UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Estudo dos efeitos dos extratos das folhas de *Bauhinia microstachya*: análise *in vitro*, potencial antioxidante e hipoglicemiante em ratos diabéticos induzidos por aloxano

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Tese submetida ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito para obtenção do grau de Doutor em Bioquímica.

Porto Alegre, 2007

“...o mundo foi feito para o homem,

ou o homem foi feito para o mundo?”

Ismael, personagem

do livro homônimo

de Daniel Quinn.

Agradecimentos

Às pessoas de minha vida, que além de agradecimentos, registro desculpas, pelas vezes de ausência, pela presença muitas vezes confusa e pela tumultuada constância, mas sempre com respeito e lealdade;

À unidade HAL-9000, pela bravura e virtudes sempre demonstradas, bem como ao Obscuro, de movimentada contribuição;

A todos os colegas do célebre Centro de Estudos em Estresse Oxidativo (Laboratório 32, Departamento de Bioquímica, UFRGS);

Ao PPG Bioquímica (UFRGS), às agências financiadoras brasileiras e riograndense de fomento à pesquisa e de bolsas de pós-graduação – CAPES, CNPq, PROPESQ/UFRGS e FAPERGS;

Aos funcionários do Departamento de Bioquímica da UFRGS;

Aos professores do PPG-Bioquímica da UFRGS;

Ao Val pela força na identificação e coleta da planta. Ao colega Guilherme Antônio Behr, pela dedicada ajuda e contribuição neste trabalho;

Ao co-orientador Felipão, por todos bons exemplos deixados para serem seguidos por nós;

Ao orientador Zé, pela amizade, compreensão e qualificação, além da oportunidade dada a mim, de ser parte de tão egrégio grupo de pesquisa.

RESUMO

Diabetes mellitus (DM) é um grupo de anomalias metabólicas caracterizadas principalmente pela hiperglicemia. Componentes macro- e micro-vasculares, além de neuropáticos inter-relacionados com a elevação da glicemia são fatores freqüentemente envolvidos em acidentes cérebro-vasculares, infartos miocárdicos, doença vascular periférica, retinopatia e nefropatia diabética. Na literatura científica atual pode-se obter um grande número de trabalhos onde o uso de plantas medicinais, extratos ou substâncias vegetais isoladas é positivamente relacionado ao tratamento do DM e/ou seus efeitos diretos e indiretos. *Bauhinia microstachya* (RADDI) MACBR. (Caesalpinaceae) é uma planta que ocorre naturalmente na região sul do Brasil onde é conhecida popularmente como “escada-de-macaco”. Algumas populações fazem uso de preparações das folhas no tratamento do DM e suas complicações. Neste trabalho propomos que os extratos aquoso e hidro-etanólico, obtidos das folhas de *B. microstachya* possuem ação antioxidante *in vitro* e que o extrato hidro-etanólico apresenta poder antioxidante *in vivo*, além de efeito hipoglicemiante, apesar de ser potencialmente hepatotóxico.

Palavras-chave: *Bauhinia microstachya*, diabetes, antioxidante, toxicidade e cardiomiopatia.

ABSTRACT

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia. Both DM types share aspects that are related to uncontrolled hyperglycaemia: alterations in the carbohydrate, fat, and protein metabolism. In addition, increased cardiovascular disease risk, retinopathy, nephropathy, and neuropathy are vascular complications associated to diabetes. In scientific literature there are several reports where the use of folk medicine is positively related to DM treatment and/or their direct and indirect effects. *Bauhinia microstachya* (RADDI) MACBR. (Caesalpinaceae) is a creeper plant that occurs naturally in southern Brazil where is popularly known as “escada-de-macaco” (monkey’s ladder) and often used as herbal antidiabetic medicine. In the present work, we propose that the extracts obtained from *B. microstachya* leaves have *in vitro* and *in vivo* antioxidant action, hypoglycemic effect, despite their hepatotoxic potential.

Keywords: *Bauhinia microstachya*, diabetes, antioxidant, toxicity e cardiomyopathy.

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SIGLAS E ABREVIATURAS

AEBM – extrato aquoso obtido a partir das folhas de *B. microstachya*

AGE's – produtos avançados de glicação

ALT – alanina aminotrasnferase

ALX – aloxano

AST – aspartato aminotrasferase

CAT – catalase

DM – diabetes mellitus

EAO – espécies ativas de oxigênio

EAN – espécies ativas de nitrogênio

ECDCDM – Expert Committee on the Diagnosis and Classification on Diabetes

GST – glutationa-S-transferase

HCAs – ácidos hidroxicinâmicos

HDL – lipoproteína de alta densidade

HEBM – extrato hidro-etanólico obtido a partir das folhas de *B. microstachya*

LDL – lipoproteína de baixa densidade

OH – grupamento hidroxil

OMS – Organização Mundial de Saúde

SOD – superóxido dismutase

VLDL – lipoproteína de baixíssima densidade

WHO – World Health Organization

2. LISTA DE FIGURAS

Parte I.

1. INTRODUÇÃO

Diabetes mellitus (DM) é um grupo de anomalias metabólicas caracterizadas principalmente pela hiperglicemia (aumento dos níveis de glicose no sangue), resultado de defeitos na secreção de insulina, em sua ação, ou em ambos. Componentes macro- e micro-vasculares, além de neuropáticos inter-relacionados com a elevação da glicemia (um dos parâmetros mais comumente monitorados para fazer o diagnóstico e avaliar a terapia) são fatores freqüentemente envolvidos em acidentes cérebro-vasculares, infartos miocárdicos, doença vascular periférica, retinopatia e nefropatia diabética. Trata-se de uma complexa enfermidade, na qual coexiste uma desordem generalizada do metabolismo dos carboidratos, lipídios e proteínas, com conseqüências diretas sobre as rotas que abrangem estas e outras biomoléculas. Apresenta uma natureza multifatorial pela existência de diferentes fatores envolvidos em sua gênese e persistência. A Organização Mundial de Saúde (OMS) estima uma prevalência de DM na população adulta de 7,9% já em 2005, com um valor esperado ao redor de 9% para 2025.

Nas últimas décadas foi alcançado um conhecimento muito mais consistente sobre a patogenia e, em alguns casos, sobre a etiologia das várias categorias de diabetes. Com base nesses novos achados, tanto o *Expert Committee on the Diagnosis and Classification on Diabetes* (ECDCDM, 1997*)*, como a Organização Mundial de Saúde (OMS) (WHO, 1997) propuseram uma nova classificação para as principais formas de diabetes:

- A categoria do diabete designado tipo 1, inclui todas formas que são causadas por destruição primária auto-imune das células β do pâncreas ou algum outro tipo de defeito primário da função deste tipo celular, que leve a uma secreção de insulina insuficiente para os tecidos periféricos alvo.

- A categoria designada tipo 2 inclui a forma mais comum de diabetes, resultante da resistência dos tecidos periféricos alvo à ação da insulina, combinada a uma secreção inadequada de tal hormônio.

1.1. DM e doença cardiovascular

Complicações vasculares como retinopatia e neuropatia são comumente encontradas em pacientes diabéticos sem controle sobre a glicemia, além disso, o risco aumentado de doença cardiovascular é fortemente associado ao DM (Clark et al., 2002; Shore, 2002; King et al., 1994). Essa doença metabólica é o principal fator para o desenvolvimento de complicações cardiovasculares e doenças relacionadas, respondendo por 80% de mortalidade em diabetes (WHO, 2004). Alguns estudos reportando o estado prejudicado da função cardíaca já foram reportados, tanto no DM experimental como na clínica (Norton, Candy & Wodddiwiss, 1996; Cai et al., 2002; Price, Verna & Li, 2003; Illarion et al., 2003).

1.2. Breve história

De acordo com que indicam os registros históricos, o DM acompanha a humanidade desde antes da era cristã. No papiro de Ebers descoberto no Egito, correspondente ao século XV antes de Cristo, já se descrevia sintomas que parecem corresponder ao DM.

Porém, foi Areteu da Capadócia quem, no século II da era cristã, deu a esta doença o nome de diabetes, que em grego significa sifão, referindo-se a um dos seus sintomas mais marcantes que é a alta taxa de eliminação de água pelos rins, sugerindo que a água entrava (polidipsia que significa sede excessiva) e saía do organismo (poliúria que é a eliminação de grande volume de urina em um determinado período) do diabético sem “fixar-se” nele. Ainda no século II, Galeno, contemporâneo de Areteu da Capadócia, também se referiu ao diabetes, atribuindo-lhe à incapacidade dos rins em reter água de maneira adequada.

Nos séculos posteriores não se encontram nos registros médicos referências a esta enfermidade até que, no século XI, Avicena fala com clara precisão desta afecção em seu famoso Cânon da Medicina.

Após um longo intervalo sem registros aparentes foi Thomas Willis quem, em 1679, fez uma descrição do diabetes, magistral para a época, ficando desde então reconhecida por sua sintomatologia como entidade clínica. Foi ele quem, referindo-se ao sabor doce da urina, lhe deu o nome de diabetes mellitus (sabor

de mel), apesar de esse fato já ter sido registrado cerca de mil anos antes na Índia, por volta do ano 500.

No entanto foi somente em 1775 que Dopson identificou a presença de glicose na urina. Frank, por essa altura também, classificou a diabetes em duas formas: diabetes mellitus (ou vera), e insípida, esta sem apresentar urina doce. A primeira observação feita através de uma necropsia em um diabético foi realizada por Cawley e publicada no London Medical Journal em 1788. Quase na mesma época o inglês John Rollo, atribuindo à doença uma causa gástrica, conseguiu melhorias notáveis com um regime rico em proteínas e gorduras e limitado a ingestão de carboidratos (Wikipedia, 2007).

1.3. A utilização de plantas medicinais

O homem primitivo foi usuário das mais diversas formas vegetais que encontrava ao seu redor, selecionando algumas para sua alimentação e rejeitando diversas outras pela existência de efeitos indesejáveis quando consumidas. A partir destas experiências empíricas de tentativas e erros surgiu a agricultura ancestral e o uso de plantas para aliviar os mais diversos males conhecidos pelos povos antigos.

Durante muito tempo as plantas medicinais e seus derivados constituíram a base da terapêutica e, cerca de 25% dos fármacos utilizados atualmente são de origem vegetal, enquanto outros 50% são de origem sintética, porém

estruturalmente relacionados às substâncias isoladas de plantas medicinais (Chechinel-Filho & Yunes, 1998). A recente literatura científica vem registrando de maneira crescente, estudos que comprovam o que se conhece empiricamente, visto que a medicina popular é rica em exemplos de plantas utilizadas para diversos fins, que substituem, muitas vezes, a prescrição médica. Acredita-se que cerca de 80% da população mundial, principalmente nos países considerados em desenvolvimento, use as plantas como primeiro recurso terapêutico (Cragg, Newman & Snader, 1997).

1.3.1. DM e as plantas

Da mesma maneira que o DM, indícios da utilização de plantas medicinais e tóxicas foram encontrados nas mais antigas civilizações. O próprio papiro de Ebers, já mencionado anteriormente, descrevia uma dieta rica em germe de trigo como terapia funcional para DM, cujo efeito hipoglicêmico foi demonstrado cientificamente (Bailey & Day, 1989). Na literatura científica atual pode-se obter um grande número de trabalhos onde o uso de plantas medicinais, extratos ou substâncias vegetais isoladas é positivamente relacionado ao tratamento do DM e/ou seus efeitos diretos e indiretos.

1.4. O gênero *Bauhinia*

Entre as inúmeras espécies vegetais de interesse medicinal, encontram-se as plantas do gênero *Bauhinia*. As plantas deste grupo pertencem à família

Caesalpinaceae que contém aproximadamente 250 espécies (Cronquist, 1981) e são encontradas, principalmente, nas áreas tropicais do planeta. Populações nativas e tradicionais destas regiões utilizam as folhas e outras partes aéreas, nas formas de infusão e outras preparações fitoterápicas, como remédios e afins (Da Silva & Cechinel-Filho, 2002; Kumar et al., 2005). A figura 1 mostra espécimes de *B. variegata*, comuns na arborização urbana.

No Brasil, as plantas do gênero *Bauhinia* mais conhecidas são as popularmente chamadas de “Pata-de-vaca” ou “Unha-de-boi”. As folhas, caules e raízes das espécies de *Bauhinia*, especialmente *B. manca*, *B. rufescens*, *B. forficata*, *B. cheitantha* e *B. splendens*, são amplamente utilizadas no Brasil e em outros países do continente, na forma de chás e outras preparações, para o tratamento de várias enfermidades, principalmente infecções, processos dolorosos e diabetes (Corrêa, 1984; Da Silva & Cechinel-Filho, 2002; Achenbach et al., 1988).

1.4.1. *Bauhinia microstachya*

Bauhinia microstachya (RADDI) MACBR. (Caesalpinaceae) é uma planta com hábito de liana (trepadeira) que ocorre naturalmente na região sul do Brasil onde é conhecida popularmente como “escada-de-macaco”. No entanto, *B. microstachya* aparece algumas vezes como planta cultivada em pequenas propriedades de agricultura familiar. Este cultivo se deve muitas vezes pela incapacidade de diferenciar esta espécie de outras espécies de *Bauhinia*, levando-

se em conta principalmente o formato característico das folhas. Esta falta de discriminação se reflete também nos usos populares e preparações da espécie. Algumas populações fazem uso de infusões das folhas ou garrafadas (preparação com cachaça ou outra bebida alcoólica e partes vegetais) no tratamento do DM e suas complicações. Entretanto, o hábito contumaz de ingestão de bebidas alcoólicas entre alguns grupos deve ser considerado ao analisar os usos de vegetais em garrafadas.

Os estudos fitoquímicos sobre as folhas de *B. microstachya* identificaram alguns compostos como glicosídeos esteroidais, triterpenos, lactonas e compostos fenólicos, principalmente flavonóides (Meyre-Silva et al., 2001; Gadotti et al., 2005; Bianco & Santos, 2003). O efeito antiespasmódico em um modelo experimental (Ramos et al., 2005) e a atividade antioxidante *in vitro* (Menezes et al., 2004) já foram registradas em trabalhos recentes sobre *B. microstachya*. A figura 2 mostra diversas partes de *B. microstachya*.

Figura 1.

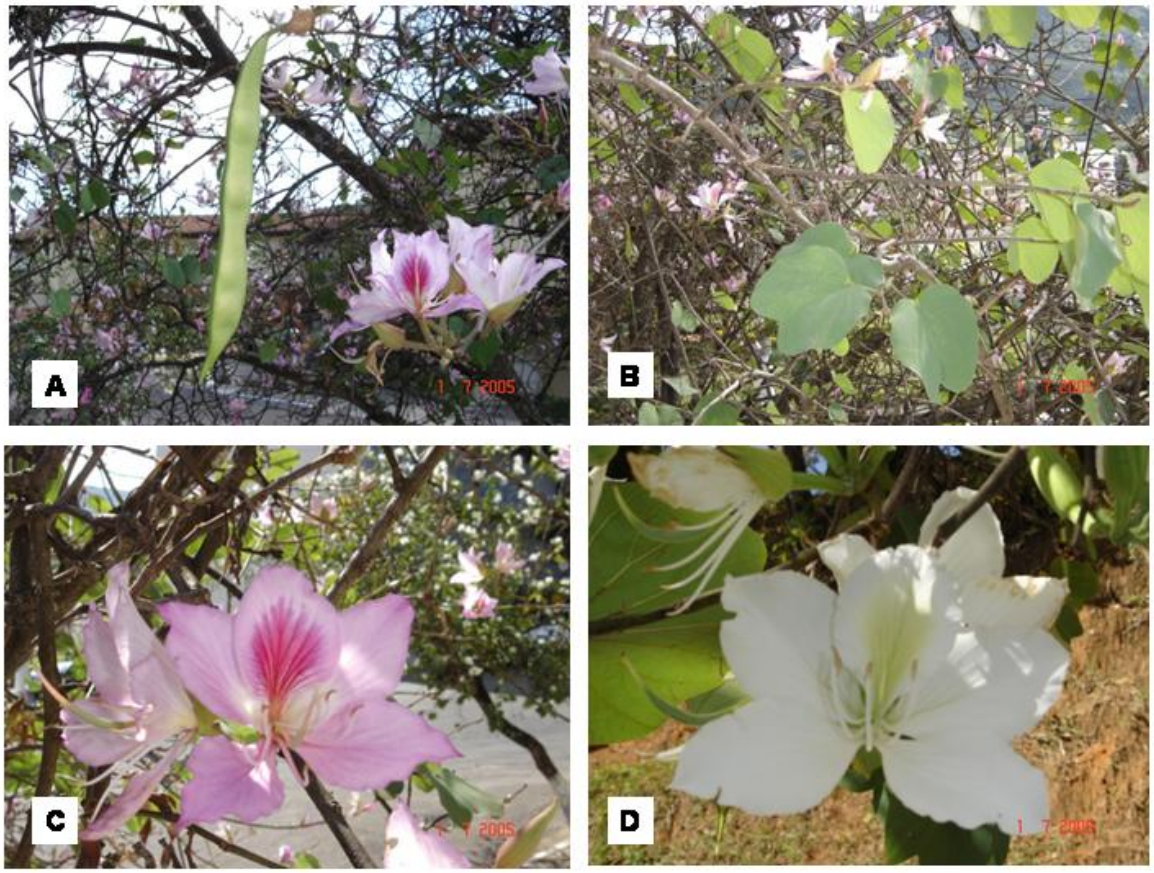

Figura 1. *B. variegata*, uma das espécies de *Bauhinia* mais comum em arborização urbana. **(A)** Vista geral com flores e frutos; **(B)** Formato típico das folhas; **(C)** Detalhe das flores; **(D)** Flores brancas ou albinas são comuns nesta espécie. Todas fotos obtidas pelos autores.

Figura 2.

Figura 2. *Bauhinia microstachya* coletada pelos autores no município de Carlos Barbosa (RS). **(A)** Inflorescências; **(B)** Hábito típico de liana; **(C)** Detalhe das folhas; **(D)** Folhas e caules em escala com caneta. Todas fotos obtidas pelos autores no local da coleta, exceto foto (A).

1.5. Compostos fenólicos

Os compostos fenólicos constituem uma grande classe de moléculas naturais onipresentes no reino vegetal. Os compostos fenólicos são substâncias químicas caracterizadas por possuir pelo menos um anel aromático (C6) carregando um ou mais grupamentos hidroxil (OH). Ácidos hidroxicinâmicos (HCAs) e flavonóides têm esqueleto básico de carbono C6-C3 e C6-C3-C6, respectivamente. HCAs e flavonóides são produzidos a partir da fenilalanina, via rota do chiquimato, rota geral de fenilpropanóides e rota específica de flavonóides, figura 3 (Herrmann, 1995).

1.5.1. Propriedade antioxidante de compostos fenólicos

Esses metabólitos secundários são reconhecidos por demonstrar uma notável variedade de interações bioquímicas que se acredita vir de suas propriedades antioxidantes (Rice-Evans et al., 1995). Essas moléculas podem modular os efeitos de espécies ativas de oxigênio (EAO) em um grande número de processos biológicos. O fato de conter um ou mais grupos OH ligado à estrutura em anel, confere a estes compostos, atividade antioxidante, porém às vezes podem ocorrer na forma glicosilada. Os polifenóis de plantas são multifuncionais e podem atuar como agentes redutores, antioxidantes doadores de elétrons (“scanvenger”), como eliminadores (“quenchers”) de oxigênio singlet e, em alguns casos, podem ser quelantes de metais (Rice-Evans, Miller & Paganga, 1996).

Figura 3.

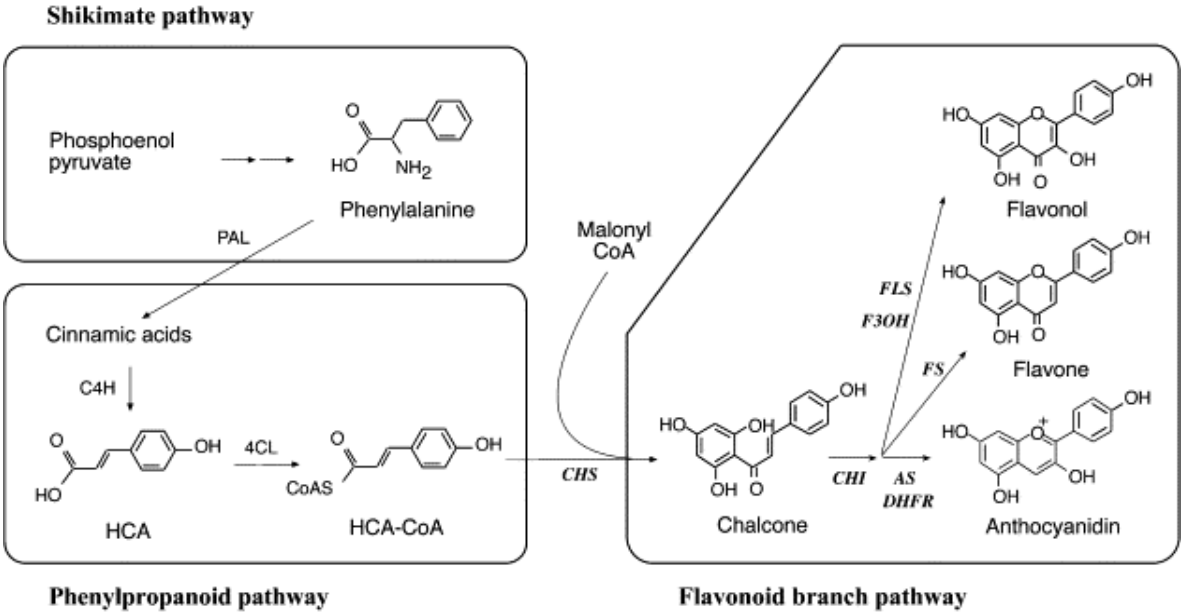

Fig. 3 - Diagrama da rota biossintética de fitofenóis. Fenilalanina amonia liase (PAL); cinamato-4-hidroxilase (C4H); 4-coumarato:CoA ligase (4CL); ácido hidroxicinâmico (HCA); hidroxicinamoil CoA (HCA - CoA); chalcona sintetase (CHS); chalcona isomerase (CHI); flavanona 3-hidroxilase (F3OH); flavona sintase (FS); flavonol sintase (FLS); dihidroflavonol redutase (DHFR) e antocianina sintase (AS). Fonte: Sakihama et al, 2002.

Para um polifenol ser definido como um antioxidante ele deve atender duas condições básicas: primeira, quando presente em baixas concentrações relativa ao substrato a ser oxidado, ele deve adiar, retardar ou prevenir a auto-oxidação ou a oxidação mediada por radicais livres (Halliwell, 1990); segunda, o radical resultante formado após a captura deve ser estável e menos reativo, diretamente pela ligação intramolecular de hidrogênio na oxidação adicional (Shahidi & Wanasundara, 1992). Por outro lado, há trabalhos que demonstram que o comportamento destes compostos como antioxidantes e até mesmo como pró-oxidantes em sistemas oxidativos, são relacionados em função de sua estrutura/atividade (Cao, Sofic & Prior, 1997; Rice-Evans, Miller & Paganga, 1996).

1.6. Oxigênio e espécies ativas de oxigênio (EAO)

O aparecimento de seres aeróbicos foi um marco na evolução biológica, pois o emprego do oxigênio aumenta consideravelmente a extração de energia dos substratos energéticos. As reações que utilizam o oxigênio no metabolismo energético são chamadas de óxido-redução, em que há trocas de elétrons entre os reagentes.

O oxigênio é parte essencial da vida aeróbica, porém uma conseqüência indesejada é a formação de radicais livres e outras EAO (Halliwell e Gutterdge, 1998). O oxigênio não é tóxico *per se*, mas os produtos originados pelas reações onde o oxigênio é parte essencial, podem ser danosos a importantes componentes celulares como lipídios, proteínas e ácidos nucléicos.

Entretanto tem sido conclusivamente demonstrado que as espécies ativas de oxigênio que sempre são formadas durante o metabolismo regular dos organismos, não são de todo danosas, servindo, por exemplo, como sinalizadores de estado redox (Halliwell e Gutterdge, 1998). A produção aumentada de EAO é considerada como um aspecto universal e comum na condição de estresse oxidativo.

1.6.1. DM e EAO

Índices aumentados na produção de EAO e, conseqüentemente de estresse oxidativo tecidual (como os medidos pela elevação das taxas de peroxidação lipídica e oxidação de proteínas), tem sido registrado em ambas as formas de diabetes retrocitadas (Sato et al., 1979; Velazquez et al., 1991; Yaqoob et al., 1994; Santini et al., 1997; Cedeberg, Basu & Eriksson, 2001). Níveis aumentados de lipoproteína de baixa densidade (LDL) oxidada ou sua sucebilidade para oxidação, também têm sido documentados em DM (Yaqoob et al., 1994; 1996; Santini et al., 1997).

No que pese diversas formas de evidências experimentais indicando que a produção aumentada de EAO e o estresse oxidativo podem determinar o início e principalmente a progressão das complicações tardias do DM (Baynes, 1991; Van Dam et al., 1995; Giugliano, Ceriello & Paolisso, 1996), há controvérsias sobre quanto o estresse oxidativo é meramente associado tardiamente ou fator causal

(ou componente de fator causal) do DM. Tais discussões originam-se parcialmente, pelas determinações de estresse oxidativo empregadas em grande parte dos estudos ser baseada em medidas indiretas e não específicas de produtos gerados por EAO, e parcialmente porque muito dos estudos em pacientes com DM foram executados em representações limitadas de grandes grupos (Laaksonen & Sen, 2000).

Os mecanismos por trás do evidenciado aumento de produção de EAO e estresse oxidativo não são completamente entendidos. A acumulação de evidências até o presente momento indica que pode haver alguns mecanismos inter-relacionados (Lyons, 1993; Cameron and Cotter, 1993; Tesfamariam, 1994; Cameron, Cotter & Hohman, 1996), produção aumentada de radicais livres como o superóxido (Nath, Chari & Rathi, 1984; Ceriello et al., 1991; Wolff, Jiang & Hunt, 1991; Dandona et al., 1996) ou diminuição do status antioxidante (Asayama et al., 1993; Tsai et al., 1994; Ceriello et al., 1997; Santini et al., 1997). Esses mecanismos incluem a glicação (ligação não enzimática da glicose a outras biomoléculas) (Hunt, Smith & Wolff, 1990; Wolff, Jiang & Hunt, 1991), a formação de produtos avançados de glicação (AGE's) (Lyons, 1993; Schleicher, Wagner and Nerlich, 1997), ativação da rota dos polióis (Cameron, Cotter & Hohman, 1996; Cameron and Cotter, 1993; Grunewald et al., 1993; Kashiwagi et al., 1994; De Mattia et al., 1994; Kashiwagi et al., 1996), metabolismo do ascorbato (Sinclair et al., 1991), redução na atividade de enzimas antioxidantes (Arai et al., 1987; Blakytny & Harding, 1992; Kawamura et al., 1992), alteração do status redox celular, assim como desequilíbrios na relação glutationa reduzida/ glutationa

oxidada (Grunewald et al., 1993; Kashiwagi et al., 1994; 1996; De Mattia et al., 1994) e perturbações no metabolismo de óxido nítrico e das prostaglandinas (Tesfamariam, 1994; Maejima et al., 2001).

2. OBJETIVOS

2.1. Geral

Levando em consideração os grandes impactos sociais, econômicos e na área da saúde, gerado pelo DM; a inexistência em nosso grupo de pesquisa, e quiçá, em nosso Departamento, de um modelo experimental de estresse oxidativo relacionado ao DM; a utilização de diversas espécies de *Bauhinia* pela população em geral no tratamento do DM (algumas reportadas cientificamente como positivas no tratamento de DM e de seus efeitos) e a inexistência de trabalhos anteriores focando a espécie nativa *Bauhinia microstachya* no tratamento de DM, consideramos como razoável ser o objetivo geral desta tese responder à questão central: o uso de folhas de *B. microstachya* contra DM é seguro e adequado? Para responder a esta questão procuramos responder as questões abaixo que se caracterizaram como nossos objetivos específicos.

2.2. Específicos

1- Os animais fornecidos pelo Biotério do Departamento de Bioquímica da UFRGS são passíveis de serem usados em um modelo de DM induzido pelo aloxano? Quais particularidades existentes?

2- Qual o perfil de constituição fitoquímica dos extratos das folhas de *B. microstachya*? Possuirão os extratos de *B. microstachya* poder antioxidante?

3- Qual o impacto do uso agudo e crônico de extratos de *B. microstachya* em animais diabéticos? Há relação com o uso popular e com outras espécies de *Bauhinia*?

4- Qual(is) efeito(s) cardíaco do uso crônico de extratos de *B. microstachya*? Qual(is) possível(is) mecanismo(s)?

Parte II.

Capítulo 1

Differences in pancreas β-cells morphology and liver antioxidant enzymes in alloxan-resistant and alloxan-susceptible Wistar rats

Manuscrito submetido à

Cell Biology and Toxicology (2007)

Editorial Manager(tm) for Cell Biology and Toxicology
Manuscript Draft

Manuscript Number:

Title: Differences in pancreas b-cells morphology and liver antioxidant enzymes in alloxan-resistant and alloxan-susceptible Wistar rats

Article Type: Original Research

Section/Category:

Keywords: Alloxan-resistant Wistar rats; oxidative stress; antioxidant enzymes; pancreatic islets; immunohistochemistry; aminotransferases.

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Manuscript Region of Origin:

Abstract: The aim of this study was to investigate biochemical and antioxidant parameters in alloxan-resistant (ALR) and alloxan-susceptible (ALS) rats. Diabetes was induced in 60-day-old male Wistar rats by a single intraperitonial injection of alloxan (AL, 150 mg/Kg). Ten days after induction a group of rats showed a significant decrease in glycemia. This group was named alloxan-resistant group. Susceptible rats showed a remarkable increase in plasma lipid content, blood glucose and HbA1. Glycogen content in liver decreased significantly in the ALS group (2.08±0.41 mg%), compared with ALR group (4.22±0.18). Aspartate aminotransferase and alanine aminotransferase activities were quantified in plasma. Interestingly, ALR rats showed a decrease in both activities (42.1±6.11, 21.7±5.54 U/mL) when compared with ALS rats (59.1±6.55,

58.1±7.28). TBARS index was significantly increased in ALS liver (0.38±0.08 nm/mg protein) when compared with ALR (0.18±0.04). Superoxide dismutase and catalase activities in ALR (230±13, 131±15 U/mg protein) liver showed a marked increase when compared with ALS (148±13, 68±5). This study also revealed that pancreatic islets of ALR rats display a different morphology amongst the groups. These results suggest a participation of antioxidant enzymes in hepatic protection of ALR rats and may be the key to resistance to alloxan.

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Differences in pancreas β-cells morphology and liver antioxidant enzymes in alloxan-resistant and alloxan-susceptible Wistar rats

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Note: The authors declare that they have no conflicts of interest.

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Brazil), Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (Fapergs) and Pró-Reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (Propesq/UFRGS).

Abstract

The aim of this study was to investigate biochemical and antioxidant parameters in alloxan-resistant (ALR) and alloxan-susceptible (ALS) rats. Diabetes was induced in 60-day-old male Wistar rats by a single intraperitonial injection of alloxan (AL, 150 mg/Kg). Ten days after induction a group of rats showed a significant decrease in glycemia. This group was named alloxan-resistant group. Susceptible rats showed a remarkable increase in plasma lipid content, blood glucose and HbA1. Glycogen content in liver decreased significantly in the ALS group (2.08±0.41 mg%), compared with ALR group (4.22±0.18). Aspartate aminotransferase and alanine aminotransferase activities were quantified in plasma. Interestingly, ALR rats showed a decrease in both activities (42.1±6.11, 21.7±5.54 U/mL) when compared with ALS rats (59.1±6.55, 58.1±7.28). TBARS index was significantly increased in ALS liver (0.38±0.08 nm/mg protein) when compared with ALR (0.18±0.04). Superoxide dismutase and catalase activities in ALR (230±13, 131±15 U/mg protein) liver showed a marked increase when compared with ALS (148±13, 68±5). This study also revealed that pancreatic islets of ALR rats display a different morphology amongst the groups. These results suggest a participation of antioxidant enzymes in hepatic protection of ALR rats and may be the key to resistance to alloxan.

Keywords: Alloxan-resistant Wistar rats; oxidative stress; antioxidant enzymes; pancreatic islets; immunohistochemistry.

1. Introduction

Reactive oxygen species (ROS) overproduction is considered to be one of the major causes of several pathological disorders. These species are continuously generated under physiological conditions and effectively eliminated by intracellular and extracellular antioxidant systems. Oxidative stress has been defined as an unbalance between ROS production and protective antioxidant systems (Halliwell and Gutteridge, 1999).

During the last two decades many studies suggested the relationship between oxidative stress and diabetes. In this syndrome, oxidative stress is probably related to an increase in ROS production, sharp reduction in antioxidant defense and altered cellular redox status (West, 1999). Uncontrolled ROS production often leads to damage in cellular macromolecules (DNA, lipids and protein) and other small antioxidant molecules (Halliwell, 1999), contributing to the progress of diabetic complications.

Experimental diabetes models can be induced by chemicals that selectively destroy the insulin-producing β-cells in the pancreas (Szkudelski, 2001). One of the most used chemicals is alloxan (AL, 2,4,5,6-tatraoxyorimidine; 5,6-dioxyuracil). This drug induces diabetes by intracellular generation of ROS formed in a cyclic reaction involving alloxan and its reduced product called dialuric acid (Munday, 1988), with subsequent inhibition of insulin synthesis and secretion.

The dose of AL required to induce diabetes depends on the animal species, route of administration and nutritional status, considering that some organisms are more resistant to AL induction. Inbred mouse strain produced in Japan by inbreeding CD-1 mice presents selective sensitivity to high or low AL concentrations generating two new mouse strains: AL-resistant (ALR/Lt) and AL-susceptible (ALS/Lt) (Mathews and Leiter, 1999). Biochemical analysis established that these two strains markedly differed in their abilities to dissipate free radical stress (Mathews et al., 2002). In the present work, we evaluated histological, biochemical and antioxidant parameters that distinguish susceptible rats from Wistar rats naturally resistant to alloxan-induced diabetes.

2. Materials and methods

2.1. Reagents and animals

Fifty 45-days-old male Wistar rats were obtained from our breeding stock. Rats were housed in plastic cages, maintained at 22±1°C, 55% relative humidity and 12 h light/dark cycle. Animals were supplied with commercial pellet food (Nuvilab ® CR-1 Curitiba, PR, Brazil) and water *ad libitum*. Alloxan (2,4,5,6-tatraoxypirimidine), thiobarbituric acid (TBA), 2,4-dinitrophenylhydrazine (DNPH), glutathione (GSH), 1-Chloro-2,4-nitrobenzene (CDNB), and

all chemicals used in the study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Diabetes induction

Diabetes was induced in forty 60-day-old male Wistar rats by a single intraperitonial injection of alloxan (150 mg/Kg) whereas the control group (n=6) received a saline injection. Both groups were injected after an 18-hour fasting period (60-70 mg/dL blood glucose). Fasting animals are more susceptible to alloxan probably due to partial protection by increased blood glucose (Szkudelski, 2001). All induced rats showed hyperglycemia (400-600 mg/dL) 48 hours after alloxan administration, 15-20% of the induced rats presented a pronounced decrease of glycemia around ten days after induction. They were called AL-resistant (ALR) group (n=7). During the experiment blood glucose level was monitored daily with Accu-Chek Active, blood glucose monitor, strips and lancing device (Roche Diagnostics, Germany). At the end of study, rats with glycemia between 400-600 mg/dL were considered diabetic and were called AL susceptible (ALS) group (n=8). Thirty days after induction all rats were weighted and killed by decapitation, and blood, pancreas and liver samples were collected. Glycated hemoglobin (HbA1) content in blood was quantified with a commercial kit (Human GmbH, Wiesbaden, Germany).

2.3. Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was performed four days before the end of study. On test day, rats were deprived of food for 12 hours (overnight fast). D-glucose solution (4 g/kg body weight) was given orally by gavage to conscious rats. Blood samples were collected by puncturing tail vein of each conscious rat sequentially before, 20, 50, 90, 150 and 310 min after OGTT.

2.4. Plasma, pancreas and liver samples

The plasma was separated to determine levels of total cholesterol, HDL, LDL, VLDL, and triglycerides. The plasma lipid profile was determined with a commercial kit (Human GmbH, Wiesbaden, Germany). Aspartate aminotransferase (AST, E.C. 2.6.1.1) and alanine aminotransferase (ALT, E.C. 2.6.1.2) plasma activities were quantified with commercial kit (In Vitro Diagnostica, Barbacena, Brazil). To immunohistochemical study, the whole pancreas from all animals was collected and fixed in 10% buffered formalin solution for 24 h, and embedded in paraffin. The liver was also removed, dried (air dried incubator, 45 ºC, 24 h), weighted, homogenized by buffer techniques and stored at - 70 ºC for further analyses.

2.5. Immunohistochemistry

For immunohistochemical insulin detection, pancreas tissue sections were incubated with citrate buffer pH 6.0 at 90°C for antigen retrieval before primary antibody application. Polyclonal guinea pig anti-insulin (Dako Diagnostics Canada, Mississauga, Canada) may be used at a dilution of 1:50 in the LSAB (Labelled Streptavidin/Biotin) amplification method. The morphology and insulin content of pancreatic islets was evaluated by optical microscopy.

2.6. Glycogen content in liver

A sample of liver tissue was weighted and digested in hot concentrated 30% KOH (100 °C for 20 minutes), precipitated with ethanol, hydrolyzed, and the glycogen content was then determined using colorimetric procedure with iodine (Krisman, 1962).

2.7. Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) widely adopted as an index of lipid peroxidation (Janero, 1990) were measured. The formation of TBARS occurs during an acid-heating reaction as previously described (Draper and Hadley, 1990). Briefly, the samples were mixed with 1mL of 10% trichloroacetic acid and 1mL of 0.67% thiobarbituric acid, and subsequently heated in a boiling-water bath for 15 minutes. The TBARS were determined by absorbance at 535 nm and were expressed as malondialdehyde equivalents (nM/mg protein).

2.8. Protein carbonyls

Protein oxidation is a marker of oxidative stress, and carbonyl content is a relevant marker of protein oxidation (Stadtman and Levine, 2000). The oxidative damage to proteins was assessed by determining carbonyl groups based on the reaction of carbonyls with dinitrophenylhydrazine (DNPH) as previously described (Levine et al., 1990). Briefly, protein liver samples were precipitated by adding 20% trichloroacetic acid and were reacted with DNPH, then the samples were redissolved in 6M guanidine hydrochloride. Carbonyl contents were determined by absorbance at 370 nm using a molar absorption coefficient.

2.9. Catalase and superoxide dismutase activities

To determine catalase (CAT, E.C. 1.11.1.6) activity, liver samples were sonicated in a 50 mM phosphate buffer in ice bath and the resulting suspension was centrifuged at 3000g for 10 min. The supernatant was used for enzyme assay. CAT activity was measured by the decreasing rate in hydrogen peroxide absorbance at 240 nm (Aebi, 1984). The superoxide dismutase (SOD, E.C. 1.15.1.1) activity in liver samples homogenate was measured spectrophotometrically by the inhibition rate of auto-catalytic adrenochrome formation in a reaction buffer containing 1 mM adrenaline/50 mM glycine-NaOH (pH 10.2)/1 mM catalase as previously described (Bannister and Calabrese, 1987).

2.10. Glutathione S-transferase activity

Glutathione S-transferase (GST, E.C. 2.5.1.18) activity was determined spectrophotometrically at 340 nm by measuring the formation of the conjugate of glutathione (GSH) and 1-Chloro-2,4-nitrobenzene (CDNB) as previously described (Habig and Jakoby, 1981). In short, liver samples were homogenized in 0.1 M potassium phosphate buffer pH 6.5. Enzyme activity was determined by adding 800 uL of buffer, 50 uL GSH 20 mM, a suitable amount of liver homogenate to a 1 mL cuvette. The reaction started by the addition of 50 uL of CDNB 20 mM was carried out at 30°C, and monitored spectrophotometrically for three minutes. Corrections of the spontaneous reaction were made by measuring and subtracting the rate in absence of enzyme.

2.11. Protein quantification

Protein content was measured as previously described (Lowry and Rosebough, 1951). Lipid peroxidation, protein carbonyls, SOD, CAT and GST activities results were standardized by protein content.

2.12. Data analysis

Results were expressed as mean±S.E.M. Statistical differences were evaluated using one-way ANOVA test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS 13.0) software. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Characterization of alloxan-resistant group

Blood glucose levels before induction showed no significant intra-group variation (60-70 mg/dL). Forty four hours after administration of AL, glycemia presented a fourfold increase ($p \leq 0.05$). Blood glucose level was monitored daily during the experiment (Fig. 1). On the tenth day 15-20% of the rats (n=7) presented significant decrease of glycemia (500±30 mg/dL to 280±29 mg/dL, $p \leq 0.05$). These rats were called AL-resistant (ALR) group. Thirty days after induction, blood glucose in control and ALR groups did not differ significantly (101±12 mg/dL and 135±15 mg/dL, respectively, $p \leq 0.05$). In our laboratory we have observed in several alloxan-induced diabetes experiments the presence of ALR group. These findings suggest that resistant rats would continue to persist in the colony.

3.2. Oral glucose tolerance test

Glucose tolerance was found to be decreased significantly in ALS group compared with the control and ALR groups (Fig. 2). No similar curves have been observed between control and

ALR groups. Resistant group showed an intermediate curve with differential blood glucose recovery profile (Fig. 2).

3.3. Biochemical parameters and plasma lipid profile

At the end of the experimental period, weight gain was significantly higher in the control and ALR groups (27±4% and 32±5%, respectively), while the ALS group showed a decrease in weight (22±5%) when compared with its baseline ($p = 0.05$). The final body weight did not differ in the control and ALR groups (321.6±10.9 g and 337±6.04 g, respectively), but it was smaller in the ALS group (210.8±10.1 g) (Table 1). Blood glucose and the proportion of glycated hemoglobin was about twice as higher in the ALS group as in the control and ALR groups (Table 1). The ALS group showed an increase in plasma lipid content in all analyzed parameters. The control and ALR groups did not differ significantly (Table 1). When taken together with other symptoms presented by diabetic rats – polyphagia, polydipsia and polyuria (data not shown) – these results confirmed the validity of alloxan-induced diabetes in rats.

3.4. Immunohistochemistry

All islets showed normal histological structures in hematoxilin-eosin observations amongst the groups (data not showed). In immunohistochemical staining using antiinsulin, ALR group presented many islet β-cells distributed in the central portion of the islets, with pronounced immunonegative peripheral cells. ALS group showed a pronounced decreased in immunoreactivity against antiinsulin in all pancreatic islet. Normal morphology was observed in control group (Fig. 3, 4).

3.5. Hepatic glycogen content and liver weight

Glycogen content in liver decreased twofold in the ALS group (2.08±0.41 mg%), compared with the control (4.23±0.21 mg%) and ALR (4.22±0.18 mg%) groups (Table 2). Absolute weight of ALS liver decreased significantly (Table 2). However, when expressed as percentage of body weight, ALS-rat liver weight showed a significant increase ($p \leq 0.05$) compared with the control and ALR groups. Similar results have been observed with dry weight (Table 2).

3.6. Oxidative stress parameters in liver

The lipid peroxidation levels in ALS liver (0.38±0.08 nM/mg protein) increased twofold when compared with the control (0.17±0.03 nM/mg protein) and ALR (0.18±0.04 nM/mg protein) groups (Table 3). No significant difference in protein oxidative damage was observed amongst the groups (Table 3). However, the ALS group showed increased values (56.71±6.92

nM/mg protein) when compared with control (28.59±9.29 nM/mg protein, p=0.11) and ALR (27.43±9.92 nM/mg protein, p=0.08) groups.

3.7. Enzyme activities

Liver SOD activity showed a threefold increase in the ALR group (23Õ±13 U/mg protein) when compared with the control (72±8 U/mg protein) group (Table 4). The ALS group also showed a twofold increase in SOD activity (148±13 U/mg protein) when compared with the control group. SOD activities in ALR and ALS groups are significantly different ($p \leq 0.05$). Liver CAT activity showed an increase in the ALR group (131±15 U/mg protein) when compared with the control (84±1 U/mg protein) group (Table 4). The ALS group showed a decrease (68±5 U/mg protein) in CAT activity when compared with the control ($p \leq 0.05$) group. No significant difference was observed in GST activities amongst control (0.035±0.003 U/mg protein), ALR (0.03Õ±0.002 U/mg protein) and ALS (0.033±0.002 U/mg protein) groups ($p \leq 0.05$) (Table 4). AST and ALT activities were quantified in plasma. The control and ALS groups did not differ significantly (Table 4). The ALR group showed a decrease in plasma activities in both aminotransferases (42±6 U/mL and 22±6 U/mL, respectively) when compared with the control group (65±7 U/mL and 43±5 U/mL, respectively) ($p \leq 0.05$).

4. Discussion

The use of AL to induce diabetes in experimental animal models has been extensively studied, and is quite well characterized now (Szkudelski, 2001). The AL mechanism of action is in part characterized by the involvement of ROS and consequently oxidative stress (Heikkila et al., 1976). One of the ROS targets is the DNA of the pancreatic islets, and its fragmentation takes place in β-cells exposed to alloxan (Sakurai and Ogiso, 1995). However, some organisms are more resistant to AL action, a dose-response study performed in tilapia islets grafts showed high-resistance to AL toxin (75, 150 and 300 mg/Kg) (Xu et al., 2004). Human islets transplanted into mice nude kidney capsule were not affected by *in vivo* AL exposure and the viability of human β-cells purified by flow cytometry was not affected by AL (5 mM) (Eizirik et al., 1994). ALR/Lt mice developed in Japan presented significant differences in the activities of multiple antioxidant enzymes when compared with closely related ALS/Lt strains (Mathews and Leiter, 1999a). In the present study, a group of Wistar rats that has become naturally resistant to alloxan diabetes induction (ALR) showed differences in the pancreatic islets morphology, liver antioxidant profile and plasma aminotransferases activities when compared with susceptible group (ALS).

Glucose tolerance was found to be significantly decreased in ALS group, that showed a pronounced decreased in immunoreactivity against antiinsulin in all pancreatic islets observed. Many studies have previously shown that rodents treated with alloxan have reduced insulin secretion and number of β-cells in islets, leading to diabetes (Szkudelski, 2001). The ALR group showed lower plasma glucose and areas under the OGTT curves and higher insulin levels than ALS group. In immunohistochemical staining using antiinsulin, ALR group presented islets with many β-cells distributed in the central portion and pronounced immunonegative peripheral cells. Similar morphology was observed in spontaneously diabetic Goto–Kakizaki rats development, before 7-days-old (Momose et al., 2006). The ability to uptake glucose by ALR group is observed in OGTT curves, these ability is relational to the high portion of insulin secreting cells shown in ALR pancreatic islets. No inflammatory infiltration have been observed in hematoxilin-eosin slides amongst the groups (data not show).

During the course of the experimental period control and ALR groups showed a gain in body weight of approximately 30%, while the ALS group showed a decrease of approximately 25%. Failure to gain body weight after AL administration has already been reported in animals (Raju et al., 2001). Five days prior to the end of the study, control and ALR groups showed no significant difference in blood glucose level, which remained unchanged until sacrifice day. Final glycemia, levels of HbA1, plasma lipid parameters, liver weight and hepatic glycogen content showed no significant difference when comparing the control group with the ALR group. The ALS group showed a hyperglycemic profile from the second day of the study on and also increased levels of HbA1 in blood and lipids in plasma. High levels of glycated proteins and alterations in lipid profile in AL diabetic animals have already been reported (Yadav et al., 2005; Chaturvedi et al., 2004). Literature on the effect of diabetes on liver weight is contradictory as some works have shown an increase in hepatic weight in rats (Vats et al., 2004) while others have reported no alterations in this parameter (Gupta et al., 1999). In the present study ALS rats showed significantly higher liver weight/100 g body weight, when compared with control and ALR rats. The data also showed a decrease in hepatic glycogen content in ALS rats. Diminished glycogen storage in diabetic rat liver has already been extensively reported in literature (Vats et al., 2004; Ong and Khoo, 2000).

Diabetes was induced in male Wistar rats by a single intraperitonial injection of alloxan. The action of this drug in the pancreas is preceded by its rapid uptake by the β-cells , although a similar uptake of alloxan also takes place in the liver (Szkudelski, 2001). The liver and other

tissues are more resistant to ROS in comparison to pancreatic β-cells and this resistance protects them against alloxan toxicity (Tiedge et al., 1997; Lenzen et al., 1996). Determination of classical markers of vertebrate liver damage (ALT and AST) in plasma has been done (El-Demerdash et al., 2005). It has already been reported that the activities of both AST and ALT were significantly increased in the plasma of AL diabetic rats as compared with control values (Mansour et al., 2002). The increment of the activities of AST and ALT in plasma may be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream, giving an indication of the hepatotoxic effect of alloxan. ALT is an index enzyme for liver damage, and it is specific to damage to this organ. AST occurs outside the liver, in organs such as the skeletal muscles, heart, kidneys, pancreas, spleen, and lungs. ALT and AST aminotransferases are tissue damage indicator enzymes. They leakage to serum not only in the case of damage to the tissue, but also when the permeability of the cell membrane increases. ALT, an enzyme occurring in the liver cytoplasm, moves more easily from the cell interiors to the serum than AST, which is mainly located inside the mitochondria (Regulska−Ilow et al., 2007). In the present work we were able to demonstrate an interesting decrease in both AST and ALT in plasma of the resistant group, and also no significant difference in both oxidative stress parameters such as TBARS and carbonyl measured in liver when compared with the control group. The results suggest the involvement of some protective mechanism in the liver of these rats. A possible explanation for the AST and ALT findings in resistant group is a decrease in the permeability of the liver cell membranes, as well in other tissues. Liver GST is an enzyme whose essential function is the biotransformation of xenobiotics (Jakoby and Habig, 1980). There is high concentration of transferases in liver tissue, i.e. approximately 10% of total soluble protein (Jakoby et al., 1976). Some works show the modulation of this enzyme in AL-inducible rats (Sheweita et al., 2002; El-Demerdash et al., 2005). To evaluate the influence of phase II drug-metabolizing system in ALR rats we determined GST enzyme activity . No significant differences in GST activities were observed amongst the groups.

Differential susceptibility amongst various inbred mouse strains to AL-induced β-cell toxicity has been well known for more than 40 years (Evan and Luft, 1980). Inbred strains show marked differences in their median effective dose for AL (Ino and Yoshikawa, 1966). In 1991, a report from Japan described the inbreeding of CD-1 (ICR) mice with selection for susceptibility versus resistance to AL. Two new inbred strains, designated alloxan-susceptible (ALS/Lt) and alloxan-resistant (ALR/Lt), were produced in this selection (Ino et al., 1991). This resistance has been associated with the unusual ability of ALR/Lt to dissipate toxic

radicals administered exogenously, a resistance extending to pancreatic islets (Mathews and Leiter, 1999b; Mathews et al., 2002).These abilities included elevated hepatic SOD, glutathione (GSH) reductase, and GSH peroxidase as well as an increase in the reduced-to-oxidized GSH ratio. The TBARS assay indicated a significantly higher constitutive level of lipid peroxides in ALS/Lt liver tissue compared with ALR/Lt mice (Mathews and Leiter, 1999a). Recent studies have demonstrated the influence of genetic differences in the resistance to AL (Mathews et al., 2005). In the present study, an unbalanced profile was found in the activities of antioxidant enzymes SOD and CAT in ALS rats, since an increase in SOD activity and decrease in CAT activity were observed, suggesting an overproduction of hydrogen peroxide that may induce the overproduction of hydroxyl radical by Fenton Reactions (Halliwell et al., 1999). The lipoperoxidation index was twofold higher in the ALS group compared with the ALR group. More interestingly, an increase was found in the activities of both SOD and CAT enzymes in liver tissue of ALR rats compared with ALS rats, and the TBARS index of ALR did not differ from control group. These findings demonstrate an ability of ALR Wistar rats to dissipate free radicals more efficiently than ALS rats, and suggest the involvement of antioxidant enzymes systems in liver protection. In our laboratory we have observed in several alloxan-induced diabetes experiments the presence of ALR group. In all alloxan-induced diabetes experiments the control group is strongly homogenous and we did not observed the presence of distinct populations in control group. Although the present report showed two populations among alloxan treated rats which emerge when they are treated whit a single intraperitonial injection of AL. These findings suggest that resistant rats would silently exist in the colony and the resistance is triggered by a single intraperitonial injection of alloxan.

In summary, many studies have previously shown that treating mice with diabetogenic drugs increases antioxidant enzyme levels and activities in response to free radical stress (Ho et al., 1998; Mathews and Leiter, 1999a; Mathews and Leiter, 1999b; Mathews et al., 2002). Increased protection from AL has been shown by the overexpression of CAT in combination with SOD, although CAT overexpression alone is ineffective (Barja et al., 1993). The present report shows an increase in SOD and CAT antioxidant enzymes in liver tissue of ALR Wistar rats. This is the first report that showed differences in pancreatic islets morphology, liver damage, oxidative profile and activities of antioxidant enzymes in ALR Wistar rats subjected to a single intraperitonial injection of AL. The results suggest that ALR liver tissue is naturally protected against the AL metabolization effects and may be the key to alloxan resistance.

Acknowledgments

We thank all colleagues for technical assistance. This work was supported by grants from: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Brazil), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Pró-Reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (PROPESQ/UFRGS).

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Table 1

Body weight, biochemical parameters and plasma lipid profile

	Control	ALR	ALS
Body weight	312.6±10.9	337±6.04	210.8±10.1 *
Glycemia	101±1.67	135±1.28	485±36.2 *
HbA1	2.05±0.08	2.24±0.09	4.21±0.13 *
HDL	24.81±1.78	22.16±1.29	35.77±3.31 *
LDL	23.56±2.04	21.63±2.31	31.64±3.09 *
VLDL	10.35±0.51	10.68±1.59	14.31±0.87 *
Cholesterol	58.71±3.08	54.47±4.87	81.71±0.51 *
Triglycerides	45.42±3.12	44.09±3.56	54.32±4.47 *

All parameters were measured on sacrifice day. Results are expressed as mean±SE. Body weight in grams. Glycemia and lipid parameters in mg/dL. HbA1 (glycated hemoglobin) in % of total hemoglobin. ($n \geq 6$) * $p \leq 0.05$ vs. control and ALR.

Table 2

Hepatic glycogen content and liver weight

	Control	ALR	ALS
Glycogen	4.23±0.21	4.22±0.18	2.08±0.41 *
Weight	10.82±0.47	10.15±0.09	8.78±0.39 *
Weight %	3.36±0.07	3.01±0.06	4.17±0.11 *
Dry weight	3.57±0.15	3.35±0.13	2.89±0.12 *
Dry weight %	1.10±0.02	0.99±0.02	1.38±0.04 *

All parameters were measured on sacrifice day. Results are expressed as mean±SE. Glycogen in mg%. Absolute and relative weight in grams. ($n \geq 6$) * $p \leq 0.05$ vs. control and ALR.

Table 3

Oxidative stress parameters in liver

	Control	ALR	ALS
TBARS	0.17±0.03	0.18±0.04	0.38±0.08 *
Carbonyl	28.59±9.29	27.43±9.92	56.71±6.92

All parameters were measured on sacrifice day. Results are expressed as mean±SE. TBARS is an index of lipid peroxidation and was expressed as malondialdehyde equivalents (nM/mg protein) ($n \geq 6$) * $p \leq 0.05$ vs. control and ALR.

Table 4

Enzymes activities

	Control	ALR	ALS
SOD	72.19±8.61	230.07±13.29 *°	148.41±13.16 *
CAT	84.19±1.04	131.04±15.41 *°	68.21±5.52 *
AST	65.2±7.05	42.1±6.11 *°	59.1±6.55
ALT	42.6±4.99	21.7±5.54 *°	58.1±7.82
GST	0.035±0.003	0.030±0.002	0.033±0.002

All parameters were measured on sacrifice day. Results are expressed as mean±SE. SOD, CAT and GST activities determined in liver samples (U/mg protein). AST and ALT activities determined in plasma (U/ml). ($n \geq 6$) * $p \leq 0.05$ vs. control. ° $p \leq 0.05$ vs. ALS.

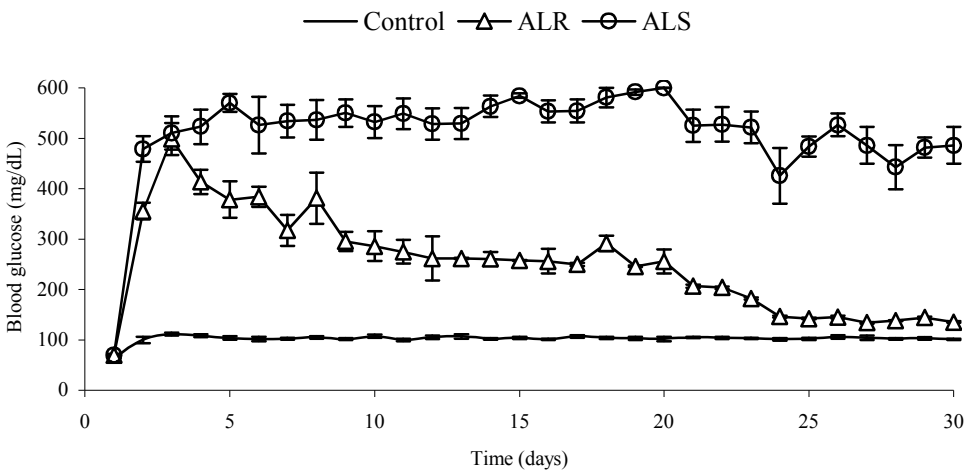

Fig. 1. Characterization of alloxan-resistant group. Alloxan-susceptible (ALS), alloxan-resistant (ALR) and control groups, were monitored for thirty days. (n≥6)

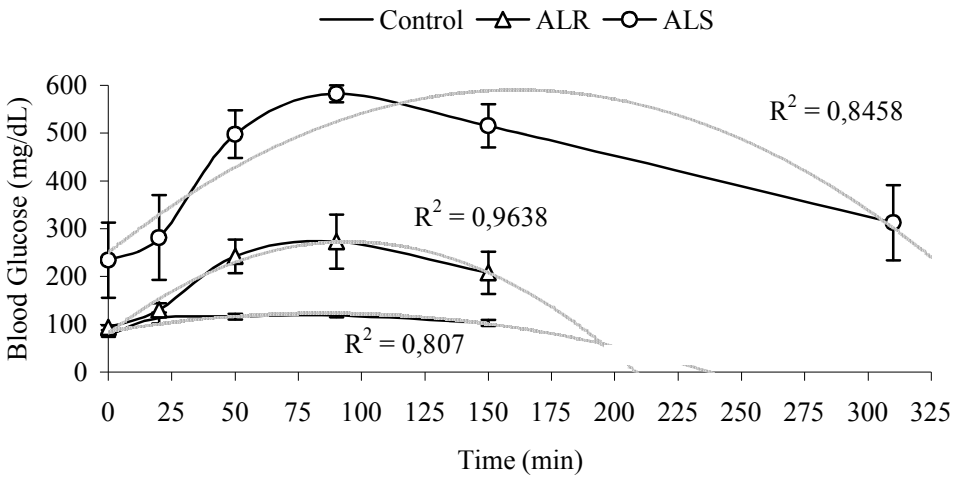

Fig. 2. Oral glucose tolerance test. OGTT was performed four days before the end of study. D-glucose solution (4 g/kg body weight) was given orally by gavage. R^2 correspond to hypothetical curves. ($n \geq 6$)

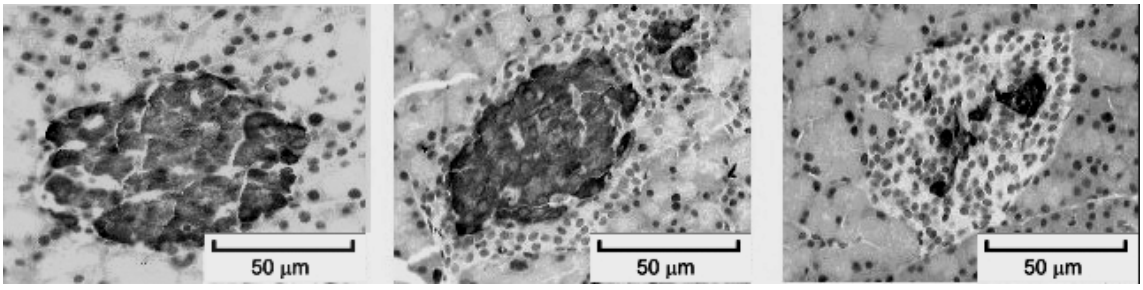

Fig. 3. Pancreas tissue immunohistochemical staining using antiinsulin. Left, control; center, ALR; right, ALS.

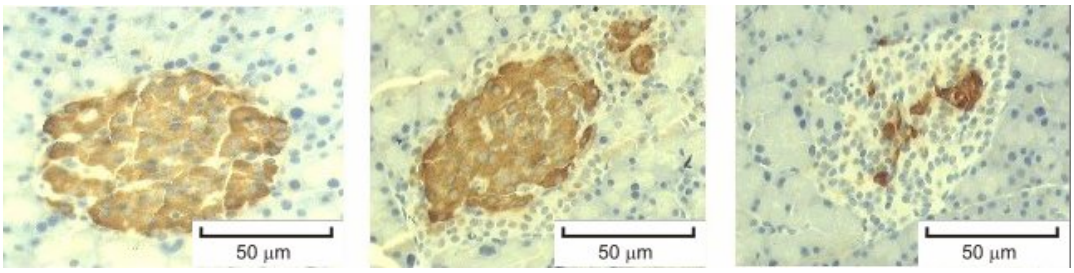

Fig. 3. Pancreas tissue immunohistochemical staining using antiinsulin. Left, control; center, ALR; right, ALS.

Capítulo 2

Antioxidant activities and free radical scavenging potential of *Bauhinia microstachya* (Raddi) Macbr. (Leguminosae) extracts linked to their polyphenol content

Artigo Publicado em

Biological & Pharmaceutical Bulletin

30(8) 1488—1496 (2007)

Antioxidant Activities and Free Radical Scavenging Potential of *Bauhinia microstachya* (Raddi) Macbr. (Caesalpinaceae) Extracts Linked to Their Polyphenol Content

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Received November 29, 2006; accepted April 27, 2007

Aqueous and hydro-ethanolic extracts of *Bauhinia microstachya* leaves (AEBM and HEBM) were investigated for their phenolic content and phytochemical profile (by spectrophotometry and HPLC), and for their antioxidant activities and free radical scavenging potential in different *in vitro* systems (TRAP, TEAC, TBARS, nitric oxide, superoxide and hydroxyl radical). HEBM presented a 27.4% higher content of phenolics when compared to AEBM and a distinct phytochemical profile was observed. Our work suggests that both extracts have potent antioxidant activities and that their antioxidant capacity and efficiency vary according to the radical-generating system. In general, HEBM was more effective than AEBM in avoiding ROS-generating damage and in scavenging the various radicals formed. Nevertheless, when results were normalized to total phenolic content, a different profile of antioxidant activities and free radical scavenging potential was observed, particularly against oxidative lipid damage and superoxide radical. *B. microstachya* extracts may be considered an interesting source of natural antioxidants as well as other phenolic-rich plants.

Key words *Bauhinia microstachya*; phenolic compound; antioxidant; HPLC analysis; *in vitro* system

Bauhinia belongs to the Caesalpinaceae, a plant family that comprises approximately 250 species.[1] Native peoples from tropical regions use *Bauhinia* leaves and bark as medicine in infusions and other phytotherapic preparations.[2,3] Some native *Bauhinia* species in Brazil are known as "pata-de-vaca" (cow's hoof) and are widely used in folk medicine to treat various ailments, mainly infections and diabetes. Some reports have demonstrated positive effects on factors related to diseases and pathological states.[2—5] *Bauhinia microstachya* (Raddi) Macbr. (Caesalpinaceae) is a creeper plant that occurs naturally in southern Brazil where is popularly known as "escada-de-macaco" (monkey's ladder). *B. microstachya* is often cultivated in small areas and its leaves are used as herbal antidiabetic medicine. Traditionally the leaf infusion or *garrafada* (a preparation with *cachaça*—the popular local sugar cane brandy—and leaves) is drunk after meals to help control blood sugar levels and other diabetic disorders. Phytochemical investigations with *B. microstachya* leaves have identified compounds such as steroidal glycosides, triterpenes, lactones and phenolic compounds, mainly flavonoids.[6—8]

Plant phenolics form a large group of natural compounds, ubiquitous in the plant kingdom. It is known that these secondary metabolites display a remarkable array of biochemical interactions, probably due to antioxidant properties.[9] These substances may act as potent metal chelators and/or free radical scavengers,[10] however, it has been reported that the performance of these compounds in oxidative systems depends on activity–structure relationships.[11,12] As an unfortunate consequence of aerobic life, free radicals and other reactive oxygen species (ROS) are formed by biological redox reactions.[13] The role of free radicals reactions in biology and medicine has become an area of intense interest due to their relationship to chronic diseases.[13] It is generally accepted that free radicals play an important role in the development of tissue damage and pathological events in living organisms.[13] Lipids containing polyunsaturated fatty acids can be oxidized by free radical-mediated reactions. In addition, when oxygen is supplied in excess or their reduction is insufficient, this generates endogenous ROS imbalance with formation of hydroxyl ($^{\cdot}OH$) and superoxide ($O_2^{\cdot -}$) radicals.[13] In inflammation and endothelial damage, NO plays a major role as precursor of peroxynitrite ($ONOO^-$).[14] If the endogenous response system, such as antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), is inadequate in scavenging ROS, damage to important macromolecules can be established and generate cellular oxidative stress.[13] In addition, the non-enzymatic antioxidants (such as vitamin C, vitamin E and phenolic compounds found in a vegetable-rich diet) play a significant role in the physiological redox balance together with enzymatic defenses.[13]

Considering the increasing interest in antioxidants, the potential antioxidant effects of phenolic compounds and the utilizations of *B. microstachya*, this study aims at investigating the phytochemical profile of aqueous and hydro-ethanolic extracts from *B. microstachya* leaves associated with their performance against free radical production in different *in vitro* systems.

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MATERIALS AND METHODS

Chemicals AAPH (2,2′-azobis[2-methylpropionamidine]dihydrochloride), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), 2-deoxyribose, glycine (aminoacetic acid), Folin-Ciocalteau (phenol reagent), Griess reagent, sodium nitroprusside, ascorbic acid, rutin, ellagic acid, 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol), gallic acid, pyrogallol, (+)-catechin, hydrogen peroxide, tannic acid, NBT (nitrotetrazolium blue chloride), xanthine, xanthine oxidase, adrenaline, catalase and SOD (superoxide dismutase) were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid), was purchased from Aldrich Chemicals Co. (Milwaukee, WI, U.S.A.). Cellulose nitrate membrane, methanol and acetic acid were purchased from Merck Chemicals KGaA (Darmstadt, Germany). Water was purified using a Milli-Q system from Millipore (Milford, MA, U.S.A.) and all other reagents used in this study were of analytical or HPLC grade.

Plant Material and Extraction *B. microstachya* leaves were collected in February 2004 in the city of Carlos Barbosa, in the state of Rio Grande do Sul, Brazil, in an area located S 29°19′33.2″ W 51°25′54.1″. Location data were obtained with a GPS 38 (Garmin Corp., Olathe, KS). Professor Valdely Ferreira Kinupp identified the plant and the reference material (ICN 144067) is kept in the Herbarium of the Department of Botany of the Federal University of Rio Grande do Sul (UFRGS). *B. microstachya* leaves were air-dried at 35±2 °C and then reduced to powder. To prepare *B. microstachya* aqueous extract (AEBM), dried leaves powder was boiled with water (15% w/v) for 10 min. After cooling, the extract was filtered, lyophilized and frozen (−20 °C). *B. microstachya* hydro-ethanolic extract (HEBM) was obtained by macerating dried leaves powder in ethanol 40% (15% w/v) at room temperature and in darkness. After eight days the material was filtered, ethanol having been eliminated under reduced pressure, and then it was stored at −20 °C.

Total Phenolic Content The total phenolic content was determined in both AEBM and HEBM by an adapted colorimetric assay[15] in which tannic acid was used as standard. Stock solutions of both extracts were prepared immediately before use. Twenty-five millgrams of dry extract and 5 ml of solvent (distilled water or EtOH 40%) were sonicated for 40 s (4×10″) at potency 4 (Sonicator XL 2020 Heat Systems Inc., Farmingdale, NY, U.S.A.), and filtered through 0.45 μm-pore cellulose-nitrate membrane. One 0.5 ml-sample of each extract was completed with water up to 8.5 ml. Then, 0.5 ml of Folin reagent (1 N) and 1 ml of Na_2CO_3 saturated solution were added and the solution was homogenized to determine total phenolic contents. Absorbance was read 10 min later at 725 nm with spectrophotometer (Beckman DU-640, wavelength accuracy ±0.5 nm, Beckman Instruments, Inc., Fullerton, CA, U.S.A.) and the total phenolic content was expressed as tannic acid equivalents (TAE μg/mg extract).

HPLC Profile. LC Apparatus The analyses were carried out with a Waters Alliance model 2695 liquid chromatograph system (Waters Corp., Milford, MA, U.S.A.), fitted with a 4.6×75 mm Symmetry® (Waters Corp., Milford, MA, U.S.A.) RP18 (3.5 μm) column and a Waters PDA detector model 996 controlled by Empower™ Chromatography Software (Waters Corp., Milford, MA, U.S.A.). A LiChrospher® (Merck Chemicals KGaA, Darmstadt, Germany) pre-column packed with Bondapak® RP18 (10×4 mm i.d., 10 μm) (Waters Corp., Milford, MA, U.S.A.) was employed. A diode array detector Waters 996 checked the peak purity of each compound. Absorbance was measured every second from 200 to 400 nm with 4.8 nm resolution.

Chromatographic Conditions The chromatography separation was carried out using a mobile phase, with methanol as solvent A and water as solvent B, at a flow rate of 0.6 ml/min. The mobile phase was prepared daily and degassed by sonication before use. The gradient program was as follows: 0—30% A (30 min), 30—40% A (45 min), 40—50% A (50 min), 50—60% A (60 min), 60—70% A (65 min), 0—30% A (70 min). The peaks were detected at 270 and 356 nm. The chromatographic separation was achieved at room temperature (22±2 °C). Extracts were dissolved in the mobile phase for HPLC analysis (200 μg/ml). Standard solutions of authentic reference compounds pyrogallol, gallic acid, rutin and (+)-catechin were prepared using MeOH as solvent. The injected volume was 5 μl for each assay.

Chemiluminescence Methods. Total Radical-Trapping Antioxidant Parameter (TRAP) An adapted method of TRAP assay was used to determine the capacity of extracts to trap a flow of water-soluble peroxyl radical produced at constant rate, through thermal decomposition of AAPH.[16] Briefly, the reaction mixture containing 4 ml of the free radical source (AAPH 10 mM) in glycine buffer (0.1 M) pH 8.6, 10 μl of the test samples (AEBM or HEBM at different concentrations) and 10 μl luminol (4 mM) as external probe to monitoring radical production were incubated at 25 °C. Trolox (water-soluble vitamin E analogue), rutin, ascorbic acid, ellagic acid, and tannic acid were used as reference peroxyl radical scavenger molecules (positive control); negative control was the only extracts vehicle (water or EtOH 40%). The chemiluminescence produced was directly proportional to the radical generation and measured in out of coincidence mode (Wallac 1409 DSA Liquid Scintillation Counter, Wallac Oy, Turku, Finland) as counts per minute (CPM). The antioxidant potential of the samples was expressed in IC_{50}.

Trolox Equivalent Antioxidant Capacity (TEAC) An alternative approach to TRAP assay is the TEAC assay in which the kinetics quality of samples against a source of peroxyl radical may be observed using luminol as external probe.[16] The addition of 10 μl of trolox (200 nM) as standard antioxidant or different concentrations of extracts to 4 ml of the free radical source (AAPH 10 mM) in glycine buffer (0.1 M) pH 8.6, decreases the chemiluminescence proportionally to its antioxidant potential at 25 °C. After the addition of the AEBM, HEBM or trolox the chemiluminescence emission was monitored for 80 min in out of coincidence mode in a liquid scintillation counter to check the decline of free radical source and the consume of samples antioxidant potential. The TEAC was defined as the concentration of Trolox with equivalent antioxidant potential to a 10 μg/ml of the tested samples (C_{sample}). The antioxidant capacity of extracts was expressed as Trolox equivalents (TEAC) by the equation

$$TEAC=[T_{sample}/T_{trolox}]\times[C_{trolox}/C_{sample}] \quad (1)$$

where "TEAC" is the antioxidant capacity of sample, C_{Trolox} is Trolox concentration, T_{Trolox} is the lag time of the AAPH kinetic curve in the presence of Trolox, C_{sample} is sample concentration and T_{sample} is the lag time of the AAPH kinetic curve in the presence of sample.

TBARS Assay TBARS (thiobarbituric acid reactive species) assay was employed for quantify lipid peroxidation[17] and an adapted TBARS method was used to measure the antioxidant capacity of extracts using egg yolk homogenate as lipid rich substrate. Briefly, egg yolk was homogenized (1% w/v) in 20 mM phosphate buffer (pH 7.4), 1 ml of homogenate was sonicated (10 s in potency 4) and then homogenized with 0.1 ml of extracts or positive controls in different concentrations prepared immediately before use. Lipid peroxidation was induced by addition of 0.1 ml of AAPH solution (0.12 M). Trolox, rutin, ascorbic acid, ellagic acid, and tannic acid were used as reference antioxidant molecules, (positive control); negative control was only extracts vehicle (water or EtOH 40%). Reactions were carried out for 30 min at 37 °C. After cooling, samples (0.5 ml) were centrifuged with 0.5 ml of trichloroacetic acid (15%) at 1200×***g*** for 10 min. An aliquot of 0.5 ml from supernatant was mixed with 0.5 ml TBA (0.67%) and heated at 95 °C for 30 min. After cooling, samples absorbance was measure using a spectrophotometer at 532 nm. The results were expressed as IC_{50}.

Scavenging Activity of Nitric Oxide (NO) Nitric oxide was generated from spontaneous decomposition of sodium nitroprusside in 20 mM phosphate buffer (pH 7.4). Once generated NO interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction.[18] The reaction mixture (1 ml) containing 10 mM sodium nitroprusside in phosphate buffer and AEBM, HEBM or reference compounds at different concentrations were incubated at 37 °C for 1 h. A 0.5 ml aliquot was taken and homogenized with 0.5 ml Griess reagent. The absorbance of chromophore was measured at 540 nm. Trolox, rutin, ascorbic acid, ellagic acid, and tannic acid were used as positive controls. Percent inhibition of nitric oxide generated was measured by comparing the absorbance values of negative controls (only 10 mM sodium nitroprusside and vehicle) and assays preparations. Results were expressed as IC_{50}.

Hydroxyl Scavenging Activity The formation of ·OH (hydroxyl radical) from Fenton reaction was quantified using 2-deoxyribose oxidative degradation. The principle of the assay is the quantification of the 2-deoxyribose degradation product, malondialdehyde, by its condensation with 2-thiobarbituric acid (TBA).[19] Briefly, typical reactions were started by the addition of Fe^{2+} (6 μM final concentration) to solutions containing 5 mM 2-deoxyribose, 100 mM H_2O_2 and 20 mM phosphate buffer (pH 7.2). As antioxidant standards, we used trolox, rutin, ascorbic acid, ellagic acid, and tannic acid. To measure AEBM and HEBM antioxidant activity against hydroxyl radical, different concentrations of extracts were added to the system before Fe^{2+} addition. Reactions were carried out for 15 min at room temperature and were stopped by the addition of 4% phosphoric acid (v/v) followed by 1% TBA (w/v, in 50 mM NaOH). Solutions were boiled for 15 min at 95 °C, then cooled at room temperature. The absorbance was measured at 532 nm and results were expressed as IC_{50}.

Superoxide Radical Scavenging Activity. Enzymatic Assay Antiradical activity was determined spectrophotometrically by monitoring the effect of tested substances on the reduction of NBT to the blue chromogen formazan by $O_2^{\cdot-}$ Superoxide radicals were generated by xanthine/xanthine oxidase system (X/XOD) as described previously.[20] Briefly, 50 μl of AEBM, HEBM or isolated compounds (tannic and ellagic acids) were mixed with 200 μl mixture of 0.4 mM xanthine and 0.24 mM NBT in 0.1 M NaH_2PO_4 buffer (pH 7.8) containing 0.1 mM EDTA. A total of 50 μl of XOD (0.10 U/ml), dissolved in the same phosphate buffer, was added, and the resulting mixture was incubated at 37 °C for 20 min. The reaction was terminated by adding 35 μl of 6% SDS solution, and the absorbance was measured at 560 nm. The superoxide scavenging activity was calculated as percentage of NBT reduction to the formazan salt. X/XOD system was considered as 100% of superoxide production. Native SOD (50 U/ml) was used to assess assay specificity to superoxide anion.

Effect on XOD Activity The effect of AEBM and HEBM on XOD activity was evaluated by measuring the formation of uric acid from xanthine at room temperature. A total of 300 μl of XOD (0.35 U/ml) in a NaH_2PO_4 buffer (0.1 M, pH 7.8) was mixed with 100 μl of the tested samples. The reaction was started by the addition of 100 μl of xanthine (1 mM) in the phosphate buffer, and the absorbance was monitored at 295 nm for 2 min.

Nonenzymatic Assay Superoxide anion scavenger activity was evaluated by measuring the rate of inhibition of superoxide-mediated adrenaline auto-oxidation to adenochrome as described previously.[21] Fifty microliters of the tested fraction was mixed with 200 μl of 50 mM glycine buffer (pH 10.2) and 10 μl of native catalase 100 U/ml. Superoxide generation was initiated by addition of 2 mM adrenaline (50 μl) and adrenochrome formation was monitored at 480 nm for 5 min at 25 °C. Superoxide production was calculated from variation coefficient of curves and $\varepsilon = 44.0\ mM^{-1}\,cm^{-1}$ for adenochrome.

Statistical Analysis The results were expressed as mean and S.E.M. All tests were performed in quadruplicate. ANOVA followed by the Tukey test was employed to detect differences between the groups. $p<0.05$ is considered significant. Statistical calculation was performed using SPSS 8.0 software (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Total Phenolic Content Both extraction methods of *B. microstachya* leaves are usual approaches for rich phenolics derivatives. The determination of total phenolic content in AEBM and HEBM showed that 2.52% dry weight of AEBM and 3.48% dry weight of HEBM are phenolic compounds. HEBM presented 27.43% higher content of phenolics when compared to AEBM (Fig. 1).

Chromatographic Profile The phytochemical analyses were performed using RP-HPLC and the results are shown in Figs. 2, 3, 4, 5, and 6. The peaks were detected at 270 and 356 nm. The analytical parameters were selected after testing a number of solvents systems and adsorbents. The HPLC separation of the phenolic compounds was tested with authentic standards of four phenolic compounds: pyrogallol,

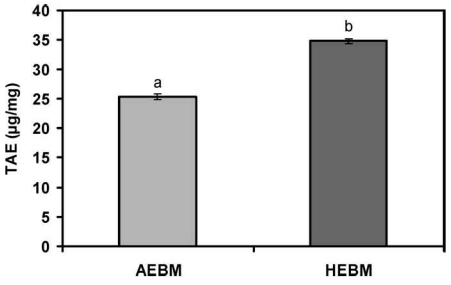

Fig. 1. Total Phenolic Content of *B. microstachya* Extracts

The results are expressed as tannic acid equivalents (TAE µg/mg extract). Bars represent the means±S.E. of triplicates from three independent experiments. Bars with no letters in common are significantly different ($p<0.05$).

gallic acid, rutin and (+)-catechin (Fig. 2). The best resolution, with sharp and symmetrical peaks, was achieved with a Symmetry® C-18 column and a linear gradient elution profile, as follows: 0—30% A (30 min), 30—40% A (45 min), 40—50% A (50 min), 50—60% A (60 min), 60—70% A (65 min), 0—30% A (70 min) where A=MeOH and B=H_2O.

In chromatograms monitored at 270 nm it was possible to detect differences in qualitative HPLC profile between AEBM and HEBM from 22 until 35 min (Figs. 3, 4), in which the main point (t_R=28.2 min in HEBM) present the same gallic acid chromophore (Fig. 4). As major component, both extracts showed a substance with the same retention

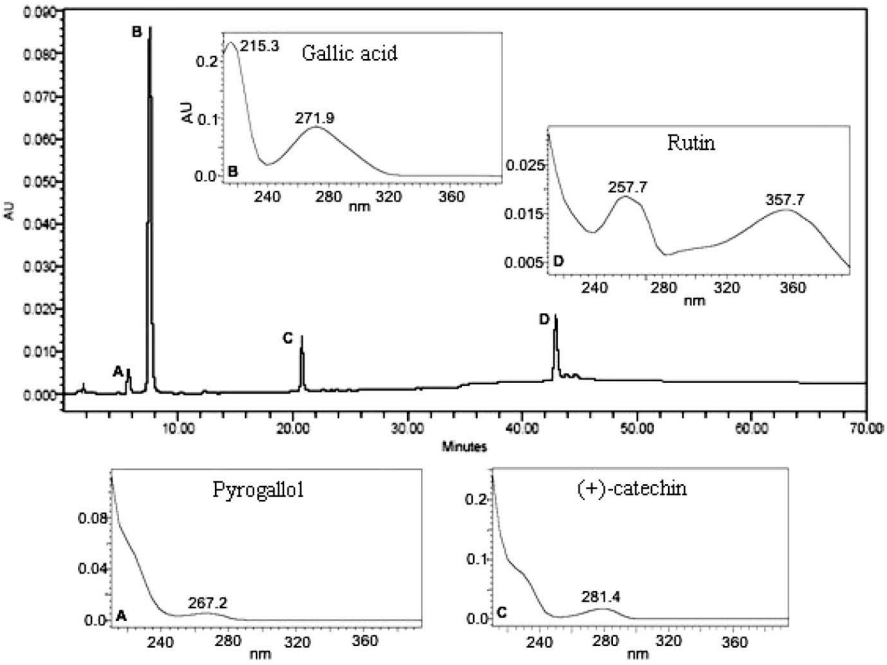

Fig. 2. HPLC Profile of Standards Detected at 270 nm

Insert graphics and graphics below of the chromatogram show the molecules UV spectra.

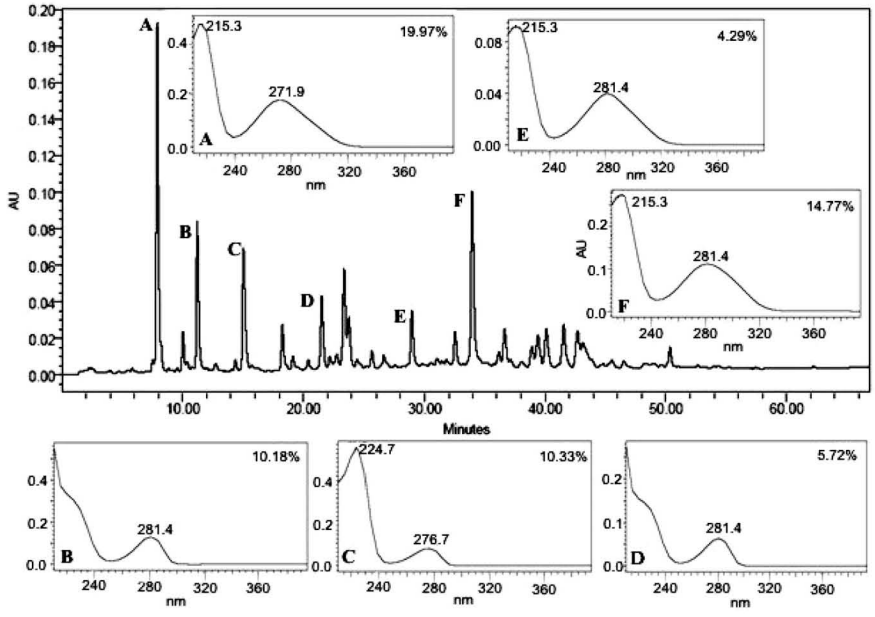

Fig. 3. HPLC Profile of AEBM Detected at 270 nm

Insert graphics and graphics below of the chromatogram show the main peaks UV spectra.

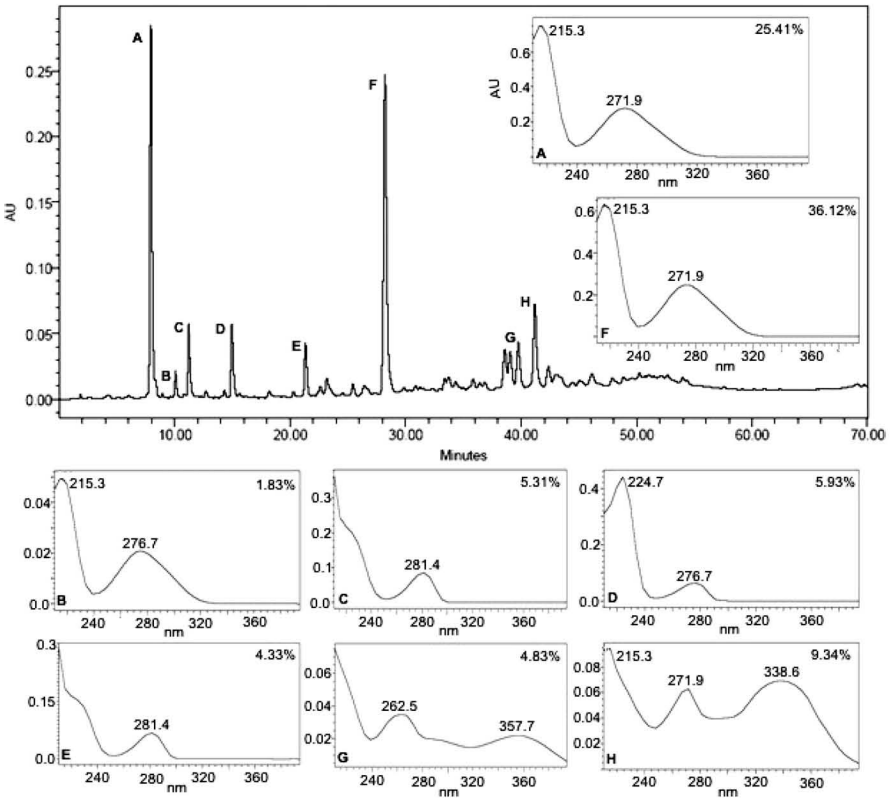

Fig. 4. HPLC Profile of HEBM Detected at 270 nm

Insert graphics and graphics below of the chromatogram show the main peaks UV spectra.

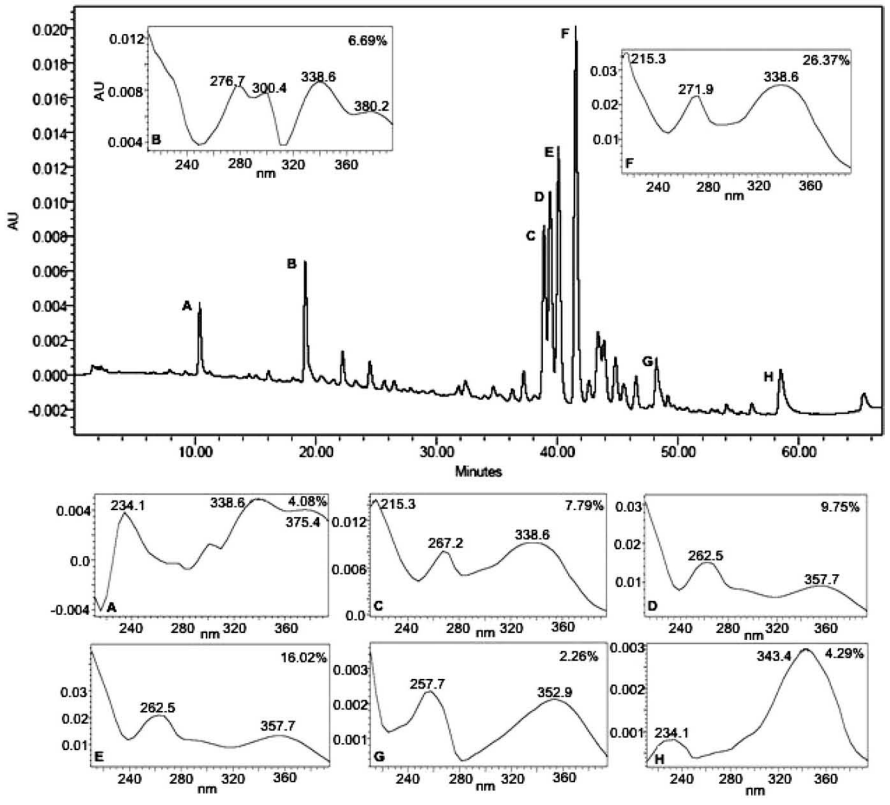

Fig. 5. HPLC Profile of AEBM Detected at 356 nm

Inserts graphics and graphics below of the chromatogram show the main peaks UV spectra.

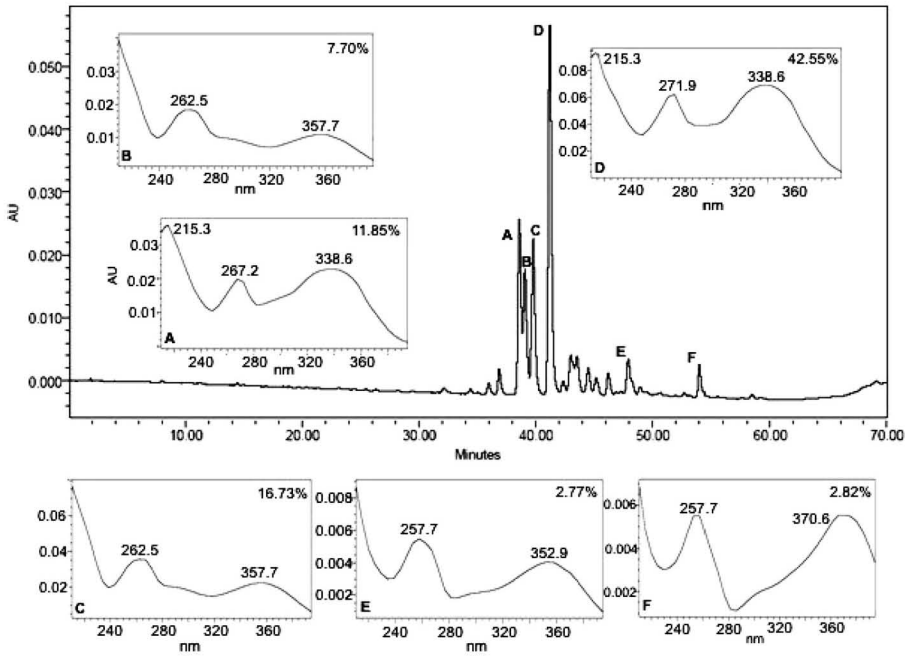

Fig. 6. HPLC Profile of HEBM Detected at 356 nm

Inserts graphics and graphics below of the chromatogram show the main peaks UV spectra.

Table 1. Antioxidant and Free Radical Scavenging Potential of *B. microstachya* in Various *in Vitro* Systems

Sample test	Scavenging activity against hydroxyl radical in IC_{50} [a)]	Inhibition of lipoperoxidation (LPO) in IC_{50} [a)]	Scavenging activity against nitric oxide radical in IC_{50} [a)]	Scavenging activity in TRAP in IC_{50} [a)]
AEBM	0.11±0.001	2.37±0.21	1.26±0.023	0.086±0.008
HEBM	0.06±0.001	0.25±0.01	0.44±0.001	0.072±0.007
Ascorbic acid	6.85±0.142	3.85±0.20	N.D.	0.273±0.071
Trolox	2.12±0.233	13.3±1.86	0.15±0.006	0.013±0.001
Tannic acid	0.03±0.001	1.64±0.006	1.16±0.082	0.626±0.057
Ellagic acid	N.D.	3.84±0.013	0.07±0.002	0.074±0.002
Rutin	1.38±0.056	4.40±0.81	1.30±0.034	0.023±0.003

a) IC_{50}: sample concentration required for 50% inhibition. AEBM and HEBM are mg/ml in hydroxyl, nitric oxide and TRAP assay; in lipoperoxidation AEBM and HEBM are μg/ml. Reference standards are mM in hydroxyl and nitric oxide assay; in lipoperoxidation and TRAP assay standards in μM. N.D. (not detected). The results are expressed as means±S.E. of triplicates from three independent experiments.

time and chromophore of gallic acid (t_R=7.9 min) (Figs. 2, 3, 4). Additionally, a substance with retention time and UV spectra similar to catechin (t_R=21 min) was present in both extracts (Figs. 2, 3, 4), while pyrogallol and rutin were not detected by HPLC-PDA analysis. Other two major substances were present, in HEBM (t_R=28.2 min) and AEBM (t_R=33.9 min) respectively, but their retention times (t_R) and HPLC-PDA analyses were different from those of the reference substances used.

The peaks detected in 356 nm suggest that AEBM presented more substances when compared with HEBM (Figs. 5, 6). However, from retention time 34 min until 50 min it was possible to detect a similar qualitative profile between AEBM and HEBM (Figs. 5, 6). In AEBM and HEBM it was possible to observe the presence of one major substance (t_R=41.2 min) but their retention time (t_R) and HPLC-PDA analyses were different from of the reference substances used. Concerning analyses at 356 nm, the HPLC profile and retention time of substances showed that AEBM has some higher polar compounds when compared to HEBM.

Total Radical-Trapping Antioxidant Parameter (TRAP) HEBM and AEBM showed antioxidant capacity in TRAP assay likewise reference peroxyl radical scavenger molecules used as positive control (Table 1). HEBM antioxidant potential was 1.19 times higher than AEBM. However, when the antioxidant parameter was corrected according to phenolic content present in the extracts, the result was altered; AEBM was 1.15 times higher when compared to HEBM (Table 2).

Trolox Equivalent Antioxidant Capacity (TEAC) Both extracts of *B. microstachya* leaves were comparable to Trolox in TEAC assay. Ten micrograms of HEBM or AEBM represent 327.75 and 236.8 μM of Trolox respectively (Fig. 7). Although HEBM was 1.38 times more equivalent to Trolox than AEBM, the result profile was similar to the result profile found in the total phenolic content (Fig. 1).

TBARS Assay Table 1 shows the antioxidant capacity of AEBM, HEBM and reference compounds in a lipoperoxidative system. All samples prevented lipid peroxidation in different concentrations, although the capacity of AEBM and HEBM was very different. HEBM presents 50% of lipid peroxidation with 11% of the AEBM concentration required to produced the same effect, IC_{50}=0.25 and 2.37 mg/ml, respectively. When taking into account the phenolic content in the

Table 2. Summary of *in Vitro* Antioxidant Potential of Extracts with Values Corrected to Phenolic Content

Sample test	Hydroxyl	TBARS	Nitric oxide	TRAP	Superoxide (enzymatic)	Superoxide (nonenzymatic)
AEBM	2.75	0.059	31.81	2.17	0.00076	0.0023
HEBM	2.3	0.00087	15.33	2.5	0.0032	0.0029

Values in IC_{50} (sample concentration required for 50% inhibition) corrected to μg of total phenolics in extracts.

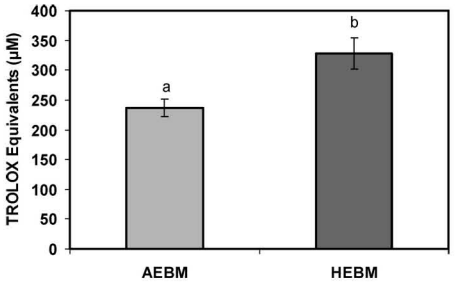

Fig. 7. Trolox Equivalent Antioxidant Capacity (TEAC) of *B. microstachya* Extracts (10 μg/ml)

The results are expressed as Trolox equivalents (μM). Bars represent the means±S.E. of triplicates from three independent experiments. Bars with no letters in common are significantly different ($p<0.05$).

extracts, the difference between AEBM and HEBM was enhanced (Table 2). HEBM presents 50% of lipid peroxidation with 1.5% of phenolics in AEBM required to produce the same effect, IC_{50}=0.00087 and 0.059 μg of phenolics present in extracts, respectively.

Scavenging Activity of the Nitric Oxide The results of the inhibitory effect of AEBM and HEBM on nitric oxide production are shown in Table 1. The HEBM ability to attenuate nitric oxide production was 2.86 times more effective than AEBM (Table 2). Nevertheless, when these values were normalized to phenolic content, this difference was reduced to 2.07 times (Table 2).

Hydroxyl Scavenging Activity Hydroxyl radicals were formed in solution and were detected by their ability to degrade 2-deoxyribose into fragments that formed a pink chromogen upon heating with TBA. When AEBM or HEBM were added to the reaction mixture, they removed hydroxyl radicals and prevented sugar degradation (Table 1). HEBM was 40% more effective (IC_{50}=0.066 mg/ml) in scavenging hydroxyl radical than AEBM (IC_{50}=0.109 mg/ml). Nevertheless, when results were normalized by phenolics concentration in extracts, the scavenging activity against hydroxyl radical was reduced to 16.36% (Table 2).

Superoxide Radical Scavenging Activity Figure 8 shows the antioxidant capacity of AEBM, HEBM and reference compounds against superoxide. In enzymatic assay (Fig. 8A) all samples can avoid superoxide production in different concentrations, although the capacity of and HEBM was different. The IC_{50} of AEBM was 30.1 μg/ml and the IC_{50} of HEBM was 92.2 μg/ml. However, AEBM not interfered in enzymatic formation of superoxide while HEBM prevented X/XOD superoxide formation by inhibiting XOD activity directly (Fig. 8B). Both extracts were similar in nonenzymatic assay of superoxide formation where the IC_{50} of AEBM was 92.2 μg/ml and the IC_{50} of HEBM was 84.6 μg/ml (Fig. 8C). The Table 2 shows the IC_{50} of AEBM and HEBM with the values corrected to total phenolic compounds present in extracts.

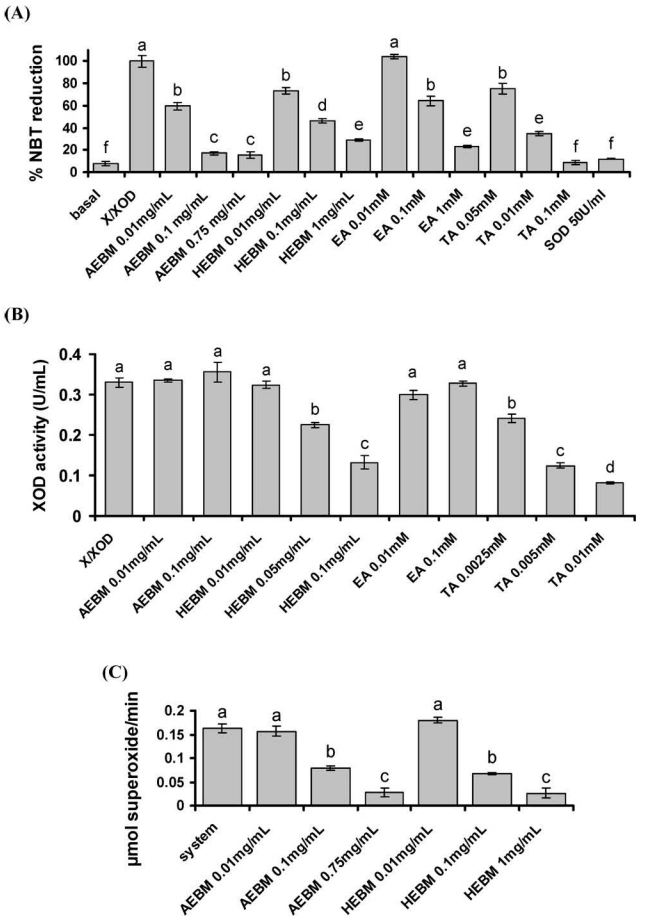

Fig. 8. Superoxide Radical Scavenging Activity of *B. microstachya* Extracts

(A) Enzymatic assay. (B) Effect on XOD activity. (C) Noenzymatic assay. EA (ellagic acid) and TA (tannic acid). Bars represent the means±S.E. of triplicates from three independent experiments. Bars with no letters in common are significantly different ($p<0.05$).

DISCUSSION

Our work suggests that AEBM and HEBM have potent antioxidant activity and that the antioxidant capacity of extracts varies according to the system-generating reactive species. In general, HEBM was more effective than AEBM in avoiding ROS-generating damage. Nevertheless, when results were normalized to total phenolic content, a different profile of antioxidant potential of extracts was observed.

It is well known that the performance of a complex mixture such as a plant extract in different antioxidant systems is related to the type of radical generated and to the polarity of the substrate system, and therefore, hard to determine. It is

recognized that the Folin-Ciocalteu method makes not distinction between the various phenolic constituents present in the extracts and that the response of these substances in the antioxidant assays is associated with their molecular structure.[11,12]

We used total phenolic content to estimate the contribution of these substances in extracts to the performance of AEBM and HEBM in different antioxidant assays. The total phenolic content of HEBM was higher than AEBM and HPLC analysis corroborates this data. In addition, the result of total phenolic content demonstrates a profile similar to the result of the TEAC assay. We observed that HEBM was more effective than AEBM in antioxidant assays (TRAP, TBARS, NO and $^{\cdot}OH$). However, when we considered the phenolic content present in the samples, these results was altered. An interesting observation was the change in TRAP assay results: an inversion in antioxidant activity was found when normalizing the dry weight to phenolic content. The antioxidant potential of these phenolic compounds is widely reported in the literature. In particular, it has been shown that these natural compounds show scavenging activity against superoxide anion radical ($O_2^{\cdot -}$). AEBM and HEBM also showed antioxidant potential against superoxide radical in enzymatic and nonenzymatic generation systems. AEBM was more effective than HEBM when we used the enzymatic generation system, once HEBM interfered in XOD activity. Considering phenolic content the superoxide scavenger activity of AEBM was 4.21 times higher than HEBM. However, the antioxidant potential of extracts was similar when the nonenzymatic generation system was used. On the other hand, in the TBARS assay, the same consideration demonstrated a significant increase in antioxidant activity of HEBM when compared to AEBM from 9.48 to 67.81 folds. In the hydroxyl and nitric oxide assay, the normalization to phenolic content did not cause any change. The coefficients calculated for extracts when considering their total phenolic content indicate that the effect of these compounds are synergistic, and therefore the effect of minor constituents may be important such as the system radical-generator and the interaction with different substrates. There are some studies showing that the total phenolic content of an extract could be positively correlated to the antioxidant potential[22—25]; on the other hand, a recent report did not find significant correlations between the total phenolic content and antioxidant activity.[26]

The results found in this study are in agreement with both observations and suggest that antioxidant activity and phenolic content correlations are dependent on the radical generation system.

The HPLC protocol described a gradient elution that was very favorable, since the results were easily reproduced. Additionally, the comparison of the retention times of the peaks in the chromatogram and the UV spectra of the associated components with poliphenolic standards confirmed the presence of a catechin-like substance and gallic acid-like substances that are acknowledged as potent antioxidants.[27] However, it is need to identify the compounds from AEBM and HEBM by using specific analyzers. Chromatographic profile suggests that there are few qualitative differences between extracts; however, these few differences were especially important in the lipoperoxidation and superoxide radical tests. HPLC results also showed that AEBM contains higher polar compounds when compared with HEBM and this characteristic could be related to superior performance of HEBM in the TBARS assay since less polar substances present better interactions with lipid-rich substrate. At the same time AEBM was superior to HEBM in the superoxide radical assay possibly by more polar profile. Further studies using appropriate equipments with radical trapping agents are needed to unravel exactly the molecular mechanisms for underlie the various antioxidant actions of AEBM and HEBM. Plants are source of antioxidant agents and epidemiologic evidence supports the concept that a diet rich in fruit, vegetables and natural beverages promote health, retard, attenuate and treat chronic diseases or pathological states.[28—30] In southern Brazil, *B. microstachya* is cultivated in small areas by family agriculture, where is used in primary health care, particularly concerning diabetes. Oxidative stress such as lipid peroxidation and protein oxidation has been shown increased in both insulin-dependent diabetes (IDDM), and non-insulin dependent (NIDDM).[31—33] When oxygen free radicals are involved in pathological conditions antioxidants could be effective in attenuating their severity, and employing plant preparations rich in phenolic compounds would be a significant alternative. These findings indicate that some of the phytochemicals present in *B. microstachya* leaves may contribute in a significant way to the intake of antioxidants and make these low-cost beverages very interesting from a nutritional point of view jointly with other phenolic-rich extracts.[34,35]

When considering HEBM, the presence of phenolics in *B. microstachya* leaves confers them a high activity against lipid damage, likewise AEBM against superoxide radical, suggesting the correct use of the preparation by the common people, and makes this low-cost beverage comparable to many foods of well-known antioxidant properties.[34—36] According to the World Health Organization, traditional experience with the respective preparation—including long-term use as well as medical history and ethnological background—should, as common practice, be taken into account when conducting phytochemical research.[37] Concluding, the data presented herein indicate that the *B. microstachya* leaf extracts have *in vitro* antioxidant activity and should be considered as a new sources of natural antioxidants jointly with other phenolic-rich plants. Further studies are needed to examine the potential use of these extracts in the prevention or treatment of pathologies where oxidative stress seems to play an important role.

Acknowledgements Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq–Brazil), Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (Fapergs) and Pró-Reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (Propesq/UFRGS).

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Capítulo 3

Hypoglycemic effect and antioxidant potential of hydro-ethanolic extract of *Bauhinia microstachya* leaves in alloxan-induced diabetic rats

Manuscrito submetido à

Clinical and Experimental Pharmacology and Physiology (2007)

HYPOGLYCEMIC EFFECT AND ANTIOXIDANT POTENTIAL OF HYDRO-ETHANOLIC EXTRACT OF *BAUHINIA MICROSTACHYA* LEAVES IN ALLOXAN-INDUCED DIABETIC RATS

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Short title: *B. microstachya* in alloxan-induced diabetic rats.

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SUMMARY

1. The efficacy of hydro-ethanolic extract of *Bauhinia microstachya* leaves (HEBM), a plant used as a treatment for diabetes and its complications, was investigated in alloxan-induced diabetic rats.

2. Diabetes was induced by a unique intraperitoneal injection of 150 mg/kg of alloxan. HEBM short-term effects on blood glucose and plasma antioxidant status were analyzed after a single oral treatment (0.01, 0.1 and 1 mg/Kg doses). The blood glucose level was measured before the animals received HEBM and at 15, 60, 120 and 180 min intervals following the treatment. HEBM long-term effects were studied in rats that received a dose (1 mg/Kg) for 28 days. Glycated hemoglobin and other biochemical analyses were carry out. The antioxidant capacity of plasma was quantified. The liver, kidney and gastrocnemius muscle were analysed. Total glycogen content and lipoperoxidation in tissues were determined. Superoxide dismutase (SOD) and catalase (CAT) activity were quantified, as well as hepatic glutathione S-transferase activity.

3. The hypoglycemic effect was found to be more pronounced 120 min after oral treatment (23%). The oral dose of 1 mg/Kg of HEBM increased the plasma antioxidant potential in the short-term experimental model.

4. The diabetes group, which was treated for 28 days with HEBM (1 mg/Kg) per day intragastrically, promoted a final weight decreased (-40.02 g) and a reduction on HbA_{1C} (9.8%). HEBM increased the triglycerides and VLDL levels. Uric acid was significantly increased in diabetic-induced animals (128%) and HEBM treatment reduced it, as well as creatinine levels. We observed that the treatment increased the

plasma AST and ALT activity. In addition, the treatment increased liver lipoperoxidation in the diabetic group and reduced GST activity (41%). HEBM treatment decreased kidney lipoperoxidation and turned SOD activity to control levels. A similar decrease in liver and kidney CAT activity was observed in control and diabetic groups that received HEBM.

5. Our findings suggest a hypoglycemic effect and kidney protection of HEBM. However, increased risk of developing heart diseases, due to impaired alterations on blood lipid profile, may be considered. The expressive reduction observed in bodyweight of diabetic animals which were treated with HEBM was a negative point found. In addition, long-term use of HEBM may induce hepatic dysfunction.

Key words: antioxidant, *Bauhinia microstachya*, diabetes, hepatic dysfunction, hypoglycemic, lipid profile.

INTRODUCTION

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia. Specific destruction of the insulin-producing pancreatic β-cells or insulin resistance in the target tissues are physiological alterations that lead to high blood glucose levels in the two most common types of DM. Both DM types share aspects that are related to uncontrolled hyperglycaemia: alterations in the carbohydrate, fat, and protein metabolism. In addition, increased cardiovascular disease risk, retinopathy, nephropathy, and neuropathy are vascular complications associated to diabetes.[1-3] Beyond these metabolic disturbances, experimental findings suggest that reactive oxygen species (ROS) have been implicated in DM.[4-6] Studies have shown that increased lipid peroxides and oxidative stress are present in diabetic subjects.[6,7] In diabetes, oxidative stress caused by increased production of ROS, reduction in antioxidant defenses, and altered cellular redox status seems to be associated to tissue damage.[4-6] It is unclear if oxidative stress precedes the appearance of diabetic complications or merely reflects the presence of hyperglycemic complications and their consequences. Moreover, the role of oxidative stress in the initiation and progression of diabetes remains uncertain.

Plants of *Bauhinia* genus have been frequently used in folk medicine to treat several ailments, especially diabetes. This group comprises approximately 250 species and is widely distributed in tropical areas.[8] Previous studies demonstrated the potential of the plants from *Bauhinia* genus against to diabetes and its effects,[9-13] except for *Bauhinia microstachya* (Raddi) Macbr. (Caesalpinaceae). *B. microstachya* is a creeper plant that occurs naturally in southern Brazil where it is popularly known as “escada-de-macaco” (monkey’s ladder) and is used as a herbal antidiabetic medicine. The leaves are used in

preparations which is drunk after meals to help control blood sugar levels and other diabetic disorders. Phytochemical investigations with *B. microstachya* leaves have identified compounds such as steroidal glycosides, triterpenes, lactones and phenolic compounds, mainly flavonoids.[14-16] This group of secondary metabolites displays a remarkable array of biochemical interactions, probably due to antioxidant properties.[17] These substances may act as metal chelators and/or free radical scavengers.[18] Our previous work showed the antioxidant properties of extracts obtained from *B. microstachya*.[19] However, the effect of *B. microstachya* on diabetes and their complications remains to be established.

Considering the previous report data showing the antidiabetic effects of plants from *Bauhinia* genus and antioxidant properties of ethanolic extract of the *B. microstachya* leaves, we decided to investigate the short- and long-term effects of the ethanolic extract of *B. microstachya* leaves on blood glucose levels, their impact on antioxidant status and some metabolic-biochemical parameters in alloxan-induced diabetic rats.

METHODS

Chemicals

AAPH (2,2'-Azobis [2-methylpropionamidine] dihydrochloride), alloxan monohydrate, 1-Chloro-2,4-nitrobenzene (CDNB), Folin-Ciocalteau (phenol reagent), glutathione (GSH), glycine, hydrogen peroxide, luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione), 2-deoxyribose, 2-thiobarbituric acid (4,6 dihydroxypyrimidine-2-thiol), adrenaline, catalase and superoxide dismutase (SOD) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxilic acid), was purchased from Aldrich Chemicals Co. (Milwaukee, WI, USA). Water was purified using a

Milli-Q system from Millipore Corp. (Billerica, MA, USA) and all other reagents used in this study were of analytical grade.

Plant material

B. microstachya leaves were collected in the city of Carlos Barbosa, in the state of Rio Grande do Sul, Brazil, in an area located S 29°19'33.2'' W 51°25'54.1''. Location data were obtained with a GPS 38 (Garmin Corp., Olathe, KS, USA). Professor Valdely Ferreira Kinupp identified the plant and the reference material (ICN 144067) is kept in the Herbarium of the Departmento de Botânica of the Universidade Federal do Rio Grande do Sul (UFRGS).

Plant extract

B. microstachya leaves were air-dried at 35 ± 2°C and then reduced to powder. *B. microstachya* hydro-ethanolic extract (HEBM) was obtained by macerating dried leaves powder in ethanol 40% (15% w/v) at room temperature and in the darkness. After eight days the material was filtered and ethanol was eliminated under reduced pressure. Then, it was stored at -20°C until the use.

Animals

Nine-week-old male Wistar rats (200-245 g) were obtained from our own breeding colony. Rats were housed in plastic cages, maintained at 22 ± 1°C, 55% relative humidity and 12 h light/dark cycle. Animals were supplied with commercial pellet food (Nuvilab CR-1 Curitiba, PR, Brazil) and water *ad libitum*. All animals were carefully monitored and

maintained in accordance with ethical recommendations of the Brazilian College for Animal Experimentation and NIH Guide for the Care and Use of Laboratory Animals.

Diabetes induction

Animals were randomly divided into two groups: control (non-induced) and diabetic-induced group. Diabetes was induced through a unique intraperitoneal injection of 150 mg/kg alloxan (prepared in 0.9% NaCl) after overnight fasting and the control group received only 0.9% NaCl.[20] Blood glucose levels were monitored thereafter by Accu-Chek Active blood glucose monitor. Ten days later, animals in which the development of hyperglycemia was confirmed (blood glucose levels higher than 300 mg/dL) and animals from control group were studied.

Immunohistochemistry

Immunohistochemical insulin detection was carried out to confirm pancreatic β cell damage 10 days after alloxan injection. In brief, pancreas tissue sections were incubated in citrate buffer pH 6.0 at 90°C for antigen retrieval before primary antibody application. Polyclonal guinea pig anti-insulin (Dako Diagnostics Canada, Mississauga, Canada) was used at a dilution of 1:50 in the LSAB (Labelled Streptavidin/Biotin) amplification method. The morphology and insulin content of pancreatic islets was evaluated in optical microscopy.

Short-term effects

In order to verify the acute effect of a unique treatment of the HEBM on blood glucose levels, we allocated the control group and alloxan-induced diabetic rats into four subgroups

each which received orally by gavage three doses of HEBM (0.01, 0.1 and 1 mg/Kg) or vehicle in the morning. The blood glucose was measured before the animals received the treatment and at 15, 60, 120 and 180 min intervals following the treatment as indicated in Figure 1. After, the rats were killed and blood was colleted.

Long-term effects

In the study concerning chronic effects of the HEBM, after hyperglycemia confirmation the animals were randomly subdivided into two subgroups. After observed the results of short-term effects, we decided to use the 1 mg/Kg dose. Rats were treated orally by gavage for 28 days with HEBM at 1 mg/Kg or vehicle (0.9% NaCl) per day. Body weight was measured twice a week and used to regulate the treatment doses. After the experimental period, the animals from different groups were weighted and analyzed 15 h after last treatment as indicated in Figure 2. Blood was collected in three different tubes, i.e. one of total blood for glycated hemoglobin determination, other with anticoagulant for plasma separation, and another without anticoagulant to separate serum for biochemical analyses. The liver, kidney, and gastrocnemius muscle were dissected out, washed in ice-cold saline, blotted dry and weighed.

Total Radical-Trapping Antioxidant Parameter (TRAP)

The total radical-trapping antioxidant parameter (TRAP) has provided a sensitive and easy tool to quantify combined non-enzymatic antioxidant capacity of plasma or tissues. The principle of TRAP measurement has been described previously.[21] An adapted method of TRAP assay was used to determine the capacity of rat plasma treated with HEBM or saline

(short and long-term effects) to trap a flow of water-soluble peroxyl radical produced at constant rate, through thermal decomposition of AAPH. Briefly, the reaction mixture containing 4 mL of the free radical source (AAPH 10 mM) in glycine buffer (0.1 M) pH 8.6 and 10 μL luminol (4 mM) as external probe to monitoring radical production was incubated at 25°C resulting in the emission of luminescence. The addition of plasma sample (150 μg of protein) decreases the luminescence in proportion to the concentration of non-enzymatic antioxidants. Luminescence was measured in out of coincidence mode in a liquid scintillation counter (Wallac 1409 DSA, Wallac Oy, Turku, Finland) as counts per minute (CPM) for 60 min after the addition of sample. Trolox (water-soluble vitamin E analogue) was used as reference peroxyl radical scavenger molecule (positive control). Results are expressed as percentage AAPH (without addition of sample = 100% emission).

Biochemical parameters

After 28 days of treatment the levels of triglycerides, total cholesterol, glycated hemoglobin (HbA_{1C}), and uric acid were determined with commercial kits produced by Human GmbH (Wiesbaden, Germany). Quantification of HDL, creatinine, AST and ALT activity were determined with commercial kits produced by In Vitro Diagnostica S/A (Itabira, Brazil). The concentrations of LDL and VLDL were obtained by Friedewald equation.[22] All colorimetric methods were performed using a spectrophotometer Beckman DU-640 with wavelength accuracy±0.5 nm (Beckman Instruments, Inc., Fullerton, CA, USA).

Tissue wet weight/ dry weight ratio

The long-term effect treatment on tissue wet weight/ dry weight ratio was investigated. After dissection different tissues were weighed, dried and maintained at 37ºC during 36 h to evaporate total water content. Afterwards the tissues were once more weighed and the ratio between fresh tissue and dry tissue was calculated. Data are show in % of dry weight presented in the sample.

Total glycogen content

To determine total glycogen content, after 28 days of treatment, tissue samples were weighed and digested in concentrated 30% KOH, boiled at 100°C for 20 minutes, precipitated with ethanol and measured through colorimetric procedure with iodine.[23]

Glutathione S-transferase (GST) activity

GST function is essential in xenobiotics biotransformation.[26] Hepatic tissue homogenate (10% w/v) was prepared in phosphate buffer 0.1 M, pH 7.4. Glutathione S-transferase (GST, E.C. 2.5.1.18) activity was determined in hepatic tissue spectrophotometrically at 340 nm by measuring the formation of the conjugate of glutathione (GSH) and 1-Chloro-2, 4-nitrobenzene (CDNB) as previously described.[27] In short, liver samples were homogenized in 0.1 M potassium phosphate buffer pH 6.5. Enzyme activity was determined by adding 800 µL of buffer, 50 µL GSH 20 mM, a suitable amount of liver homogenate to a 1 mL cuvette. The reaction started by the addition of 50 µL of CDNB 20 mM was carried out at 30°C, and monitored spectrophotometrically for three minutes. Corrections of the spontaneous reaction were made by measuring and subtracting the rate in absence of enzyme. CDNB conjugate was measured in the supernatant using the molar

extinction coefficient of 9.6 mM^{-1} cm^{-1}. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1µmole of CDNB conjugate/mg protein/min under the assay conditions.

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) widely adopted as an index of lipid peroxidation[24] were determined in tissues from rats which received the treatment for 28 days. The formation of TBARS occurs during an acid-heating reaction as previously described.[25] Briefly, the samples were mixed with 1mL of 10% trichloroacetic acid and 1mL of 0.67% thiobarbituric acid, and subsequently heated in a boiling-water bath for 15 minutes. The TBARS were determined by absorbance at 535 nm and were expressed as malondialdehyde (MDA) equivalents (nm/mg protein).

SOD activity

The superoxide dismutase (SOD, E.C. 1.15.1.1) activity in samples homogenate was measured spectrophotometrically by the inhibition rate of auto-catalytic adrenochrome formation in a reaction buffer containing 1 mM adrenaline, 50 mM glycine-NaOH (pH 10.2) and 1 mM catalase as previously described.[28]

Catalase activity

To determine catalase (CAT, E.C. 1.11.1.6) activity, samples were sonicated in a 50 mM phosphate buffer in ice bath and the resulting suspension was centrifuged at 3000g for 10

min. The supernatant was used for enzyme assay. CAT activity was measured by the decreasing rate in hydrogen peroxide absorbance at 240 nm.[29]

Protein quantification

Protein content was measured as previously described.[30] Lipid peroxidation, TRAP, SOD, CAT and GST activities results were standardized by protein content.

Statistical Analysis

The results are expressed as the mean ± SD (n=5). The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the post hoc least significant difference (LSD) test. Results were considered significantly different when $P<0.05$. All data were analyzed with SPSS for Windows version 8.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Immunohistochemistry

All islets showed normal histological structures in hematoxilin-eosin observations amongst the groups (data not show). In immunohistochemical staining using antiinsulin antibody, diabetic-induced group showed a pronounced decreased in immunoreactivity against antiinsulin in all pancreatic islet and normal morphology was observed in control group as shown in Figure 3.

Short-term effects

Blood glucose

The Figure 4 shows the blood glucose levels of control and diabetic-induced groups before and after oral administration of HEBM or saline. The administration of alloxan to the rats markedly increased blood glucose levels as compared with control animals. HEBM treatment at 0.1 and 1 mg/Kg decreased blood glucose levels at 60 and 120 min after HEBM oral administration in the diabetic rats. The hypoglycemic effect was more pronounced 120 min after oral treatment, around of 23%. After 180 min we did not observed significant difference in blood glucose levels between all animals from diabeteic-induced group.

Total Radical-Trapping Antioxidant Parameter (TRAP)

The plasma samples from control and diabetic-induced groups were antioxidant in TRAP assay, as indicated in Figure 5. However, the oral dose of 1 mg/Kg of HEBM increased the plasma antioxidant potential in both groups when compared to plasma from animals that received only saline. Doses of 0.01 and 0.1 mg/Kg HEBM were not different from saline.

Long-term effects

Blood glucose and bodyweight changes

A single i.p. injection of alloxan to the fasted rats markedly changed body weight and increased blood glucose levels as compared with non-induced rats as indicated in Table 1. The diabetic group which was treated for 28 days with HEBM at 1 mg/Kg per day did not show any decrease in blood glucose levels when compared to diabetic-induced group which had received saline. The weight variation (final weight – initial weight) shows that a

hyperglycemic status maintained during four weeks prevented the weight gain (-4.14 g) and HEBM treatment decreased significantly (-40.02 g) the final weight of the animals that received 1 mg/Kg (Table 1).

Total Radical-Trapping Antioxidant Parameter (TRAP)

The plasma samples from control and diabetic-induced groups showed the same antioxidant capacity in TRAP assay and we not observed difference among any groups (Fig. 6).

Biochemical parameters

The Table 2 shows the biochemical parameters of control and diabetic-induced groups 28 days after oral administration of HEBM or saline. There was a significant increase in HbA_{1C} from diabetic animals that received only saline. Diabetic-induced animals, treated with HEBM at 1mg/Kg per day, showed a decrease in HbA_{1C} when compared to animals of diabetic-induced group that received only saline. The HEBM treatment to diabetic-induced group increased the triglycerides and VLDL levels when compared to control group and diabetic animals which received saline. We did not observe significant difference in LDL levels among experimental groups analyzed. Total cholesterol and HDL were increased in the diabetic group and HEBM treatment did not alter these parameters. Uric acid was significantly increased in diabetic animals (128%) and HEBM treatment reduced this effect. In addition, when control animals where treated with HEBM, we observed a decrease in uric acid levels. There was a significant increase in creatinine in diabetic animals which received only saline (16%), HEBM treatment did not affect this parameter in control group and reduces creatinine to control-saline levels in diabetic animals. We observed that a single oral dose HEBM at 1 mg/Kg per day increased the levels of AST and ALT in

animals from diabetic-induced group whereas there was no significant difference between another groups.

Tissue wet weight/dry weight ratio

The kidneys from diabetic-induced animals which received saline showed a decrease in dry weight when compared to other groups (Table 3). We did not observed significant differences on liver and gastrocnemius muscle dry weight between the groups analyzed.

Total glycogen content

In Table 4, we may observe the total glycogen content in liver, kidney, and gastrocnemius muscle. Control rats that were treated with HEBM showed a reduction in glycogen content in liver (27.3%) and kidney (nearly emptied), but an increase in the glycogen content of gastrocnemius muscle (4.3 times). We also observed that diabetic induction had decreased the total glycogen content in liver (81%), and increased in kidney (2.5 times) and in gastrocnemius muscle (2.8 times). The HEBM treatment to diabetic animals induced a decrease in total glycogen content in liver (nearly depleted), and an increase in kidney (1.5 times) and in gastrocnemius muscle (1.6 times).

Glutathione S-transferase activity

Liver GST activity was quantified as an indicative of HEBM hepatotoxic potencial. Our results showed a significant reduction in GST activity (41 %) in the liver of diabetic animals that received an oral dose of HEBM per day (Fig. 7). We did not observ significant alterations in GST activity in control group animals that received the same treatment.

Lipid peroxidation

The lipoperoxidation in liver, kidney, and gastrocnemius muscle among groups are presented in Figure 8A. Animals that received HEBM treatment in control and diabetic groups showed an increase lipoperoxidation in liver when compared to control-saline group. Diabetic group showed MDA equivalents increased in kidney. However, HEBM treatment decreased it to control-saline levels. MDA equivalents levels were found decreased in gastrocnemius muscle of diabetic rats and even more decreased in diabetic animals that received HEBM.

SOD activity

Results of SOD activity in liver, kidney, and gastrocnemius muscle among groups are presented in Figure 8B. In the liver from diabetic-saline group, a significant reduction in SOD activity was observed when compared to control-saline group. Diabetic group showed increased SOD activity in kidney when compared to control-saline group. In both cases, HEBM treatment attenuated diabetic effects and turned SOD activity to control-saline levels. We did not observe significant difference in gastrocnemius muscle SOD activity among groups.

Catalase activity

CAT activity in liver, kidney, and gastrocnemius muscle are presented in Figure 8C. In the liver of control and diabetic animals that received HEBM we observed a decrease in CAT activity when compared to animals from control-saline group and diabetic-saline group.

HEBM treatment induced a decrease in kidney CAT activity of diabetic rats. In gastrocnemius muscle from control and diabetic groups treated with HEBM we observed an increase in CAT activity when compared to both control and diabetic animals treated with saline.

DISCUSSION

Experimental models of diabetes that utilizes diabetogenic agents (alloxan and streptozotocin) and induce blood glucose levels higher than 300 mg/dL have been considered as severe diabetes. Our preliminary studies concerning alloxan-induced diabetic rats showed some severe symptoms of type 1 diabetes in these animals: polydipsia, polyphagia, and polyuria which were reduced by insulin therapy (data not shown). Before HEBM treatment investigation we observed the damage to β cells (insulin source) after alloxan injection (Fig. 3). The impaired insulin secretion from β cells results in abnormal glucose homeostasis leading to type 1 diabetes.

In accordance with World Health Organization (WHO) recommendations,[31] chemotaxonomic evidences and previous reports[9-19] we utilized a plant which is described as a possible hypoglycemic agent. We observed a weak effect in blood glucose levels only until 120 min after oral treatment and that was related to increased plasma antioxidant potential at 1mg/Kg dose. This result agrees with our previous work that demonstrated the antioxidant potential of *B. microstachya* extracts *in vitro* and corroborates the hypoglycemic effect of *Bauhinia* genus.[9-13] However, in the long-term study we did not observe the hypoglycemic effect and the increased plasma antioxidant potential either. A possible explanation is the time (15 h) in which the animals were analyzed before the last

dose of HEBM. Although the absence of effect in blood glucose and plasma antioxidant potential in long-term study, the decrease in HbA_{1C} could be an indicative of blood glucose control and antioxidant potential since in the stabilization of protein glycation the free radicals formation seems to be involved.[32]

Chronic hyperglycemia is a well-recognized pathogenic factor of long-term complications in diabetes mellitus[33,34] and consumption of antioxidants from diet has been correlated to reduction in some diabetic complications[35] as well as decrease in protein glycation.[36-38] Previously, we have demonstrated the antioxidant potential and free radical scavenger ability of HEBM[19] and our present findings suggest that some of the compounds present in *B. microstachya* leaves could contribute to protein glycation reduction. Some phenolic constituents from *B. microstachya* leaves were identified as kaempferol 3-*O*-rhamnosyl, quercitrin, myricitrin, methyl gallate, and vitexin.[14-16] Our previous report identified an gallic acid-like substance as a major component (25.41%) in HEBM. Gallic acid is acknowledged as a potent antioxidant and is structurally similar to methyl gallate. In addition, Nakagawa and colleagues suggested that the presence of the gallate group is essential in the protective activity against protein oxidation and glycation, and that there is also a contribution by the hydroxyl group at the 5' position in the ring and the sterical structure of green tea tannin mixture.[39] We suggest that the gallic acid of HEBM may be essential against glycation and the consumption of preparations from *B. microstachya* leaves reduces diabetes disturbances related to glycation. However, the mechanisms by which some phenolic compounds present in HEBM inhibit glycation remain unclear.

The hyperglycemia and insulin deficiency are associated with hypercholesterolemia and hypertriglyceridemia in both clinical and experimental diabetes.[40,41] Previous studies have reported the benefice of plant extracts in hyperlipidemia treatment in experimental

animals models.[41,42] However, HEBM extract increased triglycerides, VLDL, HDL, and total cholesterol and reduced the HDL/LDL ratio from 4.93 to 2.81 in diabetic animals. This suggests that HEBM treatment can lead to an increase in the risk of developing heart diseases, because a high HDL/LDL ratio has been shown to be beneficial and is indicative of a lower risk of coronary heart disease.[43] In addition, the expressive reduction observed in final bodyweight of diabetic animals which were treated with HEBM may be considered a negative point of HEBM treatment, since that bodyweight loss is a classic symptom of type 1 diabetes. In contrast, the bodyweight loss observed in diabetic animals after HEBM treatment, allow to speculate the potential use of *B. microstachya* in type 2 diabetes, in which the overweight is a common aspect. However, specific studies using type 2 diabetes models are necessary to confirm this suggestion.

In order to investigate the potential hepatotoxic effect of HEBM we determined GST, AST, and ALT activities. Liver GST is an enzyme whose essential function is the biotransformation of xenobiotics[26] and there is a high concentration of transferases in liver tissue, i.e. approximately 10% of total soluble protein.[44] The reduction in GST activity observed in diabetic animals treated with HEBM indicate an harmful inhibitory effect on liver, since that GST is essential in phase II drug-metabolizing system and this activity reduction may results in tissue damage. Some works showed the inhibitory effects of plant polyphenols on rat liver glutathione S-transferase activity.[45,46] However, the mechanism of GST inhibition by plant polyphenols has not been fully elucidated. Das and colleagues suggested that the presence of polyhydroxylations in plant polyphenols is important for GST inhibition.[47] *B. microstachya* leaves possesses several polyphenolic compounds as constituents[14-16,19] and these hydroxyl groups could be essential to decrease GST activity reduction. On the other hand, the development of intrinsic and acquired resistance of tumor

cells against chemotherapeutic agents is a major problem in the treatment of various types of cancer. The increased GST expression and therefore enhanced GST-mediated conjugation of chemotherapeutic agents used in cancer treatment have been suggested as among the mechanisms of drugs resistance.[48,49] We suggest that some compound from HEBM could be considered as GST inhibitor in tumor cells.

The association between the reduction of the GST activity in liver of diabetic animals that were treated with HEBM and the increase in AST and ALT serum activity in the same group is an indicative of liver damage. The utility of the ratio of serum AST to ALT has been examined in several liver disorders, including alcoholic liver disease, acetaminophen hepatotoxicity, acute viral hepatitis, and fatty infiltration of the liver.[50] The reduction in AST/ALT ratio of diabetic-HEBM group (0.99) and the AST/ALT ratio > 1 of other groups may represent a hepatic dysfunction in diabetic animals treated with HEBM. It is possible that the relative activities of AST and ALT changes in disease progresses. In addition, animals from control and diabetic groups that received HEBM treatment showed an increase in MDA equivalents in liver tissue when compared to control-saline group. The increase in liver SOD/CAT ratio of groups which were treated with HEBM (control-HEBM=0.21 and diabetic-HEBM=0.19) compared to untreated groups (control-saline=0.12 and diabetic-saline=0.12) suggest that the imbalance observed in the activities of these antioxidant enzymes may be part of HEBM-induced liver damage. SOD and CAT are components of antioxidant defense system. SOD act removing superoxide anions ($O_2^{\bullet -}$) generating hydrogen peroxide (H_2O_2) which is removed by CAT. The imbalance observed in liver leads to H_2O_2 overproduction which may results in $HO^{\bullet}$ generation and tissue damage by Fenton reaction.[51]

In many countries, diabetic nephropathy is a major complication of diabetes causing disabilities and a high mortality rate. However, the mechanisms underlying the pathogenesis of diabetic nephropathy are not completely understood. Progression of diabetic nephropathy is considered a result of the interaction of multiple factors, such as high glucose, the polyol pathway, oxidative stress, non-enzymatic protein glycation, and cytokines.[52] With the onset of diabetes mellitus, there is a subsequent decrease in creatinine and urea clearance. It has also been observed that increased blood urea nitrogen and serum creatinine in diabetic rats indicates progressive renal damage.[53] Blood uric acid and creatinine levels were used as index of kidney function. The degradation of the purines nucleotides occurs mainly in the liver. The pathway of purine *in vivo* is as follows: adenosine → inosine → hypoxanthine → xanthine → uric acid. In kidney through tubular secretion uric acid is excreted in the urine. Creatinine is produced in a relatively constant rate from creatine phosphate which serves as a small reservoir of high energy phosphate and plays an important role in muscle contraction. Creatinine cannot be further metabolized. The amount of creatinine excreted in urine each day is constant. The increase in blood uric acid level (128%) and creatinine (16%) of diabetic-saline group suggest an impaired renal function in diabetic animals. In addition, the reduction in dry weight of kidney and the increase in lipoperoxidation corroborate this indication. Oxidative stress has been known to play an important role in the development and progression of diabetic nephropathy, and the formation of reactive oxygen species (ROS) is a direct consequence of hyperglycemia. The increase in kidney SOD activity suggests an overproduction of superoxide without CAT activity increment. This imbalance in antioxidant enzymes was related to lipoperoxidation increase and oxidative stress is probably involved in kidney dysfunction. The antioxidant properties and radical scavenger potential of HEBM treatment

were essential in reducing kidney damage. The renoprotective effects of HEBM seem to result from its scavenger effect on ROS. The administration of HEBM to diabetic animals resulted in a considerable reduction in the intensity and incidence of these changes. An interesting reduction in uric acid levels in animals that received HEBM was observed in control group. This data may be an indicative of reduction in velocity of purine degradation pathway. Further studies using appropriate approach and specific targets are needed to underlie the molecular mechanisms of these observations.

Skeletal muscles such as gastrocnemius are target tissues for insulin and the loss of muscular mass represents a considerable portion of bodyweight loss in type 1 diabetes. ROS, including hydrogen peroxide, are produced in almost all tissues by highly regulated enzymes and play a decisive role in numerous signaling pathways.[54] Some of these signaling pathways are involved in the control of anabolic and catabolic processes. The increased rate of protein degradation resulting from reduced insulin action may be involved in lipoperoxidation decrease observed in diabetic group, particularly in diabetic-treated group as a consequence of lipid metabolism. No difference was observed in SOD activity among the groups associated to CAT activity increased in diabetic and control-HEBM groups are indicatives of protein catabolism resulting from reduced insulin signaling in gastrocnemius muscle, similarly to reported previously.[55] These results are in accord to bodyweight reduction observed in diabetic group and animals that received HEBM treatment. We believe that HEBM treatment may accelerate the protein catabolism in skeletal muscle tissue contributing to bodyweight reduction. Unfortunately, the effect of natural products and vegetal extracts on skeletal muscle tissue physiology is few studied.

Glycogen is the primary intracellular storable form of glucose and its levels in various tissues are a direct reflection of insulin activity. Insulin promotes intracellular

glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase pathways. Paradoxically diabetes increased the total glycogen content in gastrocnemius muscle, a target tissue for insulin action. This result is in contrast to previous reports.[56,57] However, in kidney we found an increase in total glycogen content and in liver a significant reduction, in agreement with a recent report.[56] In liver of diabetic-HEBM group the glycogen was almost depleted similarly as observed in kidney of control-HEBM group. As general mode, the HEBM treatment amplified the effects of diabetes on total glycogen content in different tissues; however, we did not observed a pattern of HEBM action on different tissues of control group. The HEBM treatment modulated the total glycogen content in all tissues analyzed, including control group, indicating no relation between HEBM action and blood glucose level. In addition, no modulation pattern on different tissues suggests a tissue-specific action. This interference in glycogen metabolism indicate a signaling effect in glycogen metabolic pathway which may be modulated by phenolic-rich extracts as reported previously.[58]

In conclusion, the hydro-ethanolic extract of *B. microstachya* leaves (HEBM) had a glucose-lowering effect on alloxan-induced diabetic rats. We observed a reduction on HbA_{1C} and an increase in antioxidant potential *in vivo*. Our findings suggest kidney protective effect of HEBM and glycogen metabolism modulation by a tissue-specific mechanism. However, HEBM treatment can lead to an increase in the risk of developing heart diseases, due to impaired alterations on blood lipid profile. The expressive reduction observed in bodyweight of diabetic animals which were treated with HEBM may be considered other negative point of HEBM treatment. In addition, long-term use of HEBM may induce hepatic dysfunction. Further studies are necessary to isolate the active

compounds responsible for the different effects observed and to elucidate the exact mechanisms of action of HEBM in different tissues.

ACKNOWLEDGEMENTS

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Brazil), Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Pró-Reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (PROPESQ/UFRGS).

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Table 1.

Table 1. The effect of HEBM on blood glucose and bodyweight variation in experimental animals

Group	Blood glucose (mg/dL)	Weight variation (g)
Control + saline	98.1 ± 3.8	59.25 ± 3.19
Control + 1 mg/Kg HEBM	95.8 ± 1.52	52.66 ± 11.46
Diabetic + saline	501.85 ± 33.65*	-4.14 ± 4.03*
Diabetic + 1 mg/Kg HEBM	523.5 ± 60.96*	-40.02 ± 11.19*†

Values are expressed as the mean ± SD for five animals each group.

*$P<0.05$ compared with control-saline group. †$P<0.05$ compared with diabetic-induced treated with saline.

Table 2.

Table 2. The effect of HEBM on basic biochemical parameters in experimental animals

	Control		Diabetic-induced	
	Saline	HEBM 1 mg/Kg	Saline	HEBM 1 mg/Kg
HbA_{1C} (%)	2.59 ± 0.07	2.86 ± 0.04	4.29 ± 0.10*	3.87 ± 0.12*†
Triglycerides (mg/dL)	66.41 ± 4.12	75.81 ± 4.33	77.67 ± 12.64	167.44 ± 55.11*†
VLDL (mg/dL)	13.28 ± 0.82	15.16 ± 0.86	15.53 ± 2.52	33.48 ± 11.02*†
LDL (mg/dL)	7.03 ± 1.05	7.97 ± 1.46	7.42 ± 1.98	14.37 ± 6.86
HDL (mg/dL)	25.49 ± 0.41	27.85 ± 2.01	36.59 ± 2.76*	40.39 ± 3.71*
Total cholesterol (mg/dL)	37.62 ± 1.28	41.39 ± 3.18	51.33 ± 5.16*	57.96 ± 3.97*
Uric acid (mg/dL)	0.96 ± 0.07	0.73 ± 0.09*	2.19 ± 0.83*	0.64 ± 0.09*†
Creatinine (mg/dL)	0.44 ± 0.02	0.39 ± 0.04	0.51 ± 0.03*	0.49 ± 0.02
AST activity (U/mL)	66.06 ± 0.61	64.03 ± 1.21	68.51 ± 1.54	79.11 ± 6.69*
ALT activity (U/mL)	62.69 ± 0.77	62.15 ± 0.65	64.56 ± 2.01	79.32 ± 9.95*

Values are expressed as the mean ± SD for five animals each group. *P <0.05 compared with control-saline group. †P <0.05 compared with diabetic-induced treated with saline.

Table 3.

Table 3. The effect of HEBM on wet tissue weight/dry tissue weight ratio in experimental animals

	Control		Diabetic-induced	
	Saline	HEBM 1 mg/Kg	Saline	HEBM 1 mg/Kg
Liver	32.56 ± 0.43	32.84 ± 0.41	32.87 ± 0.53	32.14 ± 0.36
Kidney	24.36 ± 0.49	25.04 ± 0.52	22.44 ± 0.29*	23.13 ± 0.33
Gastrocnemius muscle	23.85 ± 0.62	24.1 ± 0.19	24.42 ± 0.34	23.09 ± 0.43

Data are show in % of dry weight of the sample. Values are expressed as the mean ± SD for five animals each group. *P <0.05 compared with control group.

Table 4.

Table 4. The effect of HEBM on total glycogen content in experimental animals

	Control		Diabetic-induced	
	Saline	HEBM 1 mg/Kg	Saline	HEBM 1 mg/Kg
Liver (mg/%)	2.14 ± 0.17	1.56 ± 0.21*	0.41 ± 0.06*	0.03 ± 0.017*†
Kidney (mg/%)	0.89 ± 0.07	0.01 ± 0.002*	2.21 ± 0.21*	3.33 ± 0.57*†
Gastrocnemius muscle (mg/%)	0.82 ± 0.07	3.53 ± 0.46*	2.32 ± 0.35*	3.82 ± 0.17*†

Values are expressed as the mean ± SD for five animals each group. *P <0.05 compared with control-saline group. $^{\dagger}P$ <0.05 compared with diabetic-induced treated with saline.

Figure 1.

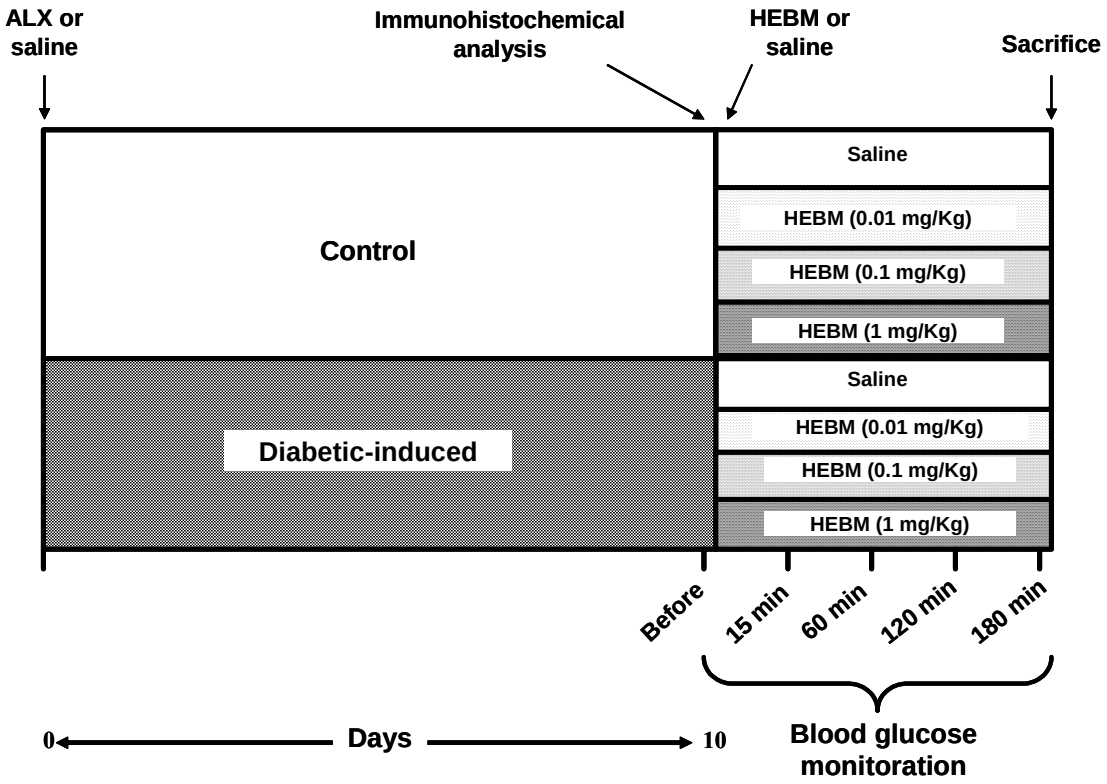
ALX or saline
Immunohistochemical analysis
HEBM or saline
Sacrifice
Control
Diabetic-induced
Saline
HEBM (0.01 mg/Kg)
HEBM (0.1 mg/Kg)
HEBM (1 mg/Kg)
Saline
HEBM (0.01 mg/Kg)
HEBM (0.1 mg/Kg)
HEBM (1 mg/Kg)
Before
15 min
60 min
120 min
180 min
0
Days
10
Blood glucose monitoration

Figure 2.

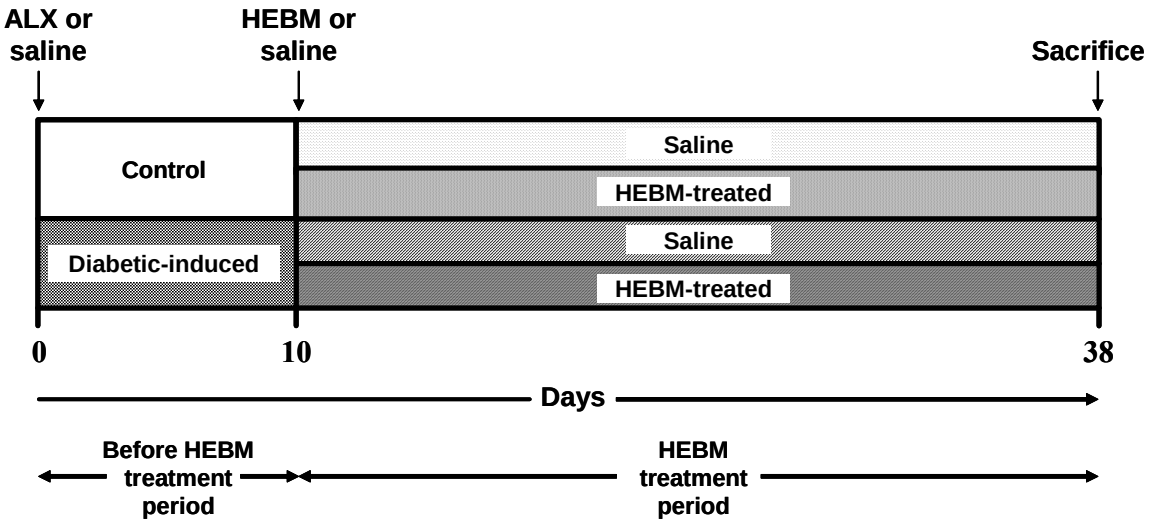
ALX or saline
HEBM or saline
Sacrifice
Control
Saline
HEBM-treated
Diabetic-induced
Saline
HEBM-treated
0
10
38
Days
Before HEBM treatment period
HEBM treatment period

Figure 3.

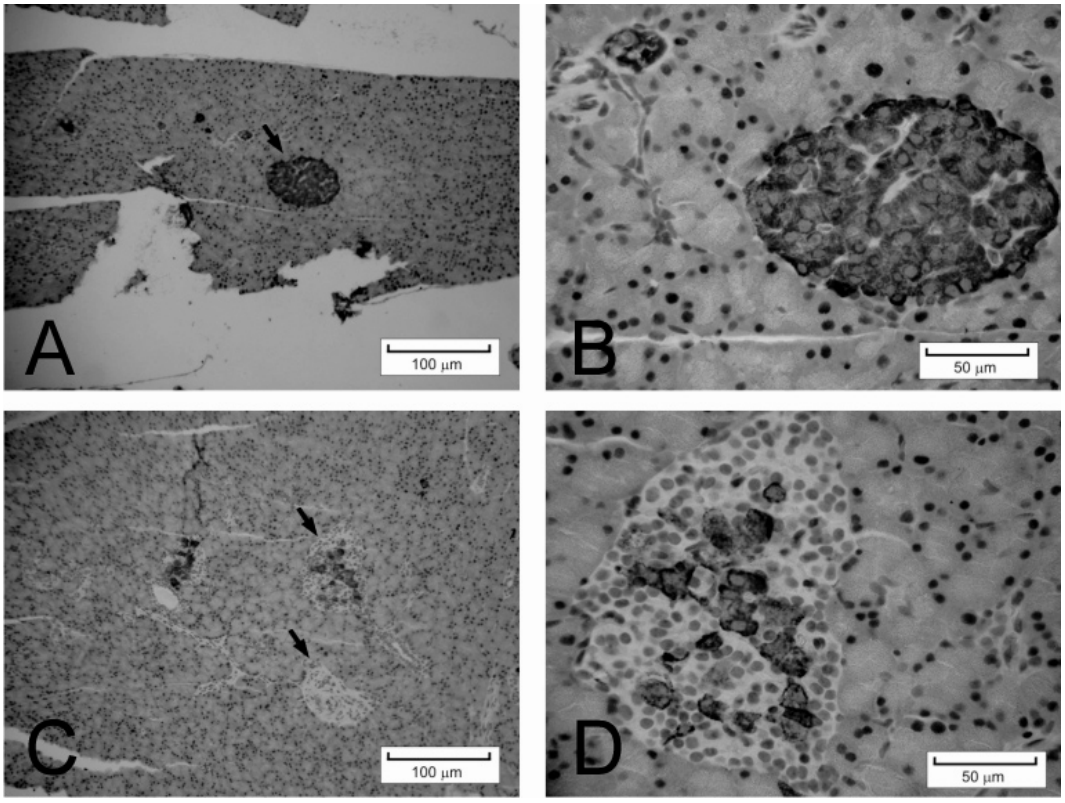

Figure 4.

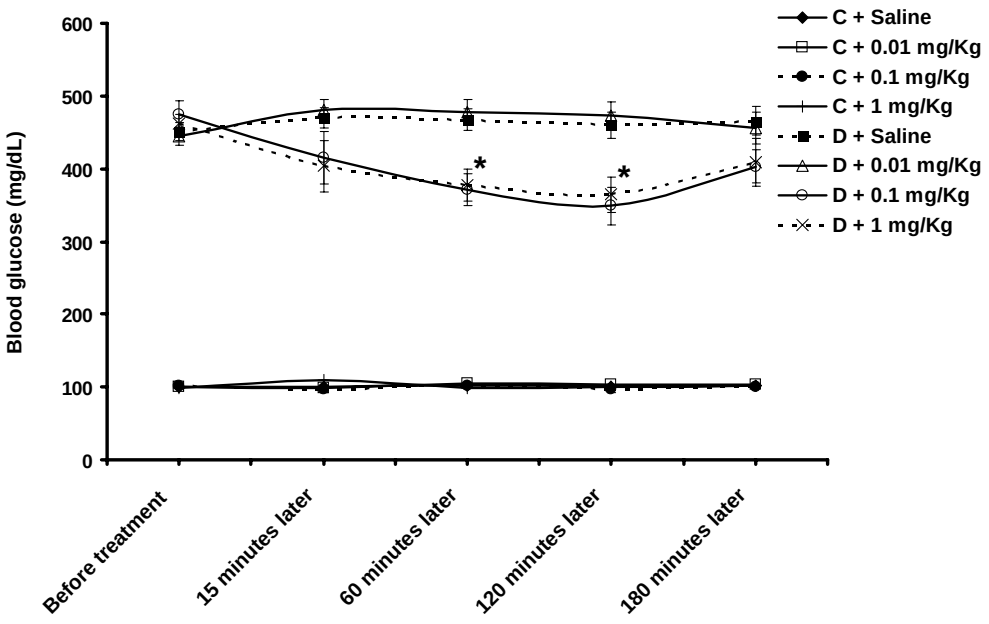
Blood glucose (mg/dL)
600
500
400
300
200
100
0
Before treatment
15 minutes later
60 minutes later
120 minutes later
180 minutes later
C + Saline
C + 0.01 mg/Kg
C + 0.1 mg/Kg
C + 1 mg/Kg
D + Saline
D + 0.01 mg/Kg
D + 0.1 mg/Kg
D + 1 mg/Kg
*
*

Figure 5.

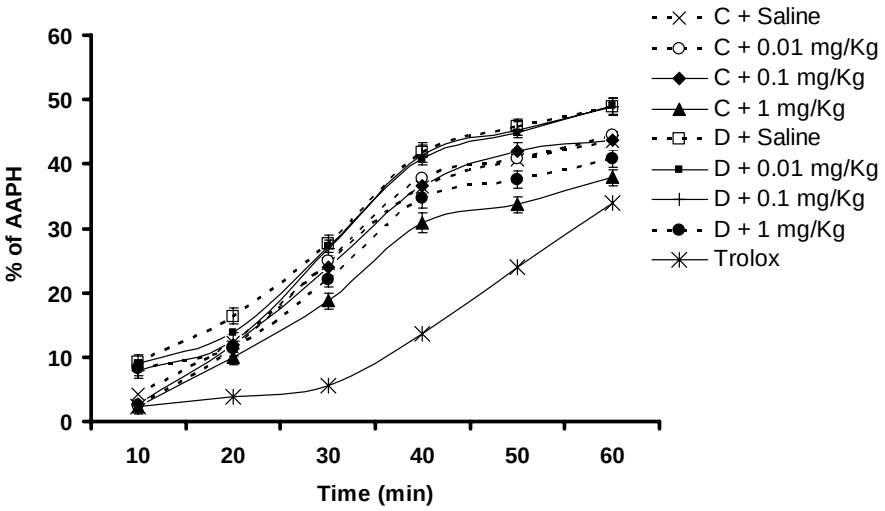
% of AAPH
60
50
40
30
20
10
0
10
20
30
40
50
60
Time (min)
C + Saline
C + 0.01 mg/Kg
C + 0.1 mg/Kg
C + 1 mg/Kg
D + Saline
D + 0.01 mg/Kg
D + 0.1 mg/Kg
D + 1 mg/Kg
Trolox

Figure 6.

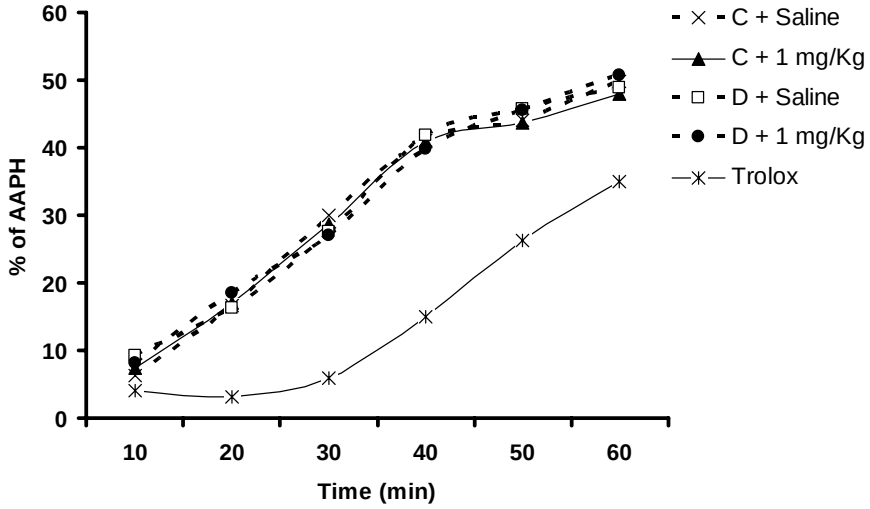
60
50
40
30
20
10
0
% of AAPH
10
20
30
40
50
60
Time (min)
C + Saline
C + 1 mg/Kg
D + Saline
D + 1 mg/Kg
Trolox

Figure 7.

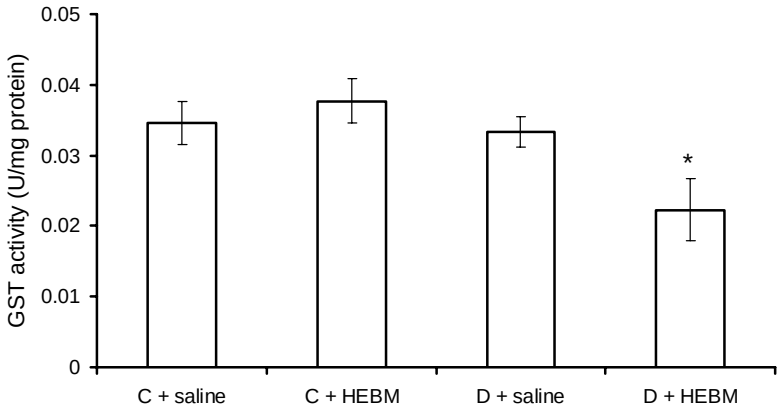
GST activity (U/mg protein)
0.05
0.04
0.03
0.02
0.01
0
*
C + saline
C + HEBM
D + saline
D + HEBM

Figure 8.

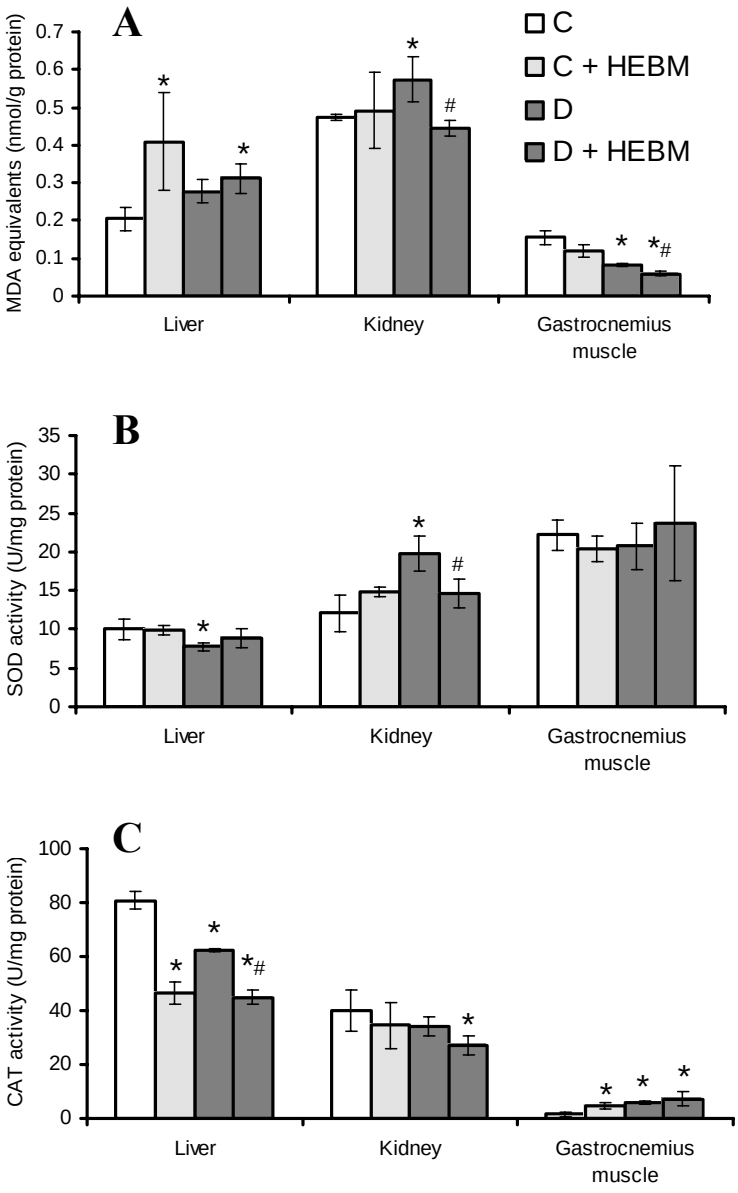
A
MDA equivalents (nmol/g protein)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C
C + HEBM
D
D + HEBM
Liver
Kidney
Gastrocnemius muscle
B
SOD activity (U/mg protein)
35
30
25
20
15
10
5
0
Liver
Kidney
Gastrocnemius muscle
C
CAT activity (U/mg protein)
100
80
60
40
20
0
Liver
Kidney
Gastrocnemius muscle

Figure legends.

Fig. 1 Experimental design of HEBM short-term effects. Animals were divided into two groups: control (non-induced) and diabetic-induced group. Diabetes was induced by an i.p. injection of 150 mg/kg of alloxan and the control group received only 0.9% NaCl. Ten days later, some animals were used in immunohistochemical insulin detection. We allocated the control group and alloxan-induced diabetic rats into four subgroups each (n=5) that received orally by gavage three doses of HEBM (0.01, 0.1 and 1 mg/Kg) or vehicle in the morning. The blood glucose was measured before the animals received the treatment and at 15, 60, 120 and 180 min intervals following the treatment. After, the rats were killed humanely and the blood was colleted.

Fig. 2 Experimental design of HEBM long-term effects. Animals were randomly divided into two groups: control (non-induced) and diabetic-induced group. Diabetes was induced by an i.p. injection of 150 mg/kg of alloxan and the control group received only 0.9% NaCl. These were treated orally by gavage for 28 days with a single intragastric dose of HEBM (1 mg/Kg) or vehicle (0.9% NaCl) per day (n=5). After the experimental period, the animals from different groups were weighted and analyzed.

Fig. 3A-D Optical microscopy of the pancreatic tissue prepared for immunohistochemical insulin detection from the control **(A-B)** and diabetic-induced groups **(C-D)**. Pancreas tissue sections were incubated in citrate buffer pH 6.0 at 90°C for antigen retrieval before primary antibody application. Polyclonal guinea pig anti-insulin was used at a dilution of 1:50 in the LSAB amplification method. The islets are indicated **(→)** in magnification x100 in control group **(A)** with islet cells insulin positive and diabetic-induced group **(C)**

showing low insulin detection (up arrow) or no insulin detected (down arrow). The same fields are showed in **(B)** and **(D)** magnification x400.

Fig. 4 Blood glucose level before and after (15, 60, 120 and 180 min) HEBM treatment. The animals received orally by gavage three doses of HEBM (0.01, 0.1 and 1 mg/Kg) or vehicle. **C** (control group) and **D** (diabetic-induced group). Values are expressed as means±S.D. (n= 5 for each group). * indicate significant difference ($P<0.05$) among diabetic-saline and diabetic-treat group (0.1 or 1 mg/Kg).

Fig. 5 Total radical-trapping antioxidant parameter in the plasma of rats. Animals were treated with an oral dose of vehicle (saline) or HEBM (0.01, 0.1 and 1 mg/Kg). 180 min after treatment animals were killed and the plasma separated for determination of total radical-trapping antioxidant parameter as described under Materials and methods. **C** (control group) and **D** (diabetic-induced group). Values are expressed as means±S.D. (n= 5 for each group).

Fig. 6 Total radical-trapping antioxidant parameter in the plasma of rats. Animals were treated with an oral dose of vehicle (saline) or HEBM (1 mg/Kg) per day. After the experimental period (28 days), the animals from different groups were killed humanely and the plasma separated for determination of total radical-trapping antioxidant parameter as described under Materials and methods. **C** (control group) and **D** (diabetic-induced group). Values are expressed as means±S.D. (n= 5 for each group).

Fig. 7 Glutathione S-transferase activity in liver of rats. Animals were treated with an oral dose of vehicle (saline) or HEBM (1 mg/Kg) per day. After the experimental period (28 days), the animals from different groups were killed humanely and hepatic tissue prepared for GST activity determination as described under Materials and methods. **C** (control group) and **D** (diabetic-induced group). Values are expressed as means±S.D. (n= 5 for each group). * indicate significant difference ($P<0.05$) among diabetic-HEBM group and other groups.

Fig. 8A-C Histograms representing lipoperoxidation **(A)**, superoxide dismutase (SOD) activity **(B)** and catalase (CAT) activity **(C)** detected in tissues after experimental period (28 days) in which animals were treated by intragrastric dose of saline or HEBM (1 mg/Kg) per day **(A)**. **C** (control group) and **D** (diabetic-induced group). Values are expressed as means±S.D. (n= 5 for each group). * indicate significant difference ($P<0.05$) when compared to control-saline group and # indicate significant when compared to diabetic-saline group.

Capítulo 4

Hydro-ethanolic extract of *Bauhinia microstachya* leaves reduces oxidative stress and modulates glycogen content by GSK-3β independent pathway in alloxan-induced diabetic rats

Manuscrito submetido à

Chemico-biological interactions (2007)

HYDRO-ETHANOLIC EXTRACT OF *BAUHINIA MICROSTACHYA* LEAVES REDUCES OXIDATIVE STRESS AND MODULATES GLYCOGEN CONTENT BY GSK-3β INDEPENDENT PATHWAY IN ALLOXAN-INDUCED DIABETIC RATS

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Abstract

Increased risk of cardiovascular disease is strongly associated to diabetes. Free radical mediated oxidative stress play an important role in subcellular remodeling and cardiac dysfunction in diabetes. Prior studies demonstrated an imbalance in antioxidant enzymes activity and increased lipid peroxidation related to diabetes cardiac dysfunction. *Bauhinia microstachya* is a creeper plant that occurs naturally in southern Brazil and often used as herbal antidiabetic medicine. Our recent work demonstrated that the *B. microstachya* leaf extracts presents effective *in vitro* antioxidant activity. The present investigation focused to evaluate the long-term effects of the ethanolic extract of *B. microstachya* leaves (HEBM) on cardiac tissue, their impact on antioxidant, morphological status and a mechanistic approach in alloxan-induced diabetic rats. We observed a reduction to the control level in protein damage, CAT and SOD activity when diabetic animals received HEBM treatment. Our results in whole mode suggest that HEBM treatment was effective in reduces oxidative stress in diabetic heart tissue and these decreases could be related to antioxidants found in HEBM. HEBM treatment reduced total glycogen content when compared to diabetic-vehicle group. The increase in the glycogen content, observed in alloxan group, could be due by the decrease of the phosphorylation and consequently activation of GSK-3β. In the other hand, the glycogen content reduction induced by HEBM treatment observed in diabetic animals was drove by other mechanism than GSK-3β, possibly by glycogen degradation and/or consumption increased.

1. Introduction

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia and alterations in the carbohydrate, fat and protein metabolism. Increased risk of cardiovascular disease is strongly associated to diabetes; in addition, retinopathy and neuropathy are other vascular complications commonly found in DM [1-3]. This metabolic disorder is the major factor for the development of cardiovascular complications and related diseases accounts for 80% of all diabetic mortality [4]. Some studies reporting impaired cardiac function have been published in both clinical and experimental diabetes [5-8].

Beyond of those increased risks and metabolic alterations, experimental findings suggest that reactive oxygen species (ROS) have been implicated in DM [9-11]. Studies have shown that increased lipid peroxides and oxidative stress are present in diabetic subjects [11,12]. In DM, oxidative stress caused by increased production of ROS, reduction in antioxidant defenses and altered cellular redox status seems to be associated to tissue damage [9-11]. Some works showed that free radical mediated oxidative stress play an important role in subcellular remodeling and cardiac dysfunction in diabetes [13,14]. The heart is a susceptible organ because it contains low levels of free radical scavengers such as metallothionein [15]. Prior studies demonstrated an imbalance in antioxidant enzymes activity and increased lipid peroxidation related to DM cardiac dysfunction [16-18]. Experimental findings suggest that antioxidants can improve cardiac function in diabetes [19].

Plants of *Bauhinia* genus have been frequently used in folk medicine to treat several ailments, especially diabetes. This group comprises approximately 250 species and is widely distributed in tropical areas [20]. Previous studies demonstrated the potential of the

plants from *Bauhinia* genus against diabetes and its effects [21-25]. *Bauhinia microstachya* (Raddi) Macbr. (Caesalpinaceae) is a creeper plant that occurs naturally in southern Brazil where is popularly known as "escada-de-macaco" (monkey's ladder) and often used as herbal antidiabetic medicine. Phytochemical investigations with *B. microstachya* leaves have identified compounds such as steroidal glycosides, triterpenes, lactones and phenolic compounds, mainly flavonoids [26-28]. This group of secondary metabolites displays a remarkable array of biochemical interactions, probably due to antioxidant properties [29]. These substances may act as metal chelators and/or free radical scavengers [30].

Our recent work demonstrated that the *B. microstachya* leaf extracts presents effective *in vitro* antioxidant activity [31]. However, the effect of *B. microstachya* on diabetes and their complications remains not studied. In spite of the vast pharmacological activities of *Bauhinia* genus, its protective effect on cardiovascular disease associated to diabetes has not been well documented. Hence, the present investigation focused to evaluate the long-term effects of the ethanolic extract of *B. microstachya* leaves on cardiac tissue, their impact on antioxidant, morphological status and a mechanistic approach in alloxan-induced diabetic rats.

2. Materials and methods

2.1. Chemicals

Alloxan monohydrate, bovine seric albumin (BSA), Folin-Ciocalteau, glycine, hydrogen peroxide, SDS, polyacrylamide, 2-thiobarbituric acid (4,6 dihydroxypyrimidine-2-thiol), β-mercaptoethanol, adrenaline and catalase (CAT) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Periodic acid Schiff (PAS) reagent was purchased from Merck Chemicals KGaA (Darmstadt, Germany). Water was purified using a Milli-Q

system from Millipore Corp. (Billerica, MA, USA) and all other reagents used in this study were of analytical grade.

2.2. Plant material

B. microstachya leaves were collected in the city of Carlos Barbosa, in the state of Rio Grande do Sul, Brazil, in an area located S 29°19'33.2'' W 51°25'54.1''. Location data were obtained with a GPS 38 (Garmin Corp., Olathe, KS, USA). Professor Valdely Ferreira Kinupp identified the plant and the reference material (ICN 144067) is kept in the Herbarium of the Departmento de Botânica of the Universidade Federal do Rio Grande do Sul (UFRGS).

2.3. Plant extract

B. microstachya leaves were air-dried at 35 ± 2°C and then reduced to powder. *B. microstachya* hydro-ethanolic extract (HEBM) was obtained by macerating dried leaves powder in ethanol 40% (15% w/v) at room temperature and in darkness. After eight days the material was filtered, ethanol having been eliminated under reduced pressure, and then it was stored at -20°C until the use.

2.4. Animals

Nine-week-old male Wistar rats (200-245 g) were obtained from Biochemical Department breeding colony. Rats were housed in plastic cages, maintained at 22 ± 1°C, 55% relative humidity and 12 h light/dark cycle. Animals were supplied with commercial pellet food (Nuvilab CR-1 Curitiba, PR, Brazil) and water *ad libitum*. All animals were

carefully monitored and maintained in accordance with ethical recommendations of the Brazilian College for Animal Experimentation and NIH Guide for the Care and Use of Laboratory Animals.

2.5 Diabetes induction

Animals were randomly divided into two groups: control (non-induced) and diabetic-induced group. Diabetes was induced by an intraperitoneal injection of 150 mg/kg of alloxan (0.9% NaCl) after overnight fasting and the control group received only 0.9% NaCl [32]. Blood glucose levels were monitored thereafter by Accu-Chek Active blood glucose monitor. Ten days later, animals in which the development of hyperglycemia was confirmed (blood glucose levels higher than 300 mg/dL) and animals from control group were analyzed.

2.6. Experimental design

In the study concerning chronic effects of the HEBM, after hyperglycemia confirmation the animals were once more randomly subdivided into two subgroups each. These were treated orally by gavage for 28 days with a single intragastric dose of HEBM (1 mg/Kg) or vehicle (0.9% NaCl) per day. Body weight was measured twice a week and used to regulate the treatment doses. After the experimental period, the animals from different groups were weighted and analyzed 15 h after last treatment. The heart was dissected out, washed in ice-cold saline, blotted dry and weighed.

2.7. Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) widely adopted as an index of lipid peroxidation [33] were determined in cardiac tissue from rats which receive the treatment for 28 days. The formation of TBARS occurs during an acid-heating reaction as previously described [34]. Briefly, the samples were mixed with 1mL of 10% trichloroacetic acid and 1mL of 0.67% thiobarbituric acid, and subsequently heated in a boiling-water bath for 15 minutes. The TBARS were determined by absorbance at 535 nm and were expressed as malondialdehyde (MDA) equivalents (nm/mg protein).

2.8. Spectrophotometric determination of carbonyl groups

Protein damage was estimated through carbonyl groups determination according to the method of Levine et al. [35]. Tissue homogenates were divided into four aliquots of 200 µl (~ 0.2 mg protein). Proteins were precipitated by the addition of 100 µl 20% trichloroacetic acid (TCA) for 5 min on ice, and centrifuged at 4000 X *g* for 5 min. The pellet was redissolved in 100 µl 0.2 M NaOH, and 100 µl of 2 M HCl or 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl added to duplicate aliquots for blanks or the derivatizing of carbonyl groups, respectively. Samples were left for 30 min at room temperature. Proteins were reprecipitated with TCA, and washed three times with 500 µl 1:1 ethanol:ethyl acetate with 15 min standing periods to remove excess DNPH. Samples were redissolved in 200 µl 20 mM KH_2PO_4, pH 2.3, and the absorbance was read at 370 nm. The carbonyl content in nmol/mg protein was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} at 370 nm after subtraction of the blank absorbance.

2.9. SOD activity

The superoxide dismutase (SOD, E.C. 1.15.1.1) activity in samples homogenate was measured spectrophotometrically by the inhibition rate of auto-catalytic adrenochrome formation in a reaction buffer containing 1 mM adrenaline, 50 mM glycine-NaOH (pH 10.2) and 1 mM catalase as previously described [36].

2.10. Catalase activity

To determine catalase (CAT, E.C. 1.11.1.6) activity, samples were sonicated in a 50 mM phosphate buffer in ice bath and the resulting suspension was centrifuged at 3000g for 10 min. The supernatant was used for enzyme assay. CAT activity was measured by the decreasing rate in hydrogen peroxide absorbance at 240 nm [37].

2.11. Protein quantification

Lipid peroxidation, SOD and CAT activities results were standardized by protein content as previously described [38]. The results of carbonyl groups quantification were standardized by protein concentration of tissue homogenates as described by the Bradford method [39]. Protein content in western blotting assay was quantified as previously described [40].

2.12. Total glycogen content

To determine total glycogen content, after 28 days of treatment, cardiac tissue samples were weighed and digested in hot concentrated 30% KOH, boiled at 100°C for 20

minutes, precipitated with ethanol and measured by colorimetric procedure with iodine [41].

2.12.1. Glycogen visualization

Heart tissue sections embedded in paraffin were cut at 4 μm and stained with the periodic acid Schiff (PAS) reagent for glycogen visualization, then were analyzed microscopically. Adobe Photoshop 5.5 (Adobe) was used for imaging and preparation of photomicrographs. Evaluation of sections was done under double-blind conditions.

2.13. Heart cytosolic fraction and western blotting assay

Heart samples were subjected to western blotting analyses for GSK-3β as previously described [42]. Cardiac tissue (80-100 μg) were homogenized in lysis buffer (4% sodium dodecylsulfate [SDS], 2.1 mM EDTA, and 50 mM Tris) with protease inhibitor and then centrifuged at 2,000g for 10 min. The supernatant was collected and kept in ice, the pellet was resuspended in lysis buffer, rehomogenized for 10 s, and centrifuged for 10 min at 2,000 g. Cytosolic fractions in the two supernatants were pooled. Aliquots were taken for protein determination and β-mercaptoethanol was added to a final concentration of 5%. Proteins were separated (40 μg per lane) on 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes using a semi-dry apparatus (Bio-Rad Trans-Blot SD, Hercules, CA, USA). Membranes were incubated for 60 min at 4°C in blocking solution (Tris-buffered saline containing 5% powdered milk and 0.1% Tween-20) and further incubated with the appropriate primary antibody dissolved in blocking solution overnight at 4°C. The primary antibodies used were anti-phosphoGSK-3β (Ser9) (pGSK-3β, 1:1000; Cell Signaling Technology) and anti-GSK-

3β (1:1000; Cell Signaling Technology). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:1000; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and the percentage of band intensity was analyzed using Optiquant software (Packard Instruments).

2.15. Statistical Analysis

The results are expressed as the mean±SD (n≥5). The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post hoc least significant difference (LSD) test. Results were considered significantly different if $P<0.05$. All data were analyzed with SPSS for Windows version 8.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Lipid peroxidation

The heart lipid peroxidation is presented in Figure 1A. We did not observed significant differences on heart lipid peroxidation among groups analyzed.

3.2. Spectrophotometric determination of carbonyl groups

The spectrophotometric determination of carbonyl groups is presented in Figure 1B. We observed in diabetic group an increase (82%) in carbonyl groups when compared to

control group. However, diabetic animals that received a diary HEBM treatment showed a reduction (32 %) in protein damage when compared to diabetic group that received saline.

3.3. SOD activity

The superoxide dismutase (SOD) activity in heart is showed in Figure 2A. Diabetic group that received saline presented a significant increase (27%) in SOD activity. However, when diabetic animals were treated with a HEBM diary dose SOD activity was reduced to control levels.

3.3. CAT activity

Catalase (CAT) activity detected in heart is presented in Figure 2B. CAT activity in diabetic group was found 32% superior to CAT activity in control group. We observed a significant reduction in CAT activity when diabetic animals were treated with HEBM. No significant difference was detected between control and diabetic-HEBM group.

3.4. Total glycogen content

The total glycogen content from heart according colorimetric determination is presented in Figure 3. Diabetic group presented the total glycogen content increased 2.8 times than total glycogen content found in control group. When diabetic animals received a HEBM diary dose the total glycogen content was reduced (35%). However, the total glycogen content from diabetic-HEBM group remained in a level 1.8 times high than total glycogen content from control group.

3.4.1. Glycogen visualization

The histological analysis of heart tissue showed similar glycogen content found in colorimetric assay. The significant increase in glycogen content in heart of diabetic animals when compared to control group was reduced when diabetic animals received HEBM treatment. Results are presented in Figure 4.

3.5. Heart cytosolic fraction and western blotting assay

The blotting results from heart cytosolic fraction are presented in Figure 5. The long term treatment and diabetic condition reduces GSK-t and p-GSK content. However, the p-GSK/GSKt ratio was reduced only in diabetic group. The HEBM treatment not alterate the p-GSK/GSKt ratio in both groups studied.

4. Discussion

Data from our laboratory reveals an increased risk of cardiac disease from HEBM chronic consumption. In order to investigate HEBM effect in cardiac tissue we analyzed the heart from these animals. Diabetic cardiomyopathy is a myocardial disease caused by diabetes mellitus independent of vascular pathology or systemic arterial hypertension. Cardiomyocyte over expressing antioxidant enzymes reduces damage to the diabetic heart in transgenic mice [10]. Functional or morphological damage to diabetic hearts can be reduced with antioxidant administration [11]. However remains unclear if oxidative stress precedes the appearance of diabetic complications or merely reflects the presence of hyperglycemic complications and their consequences. We observed a reduction to the control level in protein damage, CAT and SOD activity when diabetic animals received HEBM treatment. Our results in whole mode suggest that HEBM treatment was effective in

reduces oxidative stress in diabetic heart tissue and these decreases could be related to antioxidants found in HEBM. Both type 1 and type 2 diabetes induce damage at the level of myocyte and this damage occurs through the mechanisms utilizing reactive oxygen species [7]. The gallic acid-like substance observed as the major constituent (25.42%) in HEBM, could be important in diabetes treatment condition in both type 1 and type 2 diabetes as suggested previously [31]. We believe that antioxidants presents in HEBM were responsible in antioxidant protection in diabetic group. Gallic acid is acknowledged as a potent antioxidant and is structurally similar to methyl gallate. In addition, Nakagawa and colleagues suggested that the presence of the gallate group is essential in the protective activity against protein oxidation and glycation, and that there is also a contribution by the hydroxyl group at the 5' position in the ring and the sterical structure [43]. Interventions aimed at decreasing oxidative stress have the potential to improve cardiac efficiency [44].

The increased cardiac glycogen content in diabetic animals was identified by both PAS staining and quantitative glycogen assay as previously reported [42]. HEBM treatment reduced it when compared to diabetic-vehicle group. In order to study the mechanism of diabetic induction and HEBM in glycogen accumulation, we examined the intracellular signal protein GSK-3β. The increased GSK-3β phosphorylation leads to GSK-3β activity and thus decreasing the rate of glycogen synthesis [45]. We found GSK-3β immunocontent, as well its phosphorylation, was reduced in alloxan induced diabetic rats and HEBM treatment not revert it. These data suggest that the increase in the glycogen content, observed in alloxan group, could be due by the decrease of the phosphorylation and consequently activation of GSK-3β. In the other hand, the glycogen content reduction induced by HEBM treatment observed in diabetic animals was drove by other mechanism

than GSK-3β, possibly by glycogen degradation and/or consumption increased. However, the exact mechanism remains unclear.

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Figure 1.

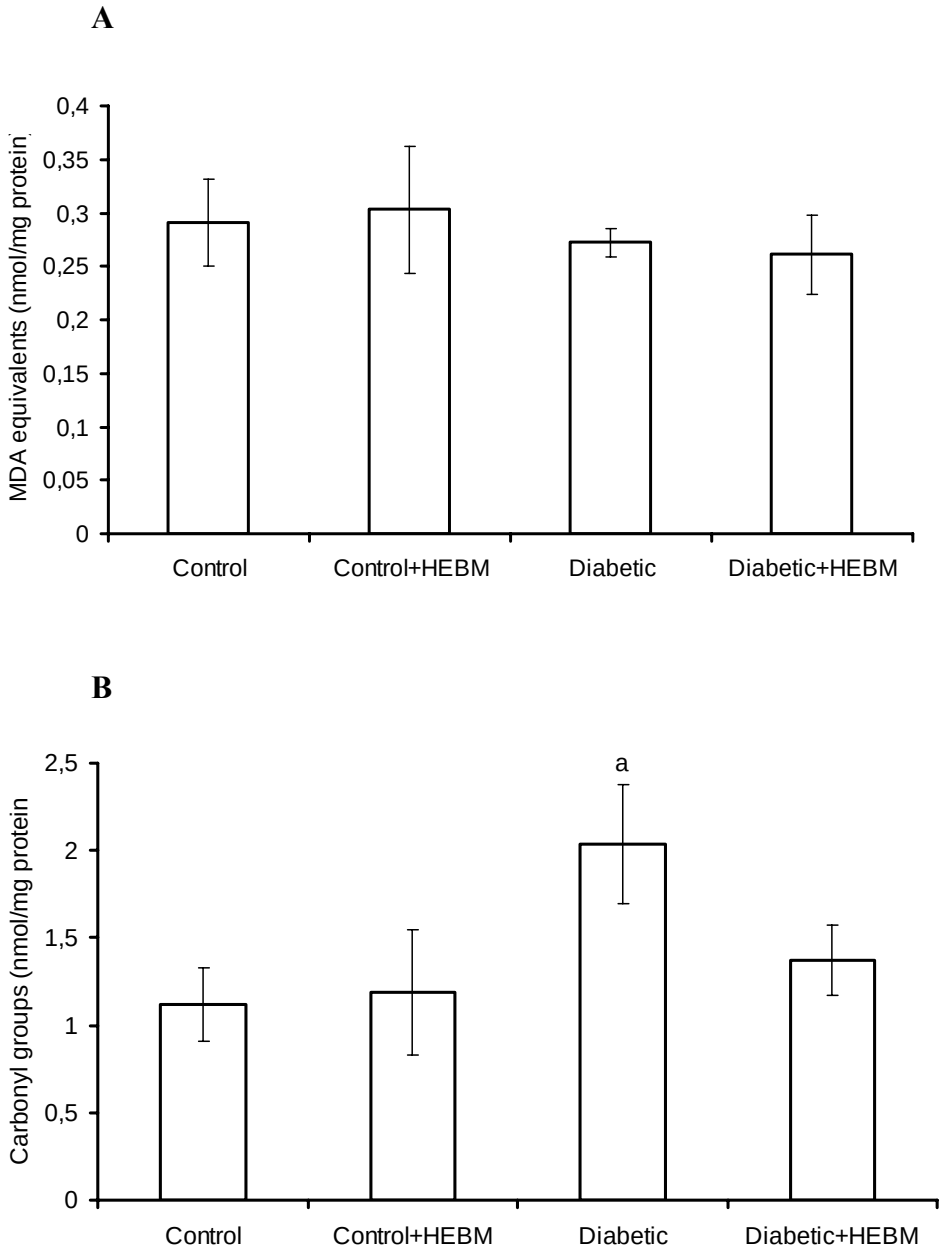

A
MDA equivalents (nmol/mg protein)
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
Control
Control+HEBM
Diabetic
Diabetic+HEBM
B
Carbonyl groups (nmol/mg protein
2,5
2
1,5
1
0,5
0
a
Control
Control+HEBM
Diabetic
Diabetic+HEBM

Figure 2.

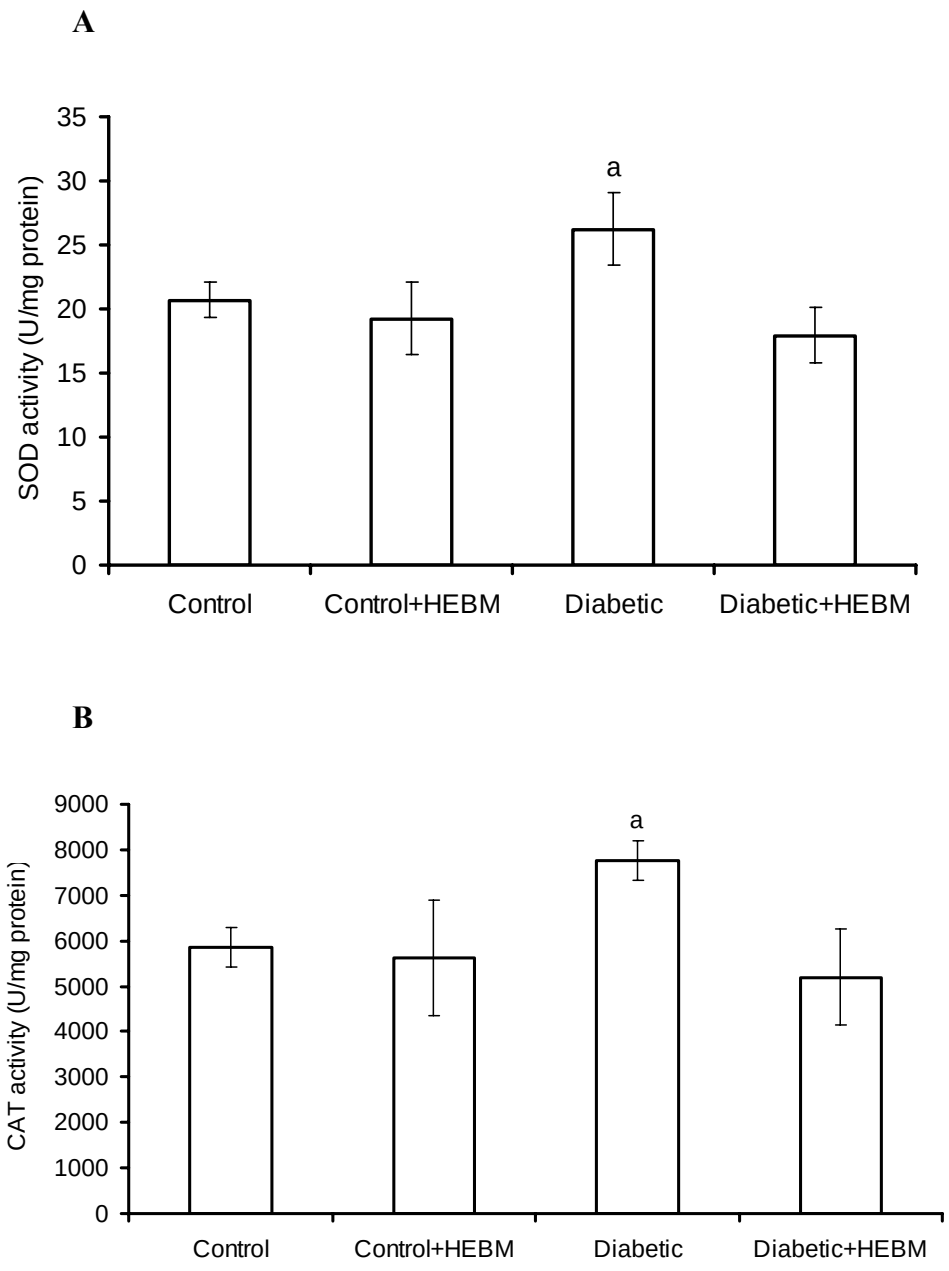

A
SOD activity (U/mg protein)
35
30
25
20
15
10
5
0
a
Control
Control+HEBM
Diabetic
Diabetic+HEBM
B
CAT activity (U/mg protein)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
a
Control
Control+HEBM
Diabetic
Diabetic+HEBM

Figure 3.

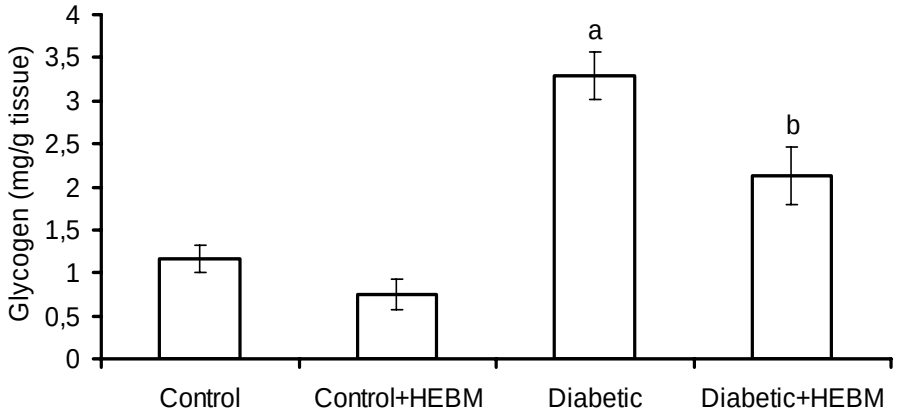

Glycogen (mg/g tissue)
4
3,5
3
2,5
2
1,5
1
0,5
0
a
b
Control
Control+HEBM
Diabetic
Diabetic+HEBM

Figure 4.

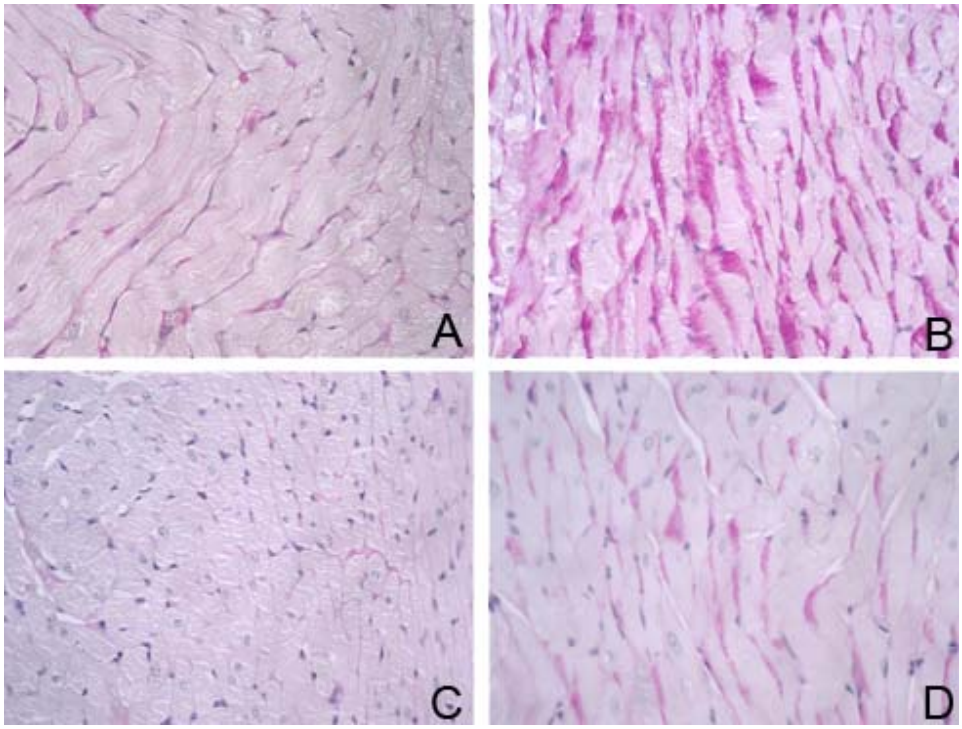
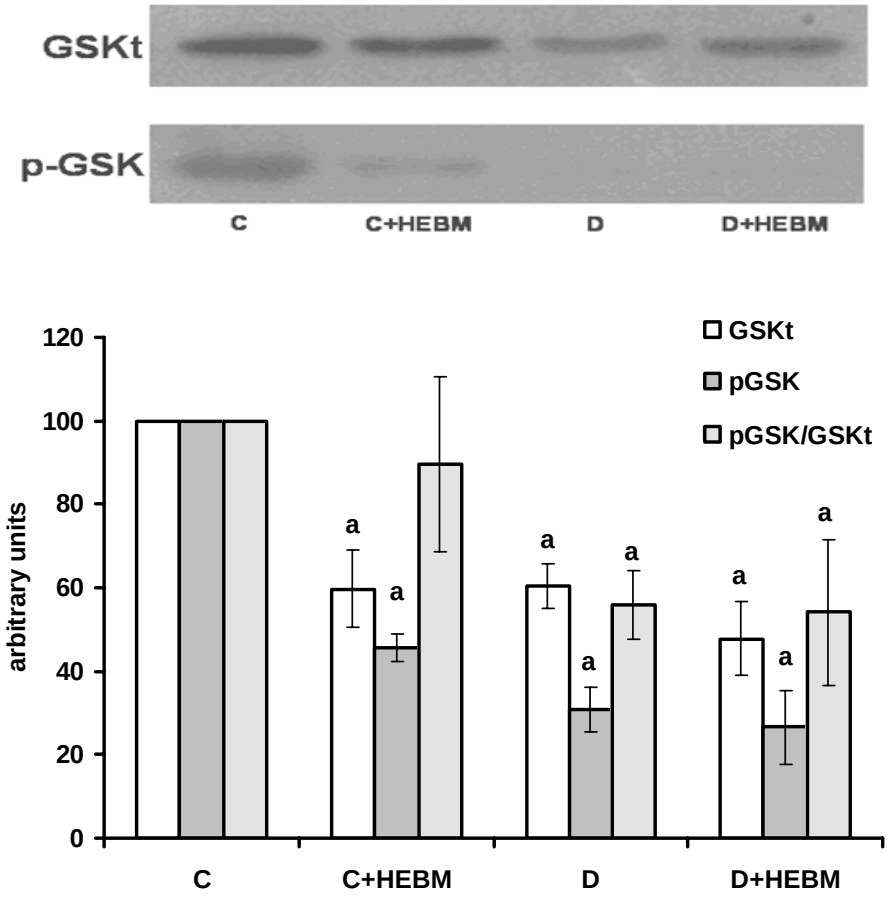


Figure 5.


GSKt
p-GSK
C
C+HEBM
D
D+HEBM
GSKt
pGSK
pGSK/GSKt
arbitrary units
120
100
80
60
40
20
0
a
C
C+HEBM
D
D+HEBM

PARTE III.

3. DISCUSSÃO

Modelos animais são largamente utilizados na pesquisa científica do DM, tanto no tipo 1, como no tipo 2 (Figura 4). Diversas espécies animais já foram utilizadas na pesquisa do DM, desde cães, gatos, porcos, primatas até as diferentes espécies de roedores. Várias técnicas fazem parte do conjunto de ferramentas para a indução e o estudo do DM em modelos animais, como por exemplo: a pancreatectomia, toxinas que destroem seletivamente as células β pancreáticas, anticorpos anti-insulina, transgenia, produção de animais "knockout" e geração de linhagens animais específicas (Rees & Alcolado, 2005). Por motivos econômicos, de disposição de animais e técnico-práticos, escolhemos o modelo de DM tipo 1 em ratos induzido pelo aloxano.

3.1. O aloxano e a indução do DM

A indução do DM experimental em animais através de toxinas que destroem seletivamente as células β do pâncreas é bastante conveniente e relativamente simples, motivo pelo qual foi escolhido neste trabalho. O aloxano (ALX) e a estreptozotocina estão entre as substâncias mais utilizadas para induzir o DM em animais, porém, outras moléculas como vacor, ditizona e 8-hidroxiquinolona já foram reportadas (Rees & Alcolado, 2005).

Figura 4.

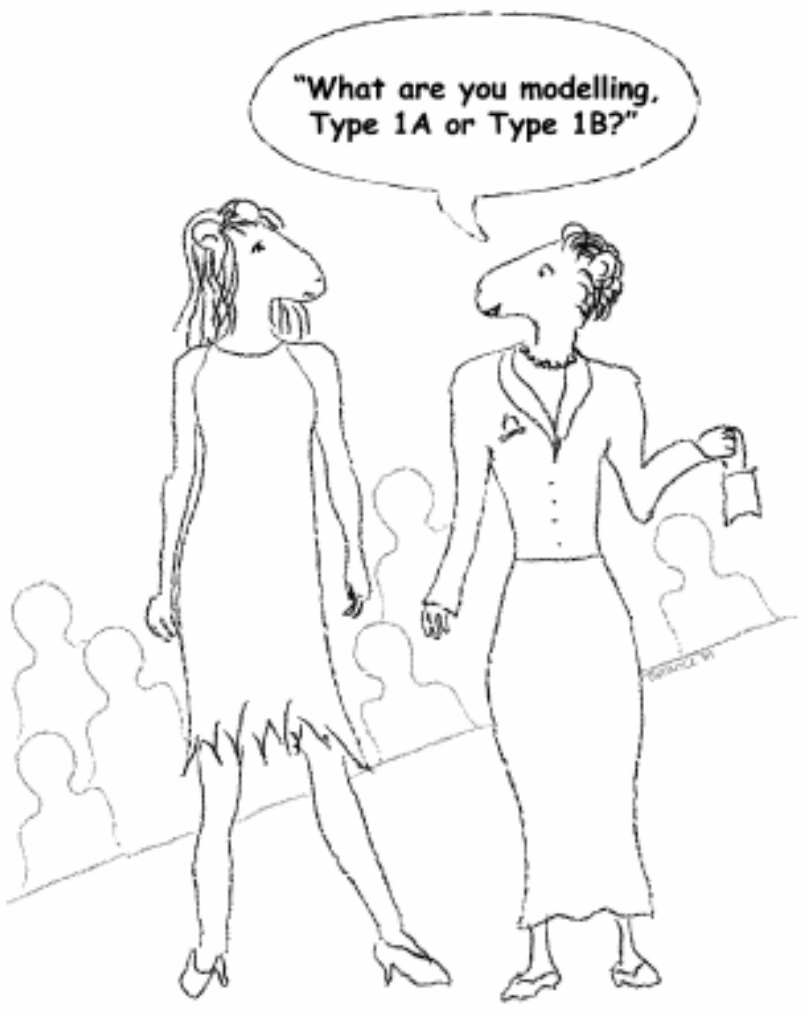


Figura 4. Qual o seu modelo? Fonte Leiter & Von Herrath, 2004.

O aloxano (2,4,5,6-tetraoxypyrimidina; 5,6-dioxyuracil) foi descrito primeiramente por Brugnatelli em 1818 (Lenzen & Panten, 1988). Wöhler e Liebig usaram o nome aloxano e descreveram sua síntese pela oxidação do ácido úrico (Lenzen & Panten, 1988). As propriedades diabetogênicas desta droga foram descritas muito tempo depois por Dunn, Sheehan e McLethie (1943) que estudaram o seu efeito administrando-a em coelhos e reportando a necrose específica das ilhotas pancreáticas. Desde então o ALX vem sendo empregado na indução de DM tipo 1.

3.1.1. Mecanismos de ação do aloxano

O ALX exerce sua ação diabetogência quando é administrado via parenteral, intravenosa, intraperitoneal ou subcutânea. A dose de ALX requerida para induzir o DM depende da espécie animal, via de administração e estado nutricional do animal. A via mais freqüente é a intravenosa, numa dose em torno de 65mg por Kg de animal (Gruppuso et al., 1990; Boylan et al., 1992). Quando o ALX é administrado intraperitonealmente ou subcutaneamente, a dose para ser efetivo deve ser 2-3 vezes maior que àquela intravenosa. Doses intraperitoneais abaixo de 150 mg/Kg podem ser insuficientes para induzir DM em ratos (Katsumata et al. 1992, 1993). Animais em jejum são mais suscetíveis à ação do ALX ((Katsumata, Katsumata & Katsumata, 1992; 1992; Szkudelski et al., 1998), enquanto que níveis mais elevados de glicemia exercem proteção parcial (Bansal, Ahmad & Kidwai, 1980; Szkudelski et al., 1998). A dose de 150 mg/Kg que foi testada e utilizada no presente trabalho foi suficiente para induzir o DM nos

animais experimentais em jejum de 24 horas, resultado este que está de acordo com trabalhos anteriores ((Katsumata, Katsumata & Katsumata, 1992; Katsumata et al., 1993). Outros parâmetros, também indicados no capítulo 1 deste trabalho, contribuem para reforçar o êxito do modelo proposto.

O mecanismo de ação do ALX é relativamente bem conhecido (Figura 5), principalmente *in vitro*. A ação do ALX no pâncreas é precedida pela sua rápida captação pelas células β (Weaver, McDaniel & Lacy, 1978; Boquist, Nelson & Lorentzon, 1983), seguida pela formação aumentada de EAO via ciclo ALX-ácido dialúrico (Heikkila et al., 1976; Munday 1988). A resistência das células β do pâncreas contra EAO é menor que a resistência de outros tecidos (Malaisse et al., 1982; Tiedge et al., 1997). Tais aspectos nos sugerem que uma vez havendo resistência à indução do DM, um conjunto de fatores relacionados à resistência contra EAO pode estar associado ao referido fenômeno. Isso é o que sugere o conteúdo contido no capítulo 1 do presente trabalho.

Desta maneira, após o uso de ALX para provocar DM em animais, deve haver um período de tempo tal antes dos experimentos que minimize os efeitos colaterais da própria droga (Szkudelski, 2001). Estabelecemos 10 dias. Além disso, deve ser enfatizado que a dose de ALX que usamos está em um limiar que provoca algumas mortes dentro do grupo experimental (em torno de 20%).

Figura 5.

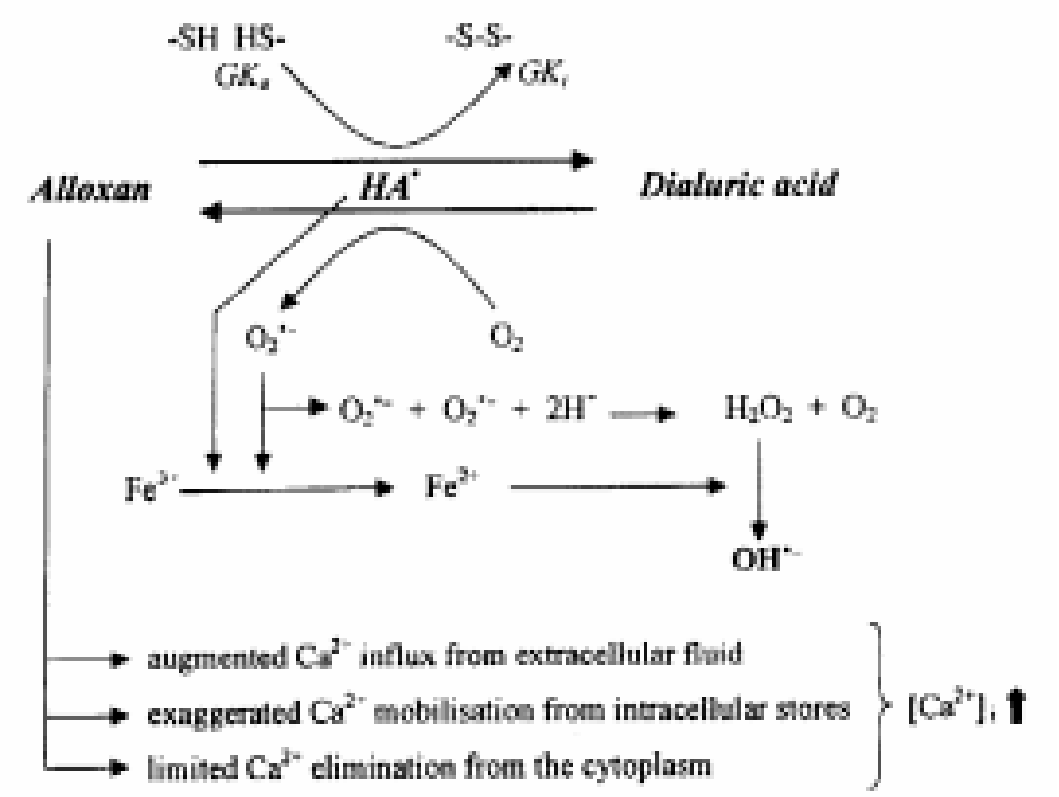


Figura 5. O mecanismo de ação do aloxano induzindo a geração de EAO nas células β do pâncreas de rato. GKa, GKi – glucocinase ativa e inativa, respectivamente; HA• – radical aloxano; [Ca2+]i – concentração intracelular de cálcio. Fonte (Szkudelski, 2001).

3.1.1. Possíveis conseqüências

O surgimento de um grupo de ratos com resistência à ação diabetogênica do ALX, dentro do conjunto de animais fornecidos pelo Departamento, foi um fato novo ao nosso conhecimento. Os dados apresentados no capítulo 1 do presente trabalho sugerem que tal grupo resistente pode estar naturalmente constituído por defesas mais eficientes que conferem tais propriedades. Tal observação associada às expressivas diferenças que os demais pesquisadores, muitas vezes, encontram dentro do mesmo grupo experimental de ratos fornecidos pelo Departamento, leva a crer que há um grupo de animais naturalmente diferenciado, possivelmente por fatores hereditários. Fenômeno semelhante foi encontrado em camundongos, onde o fator principal que distingue animais resistentes ou suscetíveis ao ALX é o status de defesas antioxidante (Mathews & Leiter, 1999), inclusive com a criação de uma linhagem estabelecida desses animais (Ino et al., 1991). Este fato nos permite supor a possibilidade de criação de uma linhagem de ratos resistentes ao ALX, com a conseqüente patente junto ao escritório de patentes de nossa instituição. Além disso, sugerimos aos demais pesquisadores, possíveis investigações com os animais obtidos no Departamento que não respondam às diferentes induções experimentais. Por outro lado, a biodiversidade existente no conjunto de animais fornecidos pelo Departamento é fator que aproxima tal grupo da população humana em geral, onde a diversidade intra-específica é considerada uma das mais acentuadas quando comparada as demais espécies animais existentes atualmente.

3.1.2. Contraponto a modelos animais de DM tipo 1

De forma bastante enfática Leiter e Von Herrath (2004) expuseram em seu trabalho toda sua discordância em comparar modelos animais de DM tipo 1 com a mesma patologia em humanos. O cerne da argumentação dos referidos pesquisadores baseia-se na limitação dos diferentes modelos existentes, em simular e/ou mimetizar de forma adequada uma doença de fundo auto-imune, como muitos casos de DM tipo 1 em humanos. Portanto, como os modelos seriam insuficientes e imperfeitos, logo os resultados seriam limitados.

O autor e demais pesquisadores que se debruçaram sobre este trabalho não discordam das observações dos autores supracitados, inclusive concordam com tais proposições. Reconhecemos as limitações de trabalhar com modelos animais que simulam doenças humanas, no entanto diversos avanços foram alcançados utilizando modelos animais e outros modelos que mimetizam patologias humanas e a literatura científica é farta nestes casos. O fator principal nestas circunstâncias encontra-se na euforia de alguns pesquisadores, em supervalorizar modelos de estudo que não possuem o poder e precisão que permitem extrapolações diretas entre o tal modelo e a versão humana da doença em questão. Afinal, esta é uma das conseqüências diretas de, aproximadamente, 65 milhões de anos de evolução biológica que derivaram as espécies. Buscamos neste trabalho não fazer extrapolações desnecessárias.

3.2. Extratos obtidos de folhas de *B. microstachya*

No capítulo 2 deste trabalho ficou demonstrado que tanto o extrato aquoso (AEBM), como o hidro-etanólico (HEBM), obtidos a partir das folhas de *B. microstachya* possuem excelente capacidade antioxidante *in vitro*. A referida capacidade parece variar de acordo com o sistema produtor de EAO ou de espécies ativas de nitrogênio (EAN) utilizado. De um modo geral, o HEBM apresentou uma performance melhor nos testes utilizados, o que está de acordo com seu conteúdo fenólico maior que o AEBM (dado corroborado pelo perfil cromatográfico obtido de ambas amostras). Esta correlação (maior conteúdo fenólico – maior poder antioxidante) já foi observada em trabalhos anteriores. Porém, quando os resultados dos testes antioxidantes foram normalizados pelo conteúdo fenólico total, um perfil diferente de poder antioxidante pode ser observado. Esta normalização teve por objetivo averiguar a qualidade dos compostos fenólicos presentes nos extratos, em evitar a formação de EAO/EAN. Porém, o teste que indica compostos fenólicos totais não distingue quais compostos e simplesmente soma substâncias possuidoras de anéis aromáticos, apesar de ser amplamente utilizado como índice de compostos fenólicos totais e ser positivamente relacionado ao poder antioxidante de extratos vegetais (Hukkanen et al., 2006; Kuti & Konuru, 2004; Valentão et al., 2002; Govindarajan et al., 2003).

A performance de misturas complexas, como extratos vegetais, em diferentes sistemas antioxidantes está relacionada ao tipo de radical gerado no

sistema e a polaridade do substrato. Os coeficientes calculados quando consideramos o conteúdo fenólico total indicam que o efeito desses compostos nos testes realizados podem ser sinergísticos, portanto, a qualidade dos constituintes em menor proporção pode ser importante para o resultado antioxidante total. Por outro lado, um trabalho prévio não encontrou correlação entre conteúdo fenólico total e poder antioxidante (Kähkönen et al., 1999). Os resultados encontrados no capítulo 2 deste trabalho estão em concordância com ambas observações.

Os vegetais são fontes naturais de agentes antioxidantes e evidências epidemiológicas suportam o conceito que dietas ricas em frutas, vegetais e bebidas naturais promovem saúde, retardam e atenuam doenças crônicas ou estados patológicos (Yen, Yeh, Chen, 2004; Goreli et al., 2005; Rimm, 2002). O estresse oxidativo aumentado, como a lipoperoxidação e oxidação de proteínas, tem sido registrado em ambos tipos (1 e 2) de diabetes (Santini et al., 1997; Tesfamariam, 1994; Cederberg, Basu & Eriksson, 2001). Portanto, o emprego de preparações vegetais ricas em compostos fenólicos pode ser uma alternativa significante no co-tratamento de DM. Nossos achados no capítulo 2 e as evidências quimiotaxônomicas suportam a proposta de que o uso de extratos de *B. microstachya* pode ser uma escolha pertinente a ser testada em um modelo animal de DM. Por questões que são preconizadas pelos princípios da Bioética (como a racionalização do número de animais a serem usados nos estudos), escolhemos o HEBM que foi o extrato com maior conteúdo fenólico total e melhor

performance nos testes contra lipoperoxidação (importante índice associado ao DM) para seguir nossos estudos em animais.

3.3. Efeitos do HEBM em animais diabéticos induzidos por ALX

Baseados em dados da literatura (Silva et al., 2002; Lino et al., 2004) e balizados pelos princípios bioéticos, realizamos primeiramente testes agudos preliminares para definir a dose a ser usada em um teste crônico. Os resultados de tais testes (contidos no capítulo 3) indicaram que a dose de 1 g/Kg poderia trazer melhores resultados, pois demonstrou que além de reduzir significativamente a glicemia de animais diabéticos, também aumentou a capacidade antioxidante total do plasma destes animais. Tais resultados estão de acordo com o reportado no capítulo 2 deste trabalho e também com evidências anteriores demonstrando a capacidade hipoglicemiante do gênero *Bauhinia* (Almeida et al., 2006; Fuentes, Aranciba-Avila, Alarcon, 2004; Lino et al, 2004; Silva et al., 2002; Pepato et al., 2004).

No estudo crônico as capacidades hipoglicemiante e a antioxidante foram parcialmente observadas, apenas na redução de hemoglobina glicada total (HbA_{1C}). A hiperglicemia crônica é reconhecidamente um dos fatores patogênicos que geram complicações no DM e o consumo de antioxidantes é correlacionado à redução de algumas complicações produzidas pelo DM (Nascimento et al., 2003) e pela glicação de proteínas (Krone & Ely, 2004; Wu & Yen, 2005; Nagasawa et al., 2003). No capítulo 2, o poder antioxidante do HEBM ficou claramente

demonstrado que os compostos presentes no extrato podem ter contribuído para a redução de hemoglobina glicada.

O HEBM aumentou os triglicerídeos, VLDL, HDL e o cholesterol total, além de reduzir a relação HDL/LDL de 4,93 para 2,81 nos animais diabéticos. Isso indica que o tratamento pode levar a um aumento do risco de desenvolver doença cardiovascular, porque a relação aumentada HDL/LDL tem sido relacionada a um baixo risco de desenvolvimento de doença cardíaca (Castelli, 1984).

A redução no peso corporal dos animais diabéticos que foram tratados com HEBM pode ser considerada um fator negativo do tratamento, pois a redução de peso em diabéticos tipo 1 é um dos sintomas clássicos da doença e também observada em modelos animais de DM tipo 1.

Muitos dos estudos anteriores sobre os efeitos de extratos vegetais em modelos animais de DM não trazem possíveis efeitos tóxicos. Objetivando averiguar este aspecto em nosso trabalho determinamos a atividade de algumas enzimas marcadoras de funcionamento hepático: GST, AST e ALT. O tratamento reduziu a atividade da GST e aumentou a atividade da AST e da ALT plasmática nos animais diabéticos, o que é um indicativo de danos ao fígado.

3.4. Efeitos do HEBM no tecido cardíaco de animais diabéticos induzidos por ALX

No capítulo 3 deste trabalho ficou sugerido que o tratamento crônico com HEBM, na dose de 1g/Kg dia, poderia aumentar o risco de doença cardíaca. Para investigar o impacto do consumo crônico de HEBM sobre o coração dos animais diabéticos realizamos o estudo sobre os parâmetros oxidativos e sobre o acúmulo e uma via relacionada ao glicogênio.

Observamos uma redução para níveis de controle na carbonilação de proteínas e na atividade das enzimas CAT e SOD no grupo de animais diabéticos que foram tratados com HEBM. Além disso, houve uma pequena redução no glicogênio total no coração dos animais que foram tratados com HEBM. Tais observações suportam a hipótese de que apesar de haver um desequilibro no perfil lipídico dos animais diabéticos que receberam HEBM, não houve aumento do estresse oxidativo destes animais, inclusive houve uma redução do estresse oxidativo gerado pela condição diabética. Não pudemos confirmar que a redução do glicogênio cardíaco foi via GSK-3β, havendo provavelmente outro mecanismo envolvido.

3.1. CONCLUSÕES

1- Os ratos fornecidos pelo Departamento de Bioquímica podem ser usados em modelo de DM induzido pelo aloxano. Aparentemente há um grupo de animais naturalmente resistente à indução do DM por aloxano e esta resistência pode estar associada a um conjunto de defesas constitutivas desses animais.

2- Os extratos de *B. microstachya* são ricos em compostos fenólicos. Ambos extratos possuem poder antioxidante *in vitro*.

3- O HEBM apresenta potencial hipoglicemiante. O uso dos extratos de *B. microstachya* é positivo na redução do estresse oxidativo provocado pelo DM, porém, há um potencial hepatotóxico e outros fatores negativos a serem considerados.

4- O HEBM foi efetivo em reduzir o estresse oxidativo cardíaco em animais diabéticos induzidos pelo aloxano. O aumento de glicogênio no tecido cardíaco dos animais diabéticos está associado à ativação da GSK-3β, mas a redução deste parâmetro nos animais diabéticos tratados com HEBM não pode ser atribuída a este mecanismo.

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4. ANEXOS

Anexo 1. Artigo - Regulation of LPS stimulated ROS production in peritoneal macrophages from alloxan-induced diabetic rats: Involvement of high glucose and PPAR-γ. Life Sciences 81 (2007) 153-159.

Anexo 2. Manuscrito - Rosiglitazone, a PPAR-γ Agonist for Type 2 Diabetes Treatment, Improves Glycemic Control and Modulates Glycogen Stores in Type 1 Diabetes Animal Model Induced by Alloxan. Submetido a Basic & Clinical Pharmacology and Toxicology (2007).

Anexo 3. Certificado de destaque na sessão de Farmacologia e Toxicologia no XVIII Salão de Iniciação Científica da Universidade Federal do Rio Grande do Sul que ocorreu de 15 a 20 de outubro de 2006, recebido por Guilherme Antônio Behr pela apresentação do trabalho “O efeito da rosiglitazona em modelo animal de diabetes mellitus tipo 1 induzida por aloxano”

Anexo 4. Certificado de “Young Investigator Award” recebido pelo trabalho “Antioxidant activity and hypoglycemic effect of *Bauhinia microstachya* leaves in alloxan-induced diabetes rats” apresentado durante o V MEETING OF SFRBM – SOUTH AMERICAN GROUP e V INTERNATIONAL CONFERENCE ON PEROXYNITRITE AND REACTIVE NITROGEN SPECIES, que ocorreram na cidade de Montevideo – Uruguai, em 02 a 06 de setembro de 2007.

Anexo 1.

Regulation of LPS stimulated ROS production in peritoneal macrophages from alloxan-induced diabetic rats: Involvement of high glucose and PPAR-γ

Artigo publicado em

Life Sciences

81 (2007) 153-159.

Available online at www.sciencedirect.com
ScienceDirect
Life Sciences 81 (2007) 153 – 159
Life Sciences
www.elsevier.com/locate/lifescie

Regulation of LPS stimulated ROS production in peritoneal macrophages from alloxan-induced diabetic rats: Involvement of high glucose and PPARγ

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Received 10 January 2007; accepted 27 April 2007

Abstract

An increased occurrence of long term bacterial infections is common in diabetic patients. Bacterial cell wall components are described as the main antigenic agents from these microorganisms and high blood glucose levels are suggested to be involved in altered immune response. Hyperglycemia is reported to alter macrophages response to lipopolysaccharide (LPS) and peroxisome proliferators activated receptor gamma (PPARγ) expression. Additionally, glucose is the main metabolic fuel for reduced nicotinamide adenine dinucleotide phosphate (NADPH) production by pentose phosphate shunt. In this work, lipopolysaccharide (LPS) stimulated reactive oxygen species (ROS) and nitrite production were evaluated in peritoneal macrophages from alloxan-induced diabetic rats. Cytosolic dehydrogenases and PPARγ expression were also investigated. LPS was ineffective to stimulate ROS and nitrite production in peritoneal macrophages from diabetic rats, which presented increased glucose-6-phosphate dehydrogenase and malate dehydrogenase activity. In RAW 264.7 macrophages, acute high glucose treatment abolished LPS stimulated ROS production, with no effect on nitrite and dehydrogenase activities. Peritoneal macrophages from alloxan-treated rats presented reduced PPARγ expression. Treating RAW 264.7 macrophages with a PPARγ antagonist resulted in defective ROS production in response to LPS, however, stimulated nitrite production was unaltered. In conclusion, in the present study we have reported reduced nitric oxide and reactive oxygen species production in LPS-treated peritoneal macrophages from alloxan-induced diabetic rats. The reduced production of reactive oxygen species seems to be dependent on elevated glucose levels and reduced PPARγ expression.

Keywords: Lipopolysaccharide; Macrophages; Alloxan; Hyperglycemia; PPAR; ROS; Nitric oxide

Introduction

Diabetes is a prevalent metabolic disorder with several secondary complications. An increased occurrence of long-term bacterial infections is common in diabetic patients, being a major complication in these individuals (Smitherman and Peacock, 1995). It was suggested that high blood glucose levels are involved in reduced bactericidal activity (Nielson and Hindson, 1989) and it has further been demonstrated that high glucose reduces interleukin 1 release from murine macrophages (Hill et al., 1998).

Bacterial cell wall components are described as the main antigenic agents from these microorganisms (Van Amersfoort et al., 2003) and their recognition by Toll-like receptors (TLR) initiates cellular responses to bacterial infections (Takeuchi and Akira, 2001). Lipopolyssacharide (LPS) is a major constituent of the outer membrane of gram negative bacteria and is recognized by animals as a molecular correlate to infection. It binds to TLR 4, triggering multiple signaling cascades (Van Amersfoort et al., 2003; Takeuchi and Akira, 2001). Macrophages are key mediators of innate immunity and their phagocytic activity in response to microbial infections and antigens produces and releases reactive oxygen species (ROS), as well as reactive nitrogen species (RNS). Both ROS and RNS have antimicrobial activity; moreover, they also act as cellular signaling molecules (Forman and Torres, 2001; Ferret et al., 2002). Additionally, exacerbated

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doi:10.1016/j.lfs.2007.04.035

ROS and RNS production in response to bacterial infection is related to inflammatory complications, as septic shock, multiple organ failure and death (Ritter et al., 2004; Andrades et al., 2005; Victor et al., 2005).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is expressed by phagocytes and is involved in superoxide production for antimicrobial activity. In non-stimulated cells, NADPH oxidase components are in different subcellular compartments, with subunits $gp91^{phox}$ and $p22^{phox}$ in the plasma membrane and subunits $p47^{phox}$/$p67^{phox}$/$p40^{phox}$ in the cytosol as a complex. Upon phagocytosis or stimulation with soluble agents, such as LPS, the cytosolic components and the small GTPase Rac1/Rac2 translocate to the plasma membrane binding to $gp91^{phox}$ and $p22^{phox}$ and initiating superoxide production by transferring one electron from NADPH to oxygen (Forman and Torres, 2001; Decoursey and Ligeti, 2005). Nitric oxide is synthesized from L-arginine by nitric oxide synthase using NADPH as a cofactor. Three nitric oxide synthases are described: neuronal and endothelial isoforms that are constitutive and calcium dependent, and the inducible isoform which is calcium independent. In stimulated macrophages, inducible nitric oxide synthase (iNOS) accounts for increased nitric oxide production, which is NADPH dependent (Forstermann and Kleinert, 1995). NADPH is an essential cellular coenzyme and its level is sustained by $NADP^+$ dependent cytosolic dehydrogenases, named glucose-6-phosphate dehydrogenase (G6PD), isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH). Impaired NADPH production by reduced G6PD activity is associated with augmented IL-10 production and redox dependent signaling in mouse peritoneal macrophages (Wilmanski et al., 2005).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear hormone receptors that have pleiotropic immune modulating effects. Peroxisome proliferator-activated receptor-γ (PPARγ) is a ligand-dependent nuclear receptor that has a critical role in adipogenesis and glucose metabolism. In macrophages, the role of PPARγ in inflammation response has been studied, showing that this nuclear receptor presents anti-inflammatory properties (Zhang and Chawla, 2004). Additionally, Sartippour et al. (Sartippour and Renier, 2000) showed that PPARs expression are regulated in macrophages by increased glucose.

Considering that increased blood glucose levels are present in diabetes and that this impairs microbial infection response, the aim of the present study was to determine peritoneal macrophage response to LPS in an animal model of diabetes by measuring ROS and nitric oxide production. As glucose is the main metabolic fuel for NADPH production by pentose phosphate shunt, we investigated the activities of cellular dehydrogenases. The modulation of PPARγ expression and the role of this transcription factor in ROS production were also evaluated.

Materials and methods

Chemicals

Alloxan monohydrate, lipopolyssacharide from *Escherichia coli* (LPS), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), β-$NADP^+$, agarose, ethidium bromide and GW9662 were purchased from Sigma (St. Louis, USA). RPMI 1640 and fungizone were purchased from Gibco™ (Invitrogen, Carlsbad, USA). The SuperScript-II RT preamplification system and dNTPs were purchased from Invitrogen (Carlsbad, USA). All other reagents were of analytical grade and were purchased from commercially available sources.

Alloxan-induced diabetic animal model

Nine-week-old male Wistar rats (190–225 g) were obtained from our breeding stock. Rats were housed in plastic cages, maintained at 22±1 °C and a 12 h light/dark cycle. Animals were supplied with commercial pellet food (Nuvilab® CR-1 Curitiba, PR, Brazil) and water *ad libitum*. Animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian College for Animal Experimentation. Animals were randomly divided into two groups: non-induced and diabetic-induced. Diabetes was induced by an intraperitoneal injection of 150 mg/kg of alloxan monohydrate (0.9% NaCl) after overnight fasting. The non-induced group received only 0.9% NaCl (Prince et al., 1998). Alloxan monohydrate injection leads to the destruction of insulin-secreting β cells of the islets of Langerhans, while other cells (α, γ, δ) are resistant. The destruction of the insulin-secreting β cells was brought about by a redox cycle with the formation of superoxide radicals established by alloxan and the product of its reduction, dialuric acid. Superoxide radicals undergo dismutation to hydrogen peroxide, which produces the reactive hydroxyl radicals by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of β cells (Szkudelski, 2001). This is accompanied by typical and permanent hypoinsulinemia and hyperglycemia (Lenzen and Panten, 1988). Blood glucose levels were monitored thereafter by Accu-Chek® Active blood glucose monitor. Ten days later animals from the diabetic-induced group, with glucose levels lower than 300 mg/dL, were not used in this study. Body weight and blood glucose were measured 30 days after alloxan injection and animals were killed by decapitation.

Peritoneal macrophages preparation

Peritoneal macrophages were isolated as described by El-Mahmoudy et al. (El-Mahmoudy et al., 2002) with modifications. Briefly, rats were sacrificed by decapitation, placed with the abdomen facing up and thoroughly wet with 70% ethanol. A transverse cut was made in the inguinal area and the skin was dissected to expose the abdominal wall which was then soaked with 70% ethanol. About 20 ml of cold Hank's balanced salt saline (HBSS) was injected. The needle was removed and the abdomen was gently massaged. The HBSS was drawn back and the peritoneal fluid was dispensed into 50 ml polypropylene tubes. This procedure was repeated two more times. The cells were centrifuged, re-suspended in RPMI 1640 medium, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/L gentamycin and 250 Units/L fungizone and counted.

Peritoneal macrophages were seeded to 24 well plates (0.5×10^6 cells for nitrite and ROS measurements) or 6 well plates (2×10^6 cells for cellular dehydrogenase activities and PPAR-γ expression) at a density of 1×10^6 cells/ml in RPMI 1640, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/L gentamycin and 250 Units/L fungizone. After 2 h, the plates were washed three times with HBSS to remove non-adherent cells. The adherent cells (97% macrophages) were them utilized for cellular dehydrogenase assay and RNA isolation or incubated for 15 h with or without LPS 1 μg/ml, at 37 °C and 5% CO_2, for nitrite and ROS measurements.

RAW 264.7 macrophages culture

RAW 264.7 macrophages were obtained from UFRJ Cell Bank, Rio de Janeiro, Brazil. Cells were maintained in RPMI 1640 medium, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/L gentamycin and 250 Units/L fungizone at 37 °C and 5% CO_2. Semi-confluent cells were scrapped and plated to 24 well plates (0.5×10^6 cells for nitrite and ROS measurements) or 6 well plates (2×10^6 cells for cellular dehydrogenase activities) at a density of 1×10^6 cells/ml in RPMI 1640, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/L gentamycin and 250 Units/L fungizone. After 24 h, the macrophages were washed with saline and incubated with or without LPS 1 μg/ml for 15 h in serum free RPMI 1640 medium. For studies on the influence of PPARγ in the response to LPS, GW9662 – a PPARγ antagonist – was added to cells 2 h before LPS treatment in serum free 1640 medium. For acute high glucose experiments, RAW 264.7 macrophages were washed with saline and incubated for 24 h with serum free RPMI 1640 supplemented with 300 mg/dL glucose (500 mg/dL total glucose concentration) before LPS treatment or cellular dehydrogenase measurement. Normal RPMI 1640 medium contains 200 mg/dL glucose.

Nitrite assay

Nitric oxide production was assayed by quantification of the stable end product of nitric oxide oxidation – nitrite (NO_2^-) – as previously described (Souza et al., 2006). Briefly, the incubation medium of peritoneal or RAW 264.7 macrophages were collected after the treatments and centrifuged at 12,000 ×*g* for 10 min. Equal volumes of supernatant and Griess reagent (1:1 0.1% naphthyl-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) were reacted for 15 min and the nitrite content was measured by absorbance at 540 nm (Beckman Instruments Spectrophotometer, Fullerton, USA). Nitrite concentration in the samples was calculated using a standard curve prepared with $NaNO_2$.

ROS measurement

Reactive oxygen species were measured as previously described (Guimaraes et al., 2006) with minor modifications. Briefly, peritoneal or RAW 264.7 macrophages in 24-well plates were incubated with the treatments and in the last 30 min of treatment 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (10 μM) was added and incubated in the dark at 37 °C. Cells were then washed thrice with PBS, harvested in PBS 0.5% TRITON X-100, centrifuged at 12,000 ×*g* for 10 min and the supernatant collected. The intensity of dichlorofluorescein (DCF) fluorescence in the supernatant was measured with an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Hitachi F2000 Fluorescence Spectrophotometer, Japan).

Cytosolic dehydrogenase assay

Peritoneal or RAW 264.7 macrophages were lysed with phosphate buffered saline plus 0.5% Triton X-100, centrifuged at 12,000 ×*g* for 10 min and the supernatants were used for enzyme measurements. Dehydrogenase activities were measured by NADPH production at 340 nm (Beckman Instruments Spectrophotometer, Fullerton, USA) at 36 °C in the presence of specific incubation mediums for each dehydrogenase, as follows:

- Glucose-6-phosphate dehydrogenase (G6PD): Tris-HCl 50 mM, $MgCl_2$ 3 mM, $NADP^+$ 0.2 mM, glucose-6-phosphate 3.2 mM, pH 7,8;
- Isocitrate dehydrogenase (IDH): Tris-HCl 80 mM, $MgCl_2$ 2 mM, $NADP^+$ 2 mM, sodium isocitric acid 5 mM, pH 7,4;
- Malate dehydrogenase (MDH): Tris-HCl 50 mM, $MgCl_2$ 1 mM, $NADP^+$ 0.5 mM, sodium malic acid 0.6 mM, pH 7,4.

Semi-quantitative RT-PCR

Total RNA was isolated from peritoneal macrophages using the Trizol Reagent (Invitrogen) as recommended by the manufacturer. Approximately 1 μg of the total RNA was added to each cDNA synthesis reaction using the SuperScript-II RT preamplification system (Invitrogen). Reactions were performed at 42 °C for 1 h using the primer T23V (5′ TTT TTT TTT TTT TTT TTT TTT TTV). Oligonucleotide primers for PPARγ were designed to amplify partial cDNA sequences (501 bp – sense 5′-TTTTCAAGGGTGCCAGTTTC-3′, antisense 5′-TCTGTGACGATCTGCCTGAG-3′). β-actin mRNA expression was determined as an internal control. PCR reactions were performed as follows: denaturation (94 °C, 30 s), extension (72 °C, 30 s) and annealing (60 °C, 30 s). The number of amplification cycles was adjusted to the non-saturated phase and the PCR products were separated on a 1% agarose gel.

Table 1
Blood glucose levels and body weight of normal and alloxan-treated animals

	Normal ($n=5$)	Alloxan ($n=5$)
Blood glucose (mg/dL)	102.08±4.71	536.04±27.16 *
Body weight (g)	289.29±8.31	230.75±9.59 *

Alloxan monohydrate (150 mg/Kg in 0.9% NaCl) or saline were injected in the peritoneal cavity of nine-week-old male Wistar rats. Blood glucose and body weight were measured 30 days after injection. Values are expressed as mean±SD.

* $p<0.05$ versus normal group (Student's *T* test).

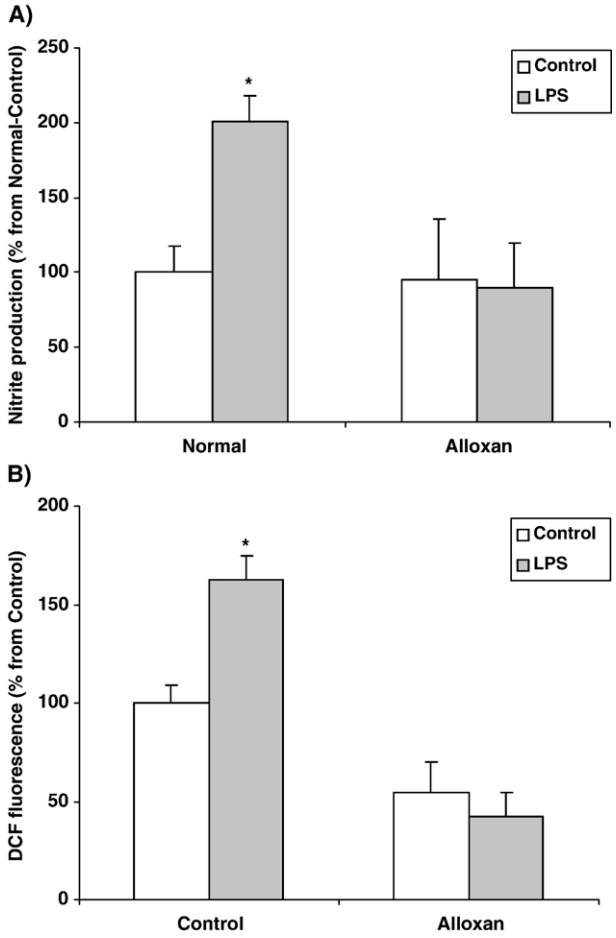

Fig. 1. Nitrite production (A) and DCF oxidation (B) in peritoneal macrophages from normal and alloxan-treated rats. Alloxan monohydrate (150 mg/Kg in 0.9% NaCl) or saline were injected in the peritoneal cavity of nine-week-old male Wistar rats. Thirty days after, peritoneal macrophages were isolated and incubated in the presence or not of LPS (1 μg/ml). After 15 h incubation, nitrite production (A) and DCF oxidation (B) were measured as described in Materials and methods. Values are expressed as percentage of control macrophages of normal rats and represent mean±SEM ($n=5$). *$p<0.05$ versus other groups (ANOVA followed by Tukey HSD test).

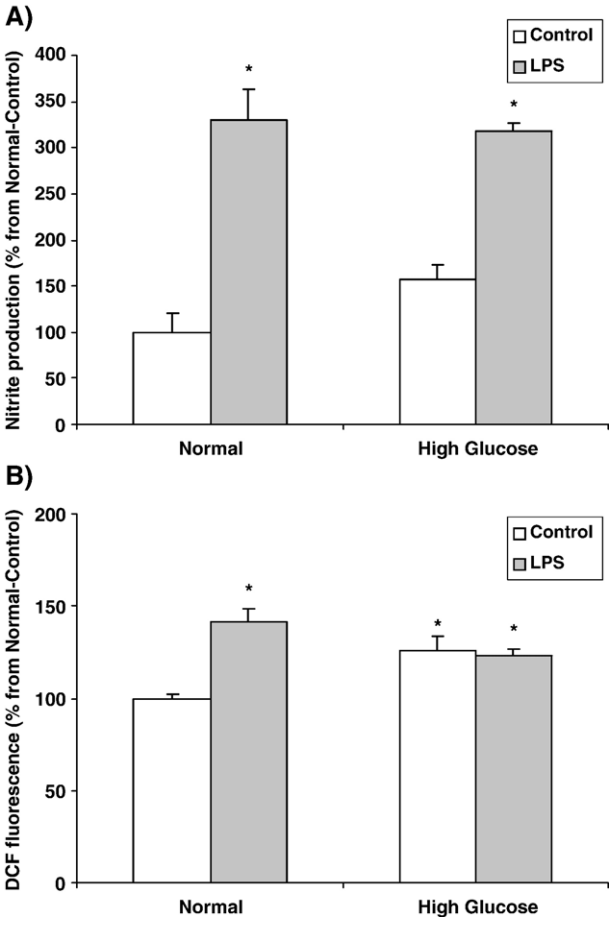

Fig. 2. Nitrite production (A) and DCF oxidation (B) in normal and high glucose-treated RAW 264.7 macrophages. Control (200 mg/dL) or 24 h high glucose (500 mg/dL)-treated RAW 264.7 macrophages were incubated in the presence or not of LPS (1 μg/ml). After 15 h incubation, nitrite production (A) and DCF oxidation (B) were measured as described in Materials and methods. Values are expressed as percentage of control macrophages incubated with normal glucose and represent mean±SEM ($n=5$). *$p<0.05$ versus other groups (ANOVA followed by Tukey HSD test).

Amplification products were visualized by ethidium bromide staining and the quantification was made using ImageJ software (NIH/USA).

Statistical analysis

Results are expressed as means+SD or SEM, as indicated in the legends of figures and tables. Differences between means were analyzed by the Student's *T* test or ANOVA with the Tukey HSD multiple comparisons test. Statistical significance was defined as $p<0.05$.

Results

Gaulton et al. (Gaulton et al., 1985) reported that the immune dysfunction associated with alloxan-diabetes is a consequence of the diabetic state, in contrast to the immune dysfunction associated with streptozotocin, which seems to be attributable to direct and irreversible impairment of lymphoid cell function and viability. Alloxan monohydrate injection leads to the destruction of insulin-secreting β cells in the islets of Langerhans, while other cells (α, γ, δ) are resistant. This is accompanied by typical and permanent hypoinsulinemia and hyperglycemia (Lenzen

Table 2
Glucose-6-phosphate and malate dehydrogenase activities in peritoneal macrophages from normal and alloxan-treated animals

	Normal ($n=4$)	Alloxan ($n=4$)
Glucose-6-phosphate dehydrogenase (μmol/min×mg protein)	57.11±3.5	74.71±5.22*
Malate dehydrogenase (μmol/min×mg protein)	5.47±1.08	7.85±0.5*

Alloxan monohydrate (150 mg/Kg in 0.9% NaCl) or saline were injected in the peritoneal cavity of nine-week-old male Wistar rats. After 30 days, peritoneal macrophages isolation and dehydrogenase activities assay were performed as described in Materials and methods. Values are expressed as mean±SEM.
* $p<0.05$ versus normal group (Student's *T* test).

and Panten, 1988). As shown in Table 1, alloxan-treated animals presented increased blood glucose levels and reduced weight. A sustained high glucose level could present both acute and chronic effects, with the later probably related to advanced glycation end products (Vlassara, 2005). LPS increased nitrite (Fig. 1A — ANOVA f value = 9.778, p = 0.026, n = 5) and ROS (Fig. 1B — ANOVA f value = 13.701, p = 0.026, n = 5) production in peritoneal macrophages from normal rats. However, in alloxan-treated animals, no effect of LPS was observed on nitrite (Fig. 1A) and ROS (Fig. 1B) production by peritoneal macrophages (Fig. 1B). To investigate if increased glucose levels could be acutely involved in altered response to LPS, we evaluated nitrite and ROS production in the murine macrophage lineage RAW 264.7 with two distinct concentrations of glucose: normal RPMI 1640 concentration, 200 mg/dL (~11 mM) or 500 mg/dL (~27 mM). As shown in Fig. 2A (ANOVA f value = 28.19, p < 0,001, n = 5), no alteration in basal or LPS stimulated nitrite production was observed in high glucose incubated macrophages. In high glucose-treated RAW 264.7 macrophages, control DCF oxidation was increased compared to normal glucose (Fig. 2B – ANOVA f value = 8.643, p = 0.003, n = 5); moreover, no effect of LPS on DCF oxidation was observed in high glucose-treated RAW 264.7 macrophages (Fig. 2B).

Glucose-6-phosphate dehydrogenase (G6PD), isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH) are essential for the maintenance of cellular NADPH levels. In peritoneal macrophages from alloxan-treated animals, G6PD and MDH activities were increased compared to the normal group (Table 2); however, no alteration in IDH activity was observed (data not shown). In high glucose-treated RAW 264.7 macrophages, no alteration of dehydrogenase activities was observed (data not shown).

PPARγ has been described as a regulator of macrophage inflammatory response by modulating the expression of cytokines and inhibiting the activity of pro-inflammatory transcription factors (Pascual et al., 2005). Reduced expression of PPARγ was observed in peritoneal macrophages from diabetic rats (Fig. 3). In order to study reduced PPARγ activity in LPS stimulation of macrophages, RAW 264.7 macrophages were incubated in the presence of GW9662, a specific PPARγ antagonist, before LPS treatment. No effect of GW9662 on LPS stimulated nitrite production was found (Fig. 4A – ANOVA f value = 34.092, p < 0.001, n = 5). However, the PPARγ antagonist abolished LPS stimulated ROS production compared to the normal group (Fig. 4B — ANOVA f value = 10.096, p = 0.001, n = 5).

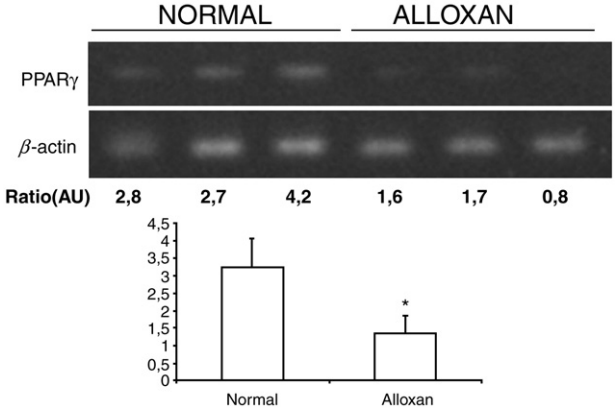

Fig. 3. PPAR-γ expression in peritoneal macrophages from normal and alloxan-treated rats. Alloxan monohydrate (150 mg/Kg in 0.9% NaCl) or saline were injected in the peritoneal cavity of nine-week-old male Wistar rats. Thirty days after, peritoneal macrophages were isolated and PPAR-γ expression measured as described in Materials and methods. Values are mean ± SD of the ratio between PPAR-γ and β-actin bands in arbitrary units (n = 3). *p < 0.05 versus normal group (Student's T test).

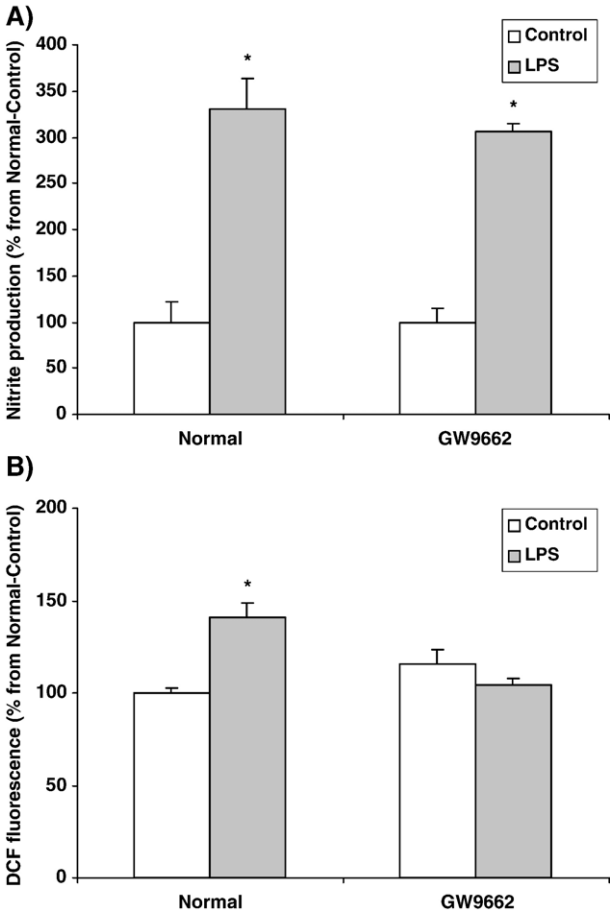

Fig. 4. Nitrite production (A) and DCF oxidation (B) in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated or not for 2 h with PPAR-γ antagonist GW9662 (10 μM). At the end of 2 h, the cells were incubated in the presence or not of LPS (1 μg/ml). After 15 h incubation, nitrite production (A) and DCF oxidation (B) were measured as described in Materials and methods. Values are expressed as percentage from control macrophages and represent mean ± SEM (n = 5). * p < 0.05 versus other groups (ANOVA followed by Tukey HSD test).

Discussion

In the present study, we reported reduced nitric oxide and ROS production in LPS-treated peritoneal macrophages from alloxan-induced diabetic rats. Altered macrophage response was already described in diabetic animals, suggesting that these cells could be involved in immune alterations observed in diabetes. Ptak et al. (Ptak et al., 1998) showed that peritoneal macrophages

from alloxan-treated animals presented impaired response to LPS, with altered cytokines and nitrite production. Increased glucose is described to be involved in some alterations of macrophages responses in diabetic animal models, including interleukin-12 expression (Wen et al., 2006) and upregulation of cyclooxygenase-II, leading to increased prostaglandin-E_2 production (Lo, 2005). Additionally, increased glucose is described to increase basal ROS production in macrophages (Guha et al., 2000). In high glucose-treated RAW 264.7 macrophages, no effect of LPS was observed on DCF oxidation, as observed in peritoneal macrophages from diabetic rats. ROS are involved in tumor necrosis factor-α (Haddad and Land, 2002); interleukin-8 (Ryan et al., 2004) and interleukin-1 β (Hsu and Wen, 2002) production in LPS stimulated macrophages. Taking into account that increased glucose levels are described to reduce interleukin 1 release from LPS-treated macrophages (Hill et al., 1998), defective ROS stimulation by LPS could be involved in altered cytokine production from macrophages in hyperglycemia and diabetes.

Acute effects of high glucose levels seem to be not involved in reduced nitrite production from LPS-treated peritoneal macrophages, since high glucose treatment presented no effect on LPS stimulated nitric oxide production on RAW 264.7 macrophages. However, chronic effects of higher glucose levels, including advanced glycation end products, could be involved in altered nitrite response to LPS of macrophages from diabetic animals. It was suggested that defective insulin signaling leads to altered nitric oxide production in stimulated macrophages (Stevens et al., 1997), but how this could be involved in alloxan-induced alterations remains to be addressed. NADPH is essential for ROS and nitric oxide production (Decoursey and Ligeti, 2005; Forstermann and Kleinert, 1995). G6PD, IDH and MDH are essential for the maintenance of cellular NADPH levels and an impaired NADPH production by reduced G6PD activity is associated with augmented IL-10 production in mouse peritoneal macrophages (Wilmanski et al., 2005). IDH activity has been described to be involved in redox buffering, maintaining NADPH levels for antioxidant enzymes, as glutathione reductase (Maeng et al., 2004). In peritoneal macrophages from alloxan-treated rats, increased G6PD and MDH activities were observed, but no alteration of cytosolic dehydrogenases was found in high glucose-treated RAW 264.7 macrophages, suggesting that acute effects of glucose altered concentration are not involved in G6PD and MDH regulation. Altered ROS production, observed in both RAW 264.7 and peritoneal macrophages, seems to be not dependent on these dehydrogenase activities, considering that they were regulated in peritoneal macrophages and not in RAW 264.7 cells. Future investigation is necessary to address the role of altered G6PD and MDH activities on altered nitrite production observed in peritoneal macrophages from diabetic rats.

PPARγ was described to regulate macrophages functions (Zhang and Chawla, 2004), and Sartippour et al. (Sartippour and Renier, 2000) showed that PPARγ expression is diminished in macrophages by increased glucose. We observed that in peritoneal macrophages from alloxan-treated rats PPARγ expression was reduced compared to normal rats (Fig. 3). The imbalance of inflammatory cytokines production during the development of diabetes could be involved in this downregulation of PPAR-γ expression. In fact, among the many features induced by alloxan treatment there is the increased plasmatic concentration of TNF-α (Ptak et al., 1998). This cytokine is known to repress PPARγ activity and expression in a variety of cell types (Kim et al., 2005; Gao et al., 2006). Additionally, pharmacological reduction of PPARγ activity in RAW 264.7 macrophages resulted in reduced ROS production in response to LPS, similarly as observed in peritoneal macrophages from diabetic rats. These results suggest that reduced PPARγ expression could be involved in impaired ROS production in LPS-treated peritoneal macrophages from diabetic rats. In opposition to our results, PPARγ agonists have been demonstrated to exert anti-inflammatory properties (Ricote et al., 1998), but many of these effects have been shown to be independent from PPARγ activation (Chawla et al., 2001; Crosby et al., 2005). More studies are necessary to investigate how reduced PPARγ expression could alter macrophage response.

In conclusion, in this work we have reported reduced nitric oxide and reactive oxygen species production in LPS-treated peritoneal macrophages from alloxan-induced diabetic rats. We have shown that reduced ROS production seems to be dependent on acute effects of elevated glucose levels and reduced PPARγ expression.

Acknowledgements

This work was supported by CAPES, CNPq, FAPERGS and PROPESQ/UFRGS.

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Anexo 2.

Rosiglitazone, a PPAR-γ Agonist for Type 2 Diabetes Treatment, Improves Glycemic Control and Modulates Glycogen Stores in Type 1 Diabetes Animal Model Induced by Alloxan.

Manuscrito Submetido à

Basic & Clinical Pharmacology and Toxicology (2007).

Rosiglitazone, a PPAR-γ Agonist for Type 2 Diabetes Treatment, Improves Glycemic Control and Modulates Glycogen Stores in Type 1 Diabetes Animal Model Induced by Alloxan

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Short title: **RGZ in alloxan-induced diabetic rats**

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Summary

Thiazolidinediones (TZDs) are antidiabetic drugs which acts as insulin sensitizers and ameliorates insulin resistance in type 2 diabetic patients. The aim of this study was to evaluate the effect of TZD (rosiglitazone) in an animal model of type 1 diabetes. Diabetes was induced in Wistar rats by alloxan (150 mg/kg). The animals were treated orally with rosiglitazone maleate (3 mg/Kg) or saline. The results in blood glucose (294.4 ± 42.74 mg/dL) and body weight increase (16.04 ± 4.56%) demonstrate a significative effect of RGZ when compared to the diabetic-vehicle group (536.04 ± 27.16 mg/dL) and (2.14 ± 6.29%) respectively. HbA_{1C} in diabetic-RGZ group was decreased (3.46 %) when compared to diabetic-vehicle group (4.29 %). Absence of adverse hepatic effects was observed. Rosiglitazone treatment reduces reactive C protein (6.0 ± 0.5 mg/L) compared to diabetic rats (9.7 ± 1.8 mg/L). The total glycogen content in tissues (liver, heart, kidney and gastrocnemius) samples from diabetic animals was modulated by RGZ. The treatment reduces glycogen content in heart and kidney (2.41 ± 0.31 and 0.41 ± 0.29 mg/%) in comparison to diabetic group (3.28 ± 0.28 and 2.21 ± 0.21 mg/%) respectively. Rosiglitazone had a positive effect on alloxan-induced diabetic rats.

Key Words: Alloxan Diabetes; Body weight changes; C-reactive protein; Diabetes Mellitus, type I; Diabetes Mellitus, type II; Drug toxicity; Peroxisome proliferator-activated receptors; Rosiglitazone.

1. Introduction

Type I diabetes is a complex syndrome with both genetic and environmental influences and is due primarily to autoimmune-mediated destruction of pancreatic β-cell islets (resulting in absolute insulin deficiency). Type II diabetes is characterized by insulin resistance and/or abnormal insulin secretion. Type 2 diabetes is numerically prevalent in the population worldwide and accounts for over 90% of cases diagnosed.[1]

Alterations in the metabolism of lipids, carbohydrates and proteins are common aspects of diabetes. Uncontrolled hyperglycemia leads to the development of severe complications in the microvasculature mainly affecting retina, renal glomerulus and peripheral nerve system.[2, 3] As a consequence of microvascular pathologies associated to this sindrome, diabetes is the leading cause of blindness, end stage renal disease and a variety of debilitating neuropathies. Diabetes is also associated with accelerated atherosclerotic macrovascular disease, affecting arteries that supply heart, brain and lower extremities.[4]

Type II diabetes mellitus is a heterogeneous and multifactorial metabolic disorder that affects approximately 6% of the adult population in Western society, probably reaching 300 million cases in 2010.[5] A newer class of antidiabetic agents—the thiazolidinediones (TZDs) including rosiglitazone (RGZ)—acts as insulin sensitizers attenuating hyperglycemia, hyperinsulinemia and dyslipidemia in type II diabetic patients,[6] thereby decreasing the risk of cardiovascular disease associated with insulin resistance. Clinical trials have demonstrated that treatment with TZDs may be associated with weight gain.[7] Increases in body weight with TZD use are positively correlated with reductions in glicated hemoglobin (HbA1c) and weight gain appears to stabilize after the initial reductions in HbA1c[7].

Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) is a drug known to generate specific necrotic processes in rat pancreatic islets,[8] therefore, being commonly used in

animal models of type I Diabetes Mellitus[9]. The aim of this study was to evaluate the effect of RGZ, a commonly administered drug for type II diabetes, in an animal model of type I diabetes induced by alloxan.

2. Materials and Methods

2.1 Chemicals

Alloxan monohydrate, glutathione (GSH) and 1-Chloro-2,4-nitrobenzene (CDNB) were purchased from Sigma Chemical Co. (St. Louis, MO). Rosiglitazone maleate (RGZ) was purchased from GlaxoSmithKline®.

2.2 Animals, experimental procedures and analysis

Nine-week-old male Wistar rats (180-215 g) were obtained from our breeding stock. Rats were housed in plastic cages, maintained at 22 ± 1°C, 55% relative humidity and 12 h light/dark cycle. Animals were supplied with commercial pellet food (Nuvilab® CR-1 Curitiba, PR, Brazil) and water *ad libitum*. All the animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian College for Animal Experimentation. Animals were randomly divided into two groups: non-induced and diabetic-induced. Diabetes was induced by an intraperitoneal injection of 150 mg/kg of alloxan monohydrate (0.9% NaCl) after overnight fasting. Non-induced group received only 0.9% NaCl.[10] Blood glucose levels were monitored thereafter by Accu-Chek® Active blood glucose monitor. Ten days later animals from the diabetic-induced group, which have exhibited glucose levels lower than 300 mg/dL, were not used in this study. Non-induced and diabetic-induced groups were once more randomly subdivided into two subgroups each. These were treated orally by gavage for 20 days with a single intragastric dose per day of rosiglitazone maleate (3 mg/Kg) or saline (0.9% NaCl), in rosiglitazone (RGZ) and non-rosiglitazone subgroups respectively. Body weight was measured twice a week and

used to regulate the treatment doses. Twenty days after the beginning of rosiglitazone treatment, animals were killed by decapitation. Tissues from liver, kidney, heart, gastrocnemius muscle and blood samples were collected for subsequent analyses. The levels of triglycerides, total cholesterol, HbA_{1C} and uric acid were determined with commercial kits produced by Human GmbH, Wiesbaden, Germany. Quantification of HDL, creatinine and TGO, TGP activity were determined with commercial kits produced by In Vitro Diagnostica, Barbacena, Brazil. The concentration of LDL and VLDL was obtained by Friedewald equation.[11] C-reactive protein (CRP) was analyzed using commercial kit from Laborclin, Curitiba, Brazil. Glutathione S-transferase (EC 2.5.1.18) activity was determined in hepatic tissue spectrophotometrically at 340 nm by measuring the formation of the conjugate of glutathione (GSH) and 1-Chloro-2, 4-nitrobenzene (CDNB) as described previously.[12] To determine total glycogen content, tissue samples were weighed and digested in hot concentrated 30% KOH, boiled at 100°C for 20 minutes, precipitated with ethanol and measured by colorimetric procedure with iodine.[13] The results were expressed as mean ± SEM. ANOVA followed by the Tukey test employed to detect differences between the groups. Difference was considered when $p< 0.05$.

3. Results and Discussion

TZDs provide many benefits to patients with type 2 diabetes by improving glycemic control and insulin sensitivity; however, the body weight gain appears to be highest when TZDs are used in combination with insulin or sulfonylureas and, lowest when used as monotherapy or in combination with metformin.[7, 14] RGZ decreased blood glucose in Alloxan-induced group (from 558.03 ± 60.56 mg/dL before treatment to 294.4 ± 42.74 mg/dL after treatment) and increased body weight significantly in alloxan-induced group (16.04 ± 4.56 %) when compared to alloxan-induced non-RGZ group (2.14 ± 6.29 %).

Recently, one of the few studies concerning TZDs and type 1 diabetes, reported a similar effect in weight gain in type 1 diabetic overweight subjects treated with rosiglitazone, despite the effects in blood glucose levels.[15] An expected consequence of blood glucose reduction was a significant decrease in HbA_{1C} levels in diabetic-induced –RGZ-treated group (3.46 %) when compared to diabetic-induced-non-RGZ-treated group (4.29 %). No statistical difference was observed in triglycerides, VLDL and LDL levels. Total cholesterol and HDL levels showed a decrease in diabetic-induced-RGZ group (43.44 ± 4.33 and 30.27 ± 2.37 mg/dL) when compared to diabetic-induced- non-RGZ-treated group (51.33 ± 5.16 and 36.59 ± 2.76 mg/dL) respectively. The changes observed in levels of total cholesterol, HDL and triglycerides differ from those found by Calkin et al,[16] that were observed in a mice model of insulin insufficiency. Rosiglitazone treatment markedly reduced uric acid levels in plasma of alloxan- induced -diabetic animals (0.62 ± 0.07 mg/dL) differing from non-diabetic non-treated group. Creatinine level was significantly increased in diabetic-induced animals (0.51 ± 0.032 mg/dL) when compared to non-induced rats; however, RGZ treatment did not affect this parameter (0.51 ± 0.021 mg/dL). Considering creatinine and uric acid levels as renal function indexes, further approaches are necessary to clarify these results.

When considering TZDs for a clinical use, one should be concerned about the potential hepatotoxicity of these compounds. In a recent review was documented that troglitazone (TGZ), but RGZ, may be hepatotoxic.[17] This data is in accordance with the present study, in which RGZ treatment used did not increase hepatic transaminase (TGO and TGP) activities in plasma (table 1). We also quantified liver glutathione S-transferase (E.C. 2.5.1.18) as an indicative for a potential hepatotoxic effect of RGZ. Liver GST is an

enzyme whose essential function is the biotransformation of xenobiotics.[12] Our results showed no significant differences in GST activities among all groups assayed (table 1).

The total glycogen content in all examined tissues samples from diabetic-induced animals was interestingly modulated by RGZ treatment (table 1). Hyperglycaemia leads to an increased in total glycogen content in heart, kidney and gastrocnemius tissues when compared to non induced-non-RGZ-treated group tissues (table 1). RGZ treatment reduces glycogen content in heart and kidney (2.41 ± 0.31 and 0.41 ± 0.29 mg/%) in diabetic – induced group when compared to non-RGZ treated - diabetic-induced group (3.28 ± 0.28 and 2.21 ± 0.21 mg/%) respectively. A different effect was observed in liver and gastrocnemius tissues. In both tissues RGZ treatment increased total glycogen content in comparison to non-treated diabetic group (table 1). Other studies have demonstrated the effect of TZD's treatment on glycogen metabolism in animal models of type 2 diabetes such as the Zucker fatty rat[19] and OLETF rat.[20] Our data demonstrated a comparable result in an animal model of type 1 diabetes. No effect of RGZ treatment was observed in glycogen content in non-diabete-induced group.

Circulating level of C-reactive protein (CRP), a sensitive systemic marker of inflammation and features of the metabolic syndrome,[21] was associated in present study with hyperglycemia in alloxan-induced diabetic rats (9.7 ± 1.8 mg/L). RGZ treatment decreased CRP level to non-diabetic non-treated level (6.0 ± 0.5 mg/L). No effect of RGZ treatment was observed in CRP content in non-diabetic-induced group.

Taken into account blood glucose decrease, HbA_{1C} level decrease, body weight increase, CRP decrease, the non-increase of TGO, TGP in plasma and GST in liver promoted by a single dose per day of RGZ (3 mg/Kg), we suggested that this compound may be particularly considered for trials aiming type 1 diabetes treatment. However, more

long-term and complex studies are needed to elucidate the mechanism action of rosiglitazone on type 1 diabetes.

4. Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Brazil), Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (Fapergs) and Pró-Reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (Propesq/UFRGS).Special thanks to Fabricia Ferraz for critical English review.

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Table 1. The effect of rosiglitazone in alloxan-induced diabetic rats

	Non-induced		Diabetic-induced	
	Untreated (n= 5)	**RGZ-treated** (n= 5)	**Untreated** (n= 5)	**RGZ-treated** (n= 5)
Blood glucose BT (mg/dL)	102.08 ± 4.71	101.34 ± 2.37	550.76 ± 37.17*	558.03 ± 60.56*
Blood glucose AT (mg/dL)	92.14 ± 3.62	91.03 ± 11.67	536.04 ± 27.16*	294.4 ± 42.74* **
HbA_{1C} (%)	2.59 ± 0.075	2.49 ± 0.092	4.29 ± 0.10*	3.46 ± 0.17* **
Body weight increase (%)	25.78 ± 3.37	23.24 ± 4.78	2.14 ± 6.29*	16.04 ± 4.56* **
Triglycerides (mg/dL)	66.41 ± 4.12	72.96 ± 6.31	77.67 ± 12.64	77.20 ± 12.19
VLDL (mg/dL)	13.28 ± 0.82	14.59 ± 1.26	15.53 ± 2.52	15.44 ± 2.43
LDL (mg/dL)	7.03 ± 1.05	8.11 ± 1.78	7.42 ± 1.98	9.06 ± 2.23
HDL (mg/dL)	25.49 ± 0.41	27.42 ± 1.96	36.59 ± 2.76*	30.27 ± 2.37* **
Total cholesterol (mg/dL)	37.62 ± 1.28	40.06 ± 3.44	51.33 ± 5.16*	43.44 ± 4.33**
Uric acid (mg/dL)	0.96 ± 0.07	0.84 ± 0.15	2.19 ± 0.83*	0.62 ± 0.07* **
Creatinine (mg/dL)	0.44 ± 0.016	0.46 ± 0.023	0.51 ± 0.032*	0.51 ± 0.021*
TGO activity (U/mL)	66.06 ± 0.61	65.93 ± 1.36	68.51 ± 1.54	65.03 ± 1.06
TGP activity (U/mL)	62.69 ± 0.77	61.55 ± 3.42	64.56 ± 2.01	59.89 ± 3.81
GST activity (U/mg protein)	0.034 ± 0.0031	0.034 ± 0.0042	0.033 ± 0.0021	0.035 ± 0.0045
Liver glycogen (mg %)	2.14 ± 0.17	1.91 ± 0.45	0.41 ± 0.06*	1.46 ± 0.37* **
Kidney glycogen (mg %)	0.91 ± 0.07	0.67 ± 0.38	2.21 ± 0.21*	0.41 ± 0.29* **
Heart glycogen (mg %)	1.16 ± 0.16	1.52 ± 0.61	3.28 ± 0.28*	2.41 ± 0.31* **
Gastrocnemius glycogen (mg %)	0.82 ± 0.07	1.02 ± 0.25	2.32 ± 0.34*	3.41 ± 0.26* **
C-reactive protein (mg/L)	5.0 ± 0.61	ND	9.7 ± 1.8*	6.0 ± 0.5**

Table 1. Physiological, biochemical and enzymatic parameters of 60-day-old rats which have received a unique intraperitoneal injection of alloxan (150 mg/Kg) or saline. 10 days later alloxan injection the diabetic-induced and non-induced groups were randomly subdivided in two subgroups which were treated by gavage for 20 days with a single intragastric dose per day of rosiglitazone maleate (3 mg/Kg) or saline. Data are expressed as mean ± S.E. Difference was considered when p= 0.05. BT (before treatment), AT (after treatment) ND (not determined).

* indicate different from control group.

** indicate different from diabetic group.

Anexo 3.

Certificado de destaque na sessão de Farmacologia e Toxicologia no XVIII Salão de Iniciação Científica da Universidade Federal do Rio Grande do Sul que ocorreu de 15 a 20 de outubro de 2006, recebido por Guilherme Antônio Behr pela apresentação do trabalho "O efeito da rosiglitazona em modelo animal de diabetes mellitus tipo 1 induzida por aloxano"

CERTIFICADO

XVIII Salão de Iniciação Científica
XV Feira de Iniciação Científica
I Salão UFRGS Jovem

Certificamos que o trabalho

O EFEITO DA ROSIGLITAZONA EM MODELO ANIMAL DE DIABETES MELLITUS TIPO I INDUZIDA POR ALOXANO

de autoria de

GUILHERME ANTONIO BEHR
EVANDRO GOMES DA SILVA
RODRIGO LORENZI
MATHEUS AUGUSTO DE BITTENCOURT PASQUALI
EMILIO LUIZ STRECK
FELIPE DAL-PIZZOL

tendo como orientador(a): JOSE CLAUDIO FONSECA MOREIRA
recebeu DESTAQUE na Sessão: Farmacologia e Toxicologia

no XVIII Salão de Iniciação Científica, promovido pela Pró-Reitoria de Pesquisa - PROPESQ, na Universidade Federal do Rio Grande do Sul, no período de 15 a 20 de outubro de 2006.

Porto Alegre, outubro de 2006

César Augusto Zen Vasconcellos
Pró-reitor de Pesquisa

UFRGS UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL SIC propesq

Anexo 4.

Certificado de "Young Investigator Award" recebido pelo trabalho "Antioxidant activity and hypoglycemic effect of *Bauhinia microstachya* leaves in alloxan-induced diabetes rats" apresentado durante o V Meeting of SFRBM – South American Group e V International Conference on Peroxynitrite and Reactive Nitrogen Species, que ocorreram na cidade de Montevideo – Uruguai, em 02 a 06 de setembro de 2007.


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in Montevideo 2007
September 2-6, 2007
V Meeting of SFRBM - South American Group
V International Conference on Peroxynitrite and Reactive Nitrogen Species
South American Group
SFRBM
We hereby certify that
EVANDRO G. DA SILVA, Brazil
Antioxidant activity and hypoglycemic effect of Bauhinia microstachya leaves in alloxan-induced diabetes rats
As a recipient of a Young Investigator Award
Free Radicals in Montevideo 2007
V Meeting of SFRBM - South American Group
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Montevideo, Uruguay
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