**UNIVERSIDADE FEDERAL DO PAMPA**

**ANDRÉIA CAROLINE FERNANDES SALGUEIRO**

# PLANTAS MEDICINAIS UTILIZADAS POR PESSOAS COM *DIABETES MELLITUS* CONTROLAM O ESTRESSE OXIDATIVO E APRESENTAM BAIXA TOXICIDADE: AVALIAÇÃO *IN SILICO, IN VITRO* E *IN VIVO* DA "PATA-DE-VACA" E "MACELA"

**TESE DE DOUTORADO**

**Uruguaiana**

**2017**

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Tese apresentada ao Programa de Pós-Graduação *Stricto sensu* em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Doutora em Bioquímica.

Orientador: Dr. Gustavo Orione Puntel

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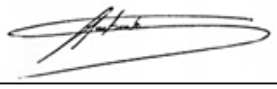
*Plantas medicinais utilizadas por pessoas com Diabetes mellitus controlam o estresse oxidativo e apresentam baixa toxicidade: avaliação in silico, in vitro e in vivo da "pata-de-vaca" e "macela"*

**Elaborada por** Andréia Caroline Fernandes Salgueiro **como requisito parcial para obtenção do grau de Doutora em Bioquímica**

**COMISÃO EXAMINADORA:**

**Gustavo Orione Puntel, Dr. (UFSM – PPG Bioq.)**

**(Presidente/Orientador)**



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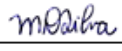

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## RESUMO

Muitas plantas são utilizadas na medicina tradicional para o tratamento do *Diabetes mellitus* (DM) e suas complicações. A hiperglicemia crônica no DM está intimamente associada ao aumento do estresse oxidativo. Assim, esta pesquisa teve como objetivos: i) investigar quais plantas são utilizadas na medicina tradicional por pessoas com DM na cidade de Uruguaiana/RS; ii) avaliar o potencial antidiabético, antioxidante e toxicológico de duas destas plantas *in silico*, *in vitro* e *in vivo*. Para isso, foi realizada uma entrevista com 105 pessoas com DM em Uruguaiana/RS, no intuito de investigar os hábitos de consumo de plantas medicinais (**Manuscrito 1**). Dos entrevistados, 67,6% afirmaram usar plantas medicinais. As quatro plantas mais utilizadas foram a pata-de-vaca (*Bauhinia*), jambolão (*Syzygium jambolanum; Syzygium cumini*), camomila (*Matricaria recutita*) e macela (*Achyrocline satureioides*). A infusão foi a forma de preparo mais utilizada e a frequência de consumo, em mais de 80% dos casos, foi diária. Predições *in silico* dos constituintes majoritários das plantas mais utilizadas mostraram a existência de provável atividade (Pa) antidiabética para alguns dos compostos analisados (**Manuscrito 1**). Na sequência, o potencial antidiabético, antioxidante e tóxico da *Bauhinia forficata* Link subsp. *pruinosa* (BF) foi testado *in silico*, *in vitro* e *in vivo*, neste último caso utilizando camundongos com hiperglicemia crônica (**Manuscrito 2**). Os resultados mostram que os fitoconstituintes previamente identificados na infusão de BF (quercetin 3-o-(2-rhamnosyl)rutinoside; kampferol 3-o-(2-rhamnosyl)rutinoside; quercetin-3-O-rutinoside; e kaempferol-3-O-rutinoside) apresentam alta predição *in silico* como antioxidantes e baixa predição como antidiabéticos e como agentes tóxicos (mutagenicidade, cardiotoxicidade e hepatotoxicidade). A predição antioxidante foi confirmada *in vitro* e *ex vivo*, sendo a BF capaz de reduzir a peroxidação lipídica e controlar os níveis de espécies reativas à diclorofluoresceína (DCF-RS) em eritrócitos de camundongos com hiperglicemia crônica. Da mesma forma, a baixa predição antidiabética foi ratificada, visto que tanto a hiperglicemia, quanto os demais sintomas clássicos do DM (poliúria, polidpsia, polifagia e perda de peso) não foram controlados pelo tratamento com a BF (**Manuscrito 2**). Além disso, a ação da BF sobre os danos hepáticos induzidos pela hiperglicemia foi avaliada em camundongos com DM (**Artigo 1**). Os resultados mostraram que os animais com DM apresentavam altos níveis de NQO-1 no pâncreas, e

de DCF-RS e lipoperoxidação no fígado, assim como diminuição da atividade da catalase hepática. O tratamento com a BF foi capaz de normalizar estes parâmetros, porém não teve efeitos sobre a hiperglicemia, a hepatomegalia, as proteínas carboniladas, os tiois não-proteicos e a atividade da *δ*- aminolevulinato desidratase (**Artigo 1**). Adicionalmente, o potencial antidiabético, antioxidante e tóxico da *Achyrocline satureioides* (AS) foi testado (**Artigo 2**). A toxicidade foi predita *in silico* e avaliada *in vitro* no ensaio de cometa com linfócitos humanos isolados, e no ensaio de determinação da dose letal mediana ($DL_{50}$) em naúplios de *Artêmia salina*. Os resultados mostraram que os componentes majoritários identificados na infusão de AS (quercetina, isoquercitrina e ácido cafeíco) apresentam alta predição como antioxidantes e baixa predição de toxicidade, exceto para a quercetina. A ação antioxidante foi confirmada tanto para o extrato aquoso bruto (infusão), quanto para os constituintes isolados. A $DL_{50}$ determinada classifica a planta como não tóxica. No ensaio de cometa não foi identificado potencial genotóxico para a infusão de AS (**Artigo 2**). Os dados apresentados confirmam o elevado uso de plantas por pessoas com DM na medicina tradicional. Da mesma forma, as análises realizadas apontam o estresse oxidativo como sendo um dos mecanismos envolvidos nos danos teciduais decorrentes do DM. As análises mostram ainda que os efeitos protetores resultantes do tratamento com a BF podem ser atribuídos à sua capacidade antioxidante, demonstrada *in vitro* e *ex vivo*, visto que não foi identificado potencial antidiabético *in silico* e *in vivo*. Nesta mesma linha, o alto potencial antioxidante e baixa predição toxicológica apontam a AS como uma alternativa para o tratamento dos danos causados pelo estresse oxidativo. Em suma, os resultados aqui apresentados permitem concluir que a planta BF, utilizada na medicina tradicional para tratar DM e suas complicações, não apresenta efeito antidiabético direto na forma como foi aqui testada. No entanto, sua importante atividade antioxidante faz, tanto da BF quanto da AS, potenciais agentes para o tratamento complementar do DM, especialmente considerando as complicações diabéticas decorrentes do incremento do estresse oxidativo. Neste contexto, abrem-se perspectivas para investigações futuras do uso dessas plantas como terapia complementar ao tratamento clínico tradicional.

**PALAVRAS-CHAVE:** Etnofarmacologia; *Diabetes mellitus;* Plantas medicinais; Estresse oxidativo; Bioinformática.

## ABSTRACT

Medicinal plants are extensively used in traditional medicine to treat *Diabetes mellitus* (DM) and its complications. Chronic hyperglycemia in DM is pointed as responsible for oxidative stress development. The aims of this study were: i) to evaluate which plants are used in traditional medicine by people with DM in Uruguaiana/RS; ii) to evaluate the antidiabetic, antioxidant and toxicological potential for two of these plants *in silico*, *in vitro* e *in vivo*. For this, a research with 105 DM patients from Uruguaiana/RS was conducted to investigate their medicinal plants consumption habits (**Manuscript 1**). From the interviewed people, 67.6% say they do use medicinal plants. The four more used plants were "cow paw" (*Bauhinia*), jambolan (*Syzygium jambolanum*; *Syzygium cumini*), chamomile (*Matricaria recutita*), and macela (*Achyrocline satureioides*). The most used form of consumption was the infusion; in more than 80% of the cases, the consumption frequency was daily. Predictions *in silico* for the major phytoconstituents of the most used plants showed that some compounds presented probable antidiabetic activity (**Manuscript 1**). In sequence, the antidiabetic, antioxidant and toxicological potential of *Bauhinia forficata* Link subsp. *pruinosa* (BF) was tested *in silico*, *in vitro* and *in vivo*, in the last case using mice with chronic hyperglycemia (**Manuscript 2**). Results show that the phytoconstituents previously identified in the BF infusion (quercetin3-o-(2-rhammosyl)rutinoside; kampferol 3-o-(2-rhammnosyl)rutinoside; quercentin-3-O-rutinoside; and kaempferol-3-o-rutinoside) show high prediction *in silico* as antioxidants and low prediction as antidiabetic agents and toxic agents (mutagenicity, cardiotoxicity and hepatotoxicity). The antioxidant prediction was confirmed *in vivo* and *ex vivo.* BF was able to reduce lipid peroxidation and to control the reactive dichlorofluroscein species (DCF-RS) levels in erythrocytes from hyperglycemic mice. In the same way, the low antidiabetic prediction was ratified, since both hyperglycemia and other classic DM symptoms (polyuria, polydipsia, polyphagia, and weight loss) were not controlled by BF treatment (**Manuscript 2**). Besides, the BF action in the hepatic damages induced by hyperglycemia was evaluated in mice with DM (**Article 1**). Diabetic mice showed high levels of NQO-1 in pancreas, and of DCF-RS and lipid peroxidation in the liver, as well as decrease in the activity of hepatic catalase. BF treatment was able of normalizing these parameters. However, it did not have any effects over hyperglycemia, hepatomegaly, carbonylated proteins, non-protein

thiols, and the *δ*- aminolevulinate dehydratase activity (**Article 1**). Additionally, the antidiabetic, antioxidant, and toxicological potential of *Achyrocline satureioides* (AS) was tested (**Article 2**). The toxicity was predicted *in silico* and, after, evaluated *in vitro* with the comet assay using human lymphocytes. The median lethal dose ($LD_{50}$) was evaluated in nauplii of *Artemia salina*. Results showed that the AS major compounds (quercetin, isoquercitrin, and caffeic acid) presents high prediction as antioxidants and low toxicity prediction, except for quercetin. The antioxidant activity was confirmed both in crude aqueous extract (infusion) and in isolated compounds. The determined $LD_{50}$ classifies the plant as non-toxic. The comet assay showed no genotoxic potential for AS infusion (**Article 2**). Our data confirm the extensive use of plants by people with DM. In the same way, our analysis point oxidative stress as one of the mechanisms involved in tissue damages resulting of DM. The analysis also showed that the protective effects resulting of the BF treatment could be assigned to its antioxidant capacity, demonstrated *in vitro* and *ex vivo*, since no antidiabetic potential was identified *in silico* and *in vivo*. In the same line, the high antioxidant potential and the low toxicological prediction, point AS as a possible alternative treatment to the damage caused by oxidative stress. In summary, the results here presented allow us to conclude that BF plant, used in traditional medicine to treat DM and its complications, does not show direct antidiabetic effects. However, the antioxidant potential observed in both, BF and AS, make them possible complementary treatments to DM, especially considering the diabetic complications resulting of the oxidative stress increase. In this context, perspectives to future investigations on the use of these plants as complementary therapy to the traditional clinic treatment are open.

**KEYWORDS:** Ethnopharmacology; *Diabetes mellitus*; Medicinal plants; Oxidative stress; Bioinformatics.

## LISTA DE FIGURAS

## LISTA DE ABREVIATURAS

δ-ALA-D – Enzima *δ*- aminolevulinato desidratase

ABTS - 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

AGEs – Produtos finais de glicação avançada

ARE – Elemento de resposta antioxidante

AS - *Achyrocline satureioides*

BF – *Bauhinia forficata* Link subsp. *pruinosa*

CAT – Catalase

DCFH-DA – Diclorofluoresceína-diacetato reduzida

DCF-RS – Diclorofluoreceína oxidada por espécies reativas

$DL_{50}$ – Dose letal mediana

DM – *Diabetes mellitus*

DPPH - 1,1-diphenyl-2-picrylhydrazyl

DTNB - 5,5′-Dithiobis(2-nitrobenzoic acid)

EROs – Espécies Reativas de Oxigênio

GSSG – Glutationa oxidada

GSH – Glutationa reduzida

GPx – Glutationa peroxidade

HOCl - Ácido hipocloroso

$HO_2^{\bullet}$ - Hidroperoxil

$H_2O_2$ – Peróxido de hidrogênio

HSF1 - *Heat Shock Factor 1*

Hsp70 – *Heat Shock Protein* 70 kDa

Hsp90 - *Heat Shock Protein 90* kDa

IDF – *International Diabetes Federation*

MDA – Malondialdeído

NQO-1 - NADPH quinone oxidoreductase 1

Nrf2 - *NF-E2-related nuclear factor erythroid-2*

$O_2^{\bullet-}$ - Ânion superóxido

$OH^{\bullet}$ - Hidroxil

PNPMF - Política Nacional de Plantas Medicinais e Fitoterápicos

$RO^{\bullet}$ - Alcoxil

$RO_2^{\bullet}$ - Peroxil

- SH – Grupos tióis

SOD – Superóxido dismutase

TBA – Ácido tiobarbitúrico

TBA-RS - Espécies Reativas ao Ácido Tiobarbitúrico

WHO - World Health Organization

**SUMÁRIO**

# APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de **Artigos Científicos** e **Manuscritos.** A formatação dos artigos e manuscritos atende às normas próprias dos periódicos onde foram publicados ou serão submetidos para publicação. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se nos próprios manuscritos e artigos, e representam a íntegra desse estudo.

O item **Discussão e Conclusões**, encontrado ao final desta tese, apresenta interpretações e comentários gerais sobre todos os resultados apresentados. As **Referências Bibliográficas** contemplam apenas as citações apresentadas na **Introdução**, **Revisão Bibliográfica** e **Discussão e Conclusões** desta tese, uma vez que os artigos e manuscritos científicos têm suas referências apresentadas na própria composição.

## 1. INTRODUÇÃO

O uso de plantas consideradas curativas na medicina tradicional é frequente e tem estimulado diversas pesquisas no ramo da etnofarmacologia (Dutra et al., 2016). No Brasil, políticas específicas como a "Política Nacional de Plantas Medicinais e Fitoterápicos" (PNPMF) e o "Programa Nacional de Plantas Medicinais e Fitoterápicos" estimulam a promoção e o reconhecimento de práticas populares de uso de plantas medicinais e remédios caseiros, assim como buscam assegurar a eficácia, segurança e qualidade no uso de plantas potencialmente medicinais (Brasil, 2006; 2009).

Essas políticas são baseadas no fato de que muitos medicamentos atualmente disponíveis no mercado são derivados de plantas medicinais. Um importante exemplo é o caso da Metformina, uma das drogas mais utilizadas para o tratamento do *Diabetes mellitus* (DM) no mundo. A Metformina é uma biguanida derivada da galegina, composto com alto poder hipoglicemiante isolado da planta *Galega officinalis* L. (Fabaceae) (Witters, 2001). Essa planta era prescrita na medicina tradicional desde a idade média para tratar a poliúria, sintoma que mais tarde se descobriria ser fortemente associado ao DM (Witters, 2001).

O DM é uma síndrome metabólica caracterizada pela não produção de insulina ou por uma incapacidade de se utilizar adequadamente a insulina produzida, resultando em elevados níveis de glicose sanguínea (IDF, 2017). Essa síndrome é considerada uma pandemia, afetando milhões de pessoas em todo o mundo (IDF, 2017). O DM está associado com o desenvolvimento de várias complicações a curto e longo prazo, muitas das quais são associadas ao aumento do estresse oxidativo (Maritim et al., 2003; Kassab & Piwowar, 2012). A nível tecidual, o aumento na produção de radicais livres associado com uma redução das defesas antioxidantes endógenas resulta em danos à lipídios, proteínas e DNA, levando à morte celular.

Nesse contexto, o uso de antioxidantes exógenos, como os flavonoides, derivados de plantas, pode ser uma importante ferramenta para o controle do dano oxidativo resultante do DM (Pandey & Rizvi, 2009; Pisochi & Pop, 2015). Experimentalmente, a avaliação da capacidade antioxidante de extratos de plantas consideradas medicinais, assim como de seus compostos isolados é possível por meio de diversos ensaios *in silico*, *in vitro*, *in vivo* e *ex vivo*. Entretanto, considerando que os extratos de plantas medicinais são misturas complexas de vários compostos com

potencial atividade medicinal, a avaliação da forma popular de utilização dos mesmos (infusão) permite uma maior compreensão de seus efeitos farmacológicos e/ou tóxicos.

Com base no exposto, a importância do presente trabalho justifica-se a medida em que se objetiva conhecer quais plantas são utilizadas por pessoas com DM na medicina tradicional, assim como investigar a eficácia e segurança de algumas destas plantas, atendendo desta forma às orientações da PNPMF.

Nesse contexto, o presente trabalho tem como proposta principal responder aos seguintes problemas de pesquisa:

- Quais plantas medicinais são utilizadas por pessoas com DM na cidade Uruguaiana/RS?
- As plantas utilizadas, em sua forma popular de preparo, apresentam potencial antidiabético, antioxidante ou tóxico?

# 2. REVISÃO BIBLIOGRÁFICA

## *2.1. Medicina Tradicional e Plantas Medicinais*

A medicina tradicional, ou medicina popular, compreende o somatório de conhecimentos, habilidades e práticas baseadas em teorias, crenças ou experiências, explicáveis ou não, de uma determinada população ou cultura, usadas na manutenção da saúde, bem como na prevenção, diagnóstico, melhoria ou tratamento de doenças físicas e mentais (World Health Organization - WHO, 2017).

As terapias da medicina tradicional podem ser classificadas como terapias de medicação, onde são utilizados medicamentos com base de ervas, partes de animais e/ou minerais, e terapias sem medicação, onde são utilizadas terapias complementares ou alternativas como Reiki, Yoga, acupuntura e outras terapias físicas (WHO, 2017). No contexto da medicina tradicional, o uso de extratos preparados à base de ervas ou plantas consideradas medicinais é uma prática comum, e inclui a utilização do material vegetal bruto, como folhas, flores, frutos, sementes, caules, casca e raízes, ou do material vegetal processado, como tinturas, óleos essenciais e outros preparados (WHO, 2017).

Notadamente, acredita-se que cerca de 80% da população de países em desenvolvimento faz uso da medicina tradicional para sua atenção primária, sendo que grande parte destes (85%) utilizam plantas ou preparações à base de plantas (Brasil, 2006). De acordo com esses dados, uma pesquisa realizada no centro-norte do estado do Rio de Janeiro apontou que 97,7% dos 1.320 entrevistados já haviam utilizado plantas para fins medicinais, e 67,9% afirmaram estar fazendo uso de alguma planta considerada medicinal durante o período em que a pesquisa foi realizada (Veiga Junior, 2008).

Com base nesse cenário, no ano de 2006, a Organização Mundial da Saúde estabeleceu a "*International Regulatory Cooperation for Herbal Medicines*" que se constitui em uma rede global de regulação do uso de medicamentos à base de plantas (WHO, 2017). A missão desta rede é proteger e promover a saúde pública e segurança através de uma melhor regulamentação para medicamentos fitoterápicos (WHO, 2017).

No Brasil, a "Política Nacional de Plantas Medicinais e Fitoterápicos" (PNPMF), estabelecida em 2006, e o "Programa Nacional de Plantas Medicinais e Fitoterápicos", estabelecido em 2008, têm como objetivos assegurar a eficácia,

segurança e qualidade no uso de plantas medicinais, assim como promover e reconhecer as práticas populares do uso de plantas consideradas medicinais e remédios caseiros (Brasil, 2006; 2009).

Nesse sentido, um dos eixos do Programa Nacional de Plantas Medicinais e Fitoterápicos propõe-se a "salvaguardar, preservar e apoiar os conhecimentos, práticas, saberes e fazeres tradicionais e populares em plantas medicinais, remédios caseiros e demais produtos para a saúde que se estruturem em princípios ancestrais" (Brasil, 2009).

Sabe-se que milhares de plantas são utilizadas popularmente como remédios naturais no Brasil. Em parte, o extenso uso de plantas deve-se ao fato de o país ser berço de grande biodiversidade vegetal, com uma infinidade de plantas sendo usadas como matéria-prima para a fabricação de fitoterápicos e outros medicamentos (Brasil, 2006; Dutra et al., 2016). Além disso, o Brasil é detentor de rica diversidade cultural e étnica que resultou em um acúmulo considerável de conhecimentos e tecnologias tradicionais, passados de geração a geração, entre os quais se destaca o vasto acervo de conhecimentos sobre manejo e uso de plantas medicinais (Brasil, 2006; Dutra et al., 2016). Assim, além de conhecer o uso popular de plantas medicinais, a PNPMF visa fomentar a pesquisa, o desenvolvimento tecnológico e a inovação com base na biodiversidade brasileira e de acordo com as necessidades epidemiológicas da população (Brasil, 2009).

Considerando as necessidades epidemiológicas da população, sabe-se que atualmente as doenças crônicas não transmissíveis, entre elas o *Diabetes mellitus* (DM), estão entre as principais causas de morte. De fato, em 2007, cerca de 72% das mortes no Brasil foram atribuídas às doenças crônicas não transmissíveis, resultando em uma importante sobrecarga para o sistema público de saúde e para o sistema previdenciário (Schmidt et al., 2011). Além disso, predições mostram que a mortalidade em indivíduos com DM é 57% mais alta do que na população em geral (Schmidt et al., 2011). Em conjunto, esses dados mostram a importância epidemiológica do DM e suas complicações no cenário de saúde atual.

Em relação à terapia farmacológica para o tratamento do DM, sabe-se que apesar do avanço na qualidade e na disponibilidade dos medicamentos utilizados, uma vasta gama de plantas consideradas medicinais é ainda utilizada empiricamente pela população para o tratamento primário ou complementar da síndrome. Entre essas

plantas, podemos citar a *Bauhinia forficata* (BF) (Fabaceae, Leguminosae), popularmente conhecida como “pata-de-vaca” e apontada como uma das plantas mais utilizadas por pessoas com DM no sul do Brasil (Trojan-Rodrigues et al. , 2012). Outra planta largamente utilizada é a *Achyrocline satureioides* (Lam) (AS) (Asteraceae), popularmente conhecida como “macela”. Nesse contexto, um levantamento sobre o uso de plantas medicinais por pessoas com DM da cidade de Uruguaiana mostrou que a macela é a quarta planta mais utilizada, sendo a pata-de-vaca a primeira (dados não publicados).

Na medicina popular brasileira, inflorescências de AS, normalmente colhidas em um dia considerado sagrado (“sexta-feira da paixão” ou “sexta-feira santa”) são usadas para tratar variados distúrbios gastrointestinais, apresentando efeitos digestivos, antiespasmódicos, antioxidantes e anti-inflamatórios (Polydoro et al. , 2004). Além disso, um composto isolado da planta conhecido como “*achyrofuran*” demonstrou ter atividade anti-hiperglicêmica em camundongos com DM (Carney et al. , 2002).

Em relação à BF, estudos demonstraram que a planta apresenta uma importante atividade antioxidante *in vitro*, tanto no extrato bruto quanto em compostos isolados (de Sousa et al., 2004, Salgueiro et al., 2013). Da mesma forma, a atividade anti-hiperglicêmica já foi comprovada em algumas plantas do gênero *Bauhinia*, porém as evidências ainda são contraditórias no que diz respeito ao efetivo controle da hiperglicemia (de Sousa et al., 2004, Pepato et al., 2002, Volpato et al., 2008). Ademais, são desconhecidos estudos que tenham avaliado o possível efeito hipoglicemiante da *Bauhinia forficata* Link subsp. *pruinosa*.

Com efeito, apesar da larga utilização destas plantas como alternativa terapêutica para o DM e suas complicações, pouco se sabe sobre seu potencial farmacológico e toxicológico, especialmente na forma como são utilizados popularmente (chás ou infusões).

### *2.2. Diabetes mellitus*

O *Diabetes mellitus* (DM) é um distúrbio metabólico que afeta atualmente mais de 415 milhões de pessoas no mundo, com previsão de chegar a 642 milhões em 2040 (IDF, 2017). Essa síndrome é caracterizada pela falta absoluta ou relativa da produção pancreática de insulina, ou ainda por uma incapacidade de utilização da insulina

produzida, resultando em elevação dos níveis sanguíneos de glicose (IDF, 2017). A hiperglicemia crônica, presente no DM, é a principal causa das complicações diabéticas que, não somente diminuem a qualidade e expectativa de vida, mas também causam sobrecarga nos sistemas de saúde (Nowotny et al., 2015).

Classicamente, o DM tem sido dividido em três principais subtipos: DM tipo 1 ou insulino-dependente, DM tipo 2 ou insulino-independente, e DM gestacional. O DM tipo 1 representa cerca de 10–15% dos casos de DM, e apresenta um risco de predisposição genética relativamente baixo, afetando 3%, 5% e 8% das crianças cuja mãe, pai ou irmãos apresentem a síndrome, respectivamente (Pociot & Lernmark, 2016). Por outro lado, o DM tipo 1 está fortemente relacionado com a presença dos haplótipos HLA-DR3-DQ2 e HLA-DR4-DQ8, sendo que mais de 90% das crianças com DM tipo 1 apresentam esses haplótipos em conjunto ou isolados (Pociot & Lernmark, 2016). Assim, o desencadeamento do DM tipo 1 dá-se por interações multifatoriais entre predisposição genética e fatores ambientais.

Entre os fatores ambientais propostos, pode-se destacar infecções, fatores dietéticos, poluição, vacinas, ambiente familiar e estresse (Butalia et al., 2016). Nesse contexto, a fisiopatologia conhecida até o momento para o desenvolvimento do DM tipo 1 envolve diferentes níveis de ataque autoimune às células β-pancreáticas, mediado por um desencadeador ambiental conhecido ou idiopático (Butalia et al., 2016; Pociot & Lernmark, 2016). Esse ataque autoimune resulta na destruição em massa dessas células levando a falha total ou quase total da produção de insulina, com consequente hiperglicemia que, se não tratada, pode ser fatal (Butalia et al., 2016; Pociot & Lernmark, 2016; IDF, 2017).

Já o DM tipo 2 representa em torno de 90% dos casos de DM diagnosticados, sendo a predisposição genética (histórico familiar de DM tipo 2) um importante fator de risco, especialmente quando associado com fatores ambientais (Kahn et al., 2014; IDF, 2017). Entre os fatores ambientais mais determinantes estão a prática de maus hábitos alimentares, aumento da circunferência abdominal e inatividade física (IDF, 2017). A fisiopatologia do DM tipo 2 envolve, como primeiro evento, a resistência dos tecidos insulinodependentes à insulina produzida, devido ao excesso de peso e inatividade física em indivíduos geneticamente predispostos (Timper & Donath, 2012; Kahn et al., 2014). Para compensar a resistência insulínica, as células β-pancreáticas passam a secretar uma quantidade maior de insulina (hiperinsulinemia), o que atrasa o aparecimento dos

sintomas em estágios iniciais (Timper & Donath, 2012). Porém, mais tardiamente, as células produtoras de insulina entram em falência e a hiperglicemia passa a ser diagnosticável (Timper & Donath, 2012).

Em ambos os casos, a hiperglicemia persistente é um dos primeiros sinais detectáveis na síndrome. Além disso, quando se torna crônica, a hiperglicemia causa um aumento do estresse oxidativo que, por sua vez, resulta em dano a diversos órgãos e tecidos como coração, fígado, rins, olhos, vasos e nervos (Maritim et al. , 2003; Maiese, 2015; Nowotny et al., 2015). Experimentalmente, esse conjunto de alterações pode ser avaliado com o uso de modelos animais.

De fato, historicamente, diversos modelos animais têm sido utilizados para a investigação das alterações patofisiológicas do DM, assim como para o desenvolvimento de novas alternativas terapêuticas para a síndrome (Al-awar et al., 2016). Dentre as alternativas disponíveis, o DM pode ser induzido quimicamente utilizando-se a toxina estreptozotocina (ETZ).

A ETZ é uma toxina sintetizada pela levedura *Streptomyces achromogenes* e foi identificada como um potente indutor de hiperglicemia por causar a destruição das células β-pancreáticas (Delfino et al. , 2002; Al-awar et al., 2016). A estrutura da ETZ, similar a molécula de glicose, permite sua entrada livre nas células β-pancreáticas através dos transportadores de glicose do tipo 2 (GLUT 2) e de forma insulino-independente (Szkudelski, 2001; Al-awar et al., 2016).

Uma vez internalizada, a ETZ causa a morte das células β por alquilação do DNA, ou seja, por interferir em etapas importantes da proliferação celular formando ligações cruzadas com os filamentos de DNA que impedem a replicação das células (Almeida et al. , 2005). Neste processo, também ocorre a formação e liberação de espécies reativas de oxigênio (EROs). Em conjunto, essas duas situações resultam na morte das células β-pancreáticas e consequente incapacidade de se produzir insulina, o que leva ao desenvolvimento de uma situação clínica similar ao DM tipo 1.

Adicionalmente à lesão das células β-pancreáticas, sabe-se que o dano causado pela ETZ ou pela hiperglicemia secundária à sua administração, é capaz de afetar outros órgãos como o fígado (sítio de metabolização da ETZ e da homeostase de glicose) e rins (sítio de excreção da ETZ e do excesso de glicose circulante) (Karunanayake et al. , 1976, Maritim et al. , 2003; Kini et al., 2016).

### *2.3. Estresse Oxidativo no Diabetes mellitus*

Acredita-se que a hiperglicemia crônica no DM esteja envolvida na gênese de diversos eventos bioquímicos, os quais resultam em um desequilíbrio entre a produção de EROs e a capacidade dos sistemas de defesa antioxidante em neutralizá-las, resultando em um estado de estresse oxidativo (Halliwell & Gutteridge, 2000; Maiese, 2015). O estresse oxidativo foi primeiramente caracterizado por Helmut Sies, em 1985, como um distúrbio potencialmente danoso no balanço entre prooxidantes e antioxidantes, em favor das espécies oxidantes (Sies, 2015; Pisoschi & Pop,2015). Atualmente, este conceito foi reformulado para incluir o papel da sinalização redox neste desbalanço (Sies, 2015), como segue:

> "*Oxidative stress is an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage*" (Sies, 2015).

As EROs, de uma forma geral, podem apresentar-se sob duas formas: como radicais livres ou ainda como espécies reativas não-radicais (Pisoschi &Pop, 2015). Radicais livres podem ser definidos como quaisquer moléculas ou fragmentos moleculares que apresentem um ou mais elétrons desemparelhados no seu orbital mais externo (Halliwell, 2001; Rochette et al., 2014). Esses elétrons desemparelhados conferem um elevado grau de reatividade a estas moléculas (Rochette et al., 2014).

Entre os radicais livres, incluem-se o ânion superóxido ($O_2^{\bullet-}$) e os radicais hidroxil ($OH^{\bullet}$), peroxil ($RO_2^{\bullet}$), alcoxil ($RO^{\bullet}$) e hidroperoxil ($HO_2^{\bullet}$) (Halliwell, 2001). Por outro lado, as espécies não-radicais, a exemplo do peróxido de hidrogênio ($H_2O_2$) e do ácido hipocloroso (HOCl), não possuem elétrons desemparelhados, entretanto, são classificadas como espécies reativas devido a sua grande instabilidade (Halliwell & Gutteridge, 2000).

Em condições fisiológicas normais, as EROs são geradas como produto da redução do oxigênio molecular durante a respiração celular em uma taxa próxima a 2% do total de oxigênio consumido (Halliwell & Gutteridge, 2000). EROs também são geradas em pequenas quantidades como sinalizadores celulares ou ainda em reações de defesa contra patógenos (Pisoschi & Pop, 2015). Entretanto, em condições patológicas pode ocorrer um aumento da produção destas espécies, associada ou não a redução das defesas antioxidantes das células. De fato, a relação entre o aumento na produção de espécies reativas e muitas alterações patológicas já está bem estabelecida, sendo que o

estresse oxidativo pode estar implicado na gênese de mais de 200 doenças humanas, entre elas, o DM (Halliwell, 2005; Nowotny et al., 2015).

Nesse contexto, estudos clínicos em humanos e com modelos animais têm demonstrado que a injúria que determina a morte celular no DM é resultado do aumento da liberação de EROs (Maritim et al., 2003; Maiese, 2015). Uma vez formadas, as EROs causam a depleção dos sistemas de defesa antioxidante, tornando os tecidos mais susceptíveis ao dano oxidativo (Niedowicz & Daleke, 2005). Especificamente no DM, as maiores fontes de estresse oxidativo incluem os processos de auto oxidação da glicose, superprodução de EROs pela mitocôndria, glicação não enzimática com a formação dos "*Advanced Glycation End Products*" (AGEs) e aumento da atividade da via dos poliois (Kassab & Piwowar, 2012; Rochette et al, 2014) (Figura 1).

A auto oxidação da glicose é umas das principais fontes de radicais livres (Maritim et al., 2003). Nesse processo, a glicose, em sua forma enediol, é oxidada em uma reação metal-dependente que forma cetoaldeídos reativos e $O_2$•- (Maritim et al., 2003). Após dismutação pela SOD, o $O_2$•- é convertido em $H_2O_2$. Na ausência de peroxidades e na presença de metais de transição, o $H_2O_2$ pode participar da formação de espécies extremamente reativas, como o OH•, através da reação de Fenton (Maritim et al., 2003). Já o $O_2$•- pode reagir com óxido nítrico, formando radicais peroxinitritos. A glicose em excesso também promove a oxidação de lipoproteínas de baixa densidade, por uma via dependente de $O_2$•-, promovendo uma geração em cascata de outros radicais livres (Maritim et al., 2003).

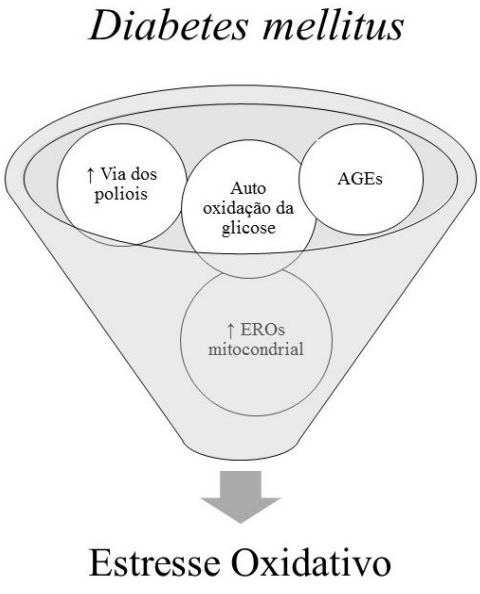


**Figura 1: Vias de estresse oxidativo no *Diabetes mellitus***

Na via dos poliois, a aldose redutase converte glicose em sorbitol às custas da coenzima nicotinamida adenina dinucleotídio fosfatada reduzida (NADPH). O fluxo aumentado de glicose pela via dos poliois leva à formação excessiva da forma oxidada da NADPH (NADP), com consequente aumento da oxidação da glutationa (GSH), requerida para a redução da NADP (Kassab & Piwowar, 2012; Hopps & Caimi, 2013; Rochette et al, 2014). A depleção de GSH, por sua vez, acaba por interferir na atividade das enzimas glutationa redutase e glutationa peroxidase (GPx), tornando as células mais susceptíveis ao dano oxidativo (Rochette et al, 2014).

Os AGEs (produtos finais de glicação avançada), por sua vez, são um grupo heterogêneo de agregados proteicos, formados a partir da reação de Maillard (glicação não-enzimática de grupamentos amino de proteínas), e em maior quantidade sob condições hiperglicêmicas (Madian & Regnier, 2010; Nowotny et al., 2015; Zilaee & Shirali, 2016). Nesse contexto, a glicação não enzimática ocorre quando açúcares redutores reagem com aminas ou com grupos aminoácidos básicos de ácidos nucleicos, sem mediação de enzimas (Zilaee & Shirali, 2016). Durante o processo de formação dos AGEs, EROs também são geradas provocando um ciclo de autoperpetuação (Kassab & Piwowar, 2012).

Dentre os AGEs mais comumente encontrados, estão os compostos carbonil altamente reativos glioxal e metilglioxal, formados durante todas as etapas da reação de Maillard, assim como por intermediários ou subprodutos da auto oxidação da glicose, peroxidação lipídica ou da via dos poliois (Nowotny et al., 2015). Mesmo que a oxidação não seja sempre necessária, muitos AGEs são formados por uma combinação de oxidação e glicação, formando produtos de glicoxidação, especialmente durante situações que envolvem hiperglicemia e estresse oxidativo (Hopps & Caimi, 2013; Nowotny et al., 2015). Assim, no DM, a formação de AGEs é acelerada devido a soma de fatores favoráveis. Uma vez formados, os AGES acumulam-se em diversos tecidos, o que tem sido correlacionado com o aparecimento das complicações decorrentes do DM, especialmente retinopatia, neuropatia e falência renal (Hopps & Caimi, 2013; Nowotny et al., 2015).

Nesse contexto, estudos anteriores já demonstraram que elevadas concentrações de glicose são capazes de lesar oxidativamente eritrócitos humanos *in vitro*, levando ao aumento da peroxidação lipídica (Salgueiro et al., 2013; Pazzini et al., 2015). Ensaios com modelos animais demonstraram aumento da lipoperoxidação no cérebro, fígado e

rins de camundongos hiperglicêmicos, assim como redução da atividade da enzima δ-aminolevulinato desidratase (δ-ALA-D), e nos sistemas de defesa antioxidante de uma forma geral (Folmer et al., 2002; Folmer et al.,2003; Maritim et al., 2003; Pisoschi &Pop, 2015). Da mesma forma, o estresse oxidativo no DM está relacionado a um aumento nos níveis de proteínas oxidadas, as quais apresentam-se diretamente envolvidas na fisiopatologia das complicações diabéticas (Hopps & Caimi, 2013). Em conjunto, esses dados revelam a importância do estresse oxidativo no desenvolvimento das alterações bioquímicas relacionadas ao DM, assim como a relevância da busca por novas alternativas terapêuticas, especialmente a terapia com antioxidantes.

### *2.3.1. Resposta Antioxidante Celular*

O estresse oxidativo é uma condição dinâmica que pode levar a alterações na expressão e função de proteínas, provocar danos a lipídios e lesões ao DNA e, por consequência, desencadear disfunção e morte das células (Rochette et al., 2014; Pisoschi &Pop, 2015). Assim, devido ao ambiente celular ser altamente propenso à ação de oxidantes, a maquinaria celular é equipada com poderosos sistemas de defesa antioxidantes, capazes de prevenir a formação de EROs, ou ainda capturar e neutralizar EROs já formadas (Pisoschi &Pop, 2015). Outra tarefa da maquinaria de defesa antioxidante da célula é a "reparação" de estruturas já danificadas ou sua degradação controlada (Nowotny et al., 2015; Pisoschi &Pop, 2015). Para isso, de uma forma geral, podem ser definidas três linhas de defesa antioxidante celular, incluindo defesas endógenas e exógenas (Figura 2).

A primeira delas contém antioxidantes de baixo peso molecular, que podem neutralizar de forma direta espécies reativas diversas, prevenindo o dano a biomoléculas (Nowotny et al., 2015). Nessa linha, encontram-se antioxidantes tais como as vitaminas C e E, os carotenoides, os flavonoides e a glutationa (GSH). Entre esses, o mais importante e abundante antioxidante celular de baixo peso molecular é a GSH, responsável por determinar o estado redox da célula, definido como a razão entre a forma oxidada da GSH (GSSG) e sua forma reduzida. Em condições fisiológicas normais, essa razão é próxima a 1:1000 (GSSG:GSH), o que proporciona um ambiente celular altamente redutor (Nowotny et al., 2015).

A segunda linha de defesa inclui os antioxidantes enzimáticos, responsáveis pela conversão de espécies altamente reativas em produtos pouco reativos ou inertes. Essa

linha inclui as enzimas superóxido dismutase (SOD) em suas isoformas cobre/zinco SOD, manganês SOD ou ainda SOD extracelular, bem como a enzima catalase (CAT).

Essas enzimas atuam em consonância, sendo a SOD responsável pela decomposição da espécie altamente reativa $O2^{\bullet -}$ em $H_2O_2$. Já a CAT, localizada nos peroxissomas, atua na decomposição do $H_2O_2$ em água e oxigênio (Pisoschi &Pop, 2015). Outras enzimas que atuam na segunda linha de defesa antioxidante incluem as glutationas peroxidase (GPx) e redutase, as tranferrinas e as peroxirredoxinas (Nowotny et al., 2015).

A terceira e última linha de defesa antioxidante celular inclui componentes responsáveis pela recuperação de biomoléculas oxidativamente danificadas, ou ainda por promover a degradação dos componentes irreversivelmente danificados. Nesses, incluem-se os proteassomas, proteases, lipases, DNAses e RNAses (Nowotny et al., 2015; Pisoschi &Pop, 2015).

Em suma, a ação conjunta destes sistemas de defesa é fundamental para a proteção celular adequada contra os danos oxidativos. Por outro lado, a regulação gênica da expressão destes componentes possui papel primordial no efetivo funcionamento desse processo. No contexto da regulação gênica da resposta antioxidante celular, o fator de transcrição *NF-E2-related nuclear factor erythroid-2* (Nrf2) desempenha papel de destaque por controlar a resposta antioxidante, sendo essencial para a manutenção da homeostase redox (Jiang et al. , 2010; Sies, 2015).

De fato, segundo Lee & Johnson (2004), o Nrf2 é um possível alvo para estudo em doenças em que o estresse oxidativo está envolvido, e já foi demonstrado que este gene tem importante papel na proteção hepática após intoxicação com acetaminofeno (Chan et al., 2001), além de proteger as células contra a apoptose induzida por diversas vias (Lee & Johnson, 2004). Assim, em situações de estresse oxidativo o fator de transcrição Nrf2 é capaz de deslocar-se do citoplasma para o núcleo e interagir com o elemento de resposta antioxidante (ARE) aumentando assim a expressão de genes antioxidantes, tais como o da GSH e das enzimas SOD, CAT, GPx e NADPH quinone oxidoreductase 1 (NQO-1) (Ma, 2013, Xu et al. , 2014).

A NQO-1 por sua vez, é uma enzima conhecida por sua ação detoxificante. Essa enzima apresenta a habilidade de reduzir quinonas reativas em compostos pouco tóxicos, como hidroquinonas (Siegel et al. , 2004). Além disso, já foi demonstrado que a NQO-1 apresenta um importante papel como *scavenger* do radical superóxido (Siegel et

al., 2004), e que sua deficiência, em camundongos *knockout*, leva a um aumento da morte das células β-pancreáticas causada por ETZ (Yeo et al., 2013).

Além do Nrf2, outro fator parece estar envolvido na resposta antioxidante celular: o *Heat Shock Factor 1* (HSF-1). Sob condições normais, o HSF1 reside de forma inativa no citoplasma celular ligado à *Heat Shock Protein 90* (Hsp90). No entanto, em condições de estresse, o HSF1 dissocia-se do complexo Hsp90, aumentado assim sua própria expressão e também a expressão da chaperona *Heat Shock Protein 70* (Hsp70) que tem por função reparar possíveis danos causados às proteínas celulares durante a situação estressora (Zhang et al. , 2011; Zilaee & Shirali, 2016). Em ratos com DM tipo 1 foi observada uma inibição na expressão da HSF-1 e da Hsp70 em diversos tecidos (Zilaee & Shirali, 2016). Por outro lado, a superexpressão da Hsp70 no DM está associada com aumento generalizado da inflamação tecidual (Zilaee & Shirali, 2016).

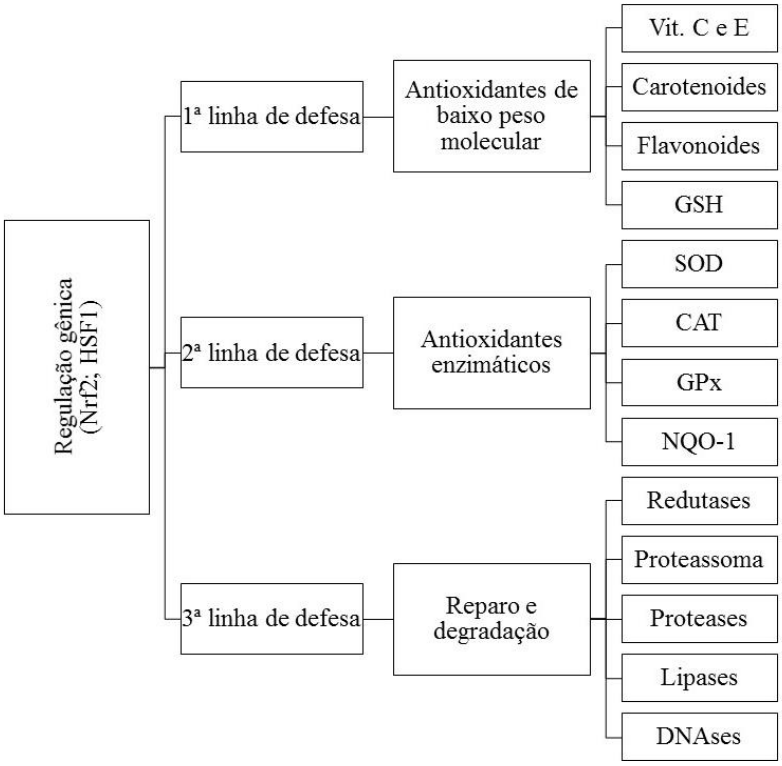


**Figura 2: Resumo das principais linhas de defesa antioxidante celular. Adaptado de Nowotny et al. (2015)**

### *2.3.2. Marcadores de Dano Oxidativo*

Quando ocorre uma superprodução de EROs associada a uma falência nos sistemas de defesa antioxidante, uma série de danos oxidativos podem ser identificados

em diferentes órgãos e tecidos. Entre os marcadores passíveis de avaliação, pode-se citar a avaliação da atividade das enzimas antioxidantes SOD e CAT, dos níveis de GSH, de proteínas carboniladas e de peroxidação lipídica, além da avaliação da expressão de fatores relacionados com a regulação gênica das defesas antioxidantes.

O tripeptídeo glutationa (g-L-glutamil-L-cisteinil-glicina), em sua forma reduzida (GSH), é o principal antioxidante intracelular, e pode ser encontrado em concentrações próximas de 10 mM (Maritim et al., 2003; Pisoschi & Pop, 2015). Além de fazer a neutralização direta de radicais livres, a GSH atua como cofator de muitas enzimas tanto antioxidantes, quanto de detoxificação de xenobióticos (Maritim et al., 2003; Pisoschi & Pop, 2015). Variações nos níveis de GSH são comumente encontradas em situações de estresse oxidativo, e podem refletir o status redox dos tecidos avaliados. Experimentalmente, os níveis de GSH podem ser avaliados através do ensaio proposto por Ellman em 1959. Neste ensaio, a reação da GSH com o ácido 5,5'-ditio-bis-(2-nitrobenzóico) (DTNB), em condições de tamponamento ideais, gera o ácido 2-nitro-5-mercapto-benzóico (TNB) de cor amarela, que pode ser quantificado espectrofotometricamente (Ellman, 1959).

A peroxidação lipídica é outro importante marcador de dano oxidativo, visto que os lipídios são particularmente vulneráveis a oxidação, sendo os componentes de membrana, os glicolipídios e o colesterol importantes alvos de radicais livres (Rochette et al., 2014; Pisoschi &Pop, 2015). Fisiologicamente, a oxidação de lipídios de membrana causa alteração da estrutura e fluidez das mesmas, fazendo com que a célula perca a capacidade de seletividade (Rochette et al., 2014), o que pode desencadear a morte celular. Durante o processo de peroxidação, uma série de produtos são liberados, entre eles o malondialdeído (MDA). Experimentalmente, os níveis de MDA podem ser quantificados espectrofotometricamente através de sua reação com o ácido tiobarbitúrico (TBA) em pH baixo e temperatura alta, através do método proposto em 1979 por Ohkawa e colaboradores.

Outras biomoléculas extensivamente susceptíveis ao dano oxidativo são as proteínas, sendo que o acúmulo de proteínas carboniladas tem sido observado em várias doenças humanas, incluindo DM (Hopps & Caimi, 2013). Nesse sentido, a carbonilação pode ser definida como um processo pós-translacional irreversível de modificação proteica induzida por EROs e outras espécies reativas (Madian & Regnier, 2010).

No contexto da avaliação dos danos oxidativos, o uso de proteínas carboniladas como biomarcadores tem algumas vantagens em relação a outros marcadores, visto que são formadas em estágios mais precoces e são mais estáveis (Dalle Donne et al., 2003; Hopps & Caimi, 2013). De fato, é sabido que a degradação de proteínas na célula ocorre de forma mais lenta do que outras biomoléculas, podendo levar de horas a dias, enquanto que os produtos da peroxidação lipídica são detoxificados em poucos minutos (Dalle Donne et al., 2003). Além disso, a estabilidade química das proteínas carboniladas permite o armazenamento das amostras a serem analisadas por até três meses a 80 °C (Dalle Donne et al., 2003).

Uma diversidade de EROs e outras espécies são capazes de danificar oxidativamente proteínas (Pisoschi & Pop, 2015). Além disso, a carbonilação de proteínas pode ocorrer por aldeídos, como 4-hydroxy-2-nonenal, MDA e 2-propenal, produzidos durante a peroxidação lipídica (Madian & Regnier, 2010; Hopps & Caimi, 2013). Outra fonte de oxidação de proteínas são os produtos carbonil-derivados como as cetoaminas e cetoaldeídos, gerados pelas reações com açucares redutores, ou ainda por glicação e/ou glicoxidação diretas, com eventual formação de AGEs (Dalle-Donne et al., 2003; Madian & Regnier, 2010).

No DM as proteínas carboniladas podem ser identificadas precocemente, e estão diretamente relacionadas com o controle glicêmico e o desenvolvimento de complicações vasculares (Hopps & Caimi, 2013). Experimentalmente, os níveis de proteínas carboniladas podem ser avaliados espectrofotometricamente após sua derivatização com 2,4-dinitrofenilhidrazina, conforme ensaio proposto em 1990 por Levine e colaboradores.

Notadamente, os níveis de peroxidação lipídica e de proteínas carboniladas podem refletir de forma indireta os níveis de EROs (Maritim et al., 2003). Outra forma indireta de estimar-se os níveis de EROs e outras espécies reativas é avaliando, fluorimetricamente, a oxidação da diclorofluoresceína (DCFH-DA), através de ensaio proposto por Myhre et al. (2003).

Da mesma forma, a avaliação da atividade das enzimas antioxidantes SOD e CAT permite inferir o *status* antioxidante dos tecidos. Isoformas da SOD podem ser encontradas no núcleo e citoplasma celulares, assim como nas mitocôndrias e no líquido extracelular (Maritim et al., 2003). A SOD catalisa a conversão do $O_2^{\bullet -}$ em $H_2O_2$, que, na sequência, é convertido pela CAT em $H_2O$ (Pisoschi & Pop, 2015).

Experimentalmente, diversos ensaios foram propostos para avaliação da cinética destas enzimas, a exemplo dos propostos por Aebi em 1984, para avaliação da atividade da CAT e por Kostyuk & Potapovich em 1989 para avaliação da atividade da SOD.

Adicionalmente, a regulação gênica da resposta antioxidante pode ser avaliada observando-se os níveis de proteínas de marcadores específicos, por técnicas de *immunoblotting*. Entre os marcadores possíveis de serem avaliados, estão o fator de transcrição Nrf2, responsável por aumentar a expressão de GSH, SOD e CAT, entre outros, e a chaperona Hsp70 (Nguyen et al., 2009).

Além dos marcadores acima elencados, outro indicador de estresse oxidativo que pode ter sua atividade avaliada é a enzima δ-ALA-D. Embora não seja uma enzima antioxidante, a δ-ALA-D é um bom biomarcador de estresse oxidativo por ser altamente sensível a agentes oxidantes. Isso ocorre devido a presença de grupamentos sulfídricos em sua estrutura que, quando oxidados, fazem com que a enzima tenha sua atividade alterada (Caballero et al., 1998; Folmer et al., 2002; Folmer et al., 2003).

Nesse sentido, sabe-se que a oxidação de grupamentos tiois por EROs ou outras espécies podem modificar de forma significativa a estrutura e função das proteínas (Hopps & Caimi, 2013). Além disso, muitos estudos têm demonstrado que a atividade da *δ*-ALA-D é reduzida em condições hiperglicêmicas, e que isso está especialmente relacionado ao aumento do estresse oxidativo (Caballero et al., 1998; Folmer et al., 2002; Folmer et al., 2003).

Outras condições como remoção do zinco do sítio catalítico e a oxidação proteica (carbonilação) também podem ser determinantes para a redução ou perda da atividade da *δ*-ALA-D (Caballero et al., 1998; Folmer et al., 2002; Folmer et al., 2003). A avaliação espectrofotométrica da atividade dessa enzima foi proposta por Sassa em 1982, e baseia-se no acompanhamento da conversão do ácido aminolevulínico em porfobilinogênio em condições de pH e tempo específicas para cada tecido.

Em conjunto, a avaliação destes biomarcadores é de grande utilidade para determinação do papel do estresse oxidativo na patofisiologia e progressão de diversas doenças humanas, assim como do papel de terapias antioxidantes no enfrentamento das mesmas.

### *2.4. Plantas Medicinais Como Fonte Exógena de Antioxidantes*

O efeito protetor de antioxidantes exógenos tem sido muito estudado *in vitro* e em modelos animais nos últimos anos, e surge como uma alternativa de tratamento para as diferentes doenças em que o estresse oxidativo está aumentado. De fato, acredita-se que a suplementação com antioxidantes exógenos pode melhorar a capacidade do organismo em conter o estresse oxidativo quando as defesas endógenas não são suficientes (Pisoschi & Pop, 2015).

Nesse contexto, antioxidantes podem ser definidos como quaisquer substâncias que, quando presentes em baixas concentrações, retardam ou inibem significativamente a oxidação de um substrato (Gutteridge & Halliwell, 2010; Pisoschi &Pop, 2015). Assim, com base na atividade dos antioxidantes, tanto EROs quanto outras espécies reativas são constantemente inativadas, através de diferentes mecanismos, de forma a impedir o dano oxidativo subjacente (Gutteridge & Halliwell, 2010).

Entre os antioxidantes exógenos mais estudados, podemos citar aqueles derivados de plantas, tais como os polifenois. Esses compostos constituem um grupo de metabólitos secundários das plantas, que são sintetizados durante o desenvolvimento normal da planta ou em resposta a estressores como radiação ultravioleta e agressão por patógenos (Corradini et al., 2011).

Nas últimas décadas, o interesse pelos potenciais benefícios do consumo de polifenois como antioxidantes tem aumentado. Isso porque diversos estudos têm sugerido que o consumo a longo prazo de dietas ricas em polifenois oferece proteção contra o desenvolvimento de câncer, doenças cardiovasculares, DM, entre outros processos patológicos onde o estresse oxidativo esteja envolvido (Arts & Hollman, 2005; Pisoschi &Pop, 2015). Além disso, no caso do DM, os polifenois podem atuar sobre a glicemia por diferentes mecanismos, incluindo a inibição da absorção intestinal de glicose (Pandey & Rizvi, 2009).

Os polifenois constituem um grupo numeroso de metabólitos secundários, sendo que mais de 8.000 variedades já foram identificadas em diversas espécies de plantas (Pandey & Rizvi, 2009). Esses compostos são classificados em diferentes grupos, em função do número de anéis fenólicos que contém e com base nos elementos estruturais que ligam estes anéis um ao outro (Pandey & Rizvi, 2009). As principais classes incluem ácidos fenólicos, flavonoides e lignanas. Entre esses, os flavonoides são os mais estudados.

Flavonoides são compostos de baixo peso molecular com mais de 6.500 variedades conhecidas, muitas responsáveis por fornecer cores atrativas a flores, frutos e folhas (Beckman, 2000; Corradini et al., 2011). Esses compostos são facilmente disponíveis no reino vegetal e possuem particular importância por seu potencial antioxidante, podendo proteger os componentes biológicos do estresse oxidativo (Corradini et al., 2011). As propriedades antioxidantes destes fitoquímicos são atribuídas especialmente:

- A sua capacidade de doar elétrons ou átomos de hidrogênio, estabilizando os radicais livres. Nessa reação, os flavonoides oxidados formam radicais estáveis e menos reativos (Nijveldt et al., 2001; Corradini et al., 2011).

$$\textbf{Flavonoide (OH) + R}^{\bullet} \rightarrow \textbf{Flavonoide (O}^{\bullet}\textbf{) + RH}$$

- A sua capacidade de quelar metais, evitando sua participação em reações como a de Fenton (Nijveldt et al., 2001; Pandey & Rizvi, 2009).

$$\textbf{H}_2\textbf{O}_2 + \textbf{Fe}^{2+}\ (\textbf{Cu}^{+}) \rightarrow {}^{\bullet}\textbf{OH} + \textbf{OH}^{-} + \textbf{Fe}^{3+}\ (\textbf{Cu}^{2+})$$

- A sua capacidade de inibir a ação de enzimas responsáveis pela produção de $O_2^{\bullet -}$, como a xantina oxidase (Nijveldt et al., 2001; Corradini et al., 2011).

Essas caraterísticas explicam em parte o grande interesse farmacológico pelos flavonoides. As subclasses de flavonoides incluem flavonol, flavanol, flavanona, flavona, isoflavona e antocianina (Pandey & Rizvi, 2009; Pisoschi &Pop, 2015). Diferenças individuais dentro de cada grupo surgem a partir da variação no número e disposição dos grupos hidroxila (Corradini et al., 2011) e estão exemplificadas na Figura 3.

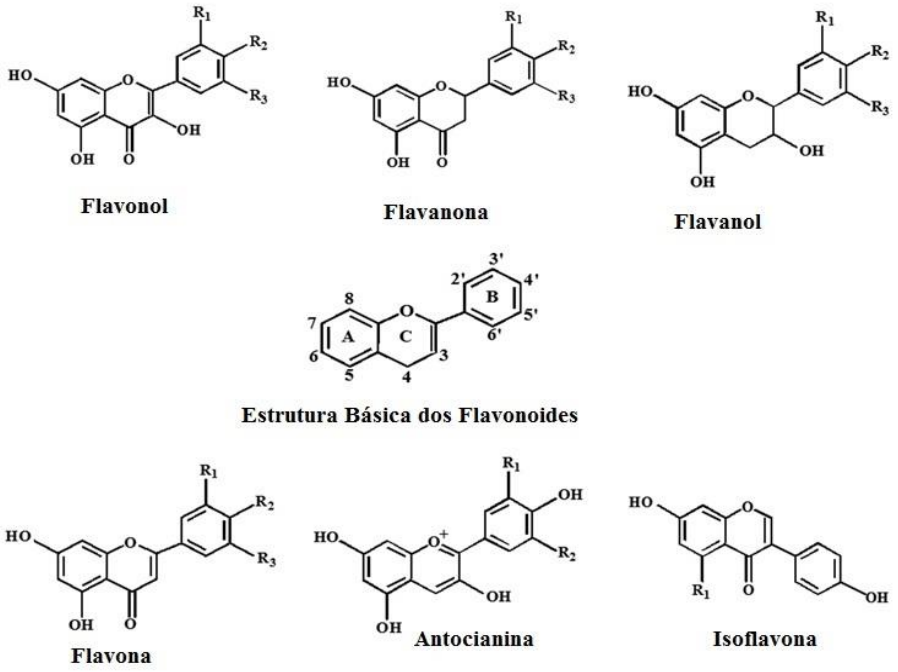


**Figura 3: Classes de flavonoides. Adaptado de Pandey & Rizvi (2009)**

Ainda, é importante destacar que as plantas medicinais fornecem misturas complexas de polifenois e flavonoides, e que o perfil fenólico é vastamente afetado pelas condições ambientais adjacentes, como tipo de solo, exposição solar e quantidade de chuvas (Pandey & Rizvi, 2009). Assim, é cabível considerar que uma mesma planta, localizada em regiões geográficas diferentes, pode apresentar conteúdo e variação de compostos fenólicos próprias das condições em que se desenvolve.

Além disso, o perfil fenólico e a capacidade antioxidante também variam de acordo com a época de colheita (fenóis estão em menor quantidade durante a maturação da planta), a parte da planta utilizada (partes aéreas apresentam maior concentração de fenóis), e o tipo de extração realizada (Beckman, 2000; Pandey & Rizvi, 2009; Corradini et al., 2011).

### *2.5. Predição de Atividade Farmacológica, Toxicidade e Potencial Antioxidante de Plantas*

Uma diversidade de conhecimentos de base empírica sobre as propriedades de plantas consideradas medicinais foi acumulada ao longo dos séculos na medicina tradicional. Atualmente, é possível dar bases científicas a esse conhecimento, utilizando

abordagens experimentais computacionais, a exemplo dos estudos *in silico* das propriedades ADMET (absorção, distribuição, metabolismo, excreção e toxicidade), e da predição de propriedades farmacológicas de compostos isolados de plantas.

Em primeira instância, a avaliação *in silico* consiste no uso de bioinformática ou simulação computacional para determinar as possíveis interações de compostos químicos com os sistemas biológicos. Essa abordagem emerge como uma alternativa economicamente viável e eticamente adequada para seleção de moléculas de interesse farmacológico. Nesse contexto, a avaliação *in silico* de compostos consiste no uso de *softwares* ou plataformas online para determinar possíveis atividades biológicas de compostos, sendo que atualmente existe uma série de plataformas com acesso licenciado ou livre.

Dentre as alternativas disponíveis para a avaliação das propriedades ADMET estão as plataformas OSIRIS® Property Explorer (http://www.organic-chemistry.org/prog/peo/), ACD/Labs team® (Toronto, Canada), admetSAR (Cheng et al., 2012), pkCSM (Pires et al., 2015) e PreADMET (https://preadmet.bmdrc.kr/). Já para a predição das atividades farmacológicas a plataforma "*Prediction of Activity Spectra for Substances*" (PASS) *online* (http://www.pharmaexpert.ru/passonline/), destaca-se como uma excelente alternativa.

Nestas plataformas, a predição de toxicidade é baseada em comparações com substâncias sabidamente mutagênicas no teste de Ames, conhecidamente cardiotóxicas no teste de inibição dos canais de potássio (teste de hERG), tóxicas para o sistema reprodutivo no teste de afinidade com receptores estrogênicos, e irritantes para a pele e olhos no teste de Draize.

O teste de Ames baseia-se na capacidade de uma molécula em induzir mutação reversa em cepas de bactérias, sendo um dos pontos mais importantes na avaliação da segurança de compostos (Xu et al., 2012). No pkCSM, a precisão para o teste de Ames está estimada em 83,8% (Pires et al., 2015). Na avaliação da cardiotoxicidade, investiga-se a capacidade de compostos em inibir receptores do tipo hERG.

Sabe-se que substâncias inibidoras de hERG podem desencadear arritmias ventriculares fatais, sendo que uma grande quantidade de drogas já foi retirada de ensaios clínicos ou do mercado devido as mortes associadas à inibição destes receptores (Rosa et al., 2016). Da mesma forma, drogas com afinidade para receptores estrogênicos

estão relacionadas com toxicidade para o sistema reprodutivo e maiores chances de desenvolvimento de câncer de mama (Ali & Coombes, 2000).

De uma forma geral, todas essas plataformas baseiam-se em bancos de dados ou bibliotecas com fragmentos de compostos já conhecidos e, por similaridade, se utilizam de algoritmos para determinar as características do composto em avaliação. Nesse contexto, alguns programas utilizam-se da determinação de relação estrutura-atividade para relacionar a estrutura físico-química de uma molécula com suas possíveis funções biológicas (Cheng et al., 2012). Desta forma, a partir das predições *in silico*, é possível selecionar compostos de interesse para testes *in vitro* e *in vivo*, uma vez que essas são abordagens complementares e interdependentes (Figura 4).

Na abordagem *in vitro*, diversos ensaios podem ser empregados na avaliação da atividade antioxidante de extratos obtidos de plantas. Entre esses, é possível empregar ensaios para avaliação da capacidade dos extratos em reduzir diferentes substratos. Entre os ensaios mais comumente utilizados, estão o de avaliação da capacidade antioxidante total, proposto por Prieto & Aguilar (1999), que avalia a redução do molibdênio (VI) a molibdênio (V), ocorrida na presença de compostos com capacidade antioxidante. Outro método similar, baseia-se na avaliação do potencial de redução do $Fe^{3+}$ a $Fe^{2+}$ na presença de antioxidantes (Pulido et al., 2000). A capacidade de reduzir diretamente radicais pode também ser avaliada nos ensaios de redução dos radicais 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) proposto por Choi et al. (2002) e 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) proposto por Re et al. (1999).

Em relação a avaliação *in vitro* da segurança de extratos e compostos naturais, o arsenal para investigação de toxicidade inclui testes para análise do potencial genotóxico e determinação de doses tóxicas, como a dose letal para metade de uma população de naúplios de *Artêmia salina*. De fato, o ensaio de determinação da $DL_{50}$ com naúplios de *A. salina* é considerado prático, barato, simples e confiável, sendo ainda uma importante ferramenta na triagem de rotina de plantas (Rosa et al., 2016).

Este ensaio, proposto por Meyer e colaboradores em 1982, apresenta ainda uma elevada correlação com ensaios de toxicidade em modelos filogeneticamente superiores (Logarto Parra et al., 2001). Já a avaliação do dano ao DNA, através do ensaio de cometa com linfócitos humanos, é um método eficaz para a investigação do potencial genotóxico de novos químicos e genotoxinas em células eucarióticas (Singh et al., 1988; Collins, 2004).

Em conjunto, todos os testes aqui mencionados são de grande utilidade para o *screening* de extratos e ou compostos isolados de extratos com atividades farmacológicas de interesse, a serem testados *in vivo*.

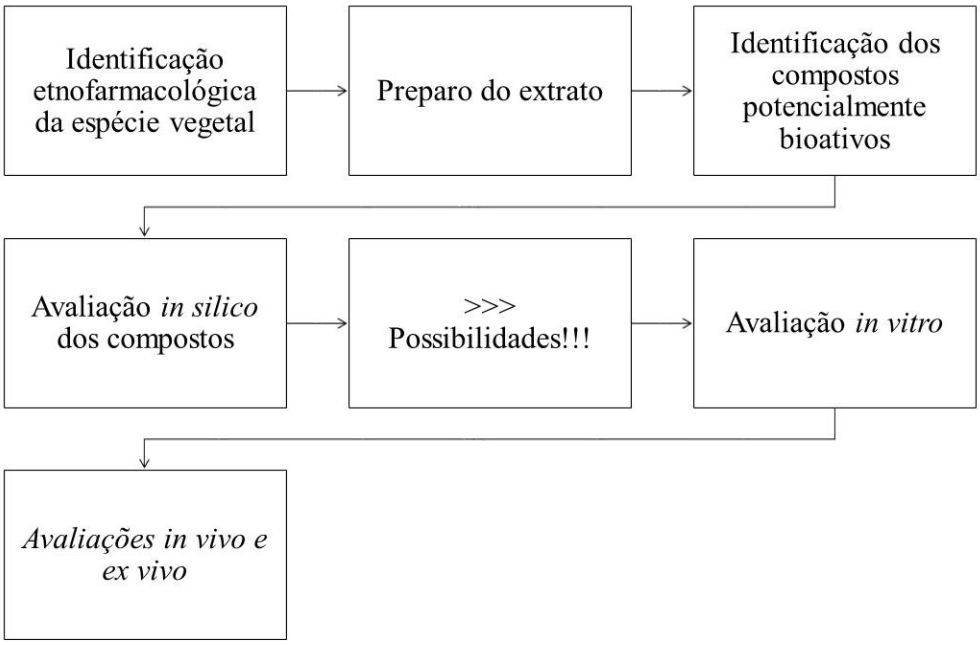


**Figura 4: Percurso metodológico para seleção e avaliação de plantas medicinais e seus compostos isolados.**

## 3. OBJETIVOS

### *Geral*

Investigar quais plantas são utilizadas por pessoas com *Diabetes mellitus* na cidade de Uruguaiana/RS e avaliar os efeitos antidiabéticos, antioxidantes e toxicológicos destas

### *Específicos*

- Conhecer as plantas consideradas medicinais utilizadas por pessoas com *Diabetes mellitus* na cidade de Uruguaiana/RS **(Manuscrito 1)**;
- Investigar as formas de preparo e uso das plantas citadas **(Manuscrito 1)**;
- Averiguar *in silico* a predição antidiabética para os principais fitoconstituintes das plantas mais utilizadas **(Manuscrito 1)**;
- Analisar a composição química das plantas "pata-de-vaca" e "macela", preparadas de acordo com a forma popular de utilização, através de cromatografia líquida de alta performance (HPLC) **(Manuscrito 2; Artigos 1 e 2)**;
- Avaliar *in silico* as predições farmacológicas e toxicológicas dos principais componentes químicos identificados nas plantas "pata-de-vaca" e "macela" **(Manuscrito 2; Artigo 2)**;
- Determinar *in vitro* a atividade antioxidante da infusão das plantas "pata-de-vaca" e "macela" **(Manuscrito 2; Artigo 2)**;
- Investigar os efeitos do tratamento com a infusão de "pata-de-vaca" sobre a glicemia e sobre os marcadores de estresse oxidativo (DCF-RS, TBA-RS, carbonilação de proteínas, níveis de tióis, e atividade das enzimas δ-ALA-D, SOD e CAT) em eritrócitos e no fígado de camundongos com DM induzido por ETZ **(Manuscrito 2; Artigo 1)**;
- Avaliar *ex vivo* os efeitos do tratamento com a "pata-de-vaca" sobre a expressão proteica dos marcadores de estresse oxidativo (Nrf2, NQO-1 e HSP70) no fígado e pâncreas de camundongos com DM induzido por ETZ **(Artigo 1)**;
- Investigar a $DL_{50}$ e o potencial genotóxico da infusão de macela **(Artigo 2)**.

## 4. RESULTADOS

Os resultados aqui apresentados estão sob a forma de dois Manuscritos Científicos e dois Artigos Científicos. Os manuscritos serão submetidos para avaliação e publicação em revistas especializadas com *Qualis* CAPES. Os artigos científicos foram publicados no ano de 2016 nas revistas **"*Oxidative Medicine and Cellular Longevity*"** (Artigo Científico 1) e ***"Journal of Ethnopharmacology"*** (Artigo Científico 2).

**4.1.** ***Manuscrito Científico 1***

# Plants Used by Persons with *Diabetes mellitus* and their Predictive Antidiabetic Activity

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**ABSTRACT**

Medicinal plants are widely used by persons with *Diabetes mellitus* (DM). Currently, there are several ways to evaluating the medicinal power of plant phytoconstituents. *In silico* evaluations consists in bioinformatics use to predict the compounds biological behavior. Thus, this study aimed: i) to investigate the use of medicinal plants for peoples with DM, as well as to know our health profile; ii) to evaluate *in silico* the probably antidiabetic activity for main phytoconstituents of the more used plants. A questionnaire was applied to persons with DM in order to know the use of plants. *In silico* predictions for antidiabetic activity was performed for the main compounds identified by a literature review to the more utilized plants. “Prediction of Activity Spectra for Substances” (PASS) platform was employed to perform *in silico* evaluations. We interviewed 105 persons with DM, most women (73.34%). Overall mean of age was 59.35 years, and 97.14% have type 2 DM. An evaluation of the routine exams of the interviewees showed that they have a poor metabolic control. Among the interviewees, 67.62% asserted make use medicinal plants. Main form of plant preparation was infusion of leaves, and consumption in association with “mate” (a typical beverage of southern Brazil). Most interviewees consume five or more cups of infusion per day, and when consumed with the mate, 1.73 liters per day. Forty-six medicinal plants were mentioned, being the most used "cow's paw" (*Bauhinia*) and "jambolão" (*Syzygium cumini*). Main objective reported for the plant use was blood glucose reduction (69.01%). Phytoconstituents PASS analysis presents six compounds with high antidiabetic prediction, especially vicenin-2, main phytochemical in *Passiflora* genus (Pa = 0.822). Our data show that many plants are used in traditional medicine, as a complementary treatment among persons with DM. Moreover, some plants used present phytoconstituents with antidiabetic potential. These data can serve as a basis for future investigations, with the objective of exploring *in vitro* and *in vivo* the effects of these plants or its compounds as antidiabetic agents.

**Keywords:** Medicinal plants; Complementary medicine; Antidiabetic; *In silico*; Bioinformatics.

## 1. INTRODUCTION

Traditional (complementary or alternative) medicine is characterized by the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and/or in the prevention, diagnosis, improvement or treatment of diseases (WHO, 2017).

In traditional medicine, many plants have been used empirically to treat numerous pathological disorders, including *Diabetes mellitus* (DM) and its complications. This use is related to folk culture that is diffused from generation to generation and, currently, there is a wide variety of plants used for their possible hypoglycemic effects (Trojan-Rodrigues et al., 2012; Arumugam et al., 2013).

In this context, DM is a group of metabolic alterations, resulting from disorders in the mechanism of production and/or action of insulin, which ultimately lead to hyperglycemia (ADA, 2010). Chronic hyperglycemia in DM is associated with several dysfunctions as dyslipidemia, vasculopathy, neuropathy, retinopathy, nephropathy and heart disease (IDF, 2017).

Prevalence surveys indicate that in 2015 there were 415 million people with DM in the world (IDF, 2017). For the year 2040, estimations suggest that the number will reach 642 million people. (IDF, 2017). Despite the high economic cost for DM treatment ($ 673 billion in 2015), there is still a large part of the population without access to pharmacotherapy, or even with the availability of drug treatment, seeking treatment options in traditional medicine.

In fact, a variety of plants with possible antidiabetic effects is used in traditional medicine. Agreeing with the popular use, about 800 plants have been identified with antidiabetic effect (Arumugam et al., 2013), and more than 200 bioactive compounds have been identified with this potential. Among these, we highlight the metformin, one of the most used drugs for DM treatment. Metformin is a biguanide derivative of galegine, compound with high hypoglycemic power isolated from *Galega officinalis* L. (Fabaceae) (Witters, 2001). This plant was prescribed in traditional medicine from the medieval times to treat polyuria, a classic DM symptom (Witters, 2001).

In this hand, it is known that the use of plants with medicinal power can be associated with several health benefits (Arumugam et al., 2013; Lima et al., 2014; Salgueiro et al., 2016a; Rosa et al., 2016). Additionally, Brazil has extremely rich in flora and there is extensive use of plants in traditional medicine, but there is a consensus

about the lack of ethnopharmacological studies to identify the most used species (Dutra et al., 2016). Furthermore, many plants are employed without any preliminary evaluation to confirm its popular use.

In this regard, our group has explored an online platform “Prediction of Activity Spectra for Substances” (PASS). PASS is performed on structural activity relationship (SAR) analysis of database containing more than 205,000 compounds exhibiting approximately 3500 biological activities. In some cases, PASS accuracy exceeds 90% (Ariffin et al 2014; Poroikov et al 2007).

Indeed, *in silico* techniques can be used to select phytochemical compounds, and to test their adverse and pharmacological effects in early stages of medicinal plants investigations (Kadir et al 2013; Singh et al. 2014). Furthermore, considering the extensive use of plants in traditional medicine, this approach appears as an alternative economically viable and ethically appropriate for planning *in vivo* experiments.

Based on the above, this study aimed to know the consumption patterns of medicinal plants by a population with clinical diagnosis of DM. Furthermore, we aimed to evaluate *in silico* the probably antidiabetic activity for main phytoconstituents compounds of the more used plants.

## 2. MATERIALS AND METHODS

### *2.1. Study Design*

Institutional Research Ethics Committee approved this study (CAAE 05027112.5.0000.5323) and all participants signed a consent form agreeing to participate. The included participants had a clinical diagnosis of type 1 or type 2 DM, and were cognitively able to understand and respond to questionnaire.

The selection of participants was made from the "snowball technique" (Snijders, 1992). Based on this technique, starting from a core of known respondents, new elements are added indicated by members of the original sample (Figure 1). This process is continued until the indicated start to repeat forming a kind of network.

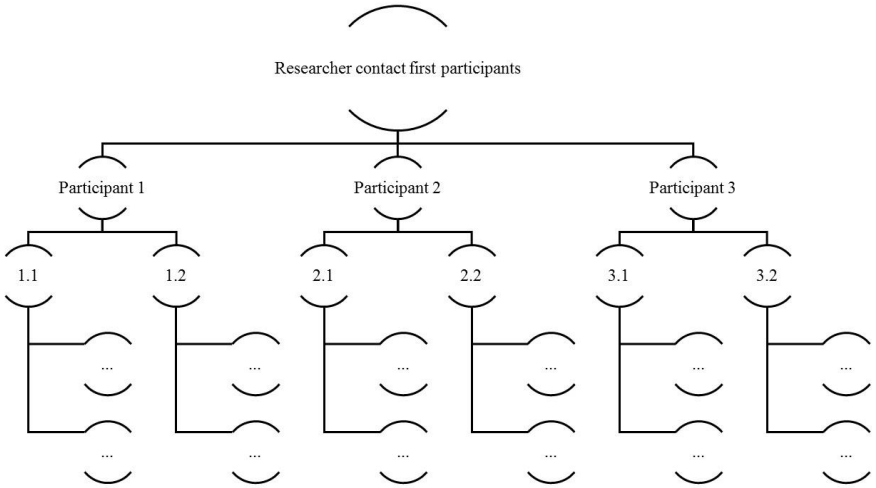


Figure 1: *Snowball technique* design.

*2.2. Questionnaire*

The questionnaire consists of a semi-structured interview, applied by the researchers, containing open and multiple-choice questions divided into four blocks:

i) *Profile of the interviewed*: in this block were investigated characteristics such as gender, age, ethnicity, occupation, marital status and education.

ii) *DM features*: in this block were investigated variables as the DM type, the time of clinical diagnosis, the forms of treatment, as well as the lifestyle (diet, exercise, alcohol or tobacco consumption) of the participants.

iii) *Use of medicinal plants*: in this block was investigated the use of medicinal plants in order to improve the control of DM or its complications, as well as the procedure of the plant preparation and the frequency of use.

iv) *Health profile*: in this block were evaluated the presence of foot lesions (ulcers, wounds, infection, swelling in the foot or ankle, change in temperature or color, fungal nail infections, skin rashes, corns, pain and feet sensibility), as well as previous history of amputations.

Researchers using stethoscope and aneroid sphygmomanometer, following a standardized procedure, measured blood pressure.

Moreover, were analyzed blood glucose, glycated hemoglobin, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides levels. These analyses were performed in authorized laboratories by a medical doctor solicitation. Only were evaluated the clinical tests performed until three months before the interview.

### *2.3. Data Analysis*

Descriptive analysis of the data obtained from interviews was performed using program INSTAT (version 3.01). The results are reported as frequency, mean ± standard deviation. For men and women metabolic profile comparison, we use unpaired T test from GraphPad Prism (version 6).

### *2.4. Prediction of antidiabetic activity from compounds of most used plants*

Prediction of antidiabetic activity from some of phytochemical compounds of most used plants was performed using bioinformatics evaluation. The compounds evaluated were selected based on scientific literature review for five most cited plants. For each selected compound, the simplified molecular-input line-entry system (SMILES) was submitted to the PASS program, available in (http://www.pharmaexpert.ru/passonline/). PASS determinates predicted activity as probable activity (Pa) and probable inactivity (Pi). Pa and Pi range between 0.000 and 1.000, where Pa > 0.7 indicates high probability to a particular property (more than 70%). Only values with Pa > Pi were considered. Briefly, data were interpreted as: (i) Pa > 0.7 = high possibility to find the predicted activity experimentally; (ii) 0.5 < Pa < 0.7 = medium chance to find the activity experimentally; and (iii) Pa < 0.5 = the chance to find the activity is weak and/or the structure is probably not similar to known pharmaceutical agents (Singh et al., 2014).

## 3. RESULTS

### *3.1. Profile of the interviewed and DM features*

One hundred and five persons with DM were interviewed. Of these, 73.34% were women with a mean age of 59.23 years. Among men (26.66%), the mean age was 61.32 years. Most of the respondents had 5-8 years of schooling (48.57%) and 32.38% less than four years. As to occupation, most (40%) has declared retired (Table 1).

**Table 1: Interviewed Profile**

| **Variables** | | ***N*** |
|---|---|---|
| **Gender (Men/Women)** | 26.66% / 73.34% | 28/77 |
| **Mean of Age (Men/Women)** | 61.32 / 58.63 | |
| **Schooling years** | | |
| 0 - 4 years | 32.38% | 34 |
| 5 - 8 years | 48.57% | 51 |
| 9 or more years | 19.04% | 20 |
| **Marital Status** | | |
| Married | 56.19% | 59 |
| Widowed | 19.04% | 20 |
| Divorced | 15.23% | 16 |
| Single | 09.52% | 10 |
| **Occupation** | | |
| Retired | 40.00% | 42 |
| Home | 30.47% | 32 |
| Working | 27.61% | 29 |
| Unemployed | 01.92% | 02 |

Regarding the type of DM, 97.14% had type 2 DM with an average of 10.88 years of clinical diagnosis. The most commonly used medications were Metformin hydrochloride (74.28%) and Glibenclamide (45.71%). In addition, 63.81% reported perform the diet control, but 69.53% do not practice exercise (Table 2).

**Table 2: DM Features and Lifestyle**

| **Variables** | | ***N*** |
|---|---|---|
| **Type 1 DM** | 00.95% | 01 |
| **Type 2 DM** | 97.14% | 102 |
| **DM type not known** | 01.91% | 02 |
| **DM medical diagnosis time (years)** | 10.88 | |
| **Treatment for Diabetes** | | |
| Yes | 96.19% | 101 |
| No | 03.81% | 04 |
| **Drugs used**[1] | | |
| Metformin hydrochloride | 74.28% | 78 |
| Glibenclamide | 45.71% | 48 |
| Insulin | 13.33% | 14 |
| Others | 03.80% | 04 |
| **Control Diet** | | |
| Yes | 63.81% | 67 |
| No | 36.19% | 38 |
| **Exercise practice** | | |
| Yes | 30.47% | 32 |
| No | 69.53% | 73 |

[1] Some interviewed use more than one drug.

*3.2. Health Profile of the Interviewed*

Data of metabolic markers evaluated in the last three months before interview are shown in Table 3. It was observed that women have mean blood glucose greater than men (177.36 mg/dL). On the other hand, the average blood pressure, both systolic (143.08 mmHg) and diastolic (84.61 mmHg) was higher in men.

**Table 3: Metabolic Markers**

| **Variables** | **Mean ± SD (mg/dL)** | | ***p* value** |
|---|---|---|---|
| | **Men** | **Women** | |
| Fasting glucose | 173.04±78.67 | 177.04±76.63 | 0.818 |
| Glycated hemoglobin | 6.29±1.22 | 12.10±2.18 | 0.005* |
| Total cholesterol | 178.40±21.41 | 205.60±69.18 | 0.405 |
| HDL cholesterol | 43.73±5.59 | 40.48±7.27 | 0.436 |
| LDL cholesterol | 109.70±13.50 | 108.64±41.76 | 0.967 |
| Triglycerides | 207.40±109.00 | 168.50±77.11 | 0.375 |
| **Blood Pressure** | **Mean ± SD (mmHg)** | | |
| Systolic | 143.10±20.55 | 142.80±22.95 | 0.950 |
| Diastolic | 84.62±13.34 | 82.89±9.35 | 0.472 |

The table 4 presents data on amputations and other DM complications. It was observed that less than 5% of the interviewed suffered amputations. However, circulatory alterations in the lower limbs have been reported by 73.33% of the interviewed, and renal and visual disorders by 28.57% and 56.19%, respectively. Of the respondents, 93.33% reported feeling discomfort or pain in the feet and legs in the last three months.

**Table 4: Amputations History and DM complications**

| Variables | | *N* |
|---|---|---|
| **Amputations history** | | |
| No | 95.23% | 100 |
| Yes | 04.77% | 05 |
| **Presence of DM complications**[1] | | |
| Circulatory disorders in the feet and legs | 73.33% | 77 |
| Visual disorders | 56.19% | 59 |
| Kidney disorders | 28.57% | 30 |
| **Uncomfortable feeling or pain in the feet and leg in the last three months** | | |
| Yes | 93.33% | 98 |
| No | 06.67% | 07 |
| **Characteristic of the painful sensation** | | |
| Numbness | 60.20% | 59 |
| Burning or heat sensation | 11.22% | 11 |
| Others | 28.57% | 28 |

[1] Some interviewees may have none or more than one complication.

*3.3. Use of medicinal plants*

Most participants (67.62%) reported the use of one or more plants, especially with glycemic control purpose (69.01%), and 82.85% consume a traditional beverage known as "mate" (*Ilex paraguariensis* St. Hill.) (Table 5). Regarding the form of plant preparation, the infusion was the most common, cited by 57.74% of respondents, and leaves are the most used plant part (60.56%). The interviewees said they consume five or more cups daily of plant preparation. When associated to mate, the mean of consumption was 1.73 liters per day. Most participants acquire the plant in local commerce (40.84%). All respondents who use medicinal plants reported positive note after the use (Table 5). In addition, 85.91% of participants did not report to their medical doctor the use of plants.

**Table 5: Frequency and Use of Medicinal Plants**

| Variables | % | N |
|---|---|---|
| **Use plants** | | |
| Yes | 67.62% | 71 |
| No | 32.38% | 34 |
| **Consumes mate** | | |
| Yes | 82.85% | 87 |
| No | 17.15% | 18 |
| **Used part of the plant** | | |
| Leaves | 60.56% | 43 |
| Stem | 11.26% | 08 |
| Seed | 09.85% | 07 |
| Flower | 08.45% | 06 |
| Fruit | 05.63% | 04 |
| Root | 04.22% | 03 |
| **Form of plant use** | | |
| Infusion | 57.74% | 41 |
| Decoction | 14.08% | 10 |
| Others | 28,16% | 20 |
| **Plant acquisition** | | |
| Local commerce | 40.84% | 29 |
| Own cultivation | 25.35% | 18 |
| Others | 33.80% | 24 |
| **Frequency of the plant use** | | |
| More than 03 times per day | 80.28% | 57 |
| Up to 03 times a day | 04.22% | 03 |
| Once a day | 15.49% | 11 |
| **Amount consumed** | | |
| 01 cup | 11.22% | 08 |
| 03 cups | 15.49% | 11 |
| 05 or more cups | 73.23% | 52 |
| **Time of the plant use (mean in months)** | 36 months | |
| **Reason to the plant use** | | |
| Glycemic control | 69.01% | 49 |
| Others[1] | 30.98% | 22 |
| **Sees positive results after plant use** | | |
| Yes | 100% | 71 |
| No | 0 | 0 |
| **Reports the use of the plant to medical doctor** | | |
| No | 85.91% | 61 |
| Yes | 14.09% | 10 |

[1] For weight loss, improve vision, circulation, and lower blood pressure.

Forty-six plants species were cited as commonly used. Ten of most frequently used plants are arranged in Table 6. Among them "cow's paw" (*Bauhinia*), the "jambolan" (*Syzygium jambolanum; Syzygium cumini*), "chamomile" (*Matricaria recutita*), and macela (*Achyrocline satureioides*) appear as the most used at 30.98%, 16.90%, 14.08%, and 11.27% of cases, respectively. It is important emphasize, whereas the mate is a typical drink from southern Brazil, primarily taken with recreational purpose, we chose to discuss it separately from the others plants reported.

**Table 6: Medicinal Plants Most Used by Persons with DM**

| Family | Scientific name | Popular name | Used part | % use[1] |
|---|---|---|---|---|
| Apiaceae | | | | |
| | Pimpinella anisum L. | Anise | Seeds | 08.45 |
| Asteraceae | | | | |
| | *Achyrocline satureioides* (Lam.) DC. | Macela | Flowers | 11.27 |
| | *Baccharis crispa Spreng.* | Carqueja | Leaves | 08.45 |
| | *Matricaria chamomilla* L. | Chamomile | Flowers | 14.08 |
| Fabaceae | | | | |
| | *Bauhinia forficata* Link. | Cow's paw | Leaves | 30.98 |
| Lamiaceae | | | | |
| | *Melissa officinalis L.* | Lemon balm | Leaves | 08.45 |
| | *Rosmarinus officinalis* L. | Rosemary | Leaves | 08.45 |
| Myrtaceae | | | | |
| | *Syzygium cumini* (L.) Skeels. | Jambolan | Stem, leaves and fruit | 16.90 |
| Moraceae | | | | |
| | *Morus alba L.* | White mulberry | Leaves | 08.45 |
| Passifloraceae | | | | |
| | *Passiflora edulis* | Yellow passion fruit | Fruit (flour of the peel) | 08.45 |

[1] All respondents use more than one plant.

*3.4. Prediction of antidiabetic activity from compounds of most used plants*

*In silico* predictions were carried to predict antidiabetic activity spectra for secondary metabolites of most used plants. All analyzed phytoconstituents from *Passiflora sp.* present high probability for antidiabetic activity (Pa > 0.700) (Table 7). Moreover, Rosmarinic acid from *Rosmarinus officinalis* L., and salvianolic acid A from *Melissa officinalis* L. also display Pa > 0.700. These main molecular structures are shown in Figure 2.

In addition, astragalin, quercetin3-O-malonylglucoside, vitalboside A, isoquercitrin, kaempferitrin, kaempferol 3-O-(2- rhamnosyl) rutinoside, quercetin 3-O-(2- rhamnosyl) rutinoside, gallic acid, and rutin presented a medium probability with 0.5 < Pa < 0.7 (Table 7).

**Table 7 – Antidiabetic PASS prediction for major compounds present in the ten most cited plants**

| Specie | Main compounds in literature | PASS-antidiabetic prediction P.a | P.i | Reference | Part used for extraction |
|---|---|---|---|---|---|
| *Achyrocline. satureioides* (Lam.) DC. | 3-Methylquercetin | 0.350 | 0.020 | Souza et al., 2002* | Inflorescence |
| | Quercetin | 0.363 | 0.018 | Carney et al., 2002* | |
| | Achyrofuran | 0.317 | 0.073 | Salgueiro et al., 2016a | |
| | Luteolin | 0.317 | 0.031 | | |
| *Baccharis crispa* Spreng. | Dicaffeoylquinic acid | 0.347 | 0.061 | Simões-Pires et al., 2005 | Aerial parts |
| | Rutin | 0.525 | 0.019 | Menezes et al., 2016 | |
| | Quercetin | 0.363 | 0.073 | | |
| *Bauhinia forficata* Link. | kaempferol-3-O-(2-rhamnosyl) rutinoside | 0.541 | 0.018 | Sousa et al., 2004* Cazarolli et al., 2013* | Leaves |
| | Kaempferitrin | 0.548 | 0.017 | Salgueiro et al., 2016b* | |
| | Quercetin-3-O-(2-rhamnosyl) rutinoside | 0.520 | 0.020 | | |
| *Matricaria chamomilla* L. | Apigenin | 0.320 | 0.029 | | Flowers |
| | Caffeic acid | 0.384 | 0.048 | Singh et al., 2011 | |
| | Chlorogenic acid | 0.340 | 0.064 | Cemek et al., 2008* | |
| | Quercetin | 0.363 | 0.073 | | |
| *Melissa officinalis* L. | Salvianolic acid A | 0.761 | 0.005 | | Leaves/Arial parts |
| | Caffeic acid | 0.384 | 0.048 | Shakeri et al., 2016 | |
| | Luteolin | 0.317 | 0.073 | Barros et al., 2013 | |
| | Oleanolic acid | 0.486 | 0.025 | | |

| | | | | | |
|---|---|---|---|---|---|
| *Morus alba* L. | Astragalin | 0.684 | 0.007 | Gryn-Rynko et al.,2016<br>Hunyadi et al., 2012*<br>Katsube et al., 2006 | Leaves |
| | Quercetin3-O-malonylglucoside | 0.542 | 0.008 | | |
| | Rutin | 0.525 | 0.018 | | |
| | Chlorogenic acid | 0.340 | 0.064 | | |
| *Passiflora* sp[#] | Vicenin-2 | 0.822 | 0.004 | Zucolotto et al., 2012<br>Colomeu et al., 2014* | Leaves/Pericarp |
| | Isovitexin | 0.810 | 0.005 | | |
| | Isoorientin | 0.801 | 0.005 | | |
| | Vitexin | 0.781 | 0.006 | | |
| *Pimpinella anisum* L. | Luteolin | 0.317 | 0.073 | Martins et al., 2016<br>Tirapelli et al., 2007 | Seeds |
| | Anethole | n.d. | n.d. | | |
| | Apigenina | 0.320 | 0.029 | | |
| | Eugenol | n.d. | n.d. | | |
| *Rosmarinus officinalis* L. | Rosmarinic acid | 0.798 | 0.005 | Sedighi et al., 2015<br>Zheng Tu et al., 2013* | Leaves |
| | Carnosic acid | 0.220 | 0.051 | | |
| | Oleanolic acid | 0.486 | 0.025 | | |
| | Ursolic acid | 0.453 | 0.031 | | |
| *Syzygium cumini* (L.) Skeels | Vitalboside A | 0.671 | 0.008 | Thiyagarajan et al., 2016*<br>Bitencourt et al., 2015* | Seeds |
| | Isoquercitrin | 0.660 | 0.008 | | |
| | Gallic acid | 0.525 | 0.018 | | |
| | Rutin | 0.404 | 0.013 | | |

*Studies that investigate the antidiabetic action from cited plants. Probable activity (Pa) and probable inactivity (Pi). (i) Pa > 0.7 high similarity to known pharmaceutical compounds and high probability of antidiabetic activity, (ii) 0.5 < Pa < 0.7 the chance to find the activity experimentally is medium (iii) Pa < 0.5 weak probability of antidiabetic activity. [#]*Passiflora* species reviewed were *P. alata* and *P. edulis*.

**Figure 2**

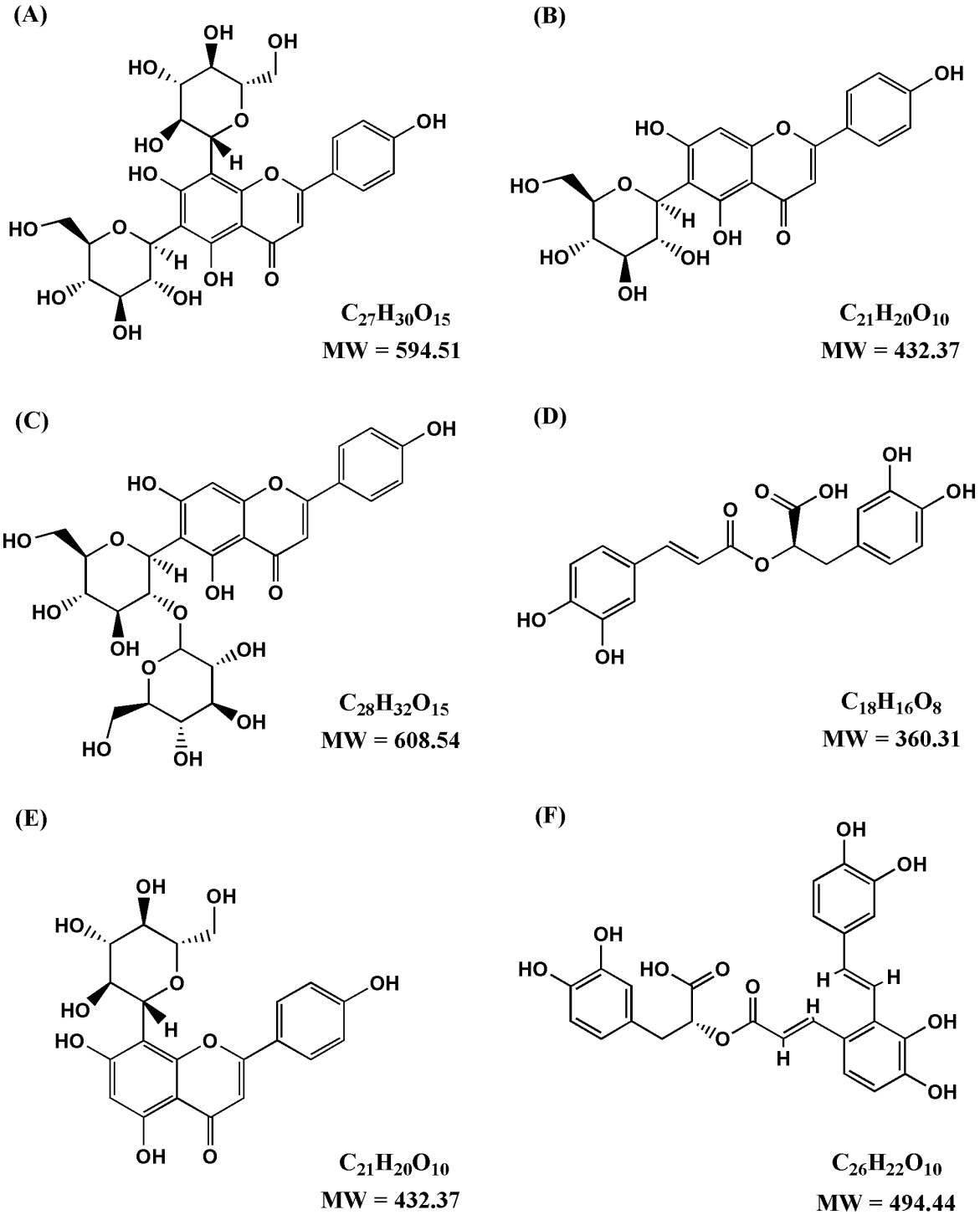


**Figure 2: Molecular structures of Vicenin-2 (A); Isovitexin (B); Isoorientin (C); Rosmarinic acid (D); Vitexin (E); Salvianolic acid-A (F)**

## 4. DISCUSSION

Ethnopharmacological identification of plants used in traditional medicine has been an important way to the discovery of new therapeutic strategies for DM treatment. Here, we investigate the medicinal plants most commonly used by people with DM, and investigate the antidiabetic prediction for some compounds identified in these plants.

In fact, the use of vegetable species with medicinal purpose is current and has recently been fomented by regulatory health agencies, as World Health Organization (WHO). According to WHO (2017), consumption of medicinal plants has been increasing in developing and developed countries since 1990s. Based on this fact, WHO seeks to promote and support the integration of traditional medicine in national health systems, in order to maintain the safety and quality, ensuring the correct use of medicinal plants and herbal medicines.

In this line, Brazil Health Ministry developed the National Policy of Medicinal Plants and Herbal Medicine (Brazil, 2006). Currently, twelve herbal medicines and eight medicinal plants are available from public health services, in "Living Pharmacies" deployed in fourteen states by the “Brazilian Unified Health System”. However, despite the regulation of some plants use by the unified health system, there are few cities served, and a greater part of the population ends up doing empirical use of plant species.

In our study, were found forty-six medicinal plants commonly used by persons with DM. However, we considered the ten plants most frequently used (Table 6). Among these, are the "cow's paw" (*Bauhinia*) and "jambolan" (*Syzygium jambolanum; Syzygium cumini*). These findings are similar to presented by Trojan-Rodrigues et al. (2012) that, from a literature review, found a wide use of medicinal plants in southern Brazil, finding 81 species in 42 families. *Bauhinia* is in fact the most consumed plant by the diabetic population (Trojan-Rodrigues et al., 2012). However, it is important to note that several *Bauhinia* species can be used in traditional medicine (Cechinel-Filho, 2009), and here we do not have conditions to accurately identify the plant species used by person with DM.

In this context, studies in the past 10 years noted the absence of hypoglycemic effect in mice with type 1 DM treated with infusion of *Bauhinia forficata* Link subsp. *pruinosa* (Salgueiro et al., 2016a), and *Bauhinia forficata* Link (Volpato et al., 2008). On the other hand, rats with type 1 and type 2 DM, treated with *Bauhinia variegata*

leaves aqueous extract, showed significant decrease in plasma glucose, cholesterol, triglyceride, creatinine and blood urea nitrogen levels (Kulkarni & Garud, 2016). A reduction in glucose levels was also observed in rats with type 1 DM treated with aqueous extract of *Bauhinia tomentosa* L. (Devaki et al., 2011), and *Bauhinia thoningii* (Ojezele & Abatan, 2011).

Contradictory results showing the antidiabetic effect of *Syzygium cumini* extracts are also numerous in scientific literature (for a review see Trojan-Rodrigues et al., 2012). Regarding the other plants cited in our study, the potential hypoglycemic activity has been previously described for *Matricaria chamomilla* L. (Cemek et al., 2008), *Rosmarinus officinalis* (Ramadan et al., 2013), and *Achyrocline satureioides*, including the identification and isolation of achyrofuran, compound responsible for the plant hypoglycemic activity (Carney et al., 2002).

Our PASS analysis indicated that most of phytochemicals evaluated from cited plants have low to medium probable antidiabetic potential. However, there were six molecules with high Pa score (> 0.7) (Fig.2), which, four (vitexin, isovitexin, vicenin and isoorientin) are present in significant concentrations in Passiflora genus (mainly *P. alata* and *P. edulis*). Vicenin-2 was the higher Pa found in our data. In previous study, this molecule showed an *in vitro* antidiabetic potential via inhibition on α-glucosidase, protein tyrosine phosphatase 1B, rat lens aldose reductase and reduced the advanced glycation end products (Islam et al., 2014). Furthermore, nanoparticles of vicenin-2 might be useful in management of DM since it regulate the intracellular glucose utilization (Chockalingam et al., 2015).

Besides this, the glycosyl flavonoids, isovitexin and vitexin, also presented high score in Pa evaluation, and both compounds showed a significant reduction in postprandial blood glucose level on normoglycemic and diabetic mice (Choo et al., 2012). Reports in literature include a significant stimulation on insulin secretion in hyperglycemic rats by isovetix (Folador et al., 2010). Isoorientin also demonstrated a high antidiabetic prediction. However, up to now, there are not studies proving the antidiabetic action of this molecule, which opens perspectives on future researches.

Nevertheless, the phytochemicals rosmarinic acid and salvianolic acid A with Pa > 0.7 are well documented in literature. Rosmarinic acid (main compound in *Rosmarinus officinalis*), reduced the hyperglycemia and ameliorated insulin sensitivity by decreasing phosphoenolpyruvate carboxykinase (PEPCK) and increasing glucose

transporter (GLUT4) expression (Runtuwene et al., 2016). Moreover, rosmarinic acid attenuated oxidative stress in a model of strepotozotocin-induced diabetic rats (Mushtaq et al., 2015). Already, salvianolic acid A, one of the major compounds in *M. officinalis*, has been experimentally validated as promising antidiabetic substance. It acting as Nrf2 (Nuclear factor E2-related factor 2) modulator preventing diabetes-associated macrovascular complications, also, it exerts an anti-apoptotic mechanism and improves cardiac function following ischemia/reperfusion injury through the JNK/PI3K/Akt signaling pathway in rats with DM (Wu et al., 2016; Chen et al., 2016).

We also highlight that compounds astragalin, vitalboside A, isoquercitrin and kaempferitrin, present in *Morus alba*, *Syzygium cumini*, *Achyrocline satureioides* and *Bauhinia forficata* respectively, have been demonstrated a significant hypoglycemic and antidiabetic potential, which are worthy of further investigation in diabetic complications (Panda and Kar 2007; Cazarolli et al., 2013; Thiyagarajan et al., 2016).

In summary, the results of PASS analysis can be a possible approach to select promising compounds. This approach may be employed with successful to investigate preliminarily the therapeutic effects reported to medicinal plants. However, we cannot discard that many reported activities in traditional medicine, can be due the synergism among different molecules in a crude plant extract (Rasoanaivo et al., 2011).

Besides antidiabetic potential, other widely exploited characteristic in medicinal plants is this antioxidant power, which may be useful in prevention of DM complications associated with oxidative stress (Nasri et al., 2015; Salgueiro et al., 2013). Thus, even plants that do not have hypoglycemic action, may improve the DM treatment as an adjunct to present antioxidant activity.

In fact, it is known that in DM and in other diseases, antioxidant defenses and production of free radicals are altered leading to a condition known as oxidative stress (Giacco & Brownlee, 2010). In this case, the excess of free radicals production associated with an antioxidant defenses reduction can cause severe damage to cells and tissues (Folmer et al., 2002; Folmer et al., 2003; Maritim et al., 2003; Salgueiro et al., 2013; Salgueiro et al., 2016a).

In this hand, Folmer et al. (2002) demonstrated an increase in thiobarbituric acid reactive species levels in kidney and liver of mice exposed to a high glucose and high starch diet, concluding that these are the main target organs to oxidative stress caused by increased blood glucose. Furthermore, increased levels of reactive oxygen species

and lipid peroxidation, and a decrease in antioxidant enzymes activity was observed by Salgueiro et al. (2016a) in liver of streptozotocin-induced diabetic mice.

In humans, it is known that over the years, accumulation of oxidative damages leads to development of DM complications such as retinopathy, neuropathy and peripheral vasculopathy (Giacco & Brownlee, 2010). Thus, the use of antioxidants from exogenous sources has been an important way to prevent or treat these complications (Nasri et al., 2015; Salgueiro et al., 2016a).

Regarding this, some findings point a strong antioxidant power for aqueous extract from *Bauhinia forficata* Link subsp. *pruinosa* and *Achyrocline satureioides* (Salgueiro et al., 2013; Salgueiro et al., 2016a; Salgueiro et al., 2016b). However, antioxidant power in plant extracts is a common finding, because of the wealth of phenolic compounds as secondary plants metabolites. Among the antioxidants usually found in plants, we highlight the phenolic compounds, especially flavonoids, able to inhibit the formation and/or eliminating free radicals, and increase or protect the endogenous antioxidant defenses (Nasri et al., 2015; Salgueiro et al., 2013; Salgueiro et al., 2016b).

The data obtained in our study indicate many of interviewees reported the use of medicinal plants in combination with 'mate' (drink traditionally consumed in southern Brazil, made from the infusion of "yerba mate" (*Ilex paraguarienses* St. Hill). However, the “mate” consumption has recreational purpose, not being used for medicinal finality.

Nevertheless, some studies indicate that yerba mate contains polyphenols and methylxanthines in its leaves, which appear to be responsible for plant antioxidant activity (Colpo et al., 2016). Furthermore, Oliveira et al. (2008) showed that yerba mate decreased gene expression of glucose transporter in intestine of rats, suggesting that yerba may modulate glucose absorption. In other studies, it was found that aqueous extract of yerba mate reduced glucose and insulin concentrations in obese rats (Arçari et al, 2009), and increased lifespan in *Caenorhabditis elegans* (Lima et al., 2014).

Currently, the high cost of industrially produced medicines and the tendency to use natural products have contributed to increased use of plants as a medicinal resource. According to data presented in Table 1, schooling level of interviewees is low, and most of them are retired. These data corroborate the findings of Salgueiro et al. (2015) that described a low education rate, and low purchasing power among person with DM at risk for "diabetic foot" development. These authors point out that low educational level

facilitates DM complications appearance, because makes it difficult the understanding of preventive measures. These findings may be related to inadequate control of DM, resulting in high rates of glucose and dyslipidemia found in our study (Table 3). Likewise, the poor DM control was related to development of others complications, as circulatory, visual and kidney disorders, as well as feet problems (Table 4).

Further, unexpectedly, it was observed that women present a poor metabolic control when compared to men (Table 3). In fact, some studies showed a tendency among women to practice more self-care than men did, because men have a passive and carefree attitude (Hjelm et al., 2002; Salgueiro et al., 2015). These metabolic decompensations become even more serious when associated with poor diet and physical inactivity (Table 2). Together, all these factors can trigger or worsen the complications related to DM (Table 4).

Taken together, our results point the high consumption of medicinal plants among persons with DM. Some plant constituents presented a prediction for antidiabetic activity. Thus, our data open the prospect to further research in order to explore *in vitro* and *in vivo* these compounds, as well as, interactions between plant compounds in conventional DM treatment. Finally, these findings are significant, considering that normally the use of plants is not notified to health professionals who treat this population.

## CONFLICT OF INTEREST

The authors declare no conflict of interests.

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***4.2. Manuscrito Científico 2***

# "Cow's paw" Aqueous Extract Protects Red Blood Cells in Mice with Chronic Hyperglycemia

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## ABSTRACT

*Bauhinia forficata* (BF) ("Cow's paw") is popularly used for *Diabetes mellitus* (DM) treatment. DM is a metabolic syndrome that classically presents as symptoms hyperglycemia, polyuria, polydipsia, polyphagia, and weight loss. Chronic hyperglycemia in DM has been related to widespread oxidative stress. Based on above, this study aimed: i) to investigate *in silico* the biological activity spectrum and the toxicological properties of BF phytoconstituents; ii) to evaluate *in vitro* the antioxidant capacity of BF extract; iii) to investigate, in mice with severe DM, the effects of BF extract on classic DM symptoms, and on markers of oxidative damage in red blood cells. For *in silico* analyses, previously identified BF compounds were evaluated via computational simulation. Antioxidant capacity was performed by total antioxidant capacity (TAC) test, ferric reducing antioxidant power (FRAP) assay, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) scavenger assay. For *in vivo* experiments, untreated male mice with chronic hyperglycemia received BF aqueous extract for 21 days. Solid/liquid intake and urine excretion were measured in metabolic cage. After the treatment, the blood was collected for analysis (glucose levels; diclorofluorescein reactive species (DCF-RS), thiobarbituric acid reactive species (TBA-RS), and non-protein thiol (NPSH) levels). Spleen was collected for weight evaluation. BF phytoconstituents Quercetin 3-o-(2-rhamnosyl)rutinoside, Kampferol 3-o-(2-rhamnosyl)rutinoside, Quercetin-3-O-rutinoside, and Kaempferol-3-O-rutinoside, presented an antidiabetic probable activity (Pa) of 0.520, 0.541, 0.527, and 0.550, respectively. In the same order, estimated Pa for antioxidant activity was 0.934, 0.891, 0.927, and 0.924. In toxicological tests, mutagenic risk was pointed by some evaluations. Antioxidant tests shows a high TAC and FRAP activity from 60µg/mL of extract ($p<0.05$), and $ABTS^{\bullet+}$ scavenger from 30µg/mL ($p<0.05$). Diabetic mice had significantly higher levels of blood glucose, increased feed and liquid intake, increased urine excretion and weight loss. BF was not effective in controlling any of these parameters. However, BF was effective in reduce the splenomegaly, and in protect red blood cells of diabetic mice against lipid peroxidation. In summary, BF has no effect against DM classic symptoms, but protects against splenomegaly and presents an important antioxidant activity in erythrocytes of mice with chronic hyperglycemia.

**Keywords:** *Bauhinia forficata*; Traditional medicine; Medicinal plants; Bioinformatics.

## INTRODUCTION

*Bauhinia forficata* (BF) is a plant of Fabaceae family, popularly known as "cow's paw". The name of the plant is due to the shape of its leaves that presents a bilobed aspect, similar to a cow hoof (Cechinel Filho, 2009). *Bauhinia* genus comprises more than 300 species, and is extensively used in popular medicine for *Diabetes mellitus* (DM) treatment (Cechinel Filho, 2009).

DM is a metabolic syndrome related to defects in insulin production, or failure to properly use the insulin produced (IDF, 2017). A variety of classic symptoms, such as persistent hyperglycemia, polyuria, polydipsia, polyphagia, and weight loss, characterizes this syndrome. Moreover, oxidative stress associated with poor glycemic control is in the genesis of DM complications development (Maiese, 2015).

In fact, in DM a range of physiological alterations triggered by chronic hyperglycemia are responsible for the increased free radicals production. Among these alterations, are the glucose autoxidation, the overproduction of free radicals by mitochondria, the non-enzymatic glycation with the production of "advanced glycation end products", and increased activity of polyols pathway (Kassab & Piwowar, 2012; Rochette et al, 2014).

In association or not with antioxidants defenses power reduction, the high free radicals levels in DM are pointed as responsible for oxidative damage in several organs and tissues (Maritim et al., 2003). The high levels of glucose can produce permanent chemical alterations in proteins and lipid peroxidation (Folmer et al., 2002). In this context, Salgueiro et al. (2016a) observed that TBA-RS levels were increased in hepatic tissue of mice with hyperglycemia, indicating that the oxygen radicals stimulate the lipid oxidation.

Of particular importance, evidences indicate that high glucose concentrations can damage oxidatively human erythrocytes (Salgueiro et al., 2013; Pazzini et al., 2015). However, we have previously demonstrated that BF subsp. *pruinosa* is able to reduce *in vitro* this damage (Salgueiro et al., 2013). In addition, *in vivo* studies suggest that others plants of *Bauhinia* specie are able to reduce glucose levels and to weight recovery in non-obese diabetic mice (Curcio et al., 2012) and in STZ-induced diabetic rats (Cunha et al., 2010). Moreover, in STZ-induced diabetic mice, the subsp. *pruinosa* of BF provides protection against hepatic oxidative damage (Salgueiro et al., 2016a).

Both antioxidant and antidiabetic effects are assigned to a variety of polyphenols and flavonoids present in *Bauhinia* plants (Cunha et al., 2010; Jorge et al., 2004; Salgueiro et al., 2013; Salgueiro et al., 2016a). Surely, natural products are an unquestionable source of antioxidants and other compounds with possible medicinal properties and, currently, many alternatives allow performing the screening of plants compounds through computer simulation (*in silico* approach).

Among other positive factors, *in silico* methods make possible to reduce the use of animals in initial tests, and to direct pharmacological research, opening perspective for screening of many plants. Thus, *in silico* evaluation can reveal biological activities of selected phytochemical compounds, their mechanisms and related side-effects (Goel et al., 2011; Rosa et al., 2016). Especially, the evaluation of compounds toxicity properties that predicts their safety, effectiveness and success therapeutically (Pires et al., 2015).

Therefore, in conjunction with *in vitro* screening, computational evaluations that can predict absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles have become an alternative approach (Cheng et al., 2012). The accuracy prediction for ADMET property by *in silico* platforms comes near to 90% (Cheng et al., 2012; Pires et al., 2015). For "Prediction of Activity Spectra for Substances" (PASS), average accuracy comes near to 95% (Goel et al., 2011). Consequently, *in silico* evaluation permits predicting the pharmacological and toxicological potential of natural compounds, serving as a refinement pathway for subsequent testing *in vivo*.

Based on the above, this study aimed:

i) To evaluate *in silico* pharmacological and toxicological potential of phytochemical compounds identified in the aqueous extract of *Bauhinia forficata* Link subsp. *pruinosa*;

ii) To test predicted antioxidant activities *in vitro*;

iii) To evaluate the BF's effects on classic DM symptoms and on oxidative stress parameters in erythrocytes of chronic hyperglycemic mice.

## MATERIAL AND METHODS

### BF Aqueous Extract Preparation

Botanical identification was performed by ICN Herbarium of Federal University of Rio Grande do Sul (number ICN167491; *B. forficata* Link subsp. *pruinosa* (Vogel) Fortunato &Wunderlin). Naturally dried BF leaves (collected in spring) were used for the aqueous extract preparation. The leaves (1mg/mL) were placed in a hot water (80ºC) for 15 minutes. After this time, the infusion was filtered and used for analysis.

### *In silico* Biological Activity Spectrum and Toxicological Properties

Computational screening of biological activity spectrum for the previous identified BF compounds (3-o-(2-rhamnosyl)rutinoside; Kampferol 3-o-(2-rhamnosyl)rutinoside; Quercetin-3-O-rutinoside; and Kaempferol-3-O-rutinoside) (Salgueiro et al., 2013) was performed in PASS online platform (http://www.pharmaexpert.ru/PASSonline/predict.php). This platform offers a quantitative structure-activity relationship constructed from the breakdown of chemical structures in 2D and/or 3D descriptors, followed by generation of models acquired from bioactive ligands (Drwal & Griffith, 2013).

Prediction results were expressed in percentage of probable activity (Pa) and probable inactivity (Pi). Pa and Pi values vary from 0.000 to 1.000. Briefly, data were interpreted as: (i) Pa > 0.7 = high possibility to find the predicted activity experimentally; (ii) 0.5 < Pa < 0.7 = medium chance to find the activity experimentally (Singh et al., 2014). In our evaluation we considered only activities with Pa > Pi, and Pa > 0.500.

For the prediction of toxicological properties, we employed the following platforms: ACD/Labs (Toronto, Canada), admetSAR server (Cheng et al., 2012), pkCSM platform (Pires et al., 2015), and PreADMET web-based (https://preadmet.bmdrc.kr/). Prediction results for mutagenicity, cardiotoxicity and hepatotoxicity were interpreted and expressed in a flexible manner: (+) low potential, (++) medium risk, (+++) high risk and non-detected risk (ND).

### BF Extract Antioxidant Activity

BF concentrations used in antioxidants tests ranged between 0 and 300 µg/mL. Total antioxidant capacity (TAC) and ferric reducing antioxidant power (FRAP) were evaluated according methods proposed by Prieto et al. (1999), and Pulido et al. (2000), respectively. Results were expressed in ascorbic acid equivalents, calculated using a

standard curve of ascorbic acid. The BF activity scavenger against the $ABTS^{\bullet+}$ was performed according Re et al. (1999). Results are expressed in percentage of scavenger activity compared with control (without BF).

**DM Induction**

To hyperglycemia induction, male mice with three months (35-40 grams) received a single streptozotocin (STZ) injection (150 mg/kg i.p.) dissolved in citrate buffer (0.01 M; pH 4.5). STZ dose was established according Animal Models of Diabetic Complications Consortium (2009), in order to induce a severe hyperglycemia in mice.

After five days of STZ injection, the blood glucose was determined using a glucometer (Accu check active – Roche ®) in a tail blood sample. Mice with glucose levels higher than 250 mg/dL remained untreated by 30 days. Treatment with BF aqueous extract (1mg/mL) started in day 31 and lasted for 21 days. BF extract was offered *ad libitum* in the drinking water. The extract was replaced daily in order to avoid changes in its composition. One week before the end of treatment, food and liquid intake and urine excretion of the mice were assessed using a metabolic cage (Figure 1).

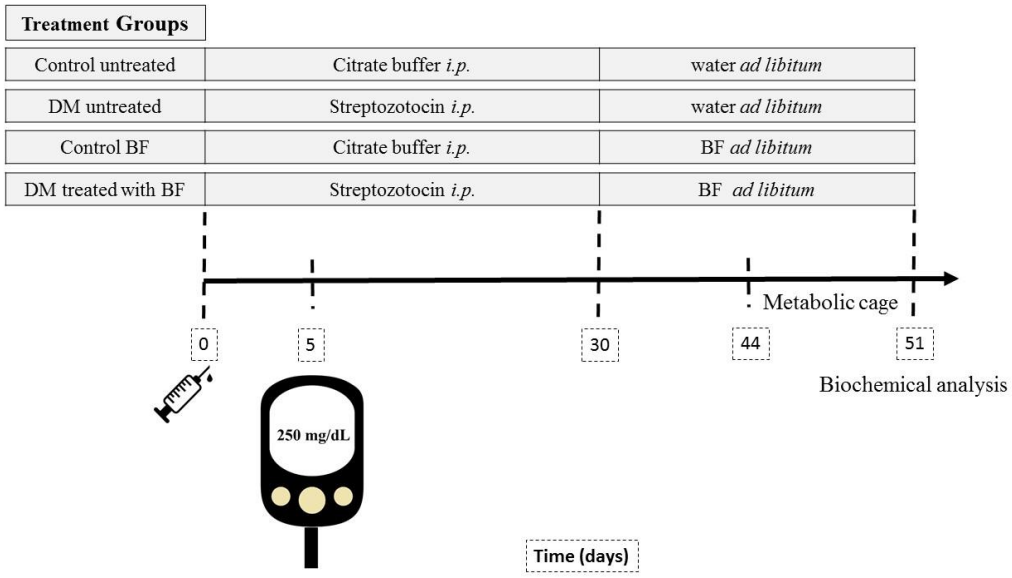



**Figure 1:** Study design.

**Sample Collection**

After the end of treatment, mice were euthanized by cardiac puncture and whole blood was collected in heparinized tubes. Were weighed euthanized bodies, spleen, and epididymal fat in order to determine the spleen/body weight and the fat/body weight index.

**Glucose Levels in Plasma**

The collected blood was centrifuged at 2000 rpm for 10 minutes at 4ºC in order to plasma obtainment. Glucose levels were determined in plasma using a commercial kit (LABTEST – MG / Brasil). Results were expressed in mg of glucose by deciliter of plasma.

**Lipid Peroxidation**

The levels of lipid peroxidation were determined in erythrocytes obtained from centrifugation of whole blood (2000 rpm for 10 minutes at 4ºC). The analysis was performed according the method of thiobarbituric acid reactive species determination, proposed by Ohkawa et al. (1979). A standard curve of malondialdehyde (MDA) was used to correct the results. Results were expressed as nmol of MDA corrected by mL of red blood cells.

**2,7-Dichlorofluorescein reactive species (DCF-RS)**

Reactive oxygen species was determined in erythrocytes as an indirect index of DCF oxidation, according Myhre et al. (2003). Briefly, an aliquot of red blood cells were added to a medium containing Tris-HCl buffer and DCFH-DA. This medium was incubated in the dark for 1 h until fluorimetric analysis (Ex: 488 nm; Em: 520 nm). The results were evaluated as DCF fluorescence intensity, corrected by mL of red blood cells, and expressed as percentage of control.

**Non-Protein Thiol Groups (NPSH)**

The levels of NPSH were determined in erythrocytes obtained from centrifugation of whole blood. In order to obtainment non-protein fraction, the red blood cells were precipitated with 40% trichloroacetic acid and centrifuged (2000 rpm for 10 minutes at 4ºC). The analysis was performed according the method proposed by

Ellman (1959). A standard curve of glutathione (GSH) was used to correct the results. Results were expressed as nmol of GSH corrected by mL of red blood cells.

**Statistical Analysis**

GraphPad prism 6 software was used for statistical analysis and for plotting graphs. Statistical analysis was performed using two-way ANOVA and Tukey *post hoc* test. Data were expressed as mean ± SEM. Values of $p<0.05$ were considered statistically significant.

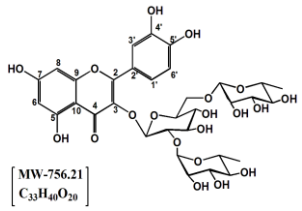
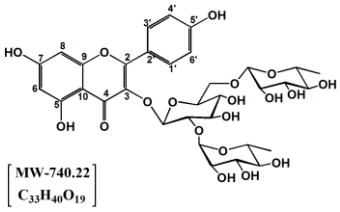
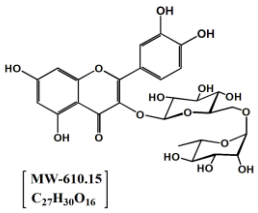
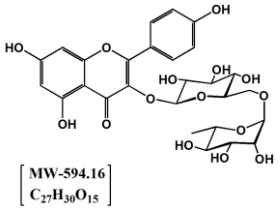
## RESULTS

***In silico* Biological Activity Spectrum and Toxicological Properties**

In previous studies of our research group (Salgueiro et al., 2013; Salgueiro et al., 2016a), we have identified four compounds (quercetin-3-O-(2-rhamnosyl) rutinoside, kaempferol-3-O-(2-rhamnosyl) rutinoside, quercetin-3-O-rutinoside and kaempferol-3-O-rutinoside) in the aqueous extract of *Bauhinia forficata* Link subsp. *pruinosa* by LC-MS. Here, we employed *in silico* analysis in order to determine the possible biological and toxicological activities for these compounds.

Table 1 shows antioxidant or free radical scavenger activity, with Pa near to or above to 0.900 for the four analyzed phytoconstituents (Pa minimum and maximum of 0.891 and 0.990, respectively). Already, for antidiabetic activity, the higher Pa found was to kaempferol-3-O-rutinoside (Pa = 0.550). Furthermore, other important activities in the context of DM were predicted as, e.g., vasoprotector, antioxidant, alpha glucosidase inhibitor, antihypercholesterolemic, antiinflammatory, aldose reductase inhibitor, and maillard reaction inhibitor, with variables Pa to four analyzed compounds (Table 1).

**Table 1- Pharmacological activities predicted for *Bauhinia forficata* Link subsp. *pruinosa* phytoconstituents**

| *Phytoconstituents* | *Main predicted properties by PASS online* | *Pa#* | *Pi#* |
|---|---|---|---|
| ***Quercetin 3-O-(2-rhamnosyl) rutinoside***   | Free radical scavenger | 0.992 | 0.001 |
| | Membrane integrity agonist | 0.979 | 0.002 |
| | Vasoprotector | 0.974 | 0.001 |
| | Antioxidant | 0.934 | 0.002 |
| | Alpha glucosidase inhibitor | 0.888 | 0.001 |
| | Antihypercholesterolemic | 0.846 | 0.004 |
| | Antiinflammatory | 0.749 | 0.010 |
| | Aldose reductase inhibitor | 0.638 | 0.002 |
| | Maillard reaction inhibitor | 0.586 | 0.004 |
| | Antidiabetic | 0.520 | 0.020 |
| ***Kampferol 3-O-(2-rhamnosyl)rutinoside***   | Free radical scavenger | 0.989 | 0.001 |
| | Cardioprotectant | 0.982 | 0.001 |
| | Hepatoprotectant | 0.972 | 0.001 |
| | Antioxidant | 0.891 | 0.002 |
| | Antihypercholesterolemic | 0.839 | 0.004 |
| | Antifungal | 0.806 | 0.006 |
| | Antithrombotic | 0.736 | 0.006 |
| | Hepatic disorders treatment | 0.718 | 0.004 |
| | Laxative | 0.595 | 0.003 |
| | Antidiabetic | 0.541 | 0.018 |
| ***Quercetin-3-O-rutinoside***   | Free radical scavenger | 0.990 | 0.001 |
| | Vasoprotector | 0.980 | 0.001 |
| | Antioxidant | 0.927 | 0.003 |
| | Antihypercholesterolemic | 0.900 | 0.003 |
| | Alpha glucosidase inhibitor | 0.858 | 0.001 |
| | Kinase inhibitor | 0.819 | 0.005 |
| | Aldose reductase inhibitor | 0.652 | 0.002 |
| | Vascular disease treatment | 0.631 | 0.004 |
| | Transcription NFkB stimulant | 0.595 | 0.009 |
| | Antidiabetic | 0.527 | 0.019 |
| ***Kaempferol-3-O-rutinoside***   | Free radical scavenger | 0.984 | 0.001 |
| | Vasoprotector | 0.979 | 0.001 |
| | Anticarcinogenic | 0.978 | 0.001 |
| | Antioxidant | 0.924 | 0.003 |
| | Alpha glucosidase inhibitor | 0.846 | 0.001 |
| | Laxative | 0.737 | 0.002 |
| | Antiinflammatory | 0.743 | 0.011 |
| | Platelet adhesion inhibitor | 0.620 | 0.007 |
| | Antiulcerative | 0.586 | 0.012 |
| | Antidiabetic | 0.550 | 0.017 |

#Pa = Probable activity; #Pi = Probable inactivity. Pa > 0.700 = probable activity greater than 70%. The PASS prediction results were interpreted as follows: (i) only activities with Pa > Pi are considered as possible for a particular compound; (ii) Pa closer to 1000 indicates high chance to find the predicted activity experimentally.

Predictions for the toxic risks (mutagenic, cardiotoxic and hepatotoxic) for BF identified compounds are shown in Table 2. The compounds evaluated presents a general low risk of toxicity, except for mutagenic risk, pointed as high by ACD/Lab platform for the four compounds. Moreover, PreADMET platform point high risk to cardiotoxicity for both kaempferol derivatives compounds (Table 2). Hepatotoxicity risk is not detected, and the median lethal acute dose estimated for rats was classified as non-toxic.

**Table 2. Toxicity prediction for *Bauhinia forficata* Link subsp. *pruinosa* phytoconstituents**

| *In silico test* / *Compound* | *Mutagenic* | *Cardiotoxic* | *Hepatotoxicity* | *$LD_{50}$ (mol/kg)** |
|---|---|---|---|---|
| ***Quercetin-3-O-rutinoside*** | (+++)[1]<br>(++)[2]<br>ND[3]<br>ND[4] | ND[1]<br>(+)[2]<br>(+)[3]<br>(+)[4] | ND[3] | 2.49[2]<br>2.49[3] |
| ***Quercetin-3-O-(2-rhamnosyl) rutinoside*** | (+++)[1]<br>(+)[2]<br>ND[3]<br>ND[4] | ND[1]<br>(+)[2]<br>(+)[3]<br>(+)[4] | ND[3] | 2.61[2]<br>2.47[3] |
| ***Kaempferol-3-O-rutinoside*** | (+++)[1]<br>(++)[2]<br>ND[3]<br>ND[4] | ND[1]<br>(+)[2]<br>(+)[3]<br>(+++)[4] | ND[3] | 2.49[2]<br>2.51[3] |
| ***Kaempferol-3-O-(2-rhamnosyl) Rutinoside*** | (+++)[1]<br>(+)[2]<br>ND[3]<br>ND[4] | ND[1]<br>(+)[2]<br>(+)[3]<br>(+++)[4] | ND[3] | 2.61[2]<br>2.46[3] |

The scale of toxicity risk ranges from low (+), medium (++), high (+++) and no detected (ND). Platforms consulted ACD/Lab[1]; admetSAR[2]; pkCSM[3]; PreADMET[4]. *Oral rat acute toxicity.

**BF Extract Antioxidant Activity**

In order to evaluate predicted antioxidant activities for BF compounds, we employed *in vitro* antioxidant tests (Figure 2). BF aqueous extract showed significant

TAC and FRAP activity from the concentration of 60 μg/mL ($p<0.05$) (Fig. 2A and 2C). In $ABTS^{\bullet+}$ assay a significant percentage of radical sequestration was found from 30 μg/mL concentration ($p<0.05$) (Fig. 2B).

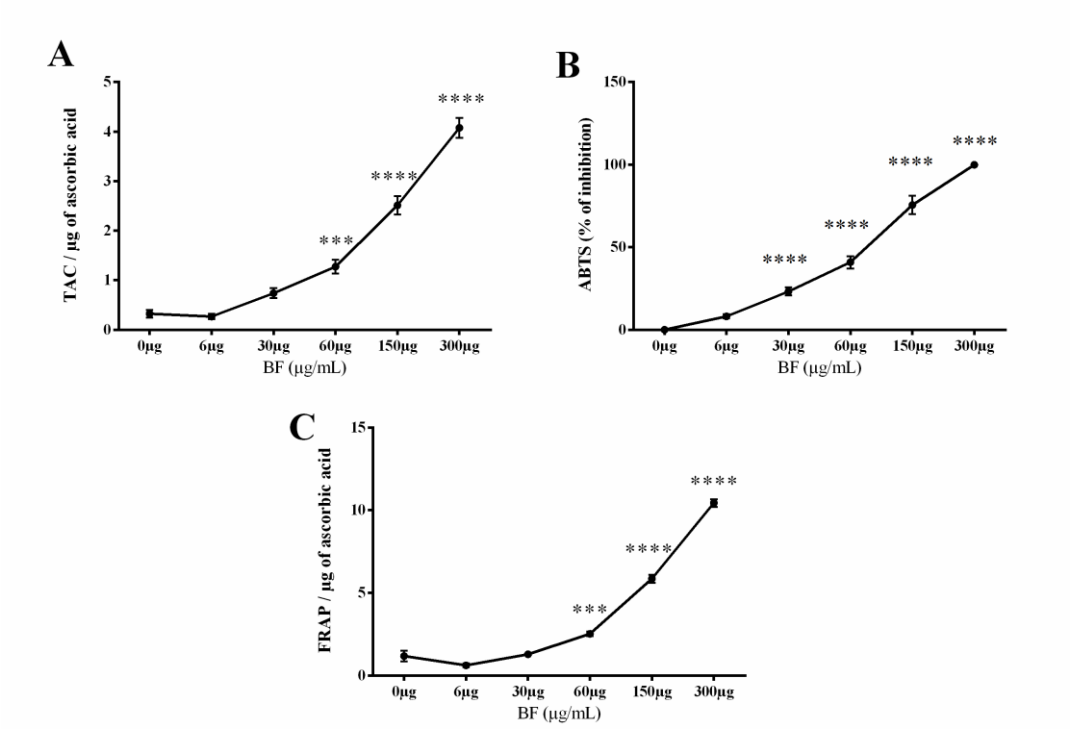



**Figure 2:** Total antioxidant capacity (A); $ABTS^{\bullet+}$ scavenger activity (B); and FRAP activity (C) of BF aqueous extract. (*) indicates significant difference in comparison to a condition without extract ($p<0.05$).

**BF Effects on DM Classic Symptoms**

To evaluate the popular use of BF in traditional medicine, and to investigate the predicted antidiabetic activities we tested, *in vivo*, the effects of BF aqueous extract on a classic DM symptoms in a model of severe diabetic mice (Figure 3). The results show that mice present all DM expected symptoms, as severe hyperglycemia with glucose values around of 400 mg/dL at the end of the experiment (Fig. 3A), polyphagia (Fig. 3B), polydipsia (Fig. 3C) , polyuria (Fig. 3D), and body fat loss (Fig 3E). BF was not effective in control any of these symptoms.

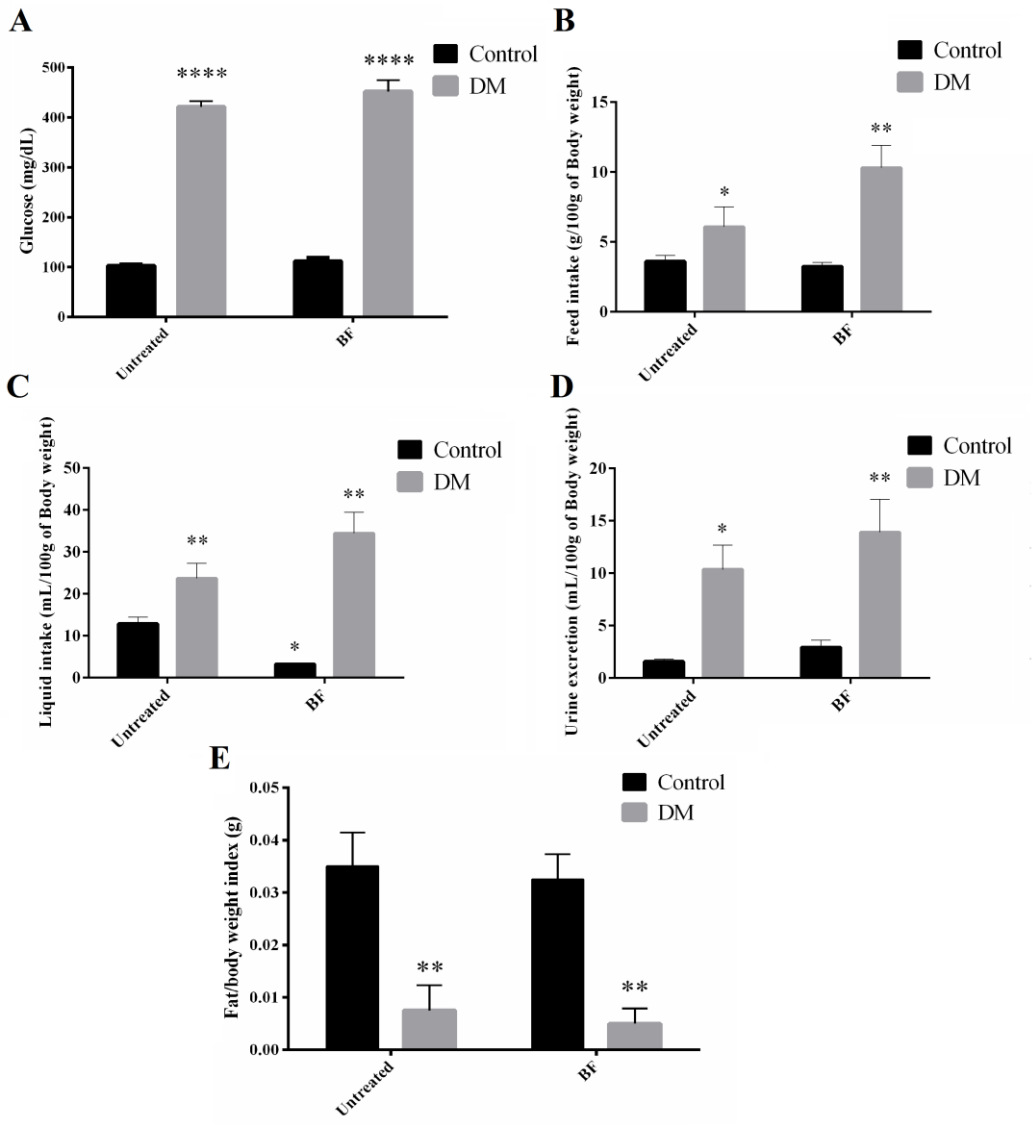



**Figure 3:** Analysis of DM classic symptoms: Hyperglycemia (A); Food intake or polyphagia (B); Liquid intake or polydipsia (C); Urine excretion or polyuria (D); and Epididymis fat weight/body weight ratio as an index of weight loss (E) in severe diabetic mice. (*) indicates significant difference in comparison to a control condition ($p<0.05$).

**BF Effects on Red Blood Oxidative Stress Markers**

The predicted antioxidants effects for BF compounds were also evaluated *ex vivo* in red blood cells of severe diabetic mice (Figure 4). Results show that lipid peroxidation, assayed by TBA-RS, and reactive oxygen and nitrogen species, assayed

by DCF-RS, were increased in erythrocytes of diabetic mice ($p<0.05$) (Fig. 4A and 4B). In both the cases, treatment with BF aqueous extract was effective in control these parameters (Fig. 4A and 4B). Thiols groups, assayed as NPSH, have not changed in any of the experimental conditions (Fig. 4C).

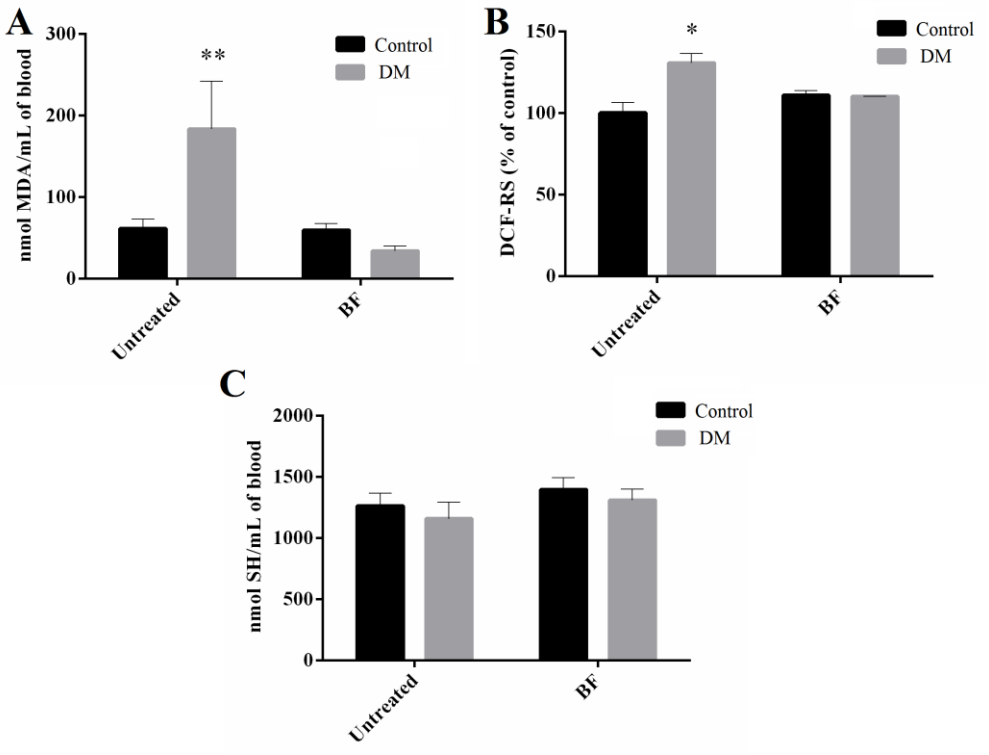



**Figure 4:** Analysis of TBA-RS levels (A); DCF-RS levels (B); and NPSH levels (C) in red blood cells of severe diabetic mice. (*) indicates significant difference in comparison to a control condition ($p<0.05$).

**BF effects on Body/Spleen Index**

The body weight/spleen weight ratio as used as an index of BF extract toxicity (Figure 5). Results show that in severe diabetic condition, this index was increased ($p<0.05$). However, BF extract was effective in reduce this index to a control situation.

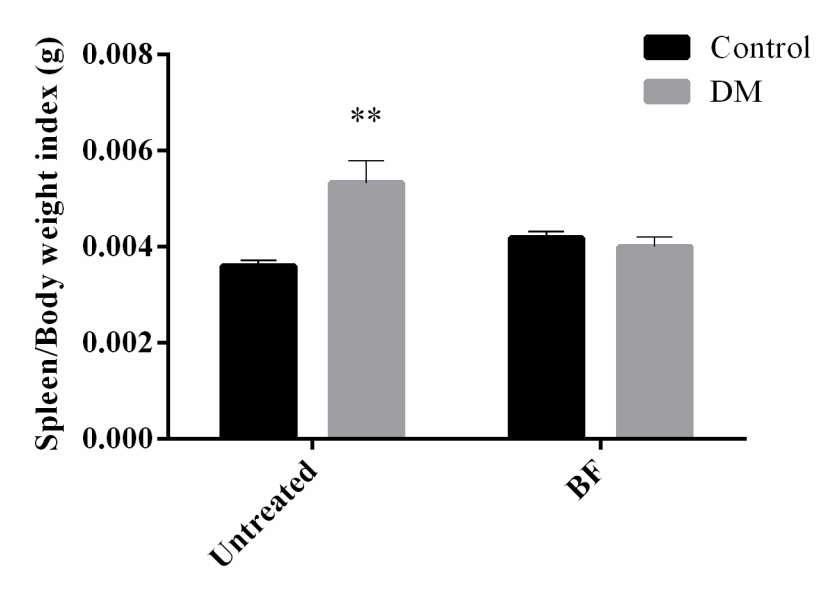



**Figure 5:** Analysis of Spleen weight/body weight ratio as an index of BF toxicity in severe diabetic mice. (*) indicates significant difference in comparison to a control condition ($p<0.05$).

## DISCUSSION

This study was performed to evaluate the biological activity spectrum of BF phytoconstituents *in silico*, as well as, to investigate the BF antioxidant and antidiabetic capacity *in vitro* and *in vivo*. Our data showed strong antioxidants predictions for BF tested compounds (Table 1). These antioxidant predictions were experimentally confirmed in crude extract (Figure 2). In this context, many assays can be used to test the antioxidant activity of plant extracts.

Among the most commonly employed tests, are the evaluation of TAC, which analyzes the reduction of molybdenum (VI) to molybdenum (V) in presence of antioxidant compounds (Prieto et al., 1999). Another similar method is based on assessment of $Fe^{3+}$ to $Fe^{2+}$ reduction (Pulido et al., 2000), and the ability to directly reduce the ABTS radical (Re et al., 1999). In plants extracts, these antioxidants proprieties are directly related to phenols presence and composition.

In fact, one of the most studied polyphenol class are flavonoids. The flavonoids are easily available in the plant kingdom and have particular importance for their

antioxidant potential, which can protect the biomolecules against oxidative damage (Corradini et al., 2011). The antioxidant properties of these phytochemicals are related to its ability to: i) donate electrons or hydrogen atoms, stabilizing free radicals; and ii) chelate transition metals, preventing their participation in reactions such as Fenton (Pandey & Rizvi, 2009; Corradini et al, 2011).

Furthermore, the major compounds of BF are kaempferol and quercetin derivatives, known to have strong antioxidant potential (Tatsimo et al., 2012; Salgueiro et al., 2013; Salgueiro et al., 2016b). Indeed, the capacity of BF crude extract for iron chelation, DPPH radical scavenger, deoxyribose degradation and lipoperoxidation protection, was previously demonstrated (Salgueiro et al., 2013).

On the other hand, toxic predictions for BF phytoconstituents were pointed by some platforms, as mutagenic risk in ACD/Lab, and cardiotoxicity risk in PreADMET. We agree that the similarity between pharmacological and toxicological predictions for quercetin and kaempferol derivatives, can be associated with its structural molecular similarity. According Crespo et al. (2008), quercetin and kaempferol are flavonols, which display minor different structural characteristics. While quercetin presents two –OH moieties on the B-ring, kaempferol presents one –OH moiety on the B-ring. This one less –OH increases kaempferol lipophilicity, when compared with quercetin (Crespo et al., 2008).

Concerning toxic predictions, quercetin is usually identified as mutagenic by the ACD/Lab platform (Salgueiro et al., 2016b). In fact, quercetin is known by this paradoxal behavior, being sometimes mutagenic *in vitro* (Resende et al., 2012). Nevertheless, *in vivo*, is quickly metabolized to non-mutagenic compounds, that can presents health beneficial properties. These characteristics can be identified by some platforms, depending on the fragments database available for structure/activity comparison.

Here, we also evaluate the effect of BF crude extract treatment in a model of mice with severe DM. Were analyzed the effects of BF on the DM symptoms (hyperglycemia, polyuria, polydipsia, polyphagia, and weight loss), and on the oxidative damage resulting from chronic hyperglycemia. Our data indicates that BF protect red blood cells against lipid peroxidation and reduce the DCF-RS levels (Figure 4). In this context, it is know that lipoperoxidation is increased in red blood cells of persons with

DM (Mallick et al., 2011). Oxidative damage in high glucose conditions also was observed in red blood cells *in vitro* (Salgueiro et al., 2013; Pazzini et al., 2015).

Indeed, red blood cells are a good maker of oxidative damage in DM because have high polyunsaturated fatty acid content in their membranes (target for oxidation), and high concentration of hemoglobin (availability of iron for generating hydroxyl radical through the Fenton reaction) (Martínez et al., 2012). Moreover, several pathological events can be related with the red blood cells lipid peroxidation, as loss of membrane selectivity and increased osmotic fragility (Yang et al., 2006, Pazzini et al., 2015).

The additional source of free radicals generated by hyperglycemia is a plausible contributing factor to the increase in TBA-RS levels. In this line, the increase in lipid peroxidation can be directly related to the increase in DCF-RS levels (Salgueiro et al., 2016a), an indirect measured of free radicals or other reactive species levels. The protection against lipid damage and the reduction of DCF-RS species by BF crude extract can be related to the high antioxidant capacity observed *in vitro* and predicted *in silico* (Figure 2; Table 1).

Similarly, the low antidiabetic activity predicted *in silico*, was confirmed by *in vivo* analyses. Diabetic mice presents high blood glucose levels and all classic DM symptoms (Figure 3). Its symptoms, in DM chemically induced model, are caused by a marked hyperglycemia. In fact, the deficient production or action of insulin is the basis of the abnormalities in carbohydrate, fat, and protein metabolism, and is the primary cause of the hyperglycemia (American Diabetes Association, 2009).

In DM, impaired insulin production or action signifies a state of reduced ability of insulin-sensitive tissues to respond to the biological action of insulin on carbohydrate metabolism (Dube et al., 2013). In this context, the cell utilization of glucose falls, and the utilization of fats and proteins increases (Ozougwu et al., 2013). This result in an uncontrolled lipolysis, elevated levels of free fatty acids in the plasma, and decreases the expression of a number of genes necessary for target tissues to respond normally to insulin (Raju & Raju, 2009). Moreover, in a situation of insulin deficiency, the protein breakdown is facilitated and the muscle wasting can be severe (Figueiredo & Cameron-Smith, 2013).

The others symptoms come from this primary disorder. The glycosuria and tissue catabolism leads to the negative caloric balance and consequent triggering of the

hunger sensation (Raju & Raju, 2009). Whereas, the DM is a catabolic state, and even with polyphagia there weight loss. Further, because glucose is an osmotic diuretic leads to polyuria, where there are loss of glucose, water and electrolyte. This results in dehydration and consequent polydipsia (Ozougwu et al., 2014).

Although the BF crude extract do not change any of these symptoms, its antioxidant properties may serve to offset oxidative stress and hence to reduce the cellular injury during DM. Oxidative stress employs multiple cellular pathways that can ultimately lead to both the onset and subsequent complications of DM (Maiese et al., 2015). Many studies have suggested that the intake of antioxidants during elevated glucose concentrations can block free radical production, preventing e.g. the advanced glycation end products production, playing a beneficial role in to reduce diabetic complications (Pandey & Rizvi, 2009).

Our data also show that BF was effective in normalizing splenomegaly in diabetic animals (Figure 5). Splenomegaly is a relative common finding in DM rodent experimental models (Al-Enazi, 2014; Yang et al., 2015). In relation to this data, we speculate that the increased spleen size may be related to the increased rate of damaged red blood cells removal. In fact, the spleen plays important roles in various vertebrate biological processes, such as removal of aged erythrocytes, recycling of iron, and elicitation of immunity (Saito et al., 2012).

Taken together, our data indicates that BF, although do not present antidiabetic effects, can be used as a DM complementary treatment because their important antioxidant protection against red blood cells oxidative damage.

## CONFLICT OF INTEREST

The authors declare no conflict of interests.

## ACKNOWLEDGMENTS

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**Figure Captions**

**Figure 1:** Study design.

**Figure 2:** Total antioxidant capacity (A); ABTS•+ scavenger activity (B); and FRAP activity (C) of BF aqueous extract. (*) indicates significant difference in comparison to a condition without extract ($p<0.05$).

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**Figure 4:** Analysis of TBA-RS levels (A); DCF-RS levels (B); and NPSH levels (C) in red blood cells of severe diabetic mice. (*) indicates significant difference in comparison to a control condition ($p<0.05$).

**Figure 5:** Analysis of Spleen weight/body weight ratio as an index of BF toxicity in severe diabetic mice. (*) indicates significant difference in comparison to a control condition ($p<0.05$).

### *4.3. Artigo Científico 1*

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*Research Article*

# Effects of *Bauhinia forficata* Tea on Oxidative Stress and Liver Damage in Diabetic Mice

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This study was designed to evaluate the effects of *Bauhinia forficata* Link subsp. *pruinosa* (BF) tea on oxidative stress and liver damage in streptozotocin (STZ)-induced diabetic mice. Diabetic male mice have remained 30 days without any treatment. BF treatment started on day 31 and continued for 21 days as a drinking-water substitute. We evaluated (1) BF chemical composition; (2) glucose levels; (3) liver/body weight ratio and liver transaminases; (4) reactive oxygen species (ROS), lipid peroxidation, and protein carbonylation in liver; (5) superoxide dismutase (SOD) and catalase (CAT) activities in liver; (6) $\delta$-aminolevulinate dehydratase ($\delta$-ALA-D) and nonprotein thiols (NPSH) in liver; (7) Nrf2, NQO-1, and HSP70 levels in liver and pancreas. Phytochemical analyses identified four phenols compounds. Diabetic mice present high levels of NQO-1 in pancreas, increased levels of ROS and lipid peroxidation in liver, and decrease in CAT activity. BF treatment normalized all these parameters. BF did not normalize hyperglycemia, liver/body weight ratio, aspartate aminotransferase, protein carbonyl, NPSH levels, and $\delta$-ALA-D activity. The raised oxidative stress seems to be a potential mechanism involved in liver damage in hyperglycemic conditions. Our results indicated that BF protective effect could be attributed to its antioxidant capacity, more than a hypoglycemic potential.

## 1. Introduction

Historically, basic therapy for treating several diseases includes the use of medicinal plants. Vegetable species with medicinal power have considered complex mixtures of biologically active products, and usually many of them are responsible for their biological properties [1]. Therefore, many plants considered medicinal have been used in folk medicine to treat *diabetes mellitus* (DM) [2]. Among these is *Bauhinia forficata* (BF) (Leguminosae, Fabaceae), popularly known as "paw of cow" [3].

In Brazil, the tea (infusion) of BF leaves is an important alternative treatment for people with DM [2]. The BF genus comprises about 300 species found especially in the tropical regions of the planet [3]. Besides their possible hypoglycemic potential, considerations about the antioxidant and hepatoprotective activities of some *Bauhinia* species have been postulated. For example, extracts of *Bauhinia forficata* Link and *Bauhinia cheilandra* showed antidiabetic activity in STZ and alloxan-induced diabetic rats [4–6]. Already, the antioxidant and hepatoprotective activity was previously demonstrated for *Bauhinia forficata* Link, *Bauhinia racemosa*

Lam, and *Bauhinia variegata* [7–9]. However, we did not find in scientific literature studies with mice or rats that investigate the same *Bauhinia* species that we use here (*Bauhinia forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin).

Biological properties of *Bauhinia* species have been attributed to its phenolic compounds. In this context, *Bauhinia forficata* Link subsp. *pruinosa* are able to scavenge reactive oxygen species (ROS) because it contains flavonoids among its constituents (especially derivatives of quercetin and kaempferol) [10, 11]. These characteristics can be extremely important in diseases where there is an increase in oxidative stress, as in DM and its complications.

Indeed, chronic hyperglycemia in DM has related to a bigger ROS production and severe oxidative damage in different tissues, including the liver (for a review see [12]). Increased ROS has been known to induce changes in expression and activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), as well as thiol oxidation and lipid peroxidation [12]. Furthermore, previous reports showed that, in experimental models of DM, the sulfhydryl-containing enzyme $\delta$-aminolevulinate dehydratase ($\delta$-ALA-D) was inhibited [13–15].

Moreover, increases in ROS production, both in liver and in pancreas, stimulate expression of factors related to cellular antioxidant response, such as NF-E2-related nuclear factor erythroid-2 (Nrf2), NADPH quinone oxidoreductase 1 (NQO-1), and heat shock protein 70 (HSP70) [16, 17]. According to Yeo et al. [18], antioxidant and chemical stress, including chemical DM induction in mice, increases NQO-1 expression.

Therefore, the aim of this study was to evaluate the effects of *Bauhinia forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin (infusion) (BF tea) treatment on oxidative stress and liver damage in diabetic mice. Among the parameters evaluated were the glycaemia, ROS production, lipid peroxidation, protein carbonylation, and nonprotein thiols levels in liver, as well as the activities of enzymes $\delta$-ALA-D, SOD, and CAT in liver. Moreover, we evaluate the Nrf2, NQO-1, and HSP70 expression in liver and, additionally, in pancreas.

## 2. Materials and Methods

*2.1. Chemicals.* Sigma-Aldrich Chemical Co. (St. Louis, MO) supplied Ellman's reagent (DTNB) and streptozotocin. Labtest (Minas Gerais, Brazil) supplied commercial kits. Other reagents were obtained from local suppliers.

*2.2. Bauhinia forficata (BF) Preparation.* BF leaves were collected in September (spring) of 2014 in southern Brazil ($29^{\circ}44'58.8''$S $57^{\circ}05'01.7''$W). Botanical identification of BF leaves samples was confirmed and a voucher specimen (number ICN 167491; *B. forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin) was deposited at ICN Herbarium of Federal University of Rio Grande do Sul (Brazil).

BF tea was prepared with naturally dried leaves in a 1 mg/mL proportion (weight of dried leaves/volume of water), described by Salgueiro et al. [11].

*2.3. Chromatographic Analyses.* Chromatographic analyses by HPLC were conducted, described in [11, 19], using a Prominence Liquid Chromatograph (Shimadzu, Kyoto, Japan). This apparatus is equipped with an SLC-10A controller, LC-20AD pump, SIL-10AF autosampler, and SPD-M10A PDA detector. An ODS-Hypersil Thermo Scientific C18 column (250 × 4.6 mm i.d., 5 $\mu$m particle size) (Bellefonte, United States) was used. Mobile phase consisted of water containing 0.05% phosphoric acid (A) and acetonitrile (B) at a flow rate of 0.8 mL min$^{-1}$ using the following gradients: 0.1–23 min, 10–40% of solvent B in A and 23.01–40 min, 10% solvent B, and 90% solvent A. Detection was done on a diode array detector (DAD) set at 340 nm and the injection volume was 20 $\mu$L. The HPLC system was operated at 25 ± 1°C. Runs were made in triplicate. The reference standard chemical composition for BF tea was established previously by our group, identifying the compounds quercetin-3-O-(2-rhamnosyl) rutinoside, kaempferol-3-O-(2-rhamnosyl) rutinoside, quercetin-3-O-rutinoside, and kaempferol-3-O-rutinoside [11, 19].

*2.4. Diabetes Mellitus (DM) Induction and BF Treatment.* Committee on the Ethics of Animal Experiments approved this study (permit number 001/2012). All experiments were conducted with the minimum number of animals and in obedience to the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology. Animals were maintained in an enriched environment with a room-controlled temperature, 12 h light-dark cycle, and food and water available *ad libitum*.

Three-month male Swiss albino mice (30–35 grams) were divided into four different groups with six animals for each group:

(1) Control received only citrate buffer intraperitoneally (*i.p.*) and drank water throughout the period.

(2) DM received a single STZ dose (150 mg/kg) *i.p.* and drank water throughout the period.

(3) BF received only citrate buffer *i.p.*, drank water during 30 days, and afterwards drank BF tea (1 mg/mL) during 21 days.

(4) DM + BF received a single STZ dose (150 mg/kg) *i.p.*, drank water during 30 days, and afterwards drank BF tea (1 mg/mL) during 21 days.

STZ was freshly prepared in citrate buffer (0.05 M, pH 4.5), and before STZ administration the animals were fasted for a period of four hours. STZ dose was established, proposed by Animal Models of Diabetic Complications Consortium [20], in order to induce a severe hyperglycemia in mice. Five days after STZ injection the hyperglycemia was confirmed by collecting a tail drop of blood and using ACCU-Check Active (Roche Diagnostics) glucometer.

The BF concentration (approximately 313 mg/kg of body weight) was established after evaluation of liquid intake of diabetic mice in metabolic cage (9.4 ± 2.24 mL of tea per day) and body weight mean (0.030 kg). This dose is in accordance with previous studies that investigated the hypoglycemic and hepatoprotective activity of other *Bauhinia* species [4–6, 8, 9].

BF treatment started on day 31 and continued for 21 days in drinking water.

*2.5. Tissue Preparation for Biochemical Analyses.* After the period of treatment, animals were killed by cardiac puncture. This procedure was performed under enough ether anesthesia to ameliorate mice suffering. Mice livers were removed and carefully washed, and part of them were weighted and homogenized in 1 : 10 ratio of tissue to cold NaCl (0.9%). The homogenates were centrifuged at 4000 g for 10 min at 4°C and the supernatants (S1) collected for biochemical analyses. All the biochemical analyses were performed in the same day of euthanasia. The liver and body weight were used to evaluate the liver weight/body weight ratio.

*2.6. Analysis of Glucose Levels and Liver Transaminases.* Blood was collected in heparinized tubes by cardiac puncture after fasting for 6 hours. After centrifugation, levels of glucose and liver transaminases were determined in plasma using a commercial kit (Labtest, Minas Gerais/Brazil).

*2.7. Assessment of 2,7-Dichlorofluorescein (DCFH) Oxidation.* Indirect quantification of reactive oxygen species (ROS) production was determined in S1 samples by evaluation of dichlorofluorescein reactive species (DCF-RS) levels, proposed by Myhre et al. [21]. Briefly, an aliquot of S1 (100 $\mu$L) were added to a medium containing Tris-HCl buffer (0.01 mM, pH 7.4) and DCFH-DA (7 $\mu$M). This medium was incubated in the dark for 1 h until fluorimetric analysis (Ex: 488 nm; Em: 520 nm). The results were stated as DCF fluorescence intensity, corrected by protein content, and expressed as percentage of control.

*2.8. Thiobarbituric Acid Reactive Species (TBA-RS) Levels.* Lipid peroxidation was assayed by adding S1 samples (100 $\mu$L) to a medium containing 8.1% sodium dodecyl sulfate, acetic acid buffer (pH 3.5), and 0.8% aqueous solution of thiobarbituric acid, proposed by Ohkawa et al. [22]. After heating at 95°C for 60 min, the red pigment produced was measured spectrophotometrically at 532 nm. The results were calculated using a standard curve constructed with malondialdehyde (MDA) at known concentrations and corrected by protein content. The results were expressed as nanomoles of MDA per milligram of protein.

*2.9. Protein Carbonyl Levels.* Protein carbonyl was measured in S1 samples, proposed by Levine et al. [23]. Briefly, an aliquot of S1 (200 $\mu$L) were derivatized using 2,4-dinitrophenylhydrazine (DNPH). DNPH reaction proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and washed three times with an ethanol/ethyl acetate mixture (1 : 1). Finally, the precipitates were dissolved in 6 M guanidine HCl solution. Protein carbonyl levels were determined spectrophotometrically at 370 nm, against blanks. The results were calculated using the molar extinction coefficient of DNPH, corrected by protein content, and expressed as nanomoles of carbonyl per milligram of protein.

*2.10. Superoxide Dismutase (SOD) Enzyme Activity.* SOD enzyme activity was determined in S1 samples, proposed by Kostyuk and Potapovich [24]. This method is based on the capacity of SOD in inhibiting quercetin autooxidation. Briefly, S1 aliquots (25 $\mu$L) were added to a medium containing 0.016 M phosphate buffer, 0.8 mM N,N,N',N'-Tetramethylethylenediamine, and 0.08 mM EDTA (final pH of the medium was 10). The kinetic analysis of SOD was measured spectrophotometrically at 406 nm after quercetin addition (1.5 mg of quercetin in 10 mL of N,N-Dimethylformamide). The results were corrected by protein content and expressed as unit per milligram of protein. One unit of SOD activity is defined as the amount of enzyme that inhibited the quercetin oxidation reaction by 50% of maximal inhibition. Fifty percent inhibition was produced by approximately 1.5 ng/mL of pure enzyme [17].

*2.11. Catalase (CAT) Enzyme Activity.* CAT enzyme activity was determined in S1 samples, proposed by Aebi [25]. This method is based on the rate of hydrogen peroxide ($H_2O_2$) degradation by the action of CAT. Briefly, S1 aliquot (20 $\mu$L) was added to a medium containing potassium phosphate buffer (50 mM, pH 7.4) and $H_2O_2$ (1 mM). The kinetic analysis of CAT was measured spectrophotometrically at 240 nm after $H_2O_2$ addition. The results were calculated using the molar extinction coefficient of $H_2O_2$, corrected by protein content, and expressed as nmol $H_2O_2$/mg protein/min.

*2.12. Delta-Aminolevulinic Acid Dehydratase ($\delta$-ALA-D) Enzyme Activity.* $\delta$-ALA-D enzyme activity was determined in S1 samples, according to Sassa [26]. This method is based in analysis of porphobilinogen (PBG) formation after $\delta$-aminolevulinic acid addition. Briefly, S1 samples (100 $\mu$L) were mixed with $\delta$-aminolevulinic acid (12 mM initial concentration). The tubes were incubated for 60 min at 37°C. The reaction was stopped by adding 10% trichloroacetic acid with 10 mM mercuric chloride. After centrifugation, an equal volume of Ehrlich reagent was added to the supernatant, and absorbance at 555 nm was recorded. The results were corrected by protein content and expressed as nanomoles of PBG per milligram of protein per hour of incubation.

*2.13. Nonprotein Thiols (NPSH) Levels.* NPSH levels were determined according to Ellman [27]. Briefly, the S1 samples were precipitated with 10% trichloroacetic acid (1 : 1) and centrifuged at 4000 g for 10 min at 4°C to obtain supernatants (S2). S2 (100 $\mu$L) samples were added to a medium containing phosphate buffer (TFK 0.25 mM, pH 7.4), and Ellman reagent (DTNB 1 mM). The yellow pigment produced was measured spectrophotometrically at 420 nm. The results were calculated in relation to a standard curve constructed with glutathione (GSH) at known concentrations and corrected by protein content. The results were expressed as nanomoles of SH per milligram of protein.

*2.14. Protein Content Determination.* Protein content was determined in S1 samples, proposed by Bradford [28], and measured spectrophotometrically at 595 nm. Bovine serum

albumin at known concentrations was used to construct a standard curve.

*2.15. Western Blot Analysis.* Western blotting was performed according to Posser et al. [29] with minor modifications. Part of the liver and pancreas were homogenized at 4°C in a medium containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM $Na_3VO_4$, 100 mM sodium fluoride, and protease inhibitor cocktail (Sigma, MO), pH 7.0. The homogenates were centrifuged at 1000 g for 10 min at 4°C and the supernatants (S1) collected. After protein determination (following Bradford [28]) using bovine serum albumin as standard, $\beta$-mercaptoethanol and glycerol were added to samples to a final concentration of 8 and 25%, respectively, and the samples were frozen in −80°C until further analysis. Proteins (2 mg/mL) were separated using SDS-PAGE with 10% gels and then electrotransferred to nitrocellulose membranes as previously described [29]. Membranes were washed in Tris-buffered saline with Tween (TBST; 100 mM Tris-HCl, 0.9% NaCl, and 0.1% Tween-20, pH 7.5) and incubated overnight (4°C) with different primary antibodies (Santa Cruz Biotechnology, TX), all produced in rabbit (anti-Nrf2, anti-NQO-1, and anti-HSP70 anti-$\beta$-actin; 1 : 1000 dilution in TBST). Following incubation, membranes were washed in TBST and incubated for 1 h at 25°C with HRP-linked anti-rabbit-IgG secondary specific antibodies (Sigma, MO). The immunoblots were visualized in the Image Station 4000MM PRO using ECL reagent (Promega, WI). Immunoreactive bands were quantified using the Scion Image software and expressed as percentage of untreated controls.

*2.16. Statistical Analysis.* Data were expressed as mean ± SEM of the number of animals used in each experiment. Statistical analysis was performed using two-way ANOVA and Tukey post hoc test. Values of $p < 0.05$ were considered statistically significant. GraphPad prism 6 software was used for statistical analysis and for plotting graphs.

## 3. Results

*3.1. Chromatographic Profile.* HPLC analysis of BF tea revealed the following main compounds kaempferol-3-O-(2-rhamnosyl) rutinoside (2) > quercetin-3-O-(2-rhamnosyl) rutinoside (1) > quercetin-3-O-rutinoside (3) > kaempferol-3-O-rutinoside (4) (Figure 1).

*3.2. Glucose Levels.* Diabetic mice had a significant increase in the serum glucose levels, which were not reduced by BF (Figure 2).

*3.3. Liver Toxicity Evaluation.* The liver/body weight ratio was increased in diabetic mice when compared to control group. These changes were not modified by BF treatment. BF *per se* did not affect this parameter (Figure 3). Diabetic mice had a significant increase in aspartate aminotransferase (AST) level (Figure 4(a)) when compared to the control group. This change was not modified by BF treatment.

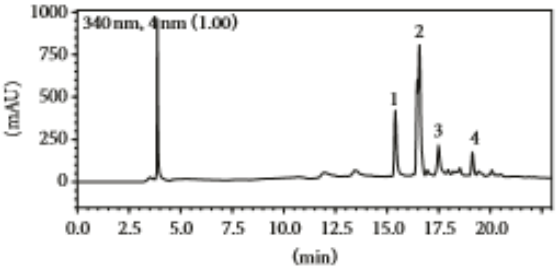


Figure 1: Chromatographic profile of *B. forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin tea. Chemical compounds identified Peak 1: quercetin-3-O-(2-rhamnosyl) rutinoside (retention time: 15.60 min); Peak 2: kaempferol-3-O-(2-rhamnosyl) rutinoside (retention time: 16.70 min); Peak 3: quercetin-3-O-rutinoside (retention time: 17.40 min); Peak 4: kaempferol-3-O-rutinoside (retention time: 19.10 min).

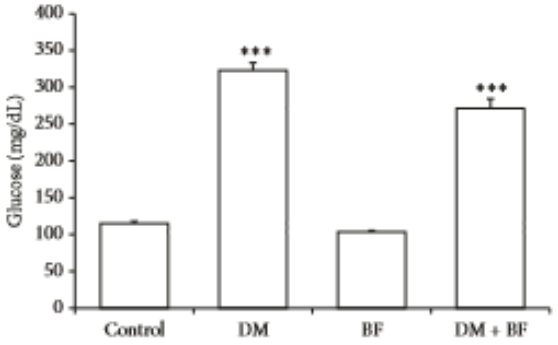


Figure 2: Glucose levels (mg/dL) at the end of treatment. The * indicates significant difference in comparison to control group ($p < 0.05$).

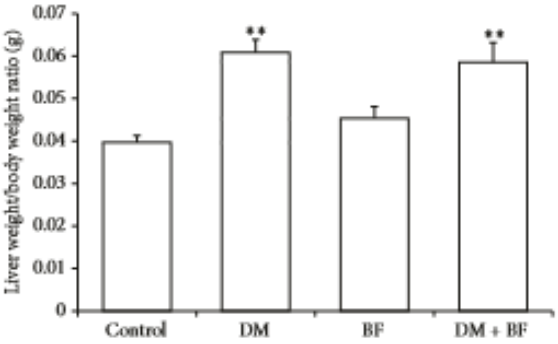


Figure 3: Liver/body weight ratio (g) of diabetic mice treated with BF. The * indicates significant difference in comparison to control group ($p < 0.05$).

Alanine aminotransferase (ALT) level (Figure 4(b)) was not changed by any treatment.

*3.4. Liver Oxidative Stress Evaluation.* BF treatment was effective in normalizing the increases in ROS (DCF-RS) and lipid

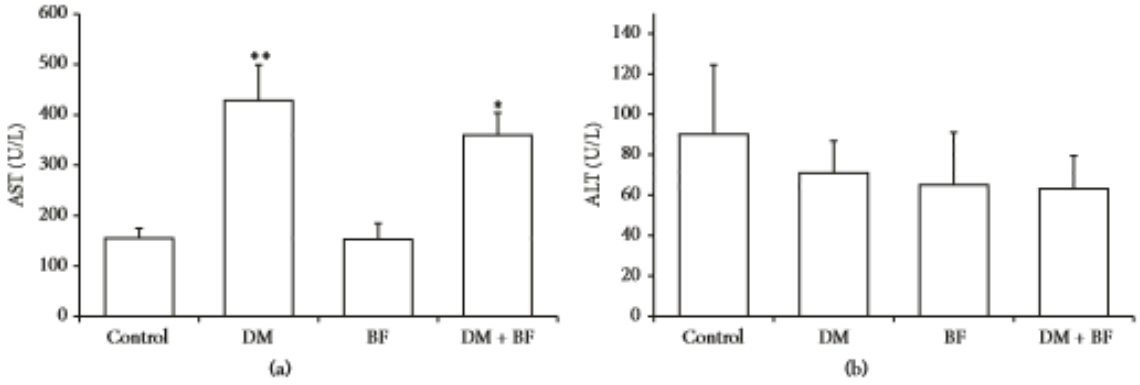


Figure 4: Alanine aminotransferase (a) and aspartate aminotransferase (b) levels (U/L) of diabetic mice treated with BF. The * indicates significant difference in comparison to control group ($p < 0.05$).

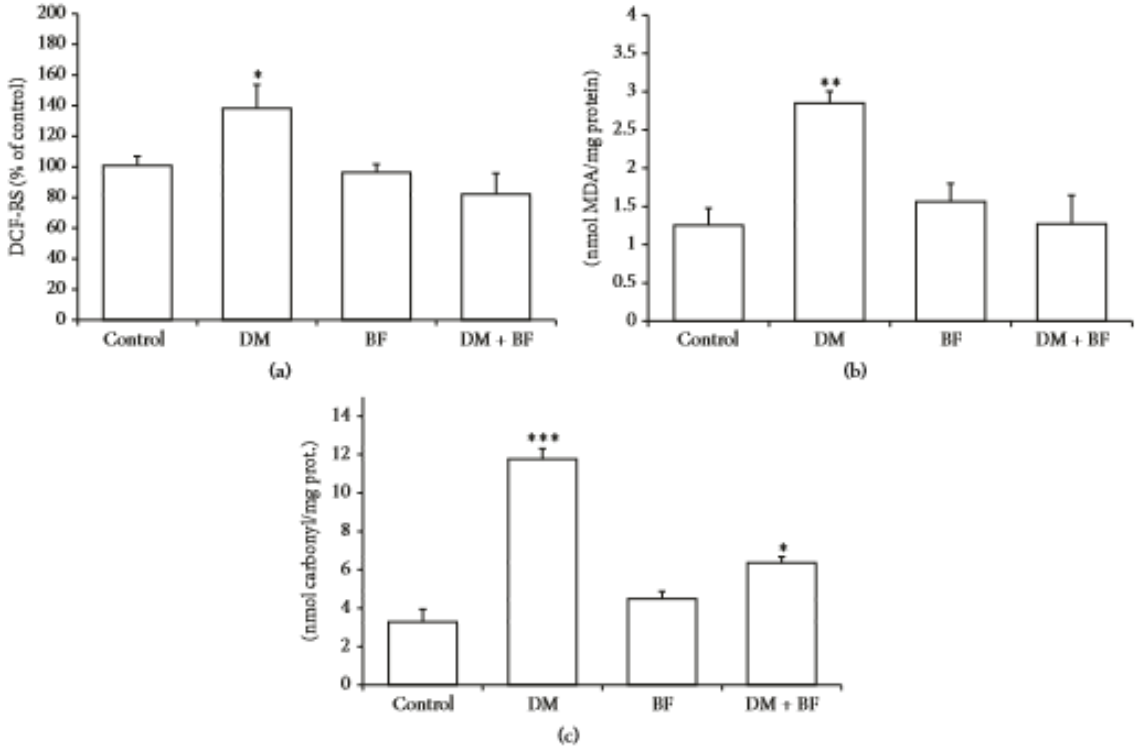


Figure 5: Liver dichlorofluorescein reactive species (DCF-RS) (a); thiobarbituric acid reactive species (TBA-RS) (b); and protein carbonyl levels (c) of diabetic mice treated with BF. The * indicates significant difference in comparison to control group ($p < 0.05$).

peroxidation (TBA-RS) levels observed in diabetic mice, to the control levels (Figures 5(a) and 5(b), resp.). Furthermore, diabetic mice had an increase in the carbonylated protein levels (Figure 5(c)) that were only partially reduced by BF treatment.

No difference in SOD activity was observed among the groups (Figure 6(a)). However, the diabetic mice had a significant decrease in CAT activity when compared to the control group. This decrease was attenuated by BF treatment (Figure 6(b)).

The activity of liver $\delta$-ALA-D was inhibited in diabetic mice. The inhibition of $\delta$-ALA-D enzyme activity was not modified by BF treatment (Figure 7(a)). Addition of a thiol donor dithiothreitol (DTT) partially reactivated $\delta$-ALA-D, however, without restoring the basal activity of $\delta$-ALA-D (data not shown). The levels of nonprotein thiol groups

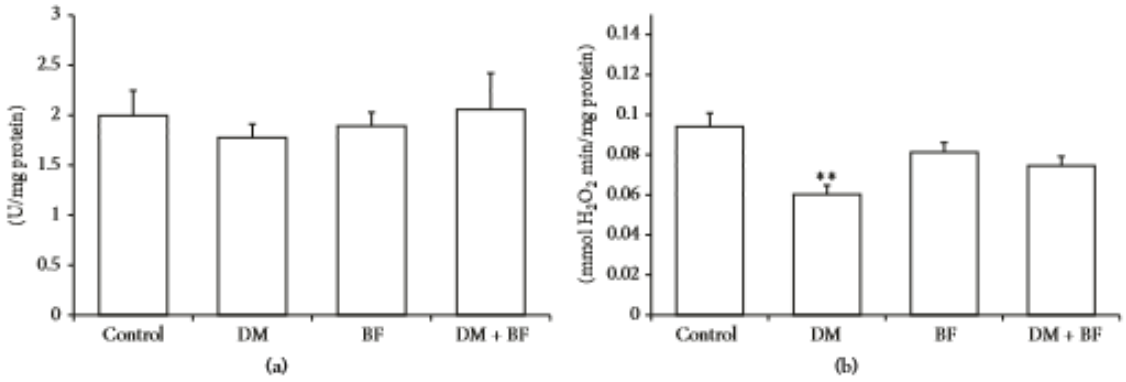


Figure 6: Liver superoxide dismutase (SOD) (a) and catalase (CAT) (b) activities of diabetic mice treated with BF. The * indicates significant difference in comparison to control group ($p < 0.05$).

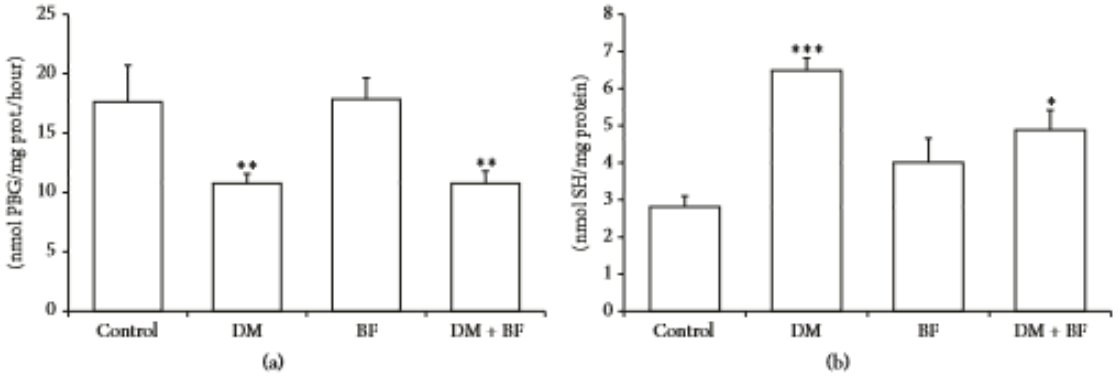


Figure 7: Liver delta-aminolevulinic acid dehydratase (δ-ALA-D) activity (a) and nonprotein SH (NPSH) levels (b) in diabetic mice treated with BF. The * indicates significant difference in comparison to control group ($p < 0.05$).

(NPSH) were increased in diabetic mice and BF treatment restores only partially this increase (Figure 7(b)).

3.5. *Western Blot Analysis.* Liver western blot showed that Nrf2, NQO-1, or HSP70 protein levels were not altered in diabetic mice or BF treatment when compared to the control (Figure 8). In pancreas, an increase in NQO-1 levels was observed, and BF treatment reduce these at levels lower than the control group (Figure 9). No differences in the levels of Nrf2 and HSP70 were observed among the groups in pancreas.

## 4. Discussion

The present study was designed to investigate the effects of *Bauhinia forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin (BF) tea against oxidative stress and liver damage in diabetic mice. In folk medicine, various species of BF have been used to treat *diabetes mellitus* (DM) [2], especially due to their possible hypoglycemic effect. Our results show BF tea reduced liver oxidative stress in diabetic mice, although it did not change the glycaemia.

In this context, the absence of hypoglycemic action of BF tea may be due to the nonextraction of some compounds in the aqueous fraction (infusion) or due to absence of kaempferitrin compound (kaempferol-3,7-O-(r)-dirhamnoside), pointed out as responsible for hypoglycemic action in other *Bauhinia* species [10].

Our results show BF tea *per se* does not determine abnormal hepatic growth or transaminases changes, indicating possible absence of toxicity (Figures 3 and 4(a), 4(b)). On the other hand, we had an increase in AST levels and in liver/body weight ratio in diabetic mice. The increase in liver/body weight ratio may be due to the reduction of body weight (data not shown), common in untreated diabetes [30]. Regarding transaminases, both AST and ALT are highly concentrated in the liver. However, ALT is localized only in the cellular cytoplasm, whereas AST is cytosolic in a minor portion and mitochondrial in a major portion. Furthermore, AST is highly concentrated in zone 3 of the hepatic acinus, and damage to this zone may indicate ischemic or toxic events, resulting in greater AST levels [31]. In case of diabetes, hepatic toxic events may occur in response to an excess in free fatty acids [32] results of insulin impairment. Known mechanisms

for hepatic toxics events that increase transaminases levels in diabetic state include cell membrane disruption, mitochondrial dysfunction, toxin formation, oxidative stress, and recruited inflammatory cells [32].

We observe an increase in reactive oxygen species (ROS) and lipid peroxidation levels (Figures 5(a) and 5(b), resp.), indicating oxidative damage in liver. The assessment of DCF-RS is well accepted to determine ROS levels, as well as reactive nitrogen species able to oxidize the DCFH, a general index of oxidative stress. Similarly, TBA-RS assay is a known biomarker used to estimate lipid damage from cells and tissues, and its increased levels are an indirect evidence of high ROS production. Although BF tea treatment did not modify the changes in liver/body weight ratio and AST levels in diabetic mice, the plant was effective in reducing DCF-RS and TBA-RS levels. These findings reinforce our previously reported antioxidant activity of BF tea even at low concentrations [11]. The antioxidant activity of BF extracts has been attributed to high levels of polyphenols and flavonoids present in its composition [11, 33]. Here, we identify four major compounds (Figure 1) that were previously reported using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) [11, 19]. Among the chemical constituents identified in our extract, the quercetin and kaempferol derivate have been extensively studied to have antioxidant properties, such as reduction of TBA-RS levels and control of antioxidant response [10, 11].

Our results also showed that there is an increase in liver carbonylated protein levels (Figure 5(c)). This increase is reduced only partially by BF treatment, and it is not related with ROS levels that were controlled by BF treatment (Figure 5(a)). Probably, a longer BF treatment might reduce the protein carbonyl levels to control levels. This is possible whereas carbonylated proteins have a long half-life and take longer to suffer degradation when compared to normal proteins.

Concerning liver antioxidant enzymes, we observed a significant decrease in CAT activity in diabetic mice, which was reverted by BF tea treatment (Figure 6(b)). No changes were observed in liver SOD activity in diabetic mice. For instance, changes in antioxidant enzymes activities or its return to normal values following a previous decrease may occur as a compensatory mechanism in response to a constant exposure to increased oxidative stress, such as those determined by prolonged hyperglycemia. This could explain the decreases in SOD activity observed by some researchers and the normal SOD activity observed by other investigators (for a review see [12]).

We also observed a decrease in $\delta$-ALA-D enzyme activity (Figure 7(a)), not related to a decrease in NPSH levels (Figure 7(b)), in diabetic mice. Several studies report that the $\delta$-ALA-D enzyme activity is reduced in hyperglycemic conditions [13, 14, 34]. This occurs due to presence of thiol groups in its structure, which are sensitive to oxidation. This characteristic explains its use as a good oxidative stress biomarker [13, 14]. In diabetic mice, we observed an increase in thiol levels, probably due to a physiological compensatory effect. In this context, the NPSH levels, glutathione (GSH) as a major compound, increase to counteract the high ROS

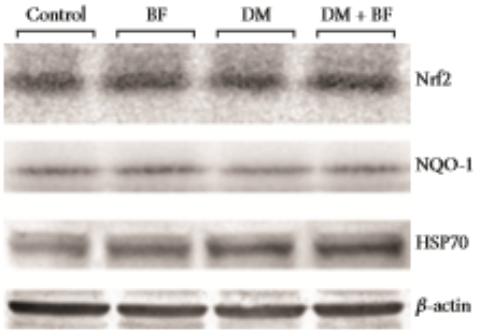


Figure 8: Liver Nrf2, NQO-1, and HSP70 protein levels in diabetic mice treated with BF. The data were normalized with $\beta$-actin expression and expressed as % of control.

production [11]. GSH is a ubiquitous cellular three-peptide antioxidant that acts as an intracellular buffer being responsible for the maintenance of the thiol redox balance [35]. In this line, mainly three functional changes may lead to a $\delta$-ALA-D enzyme activity reduction, namely, removal of divalent zinc from its catalytic site (1); oxidation of its critical thiol groups (2); or protein oxidation (3) [13, 14, 34]. Taking into account that there were no thiol levels compromising and that the SH donor dithiothreitol (DTT) only partially reactivated $\delta$-ALA-D (data not shown) we believe that the mechanism of inhibition was linked to protein oxidation. In fact, reducing sugars can interact with critical lysine residues of $\delta$-ALA-D catalytic site, oxidizing the lysine residues to disulfides and inactivating the enzyme [34]. In this context, inhibition of $\delta$-ALA-D in diabetes may be related to hyperglycemia [13, 14].

Here, the oxidative damage in liver seems to occur without changes in Nrf2, HSP70, or NQO-1 protein levels (Figure 8). Different in pancreas (target organ of STZ), we observed changes in NQO-1 expression (Figure 9) that were minimized by BF treatment. The enzyme NQO-1 is generally considered as a detoxification enzyme and has been known to protect $\beta$-cells against stressors, including the diabetogenic agent STZ [18, 36]. There is evidence that NQO-1 knockout mice present increased pancreatic $\beta$-cell death induced by STZ [18]. Furthermore, both STZ and hyperglycemia have been known to increase ROS production [12], and NQO-1 enzyme plays an important role as a superoxide scavenger that may provide an additional level of protection against ROS toxicity [36]. The increase observed in pancreas NQO-1 could be associated with a possible response against the xenobiotic injury determined by STZ. However, more studies are necessary to highlight the reasons for increased expression of NQO-1 in pancreas but not in liver.

Although we did not observe changes in Nrf2 protein levels, the NQO-1 upregulation in pancreas and the elevated levels of GSH in liver suggest an early activation of Nrf2-antioxidant response element (ARE) pathway, probably in response to increase in ROS levels. In fact, under stressing condition, the transcription factor Nrf2 interacts with ARE and upregulates antioxidative genes including NQO-1, antioxidant enzymes, and GSH levels, which are very important components of the cellular antioxidant defense

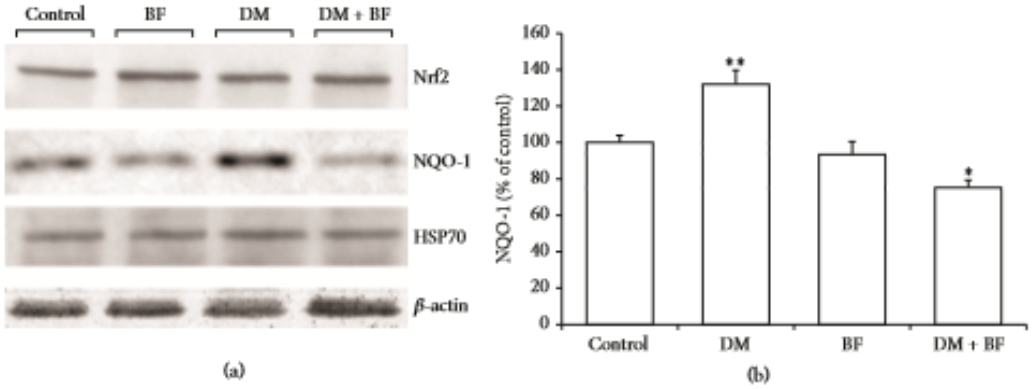


FIGURE 9: Pancreas Nrf2, NQO-1, and HSP70 protein levels in diabetic mice treated with BF (a). Graphical representation of NQO-1 pancreas protein levels (b). The data were normalized with $\beta$-actin expression and expressed as % of control. The * indicates significant difference in comparison to the control group ($p < 0.05$).

[37]. However, while NQO-1 is a stable protein (half-life greater than 18 hours in wild type cells) [38], Nrf2 is a highly unstable protein and its half-life is about 15 min under nonstress condition [39] to 100 min under stress condition [40]. In fact, according to Nguyen et al. [39], even in stress condition, Nrf2 has a short life and is still subject to a high rate of degradation. The same has been observed with the HSP70, which has a half-life of approximately 2 hours [41]. This rapid degradation rate occurs, presumably, to prevent its accumulation in an uncontrolled manner [39] and may be the reason why we cannot observe differences in the levels of this protein in our study.

We highlight that although our objective was to investigate effects of BF tea (crude aqueous extract) on liver damage in diabetic mice, some points are extremely relevant and deserve further attention in future investigations, in particular, deeper analysis of the pancreas, serum insulin concentration, analysis of BF compounds concentration in the plasma, and the role/effect of their isolated bioactive components.

## 5. Conclusion

Taken together, our observations suggested that diabetic mice present an increase in liver oxidative damage and in pancreas NQO-1 expression, which were modulated by BF treatment. Since BF tea decreased liver oxidative injury but does not change glycaemia, we believe that BF protective effect may be attributed especially to its antioxidant capacity.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## *4.4. Artigo Científico 2*

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# *In vitro* and *in silico* antioxidant and toxicological activities of *Achyrocline satureioides*

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ABSTRACT

*Ethnopharmacological relevance:* *Achyrocline satureioides* ("macela or marcela") is a medicinal plant, traditionally collected in "Good Friday" before sunrise. In traditional medicine, dried flowers of *A. satureioides* are used as anti-dyspeptic, antispasmodic and anti-inflammatory.
*Aim of the study:* To evaluate the phytochemical profile and to present an *in vitro* and *in silico* approach about toxicity and antioxidant potential of *A. satureioides* flowers extract and its major phytoconstituents.
*Materials and methods:* Plant were collected according to the popular tradition. Extract were obtained by infusion and analyzed from high-performance liquid chromatography. Toxicity was evaluated in *Artemia salina* and human lymphocytes. Extract antioxidant activity was determined with total antioxidant capacity, DPPH• and ABTS•+ scavenging, ferric reducing antioxidant power, deoxyribose degradation assay, and thiobarbituric acid reactive substances (TBA-RS) assay. TBA-RS inhibitions were evaluated in brain of rats for *A. satureioides* extract and its major phytoconstituents. Predictions of activity spectra for substances and *in silico* toxicity evaluation from major phytoconstituents were performed via computer simulation.
*Results:* Chromatographic data indicated isoquercitrin, quercetin and caffeic acid as main compounds in flowers extract. Toxicity tests demonstrated a very low toxic potential of *A. satureioides*. Extract exhibited antioxidant activities in low concentrations. Both extract and major phytochemicals standards showed protection against lipid peroxidation in brain of rats. Computer simulations pointed some biological activities in agreement with traditional use, as well as some experimental results found in this work. Moreover, *in silico* toxic predictions showed that the *A. satureioides* major compounds had low probability for toxic risk.
*Conclusion:* Our results indicate that *A. satureioides* infusion possesses low toxicological potential and an effective antioxidant activity. These findings confirm the traditional use of this plant in the folk medicine.


## 1. Introduction

Medicinal plants have been extensively used for centuries to treat several diseases. During the last decades, there has been interest in identifying metabolites from plants that can exert beneficial effects on human health. Among these metabolites, the antioxidants or free radical scavengers have received special attention for their pharmacological potential (Sen and Samanta, 2015).

*Achyrocline satureioides* (Asteraceae), popularly known as "macela or marcela", is one of the 25 Achyrocline spp. described in Brazilian territory. *A. satureioides is* a medium-sized aromatic annual herb, commonly found in tropical and subtropical America (Retta et al., 2012). In Brazilian southern, the medicinal herb *A. satureioides* is traditionally collected in "Good Friday". This collection is performed before sunrise and the naturally dried flowers are used along the year to treat several gastrointestinal disorders (Simoes et al., 1988).

*A. satureioides* is considered a promising medicinal and aromatic plant and is an official vegetable drug in the Brazilian Pharmacopoeia (Retta et al., 2012). In fact, previous *in vivo* and *in vitro* studies have confirmed the traditional use of *A. satureioides* as anti-inflammatory, hepatoprotective, antioxidant, immunomodulatory, antimicrobial,

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antitumoural and photoprotective (Arredondo et al., 2004; Cosentino et al., 2008; Kadarian et al., 2002; Morquio et al., 2005; Polydoro et al., 2004; Retta et al., 2012). Furthermore, *in vitro* examinations showed *A. satureioides* cytotoxicity at higher concentrations (Sabini et al., 2013).

Investigations about chemical composition found the flavonoids quercetin, 3-O-methylquercetin, and luteolin as the main compounds in *A. satureioides* inflorescences extracts (Carini et al., 2014). These isolated compounds have demonstrated *in vitro* some pharmacological activities, such as scavenging of reactive oxygen species (ROS) (Arredondo et al., 2004; Carini et al., 2014; Retta et al., 2012). Surely, this antioxidant property is very important considering that ROS and other reactive species have been implicated in the pathology of over 100 human diseases (Halliwell, 2001).

Considering the *A. satureioides* potential as a medicinal plant, this study aimed to identify phenolic content and to evaluate, *in silico* and *in vitro*, the antioxidant and toxicological potential of crude extract and its isolated major compounds.

## 2. Methods

### 2.1. Chemicals

Methanol, acetic acid, ascorbic acid, gallic acid, chlorogenic acid, caffeic acid, ellagic acid and ferrous sulfate were purchased from Merck (Darmstadt, Germany). Quercetin, quercitrin, isoquercitrin, rutin, luteolin, kaempferol, catechin, epicatechin, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (trolox); deoxyribose, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and histopaque® were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.2. Plant material collection

*Achyrocline satureioides* (Lam.) DC. was collected in autumn, in "Good Friday", before sunrise. The collection area (Fig. 1) is located in Brazil-Uruguay-Argentina border (29°48'02.5"S 57°00'32.7"W), in the Brazilian Pampa Biome. Botanical identification of samples was confirmed and a voucher specimen (number 085/2016) was deposited at the Herbarium of the Federal University of Pampa. The plant name has been checked with "The Plant List" (www.theplantlist.org) (accessed 1 June 2016).

### 2.3. Extract obtainment

Flowers of the plant were submitted to dryness at ambient temperature (25–30 °C) for five days. In order to reproduce the traditional use, the flowers were submitted to extraction by infusion in hot water at 80 °C, for 15 min with a plant:solvent proportion of 1:100 (w/v).

### 2.4. Phytochemical analysis

#### 2.4.1. Total phenolic and flavonoid content

Total phenolic and flavonoid content of *A. satureioides* extract was measured spectrophotometrically using the methods proposed by Nurmi et al. (1996) and Chang et al. (2002), respectively. A standard curve of gallic acid and quercetin was used to determine the polyphenols and flavonoids equivalents content.

#### 2.4.2. High performance liquid chromatography with diode array detector (HPLC-DAD) analysis

HPLC-DAD analyses were performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20 A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

Reverse phase chromatographic analyses were carried out under gradient conditions, using $C_{18}$ column (4.6 mm × 250 mm) packed with 5 μm diameter particles. The mobile phase was water containing 1% acetic acid (A) and methanol (B), and the composition gradient was: 4% (B) for 5 min; 25% (B) until 10 min; 40%, 50%, 60%, 70% and 80% (B) every 10 min (Barbosa-Filho et al., 2014). Extracts were analyzed at concentrations of 20 mg/mL. The flow rate was 0.7 mL/min and the injection volume was 40 μL. Sample and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and degassed by ultrasonic bath prior to use.

Stock solutions of standards references to *A. satureioides* extracts were prepared in the HPLC mobile phase at a concentration range of 0.020 – 0.350 mg/mL catechin, epicatechin, quercetin, isoquercitrin, quercitrin, luteolin, kaempferol and rutin, and 0.035 – 0.250 mg/mL for gallic, chlorogenic, caffeic and ellagic acids.

Quantification was carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid, 280 nm for catechin and epicatechin, 327 nm for ellagic acid, chlorogenic acid, caffeic acid and caffeic acid derivative, and 365 for quercetin, isoquercitrin, quercitrin, luteolin, rutin and kaempferol. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–500 nm). All chromatography operations were carried out at ambient temperature and in triplicate.

#### 2.4.3. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Boligon et al. (2012). LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

### 2.5. Toxicity assays

#### 2.5.1. Artemia salina toxicity bioassay

To estimate the *A. satureioides* infusion toxicity, we perform the *A. salina* toxicity bioassay (Meyer et al., 1982), with some modifications. Briefly, *A. salina* cysts were induced to hatch in aerating solution (water/sodium chloride 3%) in a conical flask at 18 °C for 24 h. The newly hatched nauplii were collected and transferred individually to a 96-well plate containing different *A. satureioides* concentrations or a control saline solution (blank). The plates were sealed and incubated. Incubation media was analyzed after 24 h in order to evaluate the number of dead nauplii. Assays were performed in triplicate and n = 30 nauplii were used in each assay.

The median lethal dose ($LD_{50}$) was the required concentration to kill 50% of nauplii. It was considered LD50 < 1000 μg/mL toxic, and ≥ 1000 μg/mL non-toxic.

#### 2.5.2. Comet assay

Comet assay was performed in human lymphocytes according Singh et al. (1988). Briefly, samples of human blood (10 mL) were collected from healthy adult volunteers by venous puncture in heparinized tubes. After four hours of incubation with *A. satureioides* (concentrations showed in the figure) or hydrogen peroxide (positive control), lymphocytes were separated with histopaque®. Separated cells were suspended in agarose low melting point, and spread into a glass microscope slide. Dry slides were incubated in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH


**Fig. 1.** Map of Brazil highlighting the geopolitical division Rio Grande do Sul state and the collection point of *A. satureioides* (red circle).

10.0%, and 1% triton X-100 with 10% DMSO). After the lysis, slides were placed on a horizontal electrophoresis unit, covered with a fresh solution (300 mM NaOH and 1 mM EDTA, pH > 13). Electrophoresis was performed for 20 min (25 V; 300 mA; 0.9 V/cm). Slides were then neutralized, washed and stained using ethidium bromide. Slides were analyzed using a microscope, and fluorescent microscopy images were processed with open source program ImageJ to enhance fluorescent cell visualization. One hundred cells from each of the three replicate slides were analyzed. Cells were visually scored according to tail length and receive scores from 0 (no migration) to 4 (maximal migration) according to tail intensity. Therefore, the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration).

#### 2.5.3. *In silico toxic risks prediction*

A computational simulation study was performed to estimate possible toxicity risks of *A. satureioides* three major compounds (isoquercitrin, quercetin and caffeic acid). For this, were employed five online computer program: ACD/Labs (Toronto, Canada), admetSAR server (Cheng et al., 2012), pkCSM platform (Pires et al., 2015), PreADMET web-based (https://preadmet.bmdrc.kr/) and OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/). The toxic risks assessed, mutagenicity, carcinogenicity, cardiotoxicity, irritant and reproductive side effects were interpreted and expressed in a flexible manner: (+) low potential, (++) medium risk, (+++) high risk and non-detected risk (ND).

### 2.6. *Antioxidant analysis*

#### 2.6.1. *Total antioxidant capacity (TAC)*

TAC of *A. satureioides* was measured through spectrophotometric method proposed by Prieto et al. (1999). For all assays described below, the samples were diluted to obtain a concentration range of 6–300 μg/mL. The controls were used in order to avoid a probable interference of extract color in results. Results are expressed in half maximal inhibitory concentration ($IC_{50}$).

#### 2.6.2. *DPPH• and ABTS•+ scavenger assay*

Measurements of *A. satureioides* activity against the radical DPPH• and ABTS•+ were performed in accordance with Choi et al. (2002) and Re et al. (1999), respectively.

Results are expressed as $IC_{50}$, based on percentage of radical inhibition in relation to the control without extract.

#### 2.6.3. *Ferric reducing antioxidant power (FRAP) assay*

FRAP assay was measured through spectrophotometric method described by Benzie and Strain (1996). The FRAP value was determined by plotting in a standard curve produced by the addition of ferrous sulfate to the FRAP reagent. Results are expressed as $IC_{50}$, based on the extract ability to reduce $Fe^{3+}$ to $Fe^{2+}$.

#### 2.6.4. *Thiobarbituric acid reactive substances (TBA-RS) assay*

The formation of TBA-RS was used as an index of deoxyribose degradation or lipid peroxidation in the experiments described below. TBA-RS was measured according Ohkawa et al. (1979), and a standard curve of malondialdehyde (MDA) was used to determine TBA-RS content.

a) **Deoxyribose degradation assay**
Deoxyribose degradation assay was performed according described by Puntel et al. (2005), using ferrous sulfate and hydrogen peroxide as oxidant agent, according proposed by Halliwell and Gutteridge (1981). Results are expressed as $IC_{50}$, based on the extract ability to inhibit deoxyribose degradation.

b) **Analysis in lipid from eggs yolks**

Lipids of eggs were prepared according to Salgueiro et al. (2013). Ferrous sulfate and hydrogen peroxide were used as oxidant agents. Results are expressed as $IC_{50}$, based on the extract ability to inhibit lipid peroxidation, corrected by mg of tissue.

c) **Analysis with brain of rats**

Animals were maintained and used in accordance with guidelines of the Committee on Care and Use of Experimental Animal Resources. Rats were euthanized by decapitation and the brain was removed, quickly homogenized in NaCl (150 mM) and kept in ice. After homogenization, samples were centrifuged at 4000g at 4 °C for 10 min to yield a low speed supernatant fraction (S1). The obtained S1 was mixed with ferrous sulfate and hydrogen peroxide in concentrations of 0.01 mM and 1 mM, respectively, with or without the extract or its isolated major compounds. To the experiments with the isolated compounds (isoquercitrin, quercetin, and caffeic acid), the curves were designed based on these concentrations in a total crude extract (16.83%, 17.79%, and 14.62%, respectively). Effects of solvent alone (ethanol or water) were analyzed in order to avoid solvent interferences. Results are expressed as $IC_{50}$, based on the extract or isolated major compounds ability to inhibit lipid peroxidation of tissue, corrected by protein content (Bradford, 1976).

### 2.7. In silico biological activity spectrum

We used Prediction of Activity Spectra for Substances (PASS, available in http://www.pharmaexpert.ru/PASSonline/predict.php) for computational screening of possible biological effects of the three identified major compounds in *A. satureioides* extract (isoquercitrin, quercetin, and caffeic acid). This tool provides quantitative structure-activity relationships based on decomposition of chemical structures in 2D and/or 3D descriptors, followed by generation of models obtained from bioactive ligands (Drwal and Griffith, 2013). Prediction results were expressed in percentage of probable activity (Pa) and probable inactivity (Pi). Pa and Pi values vary from 0.000 to 1.000, thus, in this evaluation, we considered only activities with $Pa > Pi$ and $Pa > 0.700$.

### 2.8. Statistical analysis

Statistical differences between groups were determined by ANOVA one-way with Tukey post-hoc test. Data are expressed as mean ± standard deviation for at least five independent determinations. To comet assay was used the Kruskal-Wallis test. In both, ANOVA and Kruskal-Wallis tests, values of $p \leq 0.05$ were considered the limit for significance. $LD_{50}$ determinations were performed using a logarithmic regression curve.

## 3. Results

### 3.1. Phytochemical analysis

The data show that *A. satureioides*, as popularly used, presents high levels of polyphenols and flavonoids (160.19 and 48.04 mg/g of dried flowers, respectively). HPLC profile (Fig. 2) shows that the extract contain several phenolic compounds, such as gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), epicatechin (peak 5), ellagic acid (peak 6), rutin (peak 7), quercitrin (peak 8), isoquercitrin (peak 9), quercetin (peak 10), kaempferol (peak 11) and luteolin (peak 12). Furthermore, the three identified most representative phenolic compounds were isoquercitrin, quercetin and caffeic acid, respectively (Table 1).

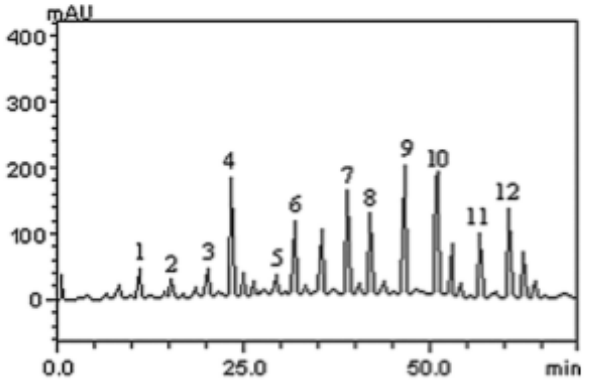



**Fig. 2.** Chromatographic analysis of *A. satureioides* (Asteraceae). Peak numbers correspond to chemical compounds gallic acid (retention time ($R_t$) = 10.35 min, peak 1); catechin ($R_t$ = 15.08 min, peak 2); chlorogenic acid ($R_t$ = 20.11 min, peak 3); caffeic acid ($R_t$ = 24.71 min, peak 4); epicatechin ($R_t$ = 29.83 min, peak 5); ellagic acid ($R_t$ = 32.57 min, peak 6); rutin ($R_t$ = 39.42 min, peak 7); quercitrin ($R_t$ = 43.01 min, peak 8); isoquercitrin ($R_t$ = 46.59 min, peak 9); quercetin ($R_t$ = 50.26 min, peak 10); kaempferol ($R_t$ = 54.69 min, peak 11) and luteolin ($R_t$ = 62.17 min, peak 12).

**Table 1**
Phenolic and flavonoids composition of *Achyrocline satureioides*.

| Compounds | *A. satureioides* | | LOD | LOQ |
|---|---|---|---|---|
| | mg/g | % | μg/mL | μg/mL |
| Gallic acid | 5.24 ± 0.03a | 0.52 | 0.023 | 0.076 |
| Catechin | 2.17 ± 0.01b | 0.21 | 0.009 | 0.031 |
| Chlorogenic acid | 5.26 ± 0.01a | 0.52 | 0.035 | 0.115 |
| Caffeic acid | 23.62 ± 0.02c | 2.36 | 0.027 | 0.094 |
| Ellagic acid | 2.07 ± 0.01b | 0.20 | 0.008 | 0.026 |
| Epicatechin | 13.61 ± 0.03d | 1.36 | 0.024 | 0.079 |
| Rutin | 17.95 ± 0.01e | 1.79 | 0.007 | 0.021 |
| Quercitrin | 14.03 ± 0.02d | 1.40 | 0.013 | 0.042 |
| Isoquercitrin | 27.18 ± 0.02f | 2.71 | 0.031 | 0.104 |
| Quercetin | 23.90 ± 0.01c | 2.39 | 0.018 | 0.061 |
| Luteolin | 12.09 ± 0.03g | 1.20 | 0.029 | 0.098 |
| Kaempferol | 14.37 ± 0.01d | 1.43 | 0.015 | 0.049 |

### 3.2. Toxicity analyses

#### 3.2.1. A. salina bioassay

*A. salina* bioassay, performed to estimate the *A. satureioides* extract toxicity, resulted in a $LD_{50}$ of 2.06 mg/mL. Moreover, in the antioxidant concentrations, *A. satureioides* extract does not present nauplii toxicity (data not show).

#### 3.2.2. Comet assay

The comet assay was performed to determine the extract genotoxic potential. Results show that *A. satureioides* did not determine DNA damage in human lymphocyte in all tested concentrations. However, the negative control was able to induce a significant increase in cells damage index ($p < 0.05$) (Table 2; Fig. 3).

#### 3.2.3. In silico toxic risks predictions

The toxic risks (mutagenic, carcinogenic, cardiotoxic, skin irritant and reproductive toxicity) for isoquercitrin, quercetin and caffeic acid were assessed by five online platforms. According to the results presented in Table 3, all the compounds, except quercetin, showed low toxicity probability. In evaluation of flavonol quercetin, the results showed a high mutagenic probability for two platforms employed and medium risk for others. However, for

**Table 2**
Number of cells with comet, distribution of damage classes and damage index.

| | CA | Comet class (mean ± SD) | | | | | DI |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | |
| **Control −** | 100 | 77.3 ± 10.2 | 18.3 ± 11.5 | 2.3 ± 0.5 | 1.7 ± 2.0 | 0.3 ± 0.5 | 29.3 ± 8.5 |
| **Control +** | 100 | 48.0 ± 7.0 | 14.7 ± 2.5 | 8.7 ± 7.4 | 11.3 ± 1.5 | 17.3 ± 3.2 | 135.3 ± 9.5* |
| **30 μg** | 100 | 70.7 ± 12.0 | 16.3 ± 6.1 | 10.0 ± 7.5 | 1.0 ± 1.0 | 2.0 ± 2.0 | 47.3 ± 14.5 |
| **60 μg** | 100 | 82.0 ± 2.6 | 11.3 ± 1.5 | 4.3 ± 1.5 | 0.3 ± 0.5 | 2.0 ± 1.0 | 29.0 ± 5.0 |
| **150 μg** | 100 | 61.3 ± 0.5 | 24.7 ± 3.0 | 8.7 ± 3.2 | 5.3 ± 1.1 | 0.0 ± 0.0 | 58.0 ± 2.0 |
| **300 μg** | 100 | 68.0 ± 2.0 | 12.0 ± 4.3 | 10.0 ± 1.0 | 5.7 ± 3.7 | 4.3 ± 1.1 | 66.3 ± 10.5 |
| **$LD_{50}$** | 100 | 74.0 ± 8.1 | 15.0 ± 7.2 | 4.3 ± 1.5 | 5.0 ± 1.0 | 1.7 ± 0.5 | 45.3 ± 8.5 |

CA: Total cells analyzed. DI: Damage index. For DI.
* indicates significant difference compared to the negative control.

isoquercitrin, three platforms did not detect mutagenic risk. In all the other parameters evaluated, both quercetin and isoquercitrin demonstrated risks ranging from low to non-toxic. Moreover, the theoretical toxicity of caffeic acid presented a very low risk prediction in all tools employed.

### 3.3. Antioxidant analysis

*A. satureioides* flowers extract presented an $IC_{50}$ of 10.4 ± 1.2 and 84.8 ± 1.8 μg/mL in TAC test and FRAP assay, respectively (Table 4). In DPPH• and $ABTS^{•+}$ assays, the $IC_{50}$ found were 112.6 ± 3.2 and 66.5 ± 1.3 μg/mL, respectively (Table 4).

Results also indicate that *A. satureioides* extract decreased the deoxyribose degradation induced by Fenton reaction at $IC_{50}$ of 211.1 ± 4.7 μg/mL. Furthermore, the higher TBA-RS levels in lipids extracted from chicken egg yolk were decreased at $IC_{50}$ of 13.1 ± 0.7 μg/mL (Table 4).

In biological tissue, *A. satureioides* flower extract and its isolated major compounds (isoquercitrin, quercetin and caffeic acid) decreased TBA-RS levels determined by Fenton reaction ($H_2O_2 + Fe^{2+}$) in brain of rats (Table 5). In this test, the $IC_{50}$ found for *A. satureioides* extract was 125.2 ± 5.5 μg/mL. For the isolated major compounds, the $IC_{50}$ determined were 13.2 ± 0.5, 111.7 ± 4.6, and 49.6 ± 3.5 μg/mL to isoquercitrin, quercetin, and caffeic acid, respectively (Table 5).

### 3.4. In silico biological activity spectrum

In order to identify potential targets and pharmacological effects related to the three *A. satureioides* major compounds, we used the online platform PASS. The number of effects displayed by the PASS, for each compound, is provided in Table 6. We selected the predicted properties with values of Pa > 0.7. Our data indicate that caffeic acid presents five properties (mucobembranous protector, hepatoprotectant, carminative, choleretic and lipid metabolism regulator), which are presented in folk medicine. Furthermore, antioxidant activity and free radical scavenger, hepatoprotectant, antihemorrhagic and hemostatic were predicted to isoquercitrin and quercetin.

## 4. Discussion

This study was performed to investigate the phytochemical, toxicological and antioxidant effects of *Achyrocline satureioides* extract, as well as to present an *in vitro* and *in silico* approach from its major phytoconstituents.

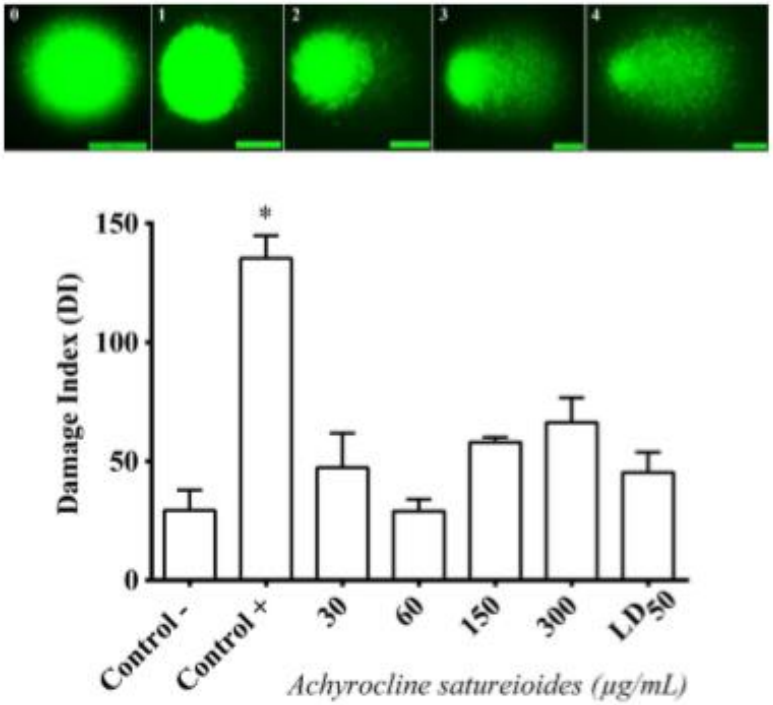



**Fig. 3.** DNA damage index (comet assay) of human lymphocytes exposed to different *A. satureioides* concentrations. Negative control (without *A. satureioides*); Positive control (hydrogen peroxide). Asterisks (*) indicates significant difference in comparison to negative control ($p < 0.05$).

**Table 3**
Toxicity prediction for the three major *Achyrocline satureioides* compounds obtained via computer simulation.

| ID substances | Toxic risk by ACD/Labs[1]; admetSAR[2]; pkCSM[3]; PreADMET[4]; OSIRIS property explorer[5] | | | | |
|---|---|---|---|---|---|
| ***Phytoconstituents*** | ***Mutagenic*** | ***Carcinogenic*** | ***Cardiotoxic*** | ***Skin irritant*** | ***Reproductive system toxicity*** |
| **Isoquercitrin** | (+)[1] | | (+)[1] | | |
| | (+)[2] | ND[2] | (+)[2] | ND[3] | (+)[1] |
| | ND[3] | ND[4] | (+)[3] | ND[5] | ND[5] |
| | ND[4] | | (+)[4] | | |
| | ND[5] | | | | |
| **Quercetin** | (+++)[1] | | (+)[1] | | |
| | (+)[2] | ND[2] | (+)[2] | ND[3] | (+)[1] |
| | (++)[3] | (+)[4] | ND[3] | ND[5] | ND[5] |
| | (++)[4] | | (+)[4] | | |
| | (+++)[5] | | | | |
| **Caffeic acid** | ND[1] | | ND[1] | | |
| | ND[2] | ND[2] | (+)[2] | ND[3] | ND[1] |
| | ND[3] | (+)[4] | ND[3] | ND[5] | (+)[5] |
| | (+)[4] | | (+)[4] | | |
| | (+)[5] | | | | |

The scale of toxicity risk ranges from low (+), medium (++), high (+++) and no detected (ND).

**Table 4**
$IC_{50}$ values to different antioxidant assays of *A. satureioides*.

| *Test* | *A. satureioides* $IC_{50}$ *(μg/mL)* |
|---|---|
| TAC | 10.4 ± 1.2 |
| DPPH• scavenger | 112.6 ± 3.2 |
| ABTS•+ scavenger | 66.5 ± 1.3 |
| FRAP | 84.8 ± 1.8 |
| Deoxyribose assay | 211.1 ± 4.7 |
| TBA-RS lipids | 13.1 ± 0.7 |

Values are expressed as mean ± SD (n=3).

**Table 5**
$IC_{50}$ values of *A. satureioides* and its isolated major compounds in TBA-RS with rat brain assay.

| | $IC_{50}$ (μg/mL) |
|---|---|
| *A. satureioides* | 125.2 ± 5.5 |
| Isoquercitrin | 13.2 ± 0.5 |
| Quercetin | 111.7 ± 4.6 |
| Caffeic acid | 49.6 ± 3.5 |

Values are expressed as mean ± SD (n=3).

HPLC analysis revealed important phenolic compounds in *A. satureioides* flowers (Fig. 2 and Table 1), including the major components isoquercitrin, quercetin, and caffeic acid, which have been detected in previous studies with *A. satureioides* (Retta et al., 2012; Sabini et al., 2013). These compounds are known to have significant antioxidant activity (Arredondo et al., 2004; Polydoro et al., 2004; Okamoto, 2005; Gulcin, 2006; Retta et al., 2012). The presence of antioxidant substances are very relevant considering that oxidative stress is involved in the pathophysiology of many human diseases (Halliwell, 2001; Salgueiro et al., 2016).

Moreover, the high content of flavonoids in *A. satureioides* and its potential as antioxidant can ratify the use of this herb as a medicinal alternative. Therefore, it is necessary investigates the toxic potential of *A. satureioides* crude extract, as popularly used. For this, we tested the flower extract toxicity in two different systems: i) determining the $LD_{50}$ in *A. salina*, and ii) assessing its DNA damage capacity in human lymphocytes, by the comet assay.

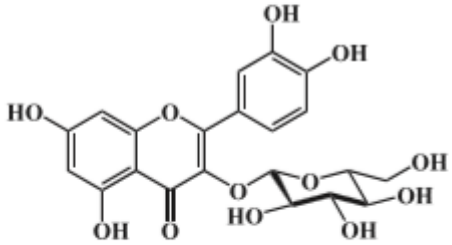
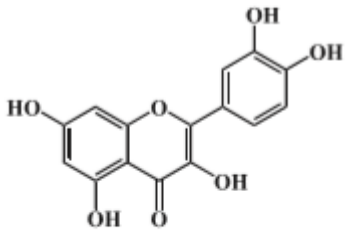
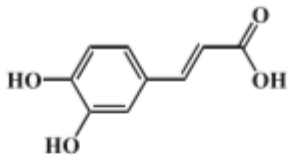
The method utilizing *A. salina* was proposed by Meyer et al. (1982) as a simple bioassay for plant extracts $LD_{50}$ determination. Nauplii of *A. salina* are used as a biological indicator of toxicity because are an obligatory non-selective filter feeder, and filters any smalls debris dissolved in water. Here, the $LD_{50}$ found to *A. salina* was 2.06 mg/mL. According Meyer et al. (1982) classification, this value is considered very low toxic for plants extracts, since the risk is greatest at concentrations $< 1000$ μg/mL. Moreover, to assure the low risk of *A. satureioides*, we tested different extract concentrations on human lymphocytes (Fig. 3). Among the tested concentrations, none of them caused damage to DNA of cells. These results indicate that *A. satureioides* extract exhibit low potential genotoxicity.

Phytochemicals like flavonoids and polyphenols related in this work are compounds with strong antioxidant capacity that offers protection against oxidative deterioration and its consequences (Arredondo et al., 2004; Polydoro et al., 2004; Okamoto, 2005; Gulcin, 2006; Retta et al., 2012). However, it is essential to consider that extracts of natural products are complex mixtures of different bioactive compounds that may act synergistically to determine its effects. For this reason, our research group believes in the importance of testing the plant products as they are popularly used (tea or infusion) in order to establish its effectiveness and security (Colpo et al., 2016; Salgueiro et al., 2013; Salgueiro et al., 2016).

Antioxidant properties of *A. satureioides* extract were assessed by different analyses, such as total antioxidant capacity determination, DPPH• and $ABTS^{•+}$ scavenger activities, and FRAP assay (Table 4). In this context, it is important to consider that the antioxidant properties of polyphenols are due to three main characteristics: i) their high reactivity as hydrogen or electron donors; ii) the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron; and iii) their ability to chelate transition metal ions (termination of the Fenton reaction) (Vertuani et al., 2004). In our study, all the proposed assays identified at different levels these abilities in *A. satureioides* infusion. Thus, it can be considered that the ability to scavenger free radicals *in vitro* can be generically compared to the ability for neutralize endogenous free radicals, such as hydroxyl radical (OH•), the most damaging of radicals found biologically.

In effect, OH• is probably the most harmful free radical (Halliwell, 2001), being continuously formed in aerobic organism as an oxygen reduction result process (Lipinski, 2011). The process of OH• formation occurs through Haber-Weiss reaction in two steps: I) reduction of $Fe^{3+}$ into $Fe^{2+}$, and II) Fenton reaction ($Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + OH•$) (Lipinski, 2011). After formed, OH• reacts fast with everything around it, which can cause

**Table 6**
Pharmacological activities predicted for *Achyrocline satureioides* major compounds.

| Phytoconstituents | Main predicted properties by PASS online | Pa[a] | Pi[a] |
|---|---|---|---|
| *Isoquercitrin*  | Membrane integrity agonist | 0.965 | 0.002 |
| | Cardioprotectant | 0.856 | 0.003 |
| | Anticarcinogenic | 0.829 | 0.004 |
| | Vasoprotector | 0.824 | 0.004 |
| | Hemostatic | 0.802 | 0.002 |
| | Hepatoprotectant | 0.800 | 0.004 |
| | Free radical scavenger | 0.795 | 0.003 |
| | Antiprotozoal (Leishimania) | 0.766 | 0.006 |
| | Antihemorrhagic | 0.758 | 0.002 |
| | Antioxidant | 0.737 | 0.004 |
| *Quercetin*  | Antioxidant | 0.945 | 0.002 |
| | Mucomembranous protector | 0.918 | 0.004 |
| | Hemostatic | 0.917 | 0.002 |
| | Free radical scavenger | 0.906 | 0.002 |
| | Antihemorrhagic | 0.883 | 0.002 |
| | Antihypercholesterolemic | 0.859 | 0.004 |
| | Hepatic disorders treatment | 0.856 | 0.004 |
| | Astringent | 0.842 | 0.001 |
| | Hepatoprotectant | 0.828 | 0.004 |
| | Vasoprotector | 0.804 | 0.006 |
| *Caffeic acid*  | Mucomembranes protector | 0.931 | 0.004 |
| | Antimutagenic | 0.886 | 0.002 |
| | Mucositis treatment | 0.844 | 0.010 |
| | Carminative | 0.787 | 0.004 |
| | Choleretic | 0.764 | 0.003 |
| | Lipid metabolism regulator | 0.762 | 0.007 |
| | Reductant | 0.755 | 0.004 |
| | Vasoprotector | 0.735 | 0.008 |
| | Antiseptic | 0.729 | 0.005 |
| | Anthelmintic (Nematodes) | 0.711 | 0.004 |

[a] Pa = Probable activity; Pi = Probable inactivity. Pa > 0.700= probable activity greater than 70%. The PASS prediction results were interpreted and used as follows: (i) only activities with Pa > Pi are considered as possible for a particular compound; (ii) if Pa > 0.7, the chance to find the activity experimentally is high.

damage to DNA, lipids and proteins (Halliwell, 2006; Salgueiro et al., 2013). On our findings, *A. satureioides* extract was effective to reduce the damage caused by $OH^{\bullet}$ generated via Fenton reaction in three different systems: deoxyribose degradation assay, lipids from egg yolk, and homogenates of rats brains (Table 4 and Table 5).

*A. satureioides* isolated major compounds were also able to protect homogenates of rat brains from lipid damage caused by $OH^{\bullet}$ (Table 5). In the crude extract, the major compounds isoquercitrin, quercetin and caffeic acid, make up 16.83%, 14.79% and 14.62%, respectively, of the total compounds identified. In our data, these compounds, in a situation of co-incubation, were able to protect brain tissue of rats to peroxidation induced by Fenton reaction, in minor concentrations when compared to crude *A. satureioides* infusion.

Moreover, in order to identify biological activities prediction for *A. satureioides* major compounds, we performed an *in silico* investigation (Table 6). *In silico* evaluations have the advantage of rapid execution, low cost and the positive side to reduce the use of animals in toxicity testing. Prediction by PASS is based on structure-activity relationships analysis of the training set containing more than 205,000 compounds exhibiting more than 3750 kinds of biological activities (Jamkhande and Barde, 2014). The PASS analysis was performed by comparison of the phytochemical compound structure with structures of well-known biologically active substance, which allows estimate if a phytochemical compound may have a particular effect.

Among the activities predicted by PASS, we found five that are reported in popular medicine for *A. satureioides* (mucobembranous protector, hepatoprotectant, carminative, choleretic and lipid metabolism regulator). This finding supports the main traditional uses of *A. satureioides* for gastrointestinal disorders, and suggests the three major compounds as contributors to produce ethnopharmacological effect. Therefore, PASS predicted antioxidant and free radical scavenger properties for isoquercitrin and quercetin (Pa > 0.7). Oxygen radical scavenger was also estimate for caffeic acid (Pa = 0.696; data not show). These properties have been previously described for these substances (Gulcin, 2006; Polydoro et al., 2004; Valentová et al., 2014). Furthermore, our data confirm the prediction for *A. satureioides* compounds as antioxidant (Table 4 and Table 5). However, our *in silico* evaluation indicates several unknown activities, which provides the basis for evaluation of unknown potential of this medicinal plant.

In this work, we also predict *in silico* toxicity for *A. satureioides* major compounds. Mutagenicity was evaluated based in AMES test against *Salmonella typhimurium* strains. Moreover, cardiotoxicity was determined with human-ether-a-go-go-related gene test (hERG). Skin irritant was assigned by Draize test, and reproductive toxicity by affinity to the estrogen receptor test. Our data indicate, in general, low toxicity probability for *A. satureioides* evaluated compounds (Table 3). Except for quercetin, that according to the evaluation, presents medium to high mutagenic potential.

In fact, some studies showed that quercetin was able to potentiate the cytotoxic effect of several chemotherapy drugs, improving the chemotherapeutical efficiency (Sak, 2012; Wang et al., 2012). Mutagenic activity of quercetin has been known, especially in *Salmonella typhimurium* mutants that reveal base-pair substitution and frameshift mutagens (Bjeldanes and Chang, 1977; Resende et al., 2012). On the other hand, the findings of quercetin-related mutagenicity or genotoxicity showed *in vitro* have not been confirmed *in vivo*, which supports the safety of quercetin use (Okamoto, 2005; Harwood et al., 2007). These findings may be explained by the "quercetin paradox", which states that structural formula of quercetin has all the structural elements necessary for both antioxidant and pro-oxidant property (Rietjens et al., 2005; Harwood et al., 2007). Furthermore, *in vivo*, quercetin have a very low order of bioavailability, and the amount available is quickly metabolized to non-mutagenic compounds, which probably contributes to the absence of mutagenicity and carcinogenicity (Harwood et al., 2007).

For isoquercitrin, one of the major glycosidic forms of the quercetin, toxicological studies were not performed and evaluation of pure isoquercitrin has not been conducted so far (Valentová et al., 2014). In this context, the preliminary assessment conducted *in silico* by us, can complement future studies on the safety of this compound. Finally, regarding the cardiotoxicity (evaluated by hERG test), while a low risk has been found for the quercetin and caffeic acid *in silico*, previous *in vitro* and *in vivo* findings showed a real risk for these compounds (Zitron et al., 2005; Choi et al., 2013; Chu et al., 2015). Thus, the *in silico* findings can be complement, but not replace the *in vitro* and *in vivo* analysis.

Taken together, our results indicate that *A. satureioides* infusion, as popularly used, has antioxidant effect in different systems and in low doses. Furthermore, *A. satureioides* infusion presents a $LD_{50}$ far above that considered toxic and has no genotoxic potential in antioxidant doses or in established $LD_{50}$. These features provide support for the popular use of this plant, and establish *A. satureioides* as a potential agent for *in vivo* testing, especially for the treatment of several disorders in which oxidative stress is involved.

## Statement of authors' contributions to manuscript

G.O.P. and V.F. designed research; A.C.F.S., H.S.R., M.T.C., A.A.B. and F.R.P conducted research and analyzed data; A.C.F.S. H.S.R. V.F. and G.O.P. wrote the paper. All authors read and approved the final manuscript.

## Conflict of interest

The authors declare no conflict of interests.

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## Glossary

ANOVA: Analysis of variance;
ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt;
DPPH: 2,2-Diphenyl-1-picrylhydrazyl;
FRAP: Ferric reducing antioxidant power;
HPLC: High-performance liquid chromatography;
DAD: Diode-array detection;
$LD_{50}$: Median lethal dose;
LOD: Limit of detection;
LOQ: Limit of quantification;
MDA: Malondialdehyde;
$OH^{\bullet}$: Hydroxyl radical;
Pa: Probable activity;
PASS: Prediction of activity spectra for substances;
Pi: Probable inactivity;
ROS: Reactive oxygen species;
$R_t$: Retention time;
S1: Low speed supernatant fraction;
TAC: Total antioxidant capacity;
TBA-RS: Thiobarbituric acid reactive substances;
TPTZ: 2,4,6-Tri(2-pyridyl)-s-triazine.

## 5. DISCUSSÃO E CONCLUSÕES

O propósito deste estudo foi investigar as plantas utilizadas na medicina tradicional por pessoas com *Diabetes mellitus* na cidade de Uruguaiana/RS (**Manuscrito 1**) e avaliar os efeitos antidiabéticos (**Manuscrito 2 e Artigo 1**), antioxidantes e toxicológicos de duas destas plantas (**Manuscrito 2, Artigos 1 e 2).**

Para responder a estas questões, investigamos, através de uma entrevista (**Anexo 3**) com 105 pessoas com DM na cidade de Uruguaiana/RS, os hábitos de consumo de plantas consideradas medicinais. Os resultados encontrados mostraram que mais de 60% dos entrevistados utilizam plantas para fins medicinais. Entre as plantas mais utilizadas estavam a "pata-de-vaca" e a "macela" (Tabela 6 - **Manuscrito 1**). A principal forma de utilização das plantas foi por infusão das folhas e flores.

Estes dados corroboram com diversas investigações que mostram o extensivo uso de plantas na medicina tradicional como alternativa terapêutica primária ou complementar, especialmente em países em desenvolvimento (Trojan-Rodrigues et al., 2012; Dutra et al., 2016). Tais achados reforçam ainda a importância de políticas, como a "Política Nacional de Plantas Medicinais e Fitoterápicos" (Brasil 2006; Brasil, 2009), que fomenta o conhecimento acerca dos saberes e práticas da medicina popular e apoia pesquisas que investiguem a eficácia e segurança destas práticas.

Entre as plantas aqui avaliadas, a *Bauhinia* (pata-de vaca) é de fato umas das mais utilizadas para o tratamento de pessoas com DM (Pepato et al., 2002, Volpato et al., 2008; Cechinel-Filho, 2009; Trojan-Rodrigues, 2012). No entanto, em decorrência da extensa quantidade de plantas pertencentes ao gênero *Bauhinia*, muitas espécies ainda carecem de estudos que avaliem seu potencial antidiabético (Cechinel-Filho, 2009). Entre essas, está a *Bauhinia forficata* Link subsp. *pruinosa* (BF).

Com base nisso, foi testado o potencial antidiabético e antioxidante da infusão dessa subespécie de BF *in silico*, *in vitro* e em um modelo experimental de hiperglicemia crônica em camundongos (**Manuscrito 2**). Os dados apresentados mostram baixa predição para atividade antidiabética dos compostos identificados na planta (Tabela 1 – **Manuscrito 2**).

Os experimentos *in vivo* confirmaram que, na forma testada, a planta não possui efeito antidiabético direto, visto que não atua sobre a hiperglicemia e sobre os demais sintomas clássicos do DM (poliúria, polidpisa, polifagia e perda de peso) (Figura 3 –

**Manuscrito 2**). Por outro lado, de acordo com a elevada predição antioxidante *in silico* e *in vitro* (Tabela 1 e Figura 2 – **Manuscrito 2**), os danos oxidativos em células vermelhas do sangue de camundongos severamente hiperglicêmicos foram amenizados pelo tratamento com a planta (Figura 4 – **Manuscrito 2**).

Nesse contexto, é conhecido que há um aumento da peroxidação lipídica nos eritrócitos de pessoas com DM (Mallick et al., 2011), possivelmente em decorrência do aumento da produção de EROs, por sua vez intimamente relacionada à hiperglicemia crônica (Kassab & Piwowar, 2012; Rochette et al, 2014). Danos oxidativos em células vermelhas do sangue já foram também identificados em eritrócitos expostos à elevadas concentrações de glicose *in vitro* (Salgueiro et al., 2013; Pazzini et al., 2015). De uma forma geral, a peroxidação lipídica aumentada nestas células determina um aumento da fragilidade osmótica, o que pode ocasionar o rompimento da célula (Pazzini et al., 2015). Dados anteriores mostraram que a infusão de BF foi efetiva em reduzir o dano oxidativo em eritrócitos *in vitro* (Salgueiro et al., 2013), entretanto, uma abordagem *ex vivo* ainda não havia sido realizada.

O DM é conhecidamente uma síndrome que afeta de forma globalizada diferentes órgãos e tecidos. Entre esses, o fígado é um dos órgãos mais afetados (Mohamed et al., 2016). De fato, um estudo *post mortem* mostrou a ocorrência de lesões generalizadas no fígado de cadáveres de pessoas com DM (Kini et al., 2016). Tanto em humanos, quanto em modelos animais de DM, essas alterações estão normalmente associadas ao desenvolvimento de esteatose hepática gordurosa não alcoólica e ao aumento do estresse oxidativo (Mohamed et al., 2016).

Para avaliar as alterações hepáticas relacionadas ao estresse oxidativo desencadeadas pelo DM, e os efeitos do tratamento com a BF sobre essas alterações, foram utilizados camundongos machos em um modelo de DM tipo 1 com estreptozotocina (**Artigo 1**). Os resultados encontrados confirmam que o DM é uma condição que leva ao desenvolvimento de danos oxidativos importantes no fígado dos animais (**Artigo 1**).

É possível inferir que os danos encontrados são decorrentes do estresse oxidativo gerado pela hiperglicemia crônica, secundária à injeção de ETZ, já que os animais permaneceram por trinta dias sem qualquer forma de tratamento. Assim, cabe destacar que a metabolização hepática e a excreção renal da ETZ ocorrem poucas horas após a aplicação da droga (Karunanayake, Hearse, 1976). Desta forma, tanto a hepatomegalia

quanto o aumento sérico das transaminases são possivelmente alterações atribuídas ao DM descompensado (Portugal et al. , 2011), e não uma resposta à toxicidade da ETZ.

De acordo com os dados apresentados, tanto os níveis de EROs, demonstrado pelo ensaio de oxidação da diclorofluoresceína, quanto os níveis de peroxidação lipídica e de proteínas carboniladas estavam aumentados nos animais hiperglicêmicos (Figura 5 - **Artigo 1**). Sabe-se que diferentes processos podem levar ao aumento da produção de EROs na hiperglicemia, entre eles estão a auto oxidação da glicose e a formação dos AGEs (Kassab & Piwowar, 2012; Rochette et al, 2014).

Considerando este quadro, as EROs geradas podem interagir com as biomembranas causando sua peroxidação e com proteínas causando sua carbonilação, além da interação direta entre glicose e proteínas (glicação e glicoxidação). Este último processo pode também explicar a inibição encontrada na atividade da enzima δ-ALA-D (Figura 7A - **Artigo 1**).

Além disso, o aumento da formação de EROs pode estar relacionado com a inibição da atividade da enzima catalase (Figura 6B - **Artigo 1**). Em grande parte dos casos, os danos foram completamente ou parcialmente revertidos pelo tratamento com a infusão da BF. Esse achado permite novamente deduzir que a proteção conferida pela BF se deve ao seu efeito antioxidante, previsto *in silico* (**Manuscrito 2**) e já demostrado *in vitro* pelo nosso grupo de pesquisa (Salgueiro et al., 2013).

Os efeitos da hiperglicemia e do tratamento com a BF também foram avaliados em relação à regulação gênica da resposta antioxidante (Figura 8 - **Artigo 1**). Entre os marcadores avaliados (Nrf2, NQO-1 e HSP70), apenas os níveis de proteínas da enzima NQO-1 foi aumentado no pâncreas de camundongos com DM, e o tratamento com a BF foi efetivo em reduzir este aumento a níveis de controle.

Sabe-se que a NQO-1 está envolvida em eventos de toxicidade e também em resposta ao estresse oxidativo, através da atuação como *scavenger* do ânion superóxido (Siegel et al., 2004). No entanto, apesar da expressão de Nrf2 não ter sido alterada, é possível afirmar que a via Nrf2-ARE provavelmente estava ativa nesta situação, já que é a via responsável pelo aumento da expressão de NQO-1.

No entanto, o aumento da expressão de NQO-1 possivelmente não foi suficiente para proteger o pâncreas contra os danos, já que, considerando a hiperglicemia apresentada pelos animais, as células β-pancreáticas permaneceram disfuncionais. Na

figura 5, abaixo, apresentamos um breve esquema dos possíveis eventos bioquímicos e fisiológicos envolvidos nos achados apresentados no **Manuscrito 2** e **Artigo 1**.

Outra planta extensivamente utilizada na medicina tradicional é a *Achyrocline satureioides* (macela). Em nossos achados, a macela foi a quarta planta mais utilizada por pessoas com DM na cidade de Uruguaiana/RS (Tabela 4 - **Manuscrito 1**). Na medicina tradicional, a macela é normalmente utilizada para o tratamento de distúrbios gastrointestinais, como antidiabética e como anti-inflamatória (Retta et al., 2012).

Porém, para além do uso do medicinal, no sul do Brasil a macela é considerada uma planta sagrada. Isso porque sua colheita é efetuada no dia conhecido como "sexta-feira santa" ou "sexta-feira da paixão", antes do nascer do sol. Suas flores, secas à sombra para evitar a perda de propriedades medicinais, são usadas durante todo o ano para o tratamento de diversas enfermidades. Além da ingestão como chá, a macela também é utilizada para a confecção de artesanatos, inclusive travesseiros para bebês, pois acredita-se que tem o poder de acalmar o sono.

Considerando essa forte tradição, as propriedades antioxidantes e o potencial tóxico da infusão das flores de macela foram testadas *in silico* e *in vitro* (**Artigo 2**). Na figura 6, abaixo, apresentamos um breve esquema dos principais achados apresentados nessa publicação.

Os dados aqui apresentados sugerem que a planta tem baixo potencial genotóxico *in silico* e em leucócitos humanos, inclusive em sua dose letal mediana, determinada em *A. salina* (Tabelas 2 e 3, Figura 3 - **Artigo 2**). Além disso, a predição antioxidante para os principais compostos identificados foi confirmada *in vitro* em diferentes ensaios, inclusive prevenindo a peroxidação induzida por ferro e peróxido de hidrogênio em cérebros de ratos (Tabelas 4, 5 e 6 - **Artigo 2**).

Tal potencial antioxidante foi identificado também em outros trabalhos, tanto para o extrato bruto, quanto para seus constituintes isolados (Retta et al., 2012). Em relação ao potencial antidiabético, a predição para os principais componentes identificados (quercetina, isoquercitrina e ácido cafeíco) é, de uma forma geral, baixa (Pa= 0,363; 0,660 e 0,384, respectivamente). A baixa predição foi encontrada inclusive para o *achyrofuran* (0,317), composto com atividade antidiabética já avaliada (Carney et al., 2002).

Nesse contexto, baixas predições podem ser falsamente identificadas em moléculas com estruturas diferenciadas em relação a outras com o efeito antidiabético já

conhecido. Isso acontece porque as plataformas de avaliação *in silico* contam com bibliotecas de fragmentos de compostos com atividades já identificadas, e, baseado na comparação da relação estrutura/atividade do composto novo com compostos já conhecidos, podem predizer as possíveis atividades de novas moléculas.

Apesar da baixa predição antidiabética, o potencial antioxidante aqui identificado faz da macela um promissor agente para o tratamento das doenças onde o estresse oxidativo esteja aumentado. Isso é especialmente interessante, considerando a larga aceitação e o extenso uso dessa planta na medicina popular. Cabe destacar que o conjunto de achados para a infusão das plantas aqui testadas são, em grande parte, ainda inéditos na literatura.

Finalmente, em relação as predições farmacológicas realizadas com o uso de bioinformática, é importante ressalvar que são abordagens complementares muito úteis na avaliação de compostos de interesse. Contudo, há de ser considerado que muitas das atividades atribuídas a plantas na medicina tradicional são possivelmente devido ao sinergismo entre os diferentes compostos presentes no extrato bruto. Assim, a separação de compostos na avaliação *in silico* pode mascarar a real atividade medicinal da planta.

Em suma, os resultados aqui apresentados culminam na tese de que a planta "pata-de-vaca", na forma testada, não apresenta atividade antidiabética direta. Por outro lado, tanto a "pata-de-vaca" quanto a "macela" demonstraram uma expressiva atividade antioxidante. Assim, essas plantas podem vir a ser potenciais agentes terapêuticos para o tratamento complementar do estresse oxidativo relacionado ao DM.

Ainda, abrimos a perspectiva para a avaliação da composição e efeitos destas plantas em outras formas de extração, visto que alguns componentes podem não ser extraídos ou ainda serem perdidos na forma popular de uso (infusão). Da mesma forma, a avaliação do potencial da macela *in vivo*, e de ambas as plantas em um modelo experimental de DM tipo 2 pode vir a ser uma interessante alternativa, visto que DM tipos 1 e 2 são entidades com diferentes fisiopatologias.

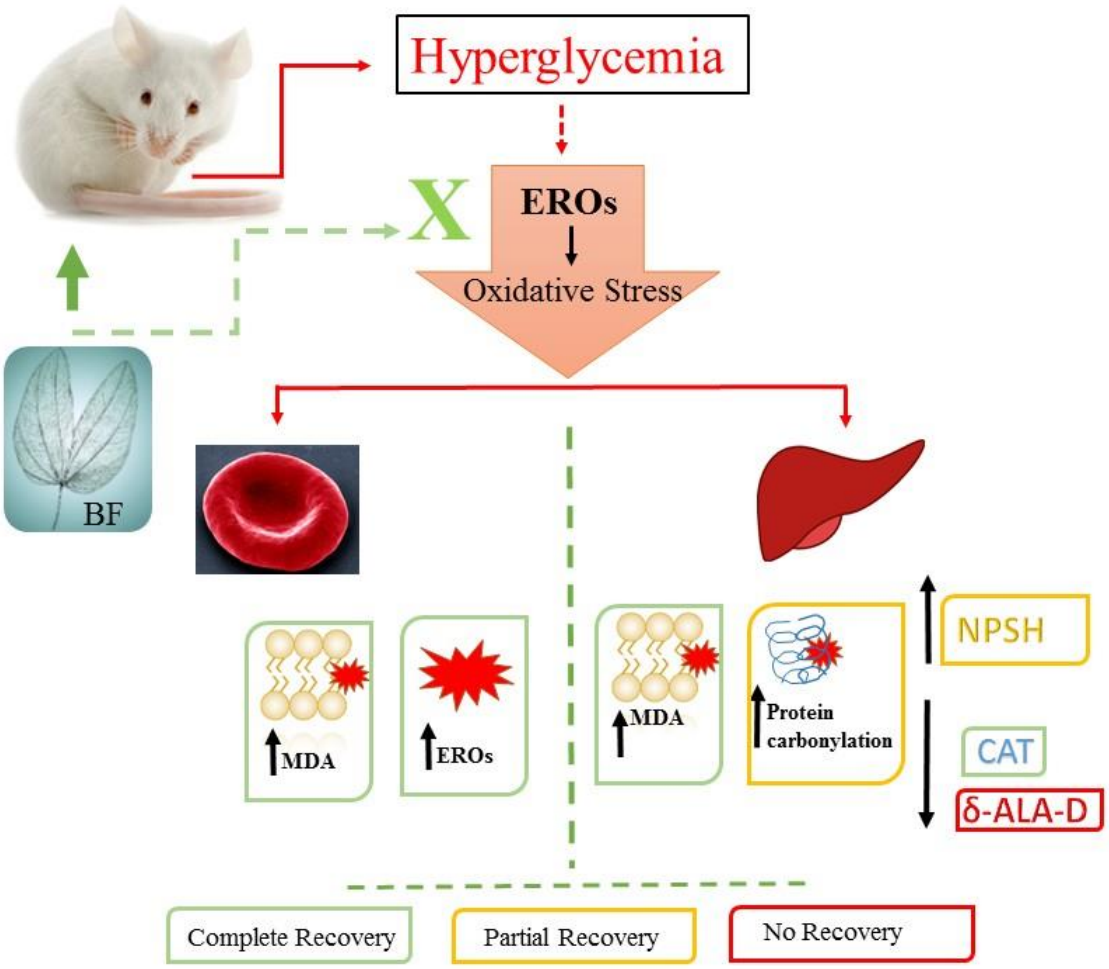


**Figura 5: Resumo dos possíveis mecanismos pelos quais o chá de *Bauhinia forficata* Link susp. *pruinosa* exerce seus efeitos**

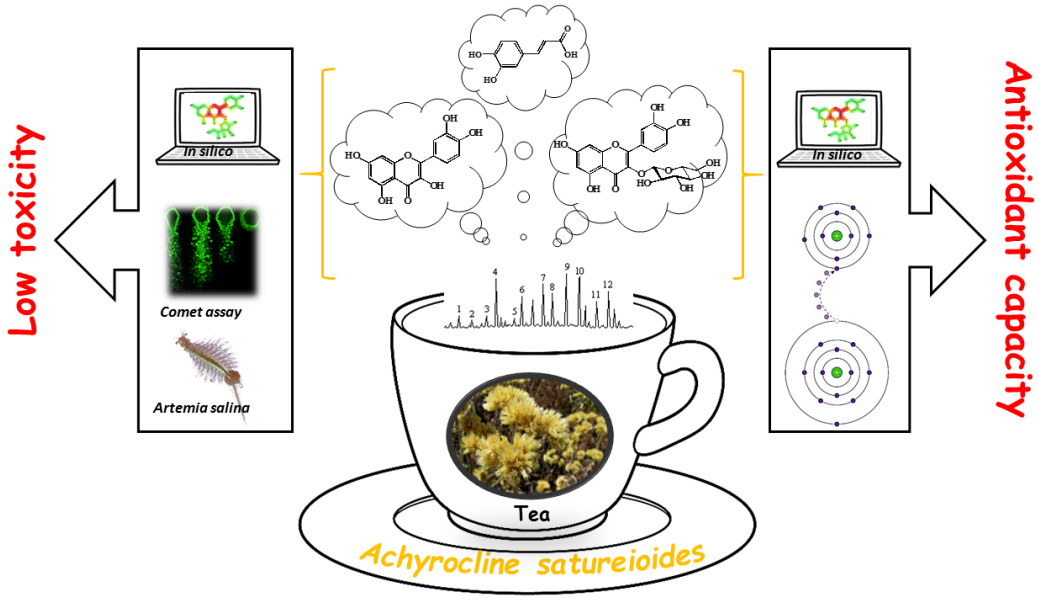


**Figura 6: Resumo dos principais achados para a *Achyrocline satureioides* (macela)**

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# 7. ANEXOS

## 7.1 Anexo 1 – Parecer do Comitê de Ética em Pesquisa (CEP)

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO PAMPA
Comitê de Ética em Pesquisa
Unipampa/CEP - Portaria n° 728/09/GR/Unipampa
Registrado na CONEP – Ofício n° 3210/CNS/GM/MS
Endereço eletrônico: cep@unipampa.edu.br


Uruguaiana, 16 de janeiro de 2012.

**CARTA DE APROVAÇÃO Nº 002 2012**

Prezado Pesquisador Responsável

Gustavo Orione Puntel

Comunicamos que o protocolo de pesquisa intitulado PERFIL BIOQUÍMICO DE INDIVÍDUOS DIABÉTICOS QUE UTILIZAM PLANTAS MEDICINAIS PARA CONTROLE GLICÊMICO: ESTUDOS IN VITRO, registro ProPesq 10.105.11.CEP, registro Unipampa/CEP 083 2011, foi avaliado por este CEP e está **aprovado** para execução a partir da presente data.

Lembramos que qualquer alteração no protocolo de pesquisa submetido a avaliação deverá ser comunicada ao Unipampa/CEP imediatamente, bem como eventos adversos, e que o relatório parcial deverá ser entregue em **julho de 2012.**

Atenciosamente,

Rosana Soibelmann Glock
Coordenadora CEP

## 7.2 Anexo 2 – Parecer do Comitê de Ética no Uso de Animais (CEUA)

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

**Pró-Reitoria de Pesquisa**

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

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**PROTOCOLO Nº 001/2012**

**Título: : Análise bioquímica dos efeitos de plantas do gênero *Bauhínia* em um modelo experimental de hiperglicemia induzida por estreptozotocina em camundongos**

**Pesquisador:** Gustavo Orione Puntel

**Campus:** Uruguaiana

**Telefone:** 55 91194886

**E-mail:** gustavopuntel@unipampa.edu.br/ gustavopuntel@yahoo.com.br

Após a análise detalhada do projeto de pesquisa a relatoria do CEUA-Unipampa emite parecer **FAVORÁVEL** para o cadastro do protocolo e execução do referido projeto.

Luiz E. Henkes
Professor Adjunto
Coordenador do CEUA/Unipampa

## 7.3 Anexo 3 – Formulário para entrevista sobre os hábitos de consumo de plantas por pessoas com DM

| |
|---|
| Entrevistado nº: Data: |
| Bairro: |
| Gênero: Idade: |
| Escolaridade: ( )Série ( ) Anos de estudo |
| Estado Civil: Ocupação: |
| Renda mensal: |
| Tempo de diagnóstico do DM: Tipo de DM: |
| Faz tratamento com medicamentos ( )Sim ( )Não<br>Quais medicamentos:<br>Usa regularmente? |
| Faz controle da alimentação ( )Sim ( )Não<br>De que forma? |
| Você pratica atividades físicas ( )Sim ( )Não<br>Com que frequência? |
| Utiliza plantas medicinais para tratar o DM ou outros sintomas*****? ( )Sim ( )Não<br>*****Descrever quais sintomas na parte específica |
| Toma chimarrão ( ) Sim ( ) Não<br>Com que frequência toma chimarrão?<br>Utiliza "jujos" no chimarrão? |
| Qual o nome da planta ou plantas que utiliza? |
| Qual parte da planta utiliza ( ) Folha ( ) Raiz ( )Semente<br>( ) Fruto ( ) Flor ( ) Caule |
| Como é feito o preparo da planta? |

| |
|---|
| Como você obtém esta planta? ( ) Cultivada ( ) Comprada ( ) Outra<br><br>Observações: |
| Com que frequência você utiliza esta planta?<br><br>( ) uma vez ao dia ( ) mais de uma vez ao dia ( ) uma vez na semana ( ) até 3 vezes na semana ( ) mais de 3 vezes na semana |
| Qual a quantidade aproximada que você consome?<br><br>( ) uma xícara / térmica por dia ( ) mais de uma xícara / térmica por dia<br><br>OBS: sublinhar se xícara ou térmica |
| Como ou através de quem ficou conhecendo as propriedades desta planta? |
| Há quanto tempo faz uso desta planta? |
| *****Para qual sintoma você utiliza esta planta? |
| Você observou alguma melhora após iniciar o uso desta planta? Explique: |
| Você observou algum efeito indesejado após iniciar o uso desta planta? Explique: |
| Seu médico sabe que você utiliza esse chá/planta? |

| |
|---|
| Você apresenta ou já apresentou:<br><br>( ) HAS<br><br>( ) Problemas visuais<br><br>( ) Problemas renais<br><br>( ) Alterações circulatórias nos pés e/ou pernas |

| |
|---|
| ( ) Alterações de sensibilidade nos pés e/ou pernas<br>( ) Alterações de temperatura nos pés e/ou pernas<br>( ) Dor, dormência ou outra sensação desagradável nos pés e/ou pernas<br>( ) Feridas de difícil cicatrização nos pés e/ou pernas |
| Já sofreu alguma amputação? ( )Sim***** ( )Não<br>*****Anotar dados |
| Solicitar autorização para exame visual dos pés e pernas*****:<br>*****Em caso de constatação de lesões fazer as orientações adequadas e avaliar necessidade de encaminhamento para especialista. |
| Você acha que algum dos problemas acima tem relação com o DM? |
| Solicitar autorização para verificar pressão arterial (observar condições necessárias): |
| Solicitar autorização para verificar peso, circunferência da cintura e quadril: |
| Verificar a existência de exames complementares (solicitar autorização para anotar dados): |