UNIVERSIDADE FEDERAL DE SERGIPE PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

POLLYANNA ALVES SECUNDO WHITE

PROPOSTA DE DUAS NOVAS TERAPÊUTICAS, EXTRATO AQUOSO DE C*hrysobalanus icaco* E RUTINA, EM MODELOS EXPERIMENTAIS DE OBESIDADE E RESISTÊNCIA À INSULINA

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Orientador: Prof. Dr. Márcio Roberto Viana Santos
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RESUMO

Proposta de duas novas terapêuticas, extrato aquoso de c*hrysobalanus icaco* e rutina, em modelos experimentais de obesidade e resistência à insulina, Pollyanna Alves Secundo White, Universidade Federal de Sergipe, 2015. A adiposidade é um grave fator de risco e está relacionada diretamente ao desenvolvimento de diversas doenças crônicas, em especial a obesidade e o diabetes tipo 2. Diversas pesquisas têm sido conduzidas a fim de desenvolver novas drogas anti-obesidade por meio de fontes à base de plantas, favorecendo a minimização de reações adversas normalmente associadas às drogas mais comumente utilizadas. Estudos prévios com o extrato aquoso da *Chrysobalanus icaco* (AECI) demonstraram redução dos níveis de glicemia e prevenção do ganho de peso e acúmulo de gordura no fígado de camundongos. O objetivo dessa tese foi de desenvolver meios de estudo para o desenvolvimento de novas terapêuticas e investigar o papel do AECI e da rutina nos respectivos modelos de obesidade e resistência à insulina. O primeiro capítulo trata da ação do AECI no perfil adiposo e glicêmico de camundongos obesos induzidos por dieta hiperlipídica. Para isso, 34 camundongos C57BL/6J machos foram aleatoriamente designados à dieta padrão ou à dieta hiperlipídica e posteriormente tratados com o AECI em duas concentrações, 0,35 mg/mL e 0,7 mg/mL. Consumo, eficiência energética, eficiência metabólica, peso corpóreo, peso dos coxins adiposos, lipídios séricos, excreção fecal lipídica, atividade locomotora, sensibilidade à insulina e tolerância à glicose foram avaliados ao final do experimento. Os resultados mostraram que o AECI em sua menor concentração promoveu o aumento da atividade locomotora ($p<0,01$) e massa muscular ($p<0,05$), redução da massa adiposa ($p<0,01$), dos níveis de triglicérides sanguíneos ($p<0.05$) e excreção fecal de lipídios ($p<0.01$), e, normalizou a sensibilidade dos tecidos à ação da insulina ($p<0.05$) e tolerância à glicose ($p<0.05$). Por outro lado, o AECI em sua maior concentração promoveu aumento do consumo ($p<0.0001$ SCA vs SC e $p<0.0001$ HFA2 vs. HFD) e da eficiência energética ($p<0.05$ SCA vs SC e $p<0.05$ HFA2 vs. HFD), não favorecendo, dessa forma, à perda de gordura corporal e homeostase glicêmica. Esses achados indicam que o AECI, em concentrações menores, pode prevenir o armazenamento de gordura ou favorecer o gasto energético, em parte, por meio do aumento da atividade locomotora, promovendo também normalização da sensibilidade à ação da insulina e tolerância à glicose. Esses efeitos podem estar ligados à atividade antioxidante do extrato e seu conteúdo polifenólico. O segundo capítulo trata do desenvolvimento de um modelo de células neuronais resistentes à ação da insulina TNF-α induzida e investigação da ação da rutina sobre esse modelo. Neste trabalho células N2a foram tratadas com TNF-α, rutina ou veículo por 30 minutos e em seguida estimuladas com insulina ou veículo por 15 minutos. Foi extraído o lisato de proteínas e quantificada a Akt fosforilada e total, assim como a IkBα total por meio de Western blot. Os resultados mostraram degradação da proteína IkBα em 64.2% ($p<0.05$) e redução significativa da fosforilação da Akt em 36.1% ($p<0.001$) após estimulação com TNF-α. A rutina, por sua vez, não foi capaz de prevenir a ativação da via NF-κB, entretanto tendeu a minimizar a atenuação da fosforilação da Akt induzida pelo TNF-α. Esses dados indicam que o TNF-α gerou um modelo de resistência insulínica mediada por inflamação em células N2a. Ademais, a rutina pode contribuir com a ativação da via Akt na minimização dos dados causados pelo TNF-α.

Descritores: obesidade; diabetes tipo 2; *Chrysobalanus icaco*; N2a; rutina.

ABSTRACT

Proposal of two new therapies, aqueous extract of *Chrysobalanus icaco* and rutin, on experimental models of obesity and insulin resistance, Pollyanna Alves Secundo White, Universidade Federal de Sergipe, 2015. Adiposity is a severe risk factor and it's directly related to the development of several chronic diseases, especially obesity and type 2 diabetes. Numerous trials have been conducted to find and develop new anti-obesity drugs through herbal sources to minimize adverse reactions associated with the present drugs used for obesity and type 2 diabetes treatment. Researches conducted with the aqueous extract of *Chrysobalanus icaco* L (AECI) have demonstrated decrease in blood glucose levels and prevention of weight gain and fat accumulation in mice liver. The aim of this thesis was to develop new study basis for the development of new therapies and investigate the role of AECI and rutin in the treatment of obesity and type 2 diabetes. The first chapter addresses the action of AECI on the adipose and glycemic profile of obese mice high-fat diet induced. To this end, a group of 34 male C57BL/6J mice were randomly assigned to standard chow or high-fat diet and and further treated with the AECI in two concentrations, 0,35 mg/mL e 0,7 mg/mL. Food intake, feed efficiency, metabolic efficiency, body weight, fat pads weight, serum lipid, fecal lipid excretion, locomotor activity and insulin and glucose sensitivity were evaluated at the end of the 14 weeks of experiment. Results showed that the AECI in the lower concentration increased locomotor activity ($p<0.01$) and lean mass ($p<0.05$), decreased fat mass gain ($p<0.01$), TG levels ($p<0.05$), and fecal lipid excretion ($p<0.01$), and normalized insulin ($p<0.05$) and glucose sensitivity ($p<0.05$). On the other hand, the AECI in the higher concentration increased food intake ($p <0.0001$ vs. SC SCA $p <0.0001$ vs. HFA2 HFD) and the feed efficiency ($p <0.05$ vs. SC SCA $p <0.05$ vs. HFA2 HFD), hindering the loss of the body fat and glucose homeostasis. These findings indicates that AECI in lower doses can prevent fat storage or enhance fat utilization, in part, due to the increase of locomotor activity, favoring the glucose homeostasis through the normalization of insulin sensitivity and glucose tolerance. These effects may be related to the antioxidant activity and polyphenol content of the extract. The second chapter addresses the development of a model of insulin resistance TNF-α induced in neuron cells. In this study N2a were treated for 30 minutes with TNF-α, rutin or vehicle and then stimulated with insulin for 15 minutes. The protein lysate was extracted and total and p-Akt were measured, as well as total IkBα through Western blot. Results showed degradation of total IkBα protein in 64.2% ($p<0.05$) and significant decrease on p-Akt of 36.1% ($p<0.001$). The rutin, on the other hand, wasn't able to prevent NF-κB activation, however, it tended to minimize the attenuation of Akt phosphorylation induced by the TNF- α. These findings indicates that TNF-α generated a N2a model of inflammation induced insulin resistance. Furthermore, rutin can contribute with the Akt activation reducing TNF- α damage in these cells.

Key words: obesity; type 2 diabetes; *Chrysobalanus icaco*; N2a; rutin.

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LISTA DE ABREVIAÇÕES

AECI	Extrato aquoso de *Chrysobalanus icaco* / Aqueous extract of *Chrysobalanus icaco*
AGs	Ácidos graxos
AgRP	Proteína relacionada agouti / Agouti-related protein
Akt	Proteina quinase B / Protein kinase B
AMPK	Proteína quinase ativada por AMP / AMP-activated protein kinase
AUC	Area under curve
BDNF	Gene do fator neutrófico derivado do cérebro
CART	T*ranscrito regulado* pela *cocaína e anfetamina* / Cocaine- and amphetamine-regulated transcript
C/EBPα	Proteína amplificadora ligante ao Ccaat / CCAAT-enhancer-binding protein α
CRF	fator liberador de corticotrofina / Corticotropin-releasing factor
eNOS	Óxido nítrico sintase endotelial
ERK	Proteína quinase regulada por sinais extracelulares / Extracellular signal-regulated protein kinase
GALP	Peptídeo similar à galanina / Galanin-like peptide
GLUT4	Transportadores de glicose tipo 4 / Glucose transporter type 4
GPx	Glutationa peroxidase / Glutathione peroxidase
GRd	Glutationa redutase / Glutathione reductase
GSH	Glutationa / Glutathione
GSK	Glicogênio sintase-quinase
GSK-3β	Glycogen synthase kinase-3β
GSSG	Dissulfeto de glutationa / Glutathione disulfide
GST	Glutationa S-transferase / Glutathione S-transferase
HFA1	Dieta hiperlipídica + AECI 0,35 mg/ml / High-fat diet + AECI 0.35 mg/ml
HFA2	Dieta hiperlipídica + AECI 0,7 mg/ml / High-fat diet + AECI 0.7 mg/ml
HDL	Lipoproteína de alta densidade / High-density lipoprotein
HFD	Dieta hiperlipídica / High-fat diet
IkB	Quinase inibidora do fator nuclear kB / Inhibitor of nuclear factor kB
IKK	Inibidor da quinase kappa B / Inhibitor of Kappa B Kinase
IL-6	Interleucina 6 / Interleukin 6
IL-4	Interleucina 4 / Interleukin 4

IL-10	Interleucina 10 / Interleukin 10
IL-12	Interleucina 12 / Interleukin 12
IL-13	Interleucina 13 / Interleukin 13
IMC	Índice de massa corpórea
iNOS	Óxido nítrico sintase induzida
IP	Inhibition percentage
IRS	Substrato do receptor da insulina / Insulin receptor substrate
JNK	C-Jun aminoterminal quinase / C-Jun N-terminal kinase
LDL	Lipoproteína de baixa densidade / Low-density lipoprotein
MAPK	Proteína quinase ativada por mitógeno / Mitogen-activated protein kinase
MCP-1	Proteína quimioatratora de monócitos 1 / Monocyte chemotactic protein-1
MC4R	Receptor 4 da melanocortina / Melanocortin 4 repetor
mTOR	Proteína *alvo* da *rapamicina* em *mamíferos* / Mammalian target of rapamycin
NPY	Neuropeptídeo Y / Neuropeptide Y
NF-κB	Factor nuclear κB / Nuclear factor κB
N2a	Neuro 2 a
PAI-1	Inibidor 1 do ativador de plasminogênio / Plasminogen activator inhibitor-1
PI(3)K	Fosfatidilinusitol 3 quinase / Phosphoinositide 3-kinase
PKC	Proteína quinase C / Protein kinase C
POMC	Proopiomelanocortina / Proopiomelanocortin
PPAR-α	Receptor ativado por proliferadores de peroxissoma α / Peroxisome-proliferator-activated receptor α
PPARγ2	Receptor ativado por proliferadores de peroxissoma gama 2 / Peroxisome-proliferator–activated receptor γ2
RNAm	Ácido ribonucléico mensageiro
TAS	Tecido adiposo subcutâneo
TAV	Tecido adiposo visceral
TGs	Trigicerídeos / Triglycerides
TNF-α	Fator de necrose tumoral α / Tumor necrosis factor
SC	Ração padrão / Standard chow
SCA	Ração padrão + AECI 0,7 mg/ml / Standard chow + AECI 0.7 mg/ml
SEM	Standard error of the mean
SOD	Superoxido dismutase
VLDL	Lipoproteína de muito baixa densidade / *Very-low-density lipoprotein*

1 INTRODUÇÃO

O aumento da prevalência de sobrepeso e obesidade em vários países tem sido descrito como uma pandemia global (NG et al., 2013). Atualmente, mais de 1,9 bilhões de indivíduos estão acima do peso (OMS, 2015). Em termos econômicos, essa alta prevalência é responsável por 2-7% do total dos custos dedicados ao cuidado da saúde e ainda assim é causa primária de mais de 3,4 milhões de mortes por ano (WITHROW; ALTER, 2011; NG et al., 2013).

A origem fundamental da obesidade é o desbalanço energético entre o que se consome e o que é gasto. Quando as calorias ingeridas excedem o gasto energético, esse excesso de energia é armazenado como gordura nas células adiposas. Esse excedente funciona como combustível para o funcionamento da maioria dos órgãos (HILL AND PETERS, 1998).

Porém, o armazenamento de energia não é a única função do adipócito. Quando maduros, eles funcionam como um órgão endócrino e parácrino e, por meio de uma rede de comunicação entre sistema nervoso simpático e cérebro com outros tecidos, atuam sobre o apetite, o equilíbrio de energia, a imunidade, a sensibilidade à insulina, a angiogênese, a pressão sanguínea, o metabolismo dos lípidos e a homeostase, por meio da secreção de uma vasta gama de adipocinas, fatores de crescimento e de proteínas (IBRAHIM, 2010).

À medida que o indivíduo aumenta sua massa adiposa, o equilíbrio energético e glicêmico é rompido. A secreção das adipocinas, que antes ajudavam na manutenção da homeostase, como a leptina e adiponectina, agora de forma desregulada favorece ainda mais o comprometimento da utilização e absorção dos lipídios e da glicose (IBRAHIM, 2010). O excesso de tecido adiposo prejudica ainda a função imune do tecido, favorecendo o acúmulo de células inflamatórias e liberação de citocinas e fatores de transcrição que vão perpetuar ainda mais esse estado inflamatório (YU et al., 2002; GREENBERG; OBIN 2006; CAWTHORN; SETHI, 2008). Todos esses fatores acabam culminando num estado de obesidade, caracterizado por uma inflamação crônica de baixo grau, associado à resistência dos tecidos à ação da insulina, contribuindo diretamente no desenvolvimento do diabetes tipo 2 e de diversas doenças cardiovasculares (VAN GREEVENBROEK; SCHALKWIJK; STEHOUWER, 2013).

O conhecimento acerca dos mecanismos fisiológicos que regem essa tríade é de fundamental importância para o desenvolvimento de novas terapêuticas que possam atuar na prevenção e tratamento da obesidade e do diabetes tipo 2. Dessa forma, o desenvolvimento de modelos animais e celulares que mimetizem o estado observado na obesidade humana, bem como pesquisas envolvendo novas alternativas terapêuticas tornam-se imprescindíveis.

No âmbito das drogas anti-obesidade, muito têm sido pesquisado em torno de medicamentos e suplementos à base de produtos naturais. Além do seu baixo custo e fácil acesso, a minimização dos efeitos adversos provocados pelas drogas sintéticas normalmente utilizadas, têm sido fator cada vez mais determinante na utilização de extratos ou substâncias isoladas derivadas de plantas medicinais (SIMÕES et al., 1998).

A planta medicinal proposta neste trabalho, a *Chrysobalanus icaco*, também conhecida como abajerú ou coco plum, é uma planta de região costeira, presente em toda a América e África tropical. Apesar da sua ampla localização, poucas pesquisas são encontradas acerca de suas propriedades e efeitos. Estudos fitoquímicos demonstraram a presença de antocianinas nos frutos da planta (BRITO et al., 2007), diterpenos no extrato orgânico das raízes (GUSTAFSON et al., 1991), e os flavonoides miricitrina e quercitrina no extrato hidroalcóolico de suas folhas (BARBOSA et al., 2006).

O extrato aquoso de *Chrysobalanus icaco* (AECI) é utilizado popularmente no Brasil como hipoglicemiante e diurético (PRESTA et al., 2007). Estudo desenvolvido por Barbosa e Peres (2002) demonstrou a presença de rutina nesse extrato. Quanto a seus efeitos, Presta e Pereira (1987) e Barbosa et al. (2013) comprovaram efeito hipoglicemiante e antioxidante do extrato em roedores diabéticos. Ademais, White (2010) observou prevenção do ganho de peso corpóreo e da deposição de gordura no fígado em camundongos obesos induzidos por dieta.

Em relação à rutina, muito se têm discutido na literatura científica. Flavonóide do tipo flavonol, denominada popularmente de hesperidina ou vitamina P (BRICKLEY; GIFFORD; DOMZ, 1959; KUNTIĆ; FILIPOVIĆ; VUJIC, 2011), a rutina têm sido amplamente utilizada no sistema de saúde público por apresentar uma vasta gama de atividades biológicas, elevadas margens de segurança e baixo custo (SHARMA et al., 2013). Estudos com essa substância têm demonstrado diversos benefícios sobre a perda de gordura corporal e sobre a homeostase glicêmica (PRINCE; KANNAN, 2006; HSU et al., 2009; FERNANDES et al., 2010; OLALEYE et al., 2014; KAPPEL et al., 2013). Porém, muito pouco é discutido em relação a seus mecanismos de ação.

Assim sendo, esta tese teve o propósito de investigar duas novas propostas terapêuticas, o extrato aquoso de *Chrysobalanus icaco* e a rutina, verificando seus efeitos e possíveis mecanismos de ação sobre o modelo de obesidade em camundongos C57BL/6J induzidos por dieta hiperlipídica, e outro de resistência à insulina induzida por TNF-α em células neuronais N2a.

2. Revisão da literatura

2. 1 Introdução e classificação da obesidade

A obesidade é definida como uma condição em que ocorre o acúmulo de gordura anormal ou excessivo no tecido adiposo, na medida em que a saúde pode ser prejudicada, resultante do balanço energético positivo e ganho de peso indesejável. A quantidade de gordura em excesso e sua distribuição regional pode variar entre indivíduos obesos, caracterizando seu perfil clínico (OMS, 2015).

Nos últimos 30 anos a obesidade tem sido classificada, principalmente, por meio do índice de massa corpórea (IMC). O IMC é um índice simples, resultado da divisão do peso em quilogramas pelo quadrado da altura em metros (kg/m^2), que classifica baixo peso, sobrepeso e obesidade em adultos (Quadro 1) (OMS, 2000). Embora sua precisão no diagnóstico da obesidade seja limitado, especialmente para indivíduos com IMC intermediário, em homens e idosos, esta classificação permite estimar a prevalência da obesidade em uma população e os riscos a ela associados (ROMERO-CORRAL et al., 2008).

Quadro 1 – Classificação do índice de massa corpórea.

Classificação	IMC (kg/m^2)	Risco de comorbidades
Baixo peso	< 18,50	Baixo (mas risco de outras condições clínicas aumentado)
Peso normal	18,50 – 24,99	-
Sobrepeso	≥ 25,00	
Pré-obeso	25,00 – 29,99	Aumentado
Obeso grau I	30,00 – 34,99	Moderado
Obeso grau II	35,00 – 39,99	Grave
Obeso grau III	≥ 40,00	Muito grave

WHO, 2000.

Embora o IMC seja uma importante ferramenta no diagnóstico nutricional em populações, o mesmo não diferencia a ampla variação na distribuição de gordura corpórea e

pode não indicar o grau de gordura ou riscos de saúde associados em diferentes indivíduos e populações (OMS, 2000).

Outros métodos bastante utilizados associados ao IMC são a medida da circunferência abdominal e a relação cintura-quadril (RCQ). Estes também são recursos de simples execução e baixo custo, que podem fornecer um correlato mais prático da distribuição de gordura abdominal e relação saúde-doença associados. A ressonância magnética e a tomografia computadorizada também são citadas na literatura, porém, são muito pouco utilizadas por seu custo oneroso (MANCINI, 2006).

2.2 Prevalência e mortalidade da obesidade

De acordo com a organização mundial da saúde, a obesidade é um dos problemas públicos de maior visibilidade, porém, bastante negligenciado, que ameaça oprimir tanto países desenvolvidos quanto subdesenvolvidos (WHO, 2000). Em todo o mundo, a prevalência de sobrepeso e obesidade aumentou 27,5% para adultos e 47,1% para crianças entre 1980 e 2013 (NG et al., 2013). O número de indivíduos acima de 18 anos com sobrepeso em 2014 ultrapassou 1,9 bilhões, sendo que 600 milhões destes são obesos. A proporção de homens acima do peso em 2014 foi de 38% e a proporção de mulheres foi de 40% no mesmo período (OMS, 2015). O aumento da prevalência de sobrepeso e obesidade em vários países tem sido descrito como uma pandemia global. Estimou-se em 2010, que o sobrepeso e a obesidade eram responsáveis por causar 3,4 milhões de mortes, 4% de perda na expectativa de vida e 4% dos anos de vida ajustados por incapacidade em todo o mundo (NG et al., 2013).

No Brasil a prevalência do sobrepeso em adultos de 27 cidades monitoradas pela VIGITEL (Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico) passou de 43,2% (2006) para 51,0% (2012), com uma taxa de crescimento anual de 1,3%. A prevalência da obesidade aumentou de 11,6% para 17,4%, com uma taxa de crescimento anual de 0,89%. Este aumento, tanto para sobrepeso e obesidade, foi significativa em todas as cidades, em todas as faixas etárias e todos os níveis de ensino, sendo mais pronunciado em mulheres. Se esses índices forem mantidos, em dez anos, cerca de dois terços dos adultos nas capitais brasileiras estarão acima do peso e um quarto serão obesos (MALTA et al., 2014).

Em termos econômicos, os custos da obesidade se apresentam na faixa de 2-7% do total dos custos no cuidado à saúde, atingindo valores de U$ 45.800,00 milhões nos EUA (WITHROW; ALTER, 2011). No entanto, os custos reais do tratamento em países em

desenvolvimento excedem os dos países desenvolvidos em função dos custos extras associados à aquisição de esquipamentos importados, uso de medicamentos caros e necessidade de formação de equipe de atendimento especializada (WHO , 2000). Como exemplo, no Brasil, em 2011, os custos atribuíveis à obesidade foi de R$ 487.980,00 representando 1,9% do total dos custos de cuidado à saúde de média e alta complexidade, quase quatro vezes mais do que os custos nos EUA. Neste cenário, a obesidade mórbida foi responsável por 23,8% dos custos da obesidade (R$ 116.200.000,00), apesar de sua prevalência ter sido 18 vezes menor (DE OLIVEIRA, 2013).

As consequências da obesidade para a saúde incluem desde queixas não fatais, mas prejudiciais à qualidade de vida, até risco aumentado de morte prematura (OMS, 2015). Várias comorbidades estão associadas à obesidade, incluindo diversas formas de doença cardíaca, diabetes tipo 2, hipertensão e vários tipos de câncer, como o câncer de mama, do colo do útero, ovário, bexiga, próstata e câncer do cólon (OMS, 2015). Embora avanços em pesquisas tenham destacado a importância dos fatores genéticos moleculares na determinação da suscetibilidade individual ao desenvolvimento da obesidade, a descoberta da leptina, das proteínas desacopladoras e dos neuropeptídeos envolvidos na regulação do peso corpóreo, não podem explicar a epidemia de obesidade (HILL; PETERS, 1998). A hereditariedade pode ser responsável por parte dos casos de obesidade, sendo de fato relativamente alta (maior que 0,70) quando comparada a outras doenças poligênicas como autismo e esquizofrenia (WALLEY; BLAKEMORE; FROGUEL, 2006). No entanto, o fator ambiental deve ser levado em consideração uma vez que o *pool* genético tem se mantido estável ao longo dos anos (ECKEL; GRUNDY; ZIMMET, 2005).

2.3 Desenvolvimento da adiposidade

O desenvolvimento da adiposidade é um processo complexo e multifatorial influenciado pelo estilo de vida, genética e fatores ambientais. A "obesogenicidade" dos ambientes modernos está abastecendo essa pandemia que vem se tornando a obesidade (SWINBURN; EGGER; RAZA, 1999).

O ambiente moderno é caracterizado por um abastecimento ilimitado de alimentos de alta densidade energética, altamente palatáveis, relativamente baratos e convenientemente oferecidos em grandes porções. Ainda, o aumento do uso de dispositivos, como o microondas, que poupam tempo e aumentam a comodidade do indivíduo está normalmente associado ao consumo de alimentos ricos em gordura saturada e repletos de conservantes (FAITH; KRAL,

2006). Dados sobre o suprimento alimentar e a utilização do Sistema de Marketing dos Estados Unidos indicam que a disponibilidade global de energia per capita nos EUA aumentou em 15 % entre 1970 e 1994, período durante o qual houve também um aumento da disponibilidade per capita de gordura na dieta, aumento do consumo de gorduras adicionadas (comumente encontradas em salgadinhos e doces), redução do consumo de leite e aumento da ingestão de refrigerantes. Durante este período, houve também um aumento do número de domicílios com dois ou mais aparelhos de televisão, dvd's e computadores domésticos (JEFFERY; UTTER, 2003). Tal ambiente favorece o consumo elevado de energia e baixo gasto energético, impulsionando a disseminação dessa pandemia (HILL; PETERS, 1998).

Embora fatores ambientais como dietas calóricas e o sedentarismo sejam os principais contribuintes para o desenvolvimento da obesidade, a predisposição genética também possui papel importante. Variações genéticas possuem efeito sobre o metabolismo, saciedade e tolerância à atividade física, tornando-se um fator importante no ambiente moderno, favorável ao desenvolvimento da adiposidade (FRAYLING, 2012).

Dentre os fatores genéticos, algumas mutações monogênicas têm sido associadas à obesidade severa e hiperfagia. A mutação no gene da leptina, identificada em 1997, foi uma das primeiras causas da obesidade humana (HEYMSFIELD; REITMAN, 2008). Ausência e mutação do receptor da leptina também está associado à obesidade genética, assim como do receptor 4 da melanocortina (MC4R) (SANTORO et al., 2009), do PPARγ2 (receptor ativado por proliferador de peroxissoma γ2) (RISTOW et al., 1998), do gene da proopiomelanocortina (POMC) e do gene do fator neurotrófico derivado do cérebro (BDNF) (FRIEDEL et al., 2005).

Além das mutações monogênicas, existem ainda mais de 22 associações de genes já comprovadas e descritas na literatura, responsáveis pela origem genética da obesidade. Esses genes incluem também membros da via leptina-melanocortina, citocinas pro-inflamatórias e proteínas desacopladoras (WALLEY; BLAKEMORE; FROGUEL, 2006).

2.4 Distúrbios endócrinos e metabólicos associados à obesidade

A obesidade é uma desordem heterogênea. Indivíduos obesos variam na sua distribuição de gordura, perfil metabólico e grau de risco cardiovascular e metabólico associado. Além disso, existem diferenças entre o tecido adiposo presente em áreas subcutâneas (TAS) e o tecido adiposo visceral (TAV), presente na cavidade abdominal. Anatomicamente, o TAV está presente basicamente no mesentério e no omento e drena diretamente na circulação portal para o fígado, enquanto que o TAS é vizualizado na parede anterior e posterior do abdomen e drena

nas veias sistêmicas (IBRAHIM, 2010). O TAV comparado ao TAS é mais vascular, inervado, contém um número maior de células inflamatórias e imunes, possui menor capacidade de diferenciação pré-adipocitária e uma maior porcentagem de adipócitos distendidos (MISRA; VIKRAM, 2003). O TAS é constituído de adipócitos menores, que avidamente absorvem ácidos graxos livres (AGs) e triglicérides no período pós-prandial, mas, uma vez que eles atingem sua capacidade máxima de absorção, o tecido perde seu benefício protetor e a gordura começa a se acumular em tecidos que não são apropriados para o armazenamento lipídico, como o músculo e fígado. Há mais receptores glicocorticóides, adrenérgicos e andrógenos no TAV, enquanto que o TAS apresenta mais receptores de estrogênio. Adipócitos do TAV são metabolicamente mais ativos, mais sensíveis à lipólise e resistentes à insulina, tornando-se disfuncionais à medida que vão se distendendo (MISRA; VIKRAM, 2003). Dessa forma, o TAV é melhor preditor do desenvolvimento da hipertrigliceridemia, hiperinsulinemia, intolerância à glicose e hiperglicemia.

Os adipócitos são mais que meros depósitos de gordura. Quando maduros, eles agem como um órgão endócrino e parácrino e, por meio de uma rede de comunicação entre sistema nervoso simpático e cérebro com outros tecidos, podem influenciar o apetite, o equilíbrio de energia, a imunidade, a sensibilidade à insulina, a angiogênese, a pressão sanguínea, o metabolismo dos lípidos e a homeostase (IBRAHIM, 2010). Os adipócitos contribuem para o estado pró-inflamatório aumentado durante a obesidade e o diabetes. Eles são capazes de sintetizar uma vasta gama de citocinas, incluindo adipocinas clássicas, fatores de crescimento e de proteínas envolvidas na homeostase glicêmica e angiogênese (TRAYHURN; WOOD, 2004). Adiante segue um resumo das funções de algumas citocinas mais comumente implicadas no desenvolvimento da obesidade e resistência à insulina.

2.4.1 Leptina

A leptina é um produto do gene obeso humano (OB). É basicamente produzido pelo tecido adiposo, branco e marrom, mas também pelo estômago, epitélio mamário, placenta e coração. Sua secreção é diretamente proporcional ao número e tamanho dos adipócitos. A leptina age através do seu receptor, expresso no tecido vascular, estômago, placenta, no hipotálamo e cerebelo. A leptina é liberada no sistema circulatório pelo tecido adiposo em função do armazenamento lipídico (KLOK; JAKOBSDOTTIR; DRENT, 2007). Uma vez secretada, a leptina atravessa a barreira hematoencefálica, passando a informação sobre o *status* de armazenamento. Por meio da ligação com seus receptores, a leptina influencia a atividade

de vários neurônios hipotalâmicos e na expressão de vários neuropeptídeos orexígenos e anorexígenos. O resultado é a redução na ingestão do alimento e aumento da taxa metabólica para que seja mantido o tamanho dos depósitos de gordura corpóreos (KLOK; JAKOBSDOTTIR; DRENT, 2007). Os peptídeos orexigênicos, cujos níveis são influenciados pela leptina, incluem o neuropeptídeo Y (NPY), hormônio concentrador da melanina, proteína relacionada ao agouti (AgRP), galanina e orexina. Os peptídeos anorexigênicos incluem a proopiomelanocortina (POMC), peptídeo similar à galanina (GALP), transcrito regulado pela cocaína e anfetamina (CART) e fator liberador de corticotrofina (CRF) (LEIBOWITZ; WORTLEY, 2004).

Além da regulação a longo prazo do balanço de energia, acredita-se que a leptina também possa desempenhar um papel na regulação a curto prazo da ingestão alimentar e do peso corpóreo. Uma vez que a leptina também é secretada pelo estômago, acredita-se que ela possa desempenhar um papel no controle do tamanho da refeição em cooperação com outros peptídeos responsáveis pela saciedade, como a colecistoquinina (KLOK; JAKOBSDOTTIR; DRENT, 2007).

Perifericamente, o tratamento em pré-adipócitos com leptina tem demonstrado aumento da oxidação e consequente redução no acúmulo de lípidos, redução da expressão de RNAm e da proteína ácido graxo sintase e também a transcrição e transdução da perilipina, proteína presente na superfície da gotícula de lípidio, que serve como um revestimento protetor, facilitando, assim, o armazenamento de lípidios (MINOKOSHI; KAHN, 2003; SINGH et al., 2012).

Entretanto, conforme animais e humanos se tornam obesos, o papel da leptina na regulação do peso corpóreo se torna mais complexo. Na maioria dos indivíduos obesos, a concentração da leptina já é normalmente alta em função da quantidade aumentada de tecido adiposo que a secreta (GREENBERG; OBIN, 2006). Verifica-se que em concentrações crescentes de leptina, o hormônio induz resistência à sua ação nas células-alvo, assim como sua sinalização também fica prejudicada em função do aumento de outras proteínas como, por exemplo, a caveolina-1, atenuando a redução leptina-dependente do acúmulo intracelular de lípidios (SINGH et al., 2012).

2.4.2 Adiponectina

A adiponectina, produto do gene apM1, é uma proteína com domínio semelhante ao colágeno, exclusivamente sintetizada pelo tecido adiposo branco. É induzida durante a

diferenciação dos adipócitos e circula em concentrações relativamente elevadas no soro (CHANDRAN et al., 2003). Os seus receptores AdipoR-1 e -2 são expressos em tecidos periféricos e no cérebro, onde a adiponectina medeia o metabolismo de AGs e modula a homeostase energética.

Apesar da adiponectina ser secretada apenas pelo tecido adiposo, seus níveis, paradoxalmente, estão reduzidos em obesos, contrastando com a maioria das adipocinas, cujos níveis apresentam-se aumentados em proporção ao aumento da massa de gordura corporal total. É possível que, apesar da expressão da adiponectina ser ativada durante a adipogênese, uma retro-inibição da sua produção pode ocorrer durante o desenvolvimento da obesidade, possivelmente mediada por outras citocinas também expressas em quantidades aumentadas no estado obeso, tais como o TNF-α (CHANDRAN et al., 2003).

A adiponectina tem demonstrado inibir tanto a produção como a ação do TNF-α e a modulação da sinalização do fator nuclear kB (NF-kB), fator de transcrição envolvido na resposta inflamatória, pelo menos parcialmente, por meio da via dependente de AMPc (OUCHI et al., 2000). Por outro lado, sensibilizadores de insulina e agonistas do PPAR, aparentemente, aumentam os níveis de adiponectina em camundongos e seres humanos (KADOWAKI; YAMAUCHI, 2005). Outras propriedades anti-inflamatórias incluem supressão da fagocitose e secreção de TNF-α pelos macrófagos e bloqueio da adesão de monócitos em células endoteliais *in vitro* (CHANDRAN et al., 2003).

Níveis reduzidos de adiponectina estão intimamente relacionados à resistência à insulina, hiperinsulinemia e dislipidemia. Por outro lado, níveis aumentados apresentam correlação positiva com os níveis de lipoproteína de alta densidade (HDL) e melhora da sensibilidade dos tecidos alvos à ação da insulina (HOTTA et al., 2001; MATSUBARA; MARUOKA; KATAYOSE, 2002; YU et al., 2002). A adiponectina medeia o efeito de sensibilização à insulina através da ligação aos seus receptores AdipoR1 e AdipoR2, conduzindo à ativação da AMPK e PPAR-α (YADAV et al., 2013).

2.4.3 TNF-α

O fator de necrose tumoral alfa (TNF-α) é uma citocina multifuncional que pode regular diversos processos celulares e biológicos como função imune, diferenciação celular, proliferação, apoptose e metabolismo energético. Além disso, o TNF-α pode ainda afetar, indiretamente, a homeostase energética por meio da regulação da função e expansibilidade do adipócito (CAWTHORN; SETHI, 2008).

O TNF-α medeia seus efeitos biológicos no tecido adiposo por meio de dois receptores, o receptor 1 e o receptor 2 do fator de necrose tumoral. Ambos os receptores são produzidos pelo tecido adiposo e a concentração de isoformas solúveis se correlacionam com o grau de adiposidade (MOHAMED-ALI et al., 1999). Entretanto, resultados de diversas investigações sugerem que o sinal transduzido pelo receptor 1 medeia a maioria dos efeitos do TNF-α na função do adipócito (CAWTHORN; SETHI, 2008).

Tanto adipócitos isolados quanto diferenciados podem produzir TNF-α. Inicialmente, presumiu-se que os adipócitos eram a fonte predominante dos altos níveis de TNF-α na obesidade. Porém, o tecido adiposo também apresenta uma fração vascular estromal que contém um número metabolicamente relevante de tipos celulares como pré-adipócitos, células endoteliais, fibroblastos, leucócitos e macrófagos. Estes últimos seriam os maiores responsáveis pela produção aumentada de TNF-α ao invés do adipócito em si (WEISBERG et al., 2003).

O papel inflamatório dos adipócitos está relacionado à sua expansão, hiperplasia e hipertrofia. O mesmo envolve uma variedade de estresses celulares como disfunção mitocondrial, estresse de retículo endoplasmático e estresse oxidativo que podem ser induzidos pelo TNF-α por meio da ativação das vias c-Jun aminoterminal quinase (JNK) ou NF-κB. De fato, tem-se sugerido que a produção de espécies reativas de oxigênio contribuem tanto para a ativação da via NF-κB TNF-α induzida quanto para a resistência à ação da insulina também induzida por TNF-α (TRIPATHI; PENDEY, 2012; HOUSTIS; ROSEN; LANDER, 2006).

O TNF-α está elevado no tecido adiposo de roedores obesos localmente e sistemicamente, atuando como mediador da resistência insulínica e diabetes tipo 2 relacionadas à obesidade. O mesmo suprime a expressão de diversas proteínas necessárias à captação de glicose induzida pela insulina nos adipócitos, como o receptor da insulina, o substrato 1 do receptor da insulina (IRS-1), proteína quinase B (Akt) e o transportador de glicose tipo 4 (GLUT4). O TNF-α também está envolvido na supressão da atividade e expressão do PPARy e da expressão do RNAm da C/EBPα (proteína amplificadora ligante ao Ccaat), favorecendo à redução da expressão do GLUT4 (RUAN et al., 2002). Essa ação no tecido adiposo parece ser mediada pela ativação das vias NF-κB e da proteína quinase ativada por mitógeno (MAPK), envolvendo a ERK (proteína quinase regulada por sinais extracelulares), p38 MAPK e JNK (RUAN et al., 2002; RYDÉN et al., 2002).

No tecido adiposo branco o TNF-α também é responsável pela inibição da adipogênese e ativação da lipólise, favorecendo o aumento dos AGs circulantes e promovendo deslipidemia. No tecido adiposo marrom, o mesmo pode comprometer a termogênese por promover uma atrofia ou comprometimento da diferenciação. Todos esses fatores culminam num tecido

adiposo disfuncional, com capacidade de expansão, de armazenamento lipídico e capacidade oxidativa comprometidos (CAWTHORN; SETHI, 2008; NISOLI et al., 1997).

2.5 Impacto da adiposidade na saúde

A adiposidade é fator de risco significativo para o desenvolvimento de muitas doenças crônicas não-transmissíveis, incluindo doenças cardiovasculares (hipertensão, varizes, trombose venosa profunda), doenças respiratórias (apnéia do sono, asma e doença pulmonar obstrutiva crônica), doenças metabólicas (hipercolesterolemia, diabetes tipo 2, dismenorreia), doenças gastrointestinais (esteatose hepática, cálculos biliares) e o câncer (mama pós-menopausa, esofágico, colorectal, endométrio, rim). Ainda, a adiposidade está associada a maiores condições de risco à saúde física do que o fumo, álcool e privação sócio-econômica (STURM; WELLS, 2001). A adiposidade é um fator de risco modificável e sua prevenção poderia levar a melhorias significativas na saúde, redução da morbidade e uma economia anual de milhões em custos diretos e indiretos com a saúde (VLAD, 2003).

Dentre as diversas doenças que estão relacionadas à obesidade, a resistência à insulina tem uma relação íntima de causa e consequência no desenvolvimento geral do quadro clínico da obesidade. De fato, acredita-se que a obesidade responda por 80-85% do risco de desenvolvimento do diabetes tipo 2 (HAUNER, 2010). Além disso, o sobrepeso e a obesidade, mais precisamente o acúmulo de gordura abdominal, estão associados a uma inflamação sistêmica de baixo grau. A mesma é caracterizada por altos níveis de citocinas pró-inflamatórias e ácidos graxos circulantes que interferem na função da insulina nos tecidos e, consequentemente, estão implicados nos desenvolvimento do diabetes tipo 2 (VAN GREEVENBROEK; SCHALKWIJK; STEHOUWER, 2013).

2.5.1 Diabetes mellitos tipo 2

A homeostase glicêmica é regida pelo balanço entre a absorção de glicose no intestino, produção pelo fígado e captação e metabolismo pelos tecidos periféricos. A insulina eleva a captação de glicose no músculo e tecido adiposo e inibe a gliconeogênese e a glicogenólise no fígado, atuando dessa forma como regulador primário da concentração de glicose sanguínea. A insulina também promove o armazenamento de substratos no tecido adiposo, fígado e músculo, estimulando a lipogênese, síntese de glicogênio e de proteínas, e inibindo a lipólise, a glicogenólise e a degradação de proteínas (SALTIEL; KAHN, 2000).

A resistência à insulina ou deficiência resulta em profunda desregulação desses processos e na capacidade do tecido em responder à insulina. Inicialmente, o pâncreas é capaz de aumentar, de forma adequada, a sua secreção de insulina compensando a resistência dos tecidos à ação da insulina, fazendo com que a tolerância à glicose permaneça normal. Com o tempo, no entanto, as células-β pancreáticas falham na tentativa de manter a sua elevada taxa de secreção de insulina e a insulinopenia relativa (isto é, em relação ao grau de resistência à insulina) leva ao desenvolvimento de intolerância à glicose e, eventualmente, à hiperglicemia e diabetes mellitus (DEFRONZO; BONADONNA; FERRANNINE, 1992; SALTIEL; KAHN, 2000).

2.5.2 Vias de sinalização da insulina

O receptor de insulina pertence a uma subfamília de receptores tirosina-quinase, constituído de duas subunidades α e β, em que a subunidade α inibe a atividade tirosina quinase da subunidade β. A ligação da insulina à subunidade α conduz à depressão da atividade quinase na subunidade β seguida pela transfosforilação das subunidades β e uma alteração conformacional que aumenta ainda mais sua atividade quinase. Quatro dos substratos intracelulares das quinases receptoras de insulina pertencem à família das proteínas do substrato do receptor da insulina (IRS) (PATTI; KAHN, 1998). Outros substratos incluem Gab-1, p60dok, Cbl, APS e isoformas de Shc (PESSIN; SALTIEL, 2000).

As tirosinas fosforiladas nestes substratos atuam como “locais de ancoragem” para as proteínas que contêm os domínios Src-homólogo-2, resultando em diversas vias de sinalização, incluindo a activação de PI(3)K (fosfoinositida 3-quinase) e a jusante PtdIns proteínas quinases (3,4,5) P3-dependente, o RAS e a cascata MAPK e Cbl/PAC e a ativação do TC10. Estas vias coordenam a regulação do tráfego de vesículas, a síntese de proteínas, a ativação e inativação de enzimas e a expressão gênica, resultando na regulação da glicose e do metabolismo lipídico e protéico (SALTIEL; KAHN 2000).

A resistência à insulina observada na obesidade e no diabetes do tipo 2 caracteriza-se por alterações em vários níveis, seja na redução da concentração do receptor e da atividade quinase, na atividade do IRS-1 e -2, na concentração e fosforilação da PI(3)K, na translocação dos transportadores de glicose ou na atividade de enzimas intracelulares (PESSIN; SALTIEL, 2000).

Além disso, a fosforilação inibitória, ou em serina, do receptor de insulina e dos IRS podem também reduzir a fosforilação em tirosina estimulada pela insulina e servir de

mecanismo para a *cross-talk* de outras vias que produzem resistência à insulina. Várias quinases têm sido implicadas neste processo, incluindo a PI(3)K, Akt, glicogênio sintase-quinase (GSK)-3 e a mTOR (proteína alvo da rapamicina em mamíferos) (PESSIN; SALTIEL, 2000). Dados reportados por Saltiel e Kahn (2000) indicam que a atenuação induzida pela obesidade na sinalização da insulina pode também resultar da ativação sequencial de proteína quinase C (PKC) e da quinase nuclear inibidora do fator kB (IkB), embora os pormenores desta via não tenham ainda sido completamente elucidados.

2.5.3 Obesidade, resistência à insulina e inflamação

É reconhecido que uma inflamação crônica de baixo grau e uma ativação do sistema imune estão envolvidos na patogênese da resistência insulínica relacionada à obesidade e diabetes tipo 2 (ESSER et al., 2014).

O tecido adiposo é heterogêneo na composição e contém, além de adipócitos maduros, também pré-adipócitos, células endoteliais, fibroblastos, macrófagos e outras células do sistema imunológico. Os macrófagos são em grande parte derivados da medula óssea e seu número está aumentado na obesidade. Estes macrófagos locais são a fonte primária do TNF-α e de uma parte importante da produção de IL-6 e da óxido nítrico-sintase induzida (iNOS) (WEISBERG et al., 2003).

Tanto a hiperplasia como a hipertrofia dos adipócitos podem contribuir para a expansão do tecido adiposo, apesar da hipertrofia ser predominante em adultos. Algumas das consequências da hipertrofia incluem aumento do fluxo de ácidos graxos, da secreção de leptina, hipóxia e morte dos adipócitos. Todas essas consequências são possíveis contribuintes para o início do recrutamento de macrófagos (SURMI; HASTY, 2008).

Existem dois tipos de macrófagos localizados no interior do tecido adiposo, o clássico e o alternativamente ativado. Os macrófagos classicamente ativados (M1) são considerados pró-inflamatórios, e o *status* M1 é induzido por mediadores como o TNF-α e o interferon-γ. Já os macrófagos alternativamente ativados (M2), tem a função primária de resolver ou atenuar a resposta inflamatória induzida por M1 e, portanto, são considerados anti-inflamatórios, contribuindo para melhorar a sensibilidade à insulina. O *status* M2 é induzido pelas IL4, IL-13 e IL-10. A maioria dos macrófagos presente no tecido adiposo na obesidade parece ser predominantemente polarizada no sentido do fenótipo M1 (SUN et al., 2012).

Em adição aos efeitos pró-inflamatórios induzidos pela hipoxia local, a alta taxa de síntese de proteínas, durante a expansão do tecido adiposo, pode levar a um acúmulo de

proteínas deformadas e, portanto, ao estresse do retículo endoplasmático. Este último ativa uma rede de sinalização, que envolve a ativação do IKK (inibidor da quinase kappa B), a p38 MAPK, JNK, e, posteriormente, o NF-kB, tendo como resposta reações inflamatórias e apoptóticas. O estresse do retículo endoplasmático induzido pela obesidade favorece também a fosforilação do IRS em serina e consequentemente inibição da sinalização da insulina (ZEYDA; STULNING, 2009).

Adipócitos hipertróficos também produzem citocinas pró-inflamatórias, tais como TNF-α, IL-6, resistina, MCP-1 (proteína quimiotática de monócitos-1), e PAI-1 (inibidor do ativador do plasminogênio-1). A produção destas substâncias tem efeitos locais no endotélio, aumentando a síntese de moléculas de adesão e a permeabilidade vascular, e no recrutamento de monócitos circulantes. O sinal inflamatório é aprimorado pelo *cross talk* entre células endoteliais, adipócitos e macrófagos residentes, que em conjunto contribuem para a produção de citocinas pró-inflamatórias e para a indução da resistência à insulina local e sistêmica. Acredita-se que o acúmulo de lípidos nos adipócitos iniciem esse sinal inflamatório no tecido adiposo e que os macrófagos residentes amplifiquem o sinal (BASTARD et al., 2006).

Essas citocinas também são responsáveis por induzir a produção de espécies reativas de oxigênio (ROS), gerando um processo conhecido como o stress oxidativo (SO). Existem vários mecanismos pelos quais a obesidade produz SO. O primeiro é a oxidação mitocondrial e peroxisomal de ácidos graxos, que podem produzir ROS em reações de oxidação. Outro mecanismo seria por meio do excesso de consumo de oxigênio, gerando também radicais livres na cadeia respiratória mitocondrial. Dietas ricas em lípidos, também são capazes de gerar ROS porque eles podem alterar o metabolismo do oxigênio. Ademais, o aumento de tecido adiposo também favorece a redução da atividade de enzimas antioxidantes, como a superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx), contribuindo para a disfunção endotelial, caracterizada particularmente pela redução do óxido nítrico, e induzindo ao desenvolvimento também de doenças ateroscleróticas. (FERNÁNDEZ-SÁNCHEZ et al., 2011).

Assim como as citocinas, as espécies reativas de oxigênio e os AGs são mediados por vias de sinalização intracelular específicas, envolvendo NF-kB, IkB quinase, IKK, proteína ativadora-1 e JNK. Todas essas vias podem interagir com a sinalização de insulina por meio da fosforilação do IRS em serina/treonina, diminuindo a resposta de insulina e induzindo a resistência à insulina (BASTARD et al., 2006).

Níveis aumentados de leptina também estão relacionados à resposta pró-inflamatória. Foi demonstrado que a leptina promove a função fagocítica, induz a síntese de óxido nítrico e

a produção de várias citocinas pró-inflamatórias, como o factor de necrose tumoral alfa (TNF-α), interferon gama e a interleucina 6 e 12 (IL-6 e IL-12), em macrófagos e monócitos (ZARKESH-ESFAHANI et al., 2001; LOFFREDA et al., 1998). Em neutrófilos, a leptina aumenta a produção de espécies oxidativas (CALDEFIE-CHEZET et al., 2001). A leptina também mostrou ter ação direta sobre as células T, induzindo a sua ativação e alterando a produção de citocinas, por meio da polarização da diferenciação de células T na direção de uma resposta pró-inflamatória, essencial para produção de citocinas e ativação de macrófagos (LORD et al., 1998).

A adiponectina, por outro lado, controla a produção e ativação do TNF-α pelo tecido adiposo e pelos macrófagos, atuando de forma anti-inflamatória e contribuindo para a melhora da resposta à ação da insulina (OUCHI et al., 2000; CHANDRAN et al., 2003).

2.6 Tratamento

Existem cinco pilares ou estratégias a serem utilizadas para perda e manutenção do peso. O primeiro pilar, a dietoterapia, envolve a orientação nutricional em como ajustar a dieta para reduzir o número de calorias ingeridas, ensinando o indivíduo sobre conteúdo calórico, composição dos alimentos e como prepará-los. O segundo pilar se refere à terapia comportamental e envolve a mudança de hábitos e padrões do indivíduo relacionados à dieta e atividade física, construindo uma rede de suporte para que o mesmo consiga realizar essas mudanças. A atividade física, terceiro pilar, envolve a prática de exercícios que possam ser inseridos na rotina diária do indivíduo, e, que de alguma forma, seja uma prática agradável. A terapia combinada, embora não seja exatamente um pilar, se utiliza desses três primeiros pilares, de forma a tentar atingir a maior probabilidade de sucesso no tratamento (NIH, 2000).

O quarto pilar consiste no tratamento medicamentoso, indicado para indivíduos com IMC maior ou igual a 30. O tratamento cirúrgico, considerado o quinto pilar, é indicado especialmente para obesos mórbidos (IMC maior ou igual a 40) ou obesos (IMC maior ou igual a 35) que apresentem co-morbidades associadas, em situações em que houve fracasso diante do tratamento convencional (NIH, 2000). O mesmo envolve uma variedade de procedimentos restritivos ou mal absorptivos que visam a perda rápida de peso e melhora do quadro clínico e co-morbidades associadas do indivíduo (CEELEN et al., 2003).

Até o presente momento, as abordagens no tratamento da obesidade têm sido principalmente educacional, comportamental e farmacológica, porém, com sucesso bastante limitado. A modificação do estilo de vida é cada vez mais difícil em função da realidade dos

ambientes obesogênicos e, além disso, as drogas desenvolvidas até o momento apresentam eficácia limitada e efeitos adversos importantes (CHEUNG; CHEUNG; SAMARANAYAKE, 2013).

Considerando os custos destinados ao tratamento de pacientes com sobrepeso e obesos para a saúde pública, medidas preventivas tem se tornado cada vez mais necessárias. Ambientes e comunidades de apoio são fundamentais na orientação de escolhas de alimentos mais saudáveis e no incentivo à prática de exercícios físicos regulares (acessíveis e de baixo custo) (OMS, 2015). No Brasil, algumas das iniciativas destinadas pelo governo envolvem ações e políticas de educação alimentar e nutricional, incluindo a revisão do Guia Alimentar para a População Brasileira, o Programa Saúde na Escola, políticas públicas inter-setoriais na área de segurança alimentar e nutricional, promoção e oferta de alimentos saudáveis em ambientes escolares, estruturação da nutrição na atenção básica no SUS - Sistema Único de Saúde, promoção de atividade física na comunidade, criação do programa Academia da Saúde, ações de regulação e controle de alimentos (MALTA, 2014).

No entanto, é importante ressaltar que as intervenções ambientais servem como medidas complementares e não substitutivas às abordagens individuais à obesidade, tais como tratamento com drogas e a terapia comportamental (SWINBURN; EGGER; RAZA, 1999). Nos últimos anos, inúmeros medicamentos têm sido aprovados para o tratamento da obesidade; no entanto, a maior parte foi retirada de circulação em função de seus efeitos adversos (KANG; PARK, 2012). A fenfluramina e a dexfenfluramina estão fora de circulação desde a década de 90 em função de danos à válvula cardíaca (CONNOLY et al., 1997). O rimonabanto, primeira droga bloqueadora seletiva do receptor canabinóide-1, atuando sobre a redução do apetite, apesar de prescrito em 56 estados americanos, nunca teve seu uso liberado pelo FDA (Food and Drug Administration) nos EUA em função do alto risco de desenvolvimento de distúrbios psiquiátricos como depressão, ansiedade e ideação suicida. A mesma foi retirada de circulação pela própria fabricante (CHRISTENSEN et al., 2007).

A sibutramina, inibidora seletiva da reabsorção da noradrenalina/serotonina, atuando também na inibição do apetite, foi amplamente utilizada após liberação pelo FDA americano em 1997. Inicialmente foi bem tolerada, apresentando efeitos colaterais comuns como constipação, cefaléia e insônia (KANG; PARK, 2012). Porém, estudo realizado a longo prazo com a droga (5-year Sibutramine Cardiovascular Outcomes - SCOUT) verificou a associação de alto risco ao desenvolvimento de infarto do miocárdio e acidente vascular cerebral, sendo, por esse motivo, retirada de circulação pelo mercado americano e europeu em 2010 (TORP-PEDERSEN et al., 2007; KANG; PARK, 2012).

No Brasil, a fenfluramina e a dexfenfluramina também teve seu uso suspendido pela ANVISA em 1997. A sibutramina, por sua vez, nunca teve sua utilização suspensa. A manutenção da venda do medicamento foi deferida pela ANVISA em 2014, porém, com limitação de dose e mantendo o mesmo controle já definido para a comercialização da substância, com retenção de receita, assinatura de termo de responsabilidade do prescritor e do termo de consentimento pós-informação por parte do usuário (ANVISA, 2014).

Mais recentemente, em fevereiro de 2011, o órgão responsável pela liberação de drogas nos EUA rejeitou a aprovação da combinação bupropiona/naltrexona diante do risco em potencial de desenvolvimento de eventos cardiovasculares (KANG; PARK, 2012). Até mesmo o orlistat, que apesar de ser uma droga considerada como "segura", ter seu uso liberado e ser amplamente recomendado por diversos médicos (CHANOINE et al., 2005), têm sido associado frequentemente a várias disfunções gastrointestinais como diarréia, dor abdominal e manchas fecais, e de forma menos frequente ao desenvolvimento de insuficiência renal aguda, colelitíase, hepatite e insuficiência hepática subaguda (FILIPPATOS et al., 2008). Além disso, têm sido demonstrado que o orlistat também interfere na absorção de muitos fármacos tais como varfarina, amiodarona, ciclosporina e tiroxina, bem como as vitaminas solúveis em gordura, afetando a sua biodisponibilidade e eficácia (FILIPPATOS et al., 2008).

Diversos estudos têm sido realizados a fim de investigar e desenvolver novas drogas anti-obesidade por meio de fontes à base de plantas medicinais, drogas vegetais, buscando principalmente minimizar as reações adversas associadas aos atuais medicamentos anti-obesidade sintéticos. O uso de produtos naturais têm sido documentado por centenas de anos em vários sistemas medicinais tradicionais em todo o mundo e continua em ascenção (VASUDEVA; YADAV; SHARMA, 2012).

De acordo com a Resolução RDC nº. 10/2010, que dispõe sobre a notificação de drogas vegetais junto à Agência Nacional de Vigilância Sanitária (ANVISA), as drogas vegetais são plantas medicinais ou suas partes, de venda isenta de prescrição médica destinados ao consumidor final, utilizadas apenas no preparo de infusões, decocções e macerações. As plantas medicinais, as drogas vegetais e os derivados vegetais podem ser utilizados como insumos farmacêuticos e/ou como medicamentos (ANVISA, 2010).

Uma grande variedade de produtos naturais estão atualmente disponíveis no mercado para o tratamento da obesidade (CHANDRASEKARAN et al., 2012), utilizada tanto em preparações medicamentosas como na forma de suplementos alimentares. Porém, existe uma grande confusão entre alimento, produto natural e fitoterápico. Um alimento, composto por espécies vegetais, não pode ter finalidade medicamentosa e/ou terapêutica. Quando ao produto

são apontadas indicações terapêuticas, este passa a ser considerado medicamento e a sua fabricação e/ou comercialização obedecem ao disposto na legislação específica de medicamentos. Os fitoterápicos, por sua vez, são obtidos a partir da industrialização, que seria a padronização da quantidade e forma de utilização, com emprego exclusivo de matérias-primas ativas vegetais, não podendo incluir na sua composição substâncias ativas isoladas, sintéticas ou naturais, nem as associações dessas com extratos vegetais. A eficácia e a segurança do fitoterápico devem ser validadas através de levantamentos etnofarmacológicos, documentações tecnocientíficas em bibliografia e/ou publicações indexadas e/ou estudos farmacológicos e toxicológicos pré-clínicos e clínicos (ANVISA, 2010).

Uma característica comum dos produtos naturais está relacionada ao fato de que a maioria desses compostos possuem propriedades antioxidantes e anti-inflamatória, devido à presença, principalmente, de compostos fenólicos (TRIPATHI; PENDEY, 2012). Várias combinações de produtos naturais podem resultar em uma atividade sinérgica que aumenta a sua biodisponibilidade e a ação em vários alvos moleculares, oferecendo vantagens sobre os tratamentos químicos (RAYALAM; BELLA-FERA; BAILE, 2008).

Esses compostos naturais podem induzir a perda de peso por meio de vários mecanismos. De acordo com Kazemipoor et al. (2012), suas funções podem ser classificadas em cinco categorias principais: inibição da atividade da lipase pancreática, potencialização da termogênese, prevenção da diferenciação adipocitária, aumento do metabolismo lipídico e redução do apetite. Considerando apenas a utilização de extratos e não componentes ou substâncias isoladas dos mesmos, algumas das espécies mundialmente mais utilizadas no tratamento da obesidade estão especificadas na tabela 1. Estas espécies foram as mais relatadas em estudos de revisão de literatura sobre a utilização de produtos naturais no tratamento da obesidade, de caráter experimental ou clínico, muito embora, a maior partes desses estudos tenham sido experimentais. Essa dificuldade de estudos em humanos se apoia na variedade de viéses diante da especificidade do organismo e hábitos de cada indivíduo, aceitabilidade do medicamento e aderência ao estudo (FIRENZUOLI; GORI, 2007).

Mesmo diante da frequência de determinadas espécies, existe uma grande variedade de plantas medicinais utilizadas em diversas parte do mundo de acordo com a tradição, cultura e regulamentação de cada país. No Brasil existe um guia de plantas medicinais (RENISUS - Relação Nacional de Plantas Medicinais de Interesse ao SUS), constituído de diversas espécies pré-selecionadas por regiões que referenciavam seu uso por indicações e de acordo com as categorias do Código Internacional de Doenças. Essa pré-seleção foi realizada por técnicos da ANVISA e do Ministério da Saúde, profissionais de serviços e pesquisadores da área de plantas

medicinais e fitoterápicos, vinculados à área da saúde, representando as diversas regiões brasileiras. A finalidade da RENISUS é subsidiar o desenvolvimento de toda cadeia produtiva relacionadas à regulamentação, cultivo, manejo, produção, comercialização e dispensação de plantas medicinais e fitoterápicos (BRASIL, 2009). A lista do RENISUS conta com mais de 71 espécies, destacando-se as espécies *Ananas Camosus* (abacaxi), *Baccharis trimera* (carqueija) e *Zingiber officinale* (gengibre), popularmente utilizadas no tratamento da obesidade (ARUN et al., 2012; SOUZA et al., 2011; GOYAL; KADNUR, 2006).

Tabela 1: Ervas medicinais frequentemente relatadas no tratamento da obesidade em estudos de revisão sistemática de 2009 a 2014.

Espécie	**Nome popular**	**Ação**
***Panax ginseng* (Araliaceae)**	Berry	Inibição do apetite e aumento da termogênese em camundongos (ATTELE et al., 2002)
***Camellia sinensis* (L.) Kuntze (Theaceae)**	Chá verde	Inibição do apetite (YUN, 2010) e inibidor da lipase pancreática (KOO; NOH, 2007) em camundongos.
	Chá oolong	Aumento da oxidação de gordura e inibição da lipase pancreática em camundongos (HAN et al., 1999)
Ilex Paraguariensis	Erva mate	Aumento da oxidação lipídica e inibição da lipogênese em ratos (PANG; CHOI; PARK, 2008).
Hibiscus sabdariffa	Hibisco	Aumento da oxidação lipídica em humanos (GURROLA-DÍAZ et al., 2009) e inibição da lipogênese em células 3T3-L1 (KIM et al., 2003)
***Zingiber officinale* Roscoe (Zingiberaceae)**	Gengibre	Inibição da absorção intestinal de gordura em camundongos (HAN et al., 2005) e aumento da oxidação lipídica em camundongos (GOYAL; KADNUR, 2006).

Fonte: HASANI-RANJBAR et al., 2009; YUN, 2010; KAZEMIPOOR et al., 2012; HASANI-RANJBAR; JOUYANDEH; ABDOLLAHI, 2013 e JUNG; LIM; KIM, 2014.

Além das espécies mencionadas, estudos conduzidos com a *Chrysobalanus icaco* L. mostraram redução de peso corpóreo no tratamento com suas sementes (EDEMA et al. 2007), redução dos níveis de glicemia no tratamento com o extrato aquoso (PRESTA; PEREIRA, 1987; BARBOSA et al., 2013) e atividade analgésica e anti inflamatória com o extrato metanólico (CASTILHO et al., 2000). Estudos prévios conduzidos em nosso laboratório reforçam o uso do extrato aquoso de *Chrysobalanus icaco* (AECI) na hiperglicemia e ressaltam seu potencial na prevenção de ganho de peso e de gordura no fígado em camundongos obesos induzidos por dieta hiperlipídica (WHITE, 2010).

A *Chrysobalanus icaco* L. é uma planta medicinal pertencente à família Chrysobalanaceae e é também conhecida como abajeru, icaco, coco plum ou guajiru. Tem origem na América e África Tropical, mas atualmente está adaptada em regiões similares em todo o mundo, desde ao sul dos Estados Unidos até Brasil, Equador e Venezuela, incluindo as Antilhas (BRITO et al., 2007; RAMÍREZ VILLALOBOS; URDANETA- FERNÁNDEZ; VARGAS-SIMÓN, 2004).

No Brasil, o chá de abajeru é comumente utilizado diariamente como agente hipoglicemiante e diurético (PRESTA et al., 2007). Suas raízes também são consumidas diariamente, maceradas e misturadas em água no tratamento da inflamação e diabetes (AGRA et al., 2007).

Estudos fitoquímicos demonstraram a presença de antocianinas nos frutos da planta (BRITO et al., 2007), diterpenos no extrato orgânico das raízes (GUSTAFSON et al., 1991), e flavonoides como a rutina, miricitrina e quercitrina, bem como outros derivados da quercetina e miricetina no extrato aquoso e hidroalcóolico de suas folhas (BARBOSA; PERES, 2002; BARBOSA et al., 2006).

A rutina, um dos principais compostos presente no AECI, é um flavonóide do tipo flavonol, que consiste em quercetina e rutinose dissacarídeo (ramnose e glicose) (KUNTIĆ; FILIPOVIĆ; VUJIC, 2011). Foi isolada pela primeira vez a partir do suco de limão e da pimenta vermelha húngara. Denominada de hesperidina ou "vitamina P" (BRICKLEY; GIFFORD; DOMZ, 1959), a mesma tem sido amplamente utilizada no sistema de saúde por apresentar uma vasta gama de atividades biológicas, em função de suas propriedades antioxidantes, elevadas margens de segurança e menor custo (SHARMA et al., 2013).

Estudos farmacológicos com este composto têm relatado aumento da captação de glicose e da síntese e translocação de GLUT-4 no músculo sóleo de ratos (KAPPEL et al., 2013) e efeito restaurador nas células-β pancreáticas, em função da sua habilidade em combater os radicais livres e mediar atividade enzimática antioxidante (HANHINEVA et al., 2010).

Redução dos níveis de lipídios plasmáticos (PRINCE; KANNAN, 2006; FERNANDES et al., 2010; OLALEYE et al., 2014), aumento do PPARγ em músculo esquelético de camundongos db/db (CAI et al., 2012), elevação da expressão de adiponectina ao nível da proteína em adipócitos 3T3-L1 (HSU; YEN, 2007) e diminuição do peso corpóreo, do fígado e dos coxins adiposos epididimal e peritoneal em camundongos obesos induzidos por dieta também foram verificados (HSU et al., 2009).

Ademais, o tratamento com rutina também reduziu o estresse oxidativo e os níveis de dissulfeto de glutationa (GSSG), e aumentou os níveis de glutationa (GSH), da GSH peroxidase (GPx), GSH redutase (GRd), e GSH S-transferase (GST) no tecido hepático. Esses dados demonstram que a rutina pode também ser benéfica na supressão do estresse oxidativo induzido por dieta hiperlipídica em ratos (HSU et al., 2009).

Outros compostos que poderiam estar atuando de forma sinérgica com a rutina no AECI são os flavonóis miricetrina, quercitrina e quercetina. Estudos desenvolvidos com quercetina relataram atividade antibacteriana, antimicrobiana (ADEROGBA et al., 2013), antitumoral (HOFMANN et al., 2006), anti-hipertensiva (DUARTE et al., 2001) e antioxidante (MAHESH; MENON, 2004). Além disso, Rivera et al. (2008) também demonstraram efeito anti-inflamatório por meio do aumento da expressão de eNOS e da concentração de adiponectina, e da redução da expressão de iNOS e na produção de TNF-α, juntamente com uma redução no ganho de peso corporal, em ratos Zucker obesos.

A quercitrina, glicosídeo formado a partir da quercetina, têm mostrado efeito hipoglicemiante, protegendo o pâncreas por meio da redução na infiltração de gordura nas ilhotas, enquanto reduz a glicose plasmática e os níveis de insulina em ratos diabéticos (BABUJANARTHANAM; KAVITHA; PANDIAN, 2010). Atividade antiaterogênica também foi observada por meio da supressão da expressão da proteína inflamatória de macrófagos 2 e do MCP-1, envolvido no tráfego de monócitos e migração de macrófagos, diminuindo assim a produção de fatores de crescimento endotelial vascular pró-inflamatórios e pró-aterogênicos (CHOI et al., 2010). No que diz respeito a miricetrina, Aderogba et al., (2013) demonstraram uma forte atividade antibacteriana e antimicrobiana.

Embora alguns efeitos e mecanismos de ação de alguns dos compostos do AECI tenham sido discutidos na literatura, pouco se sabe sobre o AECI *per si*. Com base nos dados apresentados e estudos previamente realizados em nosso laboratório, acredita-se que o AECI tenha várias propriedades que podem ainda ser amplamente exploradas e, possivelmente, trazer diversos benefícios não só no tratamento do diabetes de tipo 2, mas também da obesidade e de diversas outras doenças crônicas.

3. OBJETIVOS

3.1 Geral

- Investigar duas novas propostas terapêuticas, o extrato aquoso de *Chrysobalanus icaco* (AECI) e a rutina, em modelos experimentais de obesidade e resistência à ação da insulina, verificando seus efeitos e possíveis mecanismos de ação.

3.2 Específicos

- Desenvolver modelo de obesidade induzido por dieta hiperlipídica em camundongos C57BL/6J;
- Avaliar os efeitos do AECI sobre o consumo e peso corporal;
- Avaliar os efeitos do AECI sobre a homeostase glicêmica;
- Avaliar os efeitos do AECI sobre o perfil sérico lipídico;
- Avaliar os efeitos do AECI sobre o tecido adiposo;
- Investigar ação do AECI sobre absorção de lipídios no trato intestinal;
- Investigar ação do AECI sobre a atividade locomotora;
- Identificar compostos majoritários presentes no AECI;
- Investigar a atividade antioxidante do AECI;
- Densenvolver modelo de resistência à insulina em células N2a induzidas por TNF-α;
- Verificar ação da rutina sobre a via de inflamação induzida pelo TNF-α;
- Verificar ação da rutina sobre a resistência à insulina induzida pelo TNF-α.

Capítulo 1: Aqueous extract of *Chrysobalanus icaco* prevent fat gain in obese high-fat fed mice.

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Instruções para autores – apêndice E.

Aqueous extract of *Chrysobalanus icaco* prevent fat gain in obese high-fat fed mice

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Abstract

Ethnopharmacological relevance: Due to the rise in obesity, the necessity for resources and treatments that could reduce the morbidity and mortality associated to this pandemia has emerged. The development of new anti-obesity drugs through herbal sources has been increasing in the past decades which are being used not only as medicine but also as food supplements. Previous studies with the aqueous extract of *Chrysobalanus icaco* L (AECI) have demonstrated activity on lowering blood glucose levels and body weight. **Aim of the study:** Investigate *C. icaco* effects in overall adiposity and glycemic homeostasis. **Material and methods:** C57BL/6J mice were randomly assigned to standard chow (SC) or high-fat diet (HFD) and treated with AECI in 0.35 mg/mL or 0.7 mg/mL concentrations *ad libitum*. Food intake, feed efficiency, metabolic efficiency, body, fat pads and gastrocnemius weight, adiposity index, serum lipids, fecal lipid excretion, locomotor activity in the open field test and insulin and glucose tolerance tests were analyzed and compared. The major components of the extract were demonstrated through HPLC and its antioxidant activity analyzed through DPPH and lipid peroxidation. **Results:** The AECI in the 0.35 mg/mL concentration didn't affect food intake or body weight. However, it promoted lower adipose tissue gain, TG levels, and fecal lipid excretion, increased locomotor activity and lean mass weight, and normalized insulin sensitivity and glucose tolerance. Moreover, AECI showed the presence of rutin and quercitrin and demonstrated high antioxidant activity. **Conclusions:** AECI in lower concentrations can prevent fat storage or enhance fat utilization through the increase of locomotor activity. Also, this reinforces its ability to maintain glucose homeostasis through the normalization of insulin sensitivity and glucose tolerance despite the high fat diet intake. These activities could be associated to the extract's polyphenol content.

1. Introduction

The rise in obesity has resulted in the necessity for resources and treatments that could make some changes in overweight and obesity prevalence in all populations. The development of adiposity is a complex, multifactorial process that is influenced by lifestyle, genetic and environmental factors. The "obesogenicity" of modern environments is fueling the obesity pandemic (Swinburn et al., 1999).

To date, approaches to obesity, which have been mainly educational, behavioral, and pharmacological, have met with limited success. The modification in lifestyle has become increasingly more difficult due to the obesogenic environments and, in addition, the drugs developed to date have limited efficacy and substantial adverse effects (Cheung et al., 2013).

Numerous trials have been conducted to find and develop new anti-obesity drugs through herbal sources to minimize adverse reactions associated with the present anti-obesity drugs (Vasudeva et al., 2012), which are being used both as medicine and also as food supplements. Most of them possess antioxidant and anti-inflammatory properties, due to presence of high polyphenolic compounds (Tripathi and Pendey, 2012).

Chrysobalanus icaco L. is a medicinal plant that belongs to the *Chrysobalanaceae* family and is commonly used on a daily basis as a hypoglycemic and diuretic agent (Presta et al. 2007). Phytochemical studies have demonstrated the presence of flavonoids such as rutin, myricitrin, and quercitrin, as well as other minor myricetin and quercetin derivatives in the aqueous and hydroalcoholic extracts of its leaves (Barbosa et al. 2006).

Pharmacological studies have reported a decrease in blood glucose levels in rodents (Presta and Pereira 1987; Barbosa et al., 2013) with the aqueous extract of *C. icaco* (AECI), as well as analgesic, anti-inflammatory (Castilho et al., 2000) and antimicrobial activities with the methanol extract (Castilho and Kaplan, 2011). Also, previous studies conducted in our laboratory reinforce the use of AECI in hyperglycemia and highlight the potential extract's effect in preventing weight gain of diet-induced obese mice (White, 2013).

In this study some other effects of *C. icaco* in overall adiposity and glycemic homeostasis are reported and mechanisms by which the extract could prevent the weight gain are investigated.

2. Materials and methods

2.1 Plant material and preparation of the aqueous extract

C. icaco leaves were collected from the Jatoba village (Latitude: 10º 47' 50" S and Longitude: 36º 50' 44" W), Sergipe, Brazil, in February 2008, and were identified by Dr. Ana Paula Prata. A voucher specimen (ASE 11855) is deposited at the Herbarium of the Federal University of Sergipe.

The aqueous extract was obtained from *C. icaco* dried leaves. After collection, suitable leaves were selected and then dried in an oven with circulating and renewable air (Model Marconi, MA – 035/5, Brazil) at 40 ºC for 72 h. Subsequently, the leaves were triturated in an electric mill until they became a finely granulated dust. This powder was dissolved in distilled water at 100 ºC for 15 min, at a ratio of 50 g of leaves to 1 liter of water, and then vacuum-filtered, lyophilized, and stored at 4 ºC. Immediately prior to use, the extract was dissolved in water at the desired concentration.

2.2 Antioxidant assays

2.2.1 Analysis of phenolic compounds by high performance liquid chromatography–diode array detector (HPLC–DAD)

Quantification of phenolic compounds in extracts was carried out using the high performance liquid chromatography system, that consisted of two LC-6AD pumps, SPD-20A (UV/Vis) diode array detector (DAD), CTO-10AS column oven, DGU-20A_{3R} degasser, SIL-20A autoinjector and CBM-20A system controller (Shimadzu Co., Kyoto, Japan), equipped with a Kinetex Phenyl-Hexyl column (4.6 × 150 mm, 5 μm) from Phenomenex (Torrance, CA, USA) maintained at 40 °C. The injection volume was 20 μl and the following solvents in water with a flow rate of 1.0 mL/min were used: A, 0.1% formic acid (JT Baker, Philipsburg, PA, USA) in water and B, methanol (Tedia, Fairfield, OH, USA). The elution profile was a linear gradient elution for B of 25-33% for 5 min, keeping at 33% over 5 min, and then increased to 33-40% for 5 min, keeping at 40% for 5 min, increased to 40-47% for 7 min, and then 47-100% in 2 min, keeping at 100% for 1 min. Analytical HPLC was monitored at 254 and 280 nm. AECI sample was diluted in water in an 11 mg/mL concentration, while the rutin (Sigma-Aldrich, São Paulo, Brazil) and quercitrin (Sigma-Aldrich, São Paulo, Brasil) samples were dissolved in methanol at a concentration of 1.9 e 1.4 mg/mL, respectively. All samples were centrifuged at 13400 rpm for 5min (Eppendorf, Mini spin®, Hamburg, Germany) before injection in the HPLC. The data was obtained through the LC Solution software.

2.2.2 Scavenge of DPPH radical

The quantification of the antioxidant activity against the DPPH radical was carried out according to Paixão et al. (2013). Gallic acid (GA) and the AECI (0,5 mg/mL) were added to a solution of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, 40 µg/mL in methanol) to give the concentrations of 5, 10, 15, 25 and 30 µg/mL in 3 mL. The absorbances of the reaction mixtures were determined at 515 nm in the first, fifth, and tenth minute, and then every 10 min up to 60 min. The results were expressed as inhibition percentage (IP), while the antioxidant amount necessary to decrease the DPPH concentration in 50% (IC50) was calculated by plotting the percentage of DPPH remanescent (%DPPHREM) after 60 min versus extract concentrations. The antioxidant activity index (AAI) was calculated as AAI = [DPPH stock concentration (µg/mL)]/[IC_{50} (µg/mL)] (Scherer and Godoy, 2009).

2.2.3 Lipid Peroxidation

The potential of the AECI to reduce lipid peroxidation was measured using the production of thiobarbituric acid-reactive substances (TBARS) (Budni et al., 2007). Briefly, egg yolk homogenate (1% w/v, 1 mL) in phosphate buffer (pH 7.4) was sonicated (10 s) and mixed with freshly prepared solutions of the AECI and controls at 50, 100, 150 and 200 µg/mL. Lipid peroxidation was induced by adding either ferrous sulphate ($FeSO_4$, 0.17 mol/L, 0.1 mL). Trolox was used a positive control, while the negative control was the vehicle (methanol). The mixture was incubated at 37 °C for 30 min. Upon cooling, samples (0.5 mL) were centrifuged with 15% trichloroacetic acid (0.5 mL) at 1200 rpm for 10 min. Supernatant was taken (0.5 mL), mixed with 0.67% thiobarbituric acid (0.5 mL), incubated at 95 °C for 60 min and, after cooling, the formation of TBARS was measured by reading the supernatant absorbance at 532 nm. Results were expressed as inhibition percentage of TBARS formation.

2.3 Animals and diets

All procedures were approved by the Animal Research Ethics Committee of the Federal University of Sergipe, Brazil (Protocol number CEPA 51/2011) and were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (NAS 1996).

A group of 34 male C57BL/6J mice (2 months old) were obtained from Foundation Oswaldo Cruz, Salvador, Bahia, Brazil. The animals were maintained on a 12-h light–dark cycle and housed 2 or 3 per cage, at a temperature 20-23 °C. Mice were randomly assigned to standard chow (SC) or high-fat diet (HFD) *ad libitum*. SC and HFD were commercially

obtained from PragSoluções (Jaú, Brazil) and differed in their lipid and carbohydrate content. The standard chow consisted of 6.7% lipids and 78.5% carbohydrates, and the HFD contained 57.6% and 28.0% respectively. After 6 weeks, mice on standard diet were divided into 2 groups, one group continued receiving water (SC) while the other group was treated with AECI in 0.7 mg/mL concentration for 8 weeks (SCA), and mice on high-fat diet were divided into 3 groups with one group receiving water (HFD) while the other 2 groups were treated with AECI in 0.35 mg/mL (HFA1) and 0.7 mg/mL concentration (HFA2), respectively, for 8 weeks.

The AECI was mixed with the drinking water and replaced every day. This concentration was chosen based on previous research conducted in our laboratory, which demonstrated that the dose of 200 mg/kg of the extract prevented weight gain and normalized blood glucose in *swiss* high-fat fed obese mice. Hence, the lower concentration used (0.35 mg/mL) would be the equivalent to the dose of 200 mg/kg according to the mice's water intake and the higher concentration (0.7 mg/mL) the double of the first one.

2.4 Body weight and diet intake

AECI intake was measured every day, food intake every other day and body weight was measured once a week. The feeding efficiency was calculated by dividing total body weight gain (g) by total food intake (kcal), while the metabolic efficiency, by calculating the ability of ingested energy to be metabolized, which is the inverse parameter of the feed efficiency (Winzell and Ahrén 2004).

2.5 Fecal lipid analyses

Feces of each group were collected during seven days, at the end of the experiment, and stored at -20 °C. At the end of the week, the material was defrosted weighed and dried for 8 h in a ventilated oven at 40 °C and then weighed again. The amount of humid and dry feces produced by the groups during the period of seven days was weighed and compared between groups. The solid matter was extracted according to Folch's method (Folch, Lees, Stanley, 1957) and after the material was back-extracted and the organic phase was evaporated to dryness, the pellet was resuspended in 2 mL of chloroform and transferred to preweighed vials. The solvent was evaporated, and the vial was taken to a constant weight by drying in a rotaevaporator at 35 °C. The difference in weight between the starting empty vial and the vial containing the dried lipid was the fecal lipid amount, which was expressed as a percentage of the weight of the starting fecal sample.

2.6 Locomotor activity

The voluntary locomotor activity of animals was assessed in an open field (open top plexiglass box of 12 × 12 inches) during 10 min sessions. Two tests were carried out, one during the light cycle and the other during the dark cycle under red light. The total distance travelled (m) and average speed (m/s) were measured and analyzed over the test period using the ANY-maze video tracking software (Saito et al., 2014; Da Cruz et al., 2012).

2.7 Glucose and insulin sensitivity analyses

2.7.1 Glucose tolerance test (GTT)

Mice were fasted for 6 h prior to the experiment. A stock solution of 100 mg/mL D-glucose saline was prepared. After an initial fasting blood sample (time 0), 1 mg glucose/g body weight was injected intraperitoneally, and blood was sampled after 5, 15, 30, 60, and 120 min. The area under the glucose decay curve (AUC) was calculated for each mouse and the mean was calculated for each group (Faulhaber-Walter et al., 2011).

2.7.2 Insulin tolerance test (ITT)

Mice were fasted for 5 hours and then injected insulin (0.5 U/kg body weight) (Insunorm R – 100 U/ml, Cellofarm, Rio de Janeiro, Brazil) intraperitoneally. Glucose levels were measured at 0, 15, 30, 60, and 90 min after the injection. The area under the glucose decay curve (AUC) was calculated for each mouse and the mean was calculated for each group (Yuan et al., 2013).

2.8 Biochemical analysis

Three days after ITT, the animals were anesthetized with inhaled isoflurane and blood was collected through the inferior vena cava after a 12 h fast to analyze glucose, total cholesterol, triglycerides (TG), high-density lipoprotein-cholesterol (HDL-c) and urea assayed by colorimetric enzymatic method, according to the manufacturer's instructions (Labtest, Lagoa Santa, MG, Brazil).

2.9 Fat pads and gastrocnemius

After euthanasia, the gastrocnemius muscle, as well as the periepididymal, perirenal and retroperitoneal fat pads, were removed and weighed. The weight of the gastrocnemius muscle was used as an indicator of lean muscle mass. The sum of the fat pads determined the total visceral fat weight. The adiposity index was calculated as the result of the total fat pad (g)

divided by the final bodyweight × 100 and expressed as adiposity percent (Jeyakumar et al., 2009).

2.10 Data analysis

The data are displayed as mean ± standard error of the mean (SEM). Differences between groups were analyzed with one-way ANOVA and Bonferroni post-hoc test. Parameters over time were analyzed in a two-way ANOVA model and Bonferroni post-hoc. A p value of less than 0.05 was used to indicate statistical significance (GraphPad Prism 6.0, GraphPad Software, La Jolla, CA).

3. Results

3.1 Identification of major phenolic compounds of AECI

Figure 5 shows the HPLC-UV chromatographic profile of the aqueous extract of C. *icaco* (AECI), which demonstrated 8 major peaks (1 – 8). The peaks with retention time of 13.8 min (peak 5) and 18.2 min (peak 7) were consistent with the retention times of authentic samples of flavonols, demonstrated previously by Ducrey et al. (1995), more specifically, rutin and quercitrin respectively.

3.2 Antioxidant potential of AECI

In the DPPH assay, the AECI was able to scavenge DPPH radicals, showing an IC_{50} of 12.5 ± 0.8 compared with 1.2 ± 0.8 of the control galic acid and an inhibition percentage similar to the control, 96.9% vs. 92.4%, respectively. According to the antioxidant activity index, the AECI demonstrated a very high antioxidant activity (2.4 vs. 27.5 from galic acid).

Concerning the potential of the AECI in inhibiting TBARS formation, the results showed that all four concentrations presented a high inhibitory potential against ferrous sulfate and there were no significant difference between the percentages obtained. The corresponding values for the inhibitory potential against $FeSO_4$ of the 50, 100, 150 and 200 µg/mL concentrations were 40.4, 53.6, 67.6 and 69.2% respectively.

3.3 Effect of AECI on food intake, body weight and body fat

After six weeks of obesity induction, the animals from the HFD group showed significant higher weight gain, observed after the 4^{th} week on high fat diet ($p<0.05$), and

consequently higher final weight, compared to the control group (SC) (Figure 1 A). The food intake in grams as well as the food intake on kilocalories was not different between groups, however, the water intake was significantly lower than the SC group ($p<0.05$). Because there wasn't any difference in the caloric intake, there was no significant increase in the feed efficiency. The metabolic efficiency, on the other hand, was significantly lower in the HFD compared to the SC ($p<0.05$). This indicates that the weight gain observed in high-fat–fed mice was related to a reduced metabolic rate (Table 1).

Once the obesity was characterized, the animals were divided into control and treated groups. The AECI treatment started at the 7th week and went through the end of the 14th week (Figure 1 B). In this period, the HFD group continued showing higher body weight and lower metabolic efficiency compared to SC group. Additionally, food intake in kcal and feed efficiency became higher ($p<0.05$ and $p<0.001$ vs. SC, respectively). No changes were observed concerning the water intake between both groups (Table 2).

As for the treated groups, the groups that received the higher concentration of the extract, SCA and HFA2 groups, demonstrated higher AECI intake compared to their controls ($p<0.05$ vs. SC and $p<0.01$ vs. HFD) as well as higher food intake in grams and in kilocalories ($p<0.0001$ vs. SC and $p<0.0001$ vs. HFD, respectively) and consequently higher feed efficiency ($p<0.05$ vs. SC and $p<0.05$ vs. HFD). However, no change in metabolic efficiency was observed. On the other hand, the lower AECI concentration didn't alter any of the parameters observed (Table 4). Furthermore, no significant changes in the body weight evolution were observed between the treated groups and their controls (Figure 1 B)) nor was there a difference in the final body weight (Table 2).

As expected, the HFD group also showed a significant increased adipose tissue, in all of the fat pads weighed, periepidydimal, perirenal and retroperitoneal, as well as a higher adiposity index, compared to the SC (table 3). The group treated with the smaller concentration of AECI, in turn, presented significantly lighter fat pads and consequently lower adiposity index compared to the HFD group (table 3). The gastrocnemius weight didn't show any difference through ANOVA when comparing the five groups, however, when comparing only the three groups that received the high fat diet also through ANOVA an increased muscle mass in the HFA1 ($p<0.05$) when compared to the HFD (table 3) was revealed.

3.4 Effect of AECI on serum profile and fecal lipid extraction

After 14 weeks of high fat diet, there was a trend toward an increase in TG from HFD group compared to SC (84.3 ± 4.0 vs. 68.9 ± 6.2 mg/dl, p = 0.07). Interestingly, the HFA1 displayed a significant lower level of TG when compared to HFD (59.2 ± 1.9 mg/dl, $p<0.05$). As for the CT, HDL and urea levels neither the high fat diet nor the AECI induced any significant change between groups.

Concerning the weight of humid or dry feces between the groups, no significant difference was observed. Nevertheless, the difference in weight between the starting empty vial and the vial containing the dried lipid was significantly higher in the HFD group compared to the SC group and lower in all three treated groups compared to their control ($p<0.05$ and $p<0.01$ for HFD and SCA vs. SC, respectively, and $p<0.01$ for HFA1 and HFA2 vs. HFD) (figure 2).

3.5 Effect of AECI on locomotor activity

There was a significant decrease in HFD group locomotor activity when compared to the SC group, in both distance traveled and velocity, but only in the day cycle ($p<0.05$). Interestingly, the AECI treatment induced a significant increase in distance traveled as well as in velocity in all three treated groups, although in the night cycle for the SCA group, but in both cycles for the HFA1 and HFA2 groups, resulting in markedly improved locomotor activity (figure 3).

3.6 Effect of AECI on insulin and glucose sensitivity and fasting blood glucose

After 14 weeks of high-fat diet, the HFD group showed significantly higher blood glucose, in all time points measured (15, 30, 60 and 90 minutes), in the ITT test when compared to SC group. The area under the glucose decay curve (AUC) was also greater (500.9 ± 31.7 vs. 279.4 ± 39.7 from SC group). Interestingly, treatment with AECI in the lower concentration was able to significantly reduce the blood glucose levels at time points 15 and 60 minutes ($p<0.01$) and consequently lower the AUC (356.6 ± 26.5, $p<0.05$) (figure 4).

Mice from the HFD group also displayed higher blood glucose levels through the glucose tolerance test, in time points 15, 30 and 60 minutes, as well as higher AUC (1364 ± 35.3 vs. 884.2 ± 47.2, $p< 0.0001$) compared to the SC group, demonstrating impaired glucose tolerance. Although AECI showed lower blood glucose and lower AUC, the ANOVA comparing the five groups didn't reveal any significant decrease. However, when compared only the high fat fed groups, the ANOVA indicated that the AECI significantly normalized the glucose tolerance in the group that received the lower concentration of the extract (figure 4).

Concerning the serum blood glucose, after 12h fasting, neither the high fat diet nor the AECI treatment induced any significant changes (122.4 ± 15.1, 155.6 ± 10.2, 151.4 ± 22.5, 169.8 ± 17.8, 183.2 ± 8.7 for SC, SCA, HFD, HFA1 and HFA2 respectivelly). The HFD group did achieve insulin resistance and impaired glucose tolerance but didn't generated hyperglycemia or a complete state of diabetes type 2.

4. Discussion

Obesity is characterized not just by an increase in body weight, but especially by an increase in adiposity and changes in its distribution and function. The adipose tissue is the key factor in all these metabolic abnormalities associated with the development of obesity. The adipocyte has the capacity to sense, manage, and send signals to maintain energy equilibrium in the body. But during obesity, this equilibrium is disrupted and the adipocyte functionality is compromised, impairing the transcriptional regulation of the key factors that control adipogenesis and the sensitivity to external signals, as well as the signal transduction process (Vázquez-Vela, Torres, Tovar, 2008) which are also related to the development of insulin resistance, inflammation and dyslipidemia among other several metabolic risk factors.

The obesity induced in the C57BL/6J male mice by the 56.7% high fat diet in this study was characterized by an increased body weight as well as body fat and a trend toward high TG levels, which may have been achieved due not only to an increased feed efficiency but also to a decreased metabolic rate and locomotor activity. Energy expenditure is composed of basal metabolic rate, the thermic effect of food and locomotor activity (Bjursell et al., 2008). Therefore, it can be stated that the HFD group also showed decreased energy expenditure. These data corroborates the report by Bjursell et al. (2008) where C57BL/6J male mice with 8 weeks of age showed decreased locomotor activity after 21 days of 40% high fat diet, and decreased locomotor activity plus reduced energy expenditure after 7 weeks on the same diet. They also showed a positive correlation between these two parameters, estimating that 62% of the extra body weight gain in mice fed high fat diet can be explained by a reduced locomotor activity.

Interestingly, the AECI in the lower concentration (0.35 mg/mL), promoted lower body fat gain, decreased TG levels and increased muscle mass, which could be explained by the increased locomotor activity induced by the extract, promoting energy expenditure. Also, the fact that the AECI reduced fat excretion through feces, clarifies that the loss of fat in these mice isn't occurring through any anti-absorptive effect of the extract on the gastrointestinal tract.

Edema et al. (2007) observed that 10% *C. icaco* seeds induced weight reduction in rats without toxic effects. Moreover, White (2013) has shown prevention of weight gain in obese

high-fat fed mice with AECI treatment. These are the only reports to date about this plant and and weight loss. Nothing have been reported about energy expenditure or locomotor activity. However, some of AECI major compounds as rutin and quercetrin have been implicated in some anti-obesity activities as well as in an antiinflamatory and antioxidant role that are also linked to the overall response of these compounds in the obesity treatment.

Rutin has been shown to decrease serum lipids, body weight and peritoneal and epididymal fat pads weights in obese rats. Decreased hepatic TG and CT levels were also observed, related to reduced oxidative stress and glutathione disulfide (GSSG) content, and enhanced levels of glutathione (GSH), GSH peroxidase (GPx), GSH reductase (GRd), and GSH S-transferase (GST) in the hepatic tissue (Hsu et al., 2009). Likewise, Fernandes et al. (2010) associated rutin (50 mg/kg) with markedly decreased hepatic and cardiac levels of tryacylglycerols and elevated glycogen, improving hyperglycemia and dyslipidemia while inhibiting the progression of liver and heart dysfunction in streptozotocin-induced diabetic rats.

Quercetrin appears to be more implicated in an anti-inflamatory and antioxidant role. In study conducted by Choi et al. (2010), quercetrin blocked the uptake of oxidized LDL, cholesterol influx and inhibited the scavenger activity of the oxidized LDL through attenuation of PPARy activation in macrophages. Camuesco et al. (2004) have demonstrated improvement in colonic oxidative status in experimental colitis through reduction of NO synthase activity and downregultion of colonic activity of the NF-κB, along with reduced macrophage and granulocyte infiltration. Furthermore, prevention of TBARS production by different agents (potassium ferricyanide, quinolinic acid and sodium nitroprusside) was induced by quercetin treatment in vitro exhibiting a scavenger and antioxidant activity (Wagner et al., 2006).

Concerning the locomotor activity, some studies have reported that it can be regulated by some specific receptors located in the dorsomedial hypothalamus. Leptin receptors LepRb have been shown to regulate energy expenditure as well as locomotor activity and consequently body weight, without altering food intake (Rezai-Zadeh et al., 2014). The melanocortin receptors are also involved. Apparently the blockade of the melanocortin-3 and -4 receptor increased fat mass and decreased locomotor activity and energy expenditure (Chen at al., 2000; Huszar et al., 1997). The melanocortin receptor also can directly and rapidly control glucose utilization, TG synthesis, lipid deposition, and lipid mobilization in white adipose tissue (Nogueiras et al., 2007).

Taken together these data indicate that AECI in lower concentration, could be acting centrally and peripherally, increasing the locomotor activity and energy expenditure and

enhancing the utilization of fat and glucose or inhibiting further absorption and deposition of fat, or both.

Glucose homeostasis is governed by the balance between glucose absorption from the intestine, production by the liver and uptake and metabolism by peripheral tissues. Insulin increases glucose uptake in muscle and fat, and inhibits hepatic glucose production, thus serving as the primary regulator of blood glucose concentration. Insulin also promotes the storage of substrates in fat, liver and muscle by stimulating lipogenesis, glycogen and protein synthesis, and inhibiting lipolysis, glycogenolysis and protein breakdown (Saltiel and Kahn, 2000). Insulin resistance or deficiency results in profound dysregulation of these processes and in the tissue's ability to respond to insulin. Because the pancreas is able to appropriately augment its secretion of insulin to offset the insulin resistance, glucose tolerance remains normal. With time, however, the β-cell fails to maintain its high rate of insulin secretion and the relative insulinopenia (i.e., relative to the degree of insulin resistance) leads to the development of impaired glucose tolerance and eventually hyperglycemia and diabetes mellitus (DeFronzo, Bonadonna, Ferrannine, 1992).

In addition to obesity, the mice from the HFD group demonstrated insulin resistance and impaired glucose tolerance. The AECI, in turn, in the lower concentration (0.35 mg/mL), was capable to normalize the insulin sensitivity and improve the glucose tolerance after 8 weeks of treatment. Previous study by Presta and Pereira (1987) have reported a reduction of 64.2% in the intestinal absorption of glucose in normal mice treated with the aqueous extract of *C. icaco* at a concentration of 5% (or 50 mg/mL) mixed with drinking water.

Studies conducted with rutin have demonstrated the same effects observed in this study, by the increase of glucose uptake in the rat soleus muscle via the PI3K, atypical protein kinase C and mitogen-activated protein kinase (MAPK) pathways and activation of the transporter GLUT-4 synthesis (Kappel et al., 2013). Hanhineva et al. (2010) suggest that rutin might also preserve the glucose-sensing ability trough β-cell restoring effect due to enhanced ability to scavenge free radicals and mediate antioxidant enzyme activity in the pancreas.

Quercitrin has also shown decrease of plasma glucose and increase in insulin levels along with the restoration of carbohydrate metabolism in diabetic rats. These effects were related to an expansion of the pancreatic islets and decreased fatty infiltrate of the islets (Babujanarthanam, Kavitha, Pandian, 2010).

As demonstrated in this study, the AECI contains high levels of rutin and quercetrin. The presence of these polyphenols must be related to the antioxidant potential of AECI

observed here by the high inhibitory potential against ferrous sulfate and its ability to scavenge DPPH radicals.

Similar findings were observed by Barbosa et al. (2013) in which the AECI also presented potent antioxidant activity against the DPPH radicals that could be due to the presence of polyphenols and terpenoids, observed in the phytochemical screening of the extract. Furthermore, antioxidant effects are demonstrated as well by Ferreira-Machado et al (2004) where the AECI in the concentration of 0.7 mg/mL promoted a reduction in the lethality of *E. coli* strains PQ35 and PQ37 against the damage induced by stannous chloride that could be due to the prevention of reactive oxygen species formation and scavenge of free radicals generated by the stannous chloride.

These findings indicates that besides the antioxidant activity, the prevention of reactive oxygen species generation and scavenge of free radicals could also account for the overall improvements in glucose homeostasis and circulating lipid levels. Increased free fatty acids can induce ROS formation that are mediated through specific intracellular signaling pathways, involving NF-κB, IκB kinase, IKK, Activating Protein-1 (AP-1) and JNK signaling molecules (Davis et al., 2009; Bastard et al., 2006). The activation of these pathways could be responsible for the serine/threonine inhibitory phosphorylation of IRS, decreasing the insulin response and inducing insulin resistance.

Another fact that must be considered is that the lower gain of adipose tissue and reduced adiposity index shown by mice treated with AECI (0.35 mg/mL) could also influence the normalization of insulin sensitivity and improvement of glucose tolerance. Once the increased fat mass is responsible for the impairment of glucose oxidation and also the release of several adipokines that could directly result in a decreased sensitivity to insulin (DeFronzo, Bonadonna, Ferrannine, 1992), AECI, in the lower concentration, could be acting preventing this damage through inhibition of lipogenesis and/or enhanced fat utilization in high fat fed mice.

Concerning the effects observed with the AECI in the higher concentration, although an increase in the locomotor activity was also observed, the extract at 0.7 mg/mL induced a higher food intake and feed efficiency, which could probably account for the absence of significant effects on the adipose profile and in glucose homeostasis of the group HFA2. These findings indicates that not always the treatment with a higher concentration of an extract will necessarily promote a better response. Corroborating this statement, Barbosa et al. (2013) in the comparison of three doses of the AECI, observed that the best results in blood glucose and body weight of diabetic rats were observed with the lowest dose of the extract, 100 mg/kg, compared with the other two of 200 and 400 mg/kg.

5. Conclusions

These findings indicate that AECI in lower concentrations can prevent fat storage or enhance fat utilization through the increase of locomotor activity. Furthermore, these data reinforce its ability to maintain glucose homeostasis through the normalization of insulin sensitivity and glucose tolerance despite the high fat diet intake. These effects could be related to AECI polyphenol content and its ability to scavenge free radicals and mediate antioxidant activity.

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Figure captions

Figure1: Effect of high fat diet and AECI on body weight. Data represent the means ± SEM of body weights **A -** from mice fed standard chow (SC) and high fat diet (HFD) from the adaptation week (-1^{th}) to the 6^{th} week. **B -** from mice fed standard chow (SC), standard show + AECI 0.7 mg/mL concentration (SCA), high fat diet (HFD), high fat diet + AECI 0.35 mg/mL concentration (HFA1) and high fat diet + AECI 0.7 mg/mL concentration (HFA2), from the 7^{th} week, on the beginning of AECI treatment, to the 14^{th} week, end of experiment. Data are means ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. SC. One-way ANOVA with Bonferroni post hoc. N = 6-7.

Figure 2: Effect of high fat diet and AECI on fecal lipid extraction. Data represent the percentage of lipid amount extracted from mice fed standard chow (SC), standard show + AECI 0.7 mg/mL concentration (SCA), high fat diet (HFD), high fat diet + AECI 0.35 mg/mL concentration (HFA1) and high fat diet + AECI 0.7 mg/mL concentration (HFA2). Data are means ± SEM. $^{*}p<0.05$ and $^{**}p<0.01$ vs. SC, $^{\#\#}p<0.01$ vs. HFD. One-way ANOVA with Bonferroni post hoc. N = 6-7.

Figure 3: Effect of high fat diet and AECI on locomotor activity. Data represent **A** - the distance traveled (m) and **B** - velocity (m/s) of mice fed standard chow (SC), standard show + AECI 0.7 mg/mL concentration (SCA), high fat diet (HFD), high fat diet + AECI 0.35 mg/mL concentration (HFA1) and high fat diet + AECI 0.7 mg/mL concentration (HFA2). Data are means ± SEM. $^{*}p<0.05$ and $^{**}p<0,01$ vs. SC, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. HFD. One-way ANOVA with Bonferroni post hoc. N = 6-7.

Figure 4: Effect of high fat diet and AECI on insulin and glucose sensitivity. Data represent the means ± SEM of **A** - blood glucose levels (mg/dL) at time 0 and after injecting intraperitoneally 0.5 U of insulin/kg of body weight at time points 15, 30, 60 and 90 minutes and **B** - the correspondent area under the decay curve **C** - blood glucose levels (mg/dL) at time 0 and after injecting intraperitoneally 1 mg glucose/g of body weight at time points 5, 15, 30, 60 and 120 minutes and **D** - the correspondent area under the decay curve of mice fed standard chow (SC), standard show + AECI 0.7 mg/mL concentration (SCA), high fat diet (HFD), high fat diet + AECI 0.35 mg/mL concentration (HFA1) and high fat diet + AECI 0.7 mg/mL concentration (HFA2). $^{*}p<0.05$ and $^{***}p<0.001$ vs. SC, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. HFD, one-way

and two-way ANOVA with Bonferroni post hoc. $^{\#a}p<0.05$ vs. HFD, one-way ANOVA with Bonferroni post hoc compared only between HFD, HFA1 and HFA2. N = 6-7.

Figure 5. HPLC-UV chromatograms at 254 nm and 280 nm of AECI, rutin and quercitrin.

Table 1: Initial and final body weight, body weight gain, water and food intake, feed efficiency and metabolic efficiency on the 6 first weeks.

	SC	**HFD**
Initial body weight (g)	21.7 ± 0.8	22.9 ± 0.5
Final body weight (g)	27.2 ± 0.8	31.4 ± 0.7**
Body weight gain (g)	5.5 ± 0.8	8.1 ± 0.6*
Water intake (ml)	3.3 ± 0.1	2.4 ± 0.06***
Food intake (g)	3.9 ± 0.3	3.5 ± 0.04
Food intake (kcal)	41.3 ± 3.5	45.1 ± 4.3
Feed efficiency (g/kcal)	0.004 ± 0.001	0.005 ± 0.0004
Metabolic efficiency (kcal/g)	377.3 ± 59.6	208.9 ± 21.6*

Data are means ± SEM at the end of the 6th week of the experiment $^{*}p<0.05$ and $^{***}p<0.001$ vs. SC. One-way ANOVA with Bonferroni post hoc. N=13-21.

Table 2: Initial and final body weight, body weight gain, Water/AECI and food intake, feed efficiency and metabolic efficiency from the 7th to the 14th week.

	SC	SCA	HFD	HFA1	HFA2
Initial body weight (g)	27.2 ± 1.3	24.8 ± 0.8	33.3 ± 0.9**	31.3 ± 1.3	30.6 ± 1.0
Final body weight (g)	28.3 ± 0.5	29.3 ± 1.3	42.0 ± 2.1***	37.3 ± 1.3	38.6 ± 1.5
Body weight gain (g)	1,0 ± 0,9	5,2 ± 0,7*	8,8 ± 1,4***	6,4 ± 0,6	9,5 ± 1,2
Water /AECI intake (ml)	2.3 ± 0.05	2.7 ± 0.06*	2.4 ± 0.04	2.5 ± 0.04	2.6± 0.05##
Food intake (g)	5.9 ± 0.02	8.4 ± 0.51***	5.7 ± 0.06	5.9 ± 0.09	9.2 ± 0.05###
Food intake (kcal)	23.5 ± 0.1	33.2 ± 2.1***	31.3 ± 0.3***	32.7 ± 0.5	50.6 ± 1.0###
Feed efficiency (g/kcal)	0.001 ± 0.0004	0.003 ± 0.0006*	0.004 ± 0.0007***	0.003 ± 0.0004	0.006 ± 0.0005#
Metabolic efficiency (kcal/g)	1443.6 ± 542.4	549.6 ± 189.1	295.6 ± 53.6*	368.0 ± 52.8	171.1 ± 15.2

Data are means ± SEM at the end of the 14th week of the experiment *p<0.05, **p<0.01 and ***p<0.001 vs. SC; #p<0.05, ##p<0.01 and ###p<0.001 vs. HFD, one-way ANOVA with Bonferroni post hoc. N=6-7.

Table 3. Final body, fat pads and gastrocnemius weights and adiposity index

	SC	SCA	HFD	HFA1	HFA2
Final body weight (g)	26.62 ± 0.3	28.03 ± 1.3	40.14 ± 2.1***	35.39 ± 1.6	38.52 ± 1.8
Periepidydimal weight (g)	0.50 ± 0.06	0.73 ± 0.09	2.25 ± 0.3***	1.29 ± 0.03$^{\#\#\#}$	2.32 ± 0.17
Retroperitoneal weight (g)	0.14 ± 0.02	0.26 ± 0.05	0.70 ± 0.08***	0.48 ± 0.04$^{\#}$	0.74 ± 0.04
Perirenal weight (g)	0.07 ± 0.01	0.11 ± 0.01	0.42 ± 0.07***	0.23 ± 0.05$^{\#}$	0.26 ± 0.03
Adiposity index (%)	2.73 ± 0.3	4.15 ± 0.4	8.28 ± 0.7***	5.72 ± 0.2$^{\#\#}$	8.65 ± 0.2
Gastrocnemius weight (g)	0.16 ± 0.010	0.15 ± 0.005	0.15 ± 0.010	0.18 ± 0.010$^{\#a}$	0.16 ± 0.004

Data are means ± SEM at the day of euthanasia or final day of the experiment. $^{***}p<0.001$ vs. SC; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. HFD, one-way ANOVA with Bonferroni post hoc. $^{\#a}p<0.05$ vs. HFD, one-way ANOVA with Bonferroni post hoc between the 3 high fat fed groups. N=6-7.

Figure 1 A and B

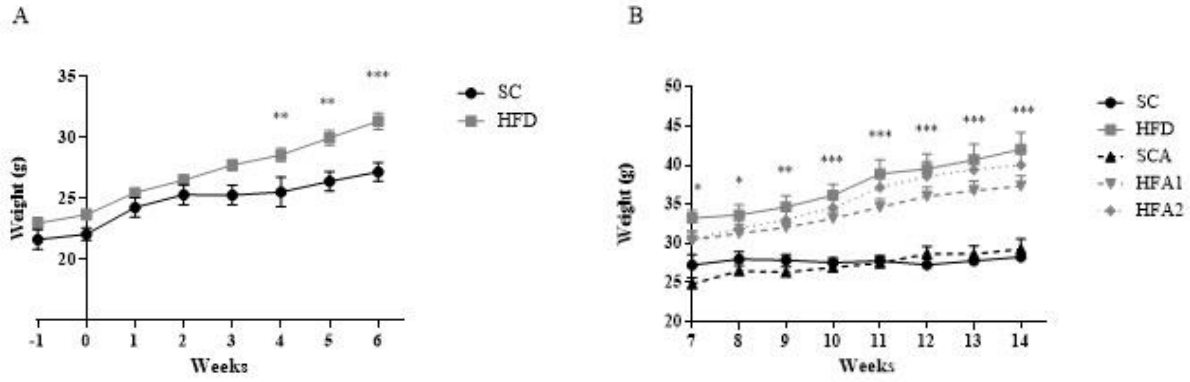


Figure 2

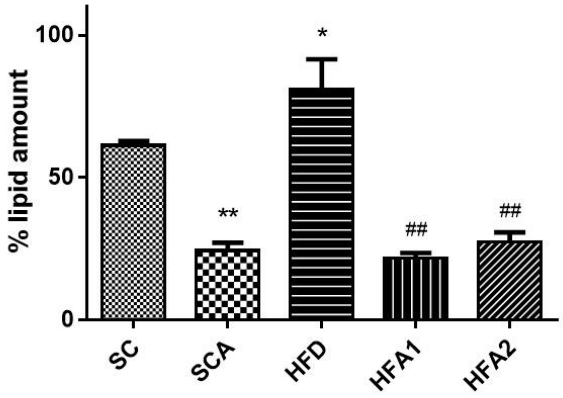
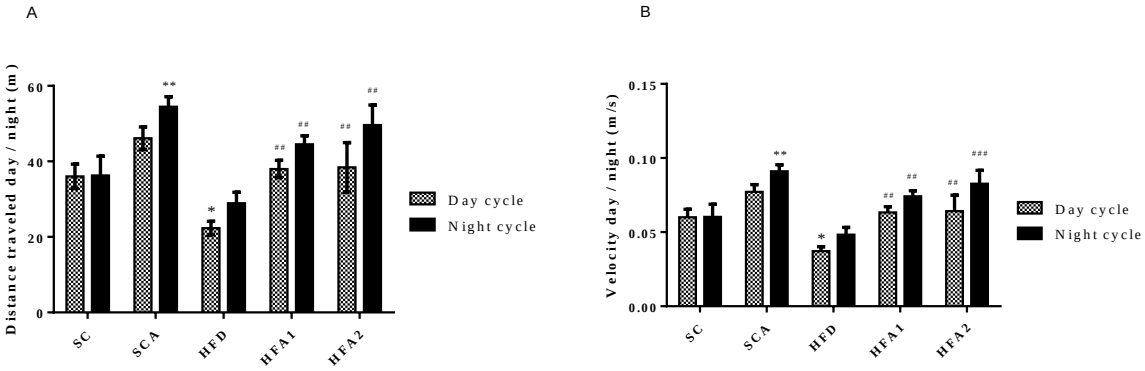


Figure 3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Velocity day / night (m/s)
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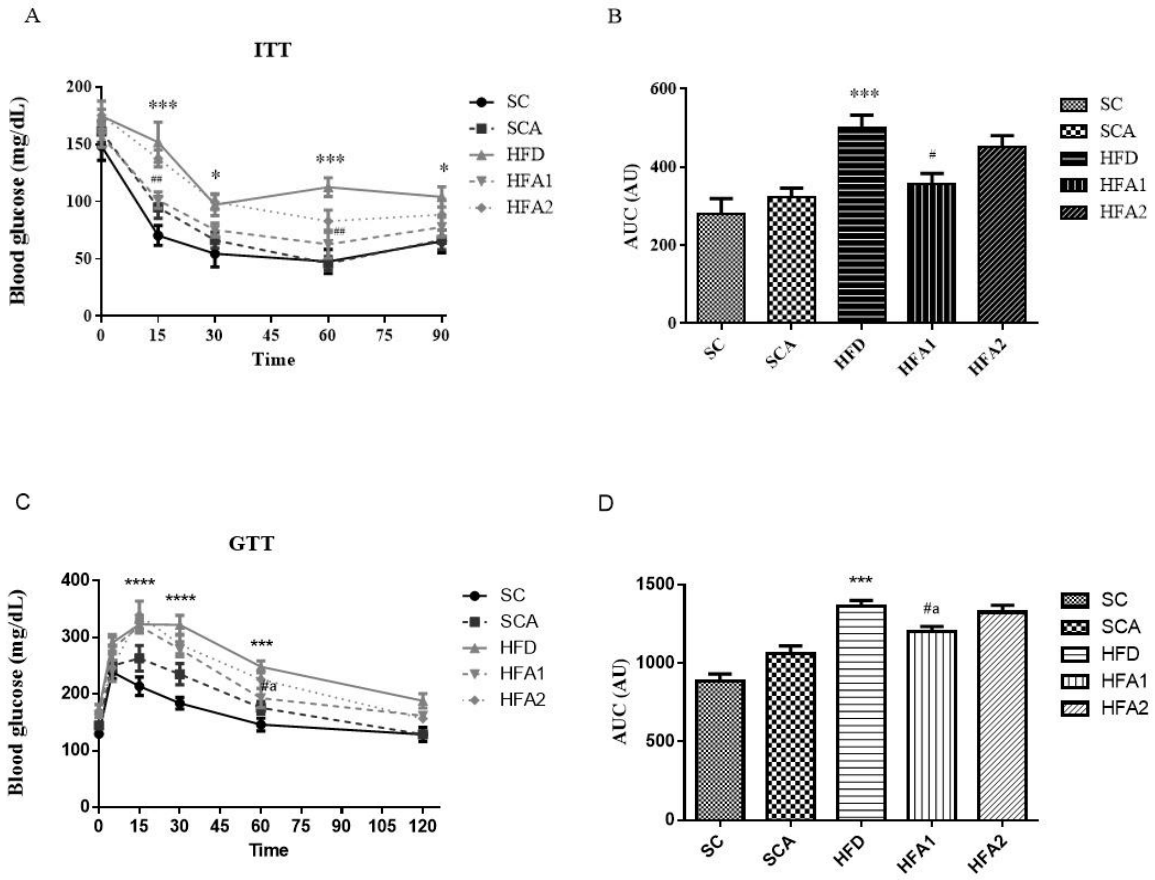
Day cycle
Night cycle

Figure 4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Blood glucose (mg/dL)
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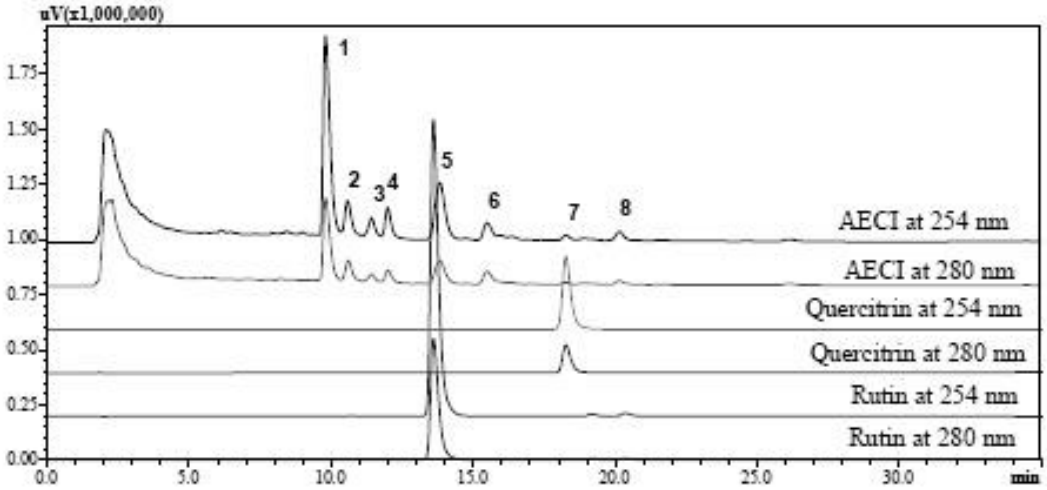
Figure 5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Quercitrin at 280 nm
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Capítulo 2: Rutin treatment of an insulin resistance TNFα-induced N2a cell model.

Artigo submetido ao periódico International Journal of Molecular Sciences, fator de impacto 2,862 - apêndice F.

Instruções para autores – apêndice G.

Abstract

Rutin can exert a protective role in oxidative stress in the brain and acts improving dyslipidemia and insulin sensitivity in peripheral tissues. However, until this date, nothing is known about its role in central insulin resistance. The aim of this study was to develop a neuron model of TNF-α induced insulin resistance and investigate rutin enrollment in signaling pathways in preventing the development of an inflammatory mediated insulin resistance. Results showed that TNF-α induced the degradation of IκB-α (64.2%, $p<0.01$) and attenuated the Akt phosphorylation (36.1%, $p<0.001$) in these neuron cells. The rutin treatment tended to prevent the attenuation of Akt phosphorylation, showing an activation 14.4% higher than the group treated with TNF-α, however, it was not statistically significant ($p = 0,063$). No significant difference was observed in the IκB-α after rutin treatment. These findings indicates that N2a cells were able to mimic the TNF-α response in the insulin signaling, generating an inflammation-induced insulin resistance model. Moreover, the rutin treatment tended to activate Akt pathway, however it wasn't able to completely prevent the development of the inflammatory mediaded insulin resistance.

1. Introduction

The central nervous system (CNS) is a key determinant of both glucose and energy homeostasis (Porte, Baskin, Shwartz, 2005). This regulation is primarily mediated in the hypothalamus by orexigenic neuropeptides including melanin concentrating hormone, agouti-related protein (AgRP), neuropeptide Y (NPY), galanin and orexin, and by anorexigenic neuropeptides pro-opiomelanocortin (POMC), galanin-like peptide (GALP), cocaine- and amphetamine-regulated transcript (CART) and corticotropin-releasing factor (CRF) (Leibowitz and Wortley 2004). Several hormones act in these neurons to control these neuropeptides, leading to normal energy balance and the prevention of obesity as leptin and insulin. Reduced central nervous system insulin signaling from either defective secretion or action contributes to the pathogenesis of common metabolic disorders, including diabetes and obesity (Porte, Baskin, Shwartz, 2005). Thus, in the extensive search for therapeutic approaches and preventative measures, the CNS has emerged as an important target in tackling these chronic diseases.

Rutin has found application in health care system due to its wide biological activities, high safety margins and lower cost, showing a wide range of pharmacological applications due to its significant antioxidant properties (Sharma et al., 2013). It can be found in many natural products such as citrus fruits, red beans and buck-wheat but it is normally available as a supplement and consumed easily (Emura et al., 2007).

Conventionally used as antimicrobial, antifungal, and antiallergic agent, rutin has shown its multispectrum pharmacological benefits for the treatment of various chronic diseases such as Alzheimer's disease, cancer, hypertension, diabetes and hypercholesterolemia. Its use is advantageous over other flavonoids as it is a nontoxic and nonoxidizable molecule (Javed et al., 2012; Sharma et al., 2013).

In the brain, current studies demonstrated that rutin treatment attenuates oxidative stress in neuroinflamattion in rats (Javed et al., 2012; Xu et al., 2014). Reduced oxidative stress and glutathione disulfide (GSSG) content, and enhanced levels of glutathione (GSH), GSH peroxidase (GPx), GSH reductase (GRd), and GSH S-transferase (GST) was also observed in the hepatic tissue of rats along with reduction in hepatic triacylglycerol and cholesterol levels (Hsu et al., 2009). In skeletal muscle, rutin has been shown to stimulated glucose uptake via the PI3K, atypical protein kinase C and mitogen-activated protein kinase (MAPK) pathways (Kappel et al., 2013) and increase the expression of peroxisome proliferator-activated receptor (PPARγ) (Cai et al., 2012).

These findings demonstrates that rutin can exert a protective role in oxidative stress in the brain and act improving dyslipidemia and insulin sensitivity in peripheral tissues. However, until this date, nothing is known about its role in central insulin resistance. This study investigated the effects and signaling pathways involved in the rutin treatment of an insulin resistant TNF-α induced neuronal cell model.

2. Material and methods

2.1 Reagents

Akt, IkBα and Rabbit Ig with HRP conjugated antibodies were purchased from Research Products International Corp. All drugs were freshly prepared from concentrated stock solutions. Human recombinant insulin 100 U/mL (Eli Lilly, Indianapolis – IN, USA) was diluted in sterile phosphate buffered saline (PBS, pH 7.4). TNF-α (Sigma-Aldrich, St. Louis – MO, USA) was dissolved in PBS, pH 7.4 and added to culture medium at 10 ng/mL concentration. Rutin (Sigma-Aldrich, St. Louis – MO, USA) was dissolved in dimethyl sulfoxide (DMSO) and added to culture medium, being the final concentration of 200 μM.

2.2 Cell culture

N2a (mouse neuroblastoma cell line) were maintained in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose (Gibco) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals) at 37°C with 5% CO_2. For passaging the cells, 0.125% Trypsin-EDTA

(Gibco) was used as a dissociation reagent. N2a cells were split into 6 well plates and grown until they reached 70 - 80% confluence. Cells were starved for 6h by replacing the complete growth medium with nonsupplemented medium before they were treated simultaneously with TNF-α (10 ng/mL) or rutin (200 µM) or vehicle (PBS) for 30 minutes and then incubated with insulin (100 nM) or vehicle for 15 minutes. The cells were washed by ice-cold PBS and added a lysate buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM NaF, 5 mM EDTA, 1% Triton, 2 mM sodium orthovanadate and Roche cocktail protease inhibitor tablet) and left in a shaker for 30 minutes at 4 °C. The cell extracts were scraped off the plates and centrifuged at 13200 g for 30 minutes at 4 °C. Protein concentration of the cell lysate supernatant was measured using the Bio-Rad DC Protein Assay kit with a spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). Protein lysate extracted from N2a cells were mixed with loading buffer (3x1), heated for 5 min at 100 °C and then stored at -20 °C for further use.

2.3 Western blotting

The samples were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). After transfer, the membranes were blocked in 5% nonfat dry milk for 1 hour at room temperature and incubated with primary antibody diluted in a solution of 5% bovine serum albumin in TBS with 0.1% Tween 20 at 4°C overnight. The primary antibodies used were total IkB-α antibody (1:1,000; Cell Signaling Technology, Beverly, MA), p-Akt antibody (1:1,000; Cell Signaling Technology), total Akt antibody (1:1,000; Cell Signaling Technology), p-ERK antibody (1:1,000; Cell Signaling Technology) and total ERK (1:1,000; Cell Signaling Technology). The membranes were then incubated with anti-rabbit IgG conjugated with HRP for 1 hour at room temperature. Visualization was done with enhanced chemiluminescence (ECL plus or prime, General Electric). The Western blot films were scanned at 600 dpi in grayscale mode. Background signal was eliminated by selecting bands of interest using wand (tracing) tool. The band's intensity was calculated using ImageJ's gel analyzer (Muta, Morgan, Rahmouni, 2015).

2.4 Statistical analysis

Data are presented as means ± SEM. All data were analyzed by one way ANOVA with Sidak's multiple comparison test. A p-value less than 0.05 was considered statistically significant. Prism 6 (GraphPad Software, Inc.) was used for statistical analysis.

3. Results

3.1 Effect of TNF-α and rutin on inflammatory signaling in cultured N2a cells.

Results showed that TNF-α reduced IkB-α activation in 60.6% ($p<0.01$), indicating a protein degradation in these neuronal cells (Figure 1). Rutin treatment wasn't able to prevent any degradation.

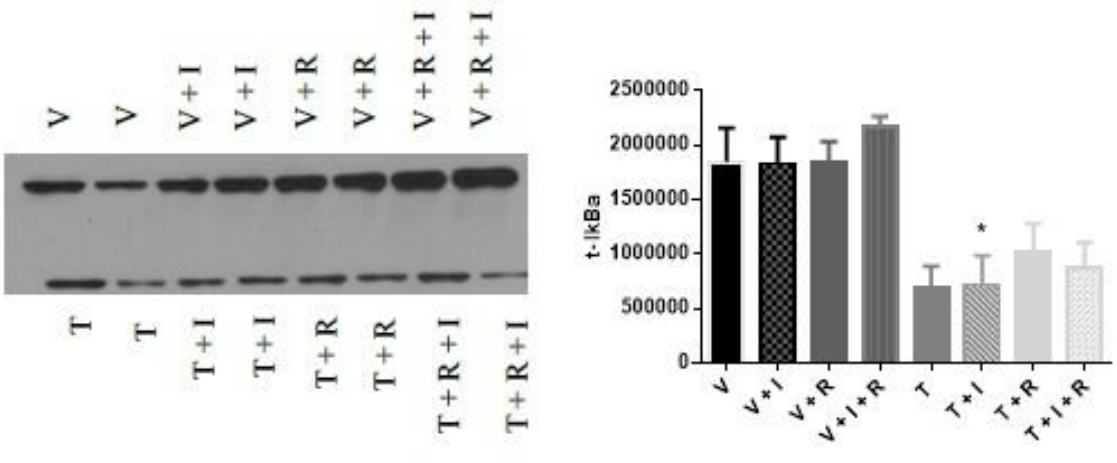

Figure 1: Effect of TNF-α and rutin on inflammatory signaling in cultured N2a cell line. Data presented are Western blots and bar graphs showing total IkBα in neuroblastoma cells treated with vehicle (V), vehicle + insulin (V + I), vehicle + rutin (V + R) and vehicle + rutin + insulin (V + R + I), TNF-α + vehicle (T + V), TNF-α + insulin (T + I), TNF-α + rutin (T + R) and TNF-α + rutin + insulin (T + R + I). Data are means ± SEM (n = 6). $^{**}p<0.01$ vs. V + I, one-way ANOVA with Sidak post hoc.

3.2 Effect of TNF-α and rutin in insulin signaling in cultured N2a cells.

As shown in Fig. 2, TNF-α reduced insulin-stimulated p-Akt production by 36.1% ($p<0.001$). Thus, cultured N2a cells are capable of developing inflammation-induced insulin resistance. Rutin treatment, on the other hand, tended to prevent the attenuation in p-Akt, showing an activation 14.4% higher than the group treated with TNF-α, however, it was not statistically significant ($p = 0,063$).

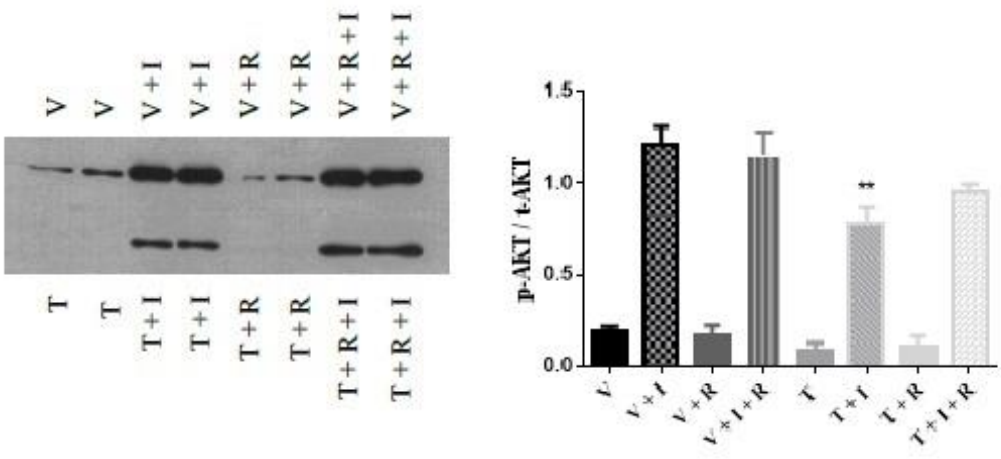

Figure 2: Effect of TNF-α and rutin in insulin signaling in cultured N2a cell line. Data presented are Western blots of p-Akt and bar graphs showing phospho/total Akt in neuroblastoma cells treated with vehicle (V), vehicle + insulin (V + I), vehicle + rutin (V + R), vehicle + rutin + insulin (V + R + I), TNF-α + vehicle (T + V), TNF-α + insulin (T + I), TNF-α + rutin (T + R) and TNF-α + rutin + insulin (T + R + I). Data are means ± SEM (n = 6). $^{***}p<0.001$ vs. V + I, one-way ANOVA with Sidak post hoc.

4. Discussion

Tumor necrosis factor-alpha (TNF-α) is a multi-functional cytokine that can regulate many cellular and biological processes such as immune function, cell differentiation, proliferation, apoptosis and energy metabolism (Cawthorn and Sethi, 2008).

The elevation in TNF-α levels is a mediator of obesity-related insulin resistance and type 2 diabetes. It suppresses the expression of many proteins that are required for insulin-stimulated glucose uptake in adipocytes, such as the insulin receptor, insulin receptor substrate-1 (IRS-1), protein kinase B (AKT) and GLUT4. TNF-α is also involved in the suppression of PPARy activity and expression and C/EBPα mRNA expression, which could be one of the causes of the GLUT-4 decreased expression (Ruan et al., 2002). Impairment of activation of IR and IRS-1 has also been shown to occur in skeletal muscle (Hotamisligil et al., 1996). In liver, TNF-α interferes with insulin signaling, thereby favoring steatosis (Crespo et al., 2001).

In the brain TNF-α is elicited following injury, infection, neurodegeneration and chemically induced neurotoxicity. The multifarious identity for this cytokine appears to be influenced by several mechanisms. Among the most prominent are the regulation of TNFα-

induced NF-κB activation by adapter proteins such as TRADD and TRAF (Sriram and O'Callaghan, 2007)

The activation of nuclear factor-kappa B (NF-κB) along with mitogen-activated protein kinase (MAPK) cascades, involving extracellular signal-regulated protein kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) are also involved in the effects observed by TNF-α in adipose tissue. These pathways could be responsible for mediating metabolic dysregulation (Ruan et al., 2002; Rydén et al., 2002).

In this study, TNF-α mediated a proinflammatory stimulus in the N2a cells, resulting in insulin resistance. It suppressed the expression of the IkBα, activating the NF-κB pathway. The NF-κB proteins regulate the transcription of a variety of cellular genes critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. In its inactive form, NF-κB is sequestered in the cytoplasm of most cells regulated by an inhibitor protein, IkBα, which binds to NF-κB via its ankyrin repeats (Baeuerle, Baltimore, 1988). Treatment with TNF-α causes the phosphorylation of IkBα, which is followed by its ubiquitination and subsequent degradation. The inhibitor of kappa B kinase (IKK) complex, a multiprotein kinase complex is responsible for the TNF-α induced phosphorylation of IkBα. These events lead to NF-κB translocation to the nucleus, binding to cognate DNA binding sites, and activation of transcription, resulting in the inflammatory response (Miyamoto et al., 1994).

Normally, in muscle cells and adipocytes the excess of saturated fatty acids induces insulin resistance through several mechanisms, including toll-like receptor-4 (Tlr4), reactive oxygen species formation, endoplasmic reticulum (ER) stress and mitochondrial dysfunction, induced by JNK and MAPK-ERK and NF-κB activation (Davis et al., 2009; Coll et al., 2006). However, little is known about the effects of excess lipids in the brain. Benoit et al. (2009) demonstrated that intracerebroventricular injection of palmitate attenuates hypothalamic insulin signaling and has been linked to changes in PKC subcellular localization, indicating that the lipoxicity may be also involved in central insulin resistance. In the study of Mayer and Belsham (2010) with mHypoE-44 neuron cells, the same saturated fatty acid also attenuated insulin signaling with a minimum of 24 h treatment. However, the stimuli in higher dose or for a longer period of time caused toxicity, inducing apoptosis of the neuron cells.

Contradictorily, in studies conducted by Choi et al. (2010), it was demonstrated that saturated fatty acid exposure does not induce inflammatory signaling or insulin resistance in cultured hypothalamic neurons. Although in this study different neuronal cell cultures were used, N43/5 and GT1–7, they were able to induce inflammation-induced insulin resistance with the TNF-α (10 ng/ml) stimuli, but for one hour.

An attempt was made to replicate Choi et al. (2010) study, however, the responses were not the same initially and the difficulty and time consuming proliferation of GT1-7 cells hindered the development of this model. In this way, the N2a cells, which are more easily manipulated and fast proliferated, were used and the results demonstrated an inflammation-induced insulin resistance neuronal cell model with the same concentration used previously but with a shorter TNF-α time induction.

Concerning the effects of rutin treatment in this inflammatory mediated insulin resistance neuronal cell model, it was observed that this compound didn't show any effect on IκB-α protein or NF-κB pathway, but had a tendency in preventing the attenuation in p-Akt caused by the TNF-α stimuli.

Rutin is a flavonoid found in many plants and has been shown to have some biological activities, but its direct effects on cells of the CNS have not been well studied. Studies by Javed et al. (2012) and Xu et al. (2014) have reported an attenuation of oxidative stress and neuroinflamation in rodent models of Alzheimer's disease, preventing cognative impairments and improving special memory. These effects were related to enhanced level of GSH, GPx, GRd as well as to decreased interleukin (IL)-1β and IL-6 levels in the brain (Xu et al., 2014). Javed et al. (2012) also observed that rutin reduced the expression of cyclooxygenase-2 (COX-2), glial fibrillary acidic protein (GFAP), interleukin-8 (IL-8), inducible nitric oxide synthase (iNOS) and NF-kB, thereby attenuating neuroinflammation.

This inhibitory effect on NF-κB and TNF-α pathway was also observed in nephrotoxicity of rats pre-treated with rutin. The compound prevented deteriorative effects induced by cisplatin through a protective mechanism that involved reduction of increased oxidative stress as well as caspase-3, TNF-α and NF-κB protein expression levels (Arjumand, Seth, Sultana, 2011).

Although these findings suggest rutin involvement in the suppression of NF-kB protein expression, its activity could be mediated through the atypical pathway which is IKK independent or indirectly by the inhibition of other proteins that would activate the NF-kB pathway. These suppositions would justify the absence in the IκB-α response observed in this study. Moreover, the experiments of the reports mentioned were conducted in rodents and with different doses.

Regarding rutin effect on insulin resistance, no reports were found with neuronal cells, however, some researches with muscle have reported some different pathways activations. In myotubes, rutin (10 μM, overnight) potentiated insulin receptor kinase (IRK) and Akt phosphorylation when IRK autophosphorylation was triggered by insulin attenuated S961-

mediated inhibition of insulin-dependent GLUT4 translocation (Hsu et al., 2014). Stimulation of glucose uptake via PI3K atypical protein kinase C and mitogen-activated protein kinase (MAPK) pathways was also observed in rat soleus muscle treatment with rutin (500 µM for 90 min) (Kappel et al., 2013). Furthermore, reduction in serum lipid profile has also directly influenciated rutin benefits in increasing insulin sensitivity in muscle and adipose cells (Fernandes et al., 2010).

In 3T3-L1 cells, Hsu and Yen (2007) observed inhibition of intracellular trigliceride, of glycerol-3-phosphate dehydrogenase (GPDH) activity, of the expression of PPARγ, CCAAT/enhancer-binding proteins (C/EBPα) and leptin, and up-regulation of the adiponectin expression at the protein level, showing the best results with rutin, 250 µM concentration, treatment for 72h.

As demonstrated in this study rutin (200 µM) treatment for 30 min tended to stimulate Akt pathway, however, it wasn't able to significantly prevent the attenuation in p-Akt induced by the TNF-α stimulation in N2a cells. In this way, it is suggested for further researches increased time of rutin treatment and / or different concentrations. Another fact that must be considered is the investigation of others protein expressions involved in the insulin signaling. These suggestions could contribute for the elucidation of rutin's role in the CNS.

5. Conclusions

These data indicates that N2a cells were able to mimic the TNF-α response in the insulin signaling, generating an inflammation-induced insulin resistance model that can provide the basis for the development of researches involving new therapeutic agents that can activate neuronal insulin signal transduction. This model has proven to be faster and viable, without causing any cell death, compared with other neuron insulin resistant cell models. Moreover, rutin treatment of these insulin resistant cells could activate Akt pathway contributing to prevent the damage induced by the TNF-α.

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6. Considerações finais e perspectivas

Esta tese consistiu no desenvolvimento de dois modelos experimentais para que se fizesse possível a realização de seu objetivo principal, a proposta de duas novas terapêuticas no tratamento da obesidade e da resistência à ação da insulina.

No primeiro capítulo foi desenvolvido um modelo de obesidade induzido por dieta hiperlipídica, em camundongos machos C57BL/6J, que reproduziu características semelhantes ao modelo humano, como aumento de peso corpóreo e de tecido adiposo associado à resistência à insulina e tolerância à glicose, possivelmente decorrente de uma maior eficiência energética, maior eficiência metabólica e menor atividade locomotora.

O tratamento com o AECI, em sua menor concentração, durante 8 das 14 semanas de experimento, promoveu, em comparação ao grupo que recebeu a mesma ração sem o extrato, menor ganho de massa adiposa e menor índice de adiposidade, menores níveis de triglicérides circulantes e maior ganho de massa magra, além de normalização da sensibilidade à insulina e tolerância à glicose. Acredita-se que esses benefícios tenham se dado, em parte, em função de uma maior atividade locomotora observada por esses animais, que possivelmente possa ter potencializado a utilização de gordura e glicose e/ou inibido seu armazenamento. O fato do extrato também ter reduzido a excreção fecal de lipídios, exclui o mecanismo de menor absorção intestinal de gordura como parte dos efeitos do extrato.

O extrato em sua maior concentração, apesar de também ter promovido aumento da atividade locomotora, tanto no grupo que recebeu dieta padrão como no que recebeu dieta hiperlipídica, favoreceu a um maior consumo de dieta e ingestão de extrato e, consequentemente, maior eficiência energética. Esse fato poderia justificar a ausência de alterações significativas sobre a adiposidade e homeostase glicêmica nos animais desses grupos.

Acredita-se que nem sempre uma maior concentração de uma droga vai exercer efeito maior ou melhor, fato que pode ser observado em outros estudos realizados, até mesmo com o uso do AECI. Outra consideração importante é que a presença dos polifenóis rutina e quercetina no extrato pode ter contribuído para sua atividade antioxidante, influenciando também na atenuação do quadro de resistência à insulina e na adiposidade instalada nos animais obesos.

A partir desse trabalho surgem diversas linhas de estudos para possíveis atuações do AECI. Algumas perspectivas para trabalhos futuros e que ajudariam a determinar o mecanismo exato de ação do extrato poderia envolver: quantificação dos AGs no sangue, fígado e nos tecidos periféricos; investigação da histologia das ilhotas pancreáticas e do fígado; observação do efeito sobre determinados fatores de transcrição como o PPRAy e a C/EBPα; investigação da influência do extrato sobre adipocinas diretamente envolvidas no quadro da obesidade e

diabetes tipo 2 como a leptina, adiponectina, TNF-α e IL-6; quantificação da insulina; investigação da ação do extrato sobre pontos específicos da via de sinalização da insulina, seja a nível da PI(3)K, Akt ou GLUT-4; e, determinação da resposta da atividade antioxidante exercida pelo extrato nos órgãos alvos envolvidos na fisiopatologia da obesidade e diabetes tipo 2.

O segundo capítulo da tese traz um modelo de células neuronais N2a resistentes à ação da insulina induzida por TNF-α e propõe o tratamento com a rutina, um dos compostos majoritários do AECI. O tratamento das células com TNF-α gerou uma degradação das proteínas IκB-α, favorecendo ativação da via NF-κB e induzindo o processo inflamatório. Paralelamente, o TNF-α também promoveu uma atenuação da fosforilação da proteína Akt, interferindo na sinalização da insulina e induzindo resistência à ação da mesma. O tratamento com rutina, realizado concomitantemente ao do TNF-α, não foi capaz de evitar a degradação da IκB-α, mas tendeu a evitar em parte a atenuação da fosforilação induzida pelo TNF-α. Dessa forma, sugere-se que a ativação da Akt possa ser uma das vias de atuação da rutina em células neuronais.

Muito pouco é discutido na literatura científica a respeito da ação da rutina sobre a resistência à insulina e a obesidade, não só em relação a seu mecanismo periférico, mas principalmente, sua ação central. Dessa forma, sugere-se que novas pesquisas sejam desenvolvidas, levando em consideração outras doses de rutina e outros períodos de tratamento, e ainda, que investiguem a ação da rutina sobre outras proteínas envolvidas na via de sinalização da insulina, seja em nível de receptor, dos substratos do receptor, PI3K e GLUT-4, ou até mesmo em vias alternativas independentes da insulina. A ação da rutina sobre a expressão das proteínas de determinadas adipocinas e citocinas também seria de grande importância.

Diante do exposto, esta tese apresenta grande relevância na literatura científica por apresentar duas novas propostas terapêuticas, o extrato aquoso de *Chrysobalanus icaco* e a rutina. O primeiro mostrou resultados importantes na redução de gordura em modelo experimental, apresentando potencial para futura aplicação no tratamento em humanos. O segundo, em contrapartida, não apresentou dados suficientes que comprovassem seus benefícios terapêuticos, porém, o modelo de resistência aqui apresentado aliado a dados prévios obtidos por outros autores, podem servir de base e direcionamento para o estudo de outras vias que possivelmente possam demonstrar o potencial da rutina na obesidade e resistência à insulina.

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Apêndices

Apêndice A: Declaração de aprovação do projeto pelo Comitê de Ética em Pesquisa com Animais da Universidade Federal de Sergipe.

UNIVERSIDADE FEDERAL DE SERGIPE
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COORDENAÇÃO DE PESQUISA
COMITÊ DE ÉTICA EM PESQUISA COM ANIMAIS (CEPA)

DECLARAÇÃO

Declaro, para os devidos fins, que o Projeto de Pesquisa intitulado **"Análise dos efeitos do extrato aquoso de abajeru (*Chrysobalanus icaco*) sobre a sensibilidade à ação da insulina e na expressão de adipocinas em camundongos obesos."**, sob coordenação do **Prof. Dr. MÁRCIO ROBERTO VIANA DOS SANTOS** (protocolo **CEPA 51/2011**) foi aprovado pelo Comitê de Ética em Pesquisa com Animais da Universidade Federal de Sergipe, em reunião realizada dia 13/06/2011.

São Cristóvão, 14 de junho de 2011

Josemar Sena Batista

Prof. Dr. Josemar Sena Batista
Vice-Presidente (em exercício) do CEPA/UFS

Cidade Universitária "Prof. Aloísio de Campos"
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Apêndice B: Artigo publicado no periódico Arquivos Brasileiros De Metabologia e Endocrinologia como produção paralela ao desenvolvimento da tese.

artigo original

Modelo de obesidade induzida por dieta hiperlipídica e associada à resistência à ação da insulina e intolerância à glicose

Model of high-fat diet-induced obesity associated to insulin resistance and glucose intolerance

Pollyanna A. S. White[1], Luana M. Cercato[2], Jéssica M. D. Araújo[2], Lucas A. Souza[2], Andréa F. Soares[2], Ana Paula O. Barbosa[1], José M. de R. Neto[3], Anderson C. Marçal[4], Ubiratan F. Machado[5], Enilton A. Camargo[1], Márcio R. V. Santos[1], Luciana C. Brito[2]

RESUMO

Objetivo: Validar um modelo de obesidade induzida por dieta hiperlipídica, de baixo custo, fácil reprodutibilidade, que mimetizasse características observadas no humano e viabilizasse posteriores proposições terapêuticas. Materiais e métodos: Dezesseis camundongos Swiss receberam dieta padrão (DP) ou dieta hiperlipídica (DH), durante 10 semanas. Resultados: Embora o grupo DP tenha apresentado maior consumo de água ($p < 0,01$) e ração ($p < 0,001$), o grupo DH apresentou maior ganho de peso corpóreo ($p < 0,5$) e aumento de coxins adiposos ($p < 0,001$), favorecendo maior índice de adiposidade ($p < 0,001$), glicemia ($p < 0,01$) e área sob a curva nos testes de tolerância à insulina ($p < 0,001$) e à glicose ($p < 0,01$). Conclusão: Validou-se um modelo de obesidade induzida por dieta hiperlipídica associada à resistência à ação da insulina e à intolerância à glicose, em um período de 10 semanas. Arq Bras Endocrinol Metab. 2013;57(5):339-45

Descritores
Obesidade; dieta hiperlipídica; resistência à insulina; intolerância à glicose; camundongos

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ABSTRACT

Objective: Validate a model of high-fat diet-induced obesity, of low cost, easy reproducibility, that could express characteristics observed in human, and would enable subsequent therapy proposals. Materials and methods: Sixteen Swiss mice received a standard diet (DP) or high-fat diet (DH) for 10 weeks. Results: Although the DP group had greater water ($p < 0.01$) and feed ($p < 0.001$) consumption, the DH group had greater body weight ($p < 0.5$) and adipose tissue gain ($p < 0.001$), favoring higher adiposity index ($p < 0.001$), glucose ($p < 0.01$), and area under the curve in the insulin ($p < 0.001$) and glucose ($p < 0.01$) tolerance tests. Conclusion: A high-fat diet-induced obesity model has been validated, which was also associated with insulin resistance and glucose intolerance after a period of 10 weeks. Arq Bras Endocrinol Metab. 2013;57(5):339-45

Keywords
Obesity; high-fat diet; insulin resistance; glucose intolerance; mice

Apêndice C: Artigo publicado no periódico *Molecules* como produção paralela ao desenvolvimento da tese.

Molecules **2014**, *19*, 14496-14527; doi:10.3390/molecules190914496

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Review

Antioxidant Activity and Mechanisms of Action of Natural Compounds Isolated from Lichens: A Systematic Review

Pollyanna A. S. White [1,*], Rita C. M. Oliveira [2,3], Aldeidia P. Oliveira [2,3], Mairim R. Serafini [4], Adriano A. S. Araújo [5], Daniel P. Gelain [6], Jose C. F. Moreira [6], Jackson R. G. S. Almeida [7], Jullyana S. S. Quintans [1], Lucindo J. Quintans-Junior [1] and Marcio R. V. Santos [1,*]

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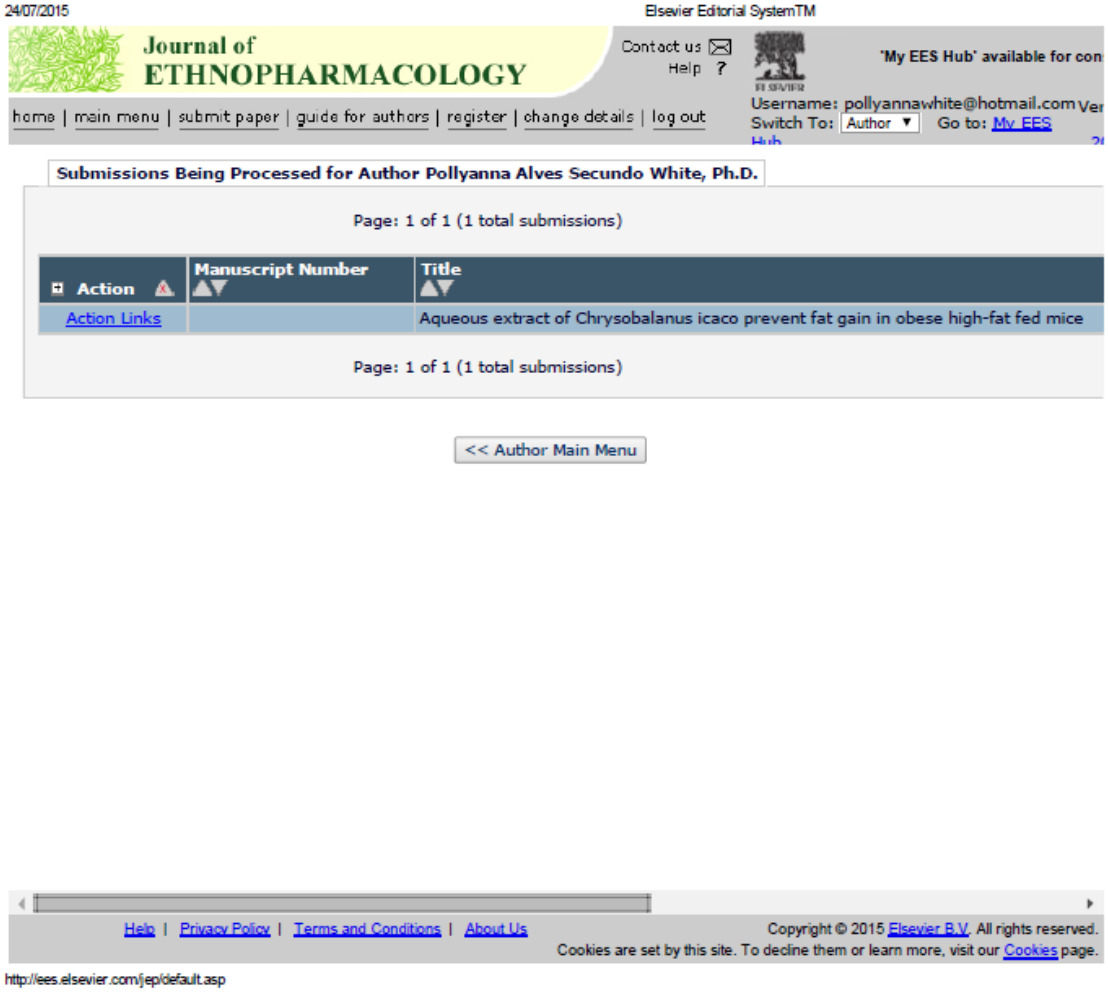
Abstract: Chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases are characterized by an enhanced state of oxidative stress, which may result from the overproduction of reactive species and/or a decrease in antioxidant defenses. The search for new chemical entities with antioxidant profile is still thus an emerging field on ongoing interest. Due to the lack of reviews concerning the antioxidant activity of lichen-derived natural compounds, we performed a review of the antioxidant potential and

Apêndice D: Artigo submetido para publicação no periódico *Journal of Ethnopharmacology*.



Apêndice E: Instruções para autores para publicação no periódico *Journal of Ethnopharmacology*.

ELSEVIER

JOURNAL OF ETHNOPHARMACOLOGY

An Interdisciplinary Journal Devoted to Indigenous Drugs

AUTHOR INFORMATION PACK

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DESCRIPTION

The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their **biological** and **pharmacological effects** based on the principles established through international conventions. Early people confronted with illness and disease, discovered a wealth of useful **therapeutic agents** in the plant and animal kingdoms. The empirical knowledge of these **medicinal substances** and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of **indigenous remedies**. Chemists continue to use **plant-derived drugs** (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

In recent years the preservation of local knowledge, the promotion of indigenous medical systems in primary health care, and the conservation of biodiversity have become even more of a concern to all scientists working at the interface of social and natural sciences but especially to ethnopharmacologists. Recognizing the sovereign rights of States over their natural resources, ethnopharmacologists are particularly concerned with local people's rights to further use and develop their autochthonous resources.

Accordingly, today's ethnopharmacological research embraces the multidisciplinary effort in the:

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- search for pharmacologically unique principles from existing indigenous remedies.

The *Journal of Ethnopharmacology* publishes original articles concerned with the observation and experimental investigation of the biological activities of plant and animal substances used in the traditional medicine of past and present cultures. The journal will particularly welcome interdisciplinary papers with an **ethnopharmacological**, an **ethnobotanical** or an **ethnochemical** approach to the study of indigenous drugs. Reports of **anthropological** and **ethnobotanical** field studies fall within the journal's scope. Studies involving **pharmacological** and **toxicological** mechanisms of action are especially welcome. Clinical studies on efficacy will be considered if contributing to the understanding of specific ethnopharmacological problems. The journal welcomes review articles in the above mentioned fields especially those highlighting the multi-disciplinary nature of ethnopharmacology. Commentaries are by invitation only.

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The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their biological and pharmacological effects based on the principles established through international conventions. Early people, confronted with illness and disease, discovered a wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of indigenous remedies. Chemists continue to use plant-derived drugs (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

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Froede, T.SA. and Y.S. Medeiros, Y.S. (2008) Animal models to test drugs with potential antidiabetic activity. Journal of Ethnopharmacology 115: 173-183. Gertsch J. (2009) How scientific is the science in ethnopharmacology? Historical perspectives and epistemological problems. Journal of Ethnopharmacology, 122: 177-183.

Chan K., et al. (2012) Good practice in reviewing and publishing studies on herbal medicine, with special emphasis on traditional Chinese medicine and Chinese Materia Medica. Journal of Ethnopharmacology 140: 469-475.

Heinrich, M., Edwards. S., Moerman. D.E.. and Leonti. M. (2009), Ethnopharmacological field studies: a critical assessment of their conceptual basis and methods. J. Ethnopharmacol, 124: 1-17.

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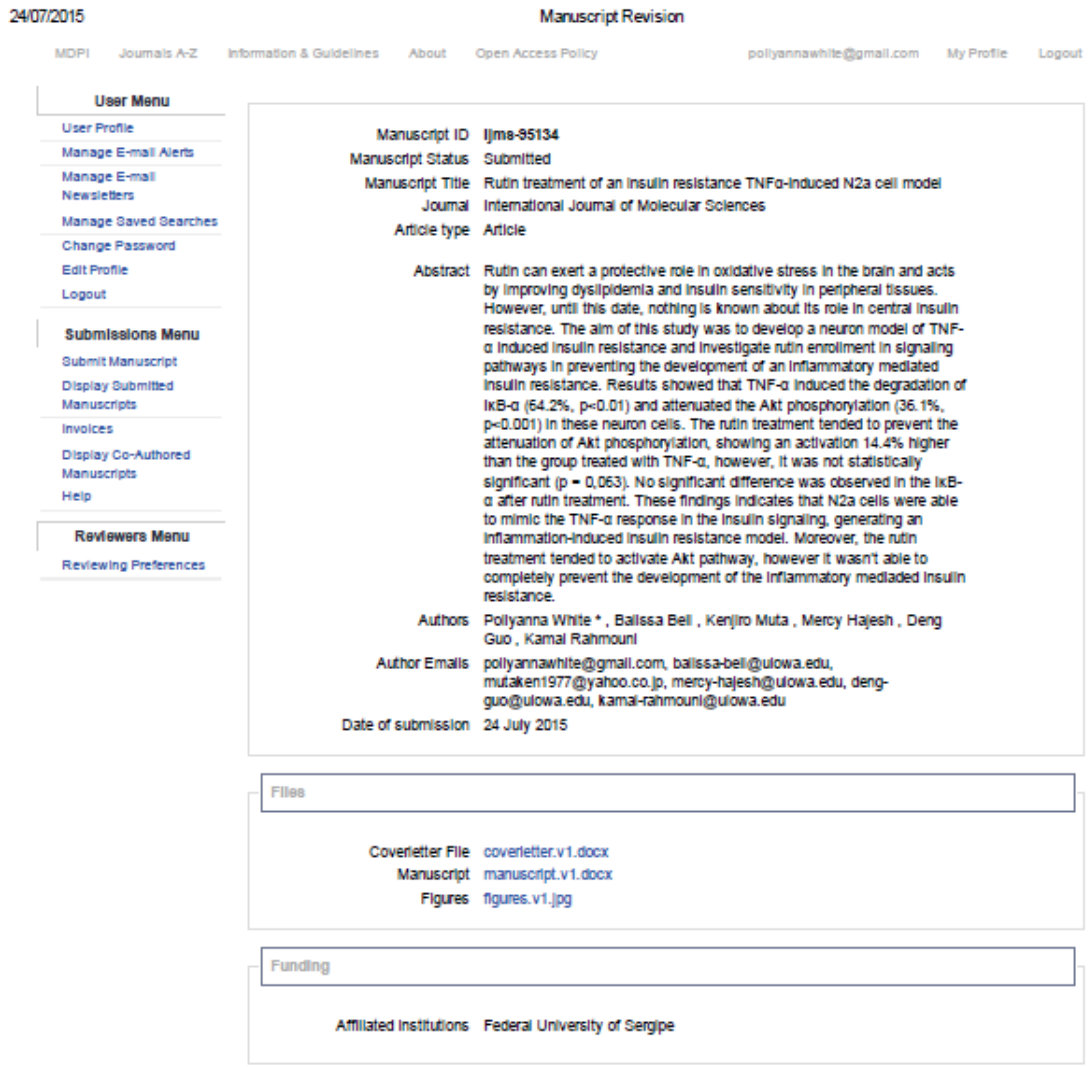

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Journal	International Journal of Molecular Sciences
Article type	Article
Abstract	Rutin can exert a protective role in oxidative stress in the brain and acts by improving dysilipidemia and insulin sensitivity in peripheral tissues. However, until this date, nothing is known about its role in central insulin resistance. The aim of this study was to develop a neuron model of TNF-α induced insulin resistance and investigate rutin enrollment in signaling pathways in preventing the development of an inflammatory mediated insulin resistance. Results showed that TNF-α induced the degradation of IκB-α (64.2%, p<0.01) and attenuated the Akt phosphorylation (36.1%, p<0.001) in these neuron cells. The rutin treatment tended to prevent the attenuation of Akt phosphorylation, showing an activation 14.4% higher than the group treated with TNF-α, however, it was not statistically significant (p = 0,063). No significant difference was observed in the IκB-α after rutin treatment. These findings indicates that N2a cells were able to mimic the TNF-α response in the insulin signaling, generating an inflammation-induced insulin resistance model. Moreover, the rutin treatment tended to activate Akt pathway, however it wasn't able to completely prevent the development of the inflammatory mediaded insulin resistance.
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