UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS DA SAÚDE
DEPARTAMENTO DE CIÊNCIAS FARMACÊUTICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

ATIVIDADE ANTI-HIPERGLICEMIANTE ORAL E SEGURANÇA DE USO DO EXTRATO AQUOSO DA CASCA DE *Caesalpinia ferrea* Martius Ex Tul (Leguminosae) EM RATOS WISTAR

CARLOS FERNANDO BRASILEIRO DE VASCONCELOS

RECIFE-PE
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RESUMO

O diabetes *mellitus* é um grave problema de saúde pública caracterizado por hiperglicemia crônica ocasionada pela ausência ou ineficiência da insulina nos tecidos-alvo. Muitas espécies de plantas têm sido usadas na medicina popular para tratar os sintomas da doença. Na etnofarmacologia, o chá da casca de *Caesalpinia ferrea* Martius Ext Tul tem sido usado no tratamento do diabetes. Nesse estudo, metabólitos secundários foram identificados e quantificados por HPLC. A fim de verificar a atividade antidiabética de *C. ferrea*, quatro grupos de ratos Wistar diabéticos induzidos por estreptozotocina (50mg/Kg) (n=7/grupo) foram tratados com o extrato aquoso da casca do caule de *C. ferrea* (EaCf) (300 e 450mg/Kg/dia), veículo e metformina (500mg/Kg/dia) durante 4 semanas. Mudanças no ganho de massa, no consumo de água e ração e nos parâmetros bioquímicos foram avaliadas. A expressão da proteína quinase B (Akt), da proteína quinase ativada por AMP (AMPK) e acetil-CoA carboxilase (ACC) foram determinadas no fígado e músculo esquelético dos animais usando *Western blot.* A avaliação da segurança de uso do EaCf foi verificada através dos ensaios de toxicidade aguda, subcrônica, crônica e reprodutiva. Para os ensaios de toxicidade aguda, o EaCf (2000mg/Kg) foi administrado em ratos Wistar de ambos os sexos (n= 5/grupo/sexo). Na toxicidade subcrônica, ratos Wistar machos (n=10/grupo) foram tratados durante 30 dias com o EaCf (300 e 1500mg/Kg/dia) e na crônica, o EaCf (300 e 1500mg/Kg/dia) foi administrado aos camundongos Swiss machos durante 90 dias. Ao final do tratamento foram realizadas análises bioquímicas, hematológicas, macro e microscópicas dos órgãos. Na toxicidade reprodutiva, o EaCf (300 e 1500mg/Kg/dia) foi administrado em ratas Wistar prenhes durante o período integral de gestação para avaliação dos parâmetros reprodutivos maternos e comportamentais da prole. O conteúdo de ácido gálico, catequina, epicatequina e ácido elágico quantificado por HPLC foi de 112,76; 17,75; 6,13 e 12,00mg/g. Os resultados ainda mostraram que EaCf reduziu os níveis glicêmicos e melhorou o estado metabólico geral dos animais diabéticos. A ativação da Akt foi observada no fígado e músculo esquelético dos animais tratados com conseqüente desativação da AMPK no músculo e ativação da ACC em ambos quando comparado aos não-tratados. Na toxicidade aguda, o tratamento não induziu nenhum sinal de toxicidade ou morte, e na subcrônica, não houve alterações macro e microscópicas dos órgãos e nem nos parâmetros hematológicos e bioquímicos, exceto a amilasemia observada. Na toxicidade crônica, houve reduções no ganho de massa corporal, nos níveis séricos de proteínas totais e albumina (em ambas as doses). Na dose de 1500mg/Kg, houve aumento da lactato desidrogenase, amilase e necrose do intestino delgado. Durante toda a gestação, houve redução no ganho de massa corporal (1500mg/Kg) e na prole foram observadas reduções na massa corporal no 1º e 4º dias de vida e no comprimento, no 21º dia de vida pós-natal (1500mg/Kg). Os resultados sugerem que EaCf apresenta atividade antidiabética e age, possivelmente, regulando a captação hepática e muscular de glicose via Akt. O tratamento crônico sugere toxicidade principalmente na maior dose, provavelmente pela ação dos taninos que comprometem a absorção de macronutrientes.

Palavras-chave: *Caesalpinia ferrea* Martius Ext Tul., Diabetes *mellitus*, Proteína quinase B (Akt), Proteína quinase ativada por AMP (AMPK), Acetil-CoA carboxilase (ACC), Toxicidade.

ABSTRACT

Diabetes is a serious public health problem characterized by chronic hyperglycemia caused by the absence or inefficiency of insulin in target tissues. Many plant species have been used in folk medicine to treat the symptoms of the disease. In the ethnopharmacology, the tea from the bark of *Caesalpinia ferrea* Martius Ex Tul has been used in the treatment of diabetes. In this study, the secondary metabolites were identified and quantified by HPLC. In order to verify the antidiabetic activity of *C. ferrea*, four groups of rats with diabetes induced by streptozotocin (50mg/kg) (n = 7/group) were treated with stem bark aqueous extract of *C. ferrea* (Cfae) (300 and 450mg/kg/day), vehicle and metformin (500mg/kg/day) for 4 weeks. Changes in mass gain, water and food intake and biochemical parameters were evaluated. The expression of protein kinase B (Akt), AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) were determined in liver and skeletal muscle of animals using Western blot. The safety assessment of use of Cfae was verified through tests of acute, subacute, chronic and reproductive toxicities. For acute toxicity tests, the Cfae (2000mg/kg) was administered to rats of both sexes (n = 5/grupo/sex). In subacute toxicity, male Wistar rats (n = 10) were treated for 30 days with Cfae (300 and 1500mg/kg/day) and in chronic, Cfae (300 and 1500mg/kg/day) was administered to Swiss male mice for 90 days. At the end of treatment were analyzed biochemical and haematological parameters and evaluated macro and microscopic aspects of organs. In the reproductive toxicity, Cfae (300 and 1500mg/kg/day) was administered to pregnant Wistar rats during the whole period of gestation for evaluation of reproductive parameters and offspring behavioral development. The contents of gallic acid, catechin, epicatechin and ellagic acid quantified by HPLC were 112.76, 17.75, 6.13 and 12.00mg/g. The results also showed that Cfae reduced blood glucose levels and improved the overall metabolic state of diabetic animals. Activation of Akt was observed in liver and skeletal muscle of treated animals with consequent de-activation of AMPK in the muscle and activation of ACC in both compared to untreated. In the acute toxicity, the treatment did not induce any signs of toxicity or death. In subacute, no changes in macro or microscopic aspects of organs and in haematological and biochemical parameters were observed except increased serum amylase. In chronic toxicity, there were reductions in body mass gain, serum levels of total protein and albumin (both doses). At a dose of 1500mg/kg, there were an increase of lactate dehydrogenase, amylase and necrosis of small intestine. Throughout gestation, there were a decrease in maternal body mass gain (1500mg/kg) and reductions in fetuses body mass on 1 and 4 days of life and in the length on 21 day of postnatal life (1500mg/kg). The results suggest that Cfae presentes antidiabetic activity and it acts possibly regulating the hepatic and muscle uptake of glucose mediated by Akt. Chronic treatment suggests toxicity mainly at the highest dose, probably by the action of tannins that impair the absorption of macronutrients.

Keywords: *Caesalpinia ferrea* Martius Ext Tul., Diabetes, Protein kinase B (Akt), AMP-activated protein kinase (AMPK), Acetyl-CoA carboxylase (ACC), Toxicity.

SUMÁRIO

1. Introdução

1.0. Introdução

O diabetes *mellitus* é um significante problema de saúde pública devido a sua alta prevalência, morbidade e mortalidade (MENDES et al., 2010). A insulina produzida pelas células β-pancreáticas facilita a homeostase da glicose ao promover sua captação e estoque no músculo esquelético, fígado e adipócitos. A falta ou a ineficiência do uso da insulina nos alvos periféricos estão juntas no diabetes, uma doença crônica caracterizada por hiperglicemia, que ao longo do tempo, pode ocasionar uma série de alterações metabólicas e neurais (KAHN; HULL; UTZSCHNEIDER, 2006).

Os dois principais tipos de diabetes *mellitus* são o tipo 1, que corresponde a 10-20% dos casos e, o tipo 2, correspondendo 80-90% dos casos. Esses dois tipos distinguem-se pela apresentação clínica, origem genética, patogênese, lesão das ilhotas pancreáticas e resposta à insulina (CONTRERAS, 2004). Segundo a Federação Internacional de Diabetes (2009), a cada ano, 7 milhões de pessoas desenvolvem os sintomas de diabetes em todo o mundo.

Estima-se que 25% de todos os medicamentos modernos são derivados, direta ou indiretamente, de plantas medicinais (SUCHER; CARLES, 2008). Grande número de espécies vegetais tem sido usado experimentalmente para tratar os sintomas do diabetes *mellitus.* Portanto, para que uma planta com indícios de ação farmacológica anti-hiperglicemiante, baseados em observações empíricas, tenha seu potencial terapêutico comprovado são necessários estudos prévios relativos aos aspectos botânicos, fitoquímicos, farmacológicos e toxicológicos, assegurados por metodologias científicas adequadas que visam ampliar as alternativas terapêuticas disponíveis no tratamento do diabetes (NEGRI et al., 2005).

Na etnofarmacologia, o chá da casca do caule de *Caesalpinia ferra* Martius Ext Tul. é usado no tratamento do diabetes, contudo, inexiste respaldo científico para tal utilização. Visando comprovar a ação anti-hiperglicemiante desta planta e investigar os prováveis mecanismos moleculares envolvidos, as expressões da proteína quinase B (PKB/Akt), presente na via de sinalização da insulina, e da proteína quinase ativada por AMP (AMPK), proteína mediadora da regulação metabólica, foram avaliadas nos animais diabéticos tratados com *Caesalpinia ferrea.*

Estudos de toxicidade também foram realizados a fim de verificar a segurança do uso do extrato.

2. Revisão Bibliográfica

2.0. Revisão Bibliográfica

2.1. Diabetes *mellitus* – Considerações gerais

O diabetes *mellitus* é considerado uma síndrome que é, quanto à etiologia e patogênese, caracterizada por alterar a homeostase do organismo, devido aos distúrbios metabólicos complexos e primários dos carboidratos, que envolvem secundariamente, porém de forma importante, o metabolismo de lipídeos e proteínas. A hiperglicemia é associada com disfunção, deficiência e falência ao longo prazo de vários órgãos, especialmente, olhos, rins, nervos, coração e vasos sanguíneos (ORTIZ-ANDRADE et al., 2005).

O diabetes *mellitus* está associado a uma série de sintomas comuns tais como polifagia e polidpsia excessivas, fraqueza muscular, perda de peso, elevação da glicemia e glicosúria (SHOELSON, 1995; SAID et al., 2002). Existem dois tipos comuns de diabetes *mellitus.* O tipo 1 também chamado de diabetes *mellitus* dependente de insulina e o tipo 2, também conhecido como diabetes *mellitus* não dependente de insulina (FULLER et al*.,* 1980).

O diabetes tipo 1 é uma doença autoimune debilitante causada pela destruição progressiva das células β-pancreáticas, levando à ausência da produção de insulina (CARR; BRUNZELL, 2004; GRUNDY, 2004; KAHN; HULL; UTZSCHNEIDER, 2006). Esquemas de reposição de insulina que visam reproduzir a faixa fisiológica dos níveis glicêmicos são os tratamentos de escolha para esses pacientes (TAUBES, 2008). Enquanto que o diabetes tipo 2 é uma doença crônica progressiva que acomete aproximadamente 90% dos pacientes diabéticos (CLARK, 2008) e pode manifestar-se em qualquer idade, em grande parte devido aos distúrbios metabólicos persistentes que envolvem fatores internos e externos, incluindo dieta e o estilo de vida (OWENS; ZINMAN; BOLLI, 2001; TAUBES, 2008).

Aumentos episódicos na secreção de insulina basal e na sua secreção pós-prandial devido aos fatores internos e ambientais conduzem gradualmente a uma insensibilidade do receptor de insulina, resistência insulínica e diminuição dos eventos subseqüentes à ligação da insulina ao seu receptor. Sob essas condições, a hipersecreção insulínica que ocorre para normalizar os níveis glicêmicos contribui para a disfunção e perda das células β-pancreáticas, levando a uma hiperglicemia

incessante, caracterizando o diabetes tipo 2 (LAZAR, 2005; BOGHOSSIAN et al., 2006; KALRA, 2009).

Uma forma incomum de diabetes que ocorre em torno de 1% dos pacientes diabéticos é o tipo MODY (*Maturity Onset Diabetes of the Young* – diabetes da maturidade implantado em jovens). O MODY é uma forma de diabetes familiar de transmissão autosssômica dominante, revelado pela presença de três gerações de mesma linhagem afetadas e diagnóstico precoce (infância, adolescência ou adultos jovens). Enquanto o diabetes tipo 2 é poligênico, o tipo MODY é devido a um único defeito no gene (ADA, 2004).

Há seis subtipos de MODY secundários a 6 genes diferentes. Esses genes codificam a enzima glicoquinase (MODY2) ou fatores de transcrição para o desenvolvimento normal e funcional das células β-pancreáticas. Estes fatores de transcrição incluem o fator hepatocítico nuclear 4α (HNF-4α /MODY1); fator hepatocítico nuclear 1α (HNF-1α /MODY3); fator promotor da insulina (IPF1/MODY4); fator hepatocítico nuclear 1β (HNF-1β /MODY5) e $NeuroD_1$ (MODY6). Esses pacientes podem apresentar desde uma hiperglicemia leve a uma hiperglicemia severa desde o nascimento (ADA, 2004).

Diabetes *mellitus* gestacional é definido como qualquer nível de intolerância a carboidratos, resultando em hiperglicemia de gravidade variável, com início ou diagnóstico durante a gestação. Sua fisiopatologia é explicada pela elevação de hormônios contra-reguladores da insulina, pelo estresse fisiológico imposto pela gravidez e a fatores predeterminantes (genéticos ou ambientais). O principal hormônio relacionado com a resistência à insulina durante a gravidez é o hormônio lactogênico placentário, contudo, sabe-se que outros hormônios hiperglicemiantes como cortisol, estrógeno, progesterona e prolactina também estão envolvidos (MIRANDA; REIS, 2006). O diabetes gestacional acomete de 2-10% das mulheres americanas grávidas. Outros tipos de diabetes resultantes de cirurgia, medicações, infecções e doenças pancreáticas correspondem de 1-5% de todos os casos diagnosticados de diabetes (CDC, 2011).

Radicais livres e o estresse oxidativo estão entre os fatores envolvidos na patogênese do diabetes e, particularmente, parecem ter um papel importante na destruição das células β-pancreáticas e no desenvolvimento das complicações crônicas da doença (BAYNES, 1991). Em pacientes diabéticos, a auto-oxidação da

glicose e a glicação não-enzimática de proteínas são conhecidos como importantes fatores produtores de espécies reativas de oxigênio (WOLFF, 1993; VARVAROVSKA et al., 2004). Em adição, a hiperglicemia leva à ativação da aldose redutase e à depleção do NADPH, que por sua vez, provoca transtornos no ciclo de redução da glutationa e agrava o estresse oxidativo nas células (ROGER et al., 2002).

As doenças cardiometabólicas têm na obesidade um importante fator que contribui para o estabelecimento da doença (PAEK; CHUNG, 2010) e a associação entre obesidade, doenças cardiovasculares e diabetes tipo 2 tem sido observada (CAREY et al., 1997), sendo a resistência insulínica um importante elo de ligação entre elas. As doenças cardiovasculares correspondem a 50-80% das mortes nos pacientes (TOSCANO, 2004; SKYLER et al., 2009).

Dentre os fatores que contribuem para a resistência à insulina incluem-se o não uso de glicose pelo músculo esquelético, desordens no metabolismo de ácidos graxos e lipídeos, aumento da gliconeogênese hepática e decréscimo da perfusão microvascular (CLARK, 2008; TURCOTTE; FISHER, 2008). Portadores de obesidade abdominal, com maior deposição de gordura visceral, também apresentam maior risco para o desenvolvimento de diabetes tipo 2 (CHAN et al., 1994). Por isso, o índice de massa corporal tem sido um excelente indicador para doenças cardiovasculares e diabetes tipo 2 (POIRIER et al., 2005).

A gordura visceral apresenta um alto *turnover* metabólico, com expressiva atividade lipolítica, drenando expressivas concentrações de ácidos graxos livres (AGL) diretamente no fígado através da veia porta. Os AGL em nível hepático, por sua vez, reduzem o *clearance* da insulina e aumentam a produção hepática de glicose. Toda a ação que vise perda ponderal de gordura tende a reduzir a progressão dos quadros de tolerância diminuída à glicose e à glicemia de jejum alterada (COLDITZ et al., 1995).

Segundo a Associação Americana de Diabetes (2010) os critérios para diagnóstico laboratorial do diabetes compreendem pelo menos uma das seguintes condições: hemoglobina A_{1c} (Hb A_{1c}) ≥ 6,5%, glicemia de jejum ≥ 126 mg/dL (sendo considerado jejum a não ingestão calórica por pelo menos 8h), glicemia ≥ 200 mg/dL duas horas após a administração de uma carga de glicose contendo o equivalente a 75 g de glicose anidra dissolvida em água e, por último, a dosagem de uma glicemia

aleatória ≥ 200 mg/dL em pacientes com sintomas clássicos de hiperglicemia ou crise hiperglicêmica.

A Federação Internacional de Diabetes – FID – (2009) estima que mais de 245 milhões de pessoas em todo o mundo tenha a doença, sendo que a expectativa é a de que esse número cresça para 380 milhões em vinte anos. A cada ano, 7 milhões de pessoas desenvolvem os sintomas de diabetes em todo o mundo. A mesma instituição estimou que em 2007, havia no Brasil, 6.913.300 pessoas diabéticas com idades entre 20-79 anos (5,8% da população na mesma faixa etária, com projeção de aumento para 11,4% em 2025).

Nos Estados Unidos foram registrados, em 2010, 10,9 milhões de pessoas acima de 65 anos com diabetes assim como aproximadamente 1,9 milhões de jovens acima de 20 anos e cerca de 215 mil jovens abaixo de 20 anos com a doença. O diabetes foi a sétima causa de morte nos Estados Unidos em 2007 e contribuiu, como causa adicional, com a morte de mais de 160 mil pessoas nesse mesmo ano (CDC, 2011). Em 2000, dados brasileiros mostraram que a taxa de mortalidade pela doença para cada 100 mil habitantes apresentavam acentuado aumento com o progredir da idade, variando de 0,58 para a faixa etária de 0-29 anos até 181,1 para a de 60 ou mais (ROGLIC et al., 2005).

O diabetes traz também um grande impacto econômico para as nações. Só nos Estados Unidos, por exemplo, os custos diretos e indiretos com a doença em 2007 foram estimados em 174 bilhões de dólares (CDC, 2011). Estimativas do custo direto para o Brasil estão em torno de 3,9 bilhões de dólares americanos (BARCELÓ, 2003). Desse modo, o diabetes *mellitus* mostra-se como um grave problema de saúde pública no Brasil comparável aos países desenvolvidos (MENDES et al., 2010).

Reduzir o impacto do diabetes significa, antes de tudo, reduzir a incidência da doença, antecipando-se ao seu aparecimento com medidas preventivas, sobretudo em indivíduos de alto risco, tais como os portadores de tolerância diminuída à glicose e de glicemia de jejum alterada. Intervenções comportamentais e farmacológicas têm sido estudadas e implementadas com esse objetivo. Modificações no estilo de vida, tais como controle dietoterápico e prática sistemática de exercícios físicos, bem como o uso de alguns fármacos orais têm se mostrado eficazes.

2.2. Vias de sinalização da insulina

A insulina é o hormônio anabólico mais conhecido e é essencial para a manutenção da homeostase da glicose, para o crescimento e diferenciação celular. Esse hormônio é secretado pelas células β das ilhotas pancreáticas em resposta ao aumento dos níveis circulantes de glicose e aminoácidos após as refeições. A insulina regula a homeostase de glicose em vários níveis, reduzindo a produção hepática de glicose (via diminuição da gliconeogênese e glicogenólise) e aumentando a captação periférica de glicose, principalmente nos tecidos muscular e adiposo. A insulina também estimula a lipogênese no fígado e nos adipócitos e reduz a lipólise, bem como aumenta a síntese e inibe a degradação protéica (CARVALHEIRA; ZECCHIN; SAAD, 2002).

A sinalização intracelular da insulina começa com a sua ligação a um receptor específico de membrana, uma proteína heterotetramérica com atividade quinase, composta por duas subunidades α e duas subunidades β, que atuam como uma enzima alostérica na qual a subunidade α inibe a atividade tirosina quinase da subunidade β. A ligação da insulina à subunidade α permite que a subunidade β adquira atividade quinase levando à alteração conformacional e autofosforilação, que aumenta ainda mais a atividade quinase do receptor (PATTI; KAHN, 1998).

Uma vez ativado, o receptor de insulina fosforila vários substratos protéicos em tirosina. Atualmente, dez substratos do receptor de insulina já foram identificados. Quatro desses pertencem à família dos substratos do receptor de insulina, as proteínas IRS (WHITE, 1998). As funções fisiológicas do IRS-1/2 foram estabelecidas através da produção de camundongos sem os genes que codificam o IRS-1 e IRS-2 (camundongos *knockout* para IRS-1 e IRS-2) (ARAKI et al., 1994).

O camundongo que não expressa IRS-1 apresenta resistência à insulina e retardo de crescimento, mas não é hiperglicêmico (ARAKI et al., 1994). Foi demonstrado que o IRS-2 poderia compensar parcialmente a ausência de IRS-1, o que explicaria o fenótipo de resistência à insulina sem hiperglicemia do camundongo *knockout* de IRS-1. O camundongo que não expressa o IRS-2 apresenta um fenótipo diferente do camundongo sem IRS-1: hiperglicemia acentuada devido a diversas anormalidades na ação da insulina nos tecidos periféricos e a falência da atividade secretória das células β acompanhada de redução significativa da massa de células

β pancreáticas. Em contraste, camundongos *knockout* para o IRS-3 e IRS-4 têm crescimento e metabolismo de glicose quase normal (FANTIN et al., 2000).

A fosforilação em tirosina das proteínas IRS cria sítios de reconhecimento para moléculas contendo domínios com homologia a Src 2 (SH2). Dentre estas, destaca-se a fosfatidilinositol 3–quinase (PI3K). A PI3K é importante na regulação da mitogênese, diferenciação celular e transporte de glicose estimulado pela insulina (SHEPHERD; NAVE;SIDDLE, 1995).

A ligação da insulina ao seu receptor, fosfofrila o IRS-1 resultando na ativação da PI3K e forma-se, então, o fosfatidilinositol-3,4,5-trifosfato (PI-3,4,5-P3), que liga-se tanto à proteína quinase B (Akt) quanto à proteína quinase 1 dependente de fosfoinositídeo (PDK-1) justapondo-as na membrana e habilitando a PDK-1 a fosforilar e ativar a Akt. Por sua vez, a Akt fosforila proteínas envolvidas na translocação do transportador de glicose-4 (GLUT4) dos compartimentos intracelulares para a membrana plasmática (LICZANO; ALESSI, 2002; HE et al., 2007) (Figura 1).

Além da ativação do IRS, outros substratos da insulina incluem Shc, Gab-1, p60 dok, JAK2, APS e Cbl (PESSIN; SALTIEL, 2000). Outra via para a captação de glicose envolve a ativação da Cbl (RIBON; SALTIEL, 1997). Na maioria dos tecidos sensíveis à insulina, Cbl está associada à proteína adaptadora CAP (RIBON et al., 1998). Após a fosforilação, o complexo Cbl-CAP migra para a membrana celular e interage com a proteína CrkII, que também está constitutivamente associada com a proteína C3G (BAUMANN et al., 2000; CHIANG et al., 2001). A C3G é uma proteína trocadora de nucleotídeos que catalisa a troca de GDP por GTP da proteína TC10 ativando-a. Uma vez ativada, TC10 causa um segundo sinal para a translocação da proteína GLUT4, em paralelo à ativação da via da PI3K (CHIANG et al., 2001). (Figura 1).

2.2.1. Atuação da proteína quinase B (PKB/Akt) na regulação da síntese de glicogênio

A insulina estimula o acúmulo de glicogênio através do aumento do transporte de glicose no músculo, síntese de glicogênio no fígado e músculo e diminuição da glicogenólise. A ativação da Akt, via PI3K, fosforila e inativa a glicogênio-sintase-

quinase 3 (GSK-3). Visto que a GSK-3 é ativa por constituição e inibe a glicogênio-sintetase, sua inativação permite a síntese de glicogênio. A insulina também ativa a proteína fosfatase 1, por um processo dependente da PI3K quinase, que desfosforila a glicogênio-sintetase diretamente (BRADY; NAIRN, SALTIEL, 1997) (Figura 2).

A insulina também inibe a produção e liberação de glicose no fígado através do bloqueio da gliconeogênese. Na inibição da gliconeogênese, a Akt uma vez ativada inibe fatores de transcrição (família *forkhead* - FOXO1) de genes que codificam a fosfoenolpiruvato carboxiquinase (PEPCK) e a glicose 6-fosfatase, enzimas- chave no controle desse processo (ARMONI; HAREL; KARNIELI, 2007) (Figura 2).

A insulina diminui a taxa de transcrição do gene que codifica a frutose-1,6-bifosfatase e aumenta a transcrição de genes de enzimas glicolíticas como a glicoquinase e a piruvato quinase (SUTHERLAND, O'BRIEN, GRANER, 1996). As vias de sinalização que regulam a transcrição desses genes envolvem ainda o co-ativador do PPAR γ (*peroxisome proliferator-activated receptor gamma)* e PGC-1α (PPAR γ *coactivator 1-alpha*).

A insulina também altera a quantidade de ácidos graxos livres liberados da gordura visceral (BERGMAN, 1997). O fluxo direto de ácidos graxos na veia porta para o fígado modula a sensibilidade à insulina nesse órgão regulando a produção de glicose (CARVALHEIRA; ZECCHIN; SAAD, 2002).

2.2.2. Efeitos da insulina sobre o crescimento e diferenciação celular

Semelhante a outros fatores de crescimento, a insulina estimula a *mitogen-activated protein* (MAP) quinase. Essa via inicia-se com a fosforilação das proteínas IRS e/ou Shc, que interagem com a proteína Grb2 (PAEZ-ESPINOSA et al., 1999). A Grb2 está constitutivamente associada à SOS, proteína que troca GDP por GTP da Ras ativando-a. A ativação da Ras requer a participação da SHP2. Uma vez ativada, a Ras estimula a fosforilação em serina da cascata da MAPK que leva à proliferação e diferenciação celulares. O bloqueio farmacológico dessa via previne a ação da insulina no crescimento celular, mas não tem efeito nas ações metabólicas do hormônio (LAZAR et al., 1995) (Figura 1).

A insulina aumenta a síntese e bloqueia a degradação de proteínas através da ativação da mTOR. mTOR controla a translação de proteínas diretamente através da fosforilação da p70- ribossomal S6 quinase ($p70^{rsk}$), que ativa a síntese ribossomal de proteínas através da fosforilação da proteína S6. A mTOR também fosforila a PHAS1, que aumenta a síntese protéica via aumento da translação de proteínas (MIRON et al., 2001) (Figura 1).

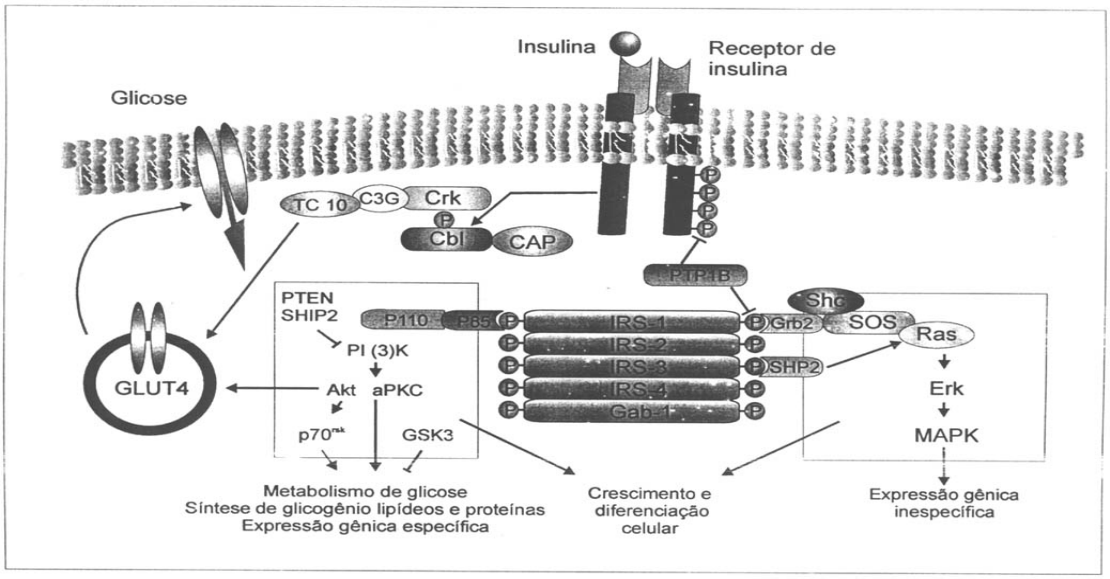

Figura 1: Vias de sinalização da insulina (CARVALHEIRA; ZECCHIN; SAAD, 2002).

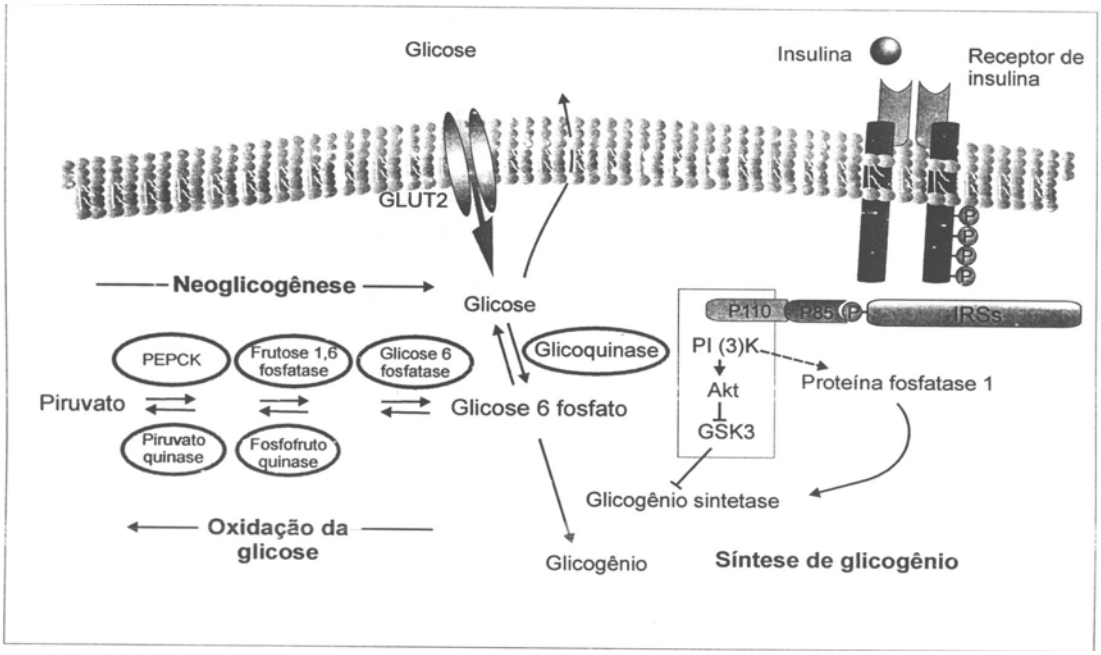

Figura 2: Regulação do metabolismo da glicose no fígado (CARVALHEIRA; ZECCHIN; SAAD, 2002).

2.2.3. Efeitos da insulina sobre o metabolismo lipídico

A homeostase de lípides em células de vertebrados é regulada por uma família de fatores de transcrição designada SREBP (*sterol regulatory element-binding proteins*). SREBPs ativam diretamente a expressão de aproximadamente 30 genes que se dedicam à síntese e captação de colesterol, ácido graxo, triglicérides e fosfolípides, assim como a de NADPH, um cofator requerido para a síntese dessas moléculas (SAKAKURA et al., 2001). No fígado, três SREBPs regulam a produção de lipídeos. SREBP-1c aumenta preferencialmente a transcrição de genes envolvidos na síntese de ácido graxo, entre eles a acetil-CoA carboxilase (ACC), que converte a acetil-CoA em malonil-CoA e a ácido graxo sintetase, que converte a malonil-CoA em palmitato (FORETZ et al., 1999) (Figura 3).

Uma ação clássica da insulina é estimular a síntese de ácido graxo no fígado em períodos de excesso de carboidratos. Várias evidências sugerem que esses efeitos da insulina são mediados pelo aumento do SREBP-1c (FORETZ et al., 1999). *In vivo*, a quantidade total de SREBP-1c no fígado é reduzida pelo jejum, que suprime a secreção de insulina e aumenta com a realimentação. De forma semelhante, os níveis de RNAm do SREBP-1c diminuem em animais com diabetes induzido por estreptozotocina e aumentam após tratamento com insulina. A hiperexpressão do SREBP-1c, no fígado de animais transgênicos, previne a redução do RNAm das enzimas lipogênicas (CARVALHEIRA; ZECCHIN; SAAD, 2002) (Figura 3).

Muitos indivíduos com obesidade e resistência à insulina apresentam esteatose hepática. As evidências indicam que a esteatose hepática causa resistência à insulina devido ao acúmulo de SREBP-1c, que está elevada em resposta aos altos níveis circulantes de insulina. De maneira semelhante, os níveis de SREBP-1c estão elevados no fígado de camundongos *ob/ob* (SHIMOMURA et al., 1999). Apesar da presença de resistência à insulina nos tecidos periféricos, a insulina continua a ativar a transcrição do SREBP-1c no fígado desses camundongos. O nível elevado de SREBP-1c nuclear aumenta a expressão de genes lipogênicos, a síntese de ácido graxo e o acumulo de triglicérides. Nos adipócitos, a insulina também reduz a lipólise através da inibição da lipase hormônio sensível (SHIMOMURA et al., 2000). Esta enzima é ativada pela PKA (proteína quinase A). A insulina inibe a atividade da PKA, ativando a fosfodiesterase AMP-

cíclico específica (PDE3B), que reduz os níveis de AMP-cíclico nos adipócitos (KITAMURA et al., 1999). A ativação da PDE3B é dependente e distal à ativação da PI 3-quinase e Akt pela insulina (CARVALHEIRA; ZECCHIN; SAAD, 2002) (Figura 3).

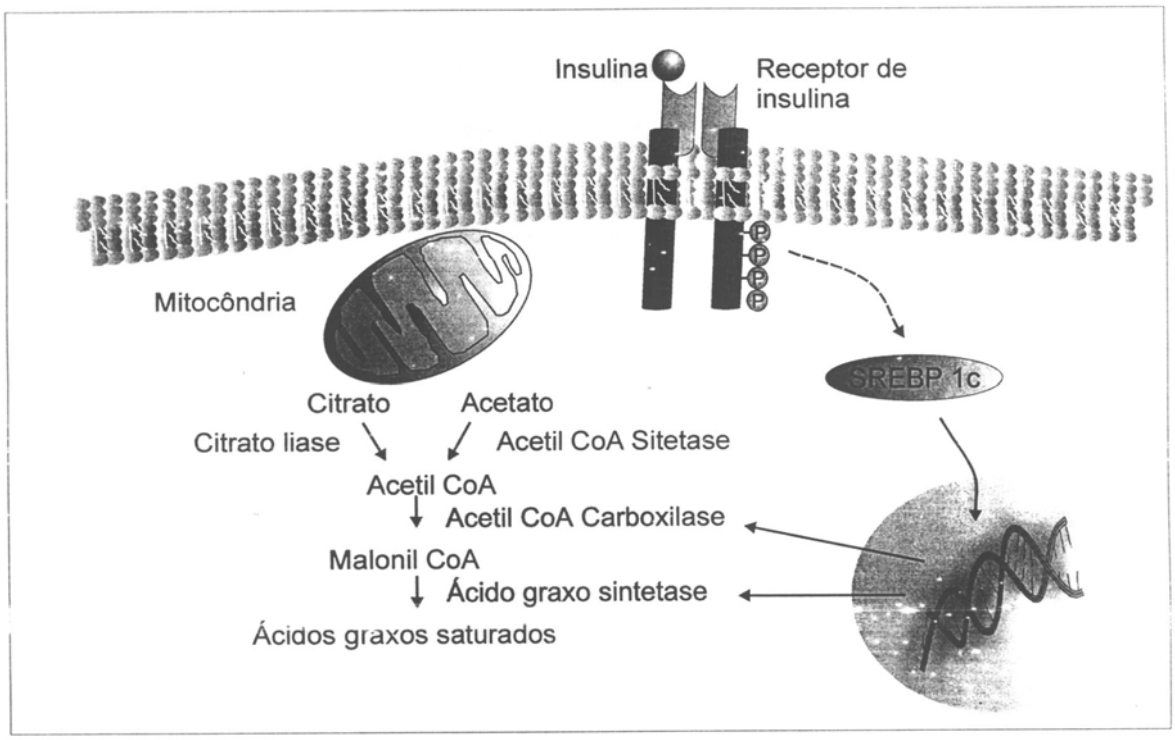

Figura 3: Regulação do metabolismo de lipídeos no fígado (CARVALHEIRA; ZECCHIN; SAAD, 2002).

2.3. Proteína quinase ativada por adenosina monofosfato (AMPK)

A proteína quinase ativada por adenosina monofosfato (AMPK) é uma via para o transporte de glicose e a translocação do GLUT4, independente da via clássica que envolve PI3K e Akt (BANDYOPADHYAY et al., 2001). A AMPK é uma enzima que induz uma cascata de eventos intracelulares em resposta a mudança da carga energética celular (HARDIE, 2003; CARLING, 2004). A função da AMPK no metabolismo celular é a manutenção da homeostasia energética (CARLING, 2004). Todas as células vivas devem continuadamente manter alta relação entre ATP e ADP para sobreviver. Isso é obtido por intermédio do catabolismo que aumenta a energia celular convertendo ADP e fosfato em ATP, enquanto o anabolismo diminui o componente energético celular, por converter ATP em ADP e fosfato. Convém

ressaltar o fato de que a relação ATP–ADP nas células geralmente permanece quase constante, indicando que o mecanismo que regula esse processo é muito eficiente. A AMPK é um componente-chave desse equilíbrio fisiológico (SCHIMMACK; DEFRONZO; MUSI, 2006).

AMPK é ativada pela fosforilação do resíduo de treonina 172, localizado no sítio de ativação do domínio catalítico da subunidade β (HARDIE, 2003; CARLING, 2004). Esse mecanismo de ativação depende da ação de uma ou mais quinases regulatórias, denominadas AMPK quinase (AMPKK) (WINDER; HARDIE, 1999; CARLING, 2004). Diversas tentativas para determinar a identidade dessa enzima já foram feitas, e a principal candidata é a LKB1 (*liver kinase 1*). Essa proteína fosforila e ativa a AMPK *in vitro* e na sua ausência ou inibição a ativação da AMPK é bloqueada (CARLING, 2004). Além disso, como já está implicado no próprio nome, AMPK também é ativada por AMP. A ligação desse nucleotídeo à AMPK tem duas conseqüências: aumenta a velocidade catalítica da enzima e torna a AMPK um substrato inadequado para desfosforilação. Isso torna a cascata de ativação da AMPK ultra-sensível, ou seja, pequenas mudanças nos níveis de AMP induzem um grande aumento na atividade da AMPK (WINDER; HARDIE, 1999).

O sistema da AMPK é, portanto, ativado por qualquer estresse que cause aumento na relação intracelular AMP–ATP, tanto aqueles que interferem com a produção de ATP quanto também àqueles que aumentam o consumo de ATP. Esses estímulos ativadores são os mais diversos e podem ser fisiológicos como exercício físico e contração muscular ou patológicos como deprivação de glicose, hipóxia, estresse oxidativo, choque osmótico, choque térmico, envenenamento metabólico, isquemia, diminuição do pH, inibição da glicólise e desacopladores da fosforilação oxidativa (HARDIE, 2003).

A AMPK é regulada, da mesma forma que visto anteriormente, por alterações que modificam a relação creatina–fosfocreatina (HARDIE, 2003). Também é ativada por hormônios (via receptores acoplados a proteína G), por leptina e adiponectina (via mecanismos ainda desconhecidos) e por drogas antidiabéticas orais, como as tiazolidinedionas (glitazonas) e a metformina (CARLING, 2004). Por outro lado, AMPK é inibida alostericamente por concentrações fisiológicas de fosfocreatina, da mesma forma que seus efeitos são também antagonizados por altas concentrações de ATP. Isso está de acordo com seu papel fisiológico de medidor do combustível

celular (HARDIE, 2003). Além disso, as subunidades β da AMPK possuem domínio de ligação com o glicogênio (GBD); no músculo, altas concentrações de glicogênio reprimem a atividade da AMPK, provavelmente por interagir com esse GBD, entretanto não existem evidências diretas para confirmar essa hipótese (WINDER; HARDIE, 1999).

Uma vez ativada, AMPK exerce efeitos sobre o metabolismo da glicose e dos lipídeos, sobre expressão gênica e sobre síntese protéica. Essa enzima atua em diversos órgãos, incluindo fígado, músculo esquelético, coração, tecido adiposo e pâncreas (ZHOU et al., 2001). Sabendo que ela é ativada principalmente pela redução no conteúdo energético celular (ou seja, aumento na relação AMP–ATP), seu maior efeito é desligar vias metabólicas que consumam ATP (por exemplo, as vias anabólicas de síntese de ácidos graxos e de colesterol), ao mesmo tempo que estimula vias metabólicas que produzam ATP (por exemplo, as vias catabólicas de oxidação de glicose e de ácidos graxos) (CARLING, 2004). Esses efeitos são de curto prazo e têm como objetivo manter a homeostasia energética dentro da célula. Para realizar esses efeitos, a AMPK fosforila diretamente enzimas regulatórias envolvidas nessas vias e também atua indiretamente sobre a expressão gênica (ZHOU et al., 2001; HARDIE, 2003).

No fígado, a AMPK tem um papel essencial sobre diversas cascatas celulares. Observa-se forte associação entre a ativação da enzima por fosforilação e a inativação da acetil-CoA carboxilase (ACC) também por fosforilação. Com a inativação da ACC não há concentrações de malonil-CoA suficientes para inibir a carnitina acil-transferase e, portanto, há predominância da β-oxidação sobre a síntese de ácidos graxos, permitindo que a produção de energia prevaleça sobre o gasto (ZHOU et al., 2001).

Mas, a AMPK não atua apenas inibindo a síntese de ácidos graxos, ela também inativa a glicerol-fosfato-acil transferase (GPAT) e a HMG-CoA redutase (3-hidroxi-3-metil-glutaril-Coa redutase), enzimas-chave na síntese de triglicérides e de colesterol, respectivamente. A AMPK também atua, em longo prazo, diminuindo a expressão de genes lipogênicos (por exemplo, FAS, S14, L-PK e SREBP-1) e neoglicogênicos (por exemplo, PEPCK e glicose-6-fosfatase). Em suma, a AMPK atua no fígado diminuindo a lipogênese e estimulando a lipólise, além de bloquear a produção hepática de glicose (ZANG et al., 2004).

Na musculatura esquelética, a AMPK atua principalmente estimulando a captação de glicose, tanto pelo aumento da translocação do transportador de glicose GLUT4 quanto pelo aumento da sensibilidade à insulina (SCHIMMACK et al., 2006). Outras importantes atividades atribuídas à AMPK são a regulação da síntese de insulina e sua conseqüente secreção pelas células β das ilhotas pancreáticas, além de aprimorar a sensibilidade à insulina nos tecidos hepático e muscular. Finalmente, a AMPK também atua nas funções hipotalâmicas, modulando os eventos relacionados com a fome e a saciedade (RUDERMAN; SAHA; KRAEGEN, 2003).

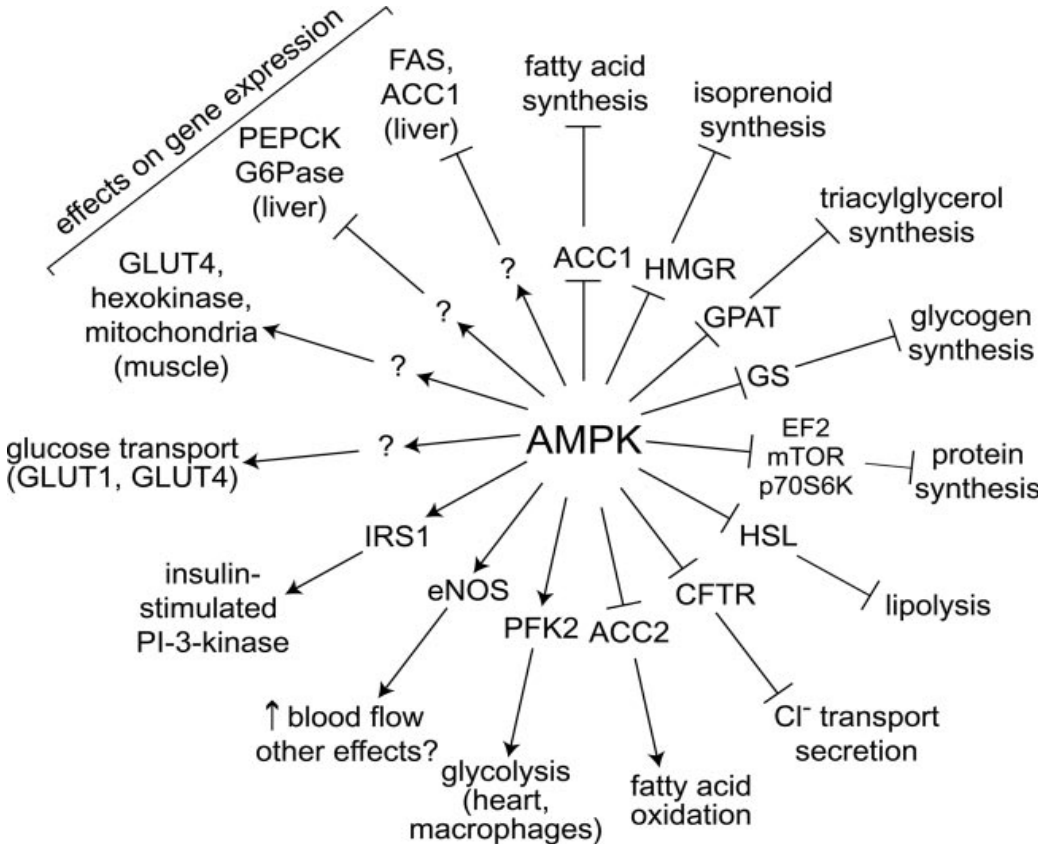

Figura 4: Proteínas-alvo e vias fisiológicas reguladas pela AMPK (HARDIE, 2003).

2.4. Tratamento farmacológico do diabetes *mellitus*

Diretrizes para o tratamento do diabetes têm sido desenvolvidas por várias sociedades profissionais, incluindo a Associação Americana de Endocrinologistas Clínicos e a Associação Americana de Diabetes (RODBARD; BLONDE; BRAITHWAITE, 2007; ADA, 2009). O tratamento é focado no controle e manutenção da glicemia em níveis fisiológicos. Os principais mecanismos utilizados para estes fins são a estimulação das células β-pancreáticas para liberação da insulina, inibição dos hormônios que aumentam a glicemia, aumento do número e sensibilidade dos receptores de insulina, diminuição da degradação do glicogênio, eliminação dos radicais livres e inibição da peroxidação dos lipídeos, melhora da microcirculação e correção do metabolismo das proteínas e lipídeos (ZHAO et al., 1999). A farmacoterapia antidiabética preconizada atualmente está resumida no quadro 1 de acordo com Skyler et al. (2009).

As Diretrizes da Federação Internacional de Diabetes (2009) recomenda para os pacientes diabéticos tipo 2 mudanças no estilo de vida aliados a um início de tratamento com metformina e, avançar para um agonista do receptor GLP-1 se os objetivos propostos pela terapia-alvo não forem alcançados em três meses. Outra diretriz baseada nas conseqüências fisiopatológicas do diabetes tipo 2 – impedimento da secreção de insulina, lipólise aumentada, decréscimo da captação de glicose e aumento da gliconeogênese – foi recentemente publicada (DEFRONZO, 2009).

Mudanças no estilo de vida associadas a um esquema triplo de tratamento constituído por tiazolidinedionas, metformina e exenatida (agonista do receptor GLP-1) diminuem de forma duradoura os níveis hiperglicêmicos sem induzir ganho de peso. O início precoce dessa terapia antidiabética deve ajudar a retardar ou evitar a disfunção das células β-pancreáticas observada nos pacientes diabéticos tipo 2 (DEFRONZO, 2009).

Quadro 1: Farmacoterapia antidiabética oral e parenteal.

Medicamento	Mecanismo de ação	Vantagens	Desvantagens

Insulina	Fisiológico. Podem ser de curta, média e longa duração	Rapidamente eficaz, não há limite de dose e melhora o perfil lipídico	Monitoramento, ganho de peso, múltiplas injeções diárias e hipoglicemia
Sulfoniluréias	Aumentam a secreção de insulina	Rapidamente eficaz	Ganho de peso e hipoglicemia (para dibenclamida e clorpropamida)
Meglitinidas	Aumentam a secreção de insulina	Rapidamente eficaz	Ganho de peso e hipoglicemia
Biguanidas (Metformina)	Aumenta a sensibilidade à insulina e inibe a gliconeogênese	Não promove ganho de peso	Distúrbio GI e é contra-indicada para pacientes com insuficiência renal
TZDs	Aumentam a sensibilidade à insulina	Melhora o perfil lipídico e diminui o risco de IM (pioglitazona)	Alto custo, ganho de peso, retenção de fluidos, ICC, fraturas ósseas e aumento do risco de IM (rosiglitazona)
Inibidores da α-glicosidase	Retarda a absorção intestinal de carboidratos	Não promove ganho de peso	Distúrbio GI
Inibidores da DPP-IV	Inibem a degradação da GLP-1 ao metabólito inativo	Não promove ganho de peso	Risco de pancreatite e falência renal
Agonistas do receptor GLP-1 (Exenatide)	Ligam-se ao receptor de GLP-1 (incretina) para potencializar a secreção de insulina	Perda de peso	Distúrbio GI, risco de pancreatite e falência renal
Análogo da amilina (pramlintide)	Suprime a secreção pós-prandial de glucagon, regula o esvaziamento gástrico e o apetite	Perda de peso	Distúrbio GI e a segurança de uso por longos períodos ainda não foi estabelecida

GI: gastrointestinal; TZDs: Tiazolidinedionas; IM: infarto do miocárdio; ICC: insuficiência cardíaca congestiva; DPP-IV: dipeptidil-peptidase IV; GLP-1: peptídeo semelhante ao glucagon 1

2.5. Modelos experimentais do diabetes *mellitus* utilizando estreptozotocina e aloxano

Vários modelos experimentais podem ser utilizados para o estudo do diabetes. Os modelos mais utilizados são aqueleas em que os roedores são tratados com estreptozotocina (STZ) ou aloxano (MARLES; FARNSWORTH, 1995). A estreptozotocina (STZ) é uma alquil nitrosuréia isolada originalmente a partir da bactéria *Streptomyces achromogenes*. Schnedl et al. (1994) demonstraram que a toxicidade da STZ dirigida às células β pancreáticas é devido a sua similaridade à molécula de glicose, o que permite que a mesma seja internalizada pelo transportador de glicose tipo 2 (GLUT 2). Evidências histológicas da lesão pancreática são observadas entre uma e sete horas da sua administração e após 24 horas o conteúdo de insulina presente nessas células está reduzido em 95%. (JUNOD et al., 1969).

O dano às células β-pancreáticas ocasionadas pela STZ pode ocorrer devido à formação de óxido nítrico decorrente da sua metabolização intracelular. O óxido nítrico causa alquilação e fragmentação do DNA, levando à apoptose (KRÖNCKE et al., 1995). Como conseqüência, há aumento da poli-polimerase com depleção intracelular do NAD^+ e dos estoques de ATP, o que pode colaborar para a diminuição da síntese e secreção de insulina (LENZEN, 2008). Confirmando a função do óxido nítrico, o bloqueio da sua produção nas ilhotas foi capaz de diminuir a fragmentação do DNA produzida pela STZ (BEDOYA et al., 1996).

Gille et al. (2002) reportaram que a geração de radicais hidroxila e de espécies reativas de oxigênio pela STZ também podem ser fatores importantes para o dano nas células β-pancreáticas. Uma dose única de STZ em ratos pode produzir o modelo experimental de diabetes *mellitus* não-insulino dependente (MARLES, FARNSWORTH, 1995). Mas, a sua administração também pode induzir um diabetes severo que muitas vezes requer insulina para que os animais sobrevivam por longos períodos (RERUP, 1970).

O aloxano, um derivado da pirimidina, é uma toxina muito seletiva para as células β-pancreáticas por causa também da formação de radicais livres. No entanto, apesar de ser um bom modelo para o diabetes *mellitus*, há muitos problemas devido à sua instabilidade química, metabolismo rápido e alguns fatores, tais como dieta e idade, que tornam quase impossível estabelecer uma relação clara entre as doses de aloxano e sua concentração efetiva no pâncreas (MARLES,

FARNSWORTH, 1995), além de apresentar pequena ação oncogênica (YAMAGAMI et al., 1985).

2.6. Plantas medicinais utilizadas no tratamento do Diabetes *mellitus*

Muitas espécies de plantas têm sido usadas pela medicina popular para tratar os sintomas do diabetes *mellitus* e algumas delas são estudadas do ponto de vista experimental (GROVER et al., 2002; SAXENA, VIKRAM, 2002; SYIEM et al., 2002; VOLPATO et al., 2002; HUO et al., 2003; ELDER, 2004). Essas plantas representam mais de 725 gêneros divididos em 183 famílias. A distância filogenética entre esses grupos de famílias sugere uma natureza variada de seus constituintes ativos (IVORRA et al., 1989; MARLES, FARNSWORTH, 1995; JOHNS, CHAPMAN, 1995; PEREIRA, 1997; PÉREZ GUTIÉRREZ et al., 1998, PÉREZ GUTIÉRREZ, 2002; LAMBA et al., 2000). Na medicina chinesa tradicional, 82 plantas medicinais têm sido usadas como medicamentos naturais para o tratamento do diabetes *mellitus* e suas complicações (LI et al., 2004). A maioria das plantas que são utilizadas como antidiabéticas ao serem avaliadas farmacologicamente demonstraram ter atividade hipoglicemiante e possuir constituintes químicos que podem ser utilizados como protótipos para novos agentes hipoglicemiantes. Entretanto, as análises posteriores revelaram que uma grande variedade de mecanismos de ação pode levar a esse efeito, porém nem todos são terapeuticamente úteis (MARLES, FARNSWORTH, 1995; SAID et al., 2002; HUO et al., 2003).

Salvia officinalis L. mostrou atividade hipoglicemiante sobre ratos diabéticos induzidos por STZ (EIDI et al., 2005). *Sclerocarya birrea* melhorou a homeostase da glicose por estimular a secreção de insulina (DIMO et al., 2006). Vijayakumar et al., (2005) demonstraram a atividade antidiabética de *Hygrophila auriculata* (K. Schum.) Heine em ratos diabéticos induzidos por STZ por inibição do dano oxidativo. O extrato etanólico das sementes de *Trigonella foenum-graecum* apresentaram efeito anti-hiperglicêmico em ratos diabéticos induzidos por aloxano (MOWLA et al., 2009) e o extrato aquoso das folhas de *Parquetina nigrescens* diminuíram o dano oxidativo à membrana do eritrócito em ratos diabéticos induzidos por aloxano (SABA; OYAGBEMI; AZEEZ, 2010).

Os efeitos dos extratos hidroalcoólicos de *Artemisia herba-alba Asso* e *Centaurium erythraea* foram avaliados em modelos de diabetes tipo 2 induzido por dieta padronizada enriquecida em gordura. O tratamento reduziu a glicemia de jejum, a concentração de triglicerídeos e os níveis de insulina sérica, diminuindo a resistência à insulina (HANZA et al., 2011). *Cecropia obtusifolia* Bertol., *Equisetum myriochaetum* Schlecht & Cham e *Acosmium panamense*, plantas tradicionalmente usadas na medicina popular mexicana, apresentaram atividade hipoglicemiante ao inibir a α-glicosidase (ANDRADE-CETTO; BECERRA-JIMÉNEZ; CÁRDENAS-VÁSQUEZ, 2008).

Os mecanismos de ação pelos quais as plantas reduzem a glicemia podem ser atribuídos aos seguintes fatores: aumento da liberação de insulina através da estimulação das células β-pancreáticas; resistência aos hormônios que aumentam a glicemia; aumento do número e da sensibilidade do sítio receptor de insulina; diminuição da glicogenólise; aumento do aporte de glicose nos tecidos e órgãos; eliminação de radicais livres; resistência à peroxidação de lipídeos; correção da desordem metabólica causada em lipídeos e proteínas e melhora da microcirculação (MARLES, FARNSWORTH, 1995; SAID et al., 2002; VOLPATO et al., 2002; HUO et al., 2003; LI et al., 2004).

Algumas plantas associadas ao tratamento do diabetes são consideradas tóxicas. Há muitos efeitos tóxicos que podem resultar em hipoglicemia, tais como, hepatotoxicidade e bloqueio dos receptores β-adrenérgicos. Detalhes tais como, identificação da planta, parte a ser usada, preparação, padronização química e biológica do extrato, estabilidade do extrato, dosagens terapêuticas, efeitos colaterais, interações medicamentosas e alimentares e contra-indicações devem ser incorporados à farmacopéia nacional. A toxicidade é influenciada pela parte da planta usada na preparação do extrato, método de preparação e rota de administração. A alergenicidade e fotossensibilização são outros aspectos de toxicidade, os quais não são revelados nos testes de toxicidade aguda e que ainda representam riscos significativos (HUO et al., 2003; LI et al., 2004).

2.7. Plantas medicinais e toxicidade

As plantas que são comumente usadas na medicina popular são tidas como naturais e, portanto, inofensivas (BNOUHAM; MERHFOUR; ELACHOUI, 2006). No entanto, esse conceito deve ser considerado com cautela uma vez que elas podem causar reações adversas, especialmente se forem usadas em doses excessivas ou se interagirem com medicamentos tradicionais (SAAD et al., 2006). A crença na naturalidade inócua das plantas medicinais não é facilmente contradita, pois as evidências científicas de ocorrência de intoxicações e efeitos colaterais relacionados ao seu uso consistem em informações que dificilmente chegam ao alcance dos usuários atendidos nos serviços de saúde pública caracterizado como indivíduos de baixa escolaridade e acervo cultural (SILVA et al., 2006; ALEXANDRE et al., 2008).

No Brasil, apesar da inexistência de dados oficiais, estima-se que 66% da população brasileira não tenham acesso aos medicamentos comercializados, fazendo uso das plantas medicinais como a única alternativa para o tratamento das suas doenças. Ou seja, o consumo de plantas medicinais no Brasil não é apenas uma questão de opção terapêutica, mas de uma necessidade de atendimento primário à saúde (DI STASI, 2007). A Organização Mundial de Saúde (1998) define planta medicinal como sendo todo e qualquer vegetal que possui, em um ou mais órgãos, substâncias que possam ser utilizadas com fins terapêuticos ou que sejam precursoras de fármacos semi-sintéticos.

Considerando o Decreto Federal nº 5.813 de 22 de Junho de 2006, que aprova a Política Nacional de Plantas Medicinais e Fitoterápicos, associado à Portaria nº 971 de 03 de Maio de 2006 do Ministério da Saúde, que aprova a Política Nacional de Práticas Integrativas e Complementares do Sistema Único de Saúde, esse tema passa a ser uma realidade nos serviços públicos de saúde e também uma alternativa terapêutica completamente legalizada (DI STASI, 2007). A Resolução da Diretoria Colegiada (RDC) da Agência Nacional de Vigilância Sanitária (Anvisa) de nº 48 de 16 de Março de 2004 define fitoterápico como sendo o medicamento obtido empregando-se exclusivamente matérias-primas ativas vegetais, cujos princípios ativos são produtos de sua extração seja ela: extrato, tintura, óleo, cera, exsudato, suco entre outros. Não sendo objeto de registro ou cadastro a planta medicinal ou as suas partes após os processos de coleta, estabilização e secagem, podendo ser íntegra, rasurada, triturada ou pulverizada (BRASIL, 2004).

Em potencial, toda substância é capaz de produzir efeitos tóxicos, dependendo apenas de fatores como dose, tempo, modo e freqüência de administração (LOOMIS; HAYES, 1996). A obtenção de dados toxicológicos em humanos é bastante limitada devido às questões éticas, morais e legais. Portanto, a maioria desses dados é obtida por meio de testes pré-clínicos que utilizam animais de laboratório em condições padronizadas (BOELSTERLI, 2003). No Brasil, o estudo toxicológico pré-clínico de plantas medicinais é regulamentado pela RDC nº 90 de 16 de Março de 2004 da Anvisa que norteia alguns ensaios de toxicidade aguda, toxicidade de doses repetidas e de longa duração (trinta dias a um ano, a depender da freqüência de uso do produto) (BRASIL, 2004).

Esses ensaios têm por objetivo comprovar a eficácia e a toxicidade das plantas visando os seus benefícios para o ser humano. Além disso, traçam o perfil dos efeitos colaterais, relacionando-os às doses e a um possível mecanismo de ação em várias espécies de animais de experimentação (SIMÕES et al., 2004). Sendo assim, na toxicidade aguda avaliam-se os efeitos tóxicos decorrentes da administração da substância, em doses única ou múltiplas, por um período de 24 horas, e também seus efeitos durante 14 dias após a administração (LARINI; OLIVEIRA, 1993). Nesse estudo, as informações obtidas devem ser usadas em estudos subsequentes de toxicidade prolongada (LOOMIS; HAYES, 1996).

A toxicidade prolongada analisa e caracteriza todos os efeitos provocados por uma determinada substância quando administrada diariamente em animais experimentais, em doses previamente estabelecidas e por longos períodos (LOOMIS; HAYES, 1996). Os principais parâmetros a serem observados durante o tratamento são as modificações na atividade motora e comportamental, na cor e textura dos pêlos e na frequência cardiorespiratória. Ao final dos experimentos, os animais devem ser sacrificados para a coleta do sangue (análise dos parâmetros bioquímicos e hematológicos) e remoção de órgãos para as análises macro e microscópica (BARNES; DOURSON, 1988).

A toxicologia do desenvolvimento engloba a teratologia e a toxicologia da reprodução. A teratologia estuda as alterações induzidas durante o desenvolvimento, entre a concepção e o nascimento. Enquanto que a toxicologia da reprodução estuda os efeitos adversos que ocorrem nos sistemas reprodutores masculino e feminino resultantes da exposição aos agentes químicos (BARROS;

DAVINO, 1996). Os parâmetros toxicocinéticos são fundamentais para determinar o grau de embriofetotoxicidade de um xenobiótico. A placenta, por ser uma estrutura responsável pela troca de substâncias entre mãe e concepto, tem importância vital quando se quer avaliar os riscos de uma substância. A placenta atua como uma barreira seletiva capaz de sintetizar e metabolizar substâncias, mas pode funcionar como um reservatório de drogas. Alterações na estrutura e função placentárias promovidas por determinadas substâncias podem produzir necrose, reduzir o fluxo sanguíneo ou inibir a passagem de nutrientes pela placenta (SILVA et al., 2009).

2.8. Estudos farmacológicos com plantas do gênero Caesalpinia

Plantas pertencentes ao gênero Caesalpinia ocorrem principalmente nos trópicos e subtrópicos, com algumas espécies utilizadas na medicina tropical. *Caesalpinia bonducella* Flem, comum no sudeste da Índia e Sri Lanka, possui atividade antiinflamatória e antimalárica. São atribuídas a ela propriedades afrodisíacas e tônicas que ajudam no rejuvenescimento do corpo. Suas sementes são usadas para o tratamento da hanseníase, do diabetes e como antipirética. Suas sementes contêm vários constituintes químicos tais como: furanoditerpenos, β-sitosterol, flavonóides, ácido aspártico, arginina, citrulina e β-caroteno (ALI et al., 1960).

Caesalpinia bonducella Flem é uma planta extremamente usada no Caribe por suas propriedades medicinais. Investigações prévias da semente desta planta elucidaram substâncias como: α, β, γ, δ, e ε-caesalpina (ALI et al., 1960). A atividade hipoglicemiante dos extratos aquosos e etanólicos de *Caesalpinia bonducella* Flem nos modelos de diabetes tipo I e II é atribuída ao aumento da utilização de glicose para a formação do glicogênio, com o conseqüente aumento da glicogênese (CHAKRABARTI et al., 2003). Um composto isolado a partir da extração das folhas se *Caesalpinia bonducella* utilizando acetato de etila mostrou atividade contra as cepas clínicas de *Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella sp., Staphylococcus pyogenes citreus, Staphylococcus aureus, Escherichia coli, Candida albicans e Rhodotorulla sp.*(SAGAR; VIDYASAGAR, 2010).

Caesalpinia pulcherrima (L.) Sw é usada para o tratamento de reumatismo, infecções da pele e como antiulcerogênica (ASOLKAR et al., 1992). Decocção de

suas folhas, casca e raízes são usadas para infecções fúngicas, possui ações antipiréticas e abortivas. São utilizadas também para tratar afecções do fígado e úlcera da boca e garganta (QUISURNBING, 1978). Compostos derivados de quercetina e isolados de *Caesalpinia pulcherrima* apresentaram atividade antiviral (CHIANG et al., 2003).

Caesalpinia sappan L é encontrada na Índia, Peru e Malásia. É utilizada na medicina Ayurveda para o tratamento de feridas, úlceras, hanseníase, diarréia, epilepsia, menorragia e diabetes. Os extratos desta planta foram considerados potentes inibidores da biossíntese das prostaglandinas e da formação do óxido nítrico demonstrando atividade anti-inflamatória e quimioterápica (KIRTIKAR et al., 1987). Os extratos metanólicos dessa planta também mostram ação relaxante sobre a aorta torácica isolada de ratos (XIE et al., 2000). Protosappanina A, protosappanina B e brasileína isolados de *C. sappan* mostraram atividade antioxidante ao inibir a formação de malonildialdeído e atuar como captadores de radicais livres (HU et al., 2008). O extrato etanólico do cerne de *C. sappan* reduziu significativamente a artrite induzida por colágeno em modelos animais de artrite reumatóide pelo decréscimo dos níveis de IL-1β, IL-6, TNF-α e PGE-2 no soro e pela expressão reduzida da COX-2 e NF-κB na cartilagem das patas desses animais (SUN et al., 2011).

Os extratos de *Caesalpinia spinosa* (Mol.) Kuntze podem aumentar a suscetibilidade dos *Staphylococcus aureus* resistentes à meticilina, e às β-Lactamases (SHIBATA et al., 2003). Taninos presentes nessa espécie inibiram a produção de radicais livres produzidos por raio ultravioleta-B (UV-B). Esses resultados sugeriram que os taninos administrados topicamente ou intraperitonealmente reduziram os efeitos carcinogênicos dos raios (UV-B) e poderiam ser úteis como fotoprotetores (GALI-MUHTASIB; YAMOUT; SIDANI, 2000).

Caesalpinia crista Linn é uma planta medicinal largamente distribuída em regiões tropicais e subtropicais do sudeste da Ásia. Essa planta é localmente conhecida como ‘Ka-Lain” e suas sementes são usadas como anti-helmínticas, antipirética, anti-inflamatória e agente antimalárico. Na Indonésia, é conhecida como “Bagore’’ e a decocção das suas raízes tem sido usada como tônico e para o

tratamento do reumatismo e dores na coluna (YANGOON, 2001). *C. crista* também apresentou atividade anti-helmíntica *in vitro* e *in vivo* (JABBAR et al., 2007).

A infusão aquosa de *Caesalpinia pyramidalis* Tul apresenta ação antifúngica contra *Trichophyton rubrum*, *Candida guilliermondii*, *Candida albicans*, *Cryptococcus neoformans* e *Fonsecaea pedrosoi*, quando comparado ao agente antifúngico Anfotericina B (CRUZ et al., 2007). O extrato metanólico da madeira e fruto de *Caesalpinia decapetala* (Roth) indicaram significante atividade antioxidante *in vitro* (PAWAR; SURANA, 2010).

A fração aquosa do extrato hidroalcoólico das folhas de *Caesalpinia bonduc* L. (Roxb.), na dose de 250 mg/Kg de peso corporal, teve sua ação anti-psoríase avaliada na cauda de camundongos e apresentou atividade antiproliferativa *in vivo* como também inibição da lipoxigenase *in vitro* (MURUGANANTHAN et al., 2011). Quatro moléculas de diterpenos, um dímero e dois diabenzofuranos isolados de extratos da raiz de *Caesalpinia minosoides* Lamk. mostraram atividade anti-inflamatória *in vitro* sobre as linhagens de células RAW 264.7 (YODSAOUE et al., 2010).

2.9. *Caesalpinia ferrea* Martius Ex Tul.

A família Fabaceae, também conhecida como Leguminosae, possui 642 gêneros e cerca de 18.000 espécies cosmopolitas. Inclui desde árvores e arbustos até lianas e ervas (RIBEIRO et al., 1999). A espécie *Caesalpinia ferrea* Mart. pertence ao reino Plantae, filo Magnoliophyta, classe Magnoliopsida, ordem Fabales, família Fabaceae e gênero Caesalpinia (ILDIS, 2007). A espécie é nativa do Brasil, principalmente na região Amazônica e caatinga nordestina (SOUZA, 2007). *Caesalpinia ferrea* é uma espécie economicamente importante, por ter multiplicidade de usos: as suas folhas servem para forragem e a madeira, de cerne muito duro com fibras revessas, é empregada na construção civil como vigas, esteios, estacas (CORRÊA, 1984).

Caesalpinia ferrea, popularmente conhecida como “pau-ferro” ou “jucá”, é uma árvore de até 10 m de altura e 120 cm de diâmetro. Apresenta tronco liso com manchas esbranquiçadas devido a sua descamação. Suas folhas são compostas e pinadas, apresentando até cinco pares de folíolos de aproximadamente 20 cm. As

flores são de coloração amarelada e arranjada em cachos, sendo possível o florescimento da planta mesmo sem folhas. Os frutos são vagens de aproximadamente 9,6 cm de comprimento, 2,1 cm de largura, 0,9 cm de espessura e contém de três a nove sementes. As sementes são duras e lisas com cerca de 1 cm de comprimento. O florescimento ocorre entre os meses de Abril e Maio e a frutificação entre Maio e Agosto (SOUZA , 2007). Na medicina popular, a espécie também tem sua utilidade: a decocção da madeira é usada como expectorante e cicatrizante; a casca é desobstruente; as raízes são febrífugas e antidiarréicas; o fruto tem propriedades béquicas e antidiabéticas (CORRÊA, 1984). A figura 5 apresenta imagens de *Caesalpinia ferrea* utilizada neste estudo.

Giazzi et al. (1991) avaliaram os parâmetros farmacognósticos de *Caesalpinia ferrea*, com suas principais fases e partes: vegetativa (folha e casca), floração (folha, casca e flor) e frutificação (folha, casca e fruto). No estudo farmacobotânico foram realizadas as caracterizações macroscópicas e microscópicas. No farmacoquímico, a triagem mostrou a presença de polifenóis em todas as partes e fases analisadas. Na casca do caule houve predominância de taninos hidrolisáveis (ácidos gálico e elágico) e condensados (catequinas) (Figura 6). Apenas traços de saponinas, alcalóides e glicosídeos cardiotônicos foram detectados. A cromatografia em camada delgada confirmou a presença de polifenóis, sendo a maior concentração observada nas flores (2,3%).

González et al. (2004) também identificou no caule da planta a presença de flavonóides, taninos, cumarinas, esteróides e derivados antracênicos. O ácido gálico e metil-galato isolados dos frutos da *Caesalpinia ferrea*, reduziram significativamente o número de papilomas de pele induzidos por 12-O-tetra-decanoilforbol-13-acetato (TPA) em camundongos, demonstrando atividade anticancerígena (NAKAMURA et al., 2002). O pauferrol A ($C_{45}H_{34}O_{12}$.), um derivado de chalcona isolado da casca de *Caesalpinia ferrea*, apresentou importante inibição da DNA-topoisomerase II e indução da apoptose de células leucêmicas (NOZAKI et al., 2007).

Giazzi et al. (1991) mostraram que os extratos aquosos da casca de *Caesalpinia ferrea* promoveram a inibição parcial de larvas de ancilostomídeos, enquanto que os extratos hidroalcoólicos apresentaram inibição total. Menezes et al. (2007) também demonstraram que o extrato aquoso da casca de *Caesalpinia ferrea* promoveu vasodilatação da artéria mesentérica de ratos.

O extrato aquoso do fruto de *Caesalpinia ferrea* (300 mg/Kg, v.o.) apresentou atividade anti-inflamatória, anti-ulcerogênica e analgésica periférica (BACCHI, 1991). Compostos isolados do fruto demonstraram atividade antimicrobiana *in vitro* contra microorganismos patógenicos da cavidade oral (SAMPAIO et al., 2009) e também inibiram a aldose-redutase, sugerindo efeitos antidiabéticos (UEDA et al., 2004). Em um estudo de toxicidade realizado por Lucinda et al. (2010) foi demonstrado que o tratamento de ratos machos com extrato aquoso dos frutos de *Caesalpinia ferrea* na dose de 300 mg/Kg por via oral, durante um e dois ciclos espermáticos (52 e 104 dias respectivamente) não interferiu no sistema reprodutor e nem prejudicou a produção espermática desses animais.

Caesalpinia ferrea apresenta-se como uma promissora planta antidiabética (BALBACH, 1972). No Brasil, o chá da casca do caule de *Caesalpinia ferrea* tem sido comumente usado na medicina popular para o tratamento do diabetes *mellitus* (BRAGANÇA, 1996). Dados etnofarmacológicos relataram que indivíduos que fizeram uso desse chá tiveram sua glicemia reduzida. Dessa forma, a investigação farmacológica detalhada da atividade anti-hiperglicemiante associada à segurança de uso da planta torna-se necessária para a obtenção de um fitomedicamento a ser usado no tratamento do diabetes.

Figura 5: (A) – Árvore de *Caesalpinia ferrea*; (B) – Flores de *Caesalpinia ferrea*. Fonte: Carlos Fernando Brasileiro de Vasconcelos.

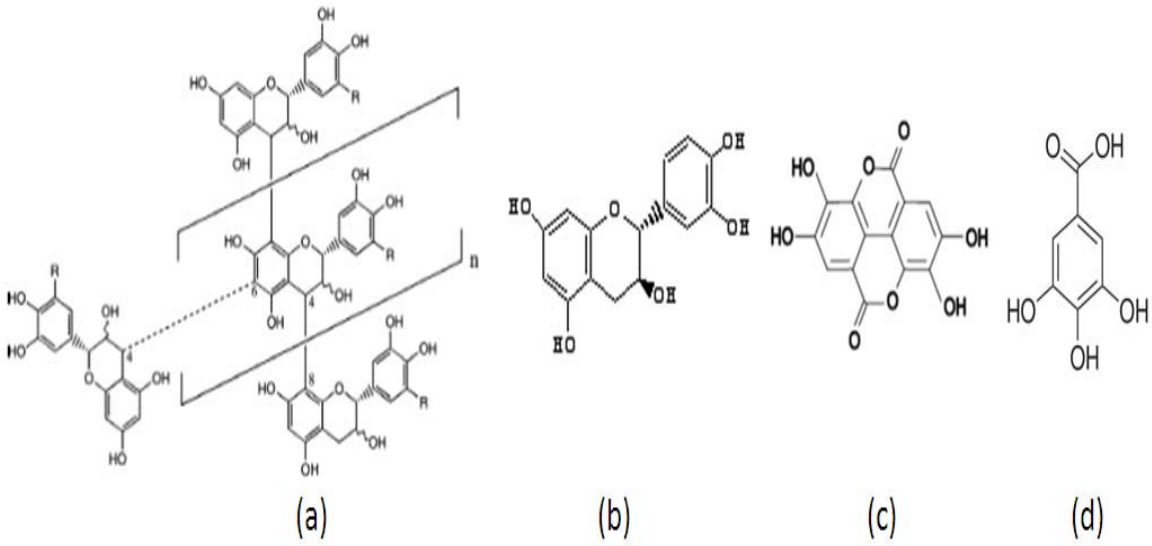

Figura 6: Estruturas químicas de taninos. (a) tanino condensado, (b) catequina, (c) ácido elágico e (d) ácido gálico.

3. Objetivos

3.0. Objetivos

3.1. Objetivo geral

Investigar os efeitos do extrato aquoso da casca do caule de *Caesalpinia ferrea* (EaCf) Martius Ex Tul., sobre o metabolismo de carboidratos, lipídeos e proteínas de ratos diabéticos induzidos por estreptozotocina (STZ) e em paralelo avaliar a segurança do seu uso.

3.2. Objetivos específicos

- Realizar a identificação dos metabólitos secundários presentes na casca de *Caesalpinia ferrea* por cromatografia em camada delgada e quantificar os principais constituintes por espectrofotometria no ultravioleta e cromatografia líquida de alta eficiência (CLAE);

- Analisar os efeitos do tratamento subcrônico (28 dias), por via oral (v.o.), do EaCf sobre a expressão da proteína quinase B (PKB/Akt) e da proteína quinase ativada por adenosina monofosfato – (AMPK), em ratos diabéticos induzidos por STZ;

- Investigar a ação antioxidante do tratamento subcrônico, v.o., do EaCf através da quantificação de substâncias reativas com o ácido tiobarbitúrico (TBARS) presentes no soro e tecido hepático de ratos Wistar diabéticos;

- Avaliar os efeitos do tratamento subcrônico, v.o., do EaCf sobre os parâmetros bioquímicos de ratos diabéticos induzidos por STZ;

- Verificar a influência do tratamento subcrônico, v.o., do EaCf sobre as massas de órgãos e tecidos de ratos diabéticos induzidos por STZ;

- Estudar o efeito do EaCf sobre a absorção intestinal de glicose em ratos Wistar normoglicêmicos;

- Avaliar a toxicidade aguda do EaCf, v.o., em ratos Wistar de ambos os sexos;

- Verificar a toxicidade subcrônica da administração oral do EaCf sobre os parâmetros hematológicos, bioquímicos e sobre a morfologia macro e microscópica dos órgãos de ratos Wistar;

- Verificar a toxicidade crônica da administração oral do EaCf sobre os parâmetros hematológicos, bioquímicos e sobre a morfologia macro e microscópica dos órgãos de camundongos Swiss machos;

- Investigar os efeitos da administração, v.o., do EaCf durante o período de gestação de ratas Wistar e sobre o desenvolvimento da sua prole.

4. Artigo I

Hypoglycaemic activity and molecular mechanisms of *Caesalpinia ferrea* Martius bark extract on streptozotocin-induced diabetes in Wistar rats

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Hypoglycaemic activity and molecular mechanisms of *Caesalpinia ferrea* Martius bark extract on streptozotocin-induced diabetes in Wistar rats

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Abstract

Ethnopharmacological relevance: The tea from the stem bark of *Caesalpinia ferrea* Martius (Leguminosae) has been popularly used in the treatment of diabetes in Brazil.

Aim of the study: To investigate the hypoglycaemic properties and to elucidate the mechanisms by which the aqueous extract of the stem bark of *Caesalpinia ferrea* reduces blood glucose levels in streptozotocin-induced diabetic rats via the enzymatic pathways of protein kinase B (PKB/Akt), AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC).

Methods: The aqueous extract of the stem bark of *Caesalpinia ferrea* (300 and 450mg/kg/day), vehicle and metformin (500mg/kg/day) were administered orally to STZ-diabetic rats (n=7/group) for 4 weeks. Changes in body weight, food and water intake, fasting glucose levels and oral glucose tolerance were evaluated. Phosphorylation (P) and the expression of Akt, AMPK and ACC in the liver and skeletal muscle were determined using Western blot.

Results: The aqueous extract of the stem bark of *Caesalpinia ferrea* reduced blood glucose levels and improved the metabolic state of the animals. P-Akt was increased in the liver and skeletal muscle of the treated animals, P-AMPK was reduced only in the skeletal muscle of these animals and P-ACC was reduced in both when compared with untreated rats.

Conclusion: The results indicate that the aqueous extract of the stem bark of *Caesalpinia ferrea* has hypoglycaemic properties and possibly acts to regulate glucose uptake in liver and muscles by way of Akt activation, restoring the intracellular energy balance confirmed by inhibition of AMPK activation.

Keywords: Caesalpinia ferrea, Leguminosae, Diabetes, Protein kinase B, AMP-actived protein kinase (AMPK), Acetyl-CoA carboxylase (ACC).

1.0. Introduction

Diabetes is a group of metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels (ADA, 2010).

The pharmacological treatment of diabetes includes oral hypoglycaemic and insulin. Although these drugs are effective in reducing glycaemia, they may cause undesirable side effects (such as weight gain, hypoglycemia, edema, gastrointestinal disturbances and insulin resistance) that can discourage patient compliance.

On the other hand, ethnopharmacological evidence has shown that the use of plants is a viable alternative for the treatment of diabetes. The advantages of herbal medicine include significant efficacy, low incidence of side effects, low cost and relative safety (Ali et al., 2006). In fact, the medicinal plants are considered an important source of molecules with potential hypoglycaemic effects. Grover et al. (2002) have reported about 800 plants with these molecules, which may act through different mechanisms, including the inhibition or stimulation of enzymatic activity and/or protein expression. The wide diversity of species has led scientists to make great efforts to bioprospect plants that may contribute to the management of diabetes.

Caesalpinia ferrea Martius (Leguminosae), popularly known as “pau-ferro” or “jucá”, is a large tree that is found mainly in the North and Northeast of Brazil. In folk medicine, the tea of the stem bark of *Caesalpinia ferrea* has been used for the treatment of diabetes (Araújo et al., 2008). Other therapeutic properties of this plant include anti-inflammatory, antiulcer (Bacchi and Sertié, 1994; Bacchi et al., 1995), analgesic (Carvalho et al., 1996), anticancer (Nozaki et al., 2007), antibacterial (Sampaio et al., 2009) and antihypertensive (Menezes et al., 2007). In view of its ethnomedicinal importance, the Brazilian Ministry of Health has included this species on the National List of Medicinal Plants important to the Health System.

Phytochemical investigation of the hydroalcoholic extract of the stem bark and leaves of *Caesalpinia ferrea* has revealed flavonoids, saponins, tannins, coumarins, steroids and other phenolic compounds (Gonzalez et al., 2004). Tannins were the

main compounds found (Souza et al., 2006). One component isolated from the fruit is ellagic acid, inhibitor of aldose reductase, which is an enzyme involved in the complications of diabetes (Ueda et al., 2001).

Although *Caesalpinia ferrea* is widely used in folk medicine, there is no experimental evidence proving its hypoglycaemic properties. The aim of this study was, therefore, to investigate the hypoglycaemic properties of the stem bark of *Caesalpinia ferrea* in streptozotocin-induced diabetic rats and to examine the effects of its aqueous extract on the phosphorylation and expression of protein kinase B (PKB/Akt), AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in skeletal muscle and the liver. The activation of these enzymes contributes to reduction of glycaemia by increasing glucose uptake in the tissues (Farese et al., 2005).

2.0. Material and Methods

2.1. Plant material and extraction

Bark from the stem of *Caesalpinia ferrea* Mart. Ex Tul. was collected from the Amazon Research National Institute (INPA) experimental culture, in the Brazilian State of Amazonas (03º 05’ 48.0”S and 59º 59’ 55.0’’W.Gr). The voucher specimen of the plant was deposited at the INPA herbarium under number 228022. The bark was collected in September 2009, March 2010 and September 2010, with approximately 3kg being harvested on each occasion. The material collected was dried, first at room temperature for 48h, and then taken to an oven with circulating air at a temperature of 45±2 °C until its weight stabilized. Subsequently, the material was ground in a 1mm mesh knife mill, thereby providing the raw material (MPV). The aqueous extract of *Caesalpinia ferrea* was prepared by raw material infusion (7.5:100, w/v) using boiling distilled water as the extractive solvent for a period of 15 minutes. The aqueous extract presented a solid soluble content of 0.6±0.02g%. The aqueous extract was dried using a MSD 1.0 Labmaq Mini Spray Dryer. The drying process was performed using the following parameters: inlet temperature of 120 °C, compressed air pressure of 2bar, diameter rotor of 0.7mm and power flow of

10mL/min. The yield of dry extract after drying of aqueous extract was 98%, representing 5.88g of dry extract per liter of aqueous extract of *Caesalpinia ferrea.*

2.2. Phytochemical analysis of Caesalpinia ferrea

2.2.1. Thin Layer Chromatographic (TLC) Analysis of Caesalpinia ferrea

The methods described by Wagner and Bladt (1996) were used to screen the dried bark extract for the hydrolysable tannins (gallic and ellagic acids), condensed tannins (catechins), flavonoids, saponins, coumarins, phenylpropanoids, cinnamic acid derivatives, alkaloids, triterpenoids/steroids, monoterpenes, sesquiterpenes, iridoids, sugars and luteolin. The phytochemical profile was drawn up using thin layer chromatography (TLC) on silica gel plates (Merck® art. 105553, UV 250–366nm) using the appropriate mobile phase, reagents and standards.

2.2.2. Estimation of total tannin content in Caesalpinia ferrea

The total tannin content was determined using the spray dried extract (SDE) of *Ceasalpinia ferrea* aqueous extract at 7.5% (w/v), by way of the difference between redissolved SDE before and after precipitation with 150mg of casein (Merck® Germany). The measurements were performed at 270nm and the total tannin content was calculated as gallic acid (mg/g of SDE). The results represent the mean of three measurements.

2.2.3. High Performance Liquid Chromatography (HPLC) analysis of Caesalpinia ferrea

The main phytochemical markers (gallic acid, ellagic acid, catechin and epicatechin) were quantified by way of LC-DAD analysis using a Shimadzu system (LC-20AT) equipped with a photo diode array detector (SPD-M20A). The chromatographic separation was performed using a Gemini RP-18 column 240 x

4mm i.d. (Phenomenex), protected by a pre-column packet of the same material. A gradient elution was performed by varying the proportion of solvent B (methanol) to solvent A (acetic acid 0.5%; w/w) at a flow rate of 0.8mL/min, according to the following gradient program: 20-40% B (10min), 40-60% B (10min), 60% B (10min), 60-40% B (10min), 40-20% B (10min). The SDE of *Caesalpinia ferrea* and standard were dissolved in methanol:water (20/80, v/v) and filtered through a 0.45µm membrane (Millipore®, USA) prior to injection of 20µL.

The peaks of each marker substance in the dried extract were initially identified by comparing the retention time and UV-spectrums. After that, the peaks were confirmed by spiking the sample with a small amount of the standards.

2.3. Animals

Male Wistar rats (*Rattus norvegicus* var. *albinus*) (aged 2 months, weighing 280–300g) were obtained from the Department of Physiology and Pharmacology at the Federal University of Pernambuco (UFPE), Pernambuco, Brazil. The animals were kept under standard environmental conditions (22 ± 2^{o} C); 12:12h dark/light cycle). Water and industrialized dry food (Labina®, Purina, Brazil) were made available *ad libitum*. The experimental protocol was approved by the Animal Experimentation Ethics Committee of UFPE (Process nº. 01411), in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

2.4. Induction of experimental diabetes

Diabetes was induced using streptozotocin (STZ) from Sigma-Aldrich®, St. Louis, MO, USA. The animals fasted overnight and diabetes was induced by way of a single intra peritoneal injection of a freshly prepared solution of STZ (50mg/kg b.w.) in a 0.1M citrate buffer (pH 4.5). On the third day of STZ-injection, the animals with fasting glycaemia higher than 200mg/dL and with signs of polyuria and polydipisia were considered to be diabetic and included in the study.

2.5. Diabetic animals

2.5.1. Treatment

In the experiment, the animals were randomly divided into five groups (n=7/group). Group 1 (NDC-non-diabetic control) and group 2 (DC-diabetic control) consisted of rats treated with vehicle (water); group 3 (MTD-diabetic rats treated with metformin 500mg/kg/day b.w.), groups 4 and 5 (diabetic rats treated with the aqueous extract of the stem bark of *Caesalpinia ferrea* 300 and 450mg/kg/day b.w).

The selected doses (300 and 450mg/kg/day of b.w.) of extract of *Caesalpinia ferrea* were based on prior study of their analgesic, anti-inflammatory and antiulcer activities (Bacchi and Sertié, 1994; Bacchi et al., 1995; Carvalho et al., 1996) and reports of the local community who use the tea 1-3 times a day before meals.

Treatment was administered orally on a daily basis in a single dose for 28 consecutive days. Fasting glucose and body weight were recorded weekly, while food and water intake were monitored daily.

2.5.2. Oral glucose tolerance test (OGTT) in STZ-diabetic rats

On the 25th day of treatment, the animals from groups 1-5 fasted for 12h. Fasting glycaemia was measured and defined as zero time. After this procedure, animals received their treatment orally and after 30 minutes, all groups received an oral load of d-glucose (2.0g/kg b.w). Blood glucose levels were measured 30, 60, 120 and 150min after glucose administration. Blood samples were obtained by retro-orbital puncture under anesthesia with Nembutal® (25mg/kg, i.p) using Architect (Abbott®) automation with Boehringer Ingelheim® biochemical kits.

2.5.3. Biochemical parameters

At the end of treatment, blood samples were centrifuged at 1500×g for 10min to obtain serum, which was stored at -20 ºC until the following parameters had been

determined: glucose; blood urea nitrogen (BUN); uric acid, aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (AlkP), total cholesterol (TC); triglycerides. Dosages were made using Architect (Abbott®) automation with Boehringer Ingelheim® biochemical kits. Liver glycogen was determined using the colorimetric method.

2.5.4. Liver and tissue mass analysis

Once blood had been collected, the animals were euthanized with an excess of Nembutal® (140mg/kg, i.p). Liver, epididymal adipose tissue, *soleus* and extensor *digitorium longus* muscles were carefully removed, individually weighed and expressed in absolute and relative terms (g and g/100g b.w., respectively).

2.5.5. Evaluation of serum and liver oxidative stress

Once blood had been collected and the liver resected, oxidative stress was evaluated using levels of thiobarbituric acid-reactive substances (TBARS). One gram of tissue was macerated in 5mL of 1.15% KCl/g in an ice bath. Subsequently, 1mL of 0.375% (w/v) thiobarbituric acid (Sigma-Aldrich®, St Louis, MO, USA) in 75% (w/v) trichloroacetic acid (TCA) (Vetec®, Rio de Janeiro, Brazil) was added for each milliliter of tissue homogenate. The same methodology was used for analysis of serum (1mL). The tubes were sealed and heated in a water bath at 100 ºC for 15min. After cooling, the protein precipitate was centrifuged at 3000×g for 10min at room temperature, the supernatant separated and the absorbance measured at 535nm.

2.5.6. Histopathological examination of the pancreas

Histopathological analyses were performed by way of optical microscopy on paraffin material. Pancreas tissue sections were fixed in 10% buffered formalin. After fixation, the sample was washed with water, immersed in 70% ethyl alcohol for 3-4

days and embedded in paraffin. Paraffin sections of 5μm were obtained from rotational microtome and stained with hematoxylin and eosin (HE).

The criteria used for scoring the injuries to the pancreas were as follows Li et al. (2001). Score 0, normal (the normal numbers and volume of the islet cells); score I, minor injury (the numbers of islet cells were slightly lower and islet cells were slightly swollen); score II, moderate injury (the numbers of islet cells were moderately lower and islet cells were moderately swollen); score III, obvious injury (the numbers of islet cells were obviously lower and islet cells were obviously swollen); score IV, severe injury (the numbers of islet cells were severely reduced and islet cells were severely swollen).

2.5.7. Determination of basal plasma insulin

Basal plasma insulin concentrations were determined by radioimmunoassay and measured with a Beckman Gamma 5500 Counter (Beckman Instruments®, Fullerton, CA). The kit (Diagnostic Products Corporation®) included human insulin as the standard and ^{125}I-labelled human insulin as the antibody, which cross-reacts with rat insulin.

2.5.8. Western blotting

Protein extraction and immunoblotting were carried out as previously reported (Rafacho et al., 2009) with modifications. Fragments of *soleus* muscle and liver were obtained from animals and first homogenized in an ice-cold cell lysis buffer (Cell Signaling®) using a Polytron PT 1200C homogenizer (Brinkmann Instruments®, NY, USA) (2 pulses of 15sec at the maximum speed) and subsequently sonicated in a cell homogenizer (Fisher Scientific®, Suwanee, GA, USA) for 2 pulses of 15sec at the intermediate speed. Protein concentration from total cell lysate was measured using the Bradford method, according to the manufacturer (Bio-Rad Laboratories®, Hercules, CA, USA). For each experiment, 150μg of protein obtained from muscle and hepatic tissue was separated by SDS-PAGE, transferred to nitrocellulose

membranes and stained with Ponceau S. The protein kinase B (Akt), AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC), in their total and phosphorylated forms (P-Akt, P-AMPK and P-ACC) were detected in the membrane after 2h of incubation at room temperature with polyclonal antibodies (Santa Cruz Biotechnology®, CA, USA). Detection was performed using enhanced chemilluminescence (SuperSignal West Pico®, Pierce, Rockford, IL) after incubation with a peroxidase-conjugated secondary antibody. Band intensities were quantified using optical densitometry (Scion Image®, Frederick, MD) of the developed autoradiogram.

2.6. Normoglycaemic animals

2.6.1. Effect of the aqueous extract of the stem bark of Caesalpinia ferrea on intestinal absorption of glucose

Healthy normoglycaemic rats fasted for 16h and were randomly divided into three groups (n=7/group). Groups 1, 2 and 3 received 1mL of water, 1mL of metformin (120mg/kg b.w.) and 1mL of the aqueous extract of the stem bark of *Caesalpinia ferrea* 450mg/kg b.w., respectively. After 30min, all groups received an oral solution of d-glucose 50% (10mL/kg b.w.) and, after 1h, all animals were euthanized with an excess Nembutal® (140mg/kg, i.p) and their small intestine removed. Then, the intestine was perfused with 50mL of water and the contents collected at the other end, centrifuged at 1500×g for 5min and the supernatant used to determine the value of glucose by way of spectrophotometry.

2.7. Statistical analysis

The results were expressed as mean ± standard error of mean (S.E.M.). Statistical analysis was performed using Graph Pad Prism 5.0® software. The difference between groups was assessed by analysis of variance (ANOVA), followed, when necessary, by Newman-Keuls test. The significance level for rejection of the null hypothesis was always ≥ 5% ($p<0.05$).

3.0. Results

3.1. Phytochemical screening of Caesalpinia ferrea

TLC for chemical identification of the constituents of *Caesalpinia ferrea* bark revealed the presence of condensed tannins (catechins) and hydrolysable tannins (gallic and ellagic acids). The total tannin content of *Caesalpinia ferrea* SDE was calculated as 266mg/g. On the other hand, the content for HPLC of gallic acid (1), catechin (2), epicatechin (3) and ellagic acid (4) was 112.76, 17.75, 6.13 and 12.00mg/g (Figure 1).

3.2. STZ-Diabetic animals

3.2.2. The effect of treatment with the aqueous extract of the stem bark of Caesalpinia ferrea on fasting blood glucose

Oral administration of *Caesalpinia ferrea* 300 and 450mg/kg/day b.w. in diabetic rats showed significant reductions in fasting glucose levels of more than 50% already in the first week of treatment when compared to DC and MTD. At the end of treatment, this reduction was of 70 and 79.5%, respectively, only in relation to DC (Figure 2).

3.2.3. The effect of treatment with the aqueous extract of the stem bark of Caesalpinia ferrea on body mass gain, food and water intake

Figures 3, 4 and 5 show the evolution of body mass gain, food and water intake in the experimental groups during the 28-day treatment, respectively. Throughout the study, body mass gain of the group receiving 450mg/kg/day of *Caesalpinia ferrea* was significantly higher than that of other groups. 300mg/kg/day of *Caesalpinia ferrea* did not show statistical differences for this parameter (Figure 3).

The results shown in Figure 4 also reveal a significant reduction in food intake in the group receiving *Caesalpinia ferrea* 450mg/kg/day from the first week of the

study until the end of the treatment when compared with DC. Statistical reduction in relation to MTD occurred since the third week. The group treated with *Caesalpinia ferrea* 300mg/kg/day showed reductions in food intake only in the first and third week of the study when compared with DC.

Polydipsia was significantly reduced in the group receiving *Caesalpinia ferrea* 450mg/kg/day already in first week of treatment compared to DC and, in the following weeks, this reduction was also significant for the DC and MTD. The group receiving *Caesalpinia ferrea* 300mg/kg/day showed significant reductions from the second week only compared to the DC (Figure 5).

As *Caesalpinia ferrea* 450mg/kg/day b.w. was the most effective during the treatment, this dose was selected for further studies.

3.2.4. The effect of the aqueous extract of the stem bark of Caesalpinia ferrea on glucose tolerance

Table 1 shows the blood glucose levels of the NDC, DC, MTD and *Caesalpinia ferrea* 450mg/kg b.w groups after oral administration of d-glucose (2.0g/kg b.w). *Caesalpinia ferrea* 450mg/kg showed significant decrease in glycaemia from 30min after glucose administration when compared to other groups. Reductions of 70, 69, 76 and 80% were observed in glycaemic levels of this group in 30, 60, 120 and 150min, respectively, when compared to DC.

3.2.5. Biochemical parameters

Biochemical parameters of *Caesalpinia ferrea* 450mg/kg/day showed statistically significant reductions in urea, uric acid, AST and ALT levels when compared to DC and significant increases in hepatic glycogen, total cholesterol and triglycerides in relation to same group. AlkP levels were statistically lower than DC and MTD (Table 2).

3.2.6. Liver and tissue mass analysis

The effects of *Caesalpinia ferrea* 450mg/kg/day on masses of liver, epididymal adipose tissue, *soleus* and extensor *digitorium longus* muscles are given in table 3. *Caesalpinia ferrea* 450mg/kg/day showed a significant increase in liver and tissues masses in relation to DC and MTD, except for the relative mass of *soleus* muscle.

3.2.7. Evaluation of serum and liver oxidative stress

Oxidative stress present in diabetic animals was measured by levels of thiobarbituric acid-reactive substances (TBARS). *Caesalpinia ferrea* 450mg/kg/day b.w showed levels of TBARS significantly lower in serum (2.60±0.15nmol MDA/mL, $p<0.05$) and liver (23.81±1.30nmol MDA/g of hepatic tissue, $p<0.05$) when compared to the diabetic control (3.34±0.25nmol MDA/mL and 27.82±0.38nmol MDA/g, respectively). The metformin group also showed levels of serum and liver TBARS significantly lower (2.80±0.10nmol MDA/mL and 24.82±0.22nmol MDA/g, respectively, $p<0.05$) in relation to the diabetic control. The non-diabetic control showed levels of serum and liver TBARS of 1.21±0.09nmol MDA/mL and 14.00±0.32nmol MDA/g, respectively).

3.2.8. Histopathological examination of the pancreas

All groups in the study had similar patterns of destruction of islets. STZ administration caused severe injury to the pancreas, such as a decrease in the number of islet cells and a reduction in the diameter of pancreatic islets. The islets were shrunken with atypical cellular changes, such as mild hyperchromasia, anysokariosis, coarse chromatin and pyknosis (Figure 6).

3.2.9. Determination of basal plasma insulin

The plasma insulin levels were drastically reduced in all groups of STZ-diabetic rats at the end of treatment and no statistical difference was found between DC (0.58±0.07ng/mL), MTD (0.74±0.08ng/mL) and *Caesalpinia ferrea* 450mg/kg/day (0.76±0.06ng/mL). The plasma insulin levels of non-diabetic control were 3.56±0.15 ng/mL.

3.2.10. Western blotting

Since glucose tolerance was markedly improved with *Caesalpinia ferrea* 450mg/kg/day b.w administration without stimulating plasma insulin secretion, we decided to investigate the role of liver and *soleus* muscle in this process. We thus analyzed the expression of total and phosphorylated levels of Akt, AMPK and ACC in both tissues of experimental rats. The *soleus* muscle of rats in the *Caesalpinia ferrea* 450mg/kg/day b.w. group displayed a 52% increase in the expression of P-Akt and reductions of 20% and 40% in terms of P-AMPK and P-ACC, respectively, when compared to the diabetic control group ($p<0.05$, Figure 7). No changes were observed in total protein content among the groups. For the liver, a 41% increase was also observed for P-Akt, but no statistical difference was found in expression of P-AMPK, although there was a 20% reduction of P-ACC in relation to the diabetic control group ($p<0.05$, Figure 8). Together, these results suggest increased glucose uptake.

3.3. Normoglycaemic animals

3.3.1. The effect of the aqueous extract of the stem bark of Caesalpinia ferrea on intestinal absorption of glucose

The intestinal absorption of glucose was significantly inhibited in normoglycaemic rats treated with aqueous extract of *Caesalpinia ferrea* 450mg/kg b.w. in relation to control animals, but it did not differ statistically from the group treated with metformin (120mg/kg b.w.) (Figure 9).

4.0. Discussion

The experiments in this study were designed to investigate the hypoglycaemic activity of the aqueous extract of the stem bark of *Caesalpinia ferrea*, as well as the possible role of the enzymatic pathways of protein kinase B (PKB/Akt) and AMP-activated protein kinase (AMPK).

The use of *Caesalpinia ferrea* in folk medicine is well known. In Brazil, many diabetic patients take tea made from the stem bark of *Caesalpinea ferrea* daily to control glycaemic levels (Teixeira and Melo, 2006). This is important from an ethnopharmacological point of view, because our study has provided the first experimental evidence of the hypoglycaemic properties of aqueous extract of the stem bark of *Caesalpinia ferrea* in streptozotocin-induced diabetic rats. Our work opens the way for further chemical, pharmacological and toxicological research into the development of traditional medicine.

The animals treated daily with *Caesalpinia ferrea* 450mg/kg had improved their metabolic state through increased glucose tolerance. This dose was therefore chosen for the other tests.

The major compounds of the aqueous extract of the stem bark of *Caesalpinia ferrea* are condensed tannins (catechins) and hydrolysable tannins (gallic acid and ellagic acid). The effect of reducing blood glucose levels could not be attributed to hydrolyzable tannins, since Ueda et al. (2004) have shown that ellagic acid obtained from the fruit of *Caesalpinia ferrea* does not reduce blood glucose of diabetic animals, but only inhibits aldose reductase, minimizing the complications of diabetes. However, the catechins present in the aqueous extract of flower of *Eugenia operculata* (500mg/kg) reduced fasting glucose in diabetic rats after 8 weeks of treatment (Mai and Chuyen, 2007). In fact, the catechins and their derivates are known for their hypoglycaemic properties and act to control diabetes (Kao et al., 2000). Thus, it is reasonable to infer that these compounds could be responsible for the hypoglycaemic effect in our study.

Our results indicate that the hipoglycaemic properties of the aqueous extract of the stem bark of *Caesalpinia ferrea* is an extra-pancreatic effect, independent of insulin secretion, since insulin levels remained low after treatment.

In order to clarify how the aqueous extract of the stem bark of *Caesalpinia ferrea* exerts its hipoglycaemic action, the molecular mechanisms that involve Akt, AMPK and acetyl-CoA carboxylase (ACC) signaling were investigated.

The activation of Akt is required for the regulation of glycogen synthesis in muscle, adipocytes, liver and for inhibition of gluconeogenesis. The increase in hepatic glycogen in treated animals, as a consequence of the activation of Akt, confirms its action on glycogen synthase as described by Farese et al. (2005). According to Liu et al. (2010), the activation of Akt in insulin-resistant rats was improved by treatment with polysaccharide from *Astragulus membranaceus.*

In skeletal muscle, the activation of Akt also increases glucose uptake by translocation of GLUT 4 from the cytosol to the plasma membrane and, as a consequence, reduces protein catabolism as seen by the increase in mass of the extensor *digitorium longus* and *soleus* muscles in treated animals. The activation of Akt is probably associated with the presence of catechins in the aqueous extract of the stem bark of *Caesalpinia ferrea.* Daisy et al. (2010) have shown that diabetic animals treated with catechins isolated from *Cassia fistula* exhibited increased expression of GLUT 4 mRNA and protein (GLUT4) in skeletal muscle even in the absence of regeneration of pancreatic β cells destroyed by STZ.

The reduction in protein catabolism decreases hepatic uptake of amino acids and, consequently, the serum levels of uric acid, urea as well as ALT, AST and AlkP, as seen in treated animals. The reduction in the levels of ALT, AST and AlkP may also be explained by the hepatoprotective effect of catechins. According to Tsuchiya (2001), catechins from *Camellia sinensis* improved the fluidity of the hepatocyte membrane.

Another molecular pathway investigated was the AMP-activated protein kinase. Under conditions of energy depletion, AMPK activation inhibits ATP-consuming pathways and stimulates the generation of ATP. AMPK activation leads to the de-activation of ACC, which allows the oxidation of fatty acids, in an attempt to restore energy balance (Zhou et al., 2001). The increase in AMPK activity and

subsequent de-activation of ACC explain the low levels of total cholesterol, triglycerides and epididymal adipose tissue mass found in untreated diabetic animals.

Therefore, the reduced activation of AMPK in the skeletal muscle and increased activation of ACC in the skeletal muscle and in the liver of treated animals suggest a restoration of the energy balance. However, in the liver of the treated animals, the increase of glycogen was not sufficient to suppress the activation of AMPK. According to Winder and Hardie (1999), the β-subunits of AMPK have a glycogen binding domain and high concentrations of glycogen suppress AMPK activity.

The treatment with *Caesalpinia ferrea* decreased oxidative stress in the liver and serum of diabetic animals. Ulicná et al. (2006) showed that aqueous extract of the leaves of *Aspalantus linearis* (300mg/kg) also prevents oxidative stress in diabetic rats. Moure et al. (2001) have shown that tannins and catechins act as free radical scavengers

The catechins present in *Caesalpinia ferrea* could act to inhibit both digestive enzymes and the glucose transporter in the intestine. *In vivo* studies confirmed that catechins inhibited α-glucosidase in an oral glucose tolerance test (Li et al., 2007) and condensed tannins from black bean inhibited the sodium-dependent intestinal glucose uptake system (Carmona et al., 1996).

As noted in this study, the improvement in glucose metabolism in diabetic animals treated could be due to Akt activation, in the liver and muscle, with consequent suppression in the activation of AMPK in the muscle. Thus, our study suggests a putative molecular mechanism for the action of the aqueous extract of the stem bark of *Caesalpinia ferrea* and indicates that it is a promising alternative treatment for diabetic conditions.

Conflicts of interest

There is no conflict of interest.

Acknowledgements

The authors would like to thank Rejane de Souza for technical assistance and CNPq for financial support (Grants numbers 562948/2010-4 and 150316/2011-9).

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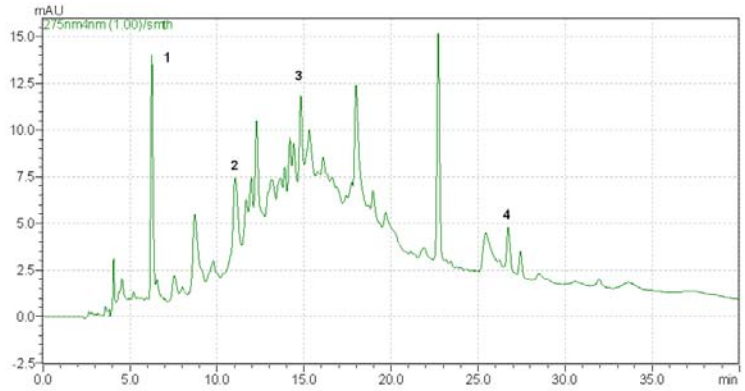

Fig. 1. Chromatogram of *Caesalpinia ferrea* SDE detected at 270 nm. Peaks: (1) gallic acid; (2) catechin; (3) epicatechin and (4) ellagic acid.

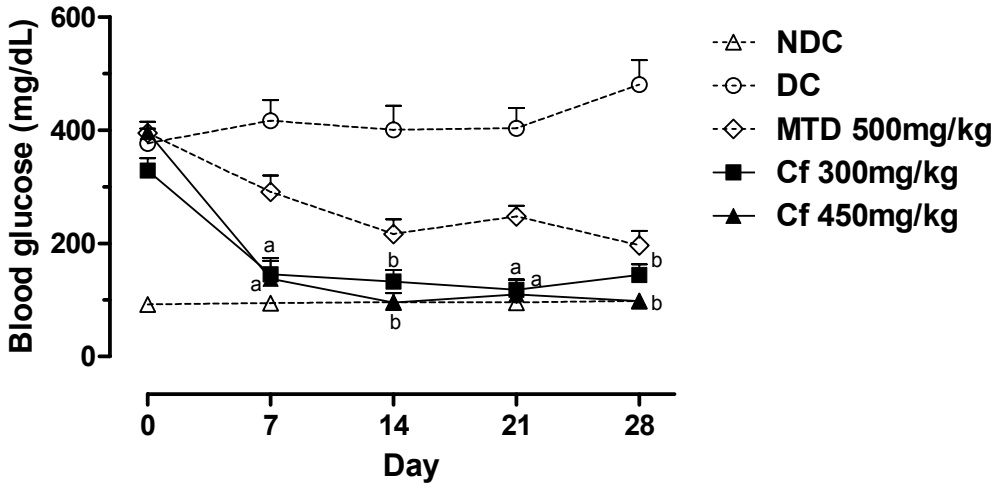

Fig. 2. Effect of aqueous extract of *Caesalpinia ferrea* (Cf) on fasting blood glucose (mg/dL) of diabetic rats. (NDC: non-diabetic control; DC: diabetic control, MTD: diabetic rats treated with metformin 500mg/kg; Cf: diabetic rats treated with aqueous extract of the stem bark of *Caesalpinia ferrea* 300 and 450mg/kg). The results are expressed as mean±S.E.M. (n=7/group). [a]Statistically different from DC and MTD, [b]Statistically different from DC (ANOVA followed by Newman-Keuls, $p<0.05$).

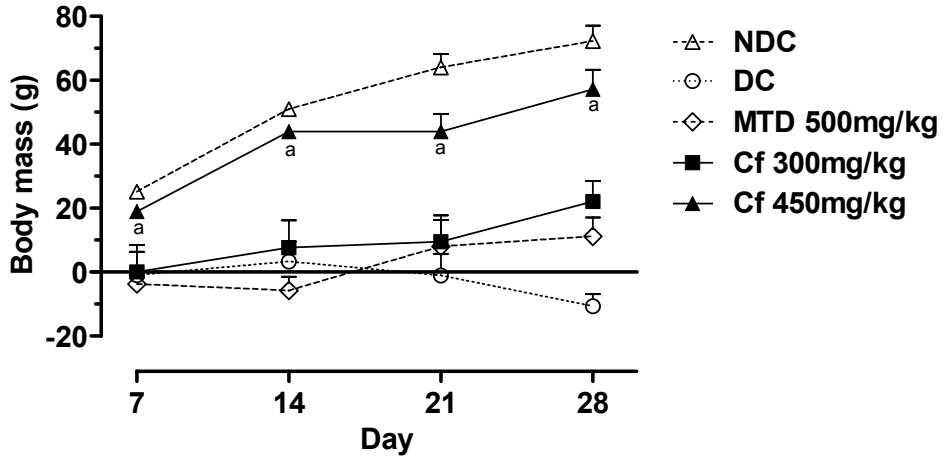

Fig. 3. Effect of aqueous extract of *Caesalpinia ferrea* (Cf) on body mass gain (g) of diabetic rats. (NDC: non-diabetic control; DC: diabetic control, MTD: diabetic rats treated with metformin 500mg/kg; Cf: diabetic rats treated with aqueous extract of the stem bark of *Caesalpinia ferrea* 300 and 450mg/kg). The results are expressed as mean±S.E.M. (n=7/group). [a]Statistically different from DC and MTD (ANOVA followed by Newman-Keuls, $p<0.05$).

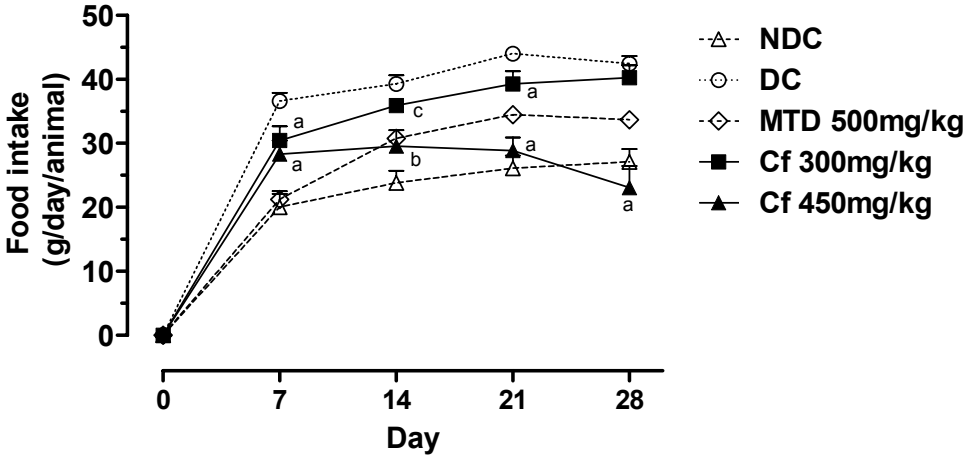

Fig. 4. Effect of aqueous extract of *Caesalpinia ferrea* (Cf) on food intake (g/day/animal) of diabetic rats. (NDC: non-diabetic control; DC: diabetic control, MTD: diabetic rats treated with metformin 500mg/kg; Cf: diabetic rats treated with aqueous extract of the stem bark of *Caesalpinia ferrea* 300 and 450mg/kg). The results are expressed as mean±S.E.M. (n=7/group).[a]Statistically different from DC and MTD,

[b]statistically different from DC, [c]statistically different from MTD (ANOVA followed by Newman-Keuls, p<0.05).

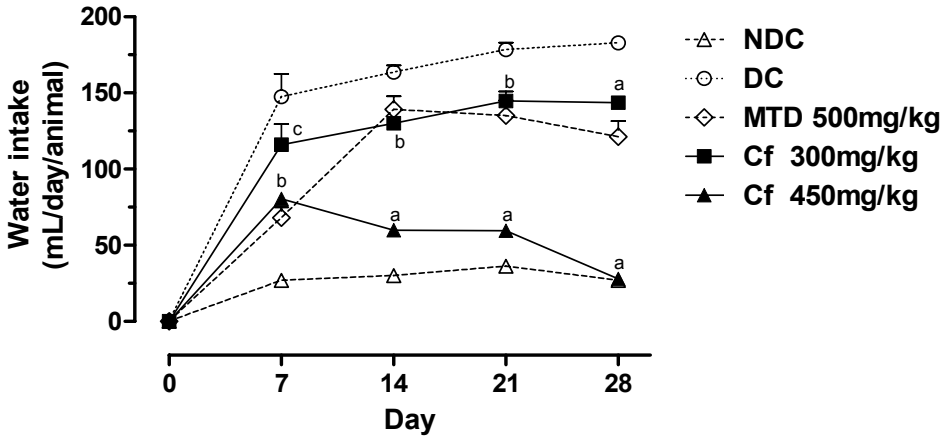

Fig. 5. Effect of aqueous extract of *Caesalpinia ferrea* (Cf) on water intake (mL/day/animal) in diabetic rats. (NDC: non-diabetic control; DC: diabetic control, MTD: diabetic rats treated with metformin 500mg/kg; Cf: diabetic rats treated with aqueous extract of the stem bark of *Caesalpinia ferrea* 300 and 450mg/kg). The results are expressed as mean±S.E.M. (n=7/group). [a]Statistically different from DC and MTD, [b]statistically different from DC, [c]statistically different from MTD (ANOVA followed by Newman-Keuls, p<0.05).

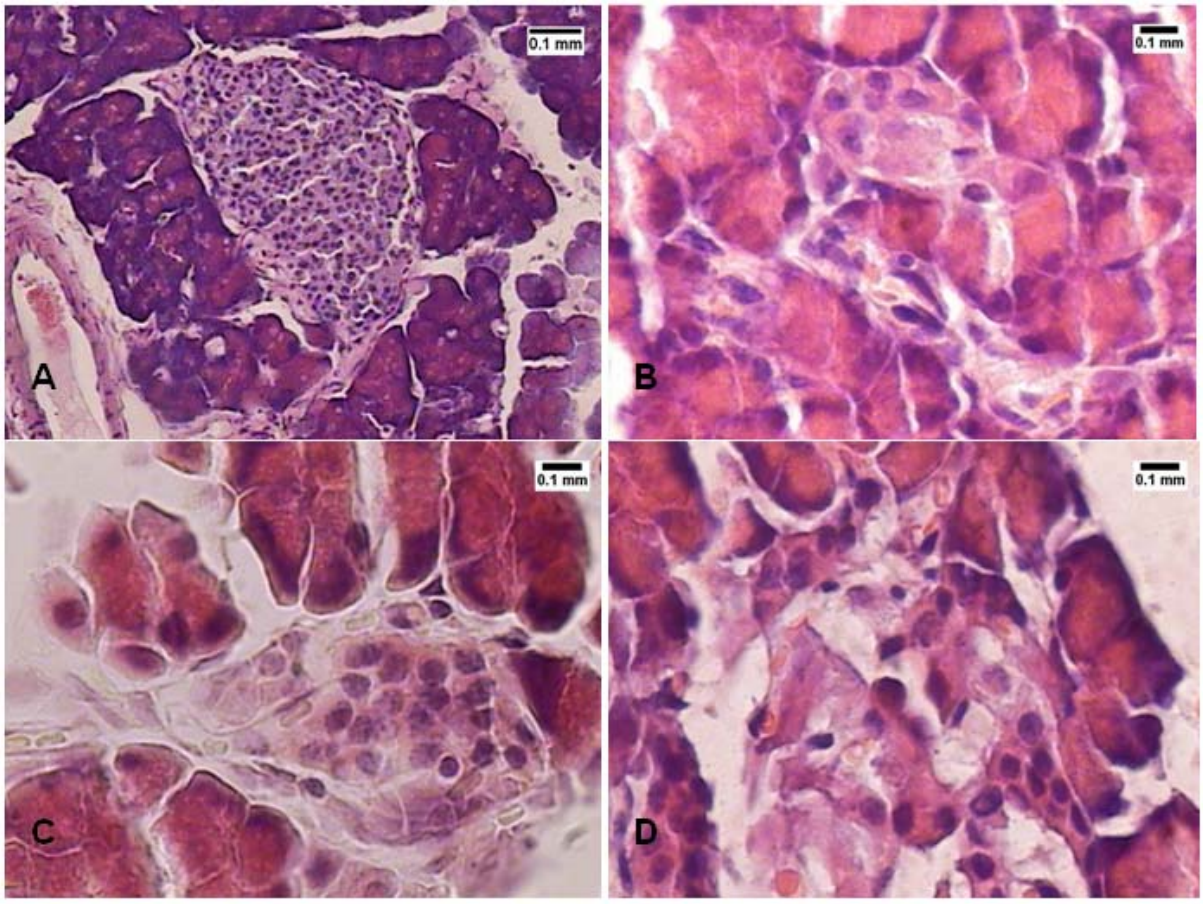

Fig. 6. Paraffin sections of pancreas (HE) of STZ-diabetic rats. (A) Non-diabetic control (magnification 100x); (B) Diabetic control (magnification 400x); (C) Diabetic rats treated with metformin 500mg/kg (magnification 400x); (D) Diabetic rats treated with aqueous extract of the stem bark of *Caesalpinia ferrea* 450mg/kg (magnification 400x).

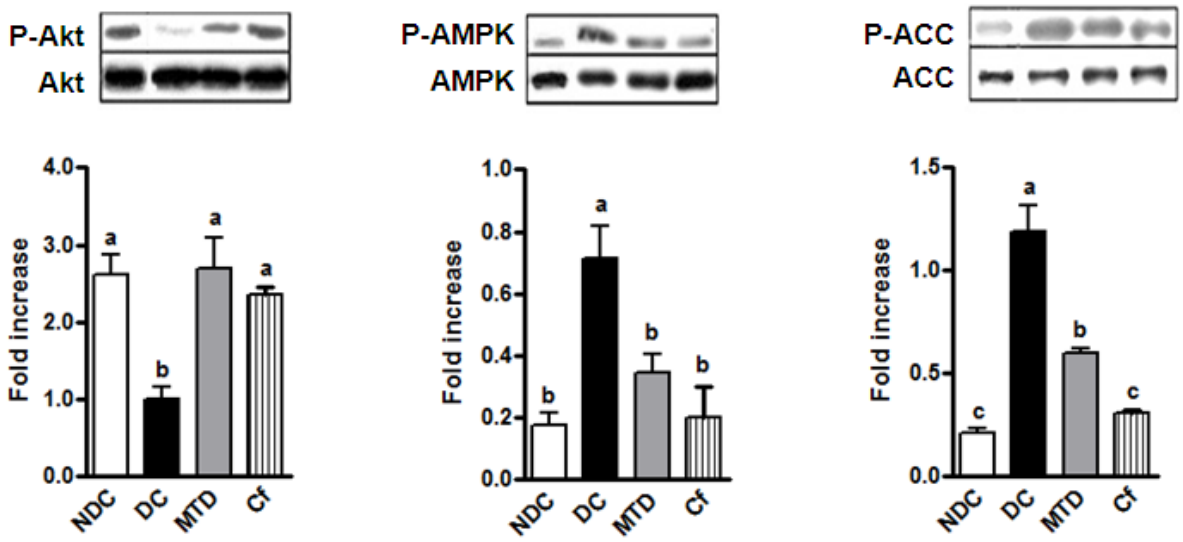

Fig. 7. Western blot analysis of Akt, AMPK and ACC in skeletal muscle of STZ-diabetic rats. (NDC: non-diabetic control; DC: diabetic control; MTD: diabetic rats treated with metformin 500mg/kg; Cf: diabetic rats treated with aqueous extract of the stem bark of *Caesalpinia ferrea* 450mg/kg). The results are expressed as mean±S.E.M and indicate the relationship between phosphorylated protein and total protein (n=7/group). Means without a common letter differ, $p<0.05$ (ANOVA followed by Newman-Keuls).

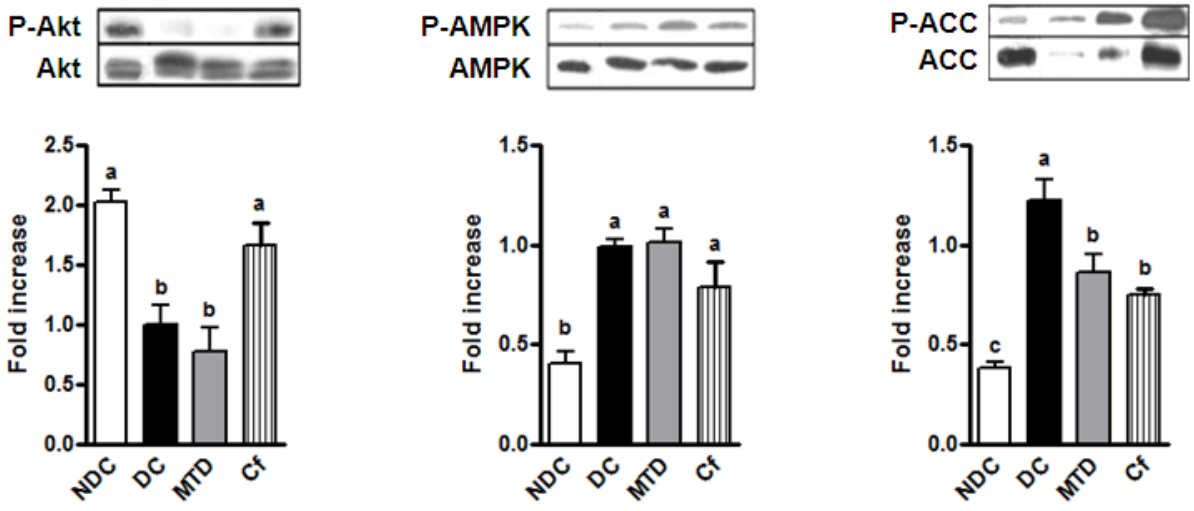

Fig. 8. Western blot analysis of Akt, AMPK and ACC in the liver of STZ-diabetic rats. (NDC: non-diabetic control; DC: diabetic control; MTD: diabetic rats treated with metformin 500mg/kg; Cf: diabetic rats treated with aqueous extract of the stem bark of *Caesalpinia ferrea* 450mg/kg). The results are expressed as mean±S.E.M and

indicate the relationship between phosphorylated protein and total protein (n=7/group). Means without a common letter differ, p<0.05 (ANOVA followed by Newman-Keuls).

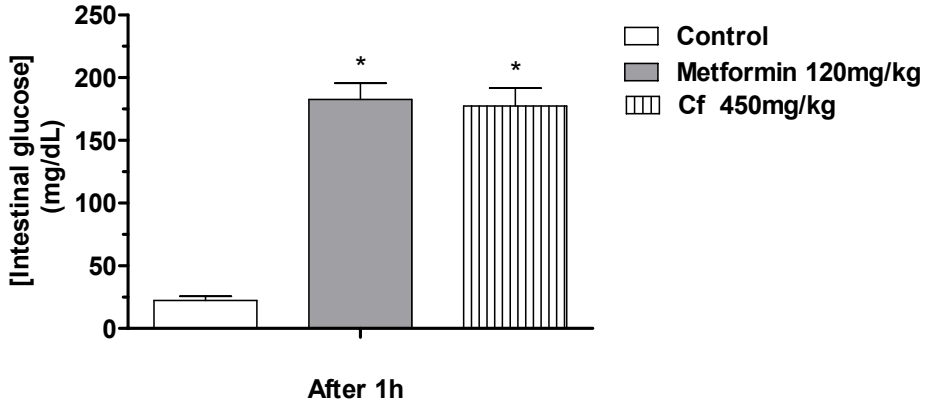

Fig. 9. Intestinal glucose concentration (mg/dL) in normoglycaemic Wistar rats. (Control: rats treated with 1mL of water; metformin: rats treated with 1mL of metformin 120mg/kg; Cf: rats treated with 1mL of aqueous extract of the stem bark of *Caesalpinia ferrea* 450mg/kg). The results are expressed as mean±S.E.M (n=7/group). *Statistically different from the control (ANOVA followed by Newman-Keuls, p<0.05).

Table 1

Effect of the aqueous extract of the stem bark of *Caesalpinia ferrea* on oral glucose tolerance test (mg/dL), after oral administration of d-glucose (2.0g/kg).

Time	NDC	DC	MTD	Cf 450
0 (Fasting)	88.6 ± 7.2	426.3 ± 19.7	297.0 ± 14.5	123.0 ± 9.0[a]
30 min	110.0 ± 5.4	642.7 ± 12.9	385.5 ± 10.5	192.0 ± 8.3[a]
60 min	104.1 ± 6.1	637.0 ± 13.9	396.0 ± 9.4	197.0 ± 8.5[a]
120 min	101.0 ± 3.4	618.3 ± 9.3	290.0 ± 8.4	149.0 ± 5.0[a]
150 min	97.0 ± 3.1	598.3 ± 4.4	260.0 ± 8.1	120.0 ± 3.0[a]

(NDC: non-diabetic control; DC: diabetic control; MTD: diabetic rats treated with metformin 500mg/kg; Cf: diabetic rats treated with aqueous extract of the stem bark of *Caesalpinia ferrea* 450mg/kg. The values are expressed as mean±S.E.M (n=7/group). [a]Statistically different from DC and MTD (ANOVA followed by Newman-Keuls, $p<0.05$).

Table 2

Biochemical parameters of normoglycaemic and diabetic rats.

Parameters	NDC	DC	MTD	Cf 450
Urea (mg/dL)	28.80 ± 1.25	101.00 ± 8.33	70.75 ± 7.46	34.20 ± 2.42[b]
UA (mg/dL)	1.10 ± 0.07	3.05 ± 0.18	0.97 ± 0.02	1.02 ± 0.03[b]
AST (U/L)	153.70 ± 8.00	596.20 ± 9.70	212.30 ± 10.40	148.60 ± 5.61[b]
ALT (U/L)	52.57 ± 2.30	406.70 ± 9.50	139.80 ± 9.44	62.80 ± 4.83[b]
AlkP (U/L)	234.00 ± 9.21	1814.00 ± 9.98	656.30 ± 6.99	179.20 ± 7.82[a]
L Gly (mg%)	2.61 ± 0.19	0.13 ± 0.06	0.53 ± 0.01	1.72 ± 0.01[b]
TC (mg/dL)	77.58 ± 5.15	60.78 ± 2.95	70.75 ± 5.63	99.20 ± 7.89[b]
Trig (mg/dL)	47.07 ± 3.94	35.77 ± 3.28	139.30 ± 7.49	70.00 ± 6.38[b]

(UA): Uric acid, (AST): aspartate aminotransferase, (ALT): alanine aminotransferase, (AlkP): alkaline phosphatase, (L Gly): liver glycogen, (TC): total cholesterol, (Trig): triglycerides. (NDC: non-diabetic control; DC: diabetic control; MTD: Diabetic rats treated with metformin 500mg/kg; Cf: diabetic rats treated with aqueous extract of the stem bark of *Caesalpinia ferrea* 450mg/kg. The values are expressed as mean±S.E.M (n=7/group). [a]Statistically different from DC and MTD, [b]statistically different from DC (ANOVA followed by Newman-Keuls, $p<0.05$).

Table 3

Effect of the aqueous extract of the stem bark of *Caesalpinia ferrea* on tissues masses of diabetic rats.

Parameters	NDC	DC	MTD	Cf 450
Liver (g)	13.56 ± 0.75	8.44 ± 0.44	8.36 ± 0.18	13.20 ± 0.47[a]
(g/100g)	4.01 ± 0.11	3.77 ± 0.22	3.83 ± 0.06	4.81 ± 0.09[a]
EAT (g)	2.25 ± 0.01	0.57 ± 0.09	0.12 ± 0.03	1.59 ± 0.30[a]
(g/100g)	0.90 ± 0.02	0.24 ± 0.09	0.05 ± 0.01	0.57 ± 0.09[a]
***Soleus* (g)**	0.158 ± 0.002	0.112 ± 0.007	0.101 ± 0.006	0.135 ± 0.003[a]
(g/100g)	0.052 ± 0.002	0.078 ± 0.027	0.046 ± 0.002	0.048 ± 0.002
EDL (g)	0.162 ± 0.003	0.089 ± 0.008	0.079 ± 0.002	0.158 ± 0.002[a]
(g/100g)	0.060 ± 0.004	0.040 ± 0.003	0.036 ± 0.001	0.058 ± 0.004[a]

EAT: Epididymal adipose tissue; *Soleus*: s*oleus* muscle, EDL: extensor *digitorium longus* muscle. (NDC: non-diabetic control; DC: diabetic control; MTD: Diabetic rats treated with metformin 500mg/kg; Cf: diabetic rats treated with aqueous extract of the stem bark of *Caesalpinia ferrea* 450 mg/kg. The values are expressed as mean±S.E.M (n=7/group). [a]Statistically different from DC and MTD (ANOVA followed by Newman-Keuls, $p<0.05$).

5. Artigo II

Acute and subacute toxicity of *Caesalpinia ferrea* stem bark extract

(Artigo a ser submetido)

Acute and subacute toxicity of *Caesalpinia ferrea* stem bark extract

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Abstract

Ethnopharmacological relevance: *Caesalpinia ferrea* Martius (Leguminosae) has long been used in Brazilian traditional medicine, especially to treat diabetes.
Aim of the study: The objective of this study was to evaluate the acute and subacute toxicity (30 days) of *Caesalpinia ferrea* via the oral route in male Wistar rats.
Materials and methods: For the acute toxicity test, the aqueous extract of the stem bark of *Caesalpinia ferrea* was administered in dose from 2000 mg/kg for both sexes (n = 5/group/sex) and in the subacute toxicity test the doses of 300 and 1500 mg/kg/day (n = 10/group) were used in male Wistar rats.
Results: In the acute toxicity test, *Caesalpinia ferrea* did not produce any toxic signs or deaths. The subacute treatment with *Caesalpinia ferrea* did not alter the body mass gain as well as analysis of organs and there were increase of water and food intake. The haematological analysis did not show significant differences in any of the parameters examined and the same was observed in the biochemical parameters, the exception was only the increase of the levels of amylase in both groups of treatment with *Caesalpinia ferrea* (300 and 1500 mg/kg), however any histological change was observed in the pancreas.
Conclusion: The acute administration of the aqueous extract of the stem bark of *Caesalpinia ferrea* did not produce toxic effects in Wistar rats. Despite the subacute treatment had not shown sign of toxicity, increased serum amylase suggests that further studies may be conducted to verify the importance of this parameter.

Keywords*: .Caesalpinia ferrea* Martius Ext Tul; Tannins, Acute toxicity, Subacute toxicity.

1.0. Introduction

Caesalpinia ferrea Martius Ext Tul (Leguminosae) is medium-sized tree widely distributed in the north and northeast regions of Brazil, where it is commonly known as “Jucá” or “Pau-ferro” (Nakamura et al, 2002). In Brazil, the tea of the stem bark of *C. ferrea* has been commonly used in the folk medicine for the treatment of diabetes, respiratory tract diseases, dysentery and liver inflammation, although the occurrence of hepatotoxicity has been reported in the literature (Di Stasi et al., 2002).

Animal experiments showed peripheral analgesic, anti-inflammatory and antiulcer activities for fruit and stem bark extracts of *Caesalpinia ferrea* (Bacchi; Sertié, 1991, 1994; Carvalho et al., 1996). An acetone extract of the stem bark of *C. ferrea* showed potent inhibitory activity against topo II and cell proliferation in HL60 cells (Nozaki et al., 2007), and Sampaio et al. (2009) show that *Caesalpinia ferrea* fruit extract can inhibit *in vitro* growth of oral pathogens. Ellagic acid isolated from fruits of *Caesalpinia ferrea* inhibited the aldose redutase, decreasing sorbitol accumulation *in vitro* and *in vivo* experiments which might relieve diabetic complications (Ueda et al., 2004). The aqueous extract of *C. ferrea* obtained from its dried stem bark showed significant hypotension effects associated to tachycardia and transient bradyarrhythmias (Menezes et al., 2007).

The crude extract of *Caesalpinia ferrea* Mart. contains anthraquinones, alkaloids, coumarins, depsides, depsidones, flavonoids, lactones, saponins, sitosterol, sugars, tannins, sesquiterpenes and triterpenes (Gonzalez et al., 2004; Souza et al., 2006). Many of these substances have toxic properties as coumarin, a well-known liver toxicant (Born et al., 2000), saponins that affect growth, food intake and reproduction in animals (Francis et al., 2002). Sitosterol, a weak estrogenic phytosterol used for lowering cholesterol was reported to have lowered sperm counts in rats (Malini; Vanithakumari,1991; Kritchevsky; Chen, 2005).

Several medicinal plants have been used for the treatment of diseases of the vascular system such as atherosclerosis, hypertension and diabetes and only some of these plants have the safety and mechanism of action confirmed (Vora; Mansoor, 2005). In addition, herbal products can produce interactions that may increase the toxic effects of traditional drugs used to treat chronic diseases (Fugh-Berman, 2000).

Although the use of *Caesalpinia ferrea* is widespred, there is a lack of detailed toxicological information in the literature. Therefore, the aim of the present study was to assess the toxicity of the aqueous extract of the stem bark of *Caesalpinia ferrea* in Wistar rats.

2.0. Material and Methods

2.1. Plant material and extraction

Bark from the stem of *Caesalpinia ferrea* Mart. Ex Tul. was collected from the Amazon Research National Institute (INPA) experimental culture, in the Brazilian State of Amazonas (03º 05' 48.0"S and 59º 59' 55.0''W.Gr). The voucher specimen of the plant was deposited at the INPA herbarium under number 228022. The bark was collected in September 2009, March 2010 and September 2010, with approximately 3kg being harvested on each occasion. The material collected was dried, first at room temperature for 48h, and then taken to an oven with circulating air at a temperature of 45±2 °C until its weight stabilized. Subsequently, the material was ground in a 1mm mesh knife mill, thereby providing the raw material (MPV). The aqueous extract of *Caesalpinia ferrea* was prepared by raw material infusion (7.5:100, w/v) using boiling distilled water as the extractive solvent for a period of 15 minutes. The aqueous extract presented a solid soluble content of 0.6±0.02g%. The aqueous extract was dried using a MSD 1.0 Labmaq Mini Spray Dryer. The drying process was performed using the following parameters: inlet temperature of 120 °C, compressed air pressure of 2bar, diameter rotor of 0.7mm and power flow of 10mL/min. The yield of dry extract after drying of aqueous extract was 98%, representing 5.88g of dry extract per liter of aqueous extract of *Caesalpinia ferrea.*

2.2. Phytochemical analysis of Caesalpinia ferrea

2.2.1. Thin Layer Chromatographic (TLC) Analysis of Caesalpinia ferrea

The methods described by Wagner and Bladt (1996) were used to screen the dried bark extract for the hydrolysable tannins (gallic and ellagic acids), condensed

tannins (catechins), flavonoids, saponins, coumarins, phenylpropanoids, cinnamic acid derivatives, alkaloids, triterpenoids/steroids, monoterpenes, sesquiterpenes, iridoids, sugars and luteolin. The phytochemical profile was drawn up using thin layer chromatography (TLC) on silica gel plates (Merck® art. 105553, UV 250–366nm) using the appropriate mobile phase, reagents and standards.

2.2.2. Estimation of total tannin content in Caesalpinia ferrea

The total tannin content was determined using the spray dried extract (SDE) of *Ceasalpinia ferrea* aqueous extract at 7.5% (w/v), by way of the difference between redissolved SDE before and after precipitation with 150mg of casein (Merck® Germany). The measurements were performed at 270nm and the total tannin content was calculated as gallic acid (mg/g of SDE). The results represent the mean of three measurements.

2.2.3. High Performance Liquid Chromatography (HPLC) analysis of Caesalpinia ferrea

The main phytochemical markers (gallic acid, ellagic acid, catechin and epicatechin) were quantified by way of LC-DAD analysis using a Shimadzu system (LC-20AT) equipped with a photo diode array detector (SPD-M20A). The chromatographic separation was performed using a Gemini RP-18 column 240 x 4mm i.d. (Phenomenex), protected by a pre-column packet of the same material. A gradient elution was performed by varying the proportion of solvent B (methanol) to solvent A (acetic acid 0.5%; w/w) at a flow rate of 0.8mL/min, according to the following gradient program: 20-40% B (10min), 40-60% B (10min), 60% B (10min), 60-40% B (10min), 40-20% B (10min). The SDE of *Caesalpinia ferrea* and standard were dissolved in methanol:water (20/80, v/v) and filtered through a 0.45µm membrane (Millipore®, USA) prior to injection of 20µL.

The peaks of each marker substance in the dried extract were initially identified by comparing the retention time and UV-spectrums. After that, the peaks were confirmed by spiking the sample with a small amount of the standards.

2.3. Animals

Adult female and male Wistar rats (*Rattus norvegicus* var. *albinus*) (aged 3 months, weighing 210 – 230 and 280 – 300 g, respectively) were obtained from the Department of Physiology and Pharmacology at the Federal University of Pernambuco (UFPE), Pernambuco, Brazil. The animals were kept under standard environmental conditions (22 ± 2^{o}C); 12:12 h dark/light cycle). Water and industrialized dry food (Labina®, Purina, Brazil) were made available *ad libitum*. The experimental protocol was approved by the Animal Experimentation Ethics Committee of UFPE (Process nº. 01411), in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

2.4. Acute toxicity

Healthy albino rats, regardless of sex, fasted overnight, but with access to water *ad libitum*, were divided into two groups (n = 5/sex). Groups were orally treated with the aqueous extract of stem bark of *Caesalpinia ferrea* with the limit test at dose of 2000 mg/kg of body weight. Animals were observed for general behavioral and body weight changes, hazardous symptoms and mortality for a period of 14 days after treatment. The lethal dose (LD_{50}) was estimated according to the method described by Bruce (1985).

2.5. Subacute toxicity

The method was performed according to the Organization for Economic Co-Operation and Development (OECD) Test Guidelines with slight modifications (OECD, 1995). Healthy male rats were randomly divided into three groups (n = 10/group). Animals were orally treated with vehicle (control group) and the aqueous extract of stem bark of *Caesalpinia ferrea* at doses of 300 and 1500 mg/kg/day for 30

consecutive days. Body weight was recorded weekly and food and water intake were monitored daily. Animals were observed for signs of abnormalities during the treatment period. At the end of the treatment, animals were fasted overnight, but allowed access to water *ad libitum*. They were then anesthetized with Nembutal® (35 mg/kg, i.p.), and blood samples were obtained by retro-orbital puncture (Waynforth, 1980) using capillary tubes for haematological and biochemical studies.

2.6. Haematological and biochemical analysis

Haematological analysis was performed using an automatic haematological analyzer (Coulter STKS, Beckman). Parameters included: red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets count and mean platelet volume (MPV). The differential leukocyte count was performed with an optical microscopy after staining and, in each case, 100 cells were counted. For biochemical analysis, blood was centrifuged at 1500×g for 10 min to obtain serum, which was stored at −20 ºC until that the following parameters were determined: glucose; blood urea nitrogen (BUN); creatinine; aspartate aminotransferase (AST); alanine aminotransferase (ALT); amylase; gamma-glutamyl transpeptidase (GGT); total cholesterol; uric acid; alkaline phosphatase and total, direct and indirect bilirubin. Dosages were made using Architect (Abott®) automation with Boehringer Ingelheim® biochemical kits.

2.7. Morphological study

After the animals were euthanized with an excess of Nembutal® (140 mg/kg, i.p.), necropsy was performed (n = 5/group) to analyze the macroscopic external features of the heart, liver, spleen, lungs, kidney, adrenal gland, stomach, and reproductive organs (testicle, epididymis, seminal vesicle and vas deferens). These organs were carefully removed and weighed individually. Organ weights were expressed in absolute and relative terms (g and g/100 g of body weight, respectively). Histological examination was performed in five animals per group/sex.

Animals were anesthetized with Nembutal® (35 mg/kg, i.p.), perfused with saline solution (for removal of blood) followed by buffered formalin solution (10%) for 10 min and the same organs were removed and fixed in Bouin solution for 48 h at room temperature. Tissue slices were processed according to the method described by Lison (1960) and stained with hematoxylin/eosin.

2.8. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 software. The difference between groups was assessed by analysis of variance (ANOVA), followed when necessary, by Newman-Keuls test. The significance level for rejection of the null hypothesis was always $\geq$ 5%.

3.0. Results

3.1. Phytochemical screening of Caesalpinia ferrea

TLC for chemical identification of the constituents of *Caesalpinia ferrea* bark revealed the presence of condensed tannins (catechins) and hydrolysable tannins (gallic and ellagic acids). The total tannin content of *Caesalpinia ferrea* SDE was calculated as 266mg/g. On the other hand, the content for HPLC of gallic acid (1), catechin (2), epicatechin (3) and ellagic acid (4) was 112.76, 17.75, 6.13 and 12.00mg/g (chromatogram not shown).

3.2. Acute toxicity

The results indicated that *Caesalpinia ferrea* acute treatment via oral route at dose of 2000 mg/kg did not produce any sign of toxicity or death in rats during 14 days of observation. Therefore, the LD_{50} could not be estimated, and it is possibly higher than 2000 mg/kg.

3.3. Subacute toxicity

No toxicity signs (such as piloerection, alteration in the locomotor activity and diarrhea) or deaths were recorded during the 30 consecutive days of treatment via oral route with *Caesalpinia ferrea* at doses of 300 and 1500 mg/kg. No significant differences were found between the initial and final body weight of the rats treated with *Caesalpinia ferrea* and control rats (Figure 1). An increased food intake was observed during all treatment for both doses when compared to control (Figure 2) and an increase in water intake was observed in the first and last weeks of treatment for both doses in relation to control (Figure 3).

3.4. Haematological and biochemical parameters

The haematological profile of the treated and control groups were presented in table 1. No statistically significant differences were recorded in any of the parameters examined. The biochemical profile of the treated and control groups were presented in table 2. The treatment with *Caesalpinia ferrea* induced an increase in the values of total and direct bilirubin (1500 mg/kg) and amylase (300 and 1500 mg/kg) in relation to control group.

3.5. Morphological parameters

The absolute and relative masses of the tissues were not changed by treatment with *Caesalpinia ferrea* (table 3). The macroscopic analysis of the target organs of the treated animals did not show significant changes in color and texture when compared with the control group. In addition, the microscopical analysis did not suggest histological alterations in any of the organs examined (data not shown), including the pancreas that remained intact despite the increased serum amylase (Figure 4).

4.0. Discussion

In the present study was checked by thin layer chromatography the presence of hydrolysable and condensed tannins as major compounds of *Caesalpinia ferrea* stem bark. According to Souza et al. (2006), the tannins are the main compounds

found in the aqueous extract of stem bark of *Caesalpinia ferrea.* Tannins (commonly referred as tannic acid) are water-soluble polyphenols that are present in many plants. Tannins have also been reported to exert physiological effects, such as to accelerate blood clotting, reduce blood pressure, decrease the serum lipid levels and modulate immunoresponses (Chung et al., 1998).

This study tested the acute and subacute toxicity of the aqueous extract of stem bark of *Caesalpinia ferrea*, which is popularly used as a natural medicine in Brazil. The results of the acute toxicity study indicated that the dose of 2000 mg/kg did not produce any sign of toxicity or death in rats, suggesting a LD_{50} above 2000 mg/kg via oral route. According to OECD (2008), substances that present LD_{50} higher than 2000 mg/kg via oral route may be considered practically non-toxic. Therefore, it may be suggested that acute toxicity of the aqueous extract of stem bark of *Caesalpinia ferrea* is practically null via oral route.

Likewise, the subacute treatment indicated that the aqueous extract of stem bark of *Caesalpinia ferrea* at doses of 300 and 1500 mg/kg/day during 30 consecutive days did not produce any deaths or clinical signs of toxicity. In addition, the body mass gain was not changed during the treatment period. However, the animals treated with both doses showed increased water and food intake during treatment. Condensed tannins have deleterious effects on food intake in cattle (Smith et al., 1995). Moreover, studies indicate that the major effect of tannins is not due to their inhibition on food intake or digestion but the decreased efficiency in converting the absorbed nutrients (Chung et al., 1998), this fact could explain the increased food intake to maintain body weight of treated animals.

No significant changes in the haematological parameters were recorded. The analysis of blood parameters is relevant for risk evaluation and some changes in the haematological system have a higher predictive value for human toxicity, when data are translated from animal studies (Olson et al., 2000).

In the biochemical parameters, a slight increase in the values of direct and total bilirubin (1500 mg/kg) was observed, which levels are within physiological limits described for the specie (Harkness and Wagner, 1993). With regard to serum amylase, it was significantly increased for both doses, but the mass and histology of the pancreas remained normal. The serum amylase levels in subjects without pancreatic disease have been shown changes following the administration of drugs

that affect carbohydrate metabolism. In general, the blood amylase levels are reduced in conditions where there is an increased use of glucose and these levels are increased after the administration of drugs that decrease the carbohydrate absorption (Dreiling et al., 1958). Thus, the catechins present in the aqueous extract of stem bark of *Caesalpinia ferrea* could reduce carbohydrate absorption and inhibit both digestive enzymes and the glucose transporter in the intestine. *In vitro* studies demonstrated a dose-dependent inhibition of α-glucosidase by catechins and *in vivo* studies confirmed this inhibition in oral glucose tolerance test by delaying the increase in blood glucose after an oral dose of glucose (Li et al., 2007).

Moreover, there was no effect on the levels of urea and creatinine as well as alanine and aspartate aminotransferases, which are good indicators of kidney and liver functions, respectively. Therefore, the blood cholesterol levels remained uneffected, the latter being an indirect indicator of liver function (Lima et al., 2009). It is reasonable to deduce that the aqueous extract of stem bark of *Caesalpinia ferrea* did not induce damage to any organs. This is further confirmed by macroscopic analysis and histological assessment of the target organs.

The data suggest that acute and subacute treatment with the aqueous extract of stem bark of *Caesalpinia ferrea* showed no signs of toxicity. The increase in serum amylase points to impairment in the absorption of carbohydrates that not interfering with mass gain and general condition of the animals. For a more reliable safety evaluation performed on the basis of the acceptable daily intake concept, data on the chronic toxicity, reproductive toxicity, genotoxicity and carcinogenicity of *Caesalpinia ferrea* also would be required.

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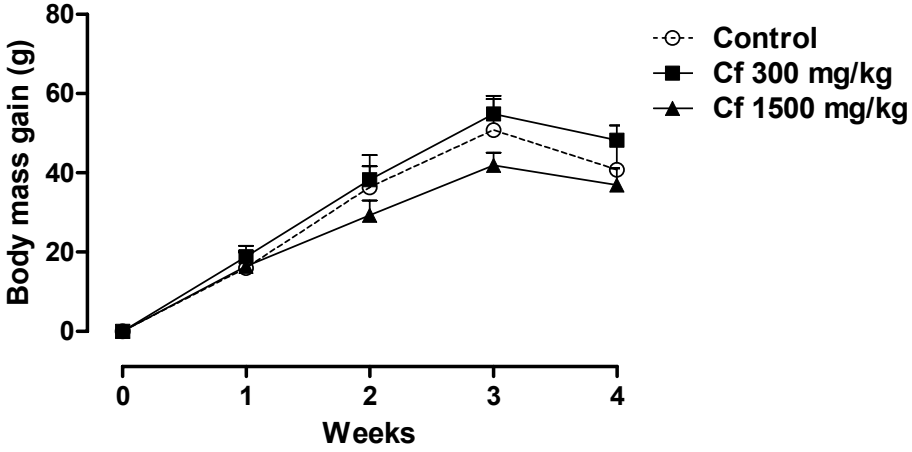

Fig. 1: Body mass gain curves of male Wistar rats treated orally with the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) for 30 consecutive days. The values are expressed as mean ± S.E.M. (n = 10/group).

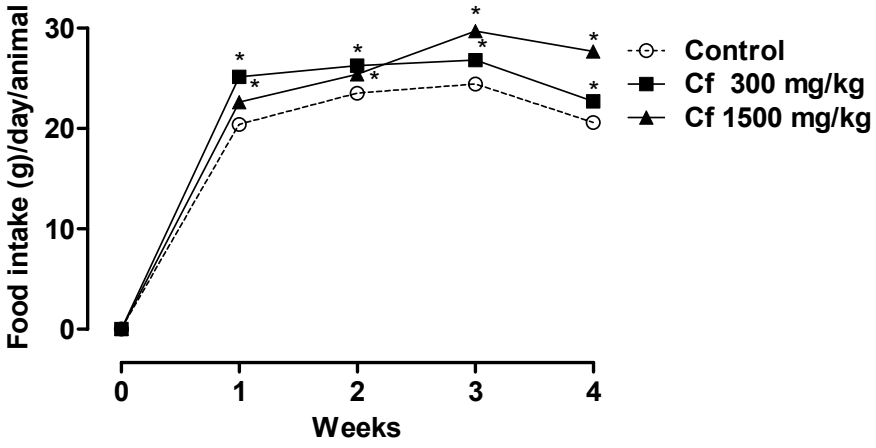

Fig. 2: Food intake curves of male Wistar rats treated orally with the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) for 30 consecutive days. The values are expressed as mean ± S.E.M. (n = 10/group). *Statistically different of the control group (ANOVA, followed by Newman-Keuls test, $p < 0.05$).

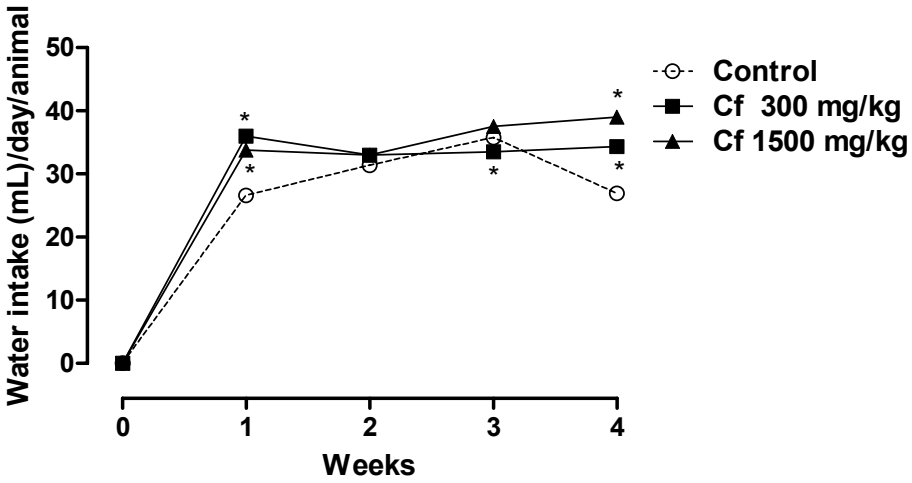

Fig. 3: Water intake curves of male Wistar rats treated orally with the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) for 30 consecutive days. The values are expressed as mean ± S.E.M. (n = 10/group). *Statistically different of the control group (ANOVA, followed by Newman-Keuls test, $p < 0.05$).

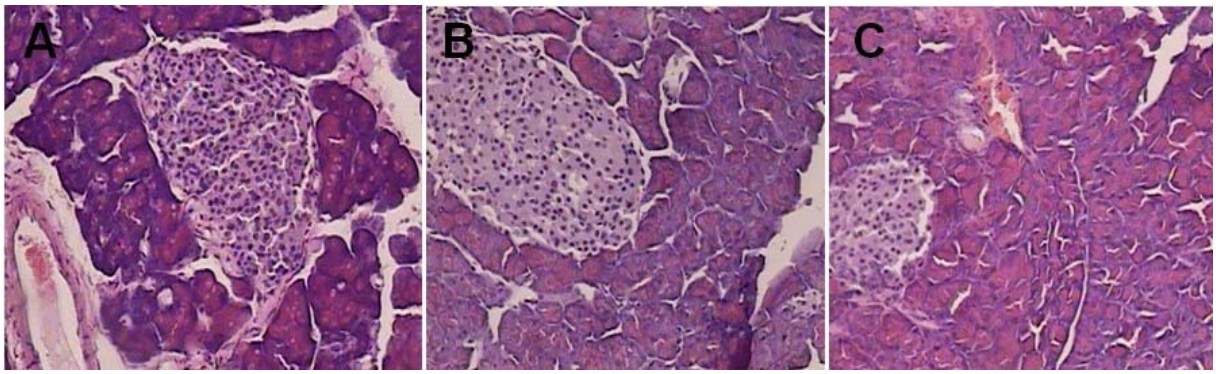

Fig. 4: Paraffin section of pancreas (HE) of male Wistar rats x 100 of (A) – Control; (B) – aqueous extract of stem bark of *Caesalpinia ferrea* 300 mg/kg; (C) aqueous extract of stem bark of *Caesalpinia ferrea* 1500 mg/kg.

Table 1: Effect of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) on haematological parameters in male Wistar rats during 30 consecutive days of treatment.

Haematological parameters	Control	Cf 300 mg/kg	Cf 1500 mg/kg
Erythrocytes (10^6/mm^3)	8.43 ± 0.13	8.52 ± 0.19	8.42 ± 0.20
Hemoglobin (g/dL)	16.07 ± 0.34	16.29 ± 0.43	15.88 ± 0.39
Hematocrit (%)	47.20 ± 0.92	47.47 ± 1.12	46.11 ± 1.04
MCV (µm^3)	56.00 ± 1.23	55.50 ± 0.70	54.90 ± 0.62
MCH (pg)	19.05 ± 0.20	19.11 ± 0.27	18.85 ± 0.21
MCHC (g/dL)	34.08 ± 0.56	34.28 ± 0.14	34.39 ± 0.15
RDW (%)	13.05 ± 0.55	13.63 ± 0.33	14.67 ± 0.52
Platelets (10^3/mm^3)	750.40 ± 28.71	716.90 ± 31.29	657.80 ± 34.07
VPM (µm^3)	6.92 ± 0.11	6.92 ± 0.16	6.99 ± 0.13
WBC (10^3/mm^3)	16.01 ± 0.99	11.46 ± 0.78*	15.41 ± 1.16
Neuthrophils (%)	10.43 ± 0.85	10.47 ± 0.83	11.15 ± 0.81
Eosinophils (%)	0.89 ± 0.31	0.26 ± 0.22	0.19 ± 0.18
Basophils (%)	0.56 ± 0.05	0.44 ± 0.08	0.35 ± 0.04*
Lynfocytes (%)	84.85 ± 1.13	85.17 ± 1.04	83.32 ± 1.44
Monocytes (%)	3.27 ± 0.39	3.66 ± 0.35	4.99 ± 0.87

Values represent the mean ± S.E.M. (n = 10/group). MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; WBC, white blood cell; MPV, mean platelet volume. *Statistically different of the control group (ANOVA, followed by Newman-Keuls test, $p < 0.05$).

Table 2: Effect of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) on biochemical parameters in male Wistar rats during 30 consecutive days of treatment.

Biochemical parameters	Control	Cf 300 mg/kg	Cf 1500 mg/kg
Glucose (mg/dL)	92.89 ± 6.12	98.78 ± 3.52	92.90 ± 2.04
BUN (mg/dL)	33.44 ± 1.16	29.56 ± 1.65	31.40 ± 1.45
Creatinine (mg/dL)	0.71 ± 0.03	0.70 ± 0.02	0.65 ± 0.02
AST (U/L)	108.20 ± 5.93	106.20 ± 6.67	108.00 ± 6.13
ALT (U/L)	50.22 ± 1.86	51.89 ± 1.70	52.10 ± 1.49
Total bilirubin (mg/dL)	0.100 ± 0.005	0.110 ± 0.002	0.120 ± 0.006*
Direct bilirubin (mg/dL)	0.07 ± 0.01	0.06 ± 0.01	0.10 ± 0.01*
Indirect bilirubin (mg/dL)	0.040 ± 0.007	0.050 ± 0.006	0.030 ± 0.004
Alkaline phosphatase (U/L)	138.90 ± 12.72	130.70 ± 5.16	121.80 ± 6.77
GGT (U/L)	6.00 ± 0.37	5.56 ± 0.18	6.00 ± 0.21
Total cholesterol (mg/dL)	99.56 ± 11.13	83.00 ± 5.91	78.20 ± 3.35
Amylase (U/L)	498.20 ± 33.22	607.40 ± 22.36*	664.30 ± 39.55*
Uric acid (mg/dL)	1.03 ± 0.06	0.96 ± 0.06	1.03 ± 0.05

Values represent the mean ± S.E.M. (n = 10/group). BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase. *Statistically different of the control group (ANOVA, followed by Newman-Keuls test, $p < 0.05$).

Table 3: Effect of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) on the mass of the organs of male Wistar rats during 30 consecutive days of treatment.

Organs	Control	Cf 300 mg/Kg	Cf 1500 mg/Kg
Adrenal (g)	0.021 ± 0.001	0.024 ± 0.002	0.024 ± 0.003
(g/100 g)	0.008 ± 0.001	0.007 ± 0.001	0.008 ± 0.001
Kidney (g)	0.97 ± 0.05	1.12 ± 0.05	1.11 ± 0.03
(g/100 g)	0.36 ± 0.02	0.34 ± 0.01	0.36 ± 0.01
Lung (g)	1.51 ± 0.09	1.39 ± 0.11	1.48 ± 0.14
(g/100g)	0.57 ± 0.05	0.42 ± 0.03	0.48 ± 0.03
Liver (g)	9.72 ± 0.72	11.16 ± 0.57	10.30 ± 0.38
(g/100 g)	3.60 ± 0.29	3.46 ± 0.16	3.38 ± 0.04
Stomach (g)	1.54 ± 0.01	1.66 ± 0.04	1.68 ± 0.04
(g/100 g)	0.51 ± 0.03	0.51 ± 0.01	0.55 ± 0.01
Heart (g)	0.93 ± 0.06	1.00 ± 0.03	1.10 ± 0.06
(g/100 g)	0.35 ± 0.03	0.31 ± 0.01	0.36 ± 0.01
Testicle (g)	1.40 ± 0.02	1.40 ± 0.06	1.46 ± 0.04
(g/100 g)	0.53 ± 0.03	0.43 ± 0.01	0.46 ± 0.01
Epididymis (g)	0.55 ± 0.04	0.60 ± 0.04	0.64 ± 0.03
(g/100 g)	0.20 ± 0.01	0.18 ± 0.01	0.19 ± 0.01
Seminal vesicle (g)	0.96 ± 0.07	1.14 ± 0.10	1.29 ± 0.06
(g/100 g)	0.34 ± 0.02	0.34 ± 0.03	0.42 ± 0.02
Vas deferens (g)	0.09 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
(g/100g)	0.035 ± 0.002	0.039 ± 0.002	0.039 ± 0.002
Spleen (g)	0.95 ± 0.16	0.66 ± 0.02	1.14 ± 0.14
(g/100 g)	0.38 ± 0.09	0.20 ± 0.01	0.37 ± 0.04
Pancreas (g)	0.84 ± 0.09	0.89 ± 0.08	0.86 ± 0.08
(g/100 g)	0.29 ± 0.02	0.28 ± 0.03	0.29 ± 0.03

Values represent the mean ± S.E.M. (n = 10/group).

6. Artigo III

Toxic effects on gastrointestinal tract from mice treated with the aqueous extract of stem bark of *Caesalpinia ferrea*

(Artigo a ser submetido)

Toxic effects on gastrointestinal tract from mice treated with the aqueous extract of stem bark of *Caesalpinia ferrea*

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Abstract

Ethnopharmacology relevance: In folk medicine, various parts of *Caesalpinia ferrea* Martius Ext Tul. are used to treat a wide range of diseases. The aqueous extract of stem bark of *Caesalpinia ferrea* present wound healing, antidiabetic and hypotensive properties.

Aim of the study: To evaluate the possible toxic effects of the chronic administration of the aqueous extract of stem bark of *Caesalpinia ferrea* in mice.

Methods: Mice were treated with vehicle or aqueous extract of stem bark *of C. ferrea* (300 and 1500 mg/kg/day) *per os* for 90 consecutive days. Changes in body mass and food and water intake were evaluated. Histopathologic studies and analysis of biochemical and haematological parameters were done.

Results: The *C. ferrea*-treated mice presented decrease in body mass probably by impairment of nutrients intestinal absorption. Their intestine presented histopathological changes. Urea, Amylase, AST and LDH levels were altered in mice treated with aqueous extract of stem bark of *C. ferrea* and the leucocyte count was augmented.

Conclusion: The results of the present study suggest toxicity of the aqueous extract of stem bark of *Caesalpinia ferrea,* mainly at the highest dose, as evidenced by impairment of rat growth, some biochemical and haematological alterations and development of enterotoxicity.

Keywords: Caesalpinia ferrea Martius Ext Tul., chronic toxicity, biochemical and haematological parameters.

1.0. Introduction

Caesalpinia ferrea Martius Ext Tul (Leguminosae), popularly known as“pau-ferro” or “jucá”, is a large tree that grows abundantly in Brazil (Di Stasi et al., 2002). In folk medicine, various parts of *C. ferrea* are used to treat a wide range of diseases.

Carvalho et al. (1996) have seen that the aqueous crude extracts from the fruit of *C. ferrea* presented anti-inflammatory action, accessed by the paw edema test in rats and by the abdominal writhes test in mice. An anti-ulcer activity was observed by Bachi et al., 1995. The fruits of *C. ferrea* showed cancer chemoprotective properties (Nakamura et al., 2002a; Nakamura et al., 2002b) and antibacterial activity (Ximenes et al., 2004). Carvalheiro et al (2009) have seen an anticoagulant activity of the seeds of *C. ferrea*.

Moreover, the stem bark has also been shown to have various pharmacological activities. Oliveira et al. (2010) have shown a wound healing activity in goats. Menezes et al. (2007) have observed that the stem bark extract induced a dose-dependent fall in blood pressure and an endothelium-independent relaxation, which involves activation of ATP-sensitive potassium channels. These authors have also observed an arrhythmogenic effect.

A preliminary phytochemical screening of hydroalcoholic extract of the stem bark and leaves demonstrated the presence of flavonoids, saponins, tannins, coumarins, steroids and phenolic compounds (Gonzalez et al., 2004). Tannins were the major compounds found (Souza et al., 2006).

Although *Caesalpinia ferrea* is widely used in folk medicine there is little toxicological information concerning the safety the use of this plant. Therefore, the aim of this study was to evaluate the possible toxic effects of the chronic administration of the aqueous extract of stem bark of *Caesalpinia ferrea* in mice.

2.0. Material and Methods

2.1. Plant material and extraction

Bark from the stem of *Caesalpinia ferrea* Mart. Ex Tul. was collected from the Amazon Research National Institute (INPA) experimental culture, in the Brazilian

State of Amazonas (03º 05’ 48.0”S and 59º 59’ 55.0’’W.Gr). The voucher specimen of the plant was deposited at the INPA herbarium under number 228022. The bark was collected in September 2009, March 2010 and September 2010, with approximately 3kg being harvested on each occasion. The material collected was dried, first at room temperature for 48h, and then taken to an oven with circulating air at a temperature of 45±2 °C until its weight stabilized. Subsequently, the material was ground in a 1mm mesh knife mill, thereby providing the raw material (MPV). The aqueous extract of *Caesalpinia ferrea* was prepared by raw material infusion (7.5:100, w/v) using boiling distilled water as the extractive solvent for a period of 15 minutes. The aqueous extract presented a solid soluble content of 0.6±0.02g%. The aqueous extract was dried using a MSD 1.0 Labmaq Mini Spray Dryer. The drying process was performed using the following parameters: inlet temperature of 120 °C, compressed air pressure of 2bar, diameter rotor of 0.7mm and power flow of 10mL/min. The yield of dry extract after drying of aqueous extract was 98%, representing 5.88g of dry extract per liter of aqueous extract of *Caesalpinia ferrea.*

2.2. Phytochemical analysis of Caesalpinia ferrea

2.2.1. Thin Layer Chromatographic (TLC) Analysis of Caesalpinia ferrea

The methods described by Wagner and Bladt (1996) were used to screen the dried bark extract for the hydrolysable tannins (gallic and ellagic acids), condensed tannins (catechins), flavonoids, saponins, coumarins, phenylpropanoids, cinnamic acid derivatives, alkaloids, triterpenoids/steroids, monoterpenes, sesquiterpenes, iridoids, sugars and luteolin. The phytochemical profile was drawn up using thin layer chromatography (TLC) on silica gel plates (Merck® art. 105553, UV 250–366nm) using the appropriate mobile phase, reagents and standards.

2.2.2. Estimation of total tannin content in Caesalpinia ferrea

The total tannin content was determined using the spray dried extract (SDE) of *Ceasalpinia ferrea* aqueous extract at 7.5% (w/v), by way of the difference between

redissolved SDE before and after precipitation with 150mg of casein (Merck® Germany). The measurements were performed at 270nm and the total tannin content was calculated as gallic acid (mg/g of SDE). The results represent the mean of three measurements.

2.2.3. High Performance Liquid Chromatography (HPLC) analysis of Caesalpinia ferrea

The main phytochemical markers (gallic acid, ellagic acid, catechin and epicatechin) were quantified by way of LC-DAD analysis using a Shimadzu system (LC-20AT) equipped with a photo diode array detector (SPD-M20A). The chromatographic separation was performed using a Gemini RP-18 column 240 x 4mm i.d. (Phenomenex), protected by a pre-column packet of the same material. A gradient elution was performed by varying the proportion of solvent B (methanol) to solvent A (acetic acid 0.5%; w/w) at a flow rate of 0.8mL/min, according to the following gradient program: 20-40% B (10min), 40-60% B (10min), 60% B (10min), 60-40% B (10min), 40-20% B (10min). The SDE of *Caesalpinia ferrea* and standard were dissolved in methanol:water (20/80, v/v) and filtered through a 0.45µm membrane (Millipore®, USA) prior to injection of 20µL.

The peaks of each marker substance in the dried extract were initially identified by comparing the retention time and UV-spectrums. After that, the peaks were confirmed by spiking the sample with a small amount of the standards.

2.3. Animals

Male Swiss mice (aged 2 months, weighing 45-50 g) were obtained from the Department of Physiology and Pharmacology at the Federal University of Pernambuco (UFPE), Pernambuco, Brazil. The animals were kept under standard environmental conditions (22 ± 2^{o}C; 12:12 h dark/light cycle). Water and industrialized dry food (Labina®, Purina, Brazil) were made available *ad libitum*. The experimental protocol was approved by the Animal Experimentation Ethics

Committee of UFPE (Process nº. 01411), in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

2.4. Treatment

The mice were divided into 3 groups (n=10/ group).The animals were treated orally with vehicle and aqueous extract of stem bark of *C. ferrea* at 300 and 1500 mg/kg daily, for 90 consecutive days.

Since the aqueous extract of stem bark of *C. ferrea* has been used in an acute toxicity assessment on the reproductive system of Wistar rats at the dose of 300 mg/kg of body weight (Reboredo et al. 2006), the same dose was selected in this experiment. This dose level also corresponds to the dose used on previous studies for the evaluation of the analgesic and anti-inflammatory properties of this plant (Carvalho et al. 1996). We have also used a dose that corresponds to five times the smaller dose.

2.5. Evaluation of the body mass gain

The animals were observed daily for abnormalities of condition or behavior. They were weighed initially and then once a week throughout the study and before necropsy.

2.6. Water and food intake

The amount of supplied and residual water and food was measured weekly in order to calculate the average consumption each week. Standard mice chow and fresh tap water were provided *ad libitum.*

2.7. Morphological study

After the animals were euthanized with an excess of Nembutal® (140 mg/kg, i.p.), necropsy was performed (n = 5/group) to analyze the macroscopic external features of the heart, liver, spleen, lungs, kidney, adrenal gland, stomach, and

reproductive organs (testicle, epididymis, seminal vesicle and vas deferens). These organs were carefully removed and weighed individually. Organ weights were expressed in absolute and relative terms (g and g/100 g of body weight, respectively). Histological examination was performed in five animals per group/sex. Animals were anesthetized with Nembutal® (35 mg/kg, i.p.), perfused with saline solution (for removal of blood) followed by buffered formalin solution (10%) for 10 min and the same organs were removed and fixed in Bouin solution for 48 h at room temperature. Tissue slices were processed according to the method described by Lison (1960) and stained with hematoxylin/eosin.

2.8. Haematological and biochemical analysis

Haematological analysis was performed using an automatic haematological analyzer (Coulter STKS, Beckman). Parameters included: red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets count and mean platelet volume (MPV). The differential leukocyte count was performed with an optical microscopy after staining and, in each case, 100 cells were counted. For biochemical analysis, blood was centrifuged at 1500×g for 10 min to obtain serum, which was stored at −20 ºC until the following parameters were determined: glucose; blood urea nitrogen (BUN); creatinine; aspartate aminotransferase (AST); alanine aminotransferase (ALT); total protein; albumin; lactate dehidrogenase (LDH) total cholesterol; triglycerides; amylase; uric acid and total, direct and indirect bilirubin. Dosages were made using Architect (Abott®) automation with Boehringer Ingelheim® biochemical kits.

2.9. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 software. The difference between groups was assessed by analysis of variance (ANOVA), followed, when necessary, by Newman-Keuls test. The significance level for rejection of the null hypothesis was always $\geq$ 5%.

3.0. Results

3.1. Phytochemical screening of Caesalpinia ferrea

TLC for chemical identification of the constituents of *Caesalpinia ferrea* bark revealed the presence of condensed tannins (catechins) and hydrolysable tannins (gallic and ellagic acids). The total tannin content of *Caesalpinia ferrea* SDE was calculated as 266mg/g. On the other hand, the content for HPLC of gallic acid (1), catechin (2), epicatechin (3) and ellagic acid (4) was 112.76, 17.75, 6.13 and 12.00mg/g (chromatogram not shown).

3.2. Body mass gain

As can be seen in Figure 1, the groups treated with *C. ferrea* did not present the body mass gain as presented by the control group. Instead, the groups treated with *C. ferrea* presented a decrease in the body mass in the initial phase of the treatment, and by the seventh week, they had their body mass stabilized.

3.3. Food and water intake

As can be seen in Figures 2 and 3, there was a variation in the food and water intake in the group receiving both doses of *C. ferrea* when compared to the control group. There were moments when the consumption was higher and moments when this consumption was lower. The eighth week onwards, the decreased food and water intake becomes clear until the end of treatment.

3.4. Morfological studies

The effects of *C. ferrea* on masses of adrenal glands, kidney, lung, liver, stomach, heart, testicles, epididymis, seminal vesicle, deferens vas, small intestine and pancreas are given in table 3.

The treatment with *C. ferrea* 300 mg/kg/day induced a significant increase in adrenal relative mass and the treatment with *C. ferrea* 1500 mg/kg/day induced a significant increase in adrenal relative as well as absolute masses.

The relative mass of the kidney and heart was increased by the treatment with *C. ferrea* 300 mg/kg/day and the heart absolute mass by the treatment with the highest dose was reduced.

The relative mass of the stomach was increased by the treatment with *C. ferrea* in the doses of 300 and 1500 mg/kg/day. The relative and absolute masses of the small intestine were augmented by the treatment with *C. ferrea* 1500 g/kg/day.

The relative mass of epididymis was higher in the mice from the group treated with *C. ferrea* 1500 mg/kg/day. The seminal vesicle absolute mass was reduced in the mice treated with *C. ferrea* 1500 mg/kg/day.

3.5. Histopathological examination of organs

All organs examined were normal, except the small intestine that presented an intraepithelial lymphocytic infiltrate and areas of necrosis (Figure 4).

3.6. Haematological and Biochemical analysis

Haematological parameters of *C. ferrea* treated mice are showed in table 1. The erythrocyte count, hematocrit and hemoglobin concentration were not altered by the treatment with *C. ferrea*. The mean corpuscular volume was decreased in the group that received *C. ferrea* 1500 mg/kg/day and the mean corpuscular hemoglobin concentration was increased in both groups treated with *C. ferrea.* The platelet count was not changed by the treatment. However, there was a decrease in mean platelet volume. The leucocyte count was higher in the group treated with *C. ferrea* 1500 mg/kg/day and the relative count of neutrophils was diminished in this group.

Table 2 shows the effects of the treatment with *C. ferrea* 300 and 1500 mg/kg/day on biochemical parameters. The mice treated with *C. ferrea* 300 mg/kg/day showed statistic reduction in urea. Both treated groups presented reduction in total proteins and albumin and an increase in amylase and AST, when compared to control group.

4.0. Discussion

Although the animals did no present behavioral or physical changes, the animals presented a decrease in body mass when treated with the aqueous extract of stem bark of *C. ferrea*. A reduction in body mass is indicative of toxic action of a substance in the organism.

Various studies show impairment in growth of animals treated with tannin-rich diet. Rats had their growth depressed when carob tannins were added to their diet (Tamir and Alumot, 1970). The authors have observed that carob tannins acted by suppression of food intake. In our study, however, the animals present a decrease in body mass probably by impairment of nutrients intestinal absorption. Joslyn and Glick (1969) have also seen that the intake of tannin acid, gallic acid or catechin induced a decrease in body mass gain in rats in a dose-dependent way and suggested that the tannins could reduce the absorption of dietary components.

In fact, in contrast to the position with ruminant animals which tannins in the diet may have considerable benefits, and in plants where tannins give partial protection against predators, in simple-stomached animals, including man, tannins in the diet are generally undesirable, except in small amounts where mild adstringency gives ‘character’ and ‘bite’ to food or drink (Mangan,1988).

The apparent digestibility of dry matter and crude protein were diminished by the presence of tannins in diet of rats (Horigome et al., 1984). Also in birds, feed conversion efficiency was reduced by tannins in diet. This feed conversion efficiency was reduced by more than a half in birds that received high amount of tannins compared to control group. Moreover, the apparent digestibility of protein was negatively correlated with tannin content of the diet (Ahmed et al., 1991).

Since tannins interact with proteins, forming complexes, the reduction in digestibility of nutrients may be due to interaction of tannins with digestive enzymes, what could inactivate the enzymes. In fact, the activities of trypsin and α-amylase were inhibited in the gut of birds as the level of seed tannins in the diet increased (Ahmed et al., 1991). Moreover, Horigome et al. (1988) have seen that the presence of tannins in the diet decreased the *in vitro* activity of trypsin and α-amylase on upper, middle and lower segments of small intestine in rats. The inhibitory effects increased with increasing polymerization, as did the protein-precipitating capacity.

These results suggest that the loss of enzyme activity was principally due to formation of an insoluble enzyme-tannin complex, which is inactive.

Another possibility for the tannins to cause reduction in digestibility of nutrients is that tannins may increase faecal excretion of some nutrients, particularly amino acids (Mangan et al., 1988). Moreover, it is possible that tannins may impair absorption of nutrients by direct damage in gastrointestinal tract. In fact, in addition to the decrease in growth, the *C. ferrea* feed mice at highest dose presented an augment in the relative mass of intestine, as well as histopathological changes in this organ. The intestine presented limphocytic intraepithelial infiltrate and areas of necrosis. An increase in relative mass of intestine was also observed in chickens treated with tannic acid (Mansoori et al. 2007). Hervas et al. (2003) have also observed necrosis of the intestinal villi, demarcated by an inflammatory infiltrate, in sheeps after feed with commercial quebracho tannin. These authors state that pigmented material in the intestine was always associated with necrotic areas and a neutrophilic infiltrate, suggesting that it appeared after damage to the intestinal epithelium had occurred.

Moreover, a hypersecretion of gastric mucus was noted in rats treated with tannic acid by Mitjavila et al. (1977). They have also seen a necrotic effect on gastric mucosa, part of the superficial mucosa was eroded. Similarly, in the duodenum, the secretion of mucus was increased with tannins. Necrosis points and eroded zones were also seen. Since there is a close association between integrity of intestine membrane and the absorption of nutrients, it is likely that the tannins exert an indirect effect on absorption through the damage cited above.

These data are reinforced by the fact that in our study, the mice treated with *C. ferrea* extract presented a decrease in plasma total proteins and albumin. This decreased can be associated with enteropathies. Other organs, such as lung, liver, testicle, deferens vas and pancreas were not affected by *C. ferrea* treatment in our study. Lucinda et al. (2010) have also seen that the treatment with the aqueous extract of the fruits of *C. ferrea* for 52 or 104 consecutive days did not change the mass of brain, pituitary, liver, lung, kidneys, testicle, epididyimis, seminal vesicle and prostate.

The relation between the toxicity of the extract and the serum enzymes comes from the fact that as the toxicity increases, the degree of tissue destruction increases

and consequently the levels of these serum enzymes, as a result of mobilization from the damaged tissues (Assi and Nasser, 1999). The mechanism whereby the plant constituents damage vital body organs cannot be derived from the present investigation, but the injury to these tissues probably contributed to the raised serum amylase, AST and LDH observed in our study. Mansoori et al. (2007) have seen that tannic acid reduced the activity of LDH, ALT and AST in chickens. On the other hand, Hervás et al. (2003) have not observed changes in the activity of ALP, GGT and AST in sheeps feed with quebracho tannin extract, but they have seen an increase in the activity of ALT. In general, the blood amylase rises due damages in the small intestine that compromise the absorption carbohydrate even without changes pancreatic (Dreiling et al., 1958).

In haematological parameters evaluation, it was observed that there were changes in mean corpuscular volume and in mean corpuscular hemoglobin concentration, but since the parameters of eritrocytes count, hematocrit and hemoglobin were not affected by the treatment with *C. ferrea*, this data do not deserve too much attention. In accordance to the histopathological changes and the biochemical findings, the mice treated with the extract of *C. ferrea* presented the leucocyte count augmented.

The results of the present study suggest toxicity of the aqueous extract of stem bark of *Caesalpinia ferrea,* mainly at the highest dose, as evidenced by impairment of rat growth, some biochemical and haematological alterations and development of enterotoxicity.

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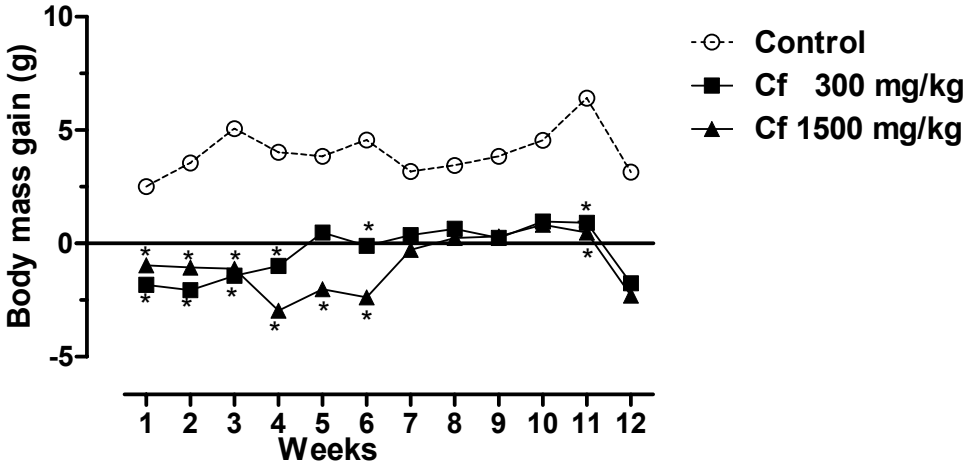

Fig. 1: Effect of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg/day) on body mass gain (g) of mice. The results are expressed as mean ± S.E.M. (n=10/group). *Statistically different from control, $p<0.05$ (one-way ANOVA, followed by Newman-Keuls).

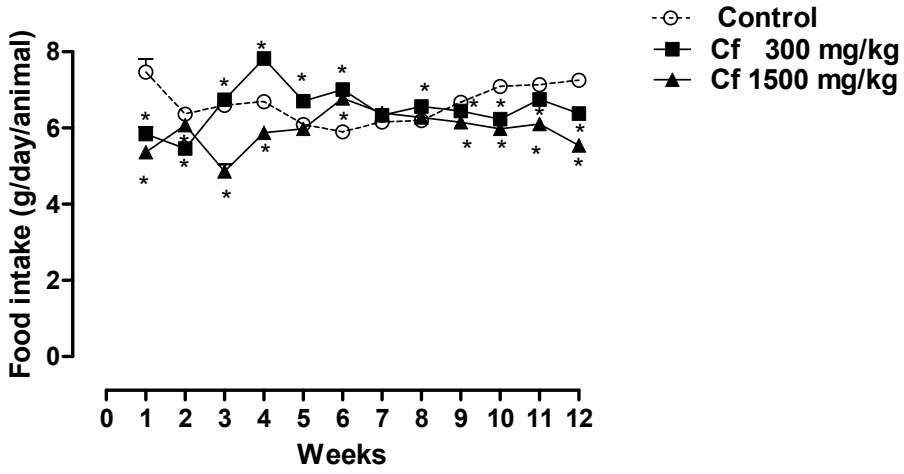

Fig. 2: Effect of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg/day) on food intake (g/day/animal) of mice. The results are expressed as mean ± S.E.M. (n=10/group). *Statistically different from control, $p<0.05$ (one-way ANOVA, followed by Newman-Keuls).

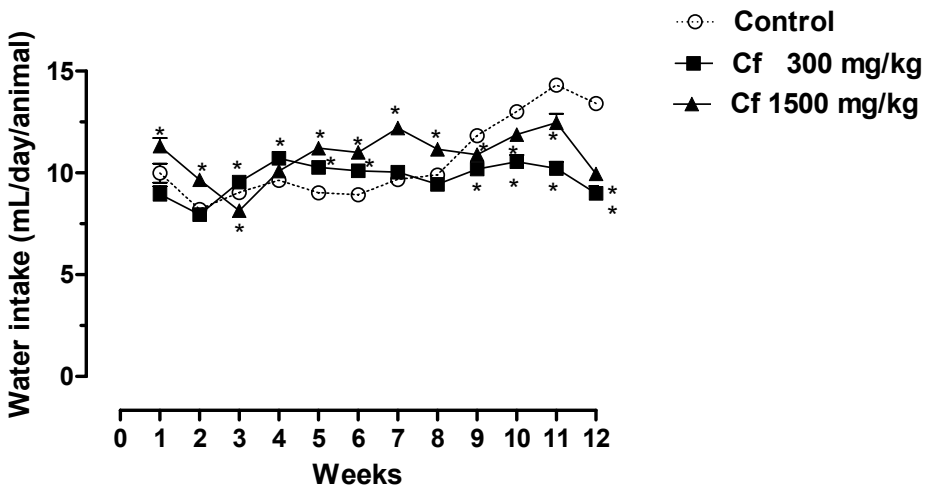

Fig. 3: Effect of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg/day) on water intake (mL/day/animal) in mice. The results are expressed as mean ± S.E.M. (n=10/group). *Statistically different from control, $p<0.05$ (one-way ANOVA followed by Newman-Keuls).

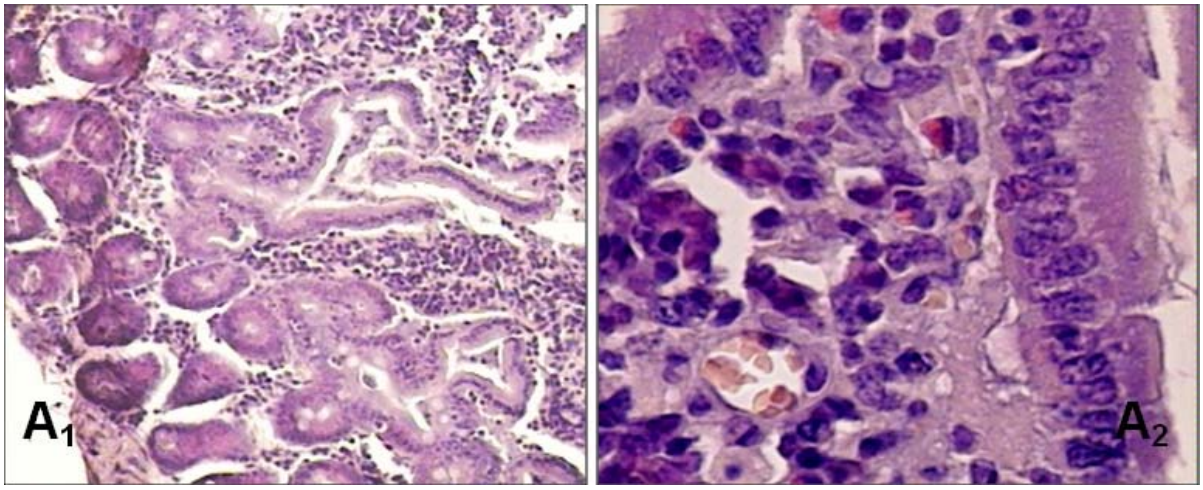

Fig. 4: Intestine histological sections of mice treated with aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 1500 mg/kg/day) (HE) – A1 and A2 (magnification: 100x e 400x): areas of lymphocytic infiltrate and necrosis in intestine.

Table 1: Effect of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) on haematological parameters in male mice during 90 consecutive days of treatment.

Haematological parameters	Control	Cf 300 mg/kg	Cf 1500 mg/kg
Erythrocytes (10^6/mm^3)	9.15 ± 0.40	9.63 ± 0.28	9.90 ± 0.15
Hemoglobin (g/dL)	14.92 ± 0.76	15.86 ± 0.38	15.67 ± 0.16
Hematocrit (%)	46.51 ± 0.67	47.63 ± 1.11	46.68 ± 0.27
MCV (µm^3)	49.75 ± 0.77	49.75 ± 0.53	46.00 ± 0.46*
MCH (pg)	16.03 ± 0.32	16.47 ± 0.17	15.50 ± 0.16
MCHC (g/dL)	32.87 ± 0.22	33.49 ± 0.05*	33.58 ± 0.21*
RDW (%)	15.10 ± 0.57	15.40 ± 0.56	15.54 ± 0.22
Platelets (10^3/mm^3)	1130 ± 71.18	1026 ± 42.40	1192 ± 36.52
MPV (µm^3)	5.10 ± 0.05	5.12 ± 0.07	4.86 ± 0.06*
WBC (10^3/mm^3)	7.40 ± 0.24	7.32 ± 0.47	12.38 ± 0.55*
Neuthrophils (%)	4.41 ± 0.50	3.95 ± 0.46	2.24 ± 0.21*
Eosinophils (%)	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Basophils (%)	0.37 ± 0.07	0.35 ± 0.07	0.34 ± 0.04
Lynfocytes (%)	94.49 ± 0.50	94.61 ± 0.46	96.69 ± 0.37
Monocytes (%)	1.04 ± 0.20	0.92 ± 0.19	0.86 ± 0.05

Values represent the mean ± S.E.M. (n = 10/group). MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; WBC, white blood cell; MPV, mean platelet volume. *Statistically different of the control group (ANOVA, followed by Newman-Keuls test, $p < 0.05$).

Table 2: Effect of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) on biochemical parameters in male mice during 90 consecutive days of treatment.

Biochemical parameters	Control	Cf 300 mg/kg	Cf 1500 mg/kg
Glucose (mg/dL)	90.13 ± 7.47	96.75 ± 5.63	90.38 ± 4.88
BUN (mg/dL)	56.25 ± 2.19	49.50 ± 1.55*	54.25 ± 1.57
Creatinine (mg/dL)	0.20 ± 0.02	0.20 ± 0.02	0.24 ± 0.02
AST (U/L)	80.75 ± 6.80	125.20 ± 11.12*	143.00 ± 11.26*
ALT (U/L)	34.63 ± 4.73	32.38 ± 2.56	42.75 ± 2.25
Total bilirubin (mg/dL)	0.16 ± 0.04	0.14 ± 0.02	0.15 ± 0.02
Direct bilirubin (mg/dL)	0.12 ± 0.02	0.11 ± 0.01	0.12 ± 0.02
Indirect bilirubin (mg/dL)	0.04 ± 0.03	0.02 ± 0.02	0.02 ± 0.01
Total proteins (g/L)	5.07 ± 0.06	4.79 ± 0.06*	4.54 ± 0.07*
Albumin (g/L)	2.86 ± 0.13	2.46 ± 0.07*	2.35 ± 0.12*
Triglycerides (mg/dL)	103.10 ± 6.19	115.10 ± 8.2	111.60 ± 6.75
Total cholesterol (mg/dL)	112.90 ± 3.91	112.30 ± 3.19	102.00 ± 3.24
Amylase (U/L)	1000.00 ± 15.50	1190.00 ± 13.09*	1375.00 ± 14.87*
Uric acid (mg/dL)	2.40 ± 0.19	2.34 ± 0.20	1.97 ± 0.01
LDH (U/L)	370.40 ± 25.29	322.10 ± 15.49	517.50 ± 19.43*

Values represent the mean ± S.E.M. (n = 10/group). BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehidrogenase.

*Statistically different of the control group (ANOVA, followed by Newman-Keuls test, $p < 0.05$).

Table 3: Effect of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) on the mass of the organs of male mice during 90 consecutive days of treatment.

Organs	Control	Cf 300 mg/Kg	Cf 1500 mg/Kg
Adrenal (g)	0.008 ± 0.001	0.012 ± 0.001	0.015 ± 0.002*
(g/100 g)	0.017 ± 0.002	0.028 ± 0.002*	0.036 ± 0.004*
Kidney (g)	0.33 ± 0.01	0.33 ± 0.01	0.30 ± 0.01
(g/100 g)	0.70 ± 0.02	0.78 ± 0.01*	0.72 ± 0.01
Lung (g)	0.28 ± 0.01	0.26 ± 0.01	0.27 ± 0.01
(g/100g)	0.57 ± 0.05	0.42 ± 0.03	0.48 ± 0.04
Liver (g)	2.35 ± 0.17	2.18 ± 0.10	2.22 ± 0.11
(g/100 g)	4.96 ± 0.46	5.09 ± 0.19	5.36 ± 0.22
Stomach (g)	0.30 ± 0.03	0.33 ± 0.01	0.34 ± 0.02
(g/100 g)	0.62 ± 0.04	0.78 ± 0.03*	0.82 ± 0.05*
Heart (g)	0.23 ± 0.01	0.23 ± 0.01	0.18 ± 0.01*
(g/100 g)	0.49 ± 0.02	0.56 ± 0.02*	0.43 ± 0.02
Testicle (g)	0.136 ± 0.007	0.122 ± 0.004	0.120 ± 0.010
(g/100 g)	0.28 ± 0.01	0.28 ± 0.01	0.33 ± 0.02
Epididymis (g)	0.054 ± 0.004	0.049 ± 0.002	0.058 ± 0.002
(g/100 g)	0.111 ± 0.007	0.119 ± 0.004	0.140 ± 0.006*
Seminal vesicle (g)	0.32 ± 0.02	0.31 ± 0.01	0.25 ± 0.01*
(g/100 g)	0.67 ± 0.05	0.68 ± 0.03	0.62 ± 0.04
Vas deferens (g)	0.019 ± 0.001	0.019 ± 0.001	0.015 ± 0.001
(g/100g)	0.042 ± 0.004	0.043 ± 0.004	0.036 ± 0.004
Small intestin (g)	2.44 ± 0.27	2.04 ± 0.11	3.14 ± 0.26*
(g/100 g)	4.35 ± 0.59	4.79 ± 0.27	7.62 ± 0.66*
Pancreas (g)	0.270± 0.024	0.29 ± 0.02	0.26 ± 0.03
(g/100 g)	0.56 ± 0.03	0.68 ± 0.06	0.62 ± 0.07

The values represent mean ± SEM. *Statistically significant from control group (one-way ANOVA, followed by Newman-Keuls, $p<0.05$).

7. Artigo IV

The role of *Caesalpinia ferrea* Martius (Leguminosae) stem bark extract on pregnancy and postnatal development of offspring of female Wistar rats

(Artigo a ser submetido)

The role of *Caesalpinia ferrea* Martius (Leguminosae) stem bark extract on pregnancy and postnatal development of offspring of female Wistar rats

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Abstract

Ethnopharmacological relevance: Caesalpinia ferrea Martius Ext Tul (Leguminosae) has long been used in traditional Brazilian medicine, especially to treat diabetes.
Aim of study: The objective of this study was investigate the toxicity of the aqueous extract of stem bark of *Caesalpinia ferrea* on pregnancy and postnatal development of offspring of female Wistar rats.
Method: The pregnant rats were divided into 3 groups (n=9/group). The control group received vehicle and other groups were treated with the aqueous extract of stem bark of *Caesalpinia ferrea* (Cfae) at doses 300 and 1500 mg/kg, by oral route, during whole pregnancy. Maternal and offspring behavioral parameters were then evaluated.
Results: Treatment with Cfae caused deaths of fetuses at dose 300 mg/kg, but it was similar to the control, and promoted reduction in the body mass gain at the highest dose, however it did not affect offspring normal development and nor caused death of pregnant rats.
Conclusion: The treatment with Cfae suggests some maternal toxicity, however further studies should be conducted to ensure its use during human gestation.

Keywords: .*Caesalpinia ferrea* Martius Ext Tul, Maternal toxicity, Offspring development.

1.0. Introduction

Caesalpinia ferrea Martius Ext Tul is a plant widely used in Brazilian folk medicine as anti-inflammatory, healing, and aid in the treatment of anemia (Carvalho et al.,1996) and it is a species selected by National Medicinal Plants and Herbal Program of Health Ministry (Brazil, 2006). Popularly, various parts of the plant (leaves, bark, seeds, roots, pieces of wood and nuts) are used in treating various diseases like diabetes. In Brazil, many people including pregnant women use either bark aqueous extract of *Caesalpinia ferrea* as herbal therapy for diabetes (Borrás et al., 2003; Di Stasi et al., 2002).

Ellagic acid isolated from fruits of *Caesalpinia ferrea* might be able to relieve diabetic complications (Ueda et al., 2004). The crude extract of *Caesalpinia ferrea* contains anthraquinones, alkaloids, coumarins, depsides, depsidones, flavonoids, lactones, saponins, sitosterol, sugars, tannins, sesquiterpenes and triterpenes. (Gonzalez et al., 2004; Souza et al., 2006). Although it contains substances such as flavonoids and coumarin, which are known by antifertility activity, male rats treated with the bark aqueous extract of *Caesalpinia ferrea* at dose 300 mg/kg of body weight, presented no impediment in spermatogenic cycles (Lucinda et al., 2010).

The treatment with oral hypoglycemic agents in gestational diabetes remains a controversial topic since even a minor degree of hypoglycemia can adversely affect the reproductive outcome (Ratnasooriya et al., 2003). Therefore, the possible harmful effects on pregnancy outcome after *in utero* exposure of the aqueous extract of stem bark of *Caesalpinia ferrea* have not been investigated. Thus, this study was conducted to investigate the effects of the oral administration of the aqueous extract of stem bark of *Caesalpinia ferrea* on pregnant Wistar rats, during whole pregnancy and on their offspring postnatal development.

2.0. Material and Methods

2.1. Plant material and extraction

Bark from the stem of *Caesalpinia ferrea* Mart. Ex Tul. was collected from the Amazon Research National Institute (INPA) experimental culture, in the Brazilian

State of Amazonas (03º 05’ 48.0”S and 59º 59’ 55.0’’W.Gr). The voucher specimen of the plant was deposited at the INPA herbarium under number 228022. The bark was collected in September 2009, March 2010 and September 2010, with approximately 3kg being harvested on each occasion. The material collected was dried, first at room temperature for 48h, and then taken to an oven with circulating air at a temperature of 45±2 °C until its weight stabilized. Subsequently, the material was ground in a 1mm mesh knife mill, thereby providing the raw material (MPV). The aqueous extract of *Caesalpinia ferrea* was prepared by raw material infusion (7.5:100, w/v) using boiling distilled water as the extractive solvent for a period of 15 minutes. The aqueous extract presented a solid soluble content of 0.6±0.02g%. The aqueous extract was dried using a MSD 1.0 Labmaq Mini Spray Dryer. The drying process was performed using the following parameters: inlet temperature of 120 °C, compressed air pressure of 2bar, diameter rotor of 0.7mm and power flow of 10mL/min. The yield of dry extract after drying of aqueous extract was 98%, representing 5.88g of dry extract per liter of aqueous extract of *Caesalpinia ferrea.*

2.2. Phytochemical analysis of Caesalpinia ferrea

2.2.1. Thin Layer Chromatographic (TLC) Analysis of Caesalpinia ferrea

The methods described by Wagner and Bladt (1996) were used to screen the dried bark extract for the hydrolysable tannins (gallic and ellagic acids), condensed tannins (catechins), flavonoids, saponins, coumarins, phenylpropanoids, cinnamic acid derivatives, alkaloids, triterpenoids/steroids, monoterpenes, sesquiterpenes, iridoids, sugars and luteolin. The phytochemical profile was drawn up using thin layer chromatography (TLC) on silica gel plates (Merck® art. 105553, UV 250–366nm) using the appropriate mobile phase, reagents and standards.

2.2.2. Estimation of total tannin content in Caesalpinia ferrea

The total tannin content was determined using the spray dried extract (SDE) of *Ceasalpinia ferrea* aqueous extract at 7.5% (w/v), by way of the difference between

redissolved SDE before and after precipitation with 150mg of casein (Merck® Germany). The measurements were performed at 270nm and the total tannin content was calculated as gallic acid (mg/g of SDE). The results represent the mean of three measurements.

2.2.3. High Performance Liquid Chromatography (HPLC) analysis of Caesalpinia ferrea

The main phytochemical markers (gallic acid, ellagic acid, catechin and epicatechin) were quantified by way of LC-DAD analysis using a Shimadzu system (LC-20AT) equipped with a photo diode array detector (SPD-M20A). The chromatographic separation was performed using a Gemini RP-18 column 240 x 4mm i.d. (Phenomenex), protected by a pre-column packet of the same material. A gradient elution was performed by varying the proportion of solvent B (methanol) to solvent A (acetic acid 0.5%; w/w) at a flow rate of 0.8mL/min, according to the following gradient program: 20-40% B (10min), 40-60% B (10min), 60% B (10min), 60-40% B (10min), 40-20% B (10min). The SDE of *Caesalpinia ferrea* and standard were dissolved in methanol:water (20/80, v/v) and filtered through a 0.45µm membrane (Millipore®, USA) prior to injection of 20µL.

The peaks of each marker substance in the dried extract were initially identified by comparing the retention time and UV-spectrums. After that, the peaks were confirmed by spiking the sample with a small amount of the standards.

2.3. Animals

Pregnant Wistar rats (*Rattus norvegicus* var. *albinus*), aged 3 months and weighing 200 – 240 g, were obtained from the Department of Physiology and Pharmacology at the Federal University of Pernambuco (UFPE), Brazil. They were maintained in standard environmental conditions (22 ± 2ºC, 12:12h dark/light cycle). Water and industrialized dry food (Labina®, Purina, Brazil) were available *ad libitum*. The experimental protocol was approved by the Animal Experimentation Ethic Committee of UFPE (Process nº. 01411), in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.4. Experimental protocol

Observation of the presence of sperm in the vaginal smear was used to establish the 1st day of pregnancy. The study was conducted from 1st to 21th day (whole pregnancy), containing 3 groups of pregnant rats randomly formed (n = 9/group). The control group received vehicle while the others were treated orally with the aqueous extract of stem bark of *Caesalpinia ferrea* at doses 300 and 1500 mg/kg. During pregnancy, the rats were evaluated for survival, altered appearance and any clinical signs of toxicity, such as changes in food and water intake, piloerection, diarrhea, change of locomotor activity and vaginal bleeding.

2.4.1. Study during whole pregnancy

During whole pregnancy, the maternal weight was recorded on days 1, 7, 14 and 21. Pregnant rats were allowed to complete their pregnancy to term. After birth, macroscopic aspects were observed in order to detect possible fetal malformations and were calculated pregnancy index (% pregnant rats with all live pups), viability index (number of live pups on day 4 of postnatal life/number of live offspring born × 100) and lactation index (number of live pups on day 21 of postnatal life / number of live pups on day 4 of postnatal life × 100). The pups body weight and length were determined on days 1, 4, 7, 14 and 21 of postnatal life (Silva et al., 2009).

2.4.2. Offspring behavioral development

After birth, one third of the offspring from each group (control, 300 and 1500 mg/kg of *Caesalpinia ferrea*) was analyzed to the following behavioral parameters: postural reflex, day of eye opening, day of adult walking and spontaneous ambulation. The pups postural reflexes were evaluated on 1st and 7th days of life. The pups were put on plane surface, in supine position, and the righting reflex was measured in seconds. The day of eye opening was determined by observation of partial displacement of the palpebral fissure at least one eye. It was considered day of adult walking when pups ambulated without dragging hind feet and without touching belly on the floor. The spontaneous ambulation was determined on 20th day

postnatal life using a square measuring 30 x 30 cm and divided in nine equal spaces. The pup was put on the central part and during two minutes was recorded the number of invaded square when it put at least three feet on this area (Costa-Silva et al., 2006).

2.4.3. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 software. The difference between groups was assessed by analysis of variance (ANOVA), followed, when necessary, by Newman-Keuls test. The significance level for rejection of the null hypothesis was always $\geq 5\%$.

3. Results

3.1. Phytochemical screening of Caesalpinia ferrea

TLC for chemical identification of the constituents of *Caesalpinia ferrea* bark revealed the presence of condensed tannins (catechins) and hydrolysable tannins (gallic and ellagic acids). The total tannin content of *Caesalpinia ferrea* SDE was calculated as 266mg/g. On the other hand, the content for HPLC of gallic acid (1), catechin (2), epicatechin (3) and ellagic acid (4) was 112.76, 17.75, 6.13 and 12.00mg/g (chromatogram not shown).

3.2. Study during whole pregnancy

The maternal reproductive parameters showed decrease in body mass gain, in the last week, for rats treated with the highest dose and an increase in the body mass of rats treated with 300 mg/kg, in second week, when compared with control group (figure 1). The gestation period in the highest dose group also showed increased compared to the control group (table 1). The same group showed no change in food and water intake during treatment. For the lowest dose no significant changes were observed for these parameters (figures 2 and 3, respectively).

3.3. Offspring behavioral development

The maternal reproductive parameters during whole pregnancy showed increase in the pups body mass on days 1st and 7th and a decrease on days 1st and 4th day postnatal life, at the lower and highest dose, respectively, when compared with control group. On the 21th day postnatal life was observed an increase and a decrease in the length of the pups to the lowest and highest doses, respectively, when compared to the control group (table 2).

The number of death fetuses observed at lower dose was similar to control group (table 1). After birth of offspring, it was not observed external abnormalities and their offspring behavioral parameters were not changed when compared with control groups (table 2).

4. Discussion

This study analyzed the reproductive toxicity of the aqueous extract of stem bark of *Caesalpinia ferrea* on pregnant rats and their offspring behavioral development. The use of *Caesalpinia ferrea* in folk medicine is well known. In Brazil, many diabetic patients take tea made from the stem bark of *Caesalpinea ferrea* daily including pregnant women (Jahangir; Terzic, 2005). The major compounds found in the aqueous extract of the stem bark of *Caesalpinia ferrea* are condensed tannins (catechins) and hydrolysable tannins (gallic acid and ellagic acid) (Souza et al. 2006).

Tannins are reported to have multiple biochemical and pharmacological activities, such as anticarcinogenic, antioxidant, antiangiogenic, antiviral and antidiabetic effects (Gomes et al., 1995; Skrzydlewska et al., 2002). Many of the mentioned beneficial effects of green tea on obesity, type-2 diabetes and cardiovascular risk factors are related to its catechins content (Lambert et al., 2003; Mandel et al., 2004)

In the other hand, condensed tannins bind to proteins in the rumen, reduce protein degradation and when dietary protein concentrations are low and fibre concentrations are high, catechins are nearly always detrimental (Waghorn, 2008). Other secondary metabolites present are hydrolysable tannins, which are potentially toxic, degradable in the rumen and they may have a greater impact than catechins

upon animal performance (Acamovic and Brooker, 2005). This could explain the decrease of body mass gain in the animals treated with the highest dose, since protein demands are highest in pregnant and lactating females.

The low protein content during gestation and lactation is frequently relation to deleterious consequences on perinatal growth. Both perinatal malnutrition and growth retardation at birth are risk factors for diabetic and cardiovascular disturbances in later life (Ravelli et al., 1998).The data suggest a deficit in absorption of proteins and carbohydrates which could explain the delay in the period of pregnancy as well as the reduced weight of offspring until the fourth day of postnatal life for the highest dose group. However, tannins are used in veterinary medicine as adstringent, anti-hemorrhagic, anti-abortive and to stimulation of milk secretion (Carrai et al., 2003). No death of fetuses was observed at the highest dose suggesting no abortive effect of the aqueous extract of stem bark of *Caesalpinia ferrea*, since the deaths found at the dose 300 mg/kg were similar to the control.

The behavioral parameters analyzed in this study were used to verify possible deleterious actions of *Caesalpinia ferrea* on behavioral and neurological development of the offspring (Carlini et al., 1988) and no changes were observed in offspring of treated animals.

In conclusion, the parameters changed by treatment with *Caesalpinia ferrea* did not cause deaths of pregnant rats, fetal malformations and nor compromised the offspring normal development. The oral administration of *Caesalpinia ferrea* could induce some maternal and fetal toxicity, mainly at the highest dose, possible due to reduction in disgestibility of nutrients caused by tannins present in the extract. This suggests that further studies should be conducted to ensure the use of the plant during human gestation.

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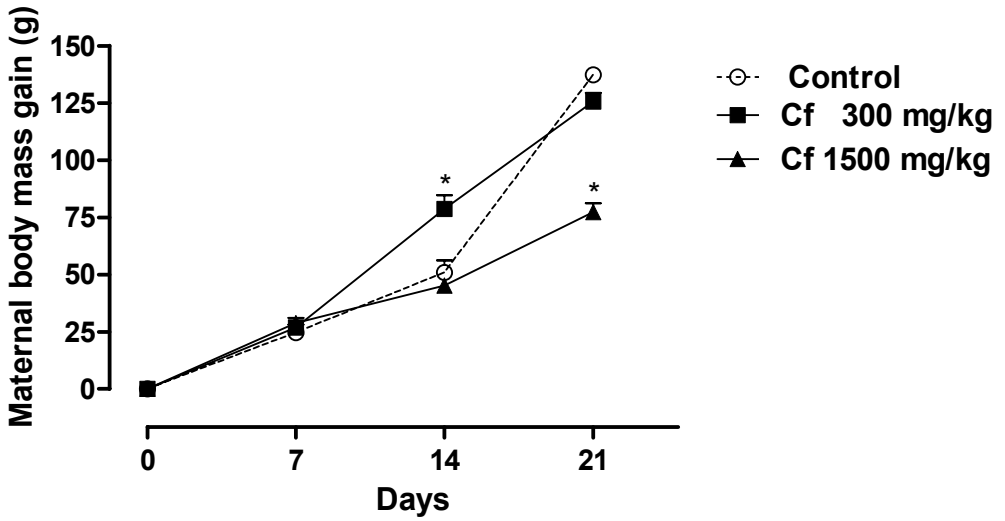

Fig. 1: Maternal body mass gain (g) of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) during whole pregnancy (1st to 21th day). The values were expressed as mean ± S.E.M. (n=9/group). * Statistically different of the control group (ANOVA, followed by Newman-Keuls test, $p < 0.05$).

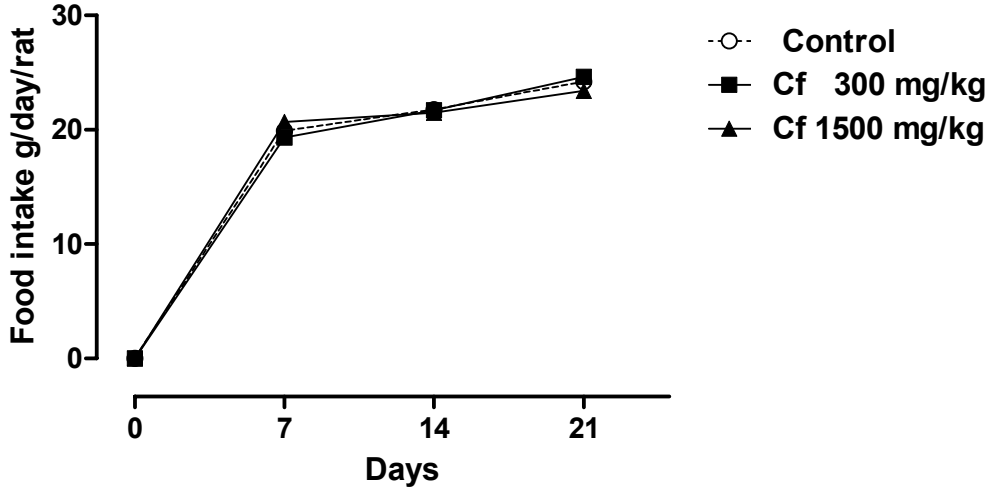

Fig. 2: Maternal food intake (g/day/rat) of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) during whole pregnancy (1st to 21th day). The values were expressed as mean ± S.E.M. (n=9/group).

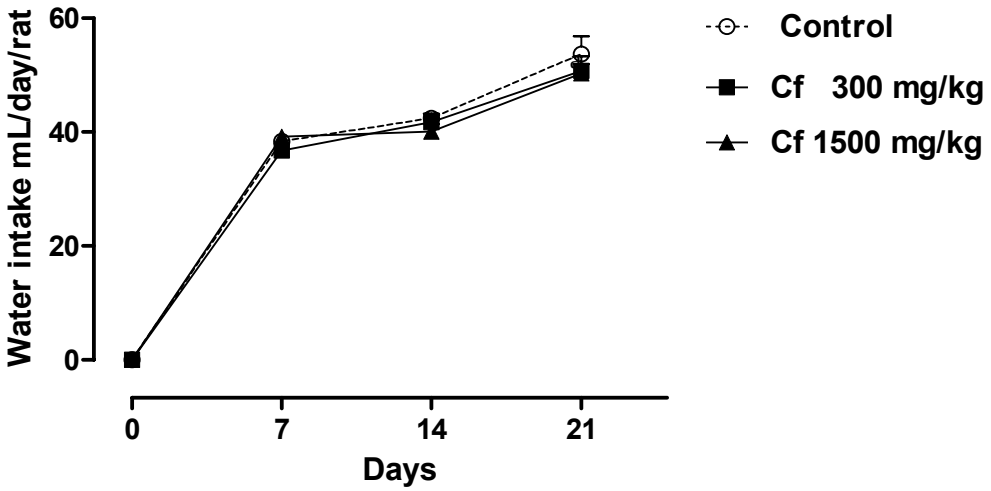

Fig. 3: Maternal water intake (mL/ day/ rat) of the aqueous extract of stem bark of *Caesalpinia ferrea* (Cf 300 and 1500 mg/kg) during whole pregnancy (1st to 21th day). The values were expressed as mean ± S.E.M. (n=9/group).

Table 1: Reproductive parameters of pregnant Wistar rats treated with *Caesalpinia ferrea* Mart. during whole pregnancy (1st to 21th day)

Parameters	Control	300 mg/kg	1500 mg/kg
Pregnant rats	09	09	09
Mass gain in the pregnancy period (g)	137.30 ± 2.51	125.6 ± 3.70	77.25 ± 3.91*
Days of pregnancy (days)	22.00 ± 0.23	21.71 ± 0.28	23.00 ± 0.37*
Number of live fetuses	103	91	106
Number of death fetuses	2	2	0
Offspring/dam relationship	11.44 ± 0.67	10.14 ± 0.86	11.78 ± 0.72
Pregnancy index (%)	97.49	91.67	100
Viability index (%)	100	100	100
Lactation index (%)	98.61	100	100
Pups body mass 1st day (g)	6.00 ± 0.04	6.18 ± 0.05*	5.75 ± 0.06*
Pups body mass 4th day (g)	8.20 ± 0.12	8.58 ± 0.12*	7.80 ± 0.13*
Pups body mass 7th day (g)	12.15 ± 0.19	13.65 ± 0.17*	11.88 ± 0.22
Pups body mass 14th day (g)	24.14 ± 0.39	24.97 ± 0.41	24.08 ± 0.29
Pups body mass 21th day (g)	38.24 ± 0.66	38.42 ± 1.01	39.02 ± 0.69
Length of the pups 1st day (cm)	6.18 ± 0.02	6.38 ± 0.03*	6.17 ± 0.04
Length of the pups 4th day (cm)	7.85 ± 0.05	7.88 ± 0.04	7.70 ± 0.06
Length of the pups 7th day (cm)	9.21 ± 0.065	9.52 ± 0.07*	9.13 ± 0.06
Length of the pups 14th day (cm)	12.67 ± 0.08	13.10 ± 0.09*	13.09 ± 0.10*
Length of the pups 21th day (cm)	17.17 ± 0.10	17.71 ± 0.18*	16.65 ± 0.12*

The values were expressed as mean ± S.E.M. *Statistically different of the control group (ANOVA, followed by Newman-Keuls test, $p < 0.05$).

Table 2: Offspring behavioral parameters of Wistar rats treated with *Caesalpinia ferrea* during whole pregnancy.

Parameters	Control	300 mg/kg	1500 mg/kg
Postural reflexes 1st day (s)	24.19 ± 3.19	22.23 ± 3.08	23.89 ± 3.05
Postural reflexes 7th day (s)	2.16 ± 0.46	1.610 ± 0.15	1.36 ± 0.10
Eye-opening Day	14.29 ± 0.21	14.56 ± 0.17	14.83 ± 0.11
Adult walking Day	14.50 ± 0.27	14.32 ± 0.20	13.84 ± 0.12
Spontaneous ambulation (number of invaded square)	20.54 ± 1.91	18.40 ± 1.59	21.30 ± 1.70

The values were expressed as mean ± S.E.M.

8.0. Conclusão

Os resultados obtidos permitem constatar os seguintes aspectos:

- Os taninos condensados e hidrolizáveis foram os compostos majoritários identificados e quantificados na casca do caule de *Caesalpinia ferrea* Mart. Ex Tul. e, provavelmente, eles são os responsáveis pela atividade antidiabética da planta;
- O extrato aquoso da casca do caule de *Caesalpinia ferrea* não teve seus efeitos antidiabéticos relacionados às concentrações plasmáticas de insulina, que permaneceram baixas nos ratos diabéticos ao final do tratamento;
- O extrato aquoso da casca do caule de *Caesalpinia ferrea* age possivelmente regulando a captação hepática e muscular de glicose em ratos diabéticos através da ativação da Akt, independente da insulina;
- Os benefícios do tratamento com o extrato aquoso da casca do caule de *Caesalpinia ferrea* nos animais diabéticos são demonstrados pela restauração do balanço energético intracelular confirmado pela inibição da ativação de AMPK;
- O aumento da massa muscular, a diminuição dos níveis séricos de transaminases bem como dos níveis de ácido úrico em ratos diabéticos tratados indicam a diminuição da gliconeogênese e proteólise;
- A redução dos níveis séricos das transaminases, da fosfatase alcalina e do ciclo da uréia nos ratos diabéticos tratados evidencia o efeito hepatoprotetor das catequinas presentes no extrato;
- O estresse oxidativo causado pelo diabetes foi reduzido no fígado e soro dos animais tratados, corroborando com os dados da literatura que demonstram que os taninos são captadores de radicais livres;
- A inibição da absorção intestinal de glicose em ratos normoglicêmicos tratados com o extrato aquoso da casca do caule de *Caesalpinia ferrea* aponta para outro mecanismo antidiabético dos taninos condensados como relatado na literatura;
- A adminstração aguda do extrato aquoso da casca do caule de *Caesalpinia ferrea* não produziu efeitos tóxicos em ratos Wistar;

- Os ensaios de toxicidde subcrônica não demonstraram sinais de toxicidade, excetuando-se o aumento dos níveis séricos de amilase, que de acordo com a literatura, relaciona-se com o comprometimento da absorção de carboidratos;
- O tratamento crônico demonstrou comprometimento da absorção de macronutrientes evidenciado pelos sinais de enterotoxicidade;
- O tratamento durante toda a gestação ocasiou redução do ganho de massa corporal de ratas prenhes tratadas com a maior dose, mas não promoveu morte das mesmas e nem comprometeu o desenvolvimento normal da sua prole.

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