR) analysis (^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC) and comparison with literature data (*18*).

Apigenin-6-C-β-L-fucopyranoside (compound **1**): Yellow amorphous powder, (drug-extract ratio = 0.36%). ^{1}H NMR (400 MHz, CD_3OD): aglycone moiety δ: 6.61 (s, H-3), 6.53 (s, H-8), 7.84 (d, *J*=8,0 *Hz*, H-2' & H-6'), 6.92 (d, *J*=8,0 *Hz*, H-3' & H-6'); sugar moiety: 4.64 (d, *J*=8.4 *Hz*, H-1"), 4.03 (t, *J*=8.7 *Hz*, H-2''), 3.97 (dd, *J*=3.0 and 6.5 *Hz*, H-3"), 3.50 (d, J=3.0 *Hz*, H-4"), 3.17 (m, H-5"), 1.44 (d, *J*=6.2 *Hz*, H-6''). ^{13}C NMR (400 MHz, CD_3OD) aglycone moiety δ: 165.1 (C-2), 99.6 (C-3), 183.1 (C-4), 161.7 (C-5), 108.0 (C-6), 163.9 (C-7), 94.0 (C-8), 161.8 (C-9), 102.8 (C-10), 121.9 (C-1'), 128.3 (C-2' and 6'), 115.9 (C-3' and 5'), 157.8 (C-4′); sugar moiety: 71.9 (C-1''), 69.1 (C-2''), 78.5 (C-3''), 70.6 (C-4''), 70.8 (C-5''), 18.1 (C-6'').

Apigenin-6-C-(2"-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (compound **2**): Yellow amorphous powder (drug-extract ratio = 0.48%). ^{1}H NMR (400 MHz, CD_3OD): aglycone moiety δ: 6.61 (s, H-3), 6.54 (s, H-8), 7.85 (d, *J*=7.6 *Hz*, H-2' & H-6'), 6.94 (d, *J*=7,6 *Hz*, H-3' & H-6'); sugar moieties: 4.91 (d, J=9.6 *Hz*, H-1''), 4.27 (t, *J*=8.8 *Hz*, H-2''), 3.75 (m, H-3''), 3.69 (sl, H-4''), 3.84 (m, H-5''), 1.28, d, *J*=6.0 *Hz*, H-6''), 5.18 (sl, H1'''), 3.78 (sl, H-2"'), 3.28 (overlapped with signals of CD_3OD, H-3'''), 3.09 (t, *J*=9.5 *Hz*, H-4'''), 2.54 (t, *J*=8.4 *Hz*, H-5'''), 0.71 (d, *J*=6.0 *Hz*, H-6'''). ^{13}C NMR (400 Mhz, CD_3OD) aglycone moiety δ: 165.1 (C-2), 104.3 (C-3), 183.0 (C-4), 161.6 (C-5), 108.8 (C-6), 163.0 (C-7), 95.0 (C-8), 159.6 (C-9), 102.8 (C-10), 122.0 (C-1'), 128.3 (C-2' and 6'), 115.9 (C-3' and 5'), 157.7 (C-4′); sugar moieties: 72.2 (C-1''), 75.1 (C-2''), 76.5 (C-3''), 72.8 (C-4''), 74.6 (C-5''), 16.0 (C-6''), 101.2 (C-1''') 71.1 (C-2'''), 70.9 (C-3'''), 72.4 (C-4'''), 68.7 (C-5'''), 16.8 (C-6''') (Figure 1).

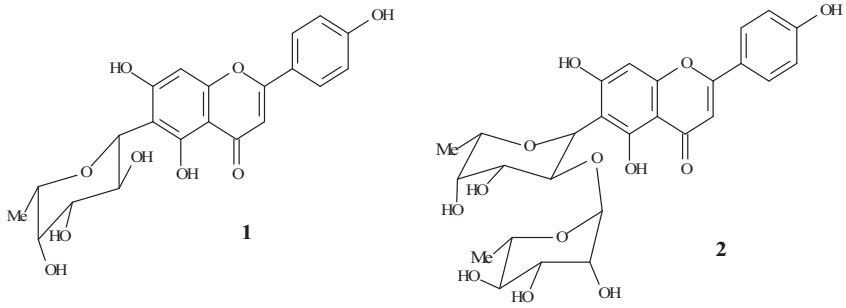

Figure 1. Chemical structure of compounds apigenin-6-C-β-L-fucopyranoside (compound **1**) and apigenin-6-C-(2"-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (compound **2**) isolated from the EtOAc fraction of *A. carambola* leaves.

Animals

Male Wistar rats (190–220 g) were used. They were bred in our animal facility and housed in an air-conditioned room (approximately 22 ± 2 ºC and during the entire experiments) 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. The animals received 50 mg/kg body weight of alloxan by a single intravenous injection. The diabetic state was assessed by measuring body weight and serum glucose levels 3 days later and a serum glucose range of 400-500 mg/dL was used for the experiment. For all oral treatments, 0.5 mL of each respective substance was given by gavage (*19*). All the animals were carefully monitored and maintained in accordance with 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 PP0007).

Study of insulin and tolbutamide effects on the serum glucose level in the oral glucose tolerance curve

Fasted rats were divided into four groups of six animals for each treatment: Group I, normal rats that received vehicle 1% EtOH-H_2O; Group II, hyperglycemic rats that received glucose (4 g/kg b.w.) plus vehicle; Group III, hyperglycemic rats that received glucose (4 g/kg) plus lispro insulin (0.05 IU) by i.p. route; Group IV, hyperglycemic rats that received glucose (4 g/kg) plus tolbutamide (100 mg/kg b.w.) by oral gavage (Table 1). Blood samples were collected just prior to and at 15, 30, 60, 120 and 180 min after the glucose loading and the serum glucose levels were measured.

Study of the effects of crude extract, fractions or isolated compounds from A. carambola *on the serum glucose level in the oral glucose tolerance curve*

Fasted rats were divided into three groups of six animals for each treatment: Group I, normal rats that received vehicle 1% EtOH-H_2O; Group II, hyperglycemic rats that received glucose (4 g/kg) plus vehicle; Group III, rats that received glucose (4 g/kg) plus crude extract (200, 400 and 800 mg/kg b.w.) (Table 1 – Group V). As shown in Figure 2, the hyperglycemic rats received EtOAc or *n*-BuOH (400 and 800 mg/kg b.w.) fractions. Also, hyperglycemic rats received isolated compounds **1** or **2** (20 and 50 mg/kg b.w.) by oral gavage (Figure 3). Blood samples were collected just prior to and at 15, 30, 60, 120 and 180 min after the glucose loading and the serum glucose levels were measured.

Study of the effects of the crude extract and fraction of A. carambola *and tolbutamide on serum glucose levels in alloxan-induced diabetic rats*

Animals in which the development of hyperglycemia was confirmed (around 90%), 72 h after the alloxan injection, were randomly allocated to different groups of six rats for each treatment: Group I, diabetic rats that received vehicle;Group II, diabetic rats that received the crude extract (400 mg/kg b.w.); Group III, diabetic rats that received EtOAc fraction (400 mg/kg b.w.); Group IV, diabetic rats that received tolbutamide (100 mg/kg b.w.). Serum glucose levels were measured at zero time (before receiving the extract, EtOAc fraction or tolbutamide) and at 1, 2 and 3 h following the treatment (Table 2).

Determination of the serum glucose concentration

Blood samples (100 µL) were collected from the tail vein of the anesthetized rat, centrifuged and the serum was used to determine the glycemia (GBC 916 UV – visible spectrophotometer) by the glucose oxidase method (*20*). The commercial kit used to determine the glycemia was from Gold Analisa (Belo Horizonte, MG, Brazil).

Glycogen content measurements

Soleus muscles and livers were harvested from normal fed or fasted rats and hyperglycemic rats treated with compounds **1** or **2** (50 mg/kg) or regular insulin (0.5 IU) and used for the assay of glycogen content immediately after 3 h of treatment (Figures 4A and 4B). Glycogen was isolated

from these tissues as described by Krisman (*21*) with minor modifications. The tissues were weighed, homogenized in 33% KOH and boiled at 100 ºC for 20 min, with occasional stirring. After cooling, 96% ethanol was added to the samples and heated to boiling followed by cooling in an ice bath to aid the precipitation of glycogen. The homogenates were centrifuged at 664 g for 15 min, the supernatant was discarded and the pellets were neutralized with saturated NH_4Cl before being heated to 100 ºC 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 were expressed as mg of glycogen/ g of tissue.

Data and statistical analysis

Data were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Bonferroni *post-test* or unpaired Student's *t*-test was used to identify significantly different groups. Differences were considered to be significant at $p \leq 0.05$. The software InStat version 3.05; Graph-Pad Software Inc., San Diego, CA was used for statistical analysis.

Results

Effect of insulin and tolbutamide on the oral glucose tolerance curve

As expected, after starting the glucose tolerance test in normal rats overloaded with glucose, the serum glucose concentration was significantly increased when compared with the zero time of this group. Also, the classical hypoglycemic effect of insulin was observed over the period studied when compared to the hyperglycemic group. The oral hypoglycemic agent, tolbutamide (100 mg/kg), produced a typical serum glucose lowering at 30 and 60 min compared to the hyperglycemic group. The normal vehicle control group (1% EtOH-H_2O) showed an unchanged profile of glycemia over the time studied (Table 1). On the other hand, tolbutamide (100 mg/kg) failed to modify the glycemia in diabetic rats, reinforcing the diabetic status of the rats (Table 2).

Effect of crude extract, EtOAc, n-BuOH fractions or apigenin-6-C-β-L-fucopyranoside and apigenin-6-C-(2"-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside compounds from A. carambola *on the oral glucose tolerance curve*

Crude extract at 200 and 400 mg/kg was effective in reducing glycemia at 30 min and/ or 60 min after oral treatment when compared with the respective hyperglycemic control group. However, oral treatment with 400 mg/kg of the EtOAc fraction in hyperglycemic rats produced a significant antihyperglycemic effect from 15 to 180 min (Figure 2A). In addition, the glycemia was maintained at basal levels from 60 to 180 min (Figure 2A). On the other hand, the 800 mg/kg dose showed a fast antihyperglycemic effect only in the first two periods studied (15 and 30 minutes) (Figure 2B). Figures 2C and 2D show the effect of the *n*-BuOH fraction of *A. carambola* at 400 and 800 mg/kg in hyperglycemic animals. Although both doses showed a slightly antihyperglycemic effect (at 60 or 30 min and 60 min, respectively) neither 400 nor 800 mg/kg doses were as powerful as the EtOAc fraction (400 mg/kg), previously demonstrated in Figure 2A. Nevertheless, treatment with the crude extract and EtOAc fraction of *A. carambola* (400 mg/kg) did not reduce the glycemic levels in diabetic animals over the period studied (Table 2).

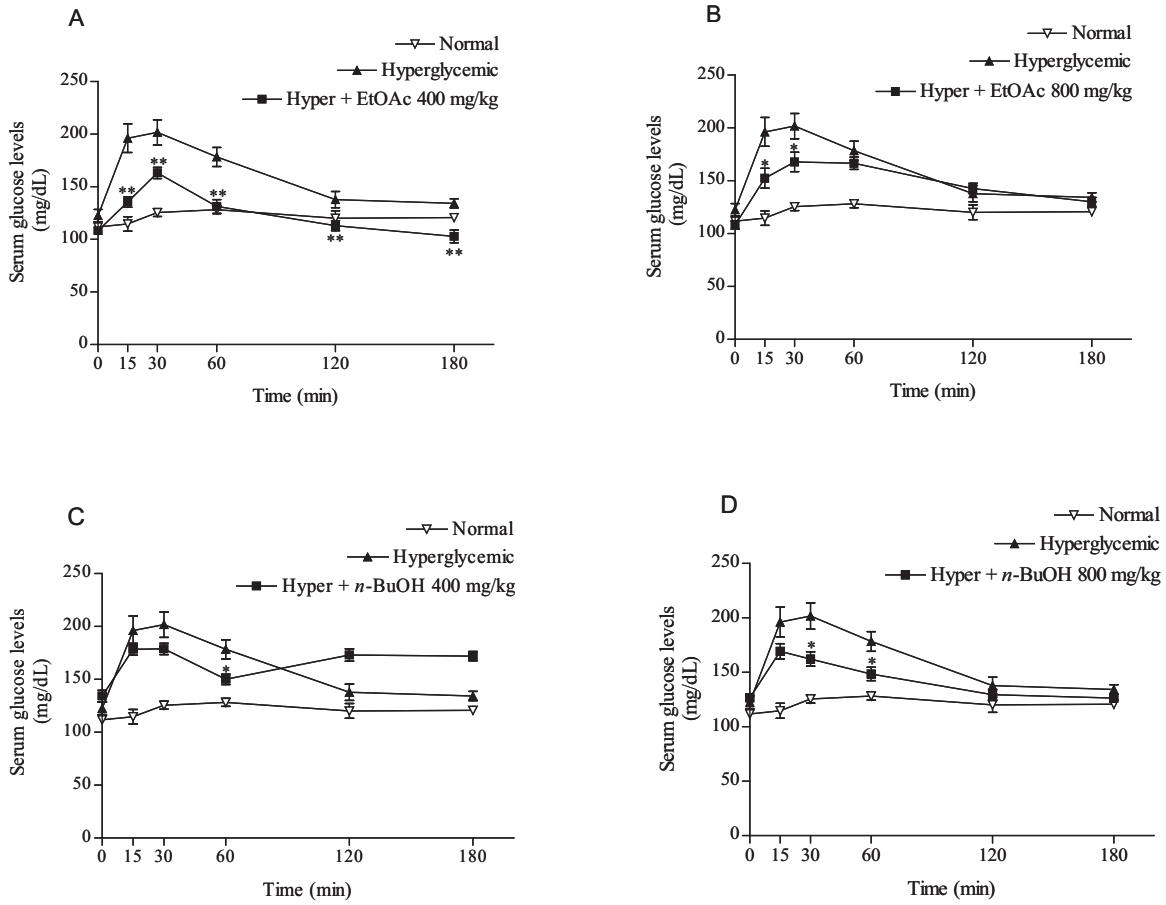

Figure 2. Effect of EtOAc and *n*-BuOH fractions from *A. carambola* on the oral glucose tolerance curve. (A and B) EtOAc 400 and 800 mg/kg, respectively; (C and D) *n*-BuOH 400 and 800 mg/kg, respectively, fractions by gavage. Values are expressed as mean ± S.E.M; n= 6 in duplicate for each treatment. Statistically significant difference to the corresponding hyperglycemic group; $*P \leq 0.05$; $**P \leq 0.001$.

The oral administration of compound **1** reduced serum glucose levels in hyperglycemic rats and the maximum reduction observed was 19% at 15 min with the higher dose (Figure 3A and 3B). The effect of compound **2** on the glucose tolerance curve was similar and is shown in Figures 3C and 3D. Oral administration with 20 and 50 mg/kg of this compound significantly reduced the glycemia by around 32%, 24% and 16% at 15, 30 and 60 min after treatment with the higher dose used. At 120 and 180 min, glycemic levels were similar to respective results for the hyperglycemic control groups.

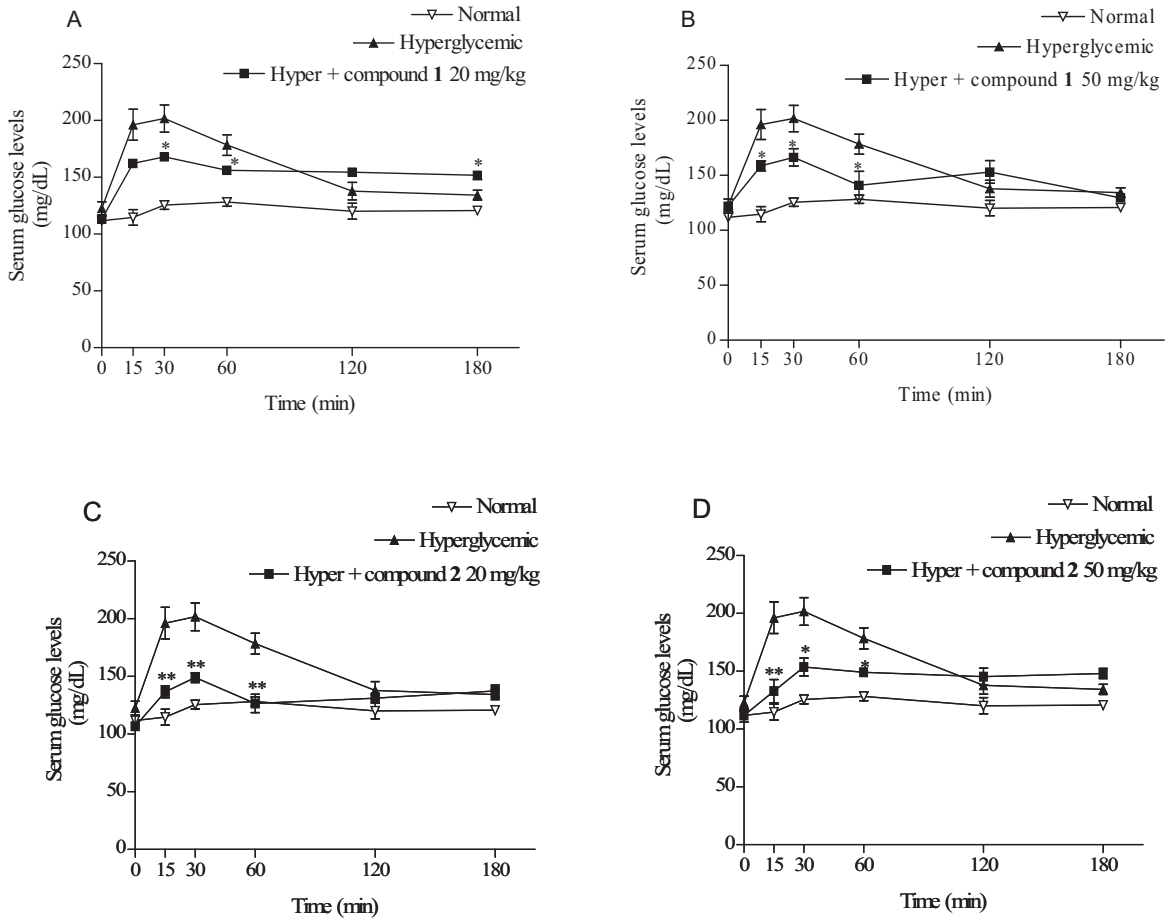

Figure 3 Effect of compound **1** and compound **2** isolated compounds from *A. carambola* on the oral glucose tolerance curve. Values are expressed as mean ± S.E.M; n= 6 in duplicate for each treatment. Statistically significant difference to the corresponding hyperglycemic group; *$P \leq 0.05$; **$P \leq 0.001$.

Effect of apigenin-6-C-β-L-fucopyranoside and apigenin-6-C-(2”-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside from A. carambola *on the glycogen content of soleus muscle and liver*

Figures 4A and 4B show that the glycogen content was significantly increased, 3 h after the administration of glucose (4 g/kg) by oral gavage, in soleus muscle compared with fed and fasted normal rats and in liver when compared with fasted normal rats. In addition, the well known insulin stimulatory effect on glycogen storage in both tissues was observed 3 h after insulin treatment (9,3 times for muscle and 19,6 times for liver) in hyperglycemic normal rats compared with fasted normal rats.

Compound **1** was able to significantly increase the glycogen content in soleus muscle when compared with fed normal (4,6 times), fasted normal (10,7 times), hyperglycemic (1,2 times) and hyperglycemic plus insulin (1,14 times) animals 3 h after treatment. Moreover, compound **1** stimulated glycogen content in the liver as well. This change was 25 times when compared with the fasted normal group at 3 h after treatment and it represented 54% of the insulin stimulatory effect.

The stimulatory effect of compound **2** on muscle glycogen content (around 1,7 and 3,9 times) was observed 3 h after treatment when compared with fed and fasted groups, respectively. However, compound 2 did not alter the glycogen content as compared with hyperglycemic group. In liver, the effect of compound **2** was around 10-fold when compared with fasted normal rats and when compared with insulin, representing 21% of the total stimulatory effect of the hormone.

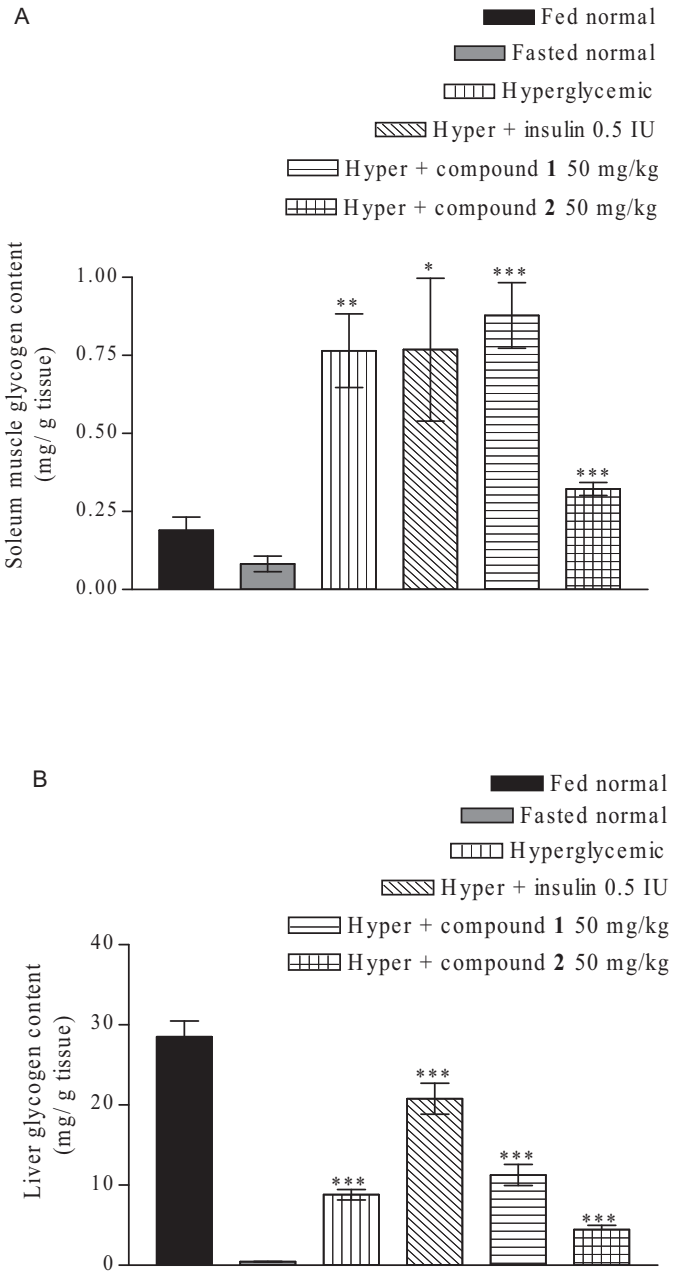

Figure 4 Effect of compound **1** and compound **2** on the glycogen content in normal hyperglycemic rats. (A) soleus muscle and (B) liver 3h after treatment by oral gavage. Values are expressed as mean ± S.E.M; n=6 in duplicate for each group. Significantly different to the corresponding fasted normal group; *$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$.

Discussion

Many plant species are known in the folk medicine of different cultures to be used for the treatment of diabetes mellitus (*3, 4*). Moreover, phytochemical and pharmacological studies that were performed in order to characterize such compounds have shown the presence of flavonoids as the active constituents in several plant extracts (*4, 20, 22, 23*).

Flavonoids are a large group of phenolic plant constituents and their bioactive potential in the treatment and prevention of diabetes and other diseases has been demonstrated (*5, 6*). They can affect glucose transport and metabolism in peripheral tissues as well as inducing insulin release from β-cells in the pancreas (*20, 24, 25, 26, 27*).

This study showed the antihyperglycemic effect of the crude extract, fractions and isolated compounds of *A. carambola* leaves in normal hyperglycemic rats following an acute treatment. Similar results showing the glucose lowering effects of the crude extract of *A. carambola* leaves in hyperglycemic normal rats were reported by Provasi et al (*9*). Also, it has been reported in the literature that the extracts of another *Averrhoa* species, *Averrhoa bilimbi*, showed antihyperglycemic effects in normal hyperglycemic and diabetic rats when compared with the respective control groups. Furthermore, chronic daily treatment with *A. bilimbi* extracts decreased glycemia in diabetic rats compared to both the control group and a metformin group (*15*). Taking this into account, the effect of the crude extract points to a mechanism involving the regulation of insulin release and/or the inhibition of intestinal glucose absorption (*5, 6, 28*).

The EtOAc and *n*-BuOH fractions were isolated from the crude extract and their potential antihyperglycemic effects were studied in normal glucose-fed rats. The antihyperglycemic effect of the EtOAc fraction was more marked than that of *n*-BuOH at 400 mg/kg and was around 26.4% at 60 min after treatment (Figures 2A and 2C). This effect was as pronounced as that caused by both tolbutamide (18.26%) (Table 1), a sulfonylurea agent that increases insulin secretion from the pancreas (*29*) and the rapid effect of lispro insulin (29.2%) during the same time-course studied (Table 1). Additionally, the absence of effect of the crude extract and EtOAc fraction in alloxan-induced diabetic rats is in accordance with the lack of tolbutamide response (used to the therapy for type 2 diabetes), observed in the same experimental model. Consequently, the effect of crude extract as well as the EtOAc fraction appears to be mediated through mechanisms involving insulin secretion from β-cells (*28, 30*). In line with these results, previous studies of Pushparaj et al (*16*) and Tan et al (*17*) showed the effect of aqueous and *n*-BuOH fractions from *A. bilimbi* leaves on glucose serum levels in diabetic and hyperglycemic normal rats. Both fractions improved the glucose tolerance curve in diabetic rats and hyperglycemic rats. Furthermore, after two weeks of treatment the fractions increased plasma insulin levels when compared with the zero time and with the diabetic control group.

Considering the EtOAc fraction's antihyperglycemic effect, two glycosylated flavonoids, compound **1** and compound **2** (C-flavones) were isolated from this fraction from *A. carambola* leaves (*31*). The oral administration of compounds **1** and **2** at 20 and 50 mg/kg resulted in significant antihyperglycemic effects in glucose-fed normal rats. Recently, Hsu et al (24) demonstrated the antihyperglycemic effect of puerarin, an isoflavone, in normal rats, hyperglycemic normal rats and diabetic rats. Puerarin reduced glycemia in normal and diabetic rats in a dose dependent manner and it was also able to attenuate the increase of plasma glucose induced by an intravenous glucose challenge in normal rats.

We have demonstrated the antihyperglycemic effect of the EtOH fraction, rich in glycosylated flavonoids, from *Bauhinia forficata* leaves as well as the major flavonoid of that fraction, kaempferitrin (*19, 23*). The antihyperglycemic activity of plant extracts rich in flavones in hyperglycemic and diabetic rats has been described (*22, 32*). The EtOAc fraction from *Gentiana olivieri* and isoorientin, a C-glycosylflavone, and the active compound isolated from this fraction, significantly reduced serum glucose levels in hyperglycemic and diabetic rats. However, the high antioxidant activity of isoorientin is suggested as a possible mechanism of action, protecting β-cells from oxidative damage and restoring plasma insulin levels (*32*).

The results observed for compounds **1** and **2** suggest that some pancreatic functions such as insulin secretion or the presence of insulin are required for the antihyperglycemic activity of such compounds since the EtOAc fraction from which compounds **1** and **2** originated had an effect in hyperglycemic normal rats but not in diabetic rats.

In mammals, carbohydrate is stored mainly in the form of glycogen, with skeletal muscle and liver as the major storage sites. Glycogen metabolism is regulated by insulin/glucagon through activation and/or inhibition of several enzymes and proteins (*33, 34*). The determination of glycogen levels in muscle and liver of hyperglycemic normal rats after acute treatments with compounds **1** and **2** revealed a significant increase in glycogen content when compared with fasted normal rats (Figures 4A and 4B). Additionally, the known effect of insulin on muscle and hepatic glycogen storage was observed. The study demonstrated that the stimulatory effect of compound **1** on glycogen storage in soleus muscle as well as in liver was greater than that of compound **2** at the same dosage (50 mg/kg). Such an effect of compound **1** was quite similar to that of insulin in muscle. The effect of this compound represented 54% of the total stimulatory effect of insulin in liver 3 h after treatment.

It is well known that glycogen deposition from glucose is regulated by insulin in two steps: firstly by controlling the uptake and transport of glucose, and secondly by regulating the phosphorylation/ dephosphorylation status of enzymes involved in the metabolism of glycogen. Flavonoids and plant extracts with proven antihyperglycemic activity have been shown to influence glycogen deposition in different tissues as well as to interact with key enzymes of the glycolytic route in rats (*25, 26, 35*).

The results found for compound **2** and specially for compound **1** (Figures 3 and 4) are closely correlated with the known insulin activity on the stimulatory effect of glucose disposal and lowering serum glucose levels in normal hyperglycemic rats (Figure 4 and Table 1). The increase in glycogen content in both liver and muscle may be due to stimulation of insulin release from β-cells (*27, 28, 30, 36*). The insulinomimetic activity of compounds **1** and/or **2** could be a consequence of direct peripheral glucose uptake, glycogen synthesis or a combination of both. However, studies are underway to elucidate the mechanism involved in the antihyperglycemic effect of these flavonoids in muscle and liver.

We conclude that the crude extract, ethyl acetate and *n*-butanol fractions of *A. carambola* leaves were effective in decreasing the serum glucose levels in hyperglycemic normal rats. The maximum antihyperglycemic effect detected was at a dose of 400 mg/kg with a very rapid response, 15 min after treatment. Also, using this approach, compound **2** (20 mg/kg), a glycosylated flavonoid isolated from the *A. carambola* EtOAc fraction, appears to be a potential natural antihyperglycemic agent. Although both compounds exhibited an antihyperglycemic effect they appear to act on the blood glucose management by different mechanisms, since compound **1** was less potent as an antihyperglycemic substance but more potent in increasing glycogen content in the soleus muscle as well as in the liver.

Considering these findings, *A. carambola*, popularly known as an antihyperglycemic plant, contains compound(s) able to manage glucose utilization, probably through different pathways. Among these compounds, **1** and **2** may be attractive adjuvants for the treatment of diabetic patients in the future.

Acknowledgements

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Table 1 Acute effect of lispro insulin, tolbutamide and crude extract of *A. carambola* on the serum glucose level in the oral glucose tolerance curve[a].

Serum Glucose Level (mg/dL)							
Time (min)	Group I vehicle (1% EtOH-H_2O)	Group II Hyperglycemic Glucose (4 g/kg) plus vehicle	Group III hyperglycemic plus lispro insulin (0.05 IU)	Group IV hyperglycemic plus tolbutamide (100 mg/kg)	Group V glucose fed hyperglycemic plus *A. carambola* crude extract		
					200 mg/kg	400 mg/kg	800 mg/kg
0	111.8 ± 2.8	122.6 ± 5.8	113.1 ± 2.6	108.4 ± 5.1	110.1 ± 3.3	111.8 ± 5.7	125.4 ± 7.8
15	114.7 ± 6.8	196.2 ± 3.6***	147.2 ± 2.1***#	168.6 ± 5.6***	178.4 ± 8.7***	174.7 ± 7.3***	172.0 ± 2.8***
30	125.5 ± 3.8	201.7 ± 1.9***	151.7 ± 4.3***#	172.0 ± 10.4***#	184.4 ± 7.6***	167.8 ± 7.3***#	182.2 ± 5.8***
60	128.3 ± 3.8	178.4 ± 9.1***	126.2 ± 4.2#	145.8 ± 6.8#	147.5 ± 5.6**#	153.2 ± 5.9***#	165.9 ± 8.9**
120	120.1 ± 6.9	137.8 ± 7.7	126.0 ± 2.7#	131.8 ± 9.5	138.4 ± 6.0	139.8 ± 5.2	151.8 ± 6.7
180	120.7 ± 2.0	134.3 ± 4.4	123.3 ± 3.6	131.8 ± 6.9	144.1 ± 4.2*	149.5 ± 4.1**	140.4 ± 6.0

[a] Values expressed as mean ± S.E.M.; n = 6 in duplicate for each treatment. Statistically significant difference to the corresponding zero time value; *$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$. Significantly different to the corresponding hyperglycemic group; # $P \leq 0.05$.

Table 2 Acute effect of crude extract and EtOAc fraction of *A. carambola* and tolbutamide on the serum glucose level in alloxan-induced diabetic rats[a].

Serum Glucose Level (mg/dL)				
Time (h)	Group I Diabetic control (vehicle 1% EtOH-H_2O)	Group II Diabetic plus Crude extract (400 mg/kg)	Group III Diabetic plus EtOAc (400 mg/kg)	Group IV Diabetic plus Tolbutamide (100 mg/kg)
0	401,6 ± 17,3	443.0 ± 13.6	446.9 ± 9.6	455.0 ± 3.9
1	438,4 ± 11,8	486.2 ± 13.2	485.5 ± 13.8	455.8 ± 22.7
2	441,4 ± 11,9	464.5 ± 14.7	486.2 ± 13.1	432.5 ± 14.8
3	429,6 ± 14,2	482.5 ± 11.2	477.7 ± 15.8	518.2 ± 1.3

[a] Values expressed as mean ± S.E.M.; n = 6 in duplicate for each treatment.

4.2 ARTIGO ACEITO PARA PUBLICAÇÃO

Periódico – European Journal of Medicinal Chemistry

CAZAROLLI, L.H.; FOLADOR, P.; MORESCO, H.H.; BRIGHENTE, I.M.C.; PIZZOLATTI, M.G.; SILVA, F.R.M.B. **Stimulatory Effect of Apigenin-6-C-β-L-fucopyranoside on Insulin Secretion and Glycogen Synthesis.**

Nota: A formatação deste artigo não corresponde extamente à forma submetida ao periódico uma vez que as figuras foram inseridas no meio do texto a fim de facilitar a leitura e compreensão.

Stimulatory Effect of Apigenin-6-C-β-L-fucopyranoside on Insulin Secretion and Glycogen Synthesis

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Abstract

In vivo and in vitro treatments were carried out to investigate the effects of apigenin-6-C-β-L-fucopyranoside (**1**), isolated from *Averrhoa carambola* L. (Oxalidaceae), on serum glucose and insulin levels in hyperglycemic rats as well as its effect on glycogen synthesis in normal rat soleus muscle. Apigenin-6-C-β-L-fucopyranoside showed an acute effect on blood glucose lowering in hyperglycemic rats and stimulated glucose-induced insulin secretion. A stimulatory effect of **1** on glycogen synthesis was observed when muscles were incubated with this flavonoid and also its effect was completely nullified by pre-treatment with insulin signal transduction inhibitors. Taking this into account, the MAPK - PP1 and PI3K – GSK3 pathways are involved in the apigenin-6-C-β-L-fucopyranoside-induced increase in glycogen synthesis in muscle. This study provides evidence for dual effects of apigenin-6-C-β-L-fucopyranoside as an antihyperglycemic (insulin secretion) as well as an insulinomimetic (glycogen synthesis) agent.

Keywords: Flavonoid; Insulin; Diabetes; Glycogen synthesis.

1. Introduction

Diabetes mellitus is a complex metabolic disorder in the endocrine system characterized by abnormalities in insulin secretion and/or insulin action that lead to progressive failure of glucose tolerance and cause chronic hyperglycemia [1]. In recent years there has been a growing interest in hypoglycemic agents from natural products, especially those derived from plants. Medicinal plants used in folk medicine for diabetes treatment may provide an alternative to the classical drugs used (insulin and oral hypoglycemic agents), with few side effects and low cost for the maintenance of treatment [2,3].

Flavonoids are naturally occurring phenolic compounds that are widely distributed in plants. They have a broad range of biological activities and numerous studies have been carried out on their potential role in the treatment of diabetes and other diseases [4,5,6]. They can exert effects on glucose transport and metabolism in peripheral tissues and ameliorate diabetic status [7,8,9,10,11]. We have previously reported the acute hypoglycemic effect of flavonoid and flavonoid-enriched fractions in diabetic rats, as well as the chalcone analogues in hyperglycemic rats [8,9,12,13,14]. Recently, it was demonstrated that crude extracts, flavonoid-enriched fractions from leaves of different species of *Averrhoa* are able to diminish serum glucose levels in hyperglycemic rats as efficiently as insulin [15,16,17].

Insulin is the most important hormone in the regulation of blood glucose concentrations. It mediates a wide spectrum of biological responses including synthesis and storage of carbohydrates, lipids and proteins, activation of specific gene transcription, and modulation of cellular growth and differentiation [18,19]. This hormone is synthesized and stored in pancreatic β cells. Glucose is the primary stimulus for insulin secretion and when blood sugar concentrations rise, insulin is secreted into the blood stream. Initially, glucose enters β-cells through the high capacity glucose transporter type 2 (GLUT 2) and is phosphorylated by glucokinase. The generation of ATP from glycolysis increases the intracellular ATP/ADP ratio. ATP binds to ATP-dependent K^+-channels on the β-cell membranes closing these channels and depolarizing the cells. The depolarization activates voltage-sensitive calcium channels causing a calcium influx triggering insulin secretion [20,21]. Among the oral antihyperglycemic agents currently used in diabetes therapy, sulphonylureas, for example glipizide, are known to stimulate insulin secretion from β-cells by inducing ATP-dependent K^+ channels to close, which activates downstream events that lead to the release of insulin-containing-vesicles [22]. Insulin released into the blood promotes glucose uptake by tissues such as fat and muscle. The binding of insulin to its receptor activates the intrinsic receptor tyrosine kinase, which results in autophosphorylation and recruitment of substrates, such as insulin receptor substrate (IRS) proteins. Specific tyrosine residues on the IRS proteins serve as docking sites for proteins that contain SH2 domains, such as the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K) [18,19]. 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 GLUT4-containing vesicles to the plasma membrane [18,19]. Most of the glucose that enters cells, especially in muscle fibers through GLUT4 in response to insulin, is converted into glycogen. This hormonal effect involves activation of glycogen synthase (GS), the enzyme that catalyzes the rate-limiting step in the conversion of intracellular glucose into glycogen [23,24]. Insulin activates GS by promoting dephosphorylation of several sites of the enzyme through the inhibition or stimulation of protein kinases and phosphatases [23,25]. Moreover, insulin also regulates GS activation by controlling the uptake and transport by GLUT4 of glucose and by regulating the phosphorylation and activation states of enzymes involved in the synthesis and degradation of glycogen [23,24].

The regulation of these cellular processes involves the activation of the PI3K-PDK-PKB pathway, similarly to the stimulation of glucose uptake. Additionally, activated PKB phosphorylates and inhibits glycogen synthase kinase 3 (GSK3). GSK3 is one of the several kinases that

phosphorylates glycogen synthase providing the enzyme is in an inactive state. The inhibition of GSK3 activity results in the dephosphorylation and activation of glycogen synthase [23,26,27]. Additional mechanisms that contribute to glycogen synthesis involve dephosphorylation of glycogen synthase by phosphatases such as protein phosphatase 1 (PP1) that is activated by phosphorylation at specific sites by insulin [23,25,28]. Moreover, it has been suggested that the MAPK/p90 ribosomal S6 kinase ($p90^{rsk}$) insulin-stimulated pathway could also be involved in insulin-induced PP1G and GSK3 phosphorylation as well as glycogen synthase activation, besides the classical metabolic PI3K pathway [29,30].

Many studies have demonstrated the hypoglycemic effects of flavonoids, as well as their action on insulin secretion [8,12,31,32,33], glucose uptake [7,9,34] and glycogen metabolism [11,35,36]. Based on the efficient antihyperglycemic effect of apigenin-6-C-β-L-fucopyranoside (**1**), compared with exogenous insulin action in hyperglycemic rats, the aim of this study was to investigate the acute effect of **1** on insulin secretion and on glycogen synthesis as well as the mechanism of action of this flavonoid in a target tissue of insulin, soleus muscle.

2. Results and Discussion

*2.1. Effect of Apigenin-6-C-β-L-fucopyranoside (**1**) on Serum Glucose and Insulin Levels in Hyperglycemic Rats*

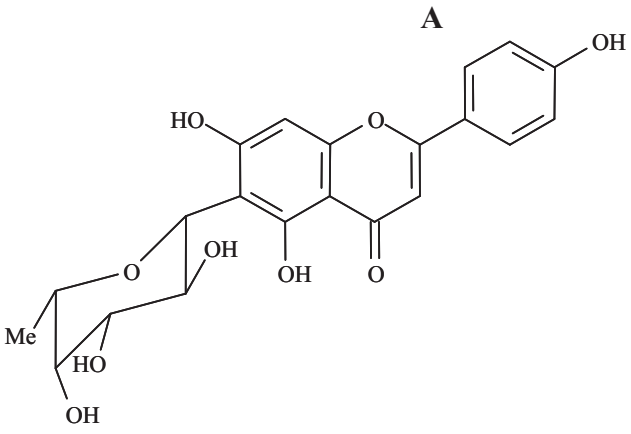
Table 1 shows the in vivo effect of apigenin-6-C-β-L-fucopyranoside (**1**) (50 mg/kg) and glipizide (10 mg/kg) on serum glucose levels in hyperglycemic normal rats following the treatment. As expected, after starting the glucose tolerance test serum glucose concentration was significantly increased when compared with zero time of the hyperglycemic group. Glipizide was able to reduce the glycemia significantly from 15 to 60 min after oral treatment and reached basal levels after that. The oral administration of **1** reduced significantly the serum glucose levels in hyperglycemic rats. This efficient and acute effect of **1** (50 mg/kg) reproduced up to 50% of the maximum glipizide action on the glucose serum lowering. Flavonoids are a large group of phenolic plant constituents and their bioactive potential as antihyperglycemic and/or hypoglycemic agents has been described [6]. We have previously demonstrated the effect of flavonoids and chalcones on serum glucose lowering in hyperglycemic and diabetic rats [8,12,14]. Furthermore, Hsu et al. [7] have demonstrated the antihyperglycemic effect of puerarin, an isoflavone, in normal rats, hyperglycemic normal rats and diabetic rats. Puerarin reduced glycemia in normal and diabetic rats in a dose-dependent manner and it was also able to attenuate the increase of plasma glucose induced by an intravenous glucose challenge in normal rats.

Table 1. Effect of apigenin-6-C-β-L-fucopyranoside (**1**) (50 mg/kg) and glipizide (10 mg/kg) on serum glucose levels in oral glucose tolerance curve [a]

Serum Glucose Levels (mg/dL)				
Time (min)	Group I Euglycemic Vehicle (1% EtOH-H_2O)	Group II Hyperglycemic Glucose (4 g/kg)	Group III Hyperglycemic plus **1** (50 mg/kg)	Group IV Hyperglycemic plus Glipizide (10 mg/kg)
0	115.76 ± 2,8	120.4 ± 6.5	121.97 ± 1.77	120.0 ± 2.10
15	114.73 ± 6,8	191.21 ± 11.91	158.62 ± 4.58*	160.0 ± 6.60*
30	125.53 ± 3,8	205.12 ± 9.75	166.39 ± 7.98*	143.0 ± 7.00*
60	128.25 ± 3,8	169.45 ± 10.21	140.98 ± 12.75*	123.0 ± 7.85*

[a] Values expressed as mean ± S.E.M.; n = 6 in triplicate for each treatment. Significant at *$p \leq 0.05$ in relation to the corresponding hyperglycemic group.

Serum insulin levels were determined in fasted rats after an oral glucose loading (4 g/kg) as shown in Fig. 1. The glucose induced-insulin secretion was increased 71% at 15 min compared with zero time and returned to the basal levels after 60 min. Biphasic insulin secretion is the normal response of β cells to a rapid and sustained increase in glucose concentration. The first phase corresponds to a prompt and marked, but transient (4-7 min), increase in the secretory rate. It is followed by a decrease to a minimum and sustained second phase that lasts as long as the glucose stimulation is maintained [21,37]. The rapid increase in insulin levels observed at 15 min after the oral glucose loading confirms, in this model, the classical profile of insulin secretion. It was observed that **1** stimulated insulin secretion at 15 min compared with the basal group and also potentiated the glucose effect after 30 and 60 min when compared to the hyperglycemic group. These results are in agreement with those reported for isoorientin, a C-glycosylflavone that significantly reduced serum glucose levels in hyperglycemic and diabetic rats due to the protection of β-cells from oxidative damage and also by restoring plasma insulin levels [31]. Furthermore, genistein and daidzein have been observed to potentiate glucose-induced insulin secretion as demonstrated by an in vitro direct action on pancreatic β cells [33,38]. Anthocyanins, anthocyanidins, quercetin and (-) epicatechin have also been described as insulin secretagogues [32,39]. The results here presented suggest that **1** is a novel flavonoid with strong antihyperglycemic characteristics.



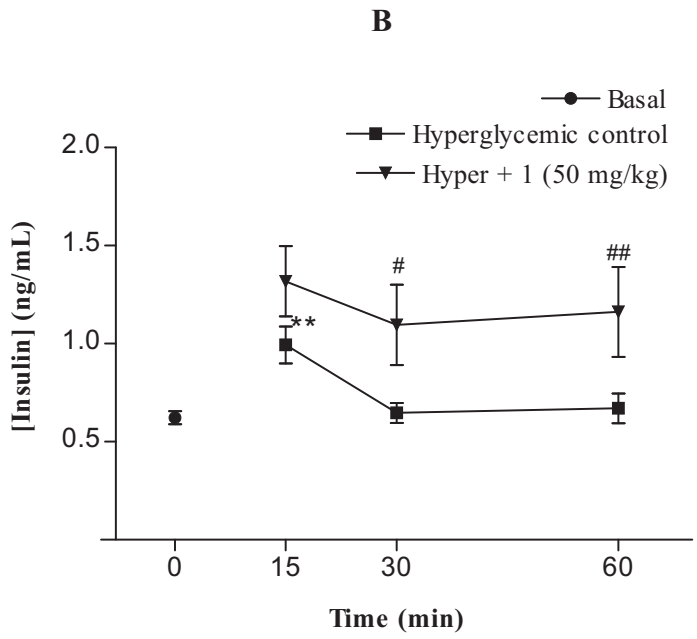


Fig. 1. Apigenin-6-C-β-L-fucopyranoside (**1**) structure (A) and effect of 50 mg/kg apigenin-6-C-β-L-fucopyranoside (**1**) on serum insulin levels in hyperglycemic rats (B). Values are expressed as mean ± S.E.M.; n = 8 in duplicate for each group. Significant at $^{**}p \leq 0.01$ in relation to zero time; $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ in relation to hyperglycemic group.

*2.2. Effect of Apigenin-6-C-β-L-fucopyranoside (**1**) on Glycogen Synthesis*

Insulin is the most important hormone that regulates glycogen synthesis in skeletal muscle [18,23]. This hormone induces its effects by phosphorylating/dephosphorylating several intracellular enzymes activating or deactivating them and subsequently stimulating GS activity [23,29,40].

It was studied the effect of apigenin-6-C-β-L-fucopyranoside **(1)** on glycogen synthesis in rat soleus muscle. As shown in Fig. 2, apigenin-6-C-β-L-fucopyranoside **(1)** at 1 and 100 μM caused a significant increase in glycogen synthesis (around 50%) in soleus muscle compared with the basal group. The stimulatory effect of apigenin-6-C-β-L-fucopyranoside **(1)** is comparable with those reported to insulin (100 nM; 113% of stimulatory effect compared with the control group) [41] although the dose of **1** was higher than that used to insulin.

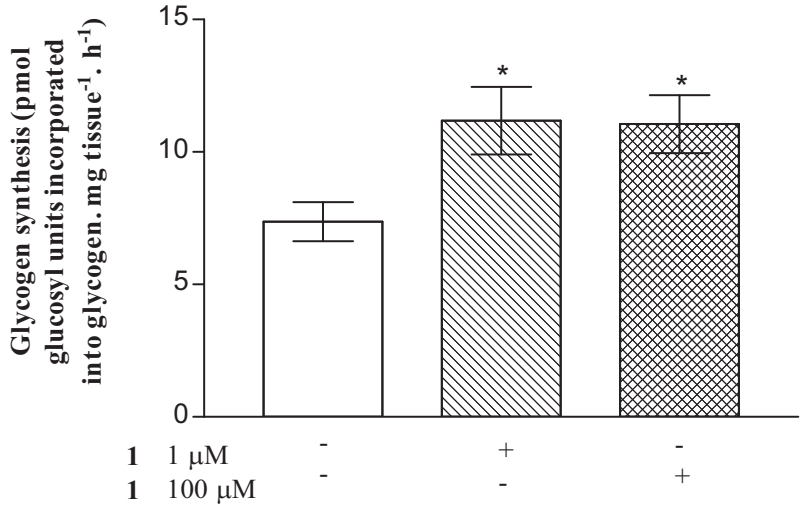


Fig. 2. Effect of apigenin-6-C-β-L-fucopyranoside (**1**) on glycogen synthesis in rat soleus muscle. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; n = 6 in duplicate for each group. Significant at $*p \leq 0.05$ in relation to basal group.

Flavonoids have been demonstrated to act on insulin signaling pathways that regulate glucose uptake and glycogen synthesis [6]. We have demonstrated the hypoglycemic effect of kaempferitrin in diabetic rats and the stimulatory effect of this flavonoid on glucose uptake in rat soleus muscle [8,9]. Also, it has been demonstrated that kaempferol 3-neohesperidoside, structurally very similar to kaempferitrin, was able to decrease blood glucose levels in diabetic rats. Additionally, it was shown to increase glucose uptake and glycogen content in soleus muscle more efficiently than kaempferitrin [9,12,42]. Catechin, myricetin and procyanidin extracts derived from grape seeds have also been shown to increase glycogen synthesis and glucose uptake in insulin responsive tissues and cells [34,35,36]. These findings of glycogen synthesis in soleus muscle support the insulinomimetic effect of apigenin-6-C-β-L-fucopyranoside.

*2.3. Effect of Various Inhibitors on the Stimulatory Action of Apigenin-6-C-β-L-fucopyranoside (**1**) on Glycogen Synthesis in Rat Soleus Muscle*

To determine the mechanism by which **1** induced glycogen synthesis in the soleus muscle, we performed the glycogen synthesis assay with 5 nM calyculin A, a specific inhibitor of PP1 activity, 50 μM PD98059, a specific inhibitor of MEK, 100 nM wortmannin, a specific inhibitor of PI3K, or 50 mM LiCl, a known inhibitor of GSK3. The inhibitor concentrations used were those previously reported in the literature [29,40,42]. Fig. 3 shows that the glycogen synthesis stimulated by **1** was inhibited by calyculin A and PD98059 pre-treatment. When only calyculin A or PD98059 were added to the muscle samples, no significant change resulted compared with the basal glycogen synthesis. Thus, the stimulatory effect of **1** on glycogen synthesis seems to be mediated, at least in part, through insulin signaling involving the MAPK and PP1 pathways. These results are in agreement with the literature concerning the role of MAPK and PP1 activity on glycogen synthesis regulated by insulin. Insulin has been demonstrated to increase PP1 activity and glycogen synthase activity in HepG2 cells. The same result has been reported with regard to L6 rat skeletal muscle cells and adipocytes [40,43]. The activation of PP1 by insulin is mediated by increasing its phosphorylation state at site 1 [23,43]. This site is readily phosphorylated in vitro by $p90^{rsk}$, a kinase that is phosphorylated and activated by MAPK when cells or tissues are incubated with insulin [30,44]. The MAPK pathway has been proposed to counteract increased glycogen synthesis stimulated by insulin in hepatocytes since PD98059 totally inhibited insulin-stimulated glucose incorporation into glycogen [29]. Furthermore, Dent et al. have suggested that the phosphorylation and activation of $p90^{rsk}$ by MAPK increase its activity toward site 1 on PP1 in rabbit skeletal muscle [30].

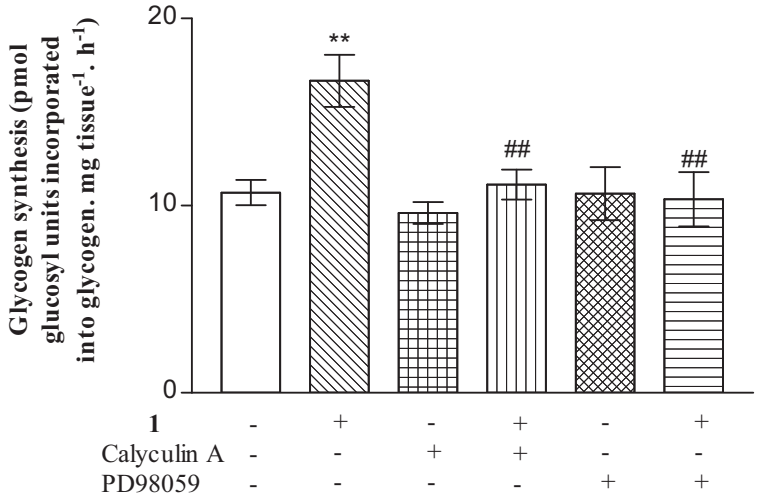


Fig. 3. Effect of 5 nM calyculin A and 50 μM PD98059 on the stimulatory action of 1 μM apigenin-6-C-β-L-fucopyranoside (**1**) on glycogen synthesis in rat soleus muscle. Basal group = no treatment. Signals (+) and (-) indicate presence and absence, respectively, of each substance in the incubation medium. 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 $^{**}p \leq 0.01$ in relation to basal group; $^{\#\#}p \leq 0.01$ in relation to apigenin 6-C-fucopyranoside (**1**) group.

Additionally, the stimulatory effect of **1** on glycogen synthesis was totally inhibited in the presence of wortmannin, as shown in Fig. 4. The involvement of the PI3K pathway in glycogen synthesis regulated by insulin has previously been demonstrated in 3T3-L1 adipocytes, hepatocytes and L6 myotubes [29,45,46]. It has been demonstrated that PI3K phosphorylates and activates PDK 1 which acts on PKB increasing its activity [26,27]. One important target of PKB is GSK3. Under basal conditions, GSK3 is constitutively active and phosphorylates and inactivates GS. The inactivation of GSK3 isoforms GSK3α and GSK3β by insulin is achieved through phosphorylation of Ser^{21} and Ser^{9}, respectively, resulting in the activation of GS and an increase in glycogen synthesis [27,40,47].

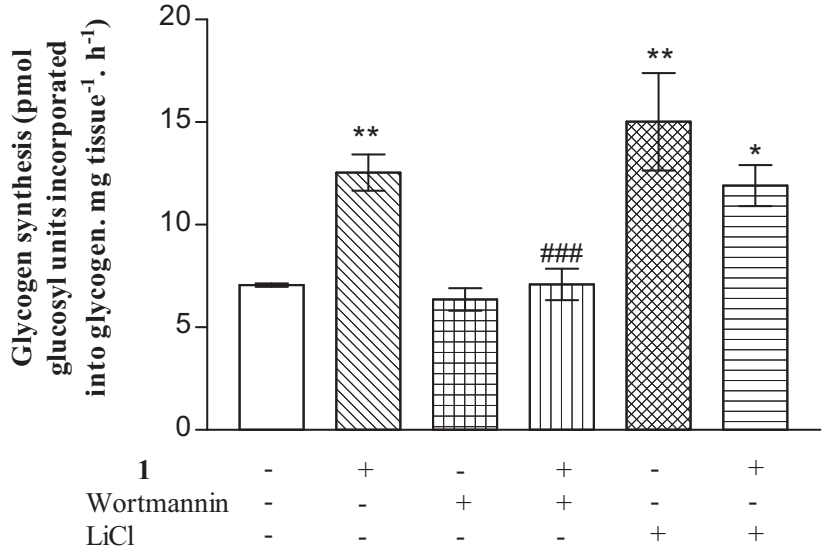


Fig. 4. Effect of 100 nM wortmannin and 50 mM LiCl on the stimulatory action of 1 μM apigenin-6-C-β-L-fucopyranoside (**1**) in glycogen synthesis in rat soleus muscle. Basal group = no treatment. Signals (+) and (-) indicate presence and absence, respectively, of each substance in the incubation medium. 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 $^{**}p \leq 0.01$ and $^{*}p \leq 0.05$ in relation to basal group. Significant at $^{\#\#\#}p \leq 0.001$ in relation to apigenin 6-C-fucopyranoside (**1**) group.

To study the involvement of GSK3 in the mechanism of action of **1** in relation to glycogen synthesis, lithium chloride (LiCl) was used. It was observed that **1** and LiCl caused similar increases in glycogen in soleus muscle (around 69% and 78%, respectively). These results suggests that, although the stimulatory effect of **1** and LiCl are very close, they may act on the same site of GSK3, since no additive effect on glycogen synthesis was observed (Fig. 4).

In hepatocytes from normal and diabetic rats lithium and insulin induced a substantial increase in glycogen accumulation as well as in GS activity. Also, lithium potentiated the effect of insulin in these events [48,49]. Additionally, in L6 myotubes and 3T3-L1 adipocytes, LiCl and insulin induced an increased inhibition of GSK3 activity, confirming the key role of GSK3 on GS

dephosphorylation and activation [50]. In agreement with this finding, it has been demonstrated that in L6 myocytes, insulin stimulates glycogen synthase and glycogen synthesis through the inactivation of GSK3 by a phosphorylation-dependent mechanism (involving the PI3K/PKB pathway) while LiCl, unlike insulin, exerts its effect through a phosphorylation-independent mechanism (involving a direct inhibitory action on GSK3) [51,52]. Since no additive effects of **1** and LiCl on glycogen synthesis were observed we can suppose that the action of the flavonoid may occurs at a distinct GSK3 site of insulin action.

3. Conclusions

We showed that apigenin-6-C-β-L-fucopyranoside stimulated insulin secretion and potentiated glucose-induced insulin secretion in hyperglycemic rats. In addition, this flavonoid stimulated glycogen synthesis in rat soleus muscle through mechanisms well known to insulin signal transduction. These results constitute the first evidence indicating that lowering blood glucose levels may occur as a consequence of the insulin secretagogue and insulinomimetic effects of apigenin-6-C-β-L-fucopyranoside.

4. Experimental

4.1. General Experimental Procedures

The inhibitors of PI3K, wortmannin, PP1, calyculin A, mitogen-activated protein kinase (MEK), PD98059 and lithium chloride, GSK-3, were purchased from Sigma-Aldrich Co (St. Louis, MO). Glipizide was purchased from Akros Ltd (USA). D - [^{14}C (U)] – glucose (^{14}C-G), specific activity 9.25 GBq/mmol and biodegradable liquid scintillation were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA). Enzyme-linked immunosorbent assay (ELISA) for 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).

4.2. Plant Material

The leaves of *A. carambola* were collected in Santo Amaro da Imperatriz, Santa Catarina, Brazil and identified by Prof. Daniel de Barcellos Falkenberg. A voucher specimen was deposited at the herbarium of the Botany Department at the Universidade Federal de Santa Catarina, Florianópolis, under the number FLOR-24.144.

4.3. Extraction and Isolation

The powdered, dried leaves (281 g) were extracted with EtOH-H_2O (4:1). The extract was concentrated to dryness by rotatory vaporization at 60^{o}C under reduced pressure (41.3 g; crude extract-dry leaves ratio = 14.7%). The concentrated extract was then suspended in EtOH-H_2O (4:1) and successively extracted with *n*-hexane, EtOAc and *n*-BuOH. The ethyl acetate soluble fraction (EtOAc) of 6.9 g was subjected to silica gel (100-200 mesh) CC and eluted with an ethyl acetate/ethanol mixture gradient to afford 36 fractions. Fractions 6-8 (150 mg) was purified by recrystallization from MeOH to give pure (HPTLC in ethyl acetate/methanol/acetic acid 80:14:6 and NMR) compound **1**. This compound was identified by NMR analysis (^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC) and comparison with literature data [53].

Apigenin-6-C-β-L-fucopyranoside (compound **1**): Yellow amorphous powder, (drug-extract ratio = 0.36%). ^{1}H NMR (400 MHz, CD_3OD): aglycone moiety δ: 6.61 (s, H-3), 6.53 (s, H-8), 7.84 (d, *J*=8,0 *Hz*, H-2' & H-6'), 6.92 (d, *J*=8,0 *Hz*, H-3' & H-6'); sugar moiety: 4.64 (d, *J*=8.4 *Hz*, H-1"), 4.03 (t, *J*=8.7 *Hz*, H-2''), 3.97 (dd, *J*=3.0 and 6.5 *Hz*, H-3"), 3.50 (d, J=3.0 *Hz*, H-4"), 3.17 (m, H-5"), 1.44 (d, *J*=6.2 *Hz*, H-6''). ^{13}C NMR (400 MHz, CD_3OD) aglycone moiety δ: 165.1 (C-2), 99.6 (C-3), 183.1 (C-4), 161.7 (C-5), 108.0 (C-6), 163.9 (C-7), 94.0 (C-8), 161.8 (C-9), 102.8 (C-

10), 121.9 (C-1'), 128.3 (C-2' and 6'), 115.9 (C-3' and 5'), 157.8 (C-4′); sugar moiety: 71.9 (C-1''), 69.1 (C-2''), 78.5 (C-3''), 70.6 (C-4''), 70.8 (C-5''), 18.1 (C-6'').

4.4. Experimental Animals

Male Wistar rats weighing 180-200 g from the Central Animal House-UFSC were used. The rats were housed in plastic cages in an air-conditioned animal room and fed on pellets with free access to tap water. Room temperature was controlled at 21 ºC with a 12 h light: 12 h dark cycle. Animals described as fasted had been deprived of food for 16 h but allowed free access to water. For all oral treatments, 0.5 mL of each respective substance was given by gavage. All the animals were monitored carefully and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/PP007).

4.5. Apigenin-6-C-β-L-fucopyranoside (1) Treatment

Fasted rats were divided into groups of six animals for each treatment: Group I, euglycemic, normal fasted rats that received the vehicle, 1% EtOH-H_2O, p.o.; Group II, hyperglycemic rats that received glucose (4 g/kg, p.o.); Group III, rats that received glucose (4 g/kg, p.o.) plus apigenin-6-C-β-L-fucopyranoside (**1**) (50 mg/kg, p.o.); Group IV, rats that received glucose (4 g/kg, p.o.) plus glipizide (10 mg/kg, p.o.); by oral gavage. Blood samples were collected just prior to and at 15, 30 and 60 min after the glucose loading. After centrifugation, serum samples were used either immediately to determine the blood glucose levels.

4.6. Determination of the Serum Insulin

Fasted rats were divided into groups of six animals for each treatment: Group I, hyperglycemic rats that received glucose (4 g/kg, p.o.); Group II, rats that received glucose (4 g/kg, p.o.) plus apigenin-6-C-β-L-fucopyranoside (**1**) (50 mg/kg, p.o.). Blood samples were collected just prior to and at 15, 30 and 60 min after the glucose loading. Blood samples from the tail vein were collected and prepared for the analysis of insulin. 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. Sensitivity value of 0.2 ng/mL. All insulin levels were estimated by means of colorimetric measurement 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}.

4.7. Glycogen synthesis on rat soleus muscle

The assays of ^{14}C-glucose incorporation into glycogen were conducted as described by Cazarolli et al., [9,41]. Slices of soleus muscle from normal rats 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 of the composition 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 1% BSA and 5 mM D-glucose and bubbled with O_2/CO_2 (95%:5%, v/v) until pH 7.4. Apigenin-6-C-β-L-fucopyranoside (**1**) (1 and 100 μM) was added to the preincubation (30 min) and incubation medium (60 min) in the presence or absence of 100 nM wortmannin, 5 nM calyculin A, 50 μM PD98059 or 50 mM lithium chloride. ^{14}C-glucose (0.15 μCi/mL) was added to each sample during the incubation period. After incubation, the muscles were removed, washed in cold KRb and dried on filter paper. The muscles were homogenized in 0.5 N KOH and boiled at 100 °C for 20 min, with occasional stirring. After cooling, 95% ethanol was added to the samples, which were heated to boiling followed by cooling in an ice bath for 20 min to aid the precipitation of glycogen. The homogenates were centrifuged at 664 g for 15 min, the supernatant was discarded, and pellets

resolubilized in water. 30 μL aliquots of the samples were placed in liquid scintillation vials 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 pmol glucosyl units incorporated into glycogen. mg tissue^{-1}. h^{-1}.

4.8. Statistical Analysis

Data were expressed as mean ± S.E.M. One or two-way analysis of variance (ANOVA), followed by the Bonferroni *post-test* or non paired Student's *t*-test, was used to identify significantly different groups. Differences were considered to be significant at the $p \leq 0.05$ level.

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4.3 ARTIGO PUBLICADO
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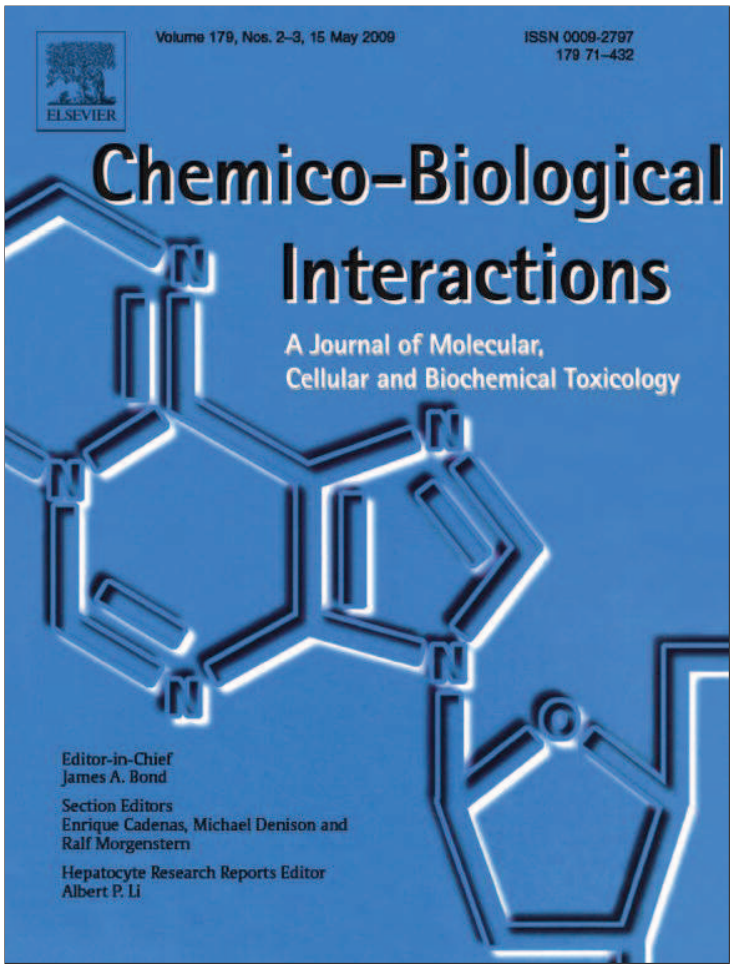

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Mechanism of action of the stimulatory effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside on ^{14}C-glucose uptake

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ABSTRACT

There has been a growing interest in hypoglycemic agents from natural products, particularly those derived from plants. Flavonoids are naturally occurring phenolic compounds with a broad range of biological activities and the beneficial effects of flavonoids have been studied in relation to diabetes mellitus, either through their capacity to avoid glucose absorption or to improve glucose tolerance. The purpose of this study was to investigate the mechanism of action of the stimulatory effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**), isolated from *Averrhoa carambola* L. (Oxalidaceae) leaves, on ^{14}C-glucose uptake. This compound (**1**) was found to have an acute effect on blood glucose lowering in diabetic rats and stimulated glucose-induced insulin secretion after oral treatment in hyperglycemic rats. A significant stimulatory effect of compound **1** on ^{14}C-glucose uptake was observed at 50 and 100 μM. The effect of compound **1** on glucose uptake was completely nullified by wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), RO318220, an inhibitor of protein kinase C (PKC), PD98059, a specific inhibitor of mitogen-activated protein kinase (MEK), cycloheximide, an inhibitor of protein synthesis, and colchicine, a microtubule-depolymerizing agent. Compound **1** (100 μM) and insulin (10 nM) did not show any synergistic effect on glucose uptake. These results suggest that the flavonoid may have a dual target of action, as an insulin-secretagogue and also as an insulin-mimetic agent.

1. Introduction

Insulin is the most important hormone that regulates energy metabolism. It mediates a wide spectrum of biological responses including synthesis and storage of carbohydrates, lipids and proteins as well as the inhibition of catabolism [1]. Glucose is the primary stimulus for insulin secretion and when blood sugar concentrations rise, insulin is secreted into the bloodstream [2,3]. Insulin stimulates glucose uptake via multiple signaling pathways. The binding of insulin to its receptor activates the intrinsic receptor tyrosine kinase, which results in autophosphorylation and recruitment of substrates, such as the insulin-receptor substrate (IRS) proteins and Cbl protein. Specific tyrosine residues on the IRS proteins serve as docking sites for proteins that contain SH2 domains, such as the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K). 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 GLUT4-containing vesicles to the plasma membrane. Alternatively, insulin also stimulates glucose uptake through the CAP–Cbl–TC10 pathway [1,4].

In both the basal and insulin-stimulated states, GLUT4 cycles between the plasma membrane and intracellular compartments. Insulin increases the amount of GLUT4 on the plasma membrane by stimulating exocytose of the intracellular GLUT4 pool and by decreasing the endocytose of the plasma membrane-associated GLUT4 protein. The overall insulin-stimulated changes in the cellular dynamics of GLUT4 protein distribution are controlled by complex and multiple vesicle trafficking events. This vesicular trafficking network includes mechanisms for the sorting, budding, trafficking, tethering, docking and fusion of the GLUT4 vesicles and they are directly regulated by intracellular signals generated from the insulin receptor [4,5]. The cytoskeleton has been implicated in various membrane trafficking events and in the retention of organelles at specific locations within cells and the actin and

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microtubule cytoskeleton networks have been implicated in the subcellular movements of the GLUT4-containing vesicles [6–8]. It is suggested that insulin stimulation could induce a release of the vesicles allowing the movement to the plasma membrane by simple diffusion, or insulin may trigger the movement of vesicles along cytoskeletal tracks, as has been observed for regulated exocytose of secretory vesicles in other cell systems [9,10]. However, the precise mechanism whereby insulin signals to the GLUT4 membrane trafficking machinery remains obscure.

An absolute or relative lack of insulin, as in the case of diabetes, leads to severe dysfunction and deregulation of insulin signaling in target tissues, such as the liver, adipose tissue and muscle. Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both [11]. The molecular defects accounting for impaired glucose utilization are not fully understood but may involve defective GLUT4 translocation, glucose uptake and aberrant insulin signal transduction. In this context, several naturally occurring polyphenols have been shown to affect glucose transport and insulin-receptor function, both of which play essential roles in diabetes [12]. It has been demonstrated that kaempferitrin is able to diminish serum glucose level and increase glucose uptake in the rat soleus muscle as efficiently as insulin [13,14]. Also, kaempferol 3-neohesperidoside has a significant hypoglycemic effect in diabetic rats and stimulate glucose uptake in rat soleus muscle mimicking insulin signaling [15,16]. It has been demonstrated by popular medicine usage in Brazil that *Averrhoa carambola* helps to balance glucose levels [17]. Recently, it was demonstrated that crude extracts, flavonoid-enriched fractions from leaves of *A. carambola* are able to diminish serum glucose levels in hyperglycemic rats as efficiently as insulin [18,19]. Considering the effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside, isolated from *A. carambola* leaves, on blood glucose levels and on glycogen content in the liver and soleus muscle from hyperglycemic rats, the aim of this study was to investigate the mechanism of action of this compound in relation to its stimulatory effect on ^{14}C-D-glucose uptake.

2. Materials and methods

2.1. Materials

Regular human insulin (Biohulin) was obtained from Biobrás, Bioquímica do Brasil S/A (Águas Claras, MG, Brazil). [U-^{14}C]-2-Deoxy-D-glucose (^{14}C-DG), specific activity 10.6 GBq/mmol, and biodegradable scintillation liquid were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA). Alloxan, wortmannin (inhibitor of phosphatidylinositol 3-kinase (PI3K)), RO318220 (inhibitor of protein kinase C (PKC)), PD98059 (inhibitor of mitogen-activated protein kinase (MEK)), cycloheximide (inhibitor of protein synthesis) and colchicine (microtubule-depolymerizing agent) were purchased from Sigma Chemical Company® (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) for 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).

2.2. Plant material

The leaves of *A. carambola* were collected in Santo Amaro da Imperatriz, Santa Catarina, Brazil and identified by Prof. Daniel de Barcellos Falkenberg. A voucher specimen was deposited at the herbarium of the Botany Department at the Universidade Federal de Santa Catarina, Florianópolis, under the number FLOR-24.144.

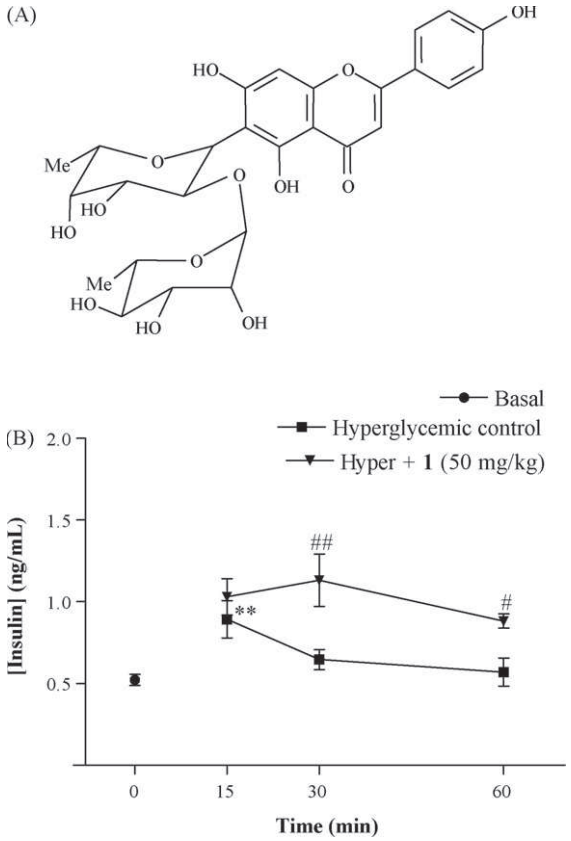

Fig. 1. (A) Apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) structure and (B) effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) 50 mg/kg on serum insulin levels in hyperglycemic rats. Values are expressed as mean ± S.E.M.; $n = 6$ in duplicate for each group. Significant at $^{**}p \leq 0.01$ in relation to zero time. Significant at $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ in relation to hyperglycemic group.

2.3. Extraction and isolation

The powdered, dried leaves (281 g) were extracted with EtOH–H_2O (4:1). The extract was concentrated to dryness by rotatory vaporization at 60 °C under reduced pressure. The concentrated extract was suspended in EtOH–H_2O (4:1) and successively extracted with *n*-hexane, EtOAc and *n*-BuOH. The ethyl acetate soluble fraction was subjected to silica gel (100–200 mesh) CC and eluted with an ethyl acetate/ethanol mixture gradient to afford fractions that were purified by recrystallization from MeOH to give isolated compounds. One of these was apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) (Fig. 1A) [20].

2.4. Experimental animals

Male Wistar rats weighing 180–200 g from the Central Animal House-UFSC were used. The rats were housed in plastic cages in an air-conditioned animal room and fed on pellets with free access to tap water. Room temperature was controlled at 21 °C with a 12 h light:12 h dark cycle. Animals described as fasted had been deprived of food for 16 h but allowed free access to water. For all oral treatments, 0.5 mL of each respective substance was given by gavage. All the animals were monitored carefully and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/PP007). Rats fasted for 16 h received 50 mg/kg body weight of alloxan (Sigma, St. Louis, MO, USA) by a single intravenous injection. We assessed the diabetic state by measuring body weight and blood glucose levels 3 days after injection.

2.5. Studies on the effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) on serum glucose levels

Fasted rats were divided into groups of six animals for each treatment: Group I, hyperglycemic rats that received glucose (4 g/kg); Group II, rats that received glucose (4 g/kg) plus apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) (50 mg/kg); Group III, alloxan-induced diabetic rats that received vehicle (1% EtOH–H_2O); and Group IV, diabetic rats that received apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) (50 mg/kg). Blood samples were collected just prior to and 60 min after treatments. Blood samples from the tail vein were collected and centrifuged, and the serum was used to determine glycemia by a glucose oxidase method. For diabetic rats, a serum glucose range of 300–400 mg/dL was used for the experiment [21].

2.6. Determination of insulin levels

Blood samples from the tail vein were collected and centrifuged, and the serum was used to determine insulin levels. Blood samples were collected just prior to, and at 15, 30 and 60 min after treatment. The insulin levels were measured by ELISA according to the manufacturer's instructions. The range of values detected by this assay was 0.2–10 ng/mL. The intra- and inter-assay coefficients of variation for insulin were 3.22 and 6.95, respectively, with a sensitivity value of 0.2 ng/mL. All insulin levels were estimated by means of colorimetric measurement at 450 nm with an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) through interpolation from a standard curve. Samples were analyzed in duplicate and results were expressed as ng of insulin serum per mL.

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

For the [U-^{14}C]-2-deoxy-D-glucose uptake experiments, soleus muscles from normal 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. Compound **1** (1, 10, 50, 100 μM and 1 mM) and insulin (10 nM) were added to the preincubation (30 min) and incubation medium (60 min) in the presence or absence of 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 muscles were placed in screw cap tubes containing 1 mL of distilled water. These were frozen at −20 °C in a freezer and then boiled for 10 min; 25 μL aliquots of tissue 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 [13].

2.8. Data and statistical analysis

Data were expressed as mean ± S.E.M. The statistical analysis was carried out using one-way analysis of variance (ANOVA), followed by the Bonferroni post-test or non-paired Student's *t*-test to identify significantly different groups. Differences were considered to be significant at $p \leq 0.05$.

3. Results and discussion

3.1. Effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) on serum glucose and insulin levels in rats

The administration of alloxan (50 mg/kg) to the fasted rats significantly increased blood glucose levels when compared with normal rats. Also, as expected, after the glucose loading serum glucose concentration was increased when compared with zero time (Table 1). The oral administration of compound **1** reduced significantly the serum glucose levels in hyperglycemic rats as well as in alloxan-induced diabetic rats after the treatments. We have previously demonstrated the effect of flavonoid-rich fractions, flavonoids and chalcones in diabetic and hyperglycemic rats [14,15,21–23]. Moreover, puerarin has been described as a hypoglycemic and antihyperglycemic agent due to its effects in hyperglycemic and diabetic rats [24].

Based on the effects of compound **1** on blood glucose levels, especially in hyperglycemic rats, serum insulin levels were determined in fasted rats after an oral glucose loading (4 g/kg) as shown in Fig. 1B. The glucose-induced insulin secretion was increased 71% at 15 min compared with zero time and returned to the basal levels after 60 min. Biphasic insulin secretion is the normal response of β cells to a rapid and sustained increase in blood glucose concentration and the rapid increase in insulin levels observed in Fig. 1B confirms the classical profile of glucose-induced insulin secretion [3,25]. The treatment with compound **1**-stimulated insulin secretion as well as potentiated the glucose effect on insulin levels in hyperglycemic rats after 30 and 60 min compared to the hyperglycemic group. Also, genistein and daidzein flavonoids have been reported as insulin-secretagogues since they increased serum insulin secretion stimulated by glucose *in vivo* and *in vitro* [26,27]. Thus, the hypoglycemic effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside seems to be a consequence of insulin secretion to manage glucose metabolism.

3.2. Effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) on ^{14}C-glucose uptake in rat soleus muscle

Fig. 2 shows the *in vitro* effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) on glucose uptake in

Table 1
Acute effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) (50 mg/kg) on serum glucose level in hyperglycemic and diabetic rats. Values expressed as mean ± S.E.M.; $n = 6$ in duplicate for each treatment.

Time (min)	Serum glucose levels (mg/dL)			
	Group I hyperglycemic glucose (4 g/kg)	Group II hyperglycemic plus **1** (50 mg/kg)	Group III diabetic plus (1% EtOH–H_2O)	Group IV diabetic plus **1** (50 mg/kg)
0	122.6 ± 5.8	111.7 ± 5.7	317.5 ± 11.9	336.0 ± 8.9
60	178.4 ± 9.1	149.0 ± 3.3[#]	372.0 ± 8.5	284.4 ± 4.5[***]

[#] Significant at $p \leq 0.05$ in relation to hyperglycemic group.
[***] Significant at $p \leq 0.001$ in relation to zero time of diabetic plus **1** group.

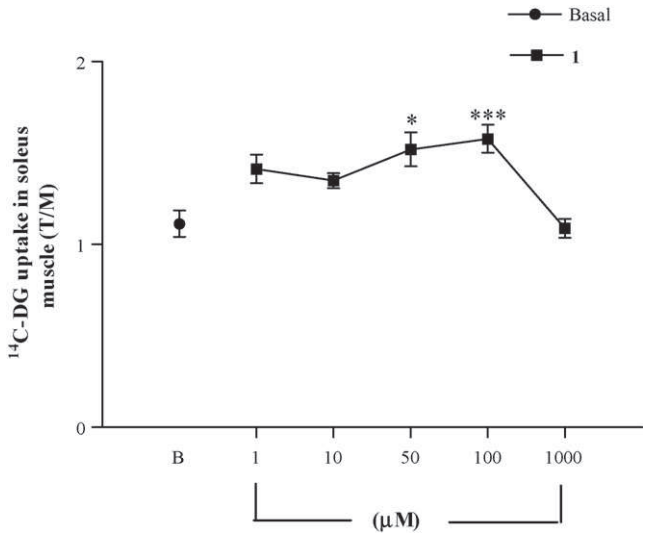

Fig. 2. Concentration–response curve of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) on ^{14}C-glucose uptake in rat soleus muscle. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; $n = 6$ in duplicate for each group. Significant at $^{***}p \leq 0.001$ and $^{*}p \leq 0.05$ in relation to basal group.

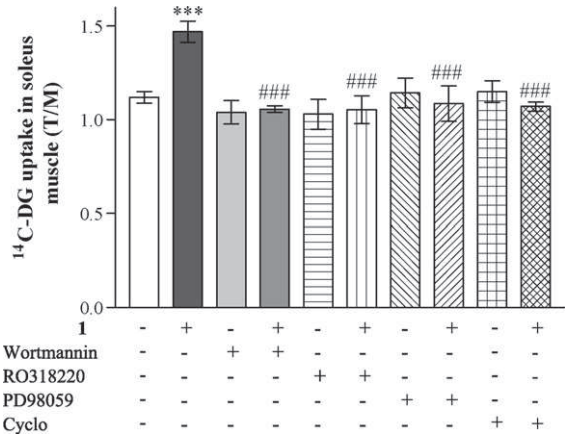

Fig. 3. Effect of enzyme inhibitors on the stimulatory action of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) on ^{14}C-glucose uptake in rat soleus muscle with 100 nM Wortmannin, 40 μM RO318220, 50 μM PD98059, 0.35 mM cycloheximide and 100 μM **1** used. Basal group = no treatment. 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 at $^{###}p \leq 0.001$ in relation to apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) group.

soleus muscle after 60 min of incubation. The stimulatory effect of compound **1** was significant at 50 and 100 μM compared to the basal group. This stimulatory effect represents 36.5% and 42% of glucose uptake compared to the basal level at 60 min, respectively. These results are in line with those recently published concerning the hypoglycemic effect of kaempferitrin and kaempferol 3-neohesperidoside (similar flavonoids to compound **1**) in diabetic rats and the stimulatory effect of these flavonoids on glucose uptake in rat soleus muscle [13–16]. The flavonoid puerarin also increased glucose utilization by stimulation of glucose uptake in soleus muscle from streptozotocin-diabetic rats [24]. These results demonstrate a potential insulinomimetic effect of compound **1** on glucose uptake in one of the insulin target tissues, soleus muscle.

3.3. Effect of various inhibitors on the stimulatory action of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) on ^{14}C-glucose uptake in rat soleus muscle

In order to study the mechanism by which compound **1** stimulates glucose uptake in soleus muscle, we performed the glucose uptake assay with 100 nM wortmannin, a specific inhibitor of PI3K, 40 μM RO318220, a specific inhibitor of PKC, 50 μM PD98059, an inhibitor of MEK, 0.35 mM cycloheximide, an inhibitor of protein synthesis or 1 μM colchicine, a microtubule-depolymerizing agent. The wortmannin, RO318220, PD98059, cycloheximide and colchicine concentrations used were those that have previously resulted in inhibition in cell and tissue assays [16,28–32].

Fig. 3 shows that the stimulation of glucose uptake by compound **1** was completely inhibited by wortmannin, RO318220, PD98059 and cycloheximide pretreatment. However, when only wortmannin, RO318220, PD98059 or cycloheximide were added to the muscle samples, no significant change resulted compared with the glucose uptake in the basal group. These results suggest that, at least in part, the stimulatory effect of compound **1** on glucose uptake is mediated through the PI3K and PKC pathways together with the involvement of the MAPK pathway as well as protein synthesis (glucose transporters?). Thus, the results here reported are in agreement with those reported in the literature concerning the key role of PI3K and GLUT4 translocation regulated by insulin [1,4]. Similarly, the stimulatory effect of kaempferol 3-neohesperidoside on glucose uptake seems to be mediated through insulin signal transduction involving the PI3K and PKC pathways. The stimulatory effect on glucose uptake has also been demonstrated for grape seed-derived procyanidins, myricetin and apigenin and its glycosides [16,30,33,34].

In contrast to the observed kaempferol 3-neohesperidoside mechanism [16], cycloheximide totally blocked the stimulation of glucose uptake induced by compound **1**. Additionally, extracts from *Aegles marmelos*, *Syzygium cumini* and *Canna indica*, plants rich in flavonoids, stimulated glucose uptake and these effects were totally inhibited in the presence of cycloheximide, suggesting that active protein synthesis is important in terms of glucose transport [32,35]. Thus, these data indicate that active protein synthesis is necessary for compound **1**-stimulated glucose transport and, coupled with the involvement of PI3K and PKC pathways, reinforces the insulinomimetic effect of this flavonoid in an insulin-sensitive tissue, soleus muscle.

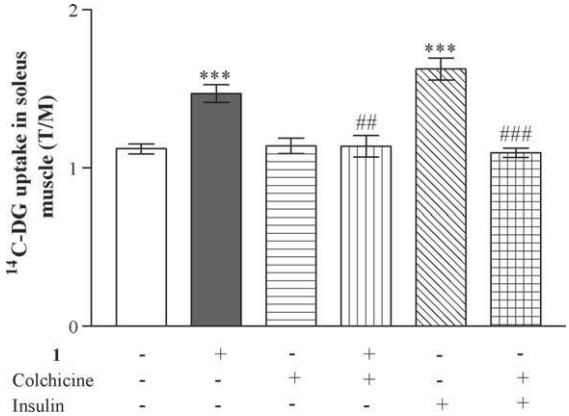

Fig. 4. Effect of colchicine (1 μM) on the stimulatory action of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) (100 μM) and insulin (10 nM) on ^{14}C-glucose uptake in rat soleus muscle. Basal group = no treatment. Signal (+) and (−) indicates presence and absence, respectively, 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 at $^{##}p \leq 0.01$ in relation to apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) group and $^{###}p \leq 0.001$ in relation to insulin group.

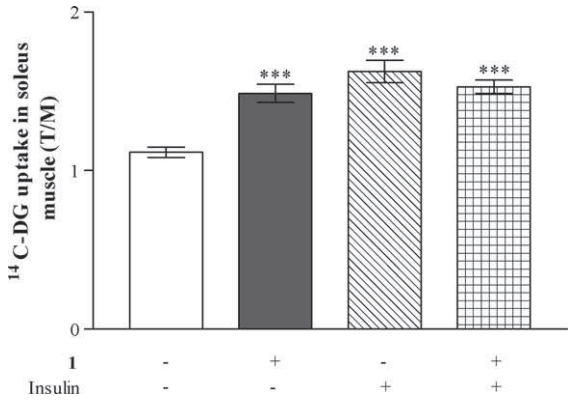

Fig. 5. Comparative effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) (100 μM) and insulin (10 nM) on ^{14}C-glucose uptake in rat soleus muscle. Basal group = no treatment. Signal (+) and (−) indicates presence and absence, respectively, of each substance in the incubation 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.

It is well known that insulin stimulates glucose uptake in skeletal muscle and adipose tissue primarily by eliciting the translocation of GLUT4 from an intracellular pool to the plasma membrane [4]. The cellular cytoskeleton structure is engaged in the efficient transport of vesicles through intracellular membrane-sorting pathways and the microtubules and actin filaments are particularly important for insulin-stimulated GLUT4 translocation [6–8]. To determine whether an intact microtubule cytoskeleton is necessary for the compound **1**-stimulated glucose uptake, the glucose uptake in the presence of colchicine was studied (Fig. 4). The stimulatory effect of compound **1** on glucose transport was completely blocked by 1 μM colchicine and when only colchicine was added to the samples no significant change was observed compared with the basal group. It has been demonstrated that the disruption of microtubules caused by colchicine and other microtubule-depolymerizing agents inhibits insulin-induced GLUT4 translocation and glucose uptake in adipocytes [10,36–39]. Taken together, these results suggest that to observe the complete and efficient effect of compound **1** on glucose uptake, the cytoskeleton integrity and cascade of PI3K–PKC and MAPK are required.

3.4. Comparative effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (*1*) and insulin on ^{14}C-glucose uptake in rat soleus muscle

Fig. 5 shows the stimulation of glucose uptake by apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**1**) (100 μM) compared to insulin (10 nM) in soleus muscle. In percentage terms, the compound was as effective as insulin in glucose uptake in this approach. On the other hand, the absence of an additive effect of compound **1** and insulin on glucose uptake clearly demonstrates that the compound **1** mechanism of action is mediated partially through the insulin transduction pathway. This result is in agreement with those reported for the effect of kaempferol 3-neohesperidoside and for grape seed-derived procyanidins on glucose uptake in insulin-sensitive cell lines and tissues, since simultaneous treatment with saturating doses of insulin and the compounds did not cause an additive effect on glucose uptake [16,30].

4. Conclusions

We have shown that apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside reduced blood glucose levels in diabetic rats, stimulated insulin secretion and potentiated glucose-induced insulin secretion in hyperglycemic rats. In addition, this flavonoid stimulated glucose uptake in rat soleus muscle through the involvement of the well-known mechanism of insulin signal transduction. These results constitute strong evidence for a dual effect of apigenin-6-C-(2″-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside characterizing it as both an insulin secretagoge and an insulin-mimetic agent.

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4.4 ARTIGO PUBLICADO
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Research paper

Signaling pathways of kaempferol-3-neohesperidoside in glycogen synthesis in rat soleus muscle

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Kaempferol 3-neohesperidoside

ABSTRACT

Kaempferol 3-neohesperidoside is one of the several compounds that have been reported to have insulin-like properties in terms of glucose lowering. We studied the effect of kaempferol 3-neohesperidoside in glycogen synthesis in rat soleus muscle through the incorporation of ^{14}C-D-glucose in glycogen. Kaempferol 3-neohesperidoside stimulates glycogen synthesis in rat soleus muscle by approximately 2.38-fold. Insulin at 100 nM showed a stimulatory effect on glycogen synthesis when compared with the control group. The stimulatory effect of kaempferol 3-neophesperidoside on glycogen synthesis was inhibited by wortmannin, the phosphatidylinositol 3-kinase (PI3K) inhibitor, and enhanced by lithium chloride, a glycogen synthase kinase 3 (GSK-3) inhibitor. Moreover, the stimulatory effect of kaempferol 3-neohesperidoside was also nullified by PD98059, a specific inhibitor of mitogen-activated protein kinase (MEK) and by calyculin A, an inhibitor of protein phosphatase 1 (PP1) activity. It was concluded that the PI3K – GSK-3 pathway and MAPK – PP1 pathway are involved in the stimulatory kaempferol 3-neohesperidoside effect on glycogen synthesis in rat soleus muscle.

1. Introduction

Insulin is the most important hormone that regulates energy metabolism. It mediates a wide spectrum of biological responses including synthesis and storage of carbohydrates, lipids and proteins as well as the inhibition of catabolism [1,2]. In mammalian tissues, carbohydrate is stored mainly in the form of glycogen and the major sites of glycogen deposition are skeletal muscle and the liver [3]. An absolute or relative lack of insulin, as in the case of diabetes, leads to severe dysfunction and deregulation of insulin signaling in target tissues such as the liver, adipose tissue and muscles. Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both [2]. In recent years, there has been a growing interest in hypoglycemic agents from natural products, especially those derived from plants [4–10]. Substances that mimic insulin action are of interest since they can act efficiently in the alleviation of insulin resistance and diabetes [10,11].

The flavonoids are a group of low-molecular-weight polyphenolic substances, qualitatively and quantitatively one of the largest groups of natural products known. Besides their roles in plants, flavonoids are important components in the human diet and are found in fruits, vegetables, seeds, nuts, grains, spices and beverages [12,13]. They have a broad range of biological activities and numerous studies have been carried out on their potential role in the treatment and prevention of diseases and especially of diabetes [13–15]. Many studies have demonstrated the hypoglycemic effects of flavonoids [16–18] as well as their action in glucose uptake [16–19], glycogen metabolism [20–22] and gluconeogenic enzyme activities [23–25].

We have previously demonstrated the acute hypoglycemic effect of isolated flavonoids as well as flavonoid-enriched fractions [5,6,26]. Recently, it was reported that kampferitrin, the major flavonoid found in *Bauhinia forficata* leaves, was able to reduce glycemia and stimulate glucose uptake in rat soleus muscle as efficiently as insulin [19]. In addition, studies with kaempferol 3-neohesperidoside, alone or complexed with vanadium IV, isolated from *Cyathea phalerata* Mart. (Cyatheaceae) stems and structurally similar to kaempferitrin, showed a significant hypoglycemic effect in diabetic rats [6]. This

Abbreviations: PI3K, phosphatidylinositol 3-kinase; GSK-3, glycogen synthase kinase 3; MEK/MAPKK, mitogen-activated protein kinase kinase; PP1, protein phosphatase 1; PKC, protein kinase C; GLUT4, glucose transporter 4; GS, glycogen synthase; LiCl, lithium chloride; MAPK, mitogen-activated protein kinase; p90^{rsk2}, p90 ribossomal S6 kinase; PKB, protein kinase B; PDK, protein kinase dependent on 3-phosphoinositides.

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flavonoid was able to increase glucose uptake as well as glycogen content in rat soleus muscle. The mechanism through which kaempferol 3-neohesperidoside stimulates glucose uptake is probably mediated by phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) pathways and, at least in part, is independent of mitogen-activated protein kinase (MEK) pathways and the synthesis of new glucose transporters [27].

Glycogen synthesis in skeletal muscle has a key role in the control of blood glucose levels by insulin since most of the postprandial glucose uptake occurs in this tissue. Most of the glucose that enters muscle fibers through glucose transporter 4 (GLUT4) in response to insulin is converted into glycogen. This hormonal effect involves activation of glycogen synthase (GS), the enzyme that catalyzes the rate-limiting step in the conversion of intracellular glucose to glycogen [3,28]. Insulin activates glycogen synthase by promoting dephosphorylation of several sites of the enzyme through the inhibition or stimulation of protein kinases and phosphatases, respectively [3,29]. Moreover, insulin also regulates glycogen synthase activation by controlling the uptake and transport by GLUT4 of glucose and by regulating the phosphorylation and activation states of enzymes involved in the synthesis and degradation of glycogen [3,28]. Based on this, the aim of this study was to investigate the effect of kaempferol 3-neohesperidoside on glycogen synthesis in rat soleus muscle and the signaling pathways involved in its mechanisms of action.

2. Material and methods

2.1. Materials

Regular human insulin (Biohulin) was obtained from Biobrás, Bioquímica do Brazil S/A (Águas Claras, MG, Brazil). Wortmannin, inhibitor of phosphatidylinositol 3-kinase (PI3K); calyculin A, inhibitor of protein phosphatase 1 (PP1); PD98059, inhibitor of mitogen-activated protein kinase (MEK) and lithium chloride, inhibitor of glycogen synthase kinase 3 (GSK-3) were purchased from Sigma–Aldrich Co. D – [^{14}C (U)] – glucose (^{14}C-glucose), specific activity 9.25 GBq/mmol and biodegradable liquid scintillation cocktails were obtained from Perkin–Elmer Life and Analytical Sciences (Boston, MA, USA). Salts and solvents were purchased from Merck AG (Darmstadt, Germany).

2.2. Plant material

C. phalerata Mart. (Cyatheaceae) was collected in March 2002 in Palhoça, Brazil, and identified by Prof. Lana da Silva Sylvestre. A voucher specimen (RBR 4287) has been deposited in the herbarium of the Botany Department at the Universidade Federal Rural do Rio de Janeiro, Seropedica, Brazil. The process of extraction and isolation of kaempferol 3-neohesperidoside was carried out as described in [27]. The flavonoid used in this study was dissolved in 1% EtOH/H_2O solution and stored aliquotted at – 20 degree Celsius.

2.3. Experimental animals

Male Wistar rats weighing 190–210 g from the Central Animal House-UFSC were used. The rats were housed in plastic cages in an air-conditioned animal room and fed on pellets with free access to tap water. Room temperature was controlled at 21 degree Celsius with a 12 h light:12 h dark cycle. Animals described as fasted had been deprived of food for 16 h but allowed free access to water. All the animals were monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/PP007).

2.4. Glycogen synthesis in rat soleus muscle

The assays of ^{14}C-glucose incorporation into glycogen were conducted as described in [19,30] with modifications. Slices (strips) of soleus muscle from normal rats were distributed (alternately left and right) between control and treated groups. Muscles were dissected, weighed, preincubated and incubated at 37 degree Celsius in Krebs Ringer-bicarbonate (KRb) buffer comprising 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ plus 1% BSA and 5 mM D-glucose and bubbled with O_2/CO_2 (95%:5%, v/v) until pH 7.4 was reached. Kaempferol 3-neohesperidoside (0.1; 1 and 10 μM) and insulin (10 and 100 nM) were added to the preincubation (30 min) and incubation (60 min) media in the presence or absence of 100 nM wortmannin, 5 nM calyculin A, 50 μM PD98059 or 50 mM lithium chloride. ^{14}C-glucose (0.15 μCi/ml) was added to each sample during the incubation period. After incubation, the muscle samples were removed, washed in cold KRb and dried on filter paper. The muscle samples were homogenized in 0.5 N KOH, and boiled at 100 degree Celsius for 20 min, with occasional stirring. After cooling, 95% ethanol was added to the samples, which were heated to boiling followed by cooling in an ice bath for 20 min to allow glycogen precipitation. The homogenates were centrifuged at 664 g for 15 min, the supernatant was discarded and pellets resolubilized in water. Aliquots (30 μl) of the samples were placed in liquid scintillation vials in an LKB rack, on a beta liquid scintillation spectrometer (model 1215; EG and G-Wallac, Turku, Finland), for the radioactivity measurements. The results were expressed as pmol glycosyl units incorporated in glycogen. mg tissue^{-1} h^{-1}.

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. One or two-way analysis of variance (ANOVA) followed by the Bonferroni post-test was used to identify significantly different groups. Differences were considered to be significant at $P \leq 0.05$.

3. Results

3.1. Studies on kaempferol 3-neohesperidoside and insulin in glycogen synthesis

As shown in Fig. 1 kaempferol 3-neohesperidoside (Fig. 1A) caused a significant increase in the *in vitro* glycogen synthesis in soleus muscle compared with the control (Fig. 1B). In percentage terms, the effect of the flavonoid at 1 μM was around 140% compared with the control. The insulin concentrations used were those previously reported by Jorge et al. (2004). As expected, insulin efficiently stimulated glucose incorporation into glycogen. This effect of insulin (10 and 100 nM) increased around 2-fold when compared with the control group (Fig. 2). The effect of kaempferol 3-neohesperidoside represents approximately twice that of insulin stimulation (10 nM) in glycogen synthesis, although the effective dose of the compound used was higher than that used for insulin.

3.2. Effect of various inhibitors on the stimulatory action of kaempferol 3-neohesperidoside in glycogen synthesis in rat soleus muscle

To determine the mechanism by which kaempferol 3-neohesperidoside induced glycogen synthesis in the soleus muscle, we performed the glycogen synthesis assay with calyculin A, a specific inhibitor of PP1 activity, PD98059, a specific inhibitor of MEK, wortmannin, a specific inhibitor of PI3K, or LiCl, a known inhibitor of GSK-3. The inhibitor concentrations used were those previously

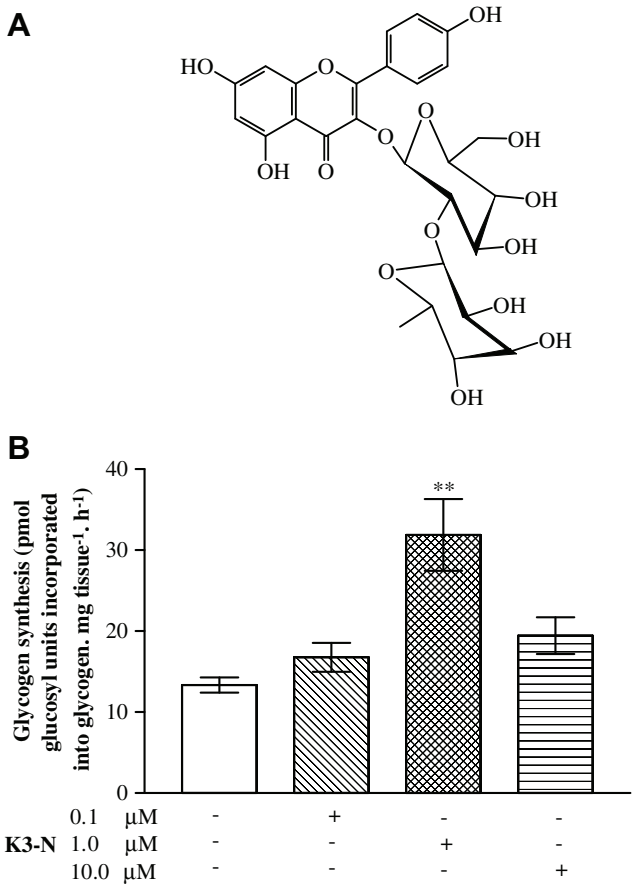

Fig. 1. Kaempferol 3-neohesperidoside (K3-N) structure (A) and dose–response curve of kaempferol 3-neohesperidoside in glycogen synthesis in the rat soleus muscle. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; $n = 6$ in duplicate for each group. Significant at $^{**}p \leq 0.01$ in relation to control group.

reported in the literature [27,31–33]. Fig. 3 shows the effect of calyculin A on the glycogen synthesis in the soleus muscle. This inhibitor was not able to reduce significantly the glycogen synthesis in soleus muscle at 1, 2.5 or 5 nM compared with the control group. On the other hand, it was observed that the stimulatory effect of

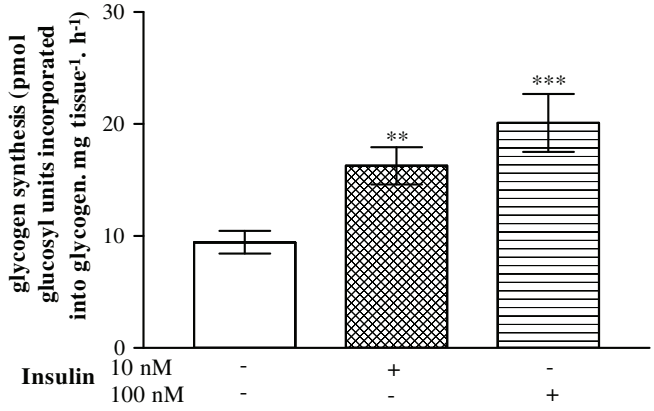

Fig. 2. - Insulin dose–response curve in glycogen synthesis in the rat soleus muscle. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; $n = 10$ in duplicate for each group. Significant at $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ in relation to control group.

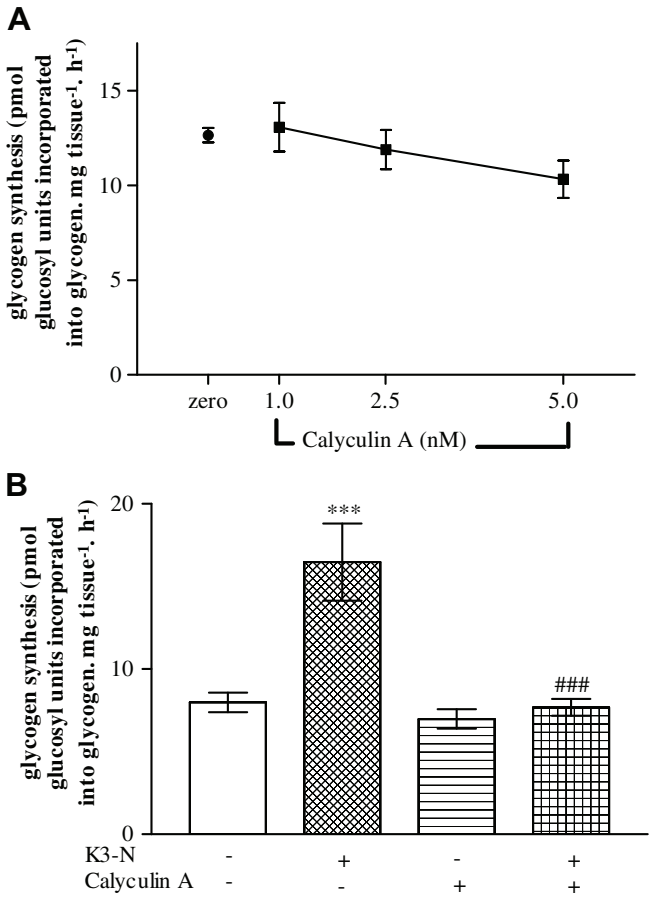

Fig. 3. - Dose–response curve of calyculin A in basal glycogen synthesis (A) and effect of 5 nM calyculin A (B) on the stimulatory action of 1 μM kaempferol 3-neohesperidoside (K3-N) in glycogen synthesis in the rat soleus muscle. Control group = no treatment. Signals (+) and (−) indicate presence and absence of each substance in the incubation medium, respectively. 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 control group and significant at $^{###}p \leq 0.001$ in relation to kaempferol 3-neohesperidoside group.

kaempferol 3-neohesperidoside on ^{14}C-glucose incorporation into glycogen in the isolated soleus muscle was completely blocked in the presence of 5 nM of calyculin (Fig. 3B).

Since the MAPK pathway may be involved in GS activation and subsequently glycogen synthesis stimulated by insulin, we studied the effect of PD98059 on the stimulatory effect of kaempferol 3-neohesperidoside in glycogen synthesis. In the presence of 50 μM PD98059 the effect of the flavonoid disappeared, suggesting an involvement of MAPK-p90^{rsk} activation in the glycogen synthesis stimulated by kaempferol 3-neohesperidoside (Fig. 4).

To elucidate whether the effects of kaempferol 3-neohesperidoside on glycogen synthesis required intact PI3K activity, wortmannin, a potent inhibitor of PI3K was used. The stimulation of glycogen synthesis by kaempferol 3-neohesperidoside was completely blocked by wortmannin with no effect on the basal synthesis (Fig. 5). Since the stimulatory effect of kaempferol 3-neohesperidoside on glycogen accumulation was mediated by PI3K we studied the influence of GSK-3, a downstream effector of PI3K in the kaempferol 3-neohesperidoside action.

Fig. 6A shows the effect of LiCl, a known inhibitor of GSK-3, on glycogen synthesis in muscle. The maximum stimulatory effect observed for lithium in glycogen synthesis was at 50 mM. In Fig. 6B, 1 μM of kaempferol 3-neohesperidoside and 50 mM of LiCl caused

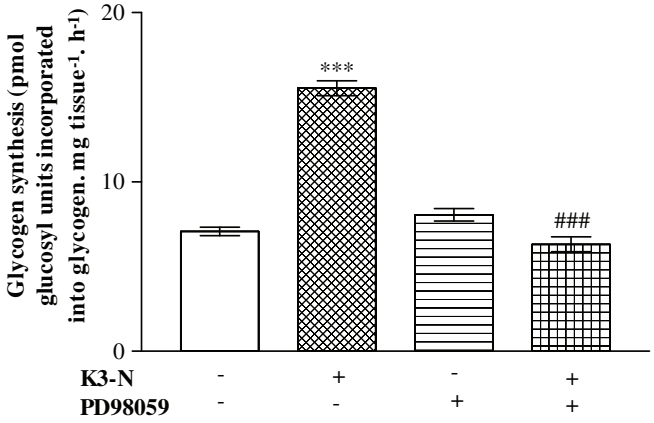

Fig. 4. Effect of 50 μM PD98059 on the stimulatory action of 1 μM kaempferol 3-neohesperidoside (K3-N) in glycogen synthesis in the rat soleus muscle. Control group = no treatment. Signals (+) and (−) indicate presence and absence, respectively, of each substance in the incubation medium. Preincubation 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 control group. Significant at $^{###}p \leq 0.001$ in relation to kaempferol 3-neohesperidoside group.

a similar increase in glycogen accumulation. Under these conditions, when incubation with LiCl plus kaempferol 3-neohesperidoside was carried out, glycogen synthesis was higher than that observed for LiCl or kaempferol 3-neohesperidoside alone.

4. Discussion

Insulin is the most important hormone that regulates glycogen synthesis in skeletal muscle [1,2]. In our study, we show that kaempferol 3-neohesperidoside stimulates the *in vitro* glycogen synthesis in soleus muscle. This effect was observed at 1 μM and represented twice that of insulin stimulation in glycogen synthesis. Previous studies have shown that flavonoids such as catechin and myricetin act in the insulin signaling pathways that regulates glucose uptake and glycogen synthesis. These flavonoids stimulated glycogen synthesis and glucose uptake in insulin target tissues, such as soleus muscle and liver tissues [16,17,21]. Also, procyanidin extract derived from grape seeds was shown to increase glycogen synthesis in 3T3-L1 adipocytes [20].

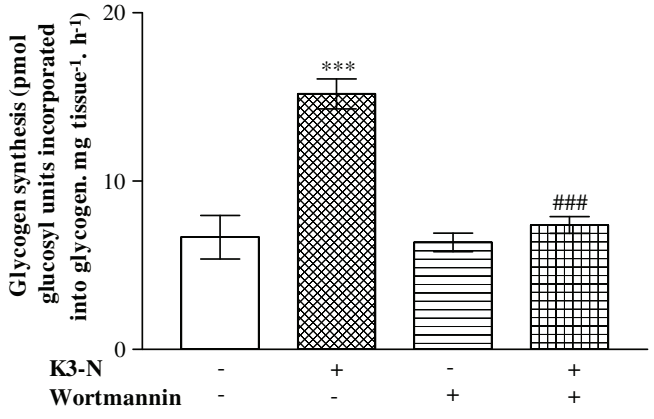

Fig. 5. Effect of 100 nM wortmannin on the stimulatory action of 1 μM kaempferol 3-neohesperidoside (K3-N) in glycogen synthesis in the rat soleus muscle. Control group = no treatment. Signals (+) and (−) indicate presence and absence, respectively, of each substance in the incubation medium. Preincubation 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 control group. Significant at $^{###}p \leq 0.001$ in relation to kaempferol 3-neohesperidoside group.

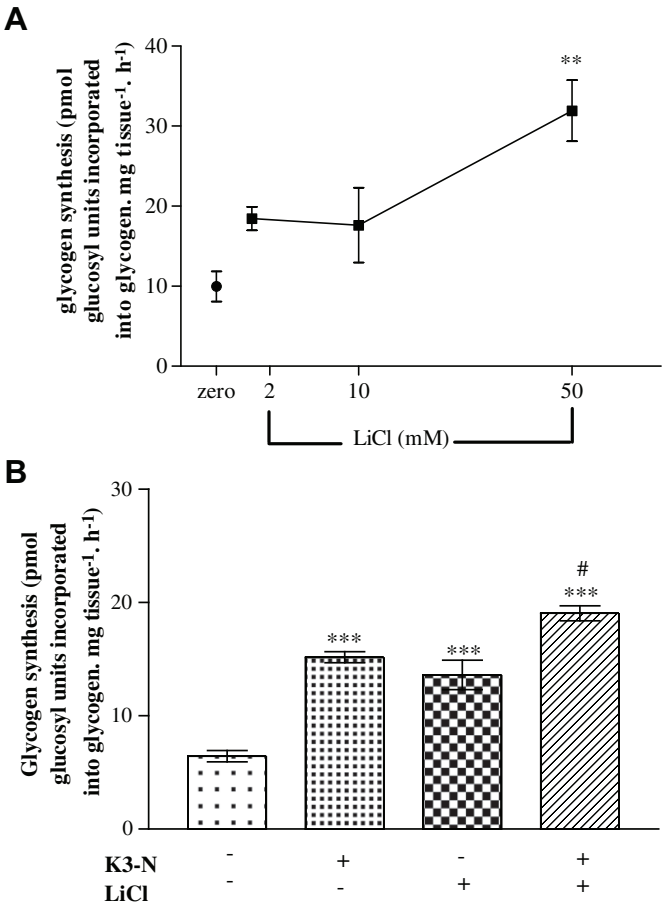

Fig. 6. Dose–response curve of LiCl in basal glycogen synthesis (A) and effect of 50 mM LiCl (B) on the stimulatory action of 1 μM kaempferol 3-neohesperidoside (K3-N) in glycogen synthesis in the rat soleus muscle. Control group = no treatment. Signals (+) and (−) indicate presence and absence, respectively, 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 at $^{**}p \leq 0.01$ in relation to control group. Significant at $^{\#}p \leq 0.05$ in relation to kaempferol 3-neohesperidoside and LiCl groups.

Furthermore, it has been demonstrated that kaempferol 3-neohesperidoside was able to decrease blood glucose levels in diabetic rats. Additionally, it was shown to increase glucose uptake and glycogen content in soleus muscle as effectively as insulin [6,27]. These results point to the insulinomimetic potential effect of kaempferol 3-neohesperidoside in tissue responsive to insulin.

It is well known that insulin induces phosphorylation/dephosphorylation of several intracellular enzymes activating or deactivating specific pathways that subsequently stimulate glycogen synthase activity [3,31,32]. These processes are regulated through insulin binding to its receptor and activating the intrinsic tyrosine kinase activity. Consequently, it promotes the phosphorylation and activation of PI3K and subsequently the phosphorylation and activation of protein kinase B (PKB) [34–37]. One important target of PKB involved in glycogen synthesis is GSK-3 and its phosphorylation induced by insulin promotes the activation of glycogen synthase [35,38–40]. Alternatively, insulin also promotes the activation of the MAPK pathway and protein phosphatase 1 (PP1) activity, the phosphatase responsible for the regulation of glycogen metabolism [31,32,41,42]. Protein phosphatase 1 has an important role in the regulation of glycogen metabolism, where it is inhibited under conditions of glycogen degradation and activated under conditions of glycogen synthesis. The basis of the regulation of the PP1 in the skeletal muscle is the ability of the catalytic subunit of

the enzyme to reversibly associate with a glycogen-binding protein, also known as the G_M subunit of PP1. As a result, the activity and specificity of the PP1 are modulated [43]. Calyculin A, a specific inhibitor of PP1 activity, was not able to change the glycogen synthesis in soleus muscle. However, it was observed that the stimulatory effect of kaempferol 3-neohesperidoside on ^{14}C-glucose incorporation into glycogen in the isolated soleus muscle was completely blocked in the presence of calyculin (Fig. 3B).

In liver derived HepG2 cells, insulin increased PP1 activity 4-fold and a quite similar effect of insulin on glycogen synthase activity was observed. These effects, either on PP1 activity or GS activity, disappeared in the presence of calyculin A. It has been suggested that insulin activates glycogen synthase through the activation of PP1. The results with HepG2 cells provide evidence that insulin acts by changing the phosphorylation state of glycogen synthase through the involvement of PP1 [32]. The same finding has been reported for L6 rat skeletal muscle cells, adipocytes and isolated skeletal muscle [41,42]. Thus, as with insulin, the stimulatory effect of kaempferol 3-neohesperidoside on glycogen synthesis in soleus muscle involves the PP1 pathway.

Additionally, insulin has been shown to activate PP1 activity in L6 rat skeletal muscle cells and fresh isolated adipocytes by increasing the phosphorylation state at site 1 of PP1 [3,41]. This site is readily phosphorylated *in vitro* by p90 ribosomal S6 kinase ($p90^{rsk2}$), a kinase that is phosphorylated and activated by MAPK when cells or tissues are incubated with insulin [44,45]. In the presence of PD98059 the effect of the kaempferol 3-neohesperidoside disappeared, suggesting an involvement of MAPK-$p90^{rsk}$ activation in the glycogen synthesis stimulated by kaempferol 3-neohesperidoside (Fig. 4). It has been proposed that the MAPK pathway increases glycogen synthesis stimulated by insulin in hepatocytes since PD98059 totally inhibits the insulin-stimulated glucose incorporation into glycogen [31]. Furthermore, Dent et al. [45] have suggested that the phosphorylation and activation of $p90^{rsk}$ by MAPK increases the PP1 activity in rabbit skeletal muscle resulting in the dephosphorylation of GS increasing the rate of glycogen accumulation.

Despite the wealth of details regarding the precise mechanism by which insulin mediates glycogen synthesis, several questions remain regarding the role of MAPK and $p90^{rsk}$ in this signal transduction [32,38,44,46–48]. The data here presented concerning the role of this flavonoid in glycogen synthesis show that the MAPK system must be active for the full kaempferol 3-neohesperidoside response in glycogen synthesis.

Besides insulin promoting glycogen synthesis through dephosphorylation of GS by PP1 activation, it also stimulates glycogen synthesis by inactivating GSK-3. In unstimulated cells, GSK-3 is active and contributes to the phosphorylation and inactivation of GS. The inactivation of GSK-3 isoforms GSK-3alpha and GSK-3beta is achieved through phosphorylation of Ser^{21} and Ser^{9}, respectively [3]. This is mediated by PKB in a PI3K-dependent model [36,37]. Taking into account the PI3K-PKB-GSK-3 downstream signaling involved in the activation of glycogen synthase and the stimulatory effect of kaempferol 3-neohesperidoside on glucose uptake blocked by LY294002 [27], the involvement of PI3K in the effect of kaempferol 3-neohesperidoside on glycogen synthesis was investigated. The stimulation of glycogen synthesis by kaempferol 3-neohesperidoside was completely blocked by wortmannin. In 3T3-L1 adipocytes, wortmannin inhibited PI3K activity as well as

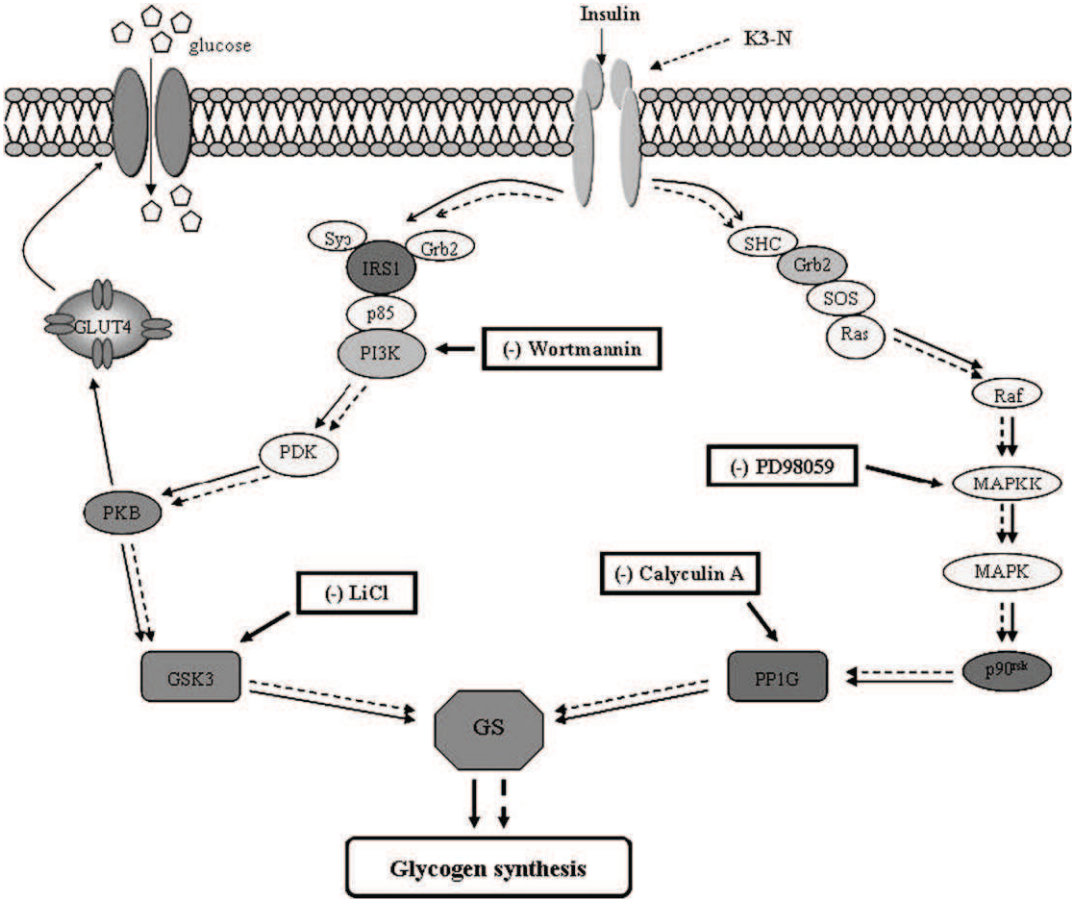

Fig. 7. Proposed mechanism of action of kaempferol 3-neohesperidoside (K3-N) in glycogen synthesis in soleus muscle.

glycogen synthase activity stimulated by insulin [49]. Moreover, the inhibitory effect of wortmannin on PI3K was also observed in hepatocytes where the basal and insulin-induced glucose incorporation into glycogen disappeared [31] as also reported for hepatocytes, myoblasts and L6 myotubes [32,38,47]. The inhibitory effect of wortmannin on kaempferol 3-neohesperidoside action is in agreement with the results reported above for insulin. This result indicates that kaempferol 3-neohesperidoside action in glycogen synthesis is mediated through PI3K in soleus muscle and probably involves the PDK-PKB-GSK-3 signaling pathway downstream of PI3K.

It has been demonstrated that PI3K activates protein kinase dependent on 3-phosphoinositides 1 (PDK 1) which acts on PKB increasing its phosphorylation and activation states [2,36,37]. Since the stimulatory effect of kaempferol 3-neohesperidoside on glycogen accumulation was mediated by PI3K we studied the influence of GSK-3, a downstream effector of PI3K in the kaempferol 3-neohesperidoside action.

Kaempferol 3-neohesperidoside and LiCl caused a similar increase in glycogen accumulation and also presented an additive action on glycogen synthesis. In hepatocytes from normal and diabetic rats, lithium induced a substantial increase in glycogen accumulation as well as in GS activity. Thus, lithium behaves as insulin, since both were able to activate GS and stimulate glycogen synthesis in hepatocytes. Thus, the additive effect of kaempferol 3-neohesperidoside and lithium on glycogen synthesis seems to be similar to that reported for lithium and insulin in hepatocytes [50,51].

In summary, we have shown that kaempferol 3-neohesperidoside promptly stimulates glycogen synthesis in soleus muscle. As hypothesized and shown in Fig. 7, the inhibitor data support the role of PI3K, GSK-3, MAPK and PP1 in the stimulatory effect of kaempferol 3-neohesperidoside on glycogen synthesis. These similarities in the involvement of protein kinases and phosphatases in the stimulation of glycogen deposition by kaempferol 3-neohesperidoside and by insulin constitute strong evidence for the insulinomimetic role of kaempferol 3-neohesperidoside in glucose homeostasis.

Acknowledgements

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4.5 ARTIGO PUBLICADO

Periódico – Mini-Reviews in Medicinal Chemistry

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Flavonoids: Prospective Drug Candidates

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Abstract: The purpose of this review is to discuss the recent developments related to the chemistry and medicinal properties of flavonoids. Major flavonoids that show well categorized structures and well defined structure function-relationships are: flavans, flavanones, flavones, flavanonols, flavonols, catechins, anthocyanidins and isoflavone. The biological properties of flavonoids include antioxidant, anti-inflamatory, antitumoral, antiviral and antibacterial, as well as a direct cytoprotective effect on coronary and vascular systems, the pancreas and the liver. These characteristics place them among the most attractive natural substances available to enrich the current therapy options.

Key Words: Flavonoids, medicinal properties, absorption, disease.

CHEMISTRY, CLASSIFICATION AND OCCURRENCE

The flavonoids belong to a large group of low-molecular-weight polyphenolic compounds biosynthesized from both the shikimic acid and acetic acid pathways. Flavonoids are the product of the condensation of the three malonyl-CoA units (C-2) and a *P*-coumaric acid unit (C-9) to give a basic nucleus (C6-C3-C6) composed of two benzene rings connected by a three-carbon unit such as an oxygen-containing pyrene ring. Their structural features comprise a fundamental skeleton of the 2-phenyl chromone with a variety of substitution patterns in the A-ring (characteristically a phloroglucinol or resorcinol hydroxylation pattern) and B-ring (usually catechol, pyrogallol or 4′-hydroxylated) [1, 2]. The oxidation level of the three-carbon unit (C-ring) gives rise to different classes of flavonoids including flavans, flavanones, flavones, flavanonols, flavonols, catechins, anthocyanidins, and isoflavone, among others (Fig. (**1**)).

Flavonoids are mainly present in nature as glycosides although free aglycones can be found as major constituents in several plants. The glycosilation of a phenolic alcoholic hydroxyl group of a flavonoid aglycone can occur through the hemiacetal bound (O-glycosides) or straight attachment to the C-1 of the sugar unit *via* a carbon-carbon bond (C-glycosides). A few monosaccharides, such as D-glucose and L-rhamnose, which are the most common, and other less frequent glucorhamnose, galactose, xylose and arabinose, or combinations of these (di- or trisaccharides), can bind to hydroxyl groups or directly to a carbon atom at different positions on the flavonoid aglycone [1, 3-5]. The O-glycosilation of the flavonoid molecule occurs most frequently at the C-3 and/or C-7 positions and for C-glycoside flavonoids the preferential glycosilation sites are at positions C-6 and C-8. The large number of flavonoids found in nature is due to the innumerable combinations between flavonoid aglycones and sugars units.

The flavonoids are very widespread in nature and are the largest group of natural products known. They are present in plants in many different glycosidic forms, for example, quercetin-3-rutinoside (rutin), quercetin-4′-glucoside and quercetin-3,4′-glucoside, which are the most common. Besides their roles in plants, flavonoids are important components in the human diet and are found in fruits, vegetables, seeds, nuts, grains, spices and beverages (wine, tea and beer). Quercetin is considered one of the most common flavonol aglycones in the human diet due its high concentration in several foods including onions, kale, french beans, broccoli, lettuce, tomatoes, apples and beverages [1, 6]. Other flavonols in the diet include kaempferol (broccoli), myricetin (berries), and isorhamnetin (onion) [1, 5, 6]. The glycoside flavanones mainly occur in citrus fruits and are represented by hesperetin-7-rutinoside and naringenin-7-rutinoside, which are the major flavonoids of oranges and mandarins. The glycosides naringenin-7-neohesperoside and narirutin are typically found in grapefruit. Pears, grapes, peaches, vegetables, tea and red wine are sources of catechins that occur as aglycones or esterified with gallic acid [7-9]. The main flavones in the diet are apigenin and luteolin and most important sources are red pepper, celery, cereal grains and aromatic herbs [9, 10]. Edible fruits, such as plums, apples, eggplant, and many berries have important contents of anthocyanidins and their glycosides, which are the compounds responsible for the red, blue or violet color. The most common anthocyanidins are pelargonidin, cyanidin, delphinidin, and malvidin [1, 11]. The predominant isoflavonoids are the genistein and daidzein

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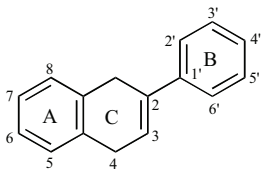
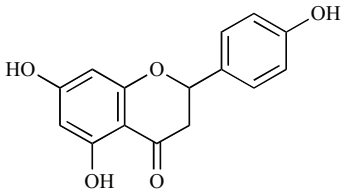
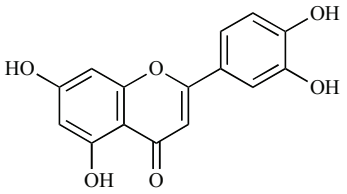


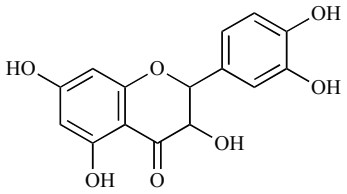
FLAVAN NUCLEUS



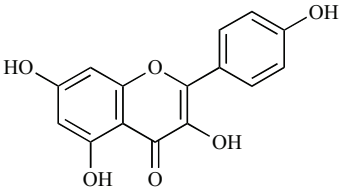
(FLAVANONE)
2,3-dihydro-5,7-dihydroxy-2-(4-dihydroxyphenyl)-
4H-1-Benzopyran-4-one (naringenin)



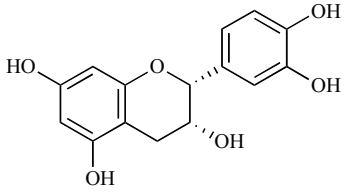
(FLAVONE)
5,7-dihydroxy-2-(3,4-hydroxyphenyl)-
4H-1-benzopyran-4-one (luteolin)



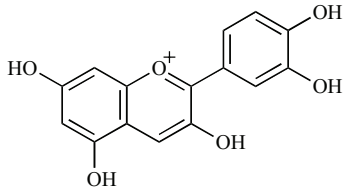
(FLAVANONOL)
2,3-dihydro-3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl)-
4H-1-benzopyran-4-one (taxifolin)



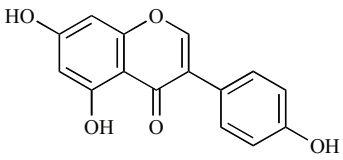
(FLAVONOL)
3,5,7-trihydroxy-2-(4-hydroxyphenyl)-
4H-1-benzopyran-4-one (kaempferol)



(FLAVANOL)
(2R,3R)-3,4-dihydro-3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl)-
4H-1-benzopyran((-)-epicatechin)



ANTHOCYANIDIN NUCLEUS
3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl)-
1-benzopirilium cyanidin



ISOFLAVONE NUCLEUS
5,7-dihydroxy-3-(4-hydroxyphenyl)-
4H-1-benzopyran-4-one (genistein)

Fig. (1). Representative structure of main classes of flavonoids.

found in high concentrations in soybean and soy products [12, 13].

ABSORPTION AND METABOLISM

An important factor in the absorption efficiency of flavonoid glycosides in the intestine is the sugar moiety, as demonstrated for quercetin glucosides, aglycone and rutin supplements [14]. Flavonoid aglycones are hydrophobic in nature and can be transported across membranes by passive diffusion. The glycoside moiety increases the hydrophilicity of the flavonoid molecule which reduces the possibility of passive transport. This leads to the theory that flavonoids are absorbed by active transport [1]. Evidence is accumulating that epithelial brush border membrane transporters play a role in the absorption of dietary (iso)flavonoids. Indirect evidence indicates that some flavonoid glycosides can be absorbed intact in the small intestine through the sodium-dependent glucose transporter 1 (SGLT-1), as reported for

quercetin-3-glucoside and quercetin-4′-glucoside [14-16] (Fig. (**2A**)).

On the other hand, the flavonoids can return to the intestinal lumen through efficient efflux transport, most likely due to the apical multi-drug resistance protein- 2 (MRP-2), a characteristic intestinal efflux pump, as in the efflux of quercetin-4′-β-glucoside, (-)-epicatechin and (-)-epigallocatechin-3-gallate [17, 18]. It has been described that before absorption flavonoids are cleaved by specific enzymes either in the lumen or inside the cells of the gut. Lactase-phlorizin hydrolase (LPH) is anchored in the brush-border membrane in the small intestine and catalyzes extracellular hydrolysis of some glucosides [19, 20]. Another enzyme, located intracellularly and with broad specificity, is the cytosolic β-glucosidase (CBG). It is found in abundance in the small intestine, liver and kidney of mammals and requires active transport of hydrophilic glucosides into the cells [20]. Concerning LPH activity, it has been shown that the enzyme cleaves some flavonols and isoflavone glycosides such as: quercetin- 4′-glucoside, quercetin-3-glucoside, quercetin-3, 4′-glucoside, 3′-methylquercetin-3-glucoside, genistein-7-glucoside, and daidzein-7-glucoside. However, quercetin-3-rhamnoglucoside (rutin) and naringenin-7-rhamnoglucoside (naringin) are not substrates for this enzyme [19, 21]. In addition, β-glucosidase (BCG) activity is reported to act on flavonoid and isoflavone glycosides according to the position and the structure of the sugar moiety attached to the flavonoid aglycone [22] (Fig. (**2A**)).

Once absorbed, flavonoids are subject to 3 main types of conjugation: methylation, sulfation and glucuronidation [23]. The metabolic steps in polyphenol metabolism are catalyzed by enzymes. The levels and sites of enzyme expression in human tissues determine the metabolic fate and the pharmacokinetics of ingested polyphenols and their glycosides [24]. The most important enzymes involved in flavonoids metabolism are: catechol-*O*-methyltransferase (COMT; EC 2.1.1.6), which methylates polyphenols and has the highest activity in the liver and kidneys [25]. Phenol sulfotransferases (P-PST, SULT; EC 2.8.2.1) are cytosolic enzymes that transfer sulfate moieties to hydroxyl groups from substrates such as iodothyronines, phenols and hydroxyarylamines mainly in the liver [23, 24, 26]. UDP glucuronosyl transferase (UDPGT, UGT; EC 2.4.1.17) catalyzes the conjugation of polyphenols to glucuronic acid in endoplasmic reticulum in the intestine, liver and kidney. In humans, the liver has the greatest capacity for glucuronidation while in rats, the highest level of glucuronyl transferase activity was observed in the intestine [26-28].

Conjugation reactions with glucuronic acid and/or sulfate seem to be the most common type of metabolic pathways for the flavonoids [1]. This conjugation first occurs in the gut barrier and these conjugates then reach the liver, where they are further metabolized [26]. For example, catechin is extensively methylated in the liver [29] and increased plasma total (+)-catechin levels were observed after hydrolysis of its glucuronide and sulphate derivatives from volunteers that ingested red wine [30]. Otake *et al.* [31] using human liver microsomes demonstrated that hepatic UDP-glucuronosyl transferase isoforms were the main factor responsible for galangin metabolism into two major glucuronides conjugated at the 7- and 3- positions. Also, Vaidyanathan and Walle [32] demonstrated no glucuronidation of (-)-epicatechin by human liver and small intestinal microsomes. However, in rats, (-)-epicatechin was efficiently metabolized by liver microsomes with formation of two glucuronides. In the same study, the authors concluded that sulfation also occurred in both the liver and intestine in human and rats.

On the other hand, glycoside flavonoids that are not absorbed in the small intestine along with the conjugated metabolites that are excreted in bile can be metabolized by microflora when they reach the colon (Fig. (**2B**)). Glycoside flavonoid-hydrolyzing enzymes have been identified in fecal flora cultures. Bokkenheuser *et al.* [33], recovered three enzyme-producing strains that, using β-glucosidases, α-rhamnosidases, and/or β-galactosidases, were capable of converting rutin to quercetin. Also, it was shown that at least some of the bacterial glycosidases are able to cleave glycosidic bonds and flavonoid-saccharide bonds in the gut [19]. The profile of metabolites has been demonstrated in studies with quercetin, rutin and naringin. The flavonoid metabolism produces aromatic acids such as phenylvaleric, phenylpropionic, phenylacetic and benzoic acids with easy absorption through the colonic barrier [34-36].

Metabolites of flavonoids in general (and also microflora metabolites), aglycones, glycosides and conjugated metabolites which are not absorbed, may follow two pathways of excretion: *via* the biliary or the urinary route. Large conjugated metabolites are more likely to be eliminated in the bile whereas small conjugates such as monosulfates are preferentially excreted in urine [27]. When excreted in bile, the flavonoids are passed to the duodenum and metabolized by intestinal bacteria, which results in the production of fragmentation products and/or the hydrolysis of glucurono- or sulfoconjugates [37]. The resulting metabolites which are released may be reabsorbed and enter an enterohepatic cycle or being excreted in feces [38, 39] (Fig. (**2B**)). For each flavonoid, the beneficial effect will be dependent upon their absorption and availability in the body. Thus, these factors should be considered in any interpretation of the potential health effects of flavonoids.

MEDICINAL PROPERTIES

1. Antioxidant Activity

The major mechanisms of action of antioxidant agents are suppression of reactive oxygen species formation, either by inhibition of enzymes or chelating trace elements involved in free radical production; scavenging of reactive oxygen species (ROS); and upregulation or protection of antioxidant defenses [40].

The antioxidant activity of flavonoids and the mechanisms involved in their action are extensively revised by Pietta [41] and Amic *et al.* [42]. The configuration and total number of hydroxyl groups are determinant factors in the mechanisms of antioxidant activity of flavonoids. Particularly, the B-ring hydroxyl configuration is significant as a scavenger of ROS and reactive nitrogen species (RNS) [43, 44]. Furthermore, a 3′,4′-catechol structure in the B-ring and the presence of 6-hydroxyl group potentiates lipid peroxidation inhibition as well as the free radical scavenger effect

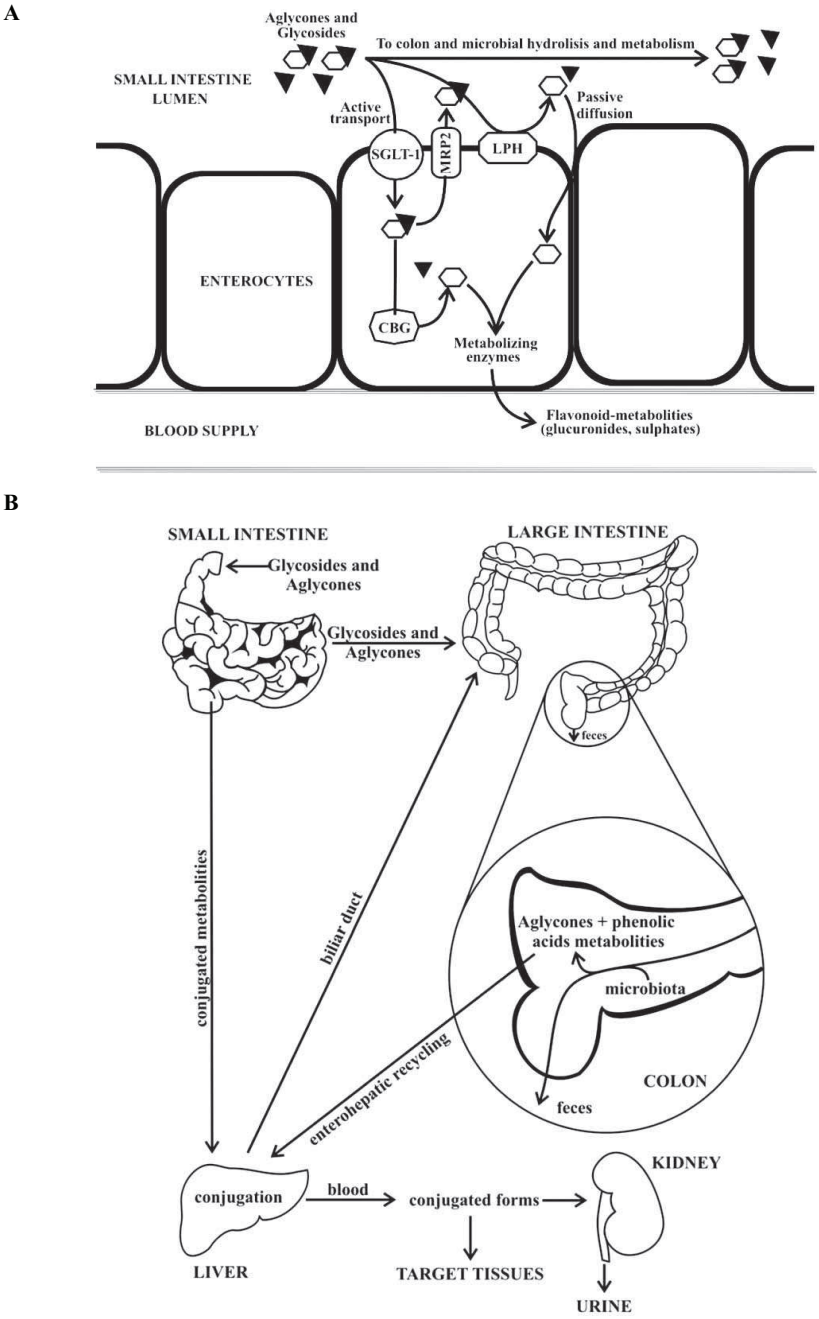


Fig. (2). Schematic representation of intestinal absorption (**A**) and metabolism of flavonoids (**B**). SGLT-1 = sodium-dependent glucose transporter-1; MRP-2 = multidrug-resistance associated protein-2; LPH = lactase phloridzin hydrolase; CBG = cytosolic β-glucosidase. Fig. (**A**) was adapted from Nemeth *et al*., (2003) [20].

[45, 46]. Different classes of compounds are able to scavenge reactive species including: catechin, luteolin, kaempferol, 3-O-methyl-quercetin, naringenin, kaempferol 3-O-D-glycoside, kaempferol-3-O-(2´´,6´´-di-O-*p*-trans-coumaroyl) glucoside, 6-hydroxyluteolin glycosides, hesperidin and quercetin [46-49].

The flavonoid heterocycle contributes to antioxidant activity by permitting conjugation between the aromatic rings and particularly through the presence of a free 3-OH which can be potentiated by the presence of a 3´,4´-catechol explaining the potent antioxidant activity of flavan-3-ols and flavon-3-ols which possess the latter feature [50, 51]. Also, the carbonyl group at C-4 and a double bond between C-2 and C-3 are important features in relation to the high antioxidant activity of flavonoids [51, 52]. Furthermore, the number of polyhydroxy or polymethoxy substituents determines the difference in the antioxidant activities of flavonoids probably due to the hydrophobicity and molecular pla-

narity induced by the substitution models [45, 50]. Another important feature of antioxidant agents is the presence, number and position of glycosides in the flavonoid structure. In general, aglycones are more potent antioxidants than the corresponding glycosides, as demonstrated by daidzein, genistein and their 7-glycosides, kaempferitrin and the flavonol glycosides in green tea [52-56].

One of the most well known antioxidant activities of flavonoids is the inhibition of the enzymes responsible for superoxide anion production, such as xanthine oxidase [57]. Also, flavonoids inhibit the enzymes of the cyclooxygenase and lipoxygenase pathway [58] and microsomal monooxygenase, glutathione *S*-transferase, mitochondrial succinoxidase, and NADH oxidase, all involved in ROS generation [59]. Another possible contributory mechanism to the antioxidant activity of flavonoids is the ability to efficiently chelate trace metals through interaction with the catechol moiety in the B-ring, 3-hydroxyl, 4-oxo groups in the heterocyclic ring, and 4-oxo, 5-hydroxyl groups between the heterocyclic and the A-rings, decreasing the availability of these metals to participate in the generation of free radicals [60, 61].

2. Anti-Inflammatory Activity

Several cellular action mechanisms have been proposed to explain the *in vivo* and *in vitro* anti-inflammatory activity of flavonoids. They can modulate the functions and activities of inflammatory and immune cells through, for instance, inhibition of histamine release from mast cells, immunosuppressive effects on T-cell proliferation and IL-2 synthesis, and regulation of secretion of IgG, IgM and IgA isotypes [58, 62]. There are also reports concerning flavonoid actions over a range of cellular types such as platelets, eosinophils, neutrophils, mast cells, basophils, macrophages and monocytes [to review see 62, 63].

In addition, one of the direct actions of flavonoids is modulating the activity of arachidonic acid (AA) metabolizing enzymes [58]. The formation of AA is the rate limiting step in the synthesis of prostaglandis, leukotrienes and the platelet activating factor. This pathway is triggered by phospholipase A_2 (PLA_2). AA undergoes metabolism to several active products (eicosanoids) by two major routes: the cyclooxygenase and lipoxygenase pathways. The immediate products of the cyclooxygenase pathway are endoperoxides (PGG_2 and PGH_2), which are converted to prostaglandins ($PGE_{2\alpha}$, PGF_2Q and PGD_2) by prostaglandins synthetase enzymes, as well as thromboxanes (TXA_2 and TXB_2) and prostacyclins (PGI_2) by the corresponding synthetases. The lipoxygenase pathway converts AA to hydroxyl fatty acids, including hydroperoxyeicosatetraenoic acids (HPETEs), which are then metabolized to hydroxyeicosatetraenoic acids (HETEs). The HETEs and their leukotriene metabolites are important mediators of inflammatory responses [64].

The first flavonoid inhibitor of PLA_2 activity discovered was quercetin, which inhibits the activity of PLA_2 from human neutrophils and several other sources. Also, other flavonoids have been reported to inhibit PLA_2 activity, for example, hesperetin, naringenin, kaempferol, myricetin and biflavonoids [64, 65].

The cyclooxygenase (COX) enzyme exists basically in two different isoforms: COX-1 and COX-2 [66, 67]. Some flavonoids such as luteolin, 3′,4′-dihydroxyflavone, galangin, and morin inhibit COX activity [68]. Inhibition was also observed for baicalin, (+)-catechin, rutin, chrysin and its derivatives, kaempferol and quercetin in different animal tissues and cells [58, 69]. Studies on flavonoid inhibition of COX-2 have been rarely reported. An inhibitory activity against COX-2 or COX-1/COX-2 are reported for some flavan-3-ols such as catechin and 4′-Me-gallocatechin and for two dihydrochalcones [70-72]. Lipooxygenase (LOXs) enzyme isoforms also act in inflammatory processes and are responsible for generating hydroxy acids and leukotrienes (LTs) from AA. Flavonols, including kaempferol, quercetin, morin and myricetin, have been found to be stronger LOX inhibitors than flavones [69, 73].

Nitric oxide (NO) is one of the cellular mediators of physiological and pathological processes involved in inflammatory events and it is biochemically synthesized from L-arginine by nitric oxide synthase (NOS) isoforms [74, 75]. Quercetin, apigenin, luteolin, genistein, kaempferol, chrysin and its derivatives were found to inhibit NO production to act against NOS isoforms both by inhibition of induced NOS (iNOS) activity or down-regulation of iNOS expression from different cell types [76, 77]. Furthermore, evidence supports the idea that certain flavonoids act as modulators of proinflammatory gene expression.

The tumor necrosis factor-α (TNF-α) and IL-1β are prominent contributors to chronic inflammatory disorders [78]. Genistein and other flavonoids are reported to inhibit IL-1β, IL-6, and TNF-α production in several kinds of cells [79, 80]. Activation of NF-κB is inhibited by genistein, apigenin, kaempferol, oroxylin A [77, 81], epigallocatechin 3-gallate and amentoflavone [82, 83] while quercetin, rutin and luteolin inhibits the expression and production of TNF-α and intercellular adhesion molecule 1 (ICAM-1) in mice [84, 85]. Another flavonoid, myricitrin, has also been described as an inhibitor of the nociceptive responses in models of acute pain by inhibiting of phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) activities, NO production, iNOS over expression and NF-kB activation [86, 87].

3. Protective Effects on Coronary Disease and Vascular Activity

Atherosclerosis is a chronic inflammatory response in the walls of arteries, in large part due to the deposition of lipoproteins (plasma proteins that carry cholesterol and triglycerides). There is much evidence that oxidized low density lipoprotein (LDL) is responsible for cholesterol loading of macrophages, foam cell formation and atherogenesis [88, 89]. LDL is oxidized by free radicals generated by endothelial cells, macrophages and smooth muscle cells. Therefore, it has been hypothesized that oxidized LDL is the responsible for the initiation and promotion of atherogenesis [90, 91].

In this regard, several aglycone and polyhydroxylated flavonoids, such as quercetin, morin, hypoleatin, setin, gossypetin and galangin, are potent inhibitors of LDL oxidation *in vitro* by macrophages or copper ions. They may reduce the formation of free radicals (for instance, chelating divalent

metal involved in the Fenton reactions), and protect the α-tocopherol present in the LDL structure from oxidative damage or regenerate it [92, 93]. Along with all of the activity of flavonoids against atherogenesis, they may inhibit adhesion and platelet aggregation as well as promote vascular smooth muscle relaxation. The antiaggregatory effects of flavonoids seems to influence the platelet activation pathway, such as the inhibition of the enzymes involved in AA metabolism as well as the inhibition of platelet aggregation by antagonizing thromboxane formation and thromboxane receptor function [94, 95].

The antioxidant actions of flavonoids appear to participate in their antithrombotic action. The antithrombotic and vasoprotective actions of quercetin, rutin, and other flavonoids have been attributed to their ability to bind to platelet membranes and scavenge free radicals, restoring the biosynthesis and action of endothelial prostacyclin and endothelial-derived relaxing factor [96-98]. Flavonoids with anti-platelet activity include isobavachalcone and neobavaisoflavone, luteolin, genistein, quercetin, apigenin and kaempferol derivatives [99-101]. One of the flavonoid mechanisms of platelet aggregation inhibition is to increase cyclic AMP (cAMP) levels through adenylate cyclase activation and phosphodiesterase inhibition [102, 103]. Quercetin-4′-O-β-D-glycoside inhibited collagen-stimulated tyrosine phosphorylation of platelet and quercetin inhibits the intracellular Ca^{2+} mobilization suggesting that these flavonoids act as inhibitors of the trigger signal for thrombus formation [104, 105]. These results have been recently confirmed in humans by Hubbard *et al*. [106].

In addition to their antiaggregatory effects, flavonoids appear to increase vasodilation by inducing vascular smooth muscle relaxation which may be mediated by the inhibition of PKC, phosphodiesterase, or by decreased cellular Ca^{2+} uptake [102]. Luteolin, naringenin and eriodictyol have been shown to promote the relaxation of rat aorta contractions induced by Ca^{2+}, noradrenaline and K^{+} [107]. Quercetin, chrysin and (-)-epicatechin also have vasorelaxant effects [108-111].

4. Antitumoral Activity

Cancer is a disease caused by a combination of exogenous and endogenous factors which results in a cellular cyclo imbalance (mitosis/apoptosis) turning normal cells into cancer cells [2].

The flavonoids are one of the most promising anticancer natural products that have been studied [112-114]. They interfere with a large number of regulatory pathways such as: cellular growth, energy metabolism, apoptosis, cell division, transcription, gene repair, neuronal transmission, inflammation, and stress response which may be involved in tumorigenesis [2, 62]. Quercetin has been reported to inhibit many biochemical events associated with tumor promotion, such as alteration in PKC activity and interactions with calmodulin [115, 116]. Also, quercetin strongly inhibits the expression of the mutated p53 (tumor suppressor gene) protein preventing the accumulation of newly synthesized p53 protein without affecting the steady-state mRNA levels of p53 in cancer cell lines [117, 118]. This flavonoid also exhibited antiproliferative effects in: drug-resistant leukemia cells *in vitro* and *in vivo*, colon and hepatocellular cancers in rats and mice and exerted growth-inhibitory effects on several malignant tumor cell lines *in vitro* [119-123].

In *in vivo* studies quercetin, kaempferol, and myricetin have been found to be able to inhibit carcinogen-induced tumors in rats and mice [124]. Other flavonoids such as catechin, epicatechin, quercetin, and resveratrol, polyphenolic compounds in red wine have been shown to inhibit growth of human breast and prostate cancer cells [125, 126]. The exposure of human epidermoid carcinoma cells to silymarin resulted in a significant decrease in ligand-induced activation of the epidermal growth factor receptor (EGFR) with an associated decrease in EGFR intrinsic kinase activity. This was accompanied by inhibition of DNA synthesis and cell growth [127].

The anticancer effects of genistein and its derivatives, such as biochanin A, daidzein, genistin and daidzin, have also been reported. They potently inhibit the growth of human breast carcinoma cell lines [128, 129]. *In vitro* studies have shown that such chemopreventive and antineoplastic effects are associated with the antioxidant activity of genistein and inhibitor activities in cell proliferation and angiogenesis [130, 131] as demonstrated recently for genistin and daidzin in M14 cells [132]. Effects of green tea on the inhibition of carcinogenesis in experimental animal models, along with its constituents, for example, (-)- epigallocatechin gallate, in all levels of cancer progression, namely initiation, promotion and transformation, have been described [133, 134].

Recently, Cárdenas *et al*. [135] studied the effects of various natural flavonoids, cinnamic acid derivatives, and a series of synthetic flavones on cell proliferation *in vitro* in established human and murine tumor cell lines. The most potent antiproliferative agents were caffeic acid *n*-butyl ester > 2′-nitroflavone > caffeic acid ethyl ester ~ 2′, 6-dinitroflavone > apigenin > 3′-bromoflavone ~ 2′-fluoro-6-bromoflavone. Some compounds showed a moderate effect, the order of cytotoxic activities being chrysin > 2′-fluoro-6-chloroflavone ~ 2′-chlorochrysin > α-naphthoflavone > β-naphthoflavone ~ 6-chloroflavone ~ 6-bromoflavone ~ 4′-nitroflavone. None of the natural or synthetic compounds tested affected the proliferation of epithelial cells derived from normal mammary glands of mice or fibroblastic cells of the mouse embryo, suggesting a selective action against tumor cells. Besides the flavonoid actions described above, they can also influence adhesion molecules, metastasis and angiogenesis as well as apoptosis, gene expression and mutagenicity, which may contribute to its anticarcinogenic activity [see review 2, 62].

5. Antiviral Effects

Viruses are obligate intracellular parasites, which contain little more than bundles of gene strands of either RNA or DNA, and may be surrounded by a lipid-containing envelope [136]. Unlike bacterial cells, which are free-living entities, viruses utilize the host cell environment to propagate new viruses. They use the reproductive machinery and metabolic pathways of cells to provide energy and building blocks for new viral particles. In an alternative stage, the viral genes

may remain silent for a prolonged period inside the host cells [136].

A recent area of research that is of particular interest is the apparent inhibitory activity of some flavonoids against human immunodeficiency virus (HIV). The mechanisms of action of compounds such as baicalin, robustoflavone and hinokiflavone, robinetin, myricetin, quercetagetin, quercetin 3-*O*-(2′′-galloyl)-α-l-arabinopyranoside and chrysin seem to involve the inhibition of entry, infection, transcription and replication in cells as well as the inhibition of enzymes involved with these processes, for instance, reverse transcriptase, HIV-1 proteinase and integrase [137-141].

The effects of green tea constituents on the HIV-1 viral life cycle have being studied. It was found that (-)- epigallocatechin gallate caused the destruction of the viral particles and inhibited viral attachment to cells, post-adsorption entry into cells, reverse transcription and viral production from chronically-infected cells [142]. Epigallocatechin-3-gallate showed a dose-dependent effect on the inhibition of the replication HIV strains [143]. An inhibitory effect on HIV-1 replication was shown for tea flavin and catechin derivatives. These tea polyphenols are able to inhibit HIV-1 entry into target cells by blocking HIV-1 envelope glycoprotein-mediated membrane fusion [144].

The structural basis for the antiviral activity of naturally occurring flavonoids appears to be associated with non glycosidic compounds and hydroxylation at the 3′, 4′, 3, 5, and 7-position is apparently a prerequisite for antiviral activity [145]. A number of possible mechanisms whereby polyphenols may exert their antiviral action have been proposed. The action of flavonoids probably derives from their direct inactivation of the virus and/or from inhibition of the virus binding to the cells. They are also known to inhibit viral replication [146].

The antiherpetic activity of several flavonoids against the herpes simplex virus type 1 and type 2 has being evaluated and morin, epicatechin, epicatechin gallate (flavanols), genistein (isoflavone), naringenin (flavanone), quercetin (flavonol), galangin, kaempferol, catechin, epigallocatechin, epigallocatechin gallate, chrysin, baicalin, fisetin, myricetin, and genistein showed significant effects [147, 148]. The antiherpetic activity and genotoxicity of catechin and some of its derivatives has also been confirmed by Savi *et al.* [149].

Homoisoflavonoids were identified as having low cytotoxicity and a good antiviral activity against Coxsackie viruses (CVB3, CVB4, CAV9) and Echovirus 30 (Echo30) which may be useful as an additional antiviral drug against these infections [150]. Furthermore, antiviral activity against the human cytomegalovirus (HCMV) has been shown for baicalein. The basic mechanism of action seems to be the blockage of HCMV infection through inhibiting it entry into the cells and its replication [151].

7. Antibacterial Activity

The use of flavonoids against bacterial, protozoan, and fungal infections has two purposes: (1) to kill the bacterial or fungal cells and (2) to counteract the spread and the effects of the bacterial toxins [152]. However, the mechanism by which this is accomplished is not known yet. Antibacterial mechanisms of action of flavonoids based on their structure-activity relationships [to review see 153] have been proposed, for example, inhibition of nucleic acid synthesis [154, 155], cytoplasmatic membrane function [156, 157] and energy metabolism [158].

Recently, the antibacterial activity of some flavonoids has been increasingly documented. Examples of such flavonoids are apigenin, galangin, chrysin, sophoraflavanone G and its derivatives, naringin and naringenin, epigallocatechin gallate and its derivatives, luteolin and luteolin 7- glucoside, quercetin, 3-*O*-methylquercetin and various quercetin glycosides, along with kaempferol and its derivatives [159-166]. Other flavones, isoflavones, flavanones, flavonols, flavonol glycosides and chalcones with antibacterial activity have also been identified [163, 167-170].

The activity of the flavonoids apigenin, baicalin and galangin against sensitive and antibiotic resistant strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli* and *Pseudomonas aeruginosa* has been investigated and galangin and apigenin were shown to have an inhibitory activity [171]. Genistein presented inhibitory effects on the growth of staphylococcal strains, *Streptococcus pasteurianus, Bacillus cereus*, and *Helicobacter pylori,* whereas *Escherichia coli* growth was not suppressed. Daidzein, which is structurally similar to genistein, also inhibited the growth of *Staphylococcus aureus*, albeit with lower potency than genistein [172]. Although advances in understanding the role played by flavonoids in each particular pathology are still required, it is now clear that structure function-relationship of glycosylated flavonoids indicates a molecular mechanism which is crucial to the drug discovery process.

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ABBREVIATIONS

AA	=	Arachidonic acid
AMP	=	3'-5'- Adenosine monophosphate
cAMP	=	3'-5'-Cyclic adenosine monophosphate
CBG	=	Cytosolic β-glucosidase
COMT	=	Catechol-*O*-methyltransferase
COX	=	Cyclooxygenase
CVB	=	Coxsackie viruses
DNA	=	Deoxyribonucleic acid
EGFR	=	Epidermal growth factor receptor

HCMV	=	Human cytomegalovirus
HETE	=	Hydroxyeicosatetraenoic acid
HIV	=	Human immunodeficiency virus
HPETE	=	Hydroperoxyeicosatetraenoic acid
ICAM-1	=	Intercellular adhesion molecule 1
IgM, IgA, IgG	=	Immunoglobulines
IL-1β	=	Interleukin 1β
Il-6	=	Interleukin 6
LDL	=	Low density lipoprotein
LOXs	=	Lipooxygenase isoforms
LPH	=	Lactase-phlorizin hydrolase
LT	=	Leucotrienes
mRNA	=	Messenger ribonucleic acid
MRP-2	=	Apical multi-drug resistance protein- 2
NADH oxidase	=	Nicotinamide adenine dinucleotide oxidase
NF-κB	=	Nuclear factor-kappa B
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
$PGE_{2\alpha}$, PGF_2Q and PGD_2	=	Prostaglandins
PGI_2	=	Prostacyclin
PI3K	=	Phosphatidylinositol 3-kinase
PKC	=	Protein kinase C
PLA_2	=	Phospholipase A_2
P-PST, SULT	=	Phenol sulfotransferases
RNA	=	Ribonucleic acid
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
SGLT-1	=	Sodium-dependent glucose transporter 1
TNF-α	=	Tumor necrosis factor-α
TXA_2 and TXB_2	=	Thromboxanes
UDPGT, UGT	=	UDP Glucuronosyl transferase

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Flavonoids: Cellular and Molecular Mechanism of Action in Glucose Homeostasis

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Abstract: The purpose of this review is to discuss the cellular and molecular mechanisms of action of flavonoids focusing on carbohydrate metabolism. The beneficial effects of flavonoids have been studied in relation to diabetes mellitus, either through their capacity to avoid glucose absorption or to improve glucose tolerance. Furthermore, flavonoids stimulate glucose uptake in peripheral tissues, regulate the activity and/or expression of the rate-limiting enzymes in the carbohydrate metabolism pathway and act *per se* as insulin secretagogues or insulin mimetics, probably, by influencing the pleiotropic mechanisms of insulin signaling, to ameliorate the diabetes status.

Key Words: Flavonoids, mechanism of action, glycemia, insulin, diabetes.

BIOLOGICAL EFFECTS OF FLAVONOIDS

In the physiological state, the maintenance of glucose homeostasis is achieved by a hormonal regulation of glucose uptake and endogenous glucose production by the muscle and liver, respectively. Diabetes mellitus is a complex metabolic disorder in the endocrine system characterized by abnormalities in insulin secretion and/or insulin action that lead to progressive deterioration of glucose tolerance and cause hyperglycemia. This disease is a major public health problem found in all parts of the world and is rapidly increasing [1]. There are basically two types of diabetes: a) type 1, insulin-dependent diabetes mellitus (IDDM), in which the body does not produce insulin, most often occurs in children and young adults; b) type 2, noninsulin-dependent diabetes mellitus (NIDDM), characterized by insulin-resistance due to an improper use of insulin. It is the most common form of the disease, occurring mainly in elderly people [1].

Recently, there has been a growing interest in hypoglycemic agents from natural products, especially those derived from plants. Flavonoids are naturally occurring phenolic compounds that are widely distributed in plants. They have a broad range of biological activities and numerous studies have been carried out on their potential role in the treatment of diabetes. Many studies have demonstrated the hypoglycemic effects of flavonoids using different experimental models and treatments. Intraperitoneal injection of epigallocatechin gallate into rats has been found to lowered blood glucose and insulin levels [2]. In the same way, green tea has been observed to improve glucose metabolism in healthy humans in oral glucose tolerance tests. Green tea also lowered blood glucose levels in diabetic db/db mice and streptozotocin-diabetic (STZ-diabetic) mice 2–6 h after administration without affecting the serum insulin level [3]. Administration of an aqueous solution of green tea in normal and alloxan-diabetic rats improved glucose tolerance and reduced blood glucose levels, respectively [4]. Furthermore, studies with procyanidins administered alone have demonstrated a significant reduction in glycemic levels and together with insulin they showed an addictive hypoglycemic effect in rats [5].

Bolus intravenous injection of puerarin, an isoflavonoid, has been reported to decrease the plasma glucose concentrations in STZ-diabetic, normal and hyperglycemic rats [6]. In addition, glucose tolerance tests with puerarin showed a significant blunting of the rise in blood glucose compared with control C57BL/6J-ob/ob mice [7]. Another isoflavonoid, genistein, has been shown to significantly decrease the blood glucose level in diabetic rats compared with the control, in glucose tolerance tests. These results were confirmed with genistein and daidzein chronic treatments in db/db mice and STZ-diabetic rats [8, 9].

Studies have been carried out on the effects of kaempferitrin, one of the compounds found in the *n*-butanol fraction of *Bauhinia forficata*, on glycemia in diabetic rats. The hypoglycemic effect of kaempferitrin in diabetic rats was evident at all doses tested and this profile was maintained throughout the study period. Additionally, in glucose-fed hyperglycemic normal rats, the kaempferitrin failed to decrease blood glucose levels [10]. Taking advantage of the hypoglycemic effect of vanadium, a vanadium-based flavonoid complex has been designed and studied. Kaempferitrin–VO(IV) (vanadium complex) as well as VO(IV) in alloxan-diabetic rats demonstrated hypoglycemic effect for 1 to 24 h when compared to the respective zero time. The kaempferol-3-neohesperidoside, a glycosylated flavonoid structurally very similar to kaempferitrin has shown an interesting hypoglycemic effect in both oral and intraperitoneal treatments in

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diabetic rats. When complexed with vanadium, the kaempferol-3-neohesperidoside–VO (IV) exhibited a powerful hypoglycemic effect throughout the post-treatment period studied when compared to zero time [11]. When complexed with vanadium, quercetin, exhibited highly potent insulin-enhancing activity in STZ-diabetic mice with no effect on the blood glucose level of normal mice, in agreement with the results for kaempferitrin and kaempferol-3-neohesperidoside-VO complexes [11, 12].

The myricetin flavonol, when injected intravenously into rats, attenuated the increase of plasma glucose levels after an intravenous glucose challenge [13]. Also, the treatment of diabetic rats with myricetin resulted in the lowering of glycemia by 50% after 2 days. Myricetin, however, did not have an effect on the serum glucose levels in normal rats [14]. Another flavonol extensively studied in relation to its potential role in diabetes is quercetin. Vessal *et al.* [15] demonstrated that quercetin reduced the blood glucose level of diabetic rats in 8–10 days of treatment. In the same study, quercetin exerted no effect on the glucose tolerance curve either in normoglycemic or in STZ-diabetic rats. These results are in agreement with those described by Shetty *et al.* [16] for hypoglycemic effects of quercetin in diabetic rats.

Anti-hyperglycemic and hypoglycemic effects have been demonstrated for various flavonoids including chrysin and its derivatives, silymarin, isoquercetrin and rutin [17-19]. Long-term studies carried out with rutin orally administered to diabetic rats showed that it decreased the plasma glucose levels by up to 60% when compared to the control group. However, oral administration of rutin to normal rats did not show any significant effect on fasting plasma glucose levels [20]. Chronic treatment with hesperidin and naringin was found to lower the blood glucose level of db/db mice compared with the control group [21]. Intraperitoneal administration of prunin (naringenin 7-O-β-D-glucoside), a glycoside from naringenin, produced a significant hypoglycemic effect in diabetic rats [22].

Several studies have demonstrated that flavonoids are absorbed in the intestine and in some cases they compete with glucose in certain absorption mechanisms. A reduction in the intestinal absorption of glucose constitutes a possible means of controlling diabetic hyperglycemia. *In vitro* studies have shown that a soybean extract containing the isoflavones genistein and daidzein inhibits glucose absorption into the intestinal brush border membrane vesicles of rabbits [23]. Naringenin, a flavonoid present in citrus fruits and juices, inhibited glucose absorption in the intestine. Naringenin showed strong inhibitory action in rat everted intestinal sleeves in a competitive manner. In the same study, the authors observed that naringenin reduced glucose uptake in the intestinal brush border membrane vesicles of diabetic rats to a level similar to that of normal rats [24]. (-)Epicatechin gallate [25], myricetin, quercetin, apigenin, (-) epigallocatechin gallate, and (-)-epigallocatechin demonstrated a marked reduction in glucose absorption, when compared with the control, by competitive inhibition of sodium-dependent glucose transporter-1. The non-glycosylated polyphenols have been shown to reduce glucose absorption under sodium-dependent conditions *in vivo* and *in vitro* in animal tissues [26, 27].

Besides reducing glucose absorption, another possible mechanism to control blood glucose levels is the inhibition of α-glucosidase activity in the intestine. Cyanidin-3-α-O-rhamnoside and pelargonidin-3-α-O-rhamnoside, two anthocyanins, have been observed to exhibit inhibitory effects on glucose absorption and on α-glucosidase activity *in vitro* by CaCo2 cells [28]. Inhibitory effects on α-glucosidase activity were demonstrated when luteolin, kaempferol, chrysin and galangin were used both *in vitro* and *in vivo* to study the potential role in the absorption and metabolism of carbohydrates [29]. The α-glucosidase inhibitory activity of flavonoids was confirmed in a study by Kim *et al.* [30], where it was shown that luteolin, amentoflavone, luteolin 7-O-glucoside and daidzein were the strongest inhibitors of the compounds tested.

Under physiological conditions, the reabsorption of glucose from renal filtrate to plasma is determined primarily by sodium-coupled glucose transporters located on the luminal membrane of the proximal tubule of the kidney. This represents important contribution to glucose homeostasis. Glucose present in urine is one of the symptoms of diabetic patients and can cause serious complications such as nephropathy. To revert this renal complication, some flavonoids have been studied due to their effect on renal glucose reabsorption and excretion. Human treatment with sylimarin can decrease glucosuria and glycemia after four months of daily ingestion of the flavonoid [18]. Naringenin has been shown to inhibit the glucose reabsorption in renal brush border membrane vesicles when compared to normal rats, while naringin was not found to have an effect [24]. In a study on quercetin, diabetic and normal rats were submitted to a long-term treatment with quercetin to evaluate urinary parameters. The quercetin-fed diabetic group showed an improvement in the polyurea state and the excretion of urine was lower throughout the study period when compared with the control groups. In addition, a quercetin-fed diabetic group demonstrated an improvement in the amount of sugar excreted in urine [16]. For kaempferitrin, no alteration in urinary glucose levels was detected in the urine of normal and diabetic rats collected for 3 h after the oral treatment [31]. Recently, flavonoids have been reported to affect renal advanced glycation end-products and protein expression, which are involved in diabetic nephropathy, for example, puerarin [32]. (-)-Epigallocatechin 3-O-gallate administration over a 50 day-period to diabetic rats has shown suppressed hyperglycemia, proteinuria and reduced renal advanced glycation end-product accumulation and its related protein expression in the kidney and pathological conditions associated with nephropathy [33]. Green tea flavonoids can attenuate urinary protein excretion and the morphological changes particular to diabetic nephropathy after long-term treatment. Also hyperglycemia, as assessed in terms of blood glucose and glycosylated protein levels, can be improved by administration of green tea flavonoids in diabetic rats [34].

MOLECULAR MECHANISM OF FLAVONOIDS COMPARED WITH INSULIN SIGNAL TRANSDUCTION

Insulin is the most important hormone in the regulation of blood glucose concentrations and is essential in the postprandial state. When blood sugar concentrations rise, insulin

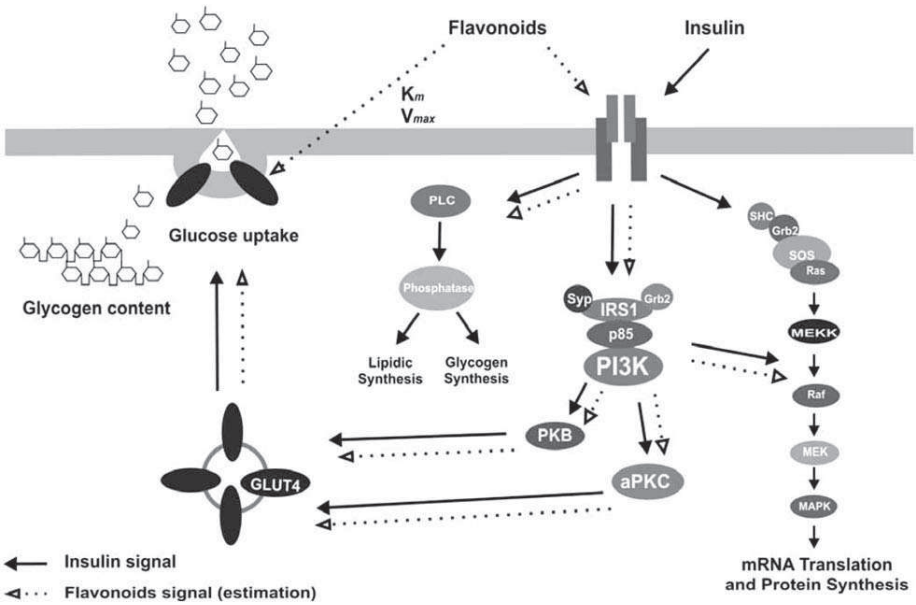

Fig. (1). Insulin binds to the insulin receptor (IR), thereby activating the intrinsic kinase activity in the β-subunit, which results in autophosphorylation and recruitment of substrates, such as insulin receptor substrate (IRS1-4) proteins, Cbl and SHC. Phosphorylated IRS proteins provide docking sites for proteins with Src Homology 2 (SH2) domains. Many of these proteins are adaptor molecules such as the regulatory subunit of PI3K, or the adaptor molecule Grb2, which associates with SOS to activate the Ras–MAPK pathway. The PI3K enzyme consists of regulatory (p85) and catalytic (p110) subunits which catalyze the formation of the lipid second messenger PIP3 in the cell, an allosteric activator of phosphoinositide-dependent kinase (PDKs). Targets of PDK include PKB/Akt and the atypical protein kinase C (PKC) isoforms. Together with PI3K, activated PKB/Akt and atypical PKCs are involved in the insulin-stimulated GLUT4 translocation, glucose uptake and glycogen synthesis.

is secreted into the blood stream by β-cells of the endocrine pancreas and glucose is the primary stimulus for insulin secretion [35]. Initially, glucose enters β-cells through the high capacity glucose transporter type 2 (GLUT 2) and is phosphorylated by glucokinase. The generation of ATP from glycolysis increases the intracellular ATP/ADP ratio [36]. ATP binds to ATP-dependent K^+-channels on the β-cell membranes closing these channels and depolarizing the cells. The depolarization activates voltage-sensitive calcium channels causing a calcium influx triggering insulin secretion [37].

Researchers have proposed flavonoids as potential antidiabetic agents since they exert multiple actions on the synthesis and release of insulin from β-cells. The supplementation of genistein increases the plasma insulin of the STZ-diabetic rats [8]. *In vitro* studies have shown that genistein can increase insulin secretion from mouse pancreatic islets in the presence of glucose. Consistent with this effect, genistein increases intracellular cAMP, probably by enhancing adenylate clyclase activity and activating protein kinase A (PKA) by a mechanism that does not involve protein tyrosine kinase **(**PTK). These findings demonstrate that genistein directly acts on pancreatic β-cells, leading to activation of the cAMP/PKA signaling cascade to exert an insulinotropic effect [38].

Oral administration of rutin in the long-term treatment of diabetic rats has been found to significantly increase plasma insulin and C-peptide levels. A histopathological study of the pancreas revealed the protective role of rutin resulting in β-cell proliferation [39]. An increase in the number of pancreatic islets has also been observed in both normoglycemic and diabetic rats treated with quercetin. This effect may be due to increased DNA replication of β-cells [15, 40]. Jayaprakasam *et al.* [41] characterized the effect of cyanidin-3-glucoside and delphinidin-3-glucoside as one of the most effective insulin secretagogues of the anthocyanins and anthocyanidins tested. Also, pelargonidin-3-galactoside and its aglycone, pelargonidin, can cause a significant increase in insulin secretion in the presence of glucose.

Once insulin is released by β-cells the molecular signaling is mediated by a complex mechanism of action. In the presence of hormones, the activated insulin receptor phosphorylates the insulin receptor substrate proteins (IRS proteins), which are linked to the activation of signaling pathways: the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B (PKB) pathway, which is responsible for most of the metabolic actions of insulin; and the Ras–mitogen-activated protein kinase (MAPK) pathway, which regulates expression of some genes and cooperates with the PI3K pathway to control cell growth and differentiation [42]. Alternatively, through specific phospholipase C activity, a second messenger (inositol phosphate glycan/IPG) can be produced which activates the protein phosphatases which in turn regulate glucose and lipid metabolism [43] (Fig. (**1**)).

Cellular glucose transport is mediated through solute carriers referred to as the family of facilitative glucose transporters (GLUTs), each one with different tissue distributions,

kinetic properties and sugar specificity. The GLUT-1 transporter is ubiquitously expressed and responsible for basal glucose uptake. The GLUT-2 isoform is primarily expressed in β-cells and in the liver, and has a relatively low affinity (high K_m) for glucose that in combination with hexokinase serves as part of the glucose sensor. GLUT-3 has the highest affinity (lowest K_m) and is expressed during fetal development and in adult neurons. Similar to the tightly controlled distribution and functionality of the other GLUT family members, GLUT-4 is predominantly restricted to fat and muscle and is responsible for insulin-stimulated glucose uptake. This process occurs through a complex and, as yet, incompletely defined signaling pathway involving the insulin receptor tyrosine kinase. The primary effect is to promote the transport of GLUT-4 from intracellular storage sites to the plasma membrane. In the basal state, GLUT-4 is localized in intracellular vesicles, while in the presence of insulin GLUT-4 is immunolocalized in the plasma membrane of fat, skeletal and cardiac muscle. The rate-limiting step at which insulin stimulates uptake of glucose in muscle and fat is the translocation of GLUT-4 transporters to the plasma membrane [43, 44].

This complex system of insulin-stimulated whole-body glucose utilization is impaired in people with diabetes. The molecular defects accounting for impaired glucose utilization are not fully understood but may involve defective GLUT4 translocation, glucose uptake and aberrant insulin signal transduction. In this context, several naturally occurring polyphenols have been shown to affect glucose transport and insulin-receptor function, both of which play essential roles in diabetes.

In vitro experiments with the rat diaphragm have shown that luteolin 5-rutinoside can increase glucose uptake and insulin secretion suggesting that this flavonoid can act as an anti-hyperglycemic and hypoglycemic agent [45]. In rat soleus muscle, kaempferitrin stimulated significantly the glucose uptake compared to the control. Kaempferitrin reportedly has no effect on protein synthesis in muscle from normal and diabetic rats treated with this compound. Considering these results, it has been proposed that kaempferitrin acts as an insulin mimetic flavonoid [31].

Rat adipocytes incubated in the presence of myricetin have shown an increased rate of both D-glucose and D-3-O-methyl-glucose uptake without affecting insulin receptor autophosphorylation, tyrosine kinase activity of the receptor or glucose transporter translocation to the plasma membrane. It has been demonstrated that the stimulatory effect of myricetin on glucose uptake involves an increase in glucose transporter V_{max} values [14, 46]. This stimulatory effect of myricetin on glucose uptake has also been observed in the soleus muscle of diabetic rats [13]. Liu *et al.* [47] demonstrated that GLUT4 mRNA and protein levels in the soleus muscle of diabetic rats were lower in comparison to the normal control rats. Additionally, chronic treatment of diabetic rats with myricetin can result in an elevation of GLUT4 gene expression and protein levels.

Hesperidin or naringin treatments have resulted in a significant reduction in the hepatic GLUT2 expression in db/db mice, while the expression of adipocyte GLUT4 level increased [48]. Puerarin, an isoflavone, in the soleus muscle of diabetic rats also enhanced the uptake of glucose. Moreover, the mRNA and GLUT4 transporter protein in soleus muscle were observed to increase after repeated administration of puerarin in diabetic rats. Probably, one of the mechanisms of puerarin in glucose uptake in muscle is mediated by a GLUT4 gene expression increase [6, 7].

The hypoglycemic effects of green tea catechins have been confirmed both *in vivo* and *in vitro*. Green tea supplementation in chronic treatment stimulated the basal and insulin-stimulated glucose uptake in adipocytes as well as GLUT4 content [49]. The effects of epigallocatechin gallate and (-) epicatechin have also been demonstrated *in vitro* [50, 51].

The antihyperglycemic effect of procyanidins has been studied in two insulin-sensitive cell lines. Procyanidin treatment caused an increase in glucose uptake in cell lines, L6E9 myotubes and 3T3-L1 adipocytes. Similarly to the action of insulin, the effect of procyanidins on glucose uptake was sensitive to wortmannin, an inhibitor of PI3K, and to SB203580, an inhibitor of p38MAPK. Procyanidins also stimulated the GLUT4 translocation to the plasma membrane suggesting that they mimic and/or influence the insulin effect by directly acting on specific components of the insulin-signaling transduction pathway [5]. Similarly, it has been demonstrated for kaempferol 3-neohesperidoside that the stimulatory effect on glucose uptake in muscle is *via* the PI3K and PKC pathways and is, at least in part, independent of the MEK pathway and the synthesis of new glucose transporters [52] (Fig. (**1**)).

In contrast with several reports regarding the stimulatory effect of flavonoids on insulin signal transduction, GLUT4 translocation and glucose transport, some natural compounds act negatively on these pathways. For example, naringenin can inhibit insulin-stimulated glucose uptake in a dose-dependent manner in adipocytes. Naringenin has been reported not to alter the phosphotyrosine status of the insulin receptor, IRS proteins, or PI3K, however, it was found to inhibit the phosphorylation of the downstream signaling molecule Akt. In an *in vitro* PI3K assay, naringenin, like wortmannin, blocked the production of PIP3 by immunoprecipitated PI3K [53]. Also, genistein inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes without affecting insulin-induced tyrosine kinase activity of the insulin receptor or activation of PKB. These results suggest that genistein can interfere with the insulin-induced glucose uptake directly and not by inhibiting GLUT4 translocation. This is in agreement with previous reports in the literature [54, 55].

In the same way, the flavonoids catechin-gallate, quercetin and myricetin can inhibit insulin-stimulated methylglucose uptake in rat adipocytes [56]. Moreover, evidence points to the fact that quercetin, myricetin and catechin-gallate inhibit glucose uptake due to a direct interaction with GLUT4, acting as competitive blockers of glucose transport [56, 57]. Interestingly, genistein, quercetin, apigenin and kaempferol have also been described as potent tyrosine kinase inhibitors [58].

The effect of insulin either on intermediate metabolism regulation or as growth factor is a consequence of the hor-

mone binding to the specific receptor at the plasma membrane in the target tissues of insulin. Post-receptor signal transduction can culminate with activated anabolic pathways as well as cell proliferation. After entering the hepatocytes or muscle cells, glucose is immediately phosphorylated by glucokinase (hexokinase IV) or hexokinase I and II in glucose-6-P. From glucose-6-phosphate, the glucose flux is directed to glycogen synthesis, glycolysis or to triglyceride synthesis [59]. Insulin inhibits gluconeogenesis and glycogenolysis through a phosphorylation mechanism and regulates the expression of genes encoding hepatic and muscular enzymes. This hormone inhibits the transcription of the gene encoding phosphoenolpyruvate carboxykinase, the rate-limiting step in gluconeogenesis [60]. Insulin also decreases transcription of the genes encoding fructose-1,6-biphosphatase and glucose-6-phosphatase (G-6-Pase), and increases transcription of glycolytic enzymes such as glucokinase and pyruvate kinase, and lipogenic enzymes such as fatty acid synthase and acetyl-CoA carboxylase [61, 62].

Glucose-6-phosphatase catalyses the dephosphorylation of glucose-6-phosphate in the liver and represents the ultimate step prior to the release of free glucose into the hepatic veins, be it derived from glycogenolysis or gluconeogenesis. As only the liver and the kidneys express glucose-6-phosphatase, only in these tissues can gluconeogenesis result in the release of free glucose to the blood stream [59].

Insulin acts directly in the accumulation and breakdown of glycogen in the liver and in the skeletal muscles. The hormone activates glycogen synthase by promoting its dephosphorylation, through the inhibition of kinases such as PKB or glycogen synthase kinase-3 (GSK-3) [63] and activation of protein phosphatase 1 (PP1) leading to increased glycogen synthesis kinase 3. On the other hand, insulin inhibits glycogenolysis, by dephosphorylating glycogen phosphorylase, through the activation of protein phosphatases [64, 65] (Fig. (**1**)).

In this regard, the supplementation of genistein to diabetic and normal rats was found to increase the glucokinase level in the diabetic rats. A significant reduction in G-6-Pase was observed in the groups treated with genistein [8]. The genistein and daidzein supplementation of diabetic (db/db) and non-diabetic mice was found to elevate hepatic glucokinase activity, while it suppressed hepatic G-6-Pase and phosphoenolpyruvate carboxykinase activity in db/db mice. Genistein and daidzein supplements enhanced hepatic glycogen in diabetic treated db/db mice when compared with diabetic db/db group and normal db/+ group [9].

It has recently been reported that epigallocatechin 3-gallate mimics the effects of insulin on the gene expression reduction of phosphoenolpyruvate carboxykinase and G-6-Pase in the mouse liver [66]. Like insulin, epigallocatechin 3-gallate increases tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1), mitogen-activated protein kinase, p70s6k, and PI3K activity, and reduces phosphoenolpyruvate carboxykinase gene expression mediated by PI3K [67]. Furthermore, epigallocatechin 3-gallate upregulates glucokinase mRNA expression in the liver of db/db mice [68].

Hesperidin and naringin supplemented groups can increase hepatic glucokinase activity and glycogen concentration. Naringin has also been observed to markedly lower the activity of hepatic G-6-Pase and phosphoenolpyruvate carboxykinase. The results suggested that hesperidin and naringin improves hyperglycemia by regulating the activity of the hepatic enzymes involved in glycolysis and gluconeogenesis [21]. Hesperidin and naringin both significantly increase the glucokinase mRNA, while naringin also lowers the mRNA expression of phosphoenolpyruvate carboxykinase and G-6-Pase in the liver [48].

In another study, oral administration of rutin to diabetic rats resulted in a decrease in plasma glucose and increase in insulin levels, and restored the glycogen content and hexokinase activity. The activity of enzymes such as G-6-Pase and fructose-1,6-bisphosphatase significantly decreased in the liver and muscles of rutin-treated diabetic rats [39].

Intraperitoneal quercetin treatment has been found to increase hexokinase and glucokinase activity in diabetic rats without effecting normal rats [15]. Also, quercetin has demonstrated a potent inhibitory effect on both glycogen phosphorylase *a* (phosphorylated, active) and *b* (unphosphorylated, inactive) in isolated muscle [69]. Also, quercetin and other flavonols inhibited the rat liver G-6-Pase activity. The highest inhibitory activity was shown by quercetin 3-*O*-α-(2"-galloyl)rhamnoside and kaempferol 3-*O*-α-(2"-galloyl) rhamnoside. Quercetin 3-*O*- α-(2"-galloyl)rhamnoside and kaempferol 3-*O*- α-(2"-galloyl)rhamnoside exhibited the lowest IC_{50} of all the flavonoids assayed. Quercetin 3-*O*- α-(2"-galloyl)rhamnoside increased the K_m for glucose-6-phosphate without changes in the V_{max} and strongly inhibited the neoglucogenic capacity of rat liver. The G-6-Pase inhibition by quercetin 3-*O*- α-(2"-galloyl)rhamnoside might explain the decrease in the liver neoglucogenesis and, in turn, the reduction in glucose levels observed in diabetic patients [70, 71].

Diabetic and normal rats treated with myricetin have shown reduced hyperglycemia after 2 days of treatment. The treatment increased the hepatic glycogen and glucose-6-phosphate levels in diabetic rats. It also increased hepatic glycogen synthase I activity without having any effect on either total glycogen synthase or hepatic phosphorylase *a* activity, however, it lowered phosphorylase *a* activity in the muscle [14]. As with myricetin, catechin can cause an increase in hepatic glycogen and in glucose uptake in rats after *in vivo* treatment. In the same way, glycogen synthase activity increases significantly whereas glycogen phosphorylase decreases, which is consistent with glycogen storage in the liver [72].

There has been considerable scientific progress over the past few years in unraveling of the effect and mechanism of action of flavonoids. The major potential benefits of flavonoids reported over the past 15-20 years discussed in this review clearly demonstrate that these exogenous substances represent an unparalleled source of molecular diversity in relation to the drug discovery process. This is of great importance given that the molecular mechanism of action of insulin is well known. However, many gaps remain in our understanding of such processes, ranging from the absorption of

flavonoids in the enterocytes to cellular behavior changes. Furthermore, we still need to define the missing steps in the flavonoid-signaling network and elucidate the mechanism of cross-talk based on the complex mechanism of insulin action, in order to provide new insights into the potential role of flavonoids in diabetes treatment.

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ABBREVIATIONS

IDDM	=	Insulin-dependent diabetes mellitus
NIDDM	=	Noninsulin-dependent diabetes mellitus
STZ	=	Streptozotocin
VO(IV)	=	Vanadium IV
GLUT	=	Glucose transporter
ATP	=	Adenosine 5'-triphosphate
ADP	=	Adenosine diphosphate
cAMP	=	3'-5'-cyclic adenosine monophosphate
PKA	=	Protein kinase A
PTK	=	Protein tyrosine kinase
DNA	=	Deoxyribonucleic acid
IRS	=	Insulin receptor substrate protein
PI3K	=	Phosphatidylinositol 3-kinase
PKB/AKT	=	Protein kinase B
MAPK	=	Ras–mitogen-activated protein kinase
mRNA	=	Messenger ribonucleic acid
p38MAPK	=	p38–mitogen-activated protein kinase
PKC	=	Protein kinase C
MEK	=	Mitogen-activated protein kinase kinase
PIP3	=	Phosphatidylinositol (3,4,5)-trisphosphate
G-6-Pase	=	Glucose-6-phosphatase
GSK-3	=	Glycogen synthase kinase 3
PP1	=	Protein phosphatase 1
p70s6k	=	p70-ribosomal S6 kinase
Cbl	=	Adaptor protein
SHC	=	Adaptor protein
PDK	=	Phosphoinositide-dependent kinase

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5 DISCUSSÃO

Em função da utilização popular da *Averrhoa carambola* no tratamento da diabetes e a presença de flavonóides, um dos objetivos deste trabalho foi estudar o efeito do extrato bruto, frações acetato de etila e *n*-butanol obtidos das folhas da *Averrhoa carambola* na glicemia de ratos normais hiperglicêmicos e diabéticos. Os resultados demonstraram que ambos, tanto o extrato bruto quanto as frações acetato de etila e *n*-butanol reduziram significativamente a glicemia de ratos normais hiperglicêmicos sem apresentar efeito em ratos diabéticos. Além disso, o efeito da fração acetato de etila foi comparável ao promovido pela tolbutamida, um agente sulfoniluréia, e pela insulina lispro. Esses resultados, presentes no item 4.1 comprovam os efeitos anti-hiperglicêmicos descritos para esta planta na literatura (MARTHA et al., 2000; PROVASI et al., 2001, 2005; DAMASCENO et al., 2002; GONÇALVEZ et al., 2005). Ainda, a *Averrhoa carambola* pode exercer os efeitos através de mecanismos que envolvam a liberação de insulina das células β pancreáticas por um ou mais componentes do extrato e/ou frações 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 (DETIMARY et al., 1995; RORSMAN, 1997; NÉMETH et al., 2003).

Da fração acetato de etila foram isoladas duas flavonas C-heterosídeos, apigenina-6-C-β-L-fucopiranosídeo (composto 1) e apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo (composto 2) (ARAHO et al., 2005). Conforme os resultados apresentados (item 4.1), ambos os flavonóides possuem importantes ações anti-hiperglicêmicas sugerindo que efeitos insulino-miméticos em tecidos alvos da insulina como músculo e tecido adiposo sejam um dos mecanismos envolvidos na regulação da glicemia pelos flavonóides 1 e 2. Como a fração acetato de etila não apresentou efeito em ratos diabéticos severos, ambos os flavonóides foram efetivos em ratos hiperglicêmicos e um deles, flavonóide 2 em animais diabéticos moderados, podemos sugerir ainda, que além da ação insulino-mimética, estes compostos exerçam os efeitos através do estímulo da secreção de insulina.

Conforme exposto nos itens 4.2 e 4.3, ambos os flavonóides da *Averrhoa carambola* potencializaram a secreção de insulina induzida por glicose após tratamento por via oral. Relatos da literatura descrevem a atividade de plantas ricas em flavonóides e de compostos isolados na captação e metabolização da glicose, seja atuando como insulino-miméticos, secretagogos de insulina ou ambos como, por exemplo, a *Gentiana olivieri* e o composto isolado, isoorientina, além de relatos para a luteolina, apigenina e os heterosídeos (ZARZUELO et al., 1996; SEZIK et al., 2005; PANDA; KAR, 2007; LI et al., 2007). Juntamente com os dados da literatura, os resultados descritos nos itens 4.2 e 4.3 reforçam a hipótese de que os flavonóides das folhas da *A. carambola* atuem de maneira dualística, como secretagogos de insulina e também como agentes insulino-miméticos em tecidos responsivos ao hormônio.

Estudos com os flavonóides canferitrina e canferol 3-neohesperidosídeo demonstraram que estes compostos atuam como agentes insulino-miméticos aumentando a captação de glicose e o conteúdo de glicogênio em um tecido alvo da ação da insulina, o músculo (JORGE et al., 2004; ZANATTA et al., 2008). Destes compostos, o canferol 3-neohesperidosídeo, isolado da *Cyathea phalerata*, foi o que apresentou melhores resultados. Em função disto, este composto foi estudado quanto ao mecanismo de ação na regulação da glicemia e foi demonstrado que o efeito estimulatório da captação de glicose em músculo sóleo ocorreu através de ações mediadas via transdução de sinal da insulina (ZANATTA et al., 2008).

Uma vez verificado o efeito insulino-mimético de compostos como a canferitrina e o canferol 3-neohesperidosídeo na captação de glicose e, além destes, os dois flavonóides isolados da *Averrhoa carambola* (compostos 1 e 2) no conteúdo de glicogênio, foi considerado de grande importância o estudo da ação do canferol 3-neohesperidosídeo e da apigenina-6-C-β-L-

fucopiranosídeo (composto 1) na síntese de glicogênio em um tecido alvo da insulina, bem como o esclarecimento do mecanismo de ação envolvido.

No presente trabalho demonstramos que o canferol 3-neohesperidosídeo aumentou significativamente a síntese de glicogênio em músculo sóleo de ratos. O canferol-3-neohesperidosídeo, mesmo com uma concentração mais elevada, promoveu um estímulo maior da síntese de glicogênio comparado à insulina, o hormônio classicamente responsável pela regulação do metabolismo do glicogênio. Este efeito corrobora o papel insulino-mimético proposto anteriormente para este flavonóide e se relaciona diretamente com a redução da glicemia e com o aumento da captação da glicose no músculo (CAZAROLLI et al., 2006; ZANATTA et al., 2008).

Além do canferol 3-neohesperidosídeo, os estudos com a apigenina-6-C-β-L-fucopiranosídeo (composto 1) revelaram um aumento significativo da síntese de glicogênio em músculo sóleo de ratos. Este flavonóide apresentou um efeito estimulatório percentualmente tão eficaz quanto à insulina reforçando a hipótese de ação insulino-mimética proposta anteriormente para este composto. Assim como o demonstrado neste trabalho para o canferol 3-neohesperidosídeo e para a apigenina-6-C-β-L-fucopiranosídeo, relatos da literatura também descrevem ações estimulatórias na síntese de glicogênio e na regulação da glicogênio sintase para os flavonóides miricetina, catequina e procianidinas (VALSA et al., 1997; ONG; KHOO, 2000; PINENT et al., 2005; LIU et al., 2005).

Uma vez constatado o efeito estimulatório do canferol 3-neohesperidosídeo e da apigenina-6-C-β-L-fucopiranosídeo (composto 1) na síntese de glicogênio no músculo, foi estudado o mecanismo de ação pelo qual estes compostos estimulam a incorporação de ^{14}C-D-glicose em glicogênio. Foram utilizados inibidores de algumas proteínas cinases que estão envolvidas no processo de transdução de sinal da insulina, uma vez que os efeitos dos flavonóides se assemelharam aos da insulina. As concentrações de cada inibidor foram escolhidas em função de trabalhos anteriores que resultaram na inibição da captação de glicose, síntese de glicogênio e/ou da atividade da glicogênio sintase em diferentes tecidos (RODRÍGUEZ-GIL et al., 1993; CARLSEN et al., 1997; SYED; KHANDELWAL, 2000; ZANATTA et al., 2008).

Uma vez que o efeito clássico da insulina está relacionado com a fosforilação e ativação da PI3K, PKB e fosforilação e inativação da GSK-3 e que os efeitos estimulatórios do canferol 3-neohesperidosídeo e da apigenina-6-C-β-L-fucopiranosídeo foram completamente inibidos pela wortmanina, podemos supor que o efeito destes flavonóides na síntese de glicogênio esteja relacionado com o aumento da fosforilação e/ou atividade dessas proteínas cinases. No entanto, para que se confirme o modo pelo qual estes flavonóides atuam sobre a via da PI3K são necessários estudos adicionais de fosforilação e expressão de proteínas que confirmem esta hipótese.

Até o momento, os resultados do presente trabalho sugerem que ambos os compostos exercem os efeitos através da via da PI3K e que atuam nas proteínas de sinalização pertencentes a esta via e envolvidas na síntese de glicogênio. Em função disto o cloreto de lítio, inibidor da GSK-3, foi utilizado para estudar os efeitos do canferol 3-neohesperidosídeo e da apigenina-6-C-β-L-fucopiranosídeo na ação da GSK-3.

Estudos com o cloreto de lítio demonstraram que o lítio estimulou a atividade da glicogênio sintase e a síntese de glicogênio em miotubos L6 e em hepatócitos similarmente à resposta da insulina. Além disso, os efeitos do lítio foram aditivos aos da insulina em diversas concentrações testadas. Ao contrário do efeito da insulina que inibe a GSK-3 através da via dependente da PI3K-PKB, o lítio inibiu diretamente a GSK-3 sem alterar o estado de fosforilação da proteína cinase. Ainda, o lítio inibiu a atividade da GSK-3 em células que haviam sido estimuladas anteriormente por insulina, ou seja, cuja atividade da GSK-3 já estava inibida. Estes resultados sugerem que os efeitos inibitórios diretos sejam independentes da fosforilação da GSK-3 pelo lítio e aditivos aos efeitos inibitórios dependentes de fosforilação promovidos pela insulina nas células L6. Isto explicaria de certa forma, o efeito aditivo de ambos no estímulo da síntese de glicogênio. (RODRÍGUEZ-GIL et al, 1993; CHOI; SUNG, 2000).

Diferentemente do observado na presença da wortmanina, o canferol 3-neohesperidosídeo e a apigenina-6-C-β-L-fucopiranosídeo apresentaram efeitos diversos na presença do cloreto de lítio. O

sinergismo de ação apresentado pelo lítio e canferol 3-neohesperidosídeo é similar ao demonstrado para a insulina e o lítio, sugerindo que este flavonóide atue de forma semelhante à insulina, ou seja, influenciando o estado de fosforilação da GSK-3. Por outro lado, a ausência de efeito aditivo entre o lítio e a apigenina-6-C-β-L-fucopiranosídeo sugere que este composto atue de forma direta na GSK-3, independente de ações que alterem o estado de fosforilação da proteína cinase e mais provavelmente através de interações com sítios de ligação na GSK-3, como o sítio de ligação do magnésio (RYVES; HARWOOD, 2001).

Além de promover a supressão da fosforilação da glicogênio sintase, a insulina também promove a desfosforilação da enzima. Diversos estudos demonstram que a inativação da GSK-3 não é suficiente para causar a ativação completa da glicogênio sintase e que mecanismos adicionais como a desfosforilação da enzima por fosfatases como a proteína fosfatase do tipo 1 (PP1) estão envolvidos. Um dos mecanismos propostos para a ativação da PP1 pela insulina envolve a fosforilação da enzima através da cascata da MAPK, levando à ativação da $p90^{rsk}$ e esta ativada, fosforila e aumenta a atividade da PP1G (SRIVASTAVA; PANDEY, 1998; RAGOLIA; BEGUM, 1998; TAHA; KLIP, 1999; BRADY; SALTIEL, 2001).

Na presença de ambos os inibidores, caliculina A, inibidor de PP1 e PD98059, inibidor de MEK, o efeito estimulatório dos flavonóides canferol 3-neohesperidosídeo e apigenina-6-C-β-L-fucopiranosídeo foi completamente abolido. Estes dados permitem inferir o envolvimento da via da MAPK e da PP1 nos efeitos estimulatórios dos flavonóides na síntese de glicogênio em músculo sóleo.

Similarmente, as ações da insulina tanto na atividade da PP1 quanto na glicogênio sintase foram completamente abolidas na presença de caliculina A em diferentes tecidos (DENT et al., 1990; BEGUM, 1995; RAGOLIA; BEGUM, 1998; SYED; KHANDELWAL, 2000). A ativação da PP1 através da fosforilação do sítio 1 da enzima é catalisado pela proteína $p90^{rsk}$ que é fosforilada e ativada através da via da MAPK após o estímulo da insulina (DENT et al., 1990; LAVOINNE et al., 1991; RAGOLIA; BEGUM, 1998). Esses resultados foram corroborados quando análogos de GTP, que estimulam a ativação da via Ras-MAPK mimetizaram os efeitos da insulina na ativação da PP1 em adipócitos isolados. (BEGUM, 1995). Adicionalmente, o envolvimento da via Ras-MAPK na síntese de glicogênio estimulada por insulina também foi comprovado em hepatócitos, uma vez que o tratamento destas células com PD98059 aboliu os efeitos da insulina (CARLSEN et al., 1997).

Entretanto, relatos na literatura questionam o papel da via da Ras-MAPK-$p90^{rsk}$ na síntese de glicogênio estimulada por insulina. Estes relatos contraditórios podem ser devidos às diferenças nas condições experimentais dos estudos sugerindo que esta via não seja essencial para a ativação inicial da atividade da glicogênio sintase nos tecidos periféricos, mas que talvez ela possa ser importante na manutenção do estímulo da insulina (LAZAR et al., 1995; CROSS et al., 1995; AZPIAZU et al., 1996; HUREL et al., 1996; PEAK et al., 1998; SYED; KHANDELWAL, 2000). Estudos complementares devem ser realizados a fim de esclarecer a importância da via da MAPK na regulação do metabolismo do glicogênio.

Todos os resultados discutidos até o momento comprovam a hipótese de que os flavonóides canferol 3-neohesperidosídeo e apigenina-6-C-β-L-fucopiranosídeo apresentam ações insulino-miméticas. O efeito hipoglicemiante destes flavonóides seria decorrente do aumento da captação de glicose pelos tecidos periféricos, principalmente músculos e a conseqüente utilização da glicose como substrato para síntese de glicogênio. Esta ação seria regulada através do envolvimento de proteínas cinase das vias de sinalização clássicas da insulina, PI3K e MAPK, como discutido e proposto por Cazarolli et al., 2009 (itens 4.2 e 4.4).

Os trabalhos desenvolvidos com o canferol 3-neohesperidosídeo na síntese de glicogênio e com a apigenina-6-C-β-L-fucopiranosídeo na glicemia, na liberação de insulina e na síntese de glicogênio demonstraram resultados relevantes quanto ao potencial hipoglicemiante e anti-hiperglicêmico destes flavonóides e os prováveis mecanismos envolvidos nessa ação. Diante disto, da similaridade estrutural entre os flavonóides, dos efeitos da apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo (composto 2) na glicemia, no conteúdo de glicogênio e na

liberação de insulina, estudamos a ação deste composto na captação de glicose em músculo sóleo e o mecanismo de ação envolvido (item 4.3).

A apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo (composto 2) estimulou a captação de glicose em músculo sóleo sendo tão efetivo quanto a insulina. Considerando que não houve efeito aditivo quando ambos, insulina e flavonóide foram estudados simultaneamente, podemos inferir que o composto 2 melhora a utilização de glicose nos tecidos periféricos através do aumento da captação de glicose, atuando provavelmente na mesma via de sinalização da insulina. Como descrito para o canferol 3-neohesperidosídeo (ZANATTA et al., 2008), o efeito estimulatório do composto 2 foi abolido na presença de inibidores da via da PI3K e PKC (wortmanina e RO318220) sugerindo que o composto 2 estimule a captação de glicose no músculo ativando proteínas cinases que fazem parte do processo de sinalização da insulina.

A insulina estimula a atividade da proteína cinase ativada por mitógeno (MAPK). A ativação desta via leva à proliferação e diferenciação celular, mas não afeta as ações metabólicas da insulina (TAHA; KLIP, 1999; SALTIEL; KAHN, 2001). No entanto, alguns relatos mostram que a p38 MAPK está relacionada com a regulação do transporte de glicose no músculo esquelético (HO et al., 2004). O efeito estimulatório do composto 2 foi completamente abolido na presença do inibidor de MEK, PD98059. Isto sugere que a via da MAPK está implicada no processo de captação de glicose estimulado pelo composto 2 e que provavelmente este flavonóide regule a expressão dos transportadores de glicose no músculo.

Ao contrário do relatado anteriormente para o canferol 3-neohesperidosídeo (ZANATTA et al., 2008) a presença da cicloheximida, inibidor de síntese protéica, bloqueou completamente o efeito do composto 2 na captação de glicose o que sugere que a ação aguda na captação de glicose seja dependente da tradução do RNAm além da expressão dos transportadores de glicose no músculo.

Ainda, a inibição induzida pela colchicina na captação de glicose estimulada pelo composto 2 e por insulina mostra que o citoesqueleto celular é importante para as ações de ambos, insulina e composto 2 na captação de glicose. A insulina estimula a captação de glicose por aumentar a translocação das vesículas de armazenamento de GLUT4 dos compartimentos intracelulares para a membrana. Os microtúbulos do citoesqueleto possuem papel fundamental no trânsito de vesículas bem como na manutenção da estrutura e posicionamento de diversas organelas intracelulares. Estudos demonstraram a importância dos microtúbulos para a translocação das vesículas de GLUT4 estimulada por insulina, uma vez que a presença de agentes desestabilizadores de microtúbulos como a colchicina e o nocodazol dispersam as vesículas no compartimento citoplasmático e inibem a captação de glicose (FLETCHER et al., 2000; CHANG et al., 2004; HUANG et al., 2005; KANZAKI, 2006; CHEN et al., 2008). Diante dos resultados com a colchicina, o efeito estimulatório na captação de glicose com o composto 2 provavelmente envolva a organização e estabilidade do citoesqueleto, permitindo assim a migração e fusão das vesículas contendo GLUT4 para a membrana plasmática das células.

Baseado nos resultados obtidos neste trabalho podemos propor que os flavonóides aqui estudados modifiquem a captação e a metabolização da glicose atuando como agentes insulino-miméticos, aumentando a utilização da glicose pelos tecidos periféricos basicamente através da captação de glicose e síntese de glicogênio. Além disso, também podem exercer ações estimulando a secreção de insulina e aumentando a sensibilidade dos tecidos periféricos ao hormônio. A especificidade de ação destes compostos apresenta-se como ferramenta de estudos importante levando-se em consideração o desenvolvimento de novas moléculas que possam ser utilizadas como fármacos bem como o aumento da segurança na utilização de plantas medicinais no tratamento da diabetes.

6 CONCLUSÕES FINAIS

A partir dos resultados obtidos, podemos concluir que:

O extrato bruto, frações acetato de etila e *n*-butanol e os flavonóides apigenina-6-C-β-L-fucopiranosídeo e apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo reduziram a glicemia de ratos normais hiperglicêmicos após período agudo de tratamento.

O extrato bruto e a fração acetato de etila não alteraram a glicemia de ratos diabéticos após período agudo de tratamento, no entanto o composto apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo reduziu a glicemia de ratos diabéticos após tratamento por via oral.

Os flavonóides apigenina-6-C-β-L-fucopiranosídeo e apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo aumentaram significativamente o conteúdo de glicogênio em músculo sóleo e fígado de ratos normais hiperglicêmicos no tempo de tratamento estudado.

Ambos os flavonóides, apigenina-6-C-β-L-fucopiranosídeo e apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo potencializaram a secreção de insulina estimulada por glicose após tratamento agudo por via oral.

A comprovação do uso popular de plantas medicinais associado à pesquisa de substâncias obtidas destas plantas com ações que mimetizem ou potencializem as ações da insulina é de grande importância para o desenvolvimento de novos fármacos. Além disso, o esclarecimento das ações dos compostos destas plantas aumenta a segurança e a eficácia da utilização, especialmente na diabetes. Os estudos com a *Averrhoa carambola* comprovaram a utilização popular desta planta uma vez que os resultados dos tratamentos se relacionam com funções fisiologicamente importantes na regulação do metabolismo de carboidratos no organismo. Este estudo evidenciou a *Averrhoa carambola* como potencial adjuvante na terapia da diabetes e também como uma nova fonte e/ou ferramenta de estudos para a elucidação de mecanismos de ação, bem como, para protótipos para síntese de novas moléculas.

O canferol-3-neohesperidosídeo estimulou significativamente a síntese de glicogênio em músculo sóleo de ratos normais com baixa concentração e este estímulo foi maior que o apresentado pela insulina.

O efeito estimulatório do canferol-3-neohesperidosídeo na síntese de glicogênio no músculo sóleo depende da atividade da PI3K, da inibição da GSK-3 (via da ativação da síntese de glicogênio) e ainda do envolvimento da via da MAPK e da ativação da PP1.

A apigenina-6-C-β-L-fucopiranosídeo estimulou significativamente a síntese de glicogênio em músculo sóleo de ratos normais e o mecanismo de ação deste flavonóide envolve a via da PI3K-PBK-GSK3 bem como a via da MAPK-PP1.

A apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo estimulou a captação de glicose em músculo sóleo de ratos normais similar ao efeito da insulina apesar de uma dose maior do flavonóide ter sido utilizada.

O efeito estimulatório da apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo na captação de glicose depende da atividade da PI3K, PKC e MAPK. Ainda, a transcrição gênica, síntese, expressão e translocação dos transportadores de glicose estão relacionados com o efeito estimulatório deste flavonóide na captação de glicose em músculo sóleo.

A apigenina-6-C-(2”-O-α-L-ramnopiranosil)-β-L-fucopiranosídeo não potencia o efeito estimulatório da insulina na captação de glicose no músculo sóleo, sugerindo uma ação na mesma via de transdução de sinais da insulina.

Os resultados da ação de flavonóides na regulação da homeostasia da glicose, em estudos *in vivo* e *in vitro*, demonstram o potencial efeito insulino-mimético e/ou anti-hiperglicêmico destes compostos. Os mecanismos propostos para os flavonóides na estimulação da captação de glicose e da síntese de glicogênio parecem envolver a atividade de cinases e/ou fosfatases através de mecanismos pleiotrópicos da sinalização insulínica intracelular. Como proposto na figura 9 os

resultados deste estudo comprovam cientificamente uma via importante do mecanismo de ação dos flavonóides na glicemia. No entanto, o esclarecimento das vias reguladas e/ou envolvidas nas ações dos flavonóides na regulação do metabolismo de carboidratos requer maiores investigações. Como perspectivas deste trabalho, estudos de cinética do transporte de glicose bem como de expressão e regulação de proteínas cinases e fosfatases terão papel fundamental na complementação dos efeitos destes compostos como agentes insulino-miméticos e/ou anti-hiperglicêmicos.

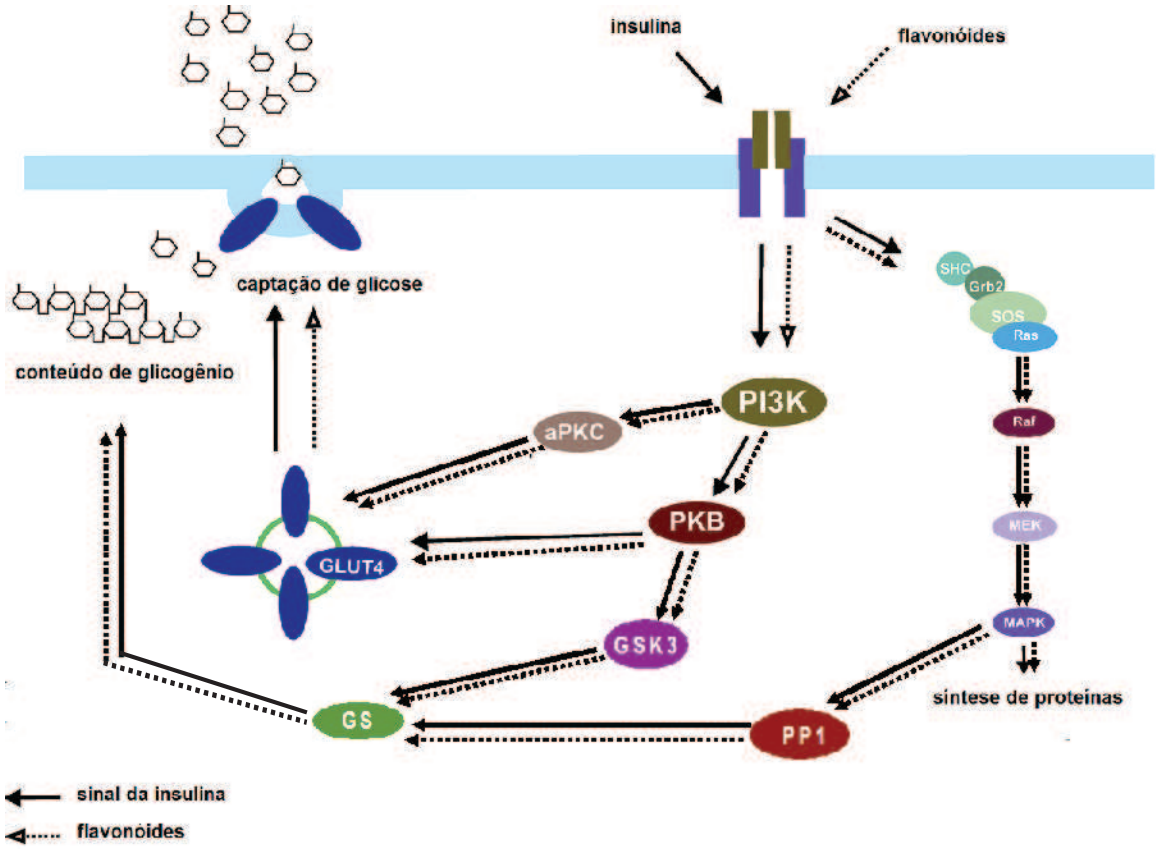

Figura 9. Modelo proposto para o mecanismo de ação geral dos flavonóides na transdução de sinais da insulina.

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8 ANEXOS

Resultado de Solicitação de Protocolo

Protocolo PP00007

Título
CARACTERIZAÇÃO DE COMPOSTOS NATURAIS E AVALIAÇÃO DA ATIVIDADE INSULINO-MIMÉTICA EM TECIDOS ALVOS DA INSULINA NO MODELO DE DIABETES EXPERIMENTAL QUIMICAMENTE INDUZIDO

Data de Entrada
20/10/2005

Resultado:
Aprovado

Data/Prazo
aprovado em 09/12/2005. Vigência de 09/12/2005 até 09/12/2007

Considerações

Ofício nº111/05/CEUA/PRPe

Da: Presidente da Comissão de Ética no Uso de Animais-CEUA

Ao(à): Prof(a) Dr(a)Fátima Regina Mena Barreto Silva.
Departamento de Bioquímica - CCB

Prezado(a) Professor(a),

Em relação ao Protocolo de Pesquisa sob sua responsabilidade, cadastrado sob o número PP00007/CEUA e intitulado: "Caracterização de compostos naturais e avaliação da atividade insulino-mimética em tecidos alvos da insulina no modelo de diabetes experimental quimicamente induzido", a Presidente da CEUA deliberou o seguinte:

- APROVADO por 2 (dois) anos, a contar desta data, por unanimidade, envolvendo o uso de 460 ratos Wistar machos, durante o período.

Por ocasião do término desse protocolo, DEVERÁ SER APRESENTADO RELATÓRIO detalhado relacionando o uso de animais no Projeto desenvolvido aos resultados obtidos, conforme formulário ON LINE CEUA.

Atenciosamente,

Profª Drª Fátima Regina Mena Barreto Silva
Presidente/CEUA/PRPe/UFSC

OBS.: Deverá ser assinado e retirado na secretaria da CEUA o "Certificado de Credenciamento" do referido protocolo para o mesmo ser CONSIDERADO APROVADO e vigente pela CEUA. Após, a retirada do Certificado de Credenciamento será enviado para o BC a aprovação do referido número de animais.

Relatório Final previsto para (90 dias após término da vigência do protocolo ou no momento da apresentação de um novo protocolo)
Data 09/03/2008

Data 09/12/2005

Resultado de Solicitação de Protocolo

Protocolo PP00146
Título
CARACTERIZAÇÃO DE COMPOSTOS NATURAIS E AVALIAÇÃO DA ATIVIDADE INSULINO-MIMÉTICA EM TECIDOS ALVOS DA INSULINA NO MODELO DE DIABETES EXPERIMENTAL QUIMICAMENTE INDUZIDO - Solicitação de prorrogação do protocolo CEUA PP007.
Data de Entrada
12/11/2007

Resultado:
Aprovado
Data/Prazo
19/03/2008

Considerações
Oficio nº 028/CEUA/PRPe/2008
Do: Presidente da Comissão de Ética no Uso de Animais-CEUA
Ao(à): Prof(a) Dr(a) Fátima Regina Mena Barreto Silva, Departamento de Bioquímica - CCB

Prezado(a) Professor(a),
Em relação ao protocolo de pesquisa sob sua responsabilidade a CEUA deliberou:

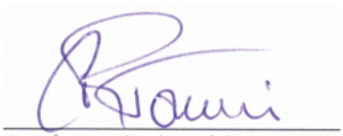
- APROVADO, por 2 (dois) anos, para a utilização de 480 ratos (Rattus novergicus). Apesar de um parecer desfavorável e outro pedindo pequenos esclarecimentos, os membros presentes entenderam que o protocolo estava adequado e com mérito.

Por ocasião do término desse protocolo, DEVERÁ SER APRESENTADO RELATÓRIO detalhado relacionando o uso de animais no Projeto desenvolvido aos resultados obtidos, conforme formulário ON LINE CEUA.

Atenciosamente,

Relatório Final previsto para (90 dias após término da vigência do protocolo ou no momento da apresentação de um novo protocolo)
Data 19/06/2010

Data 25/03/2008



Prof. Assoc. Carlos Rogério Tonussi, D.Sc.
COMISSÃO DE ÉTICA NO USO DE ANIMAIS – PRPE – UFSC
PRESIDENTE

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