UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

TESE DE DOUTORADO

EFEITOS TERAPÊUTICOS DE *ACHYROCLINE SATUREIOIDES* (LAM.): ESTUDOS *IN VIVO* E *IN VITRO*.

KARLA SUZANA MORESCO

Porto Alegre

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

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“Não se esforce para ser alguém de sucesso,
mas para ser alguém de valor."
Albert Einstein

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SUMÁRIO

APRESENTAÇÃO

A presente tese de doutorado está organizada em três partes, conforme a seguir:

Parte I: Resumo, *Abstract*, Lista de Abreviaturas, Introdução e Objetivos;

Parte II: Resultados apresentados na forma de 3 artigos científicos, dentre eles dois publicados e um submetido.

Parte III: Discussão, Conclusões, Perspectivas e Referências Bibliográficas.

PARTE 1

I. RESUMO

Achyrocline satureioides (Asteraceae) ou "Marcela" é uma planta nativa de países latino-americanos e, apesar do seu uso generalizado na medicina tradicional, as propriedades químicas e biológicas que sustentam seu uso fitoterápico, ainda não foram completamente caracterizadas. Neste trabalho apresentamos benefícios terapêuticos de extratos de *Achyrocline satureioides*, através de estudos *in vitro* e *in vivo*. Avaliou-se o perfil químico, potencial antioxidante *in vitro* e atividade antimicrobiana de três extratos de *Achyrocline satureioides*. Dentre os extratos testados, o extrato hidroetanólico exibiu atividade antioxidante em concentrações de 34 μg/mL, e apresentou um amplo espectro de atividade antimicrobiana (100 a 200 mg/mL) contra bactérias patogênicas intestinais. Os efeitos antibacterianos foram superiores aos exercidos pela amoxicilina, quando testados contra *Bacillus cereus* e *Staphylococcus aureus*. Como estudos sobre o efeito do uso indiscriminado de infusões de plantas durante a gravidez são limitados, neste trabalho, também investigamos os efeitos da suplementação em ratas wistar com dois extratos de *Achyrocline satureioides*, um extrato aquoso (47mg/Kg/dia) e um extrato hidroetanólico (35mg/Kg/dia), administrados durante a gestação e lactação. Nossos dados sugerem que tanto o extrato aquoso quanto o hidroetanólico podem induzir sintomas de toxicidade nas concentrações testadas, no que se refere ao índice de nascimentos dos filhotes, diminuição da sobrevivência neonatal, além de causar alterações significativas específicas nos tecidos quanto aos parametros enzimáticos tanto das mães como dos filhotes fornecendo evidências que podem justificar a necessidade de controle na suplementação na gestação. A administração oral de um extrato hidroetanólico de *A. satureioides* também produziu atividade anti-hiperglicêmica promissora em ratos. Sendo comparável ao fármaco controle positivo: a acarbose. Este efeito foi observado em ratos que ingeriram níveis elevados de maltose e amido, e também em modelo de indução de diabetes mellitus (DM) com estreptozotocina. Para investigar o mecanismo de ação do extrato, determinamos seus efeitos inibitórios sobre maltase e α-amilase pancreática. O nível de extrato associado a 50% de inibição enzimática (IC_{50}) foi de 185,21 μg/mL para maltase e 265,72 μg / mL para α-amilase pancreática. Uma investigação mais profunda dos constituintes de *A. satureioides* mostrou que o flavonoide, achyrobichalcona, produziu a maior inibição das glicosidases (IC_{50} de 4,74 μM para α-amilase e 6,71 μM para maltase), o que foi consistente com os nossos resultados de modelagem molecular. Estes achados sugerem uma potencial aplicação de *A. satureioides* como agente terapêutico em DM. As atividades antioxidantes, antimicrobianas e hiperglicêmica de parecem estar positivamente correlacionadas com a quantidade de flavonoides. Em conclusão, nossos resultados mostraram evidências de atividades antioxidante, antimicrobiana e hipoglicêmica convidando a pesquisas futuras sobre seu potencial uso como agente co-adjuvante terapêutico tanto para o tratamento das doenças gastrointestinais que podem apresentar altas taxas de resistência a antibióticos, bem como no DM. Contudo, são necessárias mais investigações para verificar os seus efeitos sobre a saúde.

II. ABSTRACT

Achyrocline satureioides (Asteraceae) or "Marcela" has been widely used in traditional medicine; however, chemical and biological properties supporting its phytotherapeutic usage are still poorly studied. In this work we present therapeutic benefits of extracts of *Achyrocline satureioides*, through *in vitro* and *in vivo* studies. We evaluated the chemical profiles of the dried extracts, along with antioxidant potential and antimicrobial activity against intestinal pathogenic bacteria. Our data suggest that dried *A. satureioides* extracts exert greater antibacterial effects than the antibiotic amoxicillin when tested against *Bacillus cereus* and *Staphylococcus aureus*. The antioxidant and antimicrobial activities of *A. satureioides* extracts appear to positively correlate with the amount of flavonoids measured in each extract. Our results provide evidence for the antioxidant and antimicrobial activities of *A. satureioides* extracts against intestinal pathogens. These findings justify further research regarding the potential for *A. satureioides* extracts as co-adjuvants to treat bacterial-induced intestinal diseases that may present high rates of antibiotic resistance. Studies regarding the indiscriminate use of plant infusions during pregnancy are limited. Recent reports have shown that chronic flavonoid supplementation induces toxicity in vivo and raises the mortality rates of healthy subjects. Therefore, we investigated whether supplementation of pregnant and lactating Wistar rats with two AS inflorescence extracts, consisting of an aqueous extract similar to a tea (47 mg/kg/day) and a hydroethanolic extract (35 mg/kg/day) with a higher flavonoid content, could induce redox-related side effects. Our data suggest that both aqueous extract and hydroethanolic extract of *A. satureioides* inflorescence extracts may induce symptoms of toxicity in concentrations tested in mothers regarding the delivery index and further decrease of neonatal survival. Our findings provide evidence that may support the need to control supplementation with the aqueous extract of *A. satureioides* inflorescence extracts during gestation due to potential toxicity in vivo, which might be related, at least in part, to changes in tissue-specific redox homeostasis and enzymatic activity. Oral administration of a hydroalcoholic extract from *A. satureioides* also produced promising antihyperglycemic activity in rats. This was comparable to the positive control drug: acarbose. This effect was observed in rats that ingested high levels of maltose and starch, and also in a rat streptozotocin model of DM. To investigate the mechanism of action of *A. satureioides*, we determined its inhibitory effects on maltase and pancreatic α-amylase. The level of *A. satureioides* associated with 50% enzyme inhibition (IC_{50}) was 185.21 μg / mL for maltase and 265.72 μg / mL for pancreatic α-amylase. Further investigation of AS constituents showed that flavonoid, achyrobichalcona, produced the highest inhibition of the enzyme (IC_{50} of 4.74 μM for α-amylase and 6.71 μM for maltase), which was consistent with our modeling results molecular. These findings suggest a potential application of *A. satureioides* as a therapeutic agent in DM. The antioxidant and antimicrobial and hyperglycemic activities appear to be positively correlated with the amount of flavonoids. In conclusion, our results showed evidence of antioxidant, antimicrobial and hypoglycemic activity inviting future research on its potential use as a co-adjuvant therapeutic agent both for the treatment of gastrointestinal diseases that may present high rates of antibiotic resistance as well as in DM. However, more research is needed to verify its effects on health.

III. LISTA DE ABREVIATURAS

AS = *Achyroclines satureioides*

ACB = achyrobichalcona

CAT = catalase

COMT = catecol-O-metiltransferases

DM = diabetes mellitus

ROS = espécies reativas de oxigênio

GST = glutationa S-transfersases

LFH = Lactase-Florizina Hidrolase

LUT = luteolina

MIC = Concentração mínima inibitória

3*O*MQ = *3-O-metilquercetina*

OMS= Organização Mundial da Saúde

QCT = quercetina

SGLT- 1 = Transportador de glicose dependente de sódio

SOD = Superóxido dismutase

SULTs = sulfotransferases

UGTs = UDP-glucuronosiltransferases

IV. INTRODUÇÃO

Achyrocline satureioides

Por milênios, a humanidade vem utilizando plantas para alívio de doenças, e estas sempre contribuíram e continuam desempenhando um importante papel na descoberta de novos fármacos por serem fontes para a obtenção de substâncias ativas terapeuticamente (PEI et al., 2010).

Segundo relatórios da Organização Mundial da Saúde (OMS), cerca de 80% da população mundial faz uso de fármacos de plantas medicinais (WHO, 2005). A descoberta de fármacos à base de plantas continua a ser uma área importante, pois ela pode definitivamente fornecer subsídios contra vários alvos farmacológicos (SEN; SAMANTA, 2015). Especial interesse repousa sobre as plantas que possuem uso tradicional consolidado, por apresentarem fortes indícios de efetividade (FABRICANT; FARNSWORTH, 2001)

Achyrocline satureioides, (Lam.) DC. Asteraceae (Figura 1), conhecida popularmente como macela ou marcela é uma planta amplamente utilizada no sul do Brasil, Uruguai, Argentina e Paraguai, nativa da região sudeste subtropical e temperada da América do Sul RETTA, 2012), tem sido usada em fitoterapia por muitos anos.

Caracteriza-se por ser uma planta que apresenta um ciclo de vida longo, cresce em solo arenoso, pedregoso, morros e pradarias (LORENZO et al, 2001) e pode atingir até 80 cm de altura. Suas folhas são dispostas alternadamente, com bordas lisas de aproximadamente 5 cm de comprimento, possui também numerosas inflorescências cilíndricas e pequenas, de coloração amarela a dourada, como apresentado na Figura 1. A colheita é realizada entre março e abril, geralmente por dois anos consecutivos (DAVIES; VILLAMIL, 2004; RETTA, 2012). Das 40 espécies de *Achyrocline spp*, existentes, 25 foram descritas, do Brasil, e algumas na África tropical e em Madagascar (DEBLE, 2007).

Figura 1. *Achyrocline satureioides* (Lam.) DC. Asteraceae (arquivo pessoal do autor).

Infusões das inflorescências desta planta são muito utilizadas na medicina popular no tratamento de diversas patologias. Os estudos realizados com extratos de *A. satureioides* comprovam que a mesma apresenta propriedades de hepatoproteção (KADARIAN et al., 2002), antioxidante (MORQUIO et al., 2005), (BALESTRIN et al., 2016), citoproteção (ARREDONDO et al., 2004), antiúlcera (SANTIN et al., 2010), antibacteriana (CASERO et al., 2013), (CASERO et al., 2015), antiparasitária (BALDISSERA et al., 2014), antiherpética (BIDONE et al., 2015), antiviral (SABINI et al, 2012), hipocolesterolemica (ESPIÑA et al., 2012), antioxidante (POLYDORO et al., 2004), moduladora na produção de sinais inflamatórios em células sanguíneas humanas polinucleares (COSENTINO et al., 2008) além de apresentar um papel hipoglicemiante (CARNEY et al., 2002).

Composição química da planta (*Achyrocline satureioides)*

As diversas propriedades terapêuticas atribuídas à espécie estão relacionadas, frequentemente, aos seus metabólitos secundários (RETTA et al., 2012) que podem ser divididos em três grupos principais, sendo eles: os flavonoides, compostos fenólicos e polifenóis relacionados, terpenoides e alcaloides contendo compostos de nitrogênio e de sulfuretos (RODRIGUEZ-MATEOS et al., 2014).

Na composição química da planta (*A. satureioides*) relata-se a presença principalmente de compostos como ácidos orgânicos, tais como: ácido cafeolínico, ácido clorogênico, ácido cafeico, acído ferúlico e isômeros do ácido isoclorogênico, além de óleos voláteis, cuja composição apresenta: α-pineno, β-ocimeno, β-cariofileno, 1,8 cineole, germacreno e

cariofileno-1,10-epoxido e flavonoides (RETTA et al., 2012). Dentre os flavonoides majoritários, destacam-se: quercetina (QCT), luteolina (LUT) e *3-O-metilquercetina* (3*O*MQ) e a achyrobichalcona (ACB), encontrada nas preparações extrativas hidroalcoólicas de suas inflorescências (CARINI et al., 2015).

Uma ampla gama de estudos biológicos vem sendo desenvolvidos e confirmam a bioatividade destes flavonoides, fator que justifica ainda mais, a relação destes com as propriedades terapêuticas demonstradas pela planta (HAVSTEEN, 2002), (BOOTS et al., 2008), (CARINI et al., 2013).

Biodisponibilidade dos flavonoides O-glicosídeos

As propriedades biológicas da grande maioria dos compostos são dependentes da sua biodisponibilidade. Após a ingestão, os glicosídeos dos flavonoides e polifenois podem ser modificados na cavidade oral pela atividade de hidrólise de enzimas presentes na saliva. Dentro do trato gastro intestinal, a absorção destes, também está associada à atividade hidrolítica de uma cascata de enzimas. O metabolismo ocorre em um primeiro momento no intestino delgado, só depois, os metabólitos passam pela veia porta até o fígado, onde podem sofrer novas conversões antes de entrar na circulação sistêmica e eventualmente sofrer excreção renal (RODRIGUEZ-MATEOS et al., 2014)

No intestino delgado, a clivagem da unidade de açúcar é mediada pela ação da Lactase-Florizina Hidrolase (LFH), localizada na borda escova de células epiteliais. A LFH exibe ampla especificidade para flavonoides-O-β-D-glicosídeos, as agliconas liberadas podem entrar nas células epiteliais por difusão passiva (RODRIGUEZ-MATEOS et al., 2014)

Uma segunda etapa hidrolítica alternativa associada à digestão dos carboidratos é mediada pela enzima β-glicosidase citosólica (BGC). Além desta, evidências indicam que os cotransportadores de glicose dependentes de sódio, que constituem uma família de transportadores encontrados nos enterócitos mucosa intestinal do intestino delgado (SGLT-1), também estejam envolvidos no transporte de glicosídeos de flavonoides em células epiteliais (GEE et al., 2000).

Uma vez absorvidos, os flavonoides seguem a via metabólica comum de substâncias orgânicas exógenas e, como drogas e a maioria dos xenobióticos, vão para uma segunda fase do metabolismo enzimático. Nesta fase, eles podem ser conjugados com grupos de ácido glucurônico, sulfato e metil, em reações catalisadas por UDP-glucuronosiltransferases (UGTs), sulfotransferases (SULTs) e catecol-O-metiltransferases (COMT), respectivamente. (DEL RIO

et al., 2013) (Figura 1)

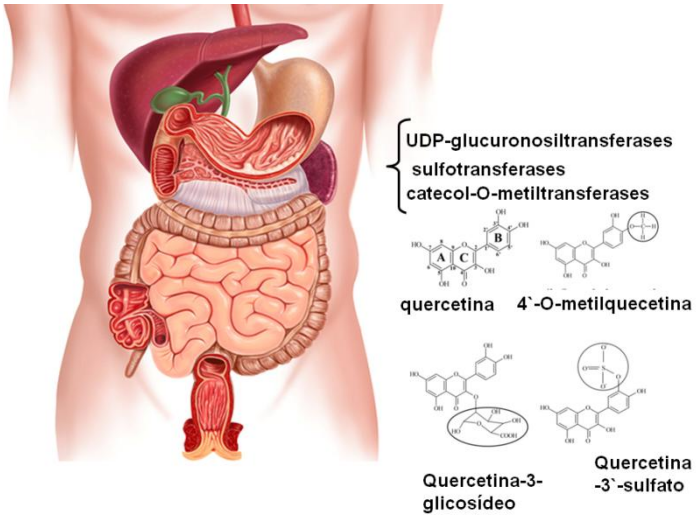

Figura 1 - Formas de conjugação da quercetina com grupos de ácido glucurônico, sulfato e metil em reações catalisadas por UDP-glucuronosiltransferases (UGTs), sulfotransferases (SULTs) e catecol-O-metiltransferases (COMT), respectivamente.

É possível também, que possa ocorrer reciclagem de metabólitos desta fase, do fígado ao intestino delgado através da recirculação enterohepática na bile. Há muitos trabalhos de especulação sobre isso na literatura, mas, até agora, há uma ausência de dados concretos e as evidências disponíveis obtidas com humanos (ACTIS-GORETTA et al. 2013; CROZIER 2013).

Além de serem absorvidos na corrente sanguínea, os metabolitos dessa segunda fase também retornam ao lúmen do intestino delgado através da família de transportadores de cassete de adenosina (MANZANO; WILLIAMSON, 2010). Estudos que analisam o líquido ileal após alimentação indicam que quantidades significativas de metabolitos que passaram pelo trato gastro intestinal, atingem o intestino grosso, onde são expostos à microbiota intestinal (BORGES et al. 2013; ERK et al., 2014).

No intestino, as bactérias intestinais podem hidrolisar glicosídeos, glucurônidos, sulfatos, amidas, ésteres, o esqueleto de flavonoides sofre fissão no anel, cujos produtos podem então ser submetidos a reações de redução, descarboxilação, desmetilação e desidroxilação (SELMA et al., 2009).

Essas modificações complexas geram catabólitos de baixo peso molecular que podem ser absorvidos eficientemente, alguns sofrendo mais metabolismo de segunda fase localmente e/ou no fígado antes de entrar na circulação, sendo excretados posteriormente na urina em quantidades que normalmente excedem a excreção de metabolitos de fenol absorvidos no trato gastrintestinal superior (CROZIER et al., 2010). Além disso, mais de metade do carbono dos glicosídeos dos flavonoides podem ser metabolizados em ácidos graxos de cadeia curta e,

portanto, estar disponíveis para o metabolismo energético pelo hospedeiro e, em última instância, ser expelido como gás carbônico (CZANK et al., 2013).

Propriedades antioxidantes dos flavonoides

Extratos vegetais que apresentam flavonoides em sua composição vêm sendo objeto de investigações visando a detecção de seu potencial antioxidante, por demonstrarem a capacidade de inativar o ânion superóxido e radicais hidroxila em sistemas enzimáticos e não enzimáticos, além da capacidade sequestrante e/ou inibição da formação de espécies reativas de oxigênio por leucócitos, e ainda, por suas interações com o óxido nítrico (DUGAS *et al*., 2000).

Os flavonoides podem modificar atividade de sistemas enzimáticos como: aldose redutase, NADPH oxidase, xantina oxidase, fosfolipase (NG et al., 2000; MOURE et al., 2001) e inibir a lipoperoxidação *in vitro* no estágio de propagação, atuando como bloqueadores das reações em cadeia provocadas pelos radicais hidroxila e peroxil (MOURE *et al*., 2001).

Ao avaliar os flavonóides: luteolina, quercetina, miricetina, genisteína, 3-hidróxi-flavona, 6-hidróxi-flavona, 3'-hidróxi-flavona, 4'-hidroxiflavona, canferol (5,7,4'-triidróxi-flavonol) quanto ao potencial antioxidante e sua relação estrutura-atividade, Cao et al., (1997) observaram que os múltiplos grupamentos hidroxila mostraram intensa atividade antioxidante sobre radical, atividade esta que não foi observada em compostos que contêm uma substituição simples na posição 5, demonstrando portanto que a metilação reduz a atividade antioxidante dos flavonoides.

Estudo que avaliou o efeito sobre a peroxidação lipídica de flavonoides como: (+) catequina, (-) epicatequina, 3-O-metilcatequina, quercetina, taxifolina, rutina, naringenina e naringina em homogeneizado de fígado demonstrou efetiva inibição da peroxidação lipídica, sendo que a estrutura 3,4-diidroxi em conexão com o anel aromático apresentou maior importância na eficiência destes compostos (YOUNES; SIEGERS, 1981).

Ao avaliar a propriedade de captar radicais livres de sete flavonoides, Robak e Gryglewski, (1988) observaram que os flavonoides mais efetivos na inibição do ânion superóxido foram a quercetina, miricetina e rutina, e quatro deles, aparentemente, também podem suprimir a atividade da xantina oxidase por um decaimento da biossíntese de ácido úrico.

Além da propriedade antioxidante, alguns flavonoides atuam como quelantes de metais, como Fe^{2+} e Cu^{2+}, inibindo a formação do radical hidroxila através da reação de Fenton, a qual é uma importante fonte de espécies reativas de oxigênico (AFANAS'EV et al, 1989).

A importância em explorar a ação antioxidante está em avaliar a capacidade de proteção celular frente ao estresse oxidativo e à formação de radicais livres, observada em algumas patologias como, por exemplo, a doença de Parkinson, doença de Alzheimer, bem como diabetes, entre outras (HALLIWELL, 2001; LIPINSKI, 2001).

Propriedades antibacterianas dos flavonoides

O uso irracional de antimicrobianos tem constituído nas últimas décadas, uma das maiores causas de surgimento de microrganismos multirresistentes. Segundo dados da WHO (2011) os custos com tratamento infecções resistentes chegaram a US$34 bilhões/ano. Este fato tem incentivado a comunidade científica na pesquisa de novos agentes antimicrobianos.

As plantas produzem uma ampla gama de metabólitos com evidências de efeito inibitório sobre bactérias patogênicas (CARPINELLA et al., 2011). O uso de extratos de plantas em medicina popular tem sido amplamente proposto como um fator importante para a seleção positiva de organismos intestinais que metabolizam ou resistem a estes compostos secundários de plantas (CARDONA et al. 2013).

Dentre os fitoterápicos atualmente estudados, os flavonoides têm merecido destaque em virtude da sua ampla gama de ações biológicas e terapêuticas demonstradas tanto em condições experimentais como em seres humanos. Muitos polifenóis por não serem absorvidos no intestino delgado pode interagir com microbiota colônica, produzindo diversos metabolitos com uma variedade de papéis fisiológicos (CARDONA, et al. 2013).

A maioria dos antibióticos existentes é derivada de produtos naturais e tendem a atingir a parede celular bacteriana, DNA ou ribossomos. Com poucas exceções, esses compostos exercem efeitos complexos na célula bacteriana e muitas vezes possuem mais de um alvo molecular (BROWN; WRIGHT, 2016).

Estudos demonstram que existe uma forte relação entre a estrutura dos flavonoides e a atividade antibacteriana (CUSHNIE; LAMB, 2005). A luteolina apresenta efeito antibacteriano frente a *E. coli*. (EUMKEB, el al., 2012). Estudos *in vitro* também tem mostrado o mecanismo dessa inibição bacteriana. A quercetina, na membrana bacteriana de patógenos, age diminuindo o seu fator de virulência, por meio da inibição da DNA girase. A epigalocatequina galato é capaz de inibir a função da membrana citoplasmática e as licochalconas são capazes de inibir o metabolismo energético (CUSHNIE; LAMB, 2005). Esses compostos tem despertado cada vez mais interesse, uma vez que o uso extensivo e indiscriminado de antibióticos convencionais tem contribuído para surgimento imediato de bactérias resistentes (FLAMM et al., 2012).

As inflorescências "Marcela" são frequentemente usadas para tratar várias doenças humanas, muitas delas relacionadas a gastrointestinais disfunção (RETTA et al., 2012). Estudos anteriores sobre a composição de *A. satureioides* demonstraram a presença de flavonoides, e outros constituintes fenólicos, incluindo achyrofurano e derivados de floroglucinol, que também mostraram algumas efeitos antibacterianos (CASERO, et al., 2015; JORAY, et al., 2013).

Estudo de Joray, et al., (2013) demonstrou a existência de uma ação concentrada dos metabólitos de flavonoides, evidenciando que a atividade antibacteriana de extratos *A. satureioides* pode ser explicada pelas interações sinérgicas entre os componentes dos extratos derivados da planta e não apenas atribuída a um único composto.

Propriedade teratogênica dos flavonoides

Devido a potenciais benefícios para a saúde e à suposição geral de que os produtos naturais são seguros, há uma tendência crescente na população em geral, incluindo gestantes, para complementar sua dieta com suplementos alimentares à base de flavonoides (ZIELINSKY et al., 2010). Como a gravidez é uma condição associada a imensas alterações fisiológicas, pode resultar na apresentação de sintomas incluindo náuseas, vômitos, constipação e azia principalmente no primeiro trimestre gestacional (LINDZON et al, 2011), o que contribui para que muitas mulheres recorram ao uso de plantas medicinais no alivio esses sintomas (KENNEDY et al., 2013). Infusões de *A. satureoides* são muito consumidas, principalmente no Brasil, Uruguai, Venezuela e Argentina (RETTA et al., 2012).

No entanto, o uso de fármacos à base de plantas não tem regulamentos estritos como outras drogas e infelizmente, os efeitos potencialmente tóxicos da ingestão excessiva de flavonoides são largamente ignorados (SKIBOLA et al., 2000). Em doses mais elevadas, os flavonoides podem atuar como mutagênicos, pró-oxidantes que geram radicais livres e como inibidores das principais enzimas envolvidas no metabolismo hormonal (HAHN et al., 2016) (SKIBOLA et al., 2000). A geração de espécies reativas de oxigênio por estes compostos implica na sinalização celular e a expressão gênica levando à apoptose e inibição da proliferação celular (GHIRINGHELLI et al., 2012).

Os flavonoides também podem atravessar a placenta para se acumular no tecido fetal (SCHRÖDER-VAN, 1998) e as adaptações feitas pelo feto para lidar com nutrição inadequada podem levar a mudanças morfológicas e fisiológicas que persistem na vida pós-natal

(VANHEES et al., 2011).

Assim, em doses elevadas, os efeitos adversos dos flavonoides podem compensar os benéficos, e deve-se ter cuidado ao ingeri-los em níveis acima daqueles que seriam obtidos a partir de uma dieta típica (SKIBOLA et al., 2000). Qualquer medicação utilizada durante a gravidez, incluindo o uso de plantas medicinais, deve sempre ter sua relação custo-benefício considerada em todas as situações.

Microbiota

A microbiota intestinal possui um papel fundamental no metabolismo de seus hospedeiros (ARUMUGAM et al., 2011). A formação da microbiota ocorre em paralelo ao processo de neurodesenvolvimento, ambos os processos caracterizados, por uma série de alterações na organização microbiana e neuronal, os quais se iniciam no útero e que continuam a ser modelados e refinados durante o período pós-natal (BORRE et al. 2014)

A colonização da microbiota começa cedo, nos recém nascidos, sendo afetada pelo modo do o nascimento, mostrando uma população maior de bactérias do epitélio em cesárea, diferente da população no parto normal, com mais bactérias do trato vaginal. Aos 3 anos de idade a população bacteriana do intestino já assume uma proporção mais parecida com a da vida adulta (YATSUNENKO et al. 2012).

O eixo microbiota-intestino-cérebro representa uma rede complexa de comunicação bidirecional entre a microbiota intestinal, o intestino e o cérebro, capaz de modular a função imune, gastrointestinal e do sistema nervoso central (CARABOTTI et al. 2015). O bom funcionamento desta rede depende de uma delicada relação sinérgica entre o hospedeiro e sua microbiota (ARUMUGAM et al., 2011).

A formação da microbiota ocorre em paralelo ao processo de neurodesenvolvimento, sendo ambos os processos caracterizados por uma série de alterações na organização microbiana e neuronal, os quais se iniciam no útero e que continuam a ser modelados e refinados durante o período pós-natal. Em comum, os dois processos possuem também janelas críticas de vulnerabilidade, onde alterações em fatores endógenos e exógenos podem potencialmente induzir mudanças importantes na comunicação intestino-cérebro (CARABOTTI et al. 2015).

Perturbações durante estes períodos críticos para a interação dinâmica entre a microbiota e o seu hospedeiro, estão amplamente associadas com sérias consequências para a saúde ao longo de todas as demais fases da vida, podendo aumentar o risco de distúrbios no desenvolvimento neural e neuropsiquiátricos (BORRE et al. 2014).

Sabemos que um dos principais fatores para a alteração da microbiota intestinal é o papel da dieta. As alterações na dieta modulam diretamente a microbiota, independente da obesidade, e essas alterações na microbiota causam uma mudança nos hábitos alimentares, afetando a quantidade de ingestão calórica (TURNBAUGH et al. 2006).

O consumo de extratos vegetais pode ser considerado um dos primeiros tratamentos medicinais da sociedade, selecionando uma porção da microbiota capaz de metabolizar ou resistir aos compostos secundários das plantas, como por exemplo: os flavonoides, catequinas e xantinas (PALMER et al. 2007)

Diabetes

O diabetes melito (DM) é uma doença crônica complexa, na qual o pâncreas não produz insulina suficiente ou o organismo não utiliza de forma eficaz a insulina produzida, resultando em hiperglicemia e sérios danos à saúde, requerendo cuidados médicos contínuos para controle glicêmico e estratégias multifatoriais de redução de riscos (WHO, 2016).

Diabetes tipo I é uma doença autoimune que leva à destruição de células beta pancreáticas. Já diabetes tipo II, que é muito mais comum, é principalmente um problema de regulação progressiva da glicose devido a uma combinação de células beta pancreáticas disfuncionais e resistência à insulina (WHO, 1999). As consequências do DM, em longo prazo, incluem danos, disfunções e falência de vários órgãos, especialmente rins, olhos, nervos, coração e vasos sanguíneos. Os sintomas clássicos do diabetes tipo I incluem perda inexplicável de peso, polidipsia e poliúria (WHO, 1999).

Em 2014, a OMS estimou a existência de 422 milhões de pessoas portadoras de DM no mundo, com prevalência mundial de 8,5% em adultos com idade ≥ 18 anos. No Brasil, esse dado foi de 8,1%. A OMS referiu que 1,5 milhões de mortes foram causadas diretamente pelo DM, em 2012. A expectativa para 2030 é de que o DM será a sétima principal causa mundial de mortalidade (WHO, 2016)

De acordo com a *International Diabetes Federation*, em 2015 havia 415 milhões de pessoas portadoras de DM com idades entre 20 e 79 anos. Nesse mesmo período ocorreram cinco milhões de mortes por essa doença. Esse mesmo levantamento revela que o Brasil ocupa a quarta posição entre os países com maior prevalência, ou seja, 14,3 milhões de pessoas portadoras de DM (INTERNATIONAL DIABETES FEDERATION, 2015)

Em relação ao impacto econômico, no Brasil, o custo com diabetes é de US$ 2,03 bilhões por ano, sendo que no primeiro trimestre de 2013 foram gastos US$ 640,9 milhões em importações de medicamentos e insumos sendo que o principal fármaco adquirido foi a insulina.

E em 2014 foi estimado um gasto global com diabetes de US$ 612 bilhões, equivalente a 11% do total de gastos em saúde com os adultos no mundo (ADA, 2016).

Muito dos fármacos sintetizados quimicamente utilizados para diminuir os efeitos adversos da DM II e suas complicações secundárias causam efeitos colaterais adversos, entre eles: ganho de peso, distúrbios gastrointestinais e insuficiência cardíaca. Atualmente, são recomendadas várias outras abordagens, entre elas, controle de dieta, exercícios físicos e uso de compostos naturais derivados de plantas com atividade hipoglicêmica no controle do DM, pois são abordagens econômicas com menor ou nenhum efeito colateral (NAVEEN; BASCARAN, 2017).

Enzimas envolvidas da digestão e absorção de carboidratos

As glicosidases, também conhecidas como glicosil hidrolases ou glicosídeo hidrolases, são enzimas encontradas amplamente nos seres vivos que catalisam a hidrólise de ligações glicosídicas entre di, oligo, polissacarídeos e glicoconjugados (GLOSTER; DAVIES, 2010).

Estas enzimas atuam sobre uma vasta gama de substratos glicosilados, uma vez que diversas moléculas biológicas podem formar ligações O-N-e S-glicosídicas, como, por exemplo, carboidratos, proteínas, lipídeos e ácidos nucleicos (CANTARAL et al., 2009). Isto permite que as glicosidases participem de eventos biológicos importantes, como a captação e armazenamento de energia, a expansão e degradação da parede celular, assim como o *turnover* de moléculas envolvidas na sinalização (DAVIES; HENRISSAT, 1995). Alterações nos níveis de glicosidases estão associadas às doenças de Pompe (GEEL et al., 2007), Gaucher (SUZUKI et al., 2009) e a distúrbios alimentares, como a hipolactasia (MATTAR; MAZO, 2010).

As glicosidases podem ser agrupadas de acordo com suas estrututras primárias em mais de 100 famílias, conforme descrito no Banco de Dados de Enzimas Ativas sobre Carboidratos, CAZY-Carbohydrate Active Enzymes Database - http://www.cazy.org (CANTARAL et al., 2009).

Além da similaridade na sequência de aminoácidos, as glicosidases de mesma família frequentemente apresentam propriedades cinéticas e estruturais conservadas, como resíduos catalíticos, mecanismo de catálise e geometria do sítio catalítico em torno da ligação glicosídica. A reação catalisada por glicosidases consiste na quebra da ligação glicosídica com retenção ou inversão da configuração anomérica (ligação α ou β-glicosídicas), o que para a maioria das glicosidases ocorre via mecanismos reacionais clássicos descritos por (KOSHLAND, 1953).

Nos seres humanos a digestão de amido, principal fonte de energia proveniente da

alimentação, envolve diversas etapas. Inicialmente, a digestão parcial pela α-amilase salivar resulta na degradação do substrato polimérico em oligômeros mais curtos. No intestino, os produtos obtidos na digestão da amilase salivar sofrem nova etapa de hidrólise pela α-amilase pancreática (α-AP), produzindo maltose, maltotriose e pequenos malto-oligossacarídeos. Em seguida, a mistura resultante alcança a mucosa da membrana com borda em escova no intestino, onde α-glicosidases adicionais irão degradar estes oligossacarídeos em glicose. A partir deste momento, a glicose pode alcançar a corrente sanguínea por meio de seu sistema de transporte específico (GLUTs). Estas α-glicosidases intestinais são exoglicosidases encontradas na superfície luminal de enterócitos contendo atividades maltase/glicoamilase e sucrase/isomaltase (QUEZADA-CALVILLO et al., 2007).

As enzimas são consideradas como um dos alvos mais atraentes para a busca de fármacos por apresentarem papéis catalíticos essenciais em muitos processos fisiológicos que podem estar alterados em situações patológicas. Consequentemente, há um amplo e crescente interesse no estudo de enzimas com o objetivo de identificar moléculas inibitórias que possam atuar como pontos de partida para a descoberta de fármacos (COPELAND, 2013). A variedade de papéis biológicos exercido pelas glicosidases tem estimulado o desenvolvimento de seus inibidores para serem utilizados como fármacos no tratamento de diversos tipos de doenças, como diabetes (DEROSA; MAFFIOLI, 2012), distúrbios de armazenamento lisossomal (SUZUKI et al., 2009), infecções virais (BUTTERS et al., 2005) e câncer (VON ITZSTEIN, 2007; SUN et al., 2009; PILI et al., 1995).

A inibição da atividade das glicosidases digestivas e a consequente diminuição da glicemia pós-prandial são de suma importância para o tratamento de DM tipo II. Por essa razão, nos últimos anos, muitos trabalhos vêm tentado descobrir novos inibidores de glicosidases, sejam estes compostos sintéticos ou naturais. Atualmente já existem fármacos inibidores de glicosidases presentes no mercado e utilizados para o tratamento da DM, como a acarbose. Este fármaco inibe as enzimas alfa-glicosidases pancreáticas e intestinais, mas possui diversos efeitos colaterais.

Efeito dos polifenóis sobre a atividade de enzimas

A maioria dos inibidores de glicosidases são açúcares ou seus derivados (LIANG et al., 2006). No entanto foi demonstrado que derivados sulfonamídicos de chalconas demonstraram atividades inibitórias satisfatórias na faixa de micro molar para glicosidases (SEO et al., 2005).

As moléculas denominadas chalconas são abundantes em plantas comestíveis e são conhecidas como precursores de flavonoides e isoflavonoides (STAR et al., 1971; STEVENS et al., 2000). Estas mesmas moléculas demonstraram uma ampla gama de atividades farmacológicas entre as quais: ação contra protozoários (NIELSEN et al., 1998; LI et al., 1995; LIU et al., 2001), anti-inflamatória (HSIEH et al., 1998; BABU et al., 2003), imunomoduladora (BARFODEt al., 2002), inibição da produção de óxido nítrico (Rojas et al., 2002), atividade anti-câncer (KUMAR et al., 2003; DUCKI et al., 1998) e anti-HIV (ARTICO et al., 1998).

Na planta *A. satureioides* isolou-se recentemente uma bichalcona que recebeu a denominação de achyrobichalcona (ACB), esta foi encontrada nas preparações extrativas hidroalcoólicas de suas inflorescências (CARINI et al., 2015).

Neste contexto, considerando o conjunto de estudos realizados com *Achyrocline satureioides* e o amplo potencial terapêutico apresentado por esta espécie vegetal, o presente trabalho apresenta como objetivo geral realizar a caracterização química bem como avaliar os efeitos terapêuticos e de toxicidade de extratos de *Achyrocline satureioides* em modelos *in vitro* e *in vivo.*

V. OBJETIVOS

Justificativa e apresentação dos objetivos

Considerando que: 1) apesar do uso generalizado de *Achyrocline satureioides* na medicina tradicional, as propriedades químicas e farmacológicas que sustentam seu uso fitoterápico, ainda não foram completamente caracterizadas; 2) estudos sobre o efeito do uso indiscriminado de infusões de *Achyrocline satureioides* durante a gravidez são limitados; 3) existem evidências que *Achyrocline satureioides* apresenta efeito hipoglicemiante, porém o mecanismo ainda não esta claro. O objetivo central desta tese foi o de **avaliar os efeitos terapêuticos e de toxicidade de extratos de *Achyrocline satureioides* em modelos *in vitro* e *in vivo***, tendo como objetivos específicos:

a) Caracterizar quimicamente três extratos de *Achyrocline satureioides;*

b) Avaliar as alterações na microbiota e atividade antimicrobiana dos extratos de *Achyrocline satureioides* frente a patógenos intestinais;

c) Avaliar o efeito da suplementação com extratos de *Achyrocline satureioides* durante a gravidez e a lactação em parâmetros redox de ratas Wistar e seus filhotes;

d) Avaliar o potencial anti-hiperglicêmico do extrato hidroalcoólico de *A. satureioides* em modelos *in vivo* diabéticos e não diabéticos e avaliar o mecanismo da inibição da atividade da glicosidases digestivas

PARTE 2 – RESULTADOS

CAPÍTULO I

Effects of *Achyrocline satureioides* inflorescence extracts against pathogenic intestinal bacteria: chemical characterization, *in vitro* tests, and *in vivo* evaluation

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Research Article

Effects of *Achyrocline satureioides* Inflorescence Extracts against Pathogenic Intestinal Bacteria: Chemical Characterization, In Vitro Tests, and In Vivo Evaluation

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Three *Achyrocline satureioides* (AS) inflorescences extracts were characterized: (i) a freeze-dried extract prepared from the aqueous extractive solution and (ii) a freeze-dried and (iii) a spray-dried extract prepared from hydroethanol extractive solution (80% ethanol). The chemical profile, antioxidant potential, and antimicrobial activity against intestinal pathogenic bacteria of AS extracts were evaluated. In vitro antioxidant activity was determined by the total reactive antioxidant potential (TRAP) assay. In vivo analysis and characterization of intestinal microbiota were performed in male Wistar rats (saline versus treated animals with AS dried extracts) by high-throughput sequencing analysis: metabarcoding. Antimicrobial activity was tested in vitro by the disc diffusion tests. Moisture content of the extracts ranged from 10 to 15% and 5.7 to 17 mg kg^{-1} of fluorine. AS exhibited antioxidant activity, especially in its freeze-dried form which also exhibited a wide spectrum of antimicrobial activity against intestinal pathogenic bacteria greater than those observed by the antibiotic, amoxicillin, when tested against *Bacillus cereus* and *Staphylococcus aureus*. Antioxidant and antimicrobial activities of AS extracts seemed to be positively correlated with the present amount of flavonoids. These findings suggest a potential use of AS as a coadjuvant agent for treating bacterial-induced intestinal diseases with high rates of antibiotic resistance.

1. Introduction

Many bacterial pathogens associated with epidemics of gastrointestinal tract disorders in humans, such as *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*, have evolved multidrug-resistant forms subsequent to antibiotic use [1]. The extensive use of antibiotics over the last decade has led to the emergence of bacterial resistance and the dissemination of resistant genes among pathogenic microorganisms [2]. In recent years, several associations between common chronic human disorders and an altered composition and function of the gut microbiome have been reported [3–6]. Numerous studies have shown that changes in some bacterial phyla of the intestinal microbiota [3, 5, 7], such as Proteobacteria and Bacteroidetes/Firmicutes, can be considered indicators of dysbiosis.

Diseases of the gastrointestinal tract are induced by oxidative stress and overproduction of reactive oxygen species (ROS), which accumulate under abnormal conditions and contribute to the rapid development of inflammation [8]. Many of the chemical constituents of plants, such as flavonoids, have been described as scavengers of the superoxide anion, hydroxyl radicals, and peroxyl radicals, as well as being inhibitors of key enzymes of mitochondrial respiration [9–11].

The use of plant extracts in folk medicine has been widely proposed as an important factor for the positive selection of intestinal organisms that metabolize or resist these secondary plant compounds. Many dietary polyphenols are not absorbed in the small intestine and can interact with colonic microbiota, producing diverse metabolites with a variety of physiological roles [12]. These phenomena may modulate gut microbiota and, consequently, alter the Bacteroidetes/Firmicutes balance [12, 13].

Infusions from *Achyrocline satureioides* (Asteraceae) or "Marcela" inflorescences are frequently used to treat several human diseases, many of them related to gastrointestinal dysfunction [14]. Previous studies of the composition of *A. satureioides* have demonstrated the presence of flavonoids, which have been shown to inhibit lipid peroxidation by scavenging ROS and chelating metal ions responsible for ROS generation [15]. Other phenolic constituents, including achyrofuran and phloroglucinol derivatives, have shown some antibacterial effects [16, 17] without causing any bacterial resistance or adverse side effects. However, to the best of our knowledge, no reports regarding the relationship between aglycone and achyrobichalcone, the major flavonoids present in *A. satureioides* inflorescences, and the intestinal activity of pathogenic bacteria have been reported.

In this context, the aim of this study was to investigate the chemical profile, the antioxidant potential in vitro, and the antimicrobial activity of *A. satureioides* inflorescence extracts against intestinal pathogenic bacteria (both gram-positive and gram-negative) as well as characterize alterations in the intestinal microbiota of rats treated with *A. satureioides* extracts.

2. Material and Methods

2.1. Plant Material. *A. satureioides* inflorescences were purchased from Centro de Pesquisas Químicas, Biológicas e Agronômicas (CPQBA, Universidade de Campinas, Brazil). The plant samples were collected and dried (room temperature) in May 2013 and identified as Cultivar CPQBA/2; they were registered at Ministério Agricultura, Pecuária e Abastecimento (MAPA-Brazil), as number 22975.

2.2. Chemicals. The following compounds were used for the experiments and were of high performance liquid chromatography (HPLC) grade: methanol (JT Baker, USA), acetonitrile (Tedia, Brazil), and phosphoric acid (Merck). Catalase (CAT, EC 1.11.1.6); superoxide dismutase (SOD, EC 1.15.1.1); thiobarbituric acid (TBA); hydrogen peroxide (H_2O_2); and the standards, quercetin, and luteolin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The flavonoid 3-O-methylquercetin was purchased from Extrasynthese (France).

2.3. Preparation of A. satureioides Extracts. Three extracts from *A. satureioides* inflorescences were prepared, including an aqueous extract, freeze-dried extract, and spray-dried extract. The plant : solvent ratio was 7.5 : 100 (w/v) for the three extracts. The aqueous extractive solution was prepared via decoction and then freeze-dried. The freeze-dried and spray-dried extracts were obtained by maceration of inflorescences in 80% ethanol (v/v). The extraction time was eight days, with occasional stirring [18]. The resulting extractive solution was filtered, and the supernatant was freeze-dried (frozen at −80°C and subsequently dried in a freeze-dryer (Edwards Modulyo 4K, Irvine, USA) at −60°C and pressure of -10^{-2} bar) or spray-dried (Spray Dryer Buchi B-290, with a two-component nozzle and current flow, under the following operating conditions: inlet temperature, 160 + 2°C; output temperature 140 + 2°C; feed rate, 3 mL/min; and spraying pressure, 2 bar [19]). The spray-dried extract contained 50% extractives, 33.4% colloidal silicon dioxide, and 16.6% polysorbate 80.

2.4. Bromatological Analysis. All chemical composition analyses of *A. satureioides* inflorescences and the corresponding extracts were performed in triplicate, according to previously described methods [20]. The protein content was quantified using the Kjeldahl method, with a converting factor of 5.75. Lipid analyses were performed by extraction with ethyl ether using a Soxhlet extractor. Reducing, nonreducing, and total sugar analyses were carried out according to the Lane-Eynon method [20]. The loss on drying analysis was conducted per the method outlined by the Association of Official Agricultural Chemists [20].

2.5. Elemental Content. The determination of cadmium, lead, chromium, and fluorine content was carried out using a contrAA® 700 model high-resolution continuum source graphite furnace atomic absorption spectrometer (HR-CS SS-GF AAS, Analytic Jena AG, Germany). The *A. satureioides* and extract powder source samples were ground in an A-11 basic micromill (IKA-Werke, Germany) and sieved through a 150 μm polyester mesh to improve particle size distribution. Larger particles that did not pass through the mesh were ground and sieved again until the entire sample passed through. The samples were then dried at 50°C for 3 h in an oven to eliminate absorbed moisture. The dried samples were stored in sealed plastic vials until further processing. The samples (0.01 to 0.30 mg) were weighed directly on the direct solid sampling (SS) platform and introduced into the HR-CS SS-GF AAS to determine cadmium, chromium, and lead concentrations. The instrumental parameters were optimized, and the method was adopted per a previous report [21].

To determine fluorine content, samples (0.01 to 0.15 mg) were weighed directly onto the SS platform and introduced into the HR-CS SS-GF MAS. Then, 10 μL of a solution containing 1500 mg L^{-1} Ca (15 μg Ca) was injected directly onto the sample to form CaF diatomic molecules. The

instrumental parameters were optimized, and the method was adopted per a previous report [22].

2.6. Flavonoid Content. Approximately 20 mg of each extract was dissolved in a liquid chromatography (LC) system (methanol : 16 mM phosphoric acid (50 : 50, v/v)) and transferred to a 100 mL volumetric flask. The solution was filtered through a 0.45 μm membrane filter (Millipore-HVHP, MA, USA), and the supernatant was injected into the LC equipment. Evaluations for each sample were repeated three times. LC analysis of *A. satureioides* extracts was performed following the method described by Bidone et al. [18]. Briefly, the LC equipment comprised a Shimadzu LC-10A system equipped with an LC-10 AD pump and a CBM- 10A system controller. The system was maintained at 30 + 1°C, and the programmed injection volume was 20 μL. Samples were diluted in methanol : 16 mM phosphoric acid (50 : 50, v/v). The method specificity was determined with a Shimadzu LC-20A system equipped with an LC-20 AT pump, a CBM-20A system controller, an SIL-20A autosampler, and an SPD-M20A diode array detector.

2.7. Total Reactive Antioxidant Potential. Total reactive antioxidant potential (TRAP) was used as an index of the nonenzymatic antioxidant capacity of *A. satureioides* extracts. Briefly, this assay is based on the ability of samples to quench peroxyl radicals generated by 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH). The reaction contained AAPH (10 mM) and luminol (35 μM) dissolved in 0.1 M glycine buffer (pH 8.6). After stabilizing for 2 h at room temperature, different concentrations of *A. satureioides* extract were added to the system to determine their antioxidant potential. Results are expressed as the area under the curve (AUC), and the total antioxidant properties (TAR) of each *A. satureioides* extract were compared to the system. A smaller AUC in relation to the system indicates a higher total reactive antioxidant potential. TAR is closely related to the quality of the antioxidants within the sample. In our study, TAR was calculated as the ratio of chemiluminescence in the absence of sample (I_o)/chemiluminescence immediately after sample addition (I). Higher TAR values represent a greater antioxidant capacity of the sample [23].

2.8. Ferric Ion Reducing Antioxidant Power (FRAP) Assay. This assay determines the ability of antioxidants to reduce iron. FRAP activity was measured according to the method of Benzie and Strain [24], with modifications. Briefly, we mixed 90 mL diluted extract (20 mg/mL) with 270 mL distilled water and 2.7 mL FRAP reagent [2.5 mL 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2.5 mL 20 mM ferric chloride, and 25 mL 0.3 M acetate buffer, pH 3.6] in a dark environment. The mixture was vortexed and incubated at 37°C for 30 min; absorbance at 595 nm was then recorded. A standard curve using a 2 mM ferrous sulfate (Fe^{+2} source) standard was created to calculate the amount of Fe^{+2} produced during the extract-induced reduction of Fe^{+3}. Equivalent concentration was used as a parameter to define the concentration of antioxidant having a ferric TPTZ-reducing ability equivalent to that of 1 mM $FeSO_4 \cdot 7H_2O$.

2.9. Fe^{2+} Chelating Activity Assay. The ferrous ion-chelating ability of the extracts was determined as previously described [25]. The reaction mixture, containing 200 mL diluted extract (20 mg/mL), 25 mL $FeSO_4$ (2 mM), and 100 mL FerroZine™ (5 mM), was shaken well and incubated for 10 min at room temperature. Ethanol (1.675 mL) was then added to the mixture to stop the reaction. Ethylenediaminetetraacetic acid (EDTA), a known chelating agent, was used to construct a standard curve (0.5–10 μg/mL final concentration). Absorbance of the FerroZine-Fe^{+2} complexes was measured at 562 nm, and the results are expressed as mg EDTA equivalents/g dry extract weight.

2.10. Hydroxyl Radical Scavenging. This assay measures the antioxidant activity of a substance against hydroxyl radicals. The formation of hydroxyl radicals from the Fenton reaction is used to quantify the oxidative degradation of 2-deoxyribose (2-DR). 2-DR is incubated with a system that generates hydroxyl radical and degrades into malondialdehyde (MDA), which condenses with thiobarbituric acid (TBA) to form a pink chromophore. The chromophore was quantified spectrophotometrically at a wavelength of 532 nm, as previously described [26].

2.11. Thiobarbituric Acid (TBARS) Assay. The TBARS assay indirectly measures lipid peroxidation in biological systems. The assay uses TBA to react with lipoperoxides such as MDA. The reaction of MDA with TBA produces a pink-red color, which is detected by absorbance at 532 nm [27].

2.12. Characterization of Intestinal Microbiota. Male Wistar rats (20 animals) were randomly divided into two groups (ten rats per group) that received saline (1 mL kg^{-1}) or *A. satureioides* extract (35 mg kg^{-1}, equivalent to the consumption of 150 mL tea per day). Rat feces were collected on the counter for handling animals in the vivarium at the Universidade Federal do Rio Grande do Sul after cleaning with 70% alcohol on day 0 (D0) and D21 after treatment. The feces were placed in wells that had been presterilized for 20 min under UV light from a laminar flow cabinet. To better represent the diversity of each group, the feces were pooled where 4 wells were made for each per group on the tubes. All experiments were approved by the Institutional Animal Care and Use Committee at the Federal University of Rio Grande do Sul (IACUC #25449).

2.13. DNA Extraction and Amplification. Total DNA was extracted immediately after collection using the QIAmp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. After extraction, DNA was stored at −20°C. Partial 16S rRNA gene fragments were amplified using universal primers 515F and 806R [28]. The PCR conditions included one initial denaturation step at 94°C for 45 s, 30 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 50°C, and extension for 1 min at 72°C, with one final extension step for 7 min at 72°C. After amplification, 16S rRNA bands were selected and purified from a 3% agarose gel using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA).

2.14. High-Throughput Sequencing Analysis, Metabarcoding. The 16S rRNA reads were generated via high-throughput sequencing and were submitted for quality control to retain sequences with a minimum length of 100 bp and trim sequences to remove low quality bases (minimum Phred score of 30) using PRINSEQ [29]. The remaining sequences were dereplicated, sorted by decreasing read abundance, and then filtered to exclude singletons using USEARCH v7.0.1090 [30]. Clusters were assembled using a minimum identity of 99%, and chimeras were removed using the Ribosomal Database Project (RDP) reference database [31]. The taxonomic assignment was obtained using Quantitative Insights into Microbial Ecology (QIIME v1.7) [32]. Sequences were clustered and assigned to operational taxonomic units (OTUs), which were selected based on 97% sequence similarity. Taxonomic data were achieved through the classification algorithm, using the 97% OTUs version of GreenGenes 13.8 [33].

2.15. Determination of Antibacterial Activity. The antibacterial activity of *A. satureioides* extracts against the bacteria *Listeria monocytogenes* (ATCC 15131), *B. cereus* (ATCC 9634), *S. aureus* (ATCC 1901), *Salmonella enteritidis* (ATCC 13076), *Listeria innocua*, *Pseudomonas*, *Aeromonas* (ATCC 27853), *Escherichia coli* (ATCC 8739), and *Corynebacterium fimi* (NCTC 7547) was determined according to the procedure of Motta and Brandelli [34], with modifications. Indicator microorganisms, at a concentration of 10^8 CFU mL^{-1} in saline solution (0.85% NaCl, w/v), were inoculated with a swab onto brain heart infusion (BHI) agar plates.

A pilot trial was initially performed with three extracts to evaluate the bacterial growth inhibiting capacity of the extracts. Aliquots of 20 μL freeze-dried extract (50, 100, and 200 mg/mL) were spotted on the freshly prepared indicator strain lawn, and plates were incubated at the optimal temperature for each test microorganism. Subsequently, zones of growth inhibition (represented by clear haloes) were measured and depicted as an inhibition zone (mm).

Based on the results of this initial experiment, antibacterial and bacteriolytic activities were observed with the freeze-dried hydroalcoholic extract only. Inhibitory capacity was the greatest against *B. cereus* (ATCC 9634), *S. aureus* (ATCC 1901), and *S. enteritidis* (ATCC13076). The reference antibiotics used as positive controls were chloramphenicol, amoxicillin, and ciprofloxacin.

Bacterial cells were cultivated in casein soy broth (17 g enzymatic digest of casein, 3 g enzymatic digest of soybean meal, 2.5 g dextrose, 5 g sodium chloride, and 2.5 g dipotassium phosphate per liter, pH 7.3) at 27°C. The overnight bacterial cultures were harvested by centrifugation (10 min, 3000 rpm) and resuspended in fresh casein soy broth. Bacterial concentrations were adjusted to approximately 5×10^7 cells/mL (OD600 ~0.2). Broth bacterial cultures and test samples were mixed for optical density measurements.

2.16. Statistical Analysis. Data obtained from animal experiments are expressed as means ± standard error of the mean. Statistical differences between the treatments and the controls were assessed by Student's *t*-test and Bonferroni. A value of $P < 0.05$ was considered statistically significant.

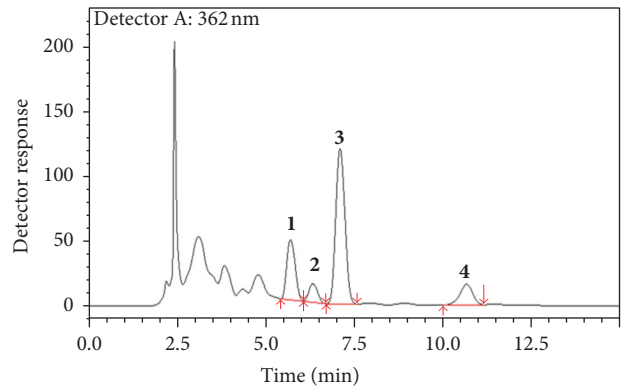


Figure 1: Chromatographic profile (Rp-HPLC) of *Achyrocline satureioides* freeze-dried extracts prepared from a hydroethanol extractive solution. (**1**) Luteolin, (**2**) quercetin, (**3**) 3-o-methylquercetin, and (**4**) achyrobichalcone are shown. Absorbance was detected at 362 nm.

3. Results

3.1. Bromatological Analysis and Composition of A. satureioides Extracts. Our results show that the level of reducing sugars and lipids in the aqueous extract was lower than that in the source plant material (Table 1). Loss on drying measurements of the extracts ranged from 5.7 to 17%, with the spray-dried extract having the lowest values. Analyses of heavy metals (lead, cadmium, and chromium) and fluorine content in the extracts and original plant material revealed that the aqueous extract contained higher levels of lead, cadmium, and fluorine than the plant material and the two extracts prepared from the hydroethanol extractive solution. The plant material and all extracts contained concentrations of heavy metals that were below harmful limits (Table 2). Finally, three major flavonoid aglycones and a chalcone were quantified, namely, quercetin, 3-*O*-methylquercetin, luteolin, and achyrobichalcone, respectively (Figure 1). Total flavonoid content of the freeze-dried extract (132 mg/g) was slightly higher than that found in the spray-dried extract (129.7 mg/g). The flavonoid content in both the freeze-dried and spray-dried extracts was approximately twofold higher than that found in the freeze-dried aqueous extract (54.23 mg/g) (Table 3). These results demonstrate that 80% ethanol (v/v) is more efficient for extracting the three flavonoid aglycones and chalcone from the inflorescences than water alone. Further, the two drying methods did not influence the content of the extracts.

3.2. Antioxidant Properties of A. satureioides Extracts. The dried *A. satureioides* extracts all exhibited antioxidant activity, especially the freeze-dried form. At a concentration of 34 μg/mL, luminescence counts (AUC) were lower for the dried extracts than for the system ($P < 0.0001$) (Figure 2). The antioxidant potential was higher for the freeze-dried extract, which showed a better quenching potential against peroxyl radical than that of the spray-dried and aqueous extracts, as determined by the TRAP assay ($P < 0.01$ and $P < 0.01$, resp.) (Figure 2(a)). When the flavonoid and chalcone content were analyzed in the extracts separately, we

Table 1: Nutritional composition of *Achyrocline satureioides* inflorescences and the corresponding extracts (g/100 g dry weight) ± standard deviation (SD).

Samples	Lipid (g/100 g)	Protein (g/100 g)	Reducing sugar (g/100 g)	Loss on drying (g/100 g)
In nature	11.0 ± 0.6^{a}	1.74 ± 0.16^{a}	17.27 ± 0.3^{a}	10.9 ± 0.4^{b}
Aqueous extract*	5.8 ± 0.15^{c}	0.97 ± 0.07^{c}	17.22 ± 0.9^{a}	17.0 ± 0.7^{a}
Freeze-dried extract**	10.8 ± 0.35^{b}	1.3 ± 0.7^{b}	16.6 ± 0.4^{a}	17.8 ± 0.35^{a}
Spray-dried extract**	11.3 ± 0.2^{a}	1.10 ± 0.2^{c}	16.2 ± 0.6^{a}	5.7 ± 0.2^{c}

* Aqueous extract: freeze-dried extract prepared from *A. satureioides* decoction. ** Freeze-dried or spray-dried extracts prepared from *A. satureioides* extractive solution were obtained by macerating inflorescences in 80% ethanol. All extractive solutions were prepared using a plant : solvent ratio of 7.5% (w/v). Spray-dried extracts contained 50% extractives, 33.4% colloidal silicon dioxide, and 16.6% polysorbate 80; excipient percentages were considered in the analysis. Different letters in the same column indicate significant differences ($P < 0.05$) determined by Tukey's test of nutritional composition between extracts and the *A. satureioides* inflorescences.

Table 2: Total metal content in *Achyrocline satureioides* inflorescences and the corresponding extracts (kg^{-1} dry weight) ± standard deviation (SD).

Samples	Lead ($mg\,kg^{-1}$)	Cadmium ($mg\,kg^{-1}$)	Chrome ($mg\,kg^{-1}$)	Fluoride ($mg\,kg^{-1}$)
In nature	0.35 ± 0.04^{b}	0.09 ± 0.01^{a}	0.62 ± 0.05^{b}	17.0 ± 1.8
Aqueous extract*	0.38 ± 0.03^{b}	0.10 ± 0.01^{a}	0.45 ± 0.02^{c}	17.0 ± 2.2
Freeze-dried extract**	0.49 ± 0.06^{a}	0.010 ± 0.001^{b}	0.47 ± 0.01^{c}	4.8 ± 0.8
Spray-dried extract**	0.13 ± 0.02^{c}	0.008 ± 0.001^{b}	0.73 ± 0.06^{a}	2.5 ± 0.3

* Aqueous extract: freeze-dried extract prepared from *A. satureioides* decoction. ** Freeze-dried or spray-dried extracts prepared from *A. satureioides* extractive solution were obtained by macerating inflorescences in 80% ethanol (v/v). All the extractive solutions were prepared with a plant : solvent ratio of 7.5% (w/v). Different letters in the same column indicate significant differences ($P < 0.05$) in the amount of metals between extracts and the *A. satureioides* inflorescences.

Table 3: Flavonoids and chalcone content in dried *Achyrocline satureioides* extracts (μg/mg dry weight).

Samples	Quercetin	3-*O*-Methylquercetin	Luteolin	Achyrobichalcone	Total flavonoid + chalcone
Aqueous extract*	15.68 ± 0.3^{c}	27.05 ± 0.1^{b}	5.5 ± 0.02^{c}	6.0 ± 2.2^{c}	54.23^{c}
Freeze-dried extract**	27.7 ± 0.6^{b}	62.3 ± 0.5^{a}	18.0 ± 0.01^{b}	24.0 ± 0.8^{a}	132.0^{a}
Spray-dried extract**	31.34 ± 0.02^{a}	60.64 ± 0.7^{a}	17.22 ± 0.06^{a}	20.5 ± 0.3^{b}	129.7^{b}

* Aqueous extract: freeze-dried extract prepared from *A. satureioides* decoction. ** Freeze-dried or spray-dried extracts prepared from *A. satureioides* extractive solution were obtained by macerating inflorescences in ethanol 80% (v/v). Spray-dried extract contained 50% extractives, 33.4% colloidal silicon dioxide, and 16.6% polysorbate 80; excipient percentages were considered in the analysis. All the extractive solutions were prepared with a plant : solvent ratio of 7.5% (w/v). Different letters in the same column indicate significant differences ($P < 0.05$) in the amount of flavonoids between extracts.

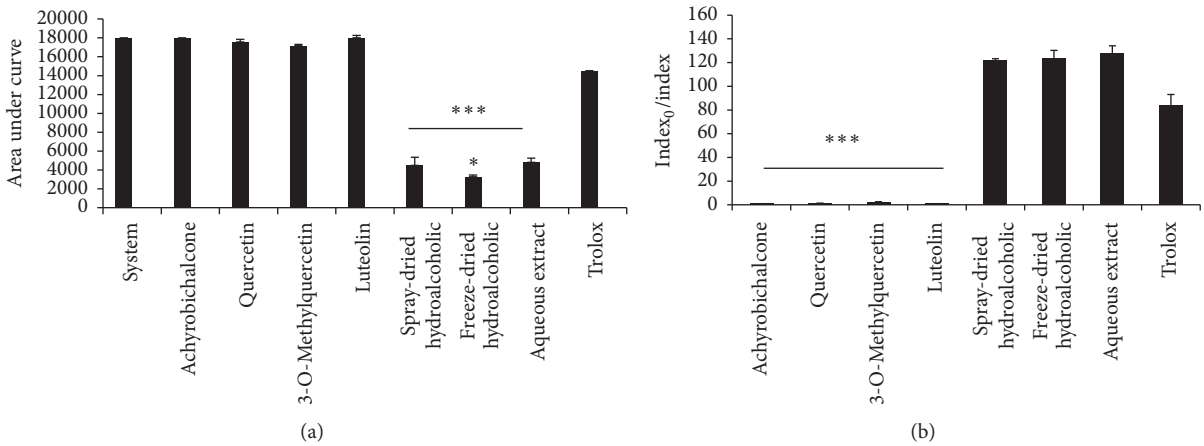


Figure 2: (a) In vitro effects of *Achyrocline satureioides* extracts on total radical-trapping antioxidant potential (TRAP). Data are presented as means ± SEM of three experiments. Black bars represent *A. satureioides* extracts and flavonoids compared to the system (i.e., solvents without sample and Trolox = control). $^{***}P < 0.0001$ extracts versus system (Student's t-test) and $^{*}P < 0.05$ freeze-dried extract versus aqueous extract and spray-dried extract. (b) Total antioxidant reactivity (TAR) index, which is related to antioxidant quality, is shown. $^{***}P < 0.0001$ flavonoids versus Trolox. Aqueous extract: freeze-dried extract prepared from *A. satureioides* decoction. Both freeze-dried and spray-dried extracts were prepared from an *A. satureioides* hydroethanol extractive solution (80% ethanol). All extractive solutions were prepared using a plant : solvent ratio of 7.5% (w/v).

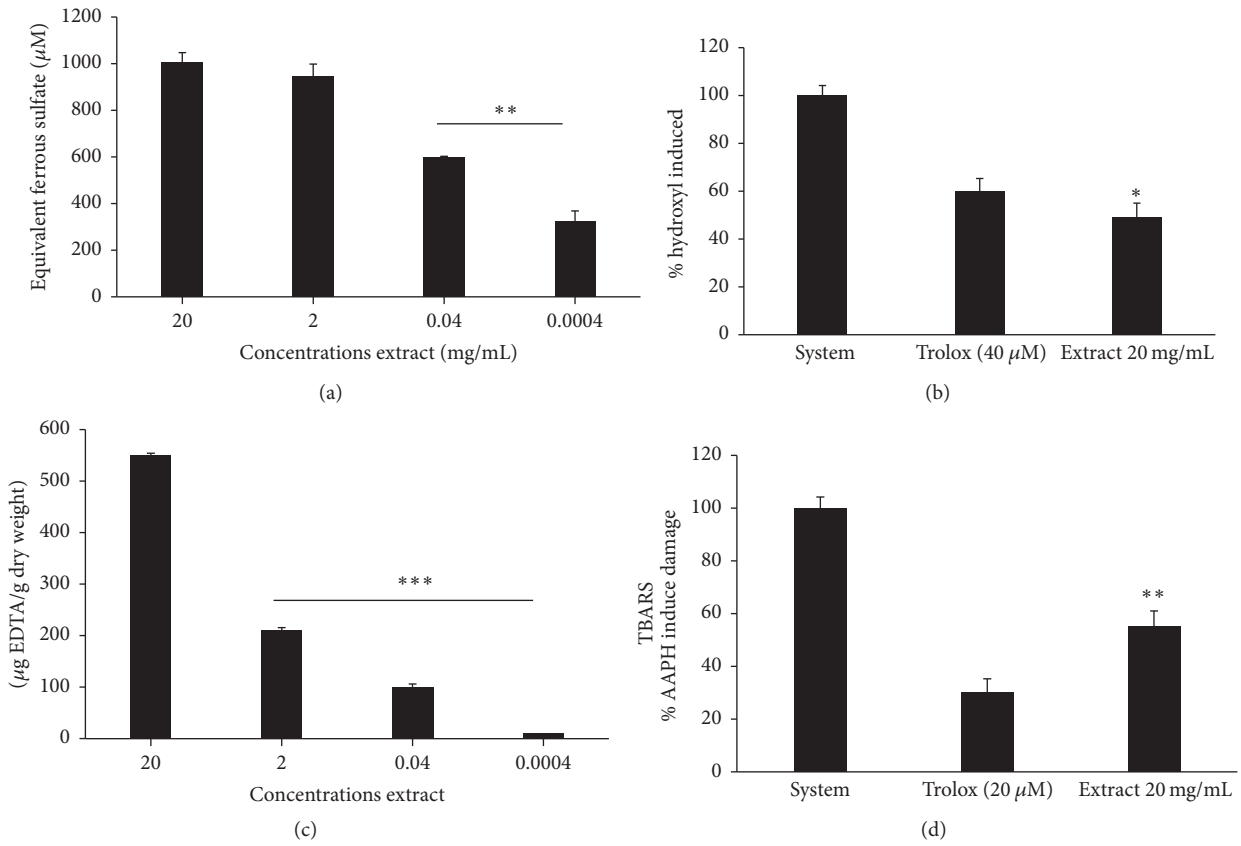


Figure 3: (a) Ferric ion reducing antioxidant power (FRAP *Assay*). Low concentrations (0.0004 and 0.004 mg/mL) of *Achyrocline satureioides* freeze-dried extract versus high concentrations (2 and 20 mg/mL) of *A. satureioides* freeze-dried extract are shown ($^{**}P < 0.005$). (b) Ferrozine assay. Results from the freeze-dried extract versus system ($^{*}P < 0.05$) are shown. Hydroxyl radical scavenging properties of (c) low *A. satureioides* extract concentrations (0.0004, 0.004, and 2 μg ethylenediaminetetraacetic acid (EDTA)/g dry weight) versus high concentrations (20 EDTA/g dry weight) ($^{***}P < 0.005$) are shown. (d) Thiobarbituric acid assay. Results from the freeze-dried extract versus system are shown ($^{**}P < 0.005$).

observed that the antioxidant activity could be attributed to synergism between the four extract compounds rather than a specific phenolic constituent. Lower AUC values represent higher antioxidant potential in the TRAP assay, whereas TAR values are directly proportional to the antioxidant properties (Figure 2(b)). Reduction equivalents of the freeze-dried extract (0.0004 mg) were equal to 325 μM ferrous ions, as determined by FRAP analyses (Figure 3(a)). In addition, the hydroxyl radical scavenging activity of the extracts was similar to that of the positive control (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid [Trolox]) (Figure 3(b)). Further, the extracts possessed significant ferrous ion-chelating properties, as determined via the FerroZine assay (Figure 3(c)). Finally, the extracts were efficient for protecting against lipid peroxidation, presenting values close to those exerted by Trolox and being slightly above those of the negative control (Figure 3(d)).

3.3. In Vivo Analysis of Intestinal Microbiota in Rats Supplemented with A. satureioides Extracts via High-Throughput Sequencing Analysis, Metabarcoding. We used high-throughput sequencing to obtain a total of 3, 217, and 215 reads, which were grouped into OTUs, with a 97% similarity cut-off level at different taxonomic levels (i.e., phylum, class, order, family, and genus). The primary gut microbiota of the rats comprised Euryarchaeota, Actinobacteria, Bacteroidetes, Cyanobacteria, Deferribacteres, Elusimicrobia, Firmicutes, Lentisphaerae Proteobacteria, Spirochaetes, Tenericutes, and TM7 (Figure 4). The abundance and diversity of the gut microbiota in rats supplemented with *A. satureioides* freeze-dried extract were not significantly different from those of the control. The proportion of bacterial phyla present in the intestinal microbiota of rats that received the freeze-dried extract was not significantly different from that of the controls, as shown in the Venn diagram displaying genotypes of the control group versus the group that received treatment (Figure 5). Bacteria from the Proteus genus were exclusively found in the group treated with *A. satureioides*.

3.4. In Vitro Testing of A. satureioides Antibacterial Properties against Intestinal Pathogens. A pilot trial was conducted using the three extracts to determine the growth inhibiting capacity for different bacteria. The freeze-dried extract prepared from the 80% ethanol (v/v) extractive

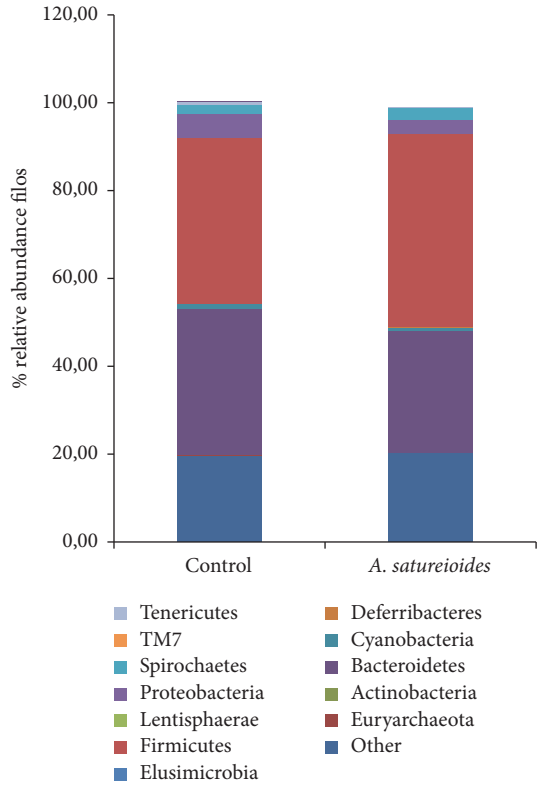


Figure 4: Profile of the intestinal microbiota of rats supplemented with saline (control) and freeze-dried extract prepared from an *Achyrocline satureioides* hydroethanol extractive solution (80% ethanol). The specific filos of the figure are Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Deferribacteres, Spirochaetes, and Other.

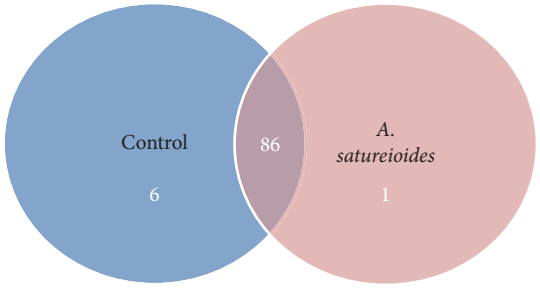


Figure 5: Venn diagram showing genotypes found in the freeze-dried extract group prepared from an *Achyrocline satureioides* hydroethanol extractive solution (80% ethanol) versus control group.

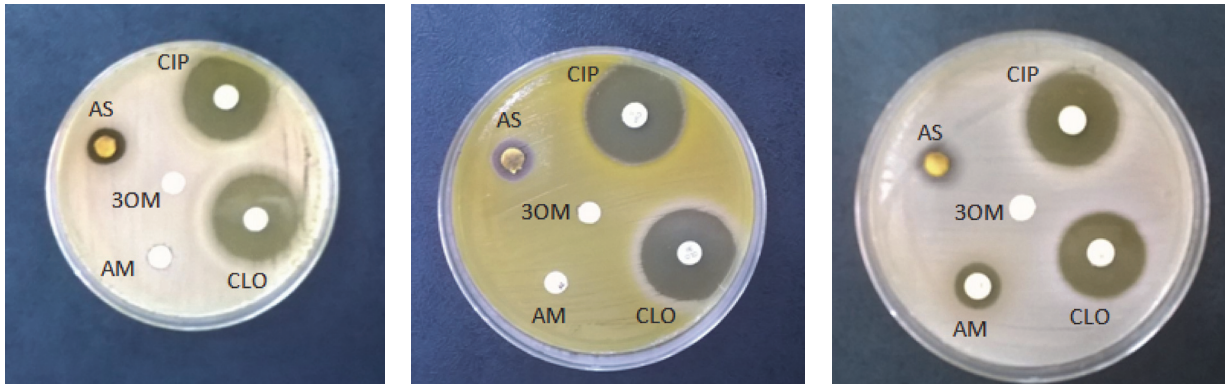
solution exhibited a broad spectrum of antimicrobial activity (100–200 mg/mL). Among all the bacteria tested, we chose the three strains showing the greatest inhibition for further testing. Specifically, the antibacterial effects of freeze-dried *A. satureioides* extracts were compared to those of chloramphenicol, amoxicillin, and ciprofloxacin (antibiotics), as well as 3-O-methylquercetin (the primary flavonoid in the *A. satureioides* extracts) in strains of *B. cereus* (ATCC 9634), *Staphylococcus aureus* (ATCC 1901), and *S. enteritidis* (ATCC 13076) (Table 4). As can be seen in (Figure 6), gram-positive strains are intrinsically resistant to amoxicillin, and evidence indicates that the antibacterial effects of *A. satureioides* extracts were higher than those exerted by amoxicillin (used as a positive control) when tested against *B. cereus* and *S. aureus*. However, additional tests are required for further confirmation.

4. Discussion

Currently, resistance to antimicrobials is a global problem of increasing importance that endangers the efficacy of antibiotics, which have transformed medicine and saved millions of lives [35]. Treatments for resistant infections cost the US health care system an estimated $21 billion to $34 billion annually [36]. In the present study, we characterized the chemical composition, antioxidant properties, and antimicrobial activities exerted by dried *A. satureioides* inflorescence extracts prepared from aqueous or hydroethanol extractive solutions. We selected the freeze-dried extract, obtained from an 80% ethanol (v/v) extractive solution, to perform both in vivo and antimicrobial activity experiments; this extract had the highest flavonoid content and significantly higher antioxidant activity than the other extracts. Furthermore, the freeze-dried extract exhibited a wide spectrum of antimicrobial activity (100 to 200 mg/mL) against intestinal pathogenic bacteria. We observed that the antibacterial effects of the freeze-dried extract were greater than those exerted by amoxicillin (an antibiotic used as a positive control) when tested against *B. cereus* and *S. aureus*. One hypothesis is that such effects are attributable to the lipophilic compounds in the extract interacting with the hydrophobic part of the bacterial membrane, thereby affecting membrane anisotropy and dipolar organization.

Screening natural products for antimicrobial activity has resulted in the discovery of higher plants as a potential source of new antibacterial agents [37], and the use of natural products derived from plants is a potential therapeutic alternative to antibiotics. Further, screening chemical compounds coming from natural products for antimicrobial activity represents an alternative strategy for the development of novel drugs. For centuries, preparations containing flavonoids as the principal physiologically active constituent have been used to treat human diseases [38]. Some researchers have reported synergy between naturally occurring flavonoids and other antibacterial agents against resistant strains of bacteria [16, 17, 38]. For instance, numerous studies have shown that chalcones are more effective against resistant strains of bacteria than flavones or flavanones and that hydroxyl groups at the 2′ position are important for the antistaphylococcal activity of these compounds. In addition, neither fluorination nor chlorination at position 4′ of the chalcones B ring is reported to significantly affect antibacterial properties [39].

Evidence indicates that among the phenol derivatives analyzed in the *A. satureioides* extracts, achyrobichalcone could be responsible for the antibacterial activity. However, other structural analogues of this same class of flavonoids would need to be synthesized and examined before the effect of halogenation upon antibacterial activity can be properly



Bacillus cereus (ATCC 9634) (a)

S. aureus (ATCC 1901) (b)

Salmonella enteritidis (ATCC 13076) (c)

Figure 6: Plate inhibition test of the *Achyrocline satureioides* (AS) freeze-dried extract (200 mg/mL), prepared from an *A. satureioides* hydroethanol extractive solution (80% ethanol), and the major flavonoid-aglycone 3-O-methylquercetin (3OM) against (a) *Bacillus cereus* (ATCC 9634), (b) *Staphylococcus aureus* (ATCC 1901), and (c) *Salmonella enteritidis* (ATCC13076). Antibiotic references used as positive controls include chloramphenicol (CLO) (30 μg), amoxicillin (AM) (30 μg), and ciprofloxacin (CIP) (5 μg).

Table 4: Antibacterial effect of freeze-dried extracts prepared from an *Achyrocline satureioides* extractive solution obtained by macerating inflorescences in 80% ethanol (v/v). Antibiotics (chloramphenicol, amoxicillin, and ciprofloxacin), as well as 3-O-methylquercetin (primary flavonoid in the extracts), were tested against strains from gram-positive and gram-negative bacteria. Antibacterial activities were classified as "no activity" (−), "modest" (+), "clear" (++), or "strong" (+++), corresponding to inhibition zones of ≤1, 2–4, 5–10, and >10 mm, respectively.

Bacteria and strains	Flavonoid extracts and antibiotics				
	Achyrocline satureioides [200 mg/mL]	3-O-methylquercetin	Amoxicillin	Chloramphenicol	Ciprofloxacin
Gram-positive					
Bacillus cereus ATCC 9634	+	−	−	+++	+++
Staphylococcus aureus ATCC 1901	+	−	−	+++	+++
Gram-negative					
Salmonella enteritidis ATCC 13 076	+	−	++	+++	+++

assessed. For instance, methoxy groups reportedly drastically decrease the antibacterial activity of flavonoids [39], and these data may explain the lack of inhibition observed in 3-*O*-methyl-quercetin, the major phenolic compound in the freeze-dried extract.

Previous reports have suggested that the antibacterial properties of flavonoids such as quercetin may play a role in inhibiting nucleic acid synthesis [40]. Further, chalcones may exert antibacterial effects by changing the permeability of cellular membranes and damaging membrane function or inhibiting energy metabolism [41].

Although there are comparatively few studies regarding the mechanisms underlying flavonoid-induced antibacterial activity, numerous studies from the literature indicate that different natural products and phytochemicals (e.g., terpenes/terpenoids) may target different components and functions of the bacterial cell [42–44].

The intestinal microbiota are considered symbiotic in nature and are involved in various processes, including the breakdown and absorption of nutrients, production of vitamins and hormones, and prevention of colonization by pathogens. Failure to achieve or maintain this equilibrium between a host and its microbiota leads to dysbiosis, which has negative consequences for both intestinal and systemic health [45]. Our results show that the abundance and diversity of the gut microbiota in rats supplemented with the freeze-dried extract prepared from a hydroethanol extractive solution were not significantly different from those of the control. This is an important result because studies have shown that several diseases that are associated with altered barrier function and increased permeability of the epithelium are linked to changes in the microbiota population or reductions in the diversity of the microbiota [46]. The antioxidant activity of the extracts at low concentrations, ferrous ion-chelating ability, reduction equivalents for ferric ions to ferrous ions (via the FerroZine assay), and antimicrobial activities may contribute to the lack of differences in abundance and diversity observed for the gut microbiota. Most of the ingested dietary polyphenols are not absorbed in the small intestine and can interact with colonic microbiota, producing diverse metabolites with a variety of physiological roles [12] that can modulate gut microbiota [12, 13].

Fluorine, found in the *A. satureioides* extracts at a concentration of 17 mg/kg, has been used worldwide to prevent

dental caries, attributable to the known in vitro inhibitory mechanisms against the production of bacterial acid [47]. Although further studies are needed, it appears that the extracts have anticarcinogenic effects depending on fluorine content.

In conclusion, we show evidence for the antimicrobial activity of *A. satureioides* freeze-dried extract obtained from a hydroethanol extractive solution against intestinal pathogens, which may be attributable, at least in part, to its antioxidant properties. Because these extracts do not produce any changes in the microbiota bacterial composition, future research is necessary regarding their potential use as coadjuvant agents for the treatment of intestinal diseases involving bacteria that present high rates of resistance to antibiotics.

Conflicts of Interest

The authors declare that no conflicts of interest exist.

Acknowledgments

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CAPÍTULO II

Supplementation with *Achyrocline satureioides* inflorescence extracts to pregnant and breastfeeding rats induces tissue-specific changes in enzymatic activity and lower neonatal survival.

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Supplementation with *Achyrocline satureioides* Inflorescence Extracts to Pregnant and Breastfeeding Rats Induces Tissue-Specific Changes in Enzymatic Activity and Lower Neonatal Survival

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Abstract: *Achyrocline satureioides* (AS, family Asteraceae) is a plant widely used in traditional medicine for stomach, digestive, and gastrointestinal disorders during pregnancy. Studies regarding the indiscriminate use of plant infusions during pregnancy are limited. Recent reports have shown that chronic flavonoid supplementation induces toxicity in vivo and raises the mortality rates of healthy subjects. Therefore, we investigated whether supplementation of pregnant and lactating Wistar rats with two AS inflorescence extracts, consisting of an aqueous (AQ) extract similar to a tea (47 mg·kg^{-1}·day) and a hydroethanolic (HA) extract (35 mg·kg^{-1}·day^{-1}) with a higher flavonoid content, could induce redox-related side effects. Total reactive antioxidant potential (TRAP), thiobarbituric reactive species (TBARS), and total reduced thiol (SH) content were evaluated. Superoxide dismutase (SOD) and catalase (CAT) activities were additionally quantified. Our data suggest that both AQ and HA of AS inflorescence extracts may induce symptoms of toxicity in concentrations of (47 mg·kg^{-1}·day) and (35 mg·kg^{-1}·day^{-1}), respectively, in mothers regarding the delivery index and further decrease of neonatal survival. Of note, significant tissue-specific changes in maternal (liver, kidney, heart, and hippocampus) and pups (liver and kidney) biochemical oxidative parameters were observed. Our findings provide evidence that may support the need to control supplementation with the AQ of AS inflorescence extracts during gestation due to potential toxicity in vivo, which might be related, at least in part, to changes in tissue-specific redox homeostasis and enzymatic activity.

Keywords: *Achyrocline satureioides*; toxicity; gestation; neonatal mortality

1. Introduction

Medicinal plants, which have contributed extensively to the development of modern medicine, have been used for centuries to treat several diseases and continue to play a significant role in

drug discovery [1]. During recent decades, interest in identifying metabolites from plants that exert beneficial effects on human health has increased. Among these metabolites, antioxidants or free radical scavengers have received particular attention for their pharmacological potential [2]. *Achyrocline satureioides* (AS, family Asteraceae), popularly known as "marcela", is one of the 25 *Achyrocline* spp. described in the Brazilian territory [2]. AS is a medium-sized aromatic annual herb, commonly found in tropical and subtropical America [3]. This plant is collected before sunrise, and the naturally dried flowers are used throughout the year to treat several gastrointestinal disorders [4].

AS is considered a promising medicinal plant, which has been used for a long time in folk medicine, and is also a designated official vegetable drug in the Brazilian Pharmacopeia [3]. In fact, previous in vivo and in vitro studies have provided evidence supporting the traditional use of AS as an anti-inflammatory, hepatoprotective, antioxidant, immunomodulatory, antimicrobial, antitumoral, and photoprotective agent [3,5–7]. Furthermore, in vitro analysis have shown that AS is cytotoxic at higher concentrations (588–653 $\mu g \cdot mL^{-1}$) [8]. Investigations of chemical composition revealed the flavonoids quercetin, 3-*O*-methylquercetin, and luteolin as the main compounds in AS inflorescence extracts [9]. These isolated compounds have demonstrated, in vitro, some pharmacological activities, such as scavenging of reactive oxygen species (ROS) [4,5,9]. This scavenger property is very important considering that ROS and other reactive species have been implicated in the pathology of over 100 human diseases [10].

The potential health benefits and general assumption that natural products are safe have increased the consumption of dietary flavonoid-based supplements by the general population, including pregnant women [11]. Pregnancy is a condition associated with large physiological changes resulting in numerous pregnancy-related symptoms, including nausea, vomiting, constipation, and heartburn [12]. Many women resort to the use of medicinal plants to alleviate these symptoms and one of the most widely consumed is *A. satureoides* infusion [3].

Herbal products are preferred over prescription medications in pregnant women because they are believed to be safer for the fetus than modern medicines are [13]. However, unlike conventional drugs, the use of herbal medications is not strictly regulated and, unfortunately, the potentially toxic effects of excessive flavonoid intake are largely ignored [14]. At higher doses, flavonoids may act as mutagens, pro-oxidants that generate free radicals, and inhibitors of key enzymes in hormone metabolism, such as kinases [15,16] and topoisomeras [17,18]. Concentrations of 50 μM of quercetin can inhibit the mitochondrial respiratory chain [19]. Unrepaired or misrepaired oxidative DNA damage can result in DNA strand breaks and mutations [20] that may lead to irreversible preneoplastic lesions. Furthermore, high intakes of these compounds may potentiate other deleterious effects due to their diverse pharmacological properties, which may alter drug and amino acid metabolism, modulate the activity of environmental genotoxicants, and alter the activity of other key metabolizing enzymes [14].

Flavonoids also act as powerful antioxidants in vitro and in vivo by scavenging diverse reactive oxygen species (ROS) or inhibiting their formation [21]. In vitro studies also showed that treatment with components of energy drinks (caffeine, taurine, and guarana) with higher doses of flavonoids exerts cytotoxic effects on human neuronal SH-SY5Y cells by decreasing ROS production [22]. Furthermore, fetuses exposed to a flavonoid-rich diet, especially during the third trimester of pregnancy, show higher ductal velocities, lower pulsatility indices, and larger right ventricles than those exposed to minimal amounts of these substances do [21]. Thus, in high doses, the adverse effects of flavonoids may outweigh their benefits and caution should be exercised in ingesting them at higher levels than would be obtained from a typical diet [14]. Any medication used during pregnancy, including medicinal plants, should always have its cost-effectiveness and benefit versus harm considered in every situation. The scarcity of data on the use of medication during pregnancy makes it even more critical. Several flavonoids have been shown to cross the hemato-placental barrier to accumulate in fetal tissue [23], and adaptations made by the fetus to cope with inappropriate nutrition may lead to morphological and physiological changes that persist into postnatal life [24].

Therefore, the aim of the present study was to evaluate the effect of supplementation with AS extract during pregnancy and lactation on redox parameters in Wistar rat dams and their offspring.

2. Experimental Section

2.1. Plant Material

AS inflorescences were purchased from Centro de Pesquisas Químicas, Biológicas e Agronômicas (CPQBA, Universidade de Campinas, Campinas, Brazil). The plant samples were collected, dried at room temperature in May 2013, and subsequently identified as cultivar CPQBA/2 registered at the Ministério Agricultura, Pecuária e Abastecimento (MAPA-Brazil) as number 22975.

2.2. Chemicals

The following chemical compounds were used: methanol (J.T., Baker, CA, USA), acetonitrile (Tedia, Aparecida de Goiânia-GO, Brazil), and phosphoric acid (Merck, Kenilworth, NJ, USA) were high-performance liquid chromatography (HPLC) grade. The standards quercetin, luteolin, and 3-O-methylquercetin were purchased from Sigma, Alfa Aesar (Karlsruhe, Germany), and Extrasynthese (Genay, France), respectively. The standard of achyrobichalcone was isolated from inflorescences of *A. satureoides* according to the method in [25].

2.3. Preparation of AS Extracts

Two AS extracts were prepared and the medicinal plant to solvent proportion used was 7.5:100 (w/v) for each extractive solution. The aqueous extract (AQ) was prepared by decoction, and the freeze-dried hydroalcoholic (HA) extract was prepared by macerating the inflorescences in ethanol 80% (v/v). The extraction time was eight days, and the extraction mixture was constantly stirred [4]. The extract obtained was filtered before use, frozen at -80 °C, and was subsequently dried in a freeze-dryer (Edwards Modulyo 4K, Irvine, CA, USA) at a temperature and pressure of -60 °C and -10^{-2} Bar, respectively.

2.4. Flavonoid Content Determination

Approximately 20 mg AS was dissolved in 20 mL 80.0% ethanol and placed in an ultrasound bath (Unique, São Paulo, Brazil) for 10 min. This solution was appropriately diluted with a methanol and 16 mM phosphoric acid solution (1:1, v/v), filtered through a 0.45-µm membrane filter (Millipore-HVHP, MA, USA), and evaluated in triplicate. Liquid chromatography (LC) analysis of the AS was carried out following a method described previously [26]. The Shimadzu LC-10A system used for the analysis was equipped with an LC-10 AD pump and a CBM-10A system controller; the system was controlled at 30 $\pm$ 1 °C, and the programmed injection volume was 20 µL. Method specificity was evaluated using a Shimadzu LC-20A system, equipped with an LC-20 AT pump, a CBM-20A system controller, an SIL-20A autosampler, and an SPD-M20A diode array detector. The limits of detection and quantitation were determined using the equations described in the International Council for Harmonization guidelines. The results are expressed as the mean of flavonoid (g) in 100 g dry extract of three analyses.

2.5. Animal Model and Experimental Design

The Federal University of Rio Grande do Sul Ethical Committee for Animal Experimentation reviewed and approved the study protocol (project number 21563, 19 July 2013). All experimental procedures were performed in accordance with the recommendations of the Brazilian Society for Science in Laboratory Animals. Male and female Wistar rats (90-day-old) were obtained from our breeding colony. The animals were housed in groups of four with free access to water and standard commercial food and were maintained on a 12-h light-dark cycle at a constant temperature

(22 ± 4 °C) and relative humidity (30–40%). These standard conditions were maintained throughout the experiments.

The pregnant rats were obtained from nulliparous females (90-day-old, weighing 200–250 g) caged with a single mature male (1 female:1 male (1F:1M)) overnight. Prior to mating, all females were checked daily for two weeks to determine their estrous cycles by direct vaginal smear examination using light microscopy and selected during the sexual receptive phase of their estrous cycles (proestrus) [27]. In the morning, the presence of a vaginal plug, viable sperm, or both in the vaginal smear was regarded as successful mating. This day was designated as gestation day 0 (GD0). The dams were allowed to litter naturally, the delivery day was defined as postnatal day 0 (PND0), and the dams were housed with their litter until euthanasia at PND42. The pregnant females were randomly divided into three groups, which were treated during pregnancy and lactation (21 days each of gestation and lactation) with of the AQ and AH extracts at concentrations equivalent to 150 mL tea from day one (47 mg·kg^{-1}·day^{-1}) and 35 mg·kg^{-1}·day^{-1}, respectively, while the control received water (Figure 1).

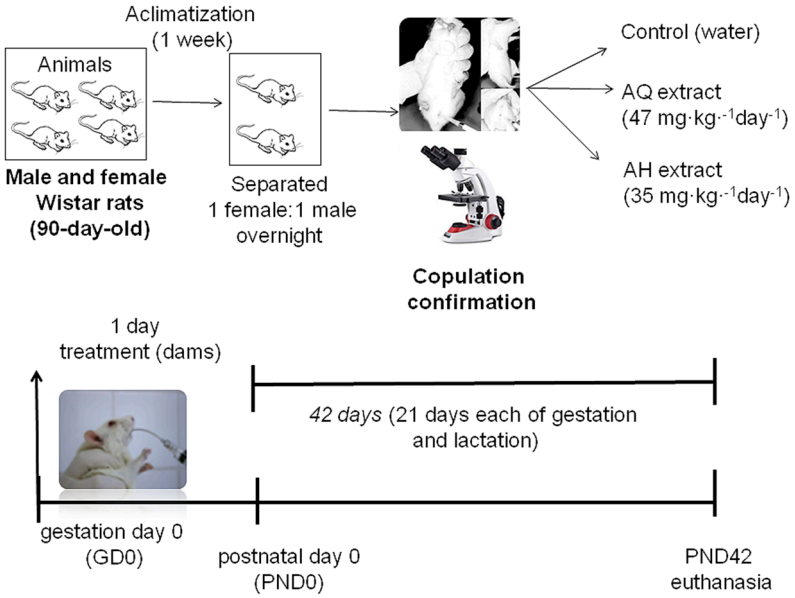

Figure 1. Workflow timeline and experimental design.

All female rats were observed for clinical symptoms of toxicity and mortality once a day throughout the study. The body weights of the dams were assessed on GD0, 7, 14, and 20 and lactation day (LD) 0, 7, 14, and 21, and the body weight gain was calculated. Rats that died during the administration period were necropsied and simply examined. On PND0, pups of both sexes were counted, weighed, and checked for the presence of external malformations and stillbirth. During the lactation period, the pups were examined daily for clinical signs and mortality. Litter sizes were determined on PND0; the litters were weighed on PND0, 7, 14, and 20; and the body weight gain was calculated on PND15 for eye-opening of the pups. The viability indices of the pups were calculated for each litter on PND0, 7, 14, and 21 and at the terminal necropsy; the females were confirmed for gestation by counting the number of uterine implantation sites.

2.6. Antioxidant Enzymes and Glutathione S-Transferase (GST)

All animals (dams and offspring rats) were euthanized by decapitation 24 h after the last extract administration, the tissues were immediately collected, and then they were frozen at −80 °C. The total protein was quantified using the Lowry assay [28] and used to normalize all the data. The catalase, superoxide dismutase (SOD), glutathione (GSH) peroxidase (GPx), and GSH S-transferase (GST) activities were quantified in the tissue homogenates of the liver, heart, kidney, cortex, hippocampus,

and cerebellum of the dam and offspring rats. SOD activity was measured by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation to adrenochrome [29]. CAT activity was evaluated by following the rate of decrease in hydrogen peroxide (H_2O_2) absorbance at 240 nm [30]. GPx activity was measured by following the decrease of NADPH at 340 nm (37 °C) [31]. GST was measured by the to produce a colored of dinitrophenyl thioether monitored at 340 nm [32]. To better understand the effect of AS extract supplementation on these free radical-detoxifying enzymes, we determined the ratio of SOD and CAT activities (SOD/CAT), two enzymes that act in sequence to reduce the superoxide anion to water.

2.7. Oxidative Damage Markers

All protein oxidative damage and effects on lipids in dams and offspring rats were analyzed in tissue samples of the liver, kidney, heart, and cortex, hippocampus, and cerebellum. The oxidative status of the thiol groups was assessed by quantification of the total reduced sulfhydryl (SH) groups. Samples were reacted with 5,5′-dithionitrobis 2-nitrobenzoic acid (10 mM) during a 60-min incubation at room temperature, and the absorbance of the solution was read using a spectrophotometer at 412 nm [33]. The carbonyl groups were determined as an index of the oxidative protein damage, based on the reaction with 2,4-dinitrophenylhydrazine (DNPH), as previously described [34]. Lipoperoxidation was determined by the quantification of TBARS generated from the reaction of the thiobarbituric acid with lipoperoxides in an acid-heating medium. After precipitation with 10% trichloroacetic acid (TCA), the supernatant was mixed with 0.67% TBA and heated in a boiling water bath for 20 min. TBARS was determined by measuring the absorbance using a spectrophotometer at 532 nm [35].

2.8. Statistical Analysis

The statistical analysis was performed using Statistical Package for the Social Sciences software (IBM), and the results were expressed as the means ± standard error of the mean (SEM). The data were evaluated using univariate analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Differences were considered significant when $p < 0.05$ for all the data.

3. Results

3.1. AS Extract Composition

The components of the AS HA and AQ extracts were identified using LC separation. The flavonoids quercetin, luteolin, 3-o-methylquercetin, and achyrobichalcone were the main components of both extracts, and their content in the HA (12.4 g 100 g^{-1} extract) and AQ (5.6 g 100 g^{-1} extract) are shown in Table 1. The HA contained 22.44 and 14.5% of quercetin and luteolin, respectively, which collectively corresponded to 36.9% of the total flavonoids present in the extract. The AQ contained 12.3% and 6.5% quercetin and luteolin, respectively, which constituted approximately half of the flavonoid content of the HA extract, demonstrating that ethanol had a higher extraction capacity than water did for the flavonoids in the inflorescences.

Table 1. Total flavonoids content of *Achyroclines satureoides* (AS) extracts (μg·mg^{-1} DW).

Samples	Quercetin (g/100 g Extract)	*3-O-methylquercetin* (g/100 g Extract)	Luteolin (g/100 g Extract)	Achyrobichalcone (g/100 g Extract)	Total Flavonoid (g/100 g Extract)
Freeze-dried hydroalcoholic	2.77 ± 0.6 ^{b}	6.23 ± 0.5 ^{a}	1.80 ± 0.01 ^{a}	1.60 ± 0.8 ^{a}	12.4 ^{a}
Aqueous extract	1.68 ± 0.3 ^{b}	2.70 ± 0.1 ^{a}	0.50 ± 0.02 ^{b}	0.60 ± 0.2 ^{b}	5.6 ^{b}

Data are means ± standard deviation SD. Different letters in the same column indicate significant differences in the total flavonoids content between of *Achyroclines satureoides* extracts (* $p < 0.05$). $n = 3$.

3.2. Reproductive, Maternal, and Litter Data in Pups

The litter sizes of the AQ and HA groups were significantly different (both $p < 0.01$) compared to that of the control group (Table 2). The change in the delivery index (relation between the number of pups delivered and the number of pups implanted multiplied by 100, $p < 0.01$ and 0.001, respectively) suggest a possible toxic effect of the AQ and HA treatment compared to the control treatment. The delivery index of the AQ group was also significantly different from that of the HA group ($p < 0.01$). During pregnancy, no differences in weight gain were observed between the groups and no malformations were observed in the pups (Table 2). The treatments did not modify the sex ratio of the litters between the groups. During lactation, the pups exhibited no intoxication symptoms related to the treatments and no treatment-induced reduction in body weights. However, we observed treatment-related differences ($p < 0.05$) in the time of eye-opening of the pups. The AQ- and HA-treated pups opened their eyes three to four days before the control group pups did (Table 2). The number of pups in the AQ group was lower ($p < 0.05$, 11 animals) than that in the control and the HA groups (24 and 20 animals, respectively), demonstrating that the AQ extract may contain teratogenic compounds.

Table 2. Reproductive data.

Achyrocline Satureioides (mg·kg^{-1}·day^{-1})			
Reproductive Parameters	**Control (Water)**	**Aqueous (AQ) Extract (47)**	**Hydroalcoholic (HA) Extract (35)**
Gestation weight gain (%)	15.8 ± 5.1	13.8 ± 7.1	14.8 ± 4.4
Lactation weight gain (%)	13.8 ± 6.1	11.5 ± 2.1	11.8 ± 2.4
Gestation length (days)	21 ± 1	23 ± 1	22 ± 1
No. of implantations	10 ± 4.1	8.5 ± 5.1	11.8 ± 2.4
Delivery index (%)	98 ± 5.1	73 ± 6.1	80 ± 5.5
Days before eye opening	13 ± 1	10 ± 2	9 ± 1*
No. of pups	24 ± 1	11 ± 6.1 *	20 ± 2.1 *,#
Viability index (%)			
Day 0	98.3 ± 1.6	70.3 ± 1.3 *	90.4 ± 3.6
Day 7	99.3 ± 2.1	88.2 ± 2.3 *	98.4 ± 2.9
Day 14	88.3 ± 3.1	85.3 ± 1.3	87.4 ± 3.3
Day 21	98.5 ± 1.6	97.3 ± 5.3	90.4 ± 5.6
Day 42	98.3 ± 5.6	96.3 ± 3.5	98.4 ± 3.7
Pup weight (g)			
Day 5	9.8 ± 1.1	8.2 ± 1.3	8.4 ± 1.9
Day 14	18.3 ± 2.2	15.3 ± 1.0	17.4 ± 2.4
Day 21	30.5 ± 5.2	29.3 ± 2.3	30.4 ± 2.6
Day 42	48.3 ± 3.1	44.3 ± 2.3	42.4 ± 3.7

* $p < 0.05$ and # $p < 0.05$ compared to control and AQ treatment, respectively; Gestation weight gain (%) = ((weight on PND0 − weight on GD0)/weight on GD0)) × 100. Lactation weight gain (%) = ((weight on PND21 − weight on PND0)/weight on PND0)) × 100; Delivery index (%) = (no. of pups delivered/No. of implantations) × 100; Viability index on postnatal day 0 (%) = (no. of live pups delivered/total no. of pups delivered) × 100; Viability index on postnatal day 7 (%) = (no. of live pups on postnatal day 7/no. of live pups delivered) × 100; Viability index on postnatal day 14 (%) = (no. of live pups on postnatal day 14/no. of live pups on postnatal day 7 after delivery) × 100; Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live pups on postnatal day 14 after delivery) × 100.

3.3. Maternal Oxidative Parameters

The biochemical data showed increased SOD, CAT, and GST activities in the liver and kidneys of dams treated with AQ and HA compared with the levels in the control group (Table 3). In the heart, we observed only a significant increase in GST activity in the treated groups compared with the control group. Oxidative lipid and protein damage was determined by assaying TBARS and protein carbonylation levels, which showed no significant difference in any of the analyzed tissues. The results of tissue sample analysis of the cerebellum, frontal cortex, and hippocampus are presented in Table 3.

No significant differences occurred in the enzyme activities, and no oxidative damage was observed in central nervous system (CNS) structures.

Table 3. Biochemical data of tissues from dams.

Achyrocline Satureioides (mg·kg^{-1}·day^{-1})			
Biochemical Parameters	**Control (Water)**	**AQ (47)**	**HA (35)**
Liver			
TBARS level (nmol MDA/mg protein)	7.21 ± 1.4	7.54 ± 1.1	6.11 ± 1.1
Carbonyl level (nmol carbonyl/mg protein)	1.52 ± 0.5	1.66 ± 0.5	1.71 ± 0.3
Total thiol content (mmol SH/mg protein)	52.6 ± 5.2	53.61 ± 2.8	55.51 ± 5.2
TRAP (under curve area)	354.3 ± 18.5	357.76 ± 61.1	338.7 ± 27.7
GST activity (U GST/mg protein)	0.217 ± 0.5	0.261 ± 0.3 *	0.278 ± 0.2 *
CAT activity (U CAT/mg protein)	42.6 ± 1.8	47.62 ± 2.5 *	49.79 ± 1.7 *
SOD activity (U SOD/mg protein)	32.76 ± 9.4	38.32 ± 1.5 *	39.09 ± 2.5 *
SOD/CAT ratio (arbitrary units)	0.76 ± 0.3	0.80 ± 0.5 *	0.78 ± 0.9 *
Kidney			
TBARS level (nmol MDA/mg protein)	5.34 ± 1.8	3.28 ± 1.6	4.3846 ± 1.5
Carbonyl level (nmol carbonyl/mg protein)	1.77 ± 0.2	1.73 ± 0.5	1.38 ± 0.3
Total thiol content (mmol SH/mg protein)	44.96 ± 5.9	45.53 ± 6.2	47.34 ± 5.3
TRAP (under curve area)	176.3 ± 27.8	224.3 ± 22.7	178.8 ± 21.79
CAT activity (U CAT/mg protein)	31.87 ± 4.1	37.48 ± 3.1 *	36.43 ± 3.4 *
SOD activity (U SOD/mg protein)	18.16 ± 2.5	19.81 ± 6.5 *	20.52 ± 1.2 *
GST activity (U GST/mg protein)	0.217 ± 0.5	0.251 ± 0.3 *	0.248 ± 0.2 *
SOD/CAT ratio (arbitrary units)	0.56 ± 0.6	0.52 ± 0.7 *	0.56 ± 0.7
Heart			
TBARS level (nmol MDA/mg protein)	2.0379 ± 0.2	2.2703 ± 0.5	2.1881 ± 0.6
Carbonyl level (nmol carbonyl/mg protein)	2.0249 ± 0.3	1.9941 ± 0.2	1.6428 ± 0.4
Total thiol content (mmol SH/mg protein)	35.01 ± 5.8	36.91 ± 8.5	43.95 ± 7.4
TRAP (under curve area)	488.45 ± 48.0	401.53 ± 22.55	407.7 ± 60.39
CAT activity (U CAT/mg protein)	6.42 ± 0.5	6.43 ± 1.6	6.3 ± 2.0
SOD activity (U SOD/mg protein)	5.25 ± 1.2	6.75 ± 2.7	6.5 ± 1.7
GST activity (U GST/mg protein)	0.217 ± 0.8	0.251 ± 0.2 *	0.248 ± 0.3 *
SOD/CAT ratio (arbitrary units)	0.81 ± 0.8	1.04 ± 0.8 *	1.03 ± 0.8 *
Cerebellum			
TBARS level (nmol MDA/mg protein)	6.81 ± 5.4	6.54 ± 1.6	6.01 ± 1.1
Carbonyl level (nmol carbonyl/mg protein)	1.2 ± 0.3	1.6 ± 0.9	1.0 ± 0.7
Total thiol content (mmol SH/mg protein)	50.6 ± 2.2	52.61 ± 8.8	49.51 ± 7.2
CAT activity (U CAT/mg protein)	4.66 ± 1.8	4.62 ± 3.5	6.79 ± 1.7
SOD activity (U SOD/mg protein)	6.76 ± 1.4	10.32 ± 4 *	11.09 ± 2.5
SOD/CAT ratio (arbitrary units)	1.45 ± 1.3	2.23 ± 0.8 *	1.63 ± 0.9
Hippocampus			
TBARS level (nmol MDA/mg protein)	5.38 ± 1.8	3.08 ± 1.6	4.46 ± 1.5
Carbonyl level (nmol carbonyl/mg protein)	1.77 ± 0.2	1.76 ± 0.5	1.28 ± 0.3
Total thiol content (mmol SH/mg protein)	44.96 ± 5.9	45.53 ± 6.2	47.34 ± 5.3
CAT activity (U CAT/mg protein)	11.87 ± 4.1	12.48 ± 2.1	10.43 ± 3.4
SOD activity (U SOD/mg protein)	8.16 ± 2.5	5.81 ± 1.5 *	5.52 ± 3.2 *
SOD/CAT ratio (arbitrary units)	0.68 ± 0.1	0.46 ± 0.1 *	0.52 ± 0.2
Cortex			
TBARS level (nmol MDA/mg protein)	2.0 ± 0.2	2.03 ± 0.5	2.81 ± 0.6
Carbonyl level (nmol carbonyl/mg protein)	2.49 ± 0.3	1.99 ± 0.2	1.68 ± 0.4
Total thiol content (mmol SH/mg protein)	35.01 ± 5.8	36.91 ± 8.5	43.95 ± 7.4
CAT activity (U CAT/mg protein)	6.42 ± 0.5	6.43 ± 1.6	6.51 ± 0.2
SOD activity (U SOD/mg protein)	5.25 ± 1.2	6.75 ± 2.7	6.31 ± 0.8
SOD/CAT ratio (arbitrary units)	0.81 ± 1.8	1.04 ± 2.8	0.96 ± 1

* Significantly different from control; $n = 6$. TBARS, thiobarbituric reactive species; TRAP, total reactive antioxidant potential; GST, glutathione *S*-transferase; CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde.

3.4. Pups' Oxidative Parameters

The biochemical data of the dams showed no increases in the levels of oxidative damage markers in the studied tissues (the liver, heart, and kidney). The liver showed significant alterations in the GST, CAT, and SOD activity in the AQ and HA extract-treated groups compared to that in the control group. SOD enzymatic activity in the AQ and HA extract-treated groups were significantly higher (25.81 and 25.52 units SOD·mg^{-1} protein, respectively, $p < 0.01$) than that in the control group. In the heart tissue, no significant difference in enzymatic activities was observed (Table 4). We also analyzed the oxidative parameters in the cerebellum, frontal cortex, and hippocampus tissues, and no significant differences were observed in the enzymes' activities, and no oxidative damage was observed in these CNS structures.

Table 4. Biochemical data of the tissues of the pups.

***Achyrocline satureioides* (AS, mg·kg^{-1}·day^{-1})**			
No. of Pups Examined	**Control (Water) 24**	**AQ (47) 11**	**HA (35) 20**
	Liver		
TBARS level (nmol MDA/mg protein)	6.81 ± 1.4	6.54 ± 1.6	6.01 ± 1.1
Carbonyl level (nmol carbonyl/mg protein)	1.62 ± 0.3	1.66 ± 0.8	1.80 ± 0.7
Total thiol content (mmol SH/mg protein)	55.6 ± 2.2	58.61 ± 8.8	53.51 ± 6.2
TRAP (under curve area)	324.3 ± 55.25	317.76 ± 29.9	318.37 ± 7.1
CAT activity (U CAT/mg protein)	42.66 ± 1.8	54.62 ± 3.5 *	56.79 ± 1.7 *
SOD activity (U SOD/mg protein)	36.76 ± 1.4	40.32 ± 1.5 *	46.09 ± 2.5 *
SOD/CAT ratio (arbitrary units)			
GST activity (U GST/mg protein)	0.217 ± 0.5	0.251 ± 0.3 *	0.248 ± 0.2 *
	Kidney		
TBARS level (nmol MDA/mg protein)	5.38 ± 1.8	3.08 ± 1.6	4.46 ± 1.5
Carbonyl level (nmol carbonyl/mg protein)	1.77 ± 0.2	1.76 ± 0.5	1.28 ± 0.3
Total thiol content (mmol SH/mg protein)	44.96 ± 5.9	45.53 ± 6.2	47.34 ± 5.3
TRAP (under curve area)	176.6 ± 27.82	124.33 ± 32.76	128.78 ± 21.79
CAT activity (U CAT/mg protein)	31.87 ± 4.1	37.48 ± 3.1	36.43 ± 3.4
SOD activity (U SOD/mg protein)	18.16 ± 2.5	25.81 ± 1.5 *	25.52 ± 3.2 *
SOD/CAT ratio (arbitrary units)	0.56 ± 0.5	0.68 ± 0.2	0.70 ± 1.0
GST activity (U GST/mg protein)	0.05 ± 0.7	0.08 ± 0.5	0.09 ± 0.6
	Heart		
TBARS level (nmol MDA/mg protein)	2.0 ± 0.2	2.03 ± 0.5	2.81 ± 0.6
Carbonyl level (nmol carbonyl/mg protein)	2.49 ± 0.3	1.99 ± 0.2	1.68 ± 0.4
Total thiol content (mmol SH/mg protein)	35.01 ± 5.8	36.91 ± 8.5	43.95 ± 7.4
TRAP (under curve area)	488.5 ± 48.57	401.5 ± 22.57	407.89 ± 60.4
CAT activity (U CAT/mg protein)	6.42 ± 0.5	6.43 ± 1.6	6.5 ± 1.3
SOD activity (U SOD/mg protein)	5.25 ± 1.2	6.75 ± 2.7	6.3 ± 2.3
SOD/CAT ratio (arbitrary units)	0.81 ± 1.7	1.04 ± 1.2	0.96 ± 2.9
GST activity (U GST/mg protein)	0.06 ± 0.7	0.05 ± 0.7	0.06 ± 0.5
	Cerebellum		
TBARS level (nmol MDA/mg protein)	6.81 ± 1.4	6.54 ± 1.6	6.01 ± 1.1
Carbonyl (nmol carbonyl/mg protein)	1.62 ± 0.3	1.66 ± 0.8	1.80 ± 0.7
Total thiol (mmol SH/mg protein)	55.6 ± 2.2	58.61 ± 8.8	53.51 ± 6.2
CAT activity (U CAT/mg protein)	2.66 ± 1.8	4.2 ± 3.5	6.79 ± 1.7
SOD activity (U SOD/mg protein)	36.76 ± 1.4	40.2 ± 1.5	46.09 ± 2.5
SOD/CAT ratio (arbitrary units)	0.86 ± 1.8	0.74 ± 1.2	0.81 ± 1.6

Table 4. *Cont.*

Achyrocline satureioides (AS, mg·kg^{-1}·day^{-1})			
No. of Pups Examined	**Control (water) 24**	**AQ (47) 11**	**HA (35) 20**
Hippocampus			
TBARS level (nmol MDA/mg protein)	3.38 ± 1.8	3.08 ± 1.6	4.46 ± 1.5
Carbonyl (nmol carbonyl/mg protein)	1.77 ± 0.2	1.76 ± 0.5	1.28 ± 0.3
Total thiol (mmol SH/mg protein)	44.96 ± 5.9	45.53 ± 6.2	47.34 ± 5.3
CAT activity (U CAT/mg protein)	4.87 ± 4.1	7.48 ± 5.1	6.43 ± 3.4
SOD activity (U SOD/mg protein)	5.16 ± 2.5	5.81 ± 5.5	5.52 ± 3.2
SOD/CAT ratio (arbitrary units)	1.05 ± 1.5	0.77 ± 2.3	0.85 ± 0.5
Cortex			
TBARS level (nmol MDA/mg protein)	2.0 ± 0.2	2.03 ± 0.5	2.81 ± 0.6
Carbonyl (nmol carbonyl/mg protein)	2.49 ± 0.3	1.99 ± 0.2	1.68 ± 0.4
Total thiol (mmol SH/mg protein)	15.01 ± 5.8	16.91 ± 8.5	13.95 ± 7.4
CAT activity (U CAT/mg protein)	3.2 ± 0.5	3.43 ± 1.6	3.5 ± 1
SOD activity (U SOD/mg protein)	3.25 ± 1.2	3.75 ± 2.7	3.31 ± 2
SOD/CAT ratio (arbitrary units)	1.01± 3.5	1.09 ± 2.2	0.94 ±3.6

* Significantly different from the control. TBARS, thiobarbituric reactive species; TRAP, total reactive antioxidant potential; GST, glutathione *S*-transferase; CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde.

4. Discussion

In this study, we supplemented pregnant and lactating rats with AS extracts in doses equivalent to the consumption of 150 mL of tea per day according to the extraction yield (AQ and HA, 47 and 35 mg·kg^{-1}·day^{-1}, respectively). The doses used were equivalent to the mean doses ingested in tea beverages by pregnant women [13]. Equivalent doses may be obtained by applying uncertainty factors of 10-fold each for species and interspecies differences [36]. At these doses and conditions, reproductive and developmental toxicity endpoints were observed with treatment-related clinical signs of maternal and offspring toxicity in the delivery index. Furthermore, treatment-related effects, including a slight delay in the eye-opening completion rate, were found in both AS extract-treated groups. Flavonoids are extensively and rapidly metabolized by the liver by methylation, sulfonation, glucuronidation, or a combination of these processes, which likely modulates the cellular bioavailability of these compounds [37]

Our results showed that AS extracts may induce symptoms of toxicity in dams in the delivery index; however, the exact mechanism has not yet been determined. Our results seems to be different to those of another study that reported no negative effects on fertility, fetal weight, or prenatal development when CD-1 mice were gavaged with 400 or 800 mg·kg^{-1}·day^{-1} green tea extract (GTE) alone (containing epigallo-catechin-gallate (EGCG) flavonoid) from GD6 to 13 [38]. The number of pups in the AQ group was lower than that in the control and the HA groups, demonstrating that the AQ may contain compounds able to modify the oxidative biochemical parameters in dam and pups, and to have a negative impact on the delivery index and neonatal survival. Levels of enzymatic activity are considered an important factor that protects organs against the deleterious effect of potential toxicants [39], and our results showed that GST, CAT, and SOD activity in maternal livers were significantly increased. In addition, GST also detoxifies endogenous electrophiles, which are usually the consequence of free-radical damage and may be an important participant in the mechanism mediating the repair of free-radical damage [40]. Alterations in GST activity likely altered the redox state and the antioxidant defenses of the tissue [41]. Finally, GST is also an endogenous switch for the control of signaling cascade pathways, and alterations in its activity may alter the regulatory balance of numerous kinase pathways [42].

SOD is a key antioxidant enzyme implicated in the regulation of ROS-mediated tissue damage. SOD plays a key role in detoxifying superoxide anions into H_2O_2 and oxygen, and CAT may degrade

H_2O_2 into water and oxygen [43]. A poor defense system allows the formation of superoxide anions and H_2O_2. The superoxide radical can react with NOx, generating the highly reactive peroxynitrite anion, which can induce lipid oxidation and inactivate several key SH-bearing enzymes, depleting the SH protein content [44].

GST is a detoxification enzyme [42], which acts to detoxify endogenous compounds, such as peroxidized lipids, and thereby enables the breakdown of xenobiotics. GST may also bind toxins and function as a transport protein, which explains the earlier term for GST, ligandin. These results corroborate those of another study that reported the hepatoprotective activity of achyrocline extract [45]. The major natural antioxidative components in AS extracts are flavonoids [3] and also phenolic compounds. The antioxidant activities of AS extracts have been reported by other studies and flavonoids, phenolic compounds, and achyrobichalcone were found to be the most powerful radical scavenging compounds in the extracts.

The cerebellum, frontal cortex, and hippocampus were chosen based on their critical role in the maintenance of basic brain activities. The cerebellum is thought to have a primary role in motor control and coordination, and this complex structure, which contains the majority of the brain's neurons, has a considerable role in cognition [46]. The frontal cortex has a crucial role in brain homeostasis during adaptive behavior through its involvement in decision-making [47]. The acquisition of new memories of events and places depends on the optimal functioning of the hippocampus [48]. In humans, the development of CNS connections occurs mainly during the intrauterine phase; however, in rats, it occurs mainly during the period from the last third of the gestation until approximately the first two weeks of the suckling phase [49].

Maternal nutrition has a significant effect on developmental processes during pregnancy and lactation. Many women resort to the use of AS infusions to alleviate symptoms of pregnancy [13] and the potential health benefits are attributed to flavonoids and phenolic compounds [45,50]. The analysis of the flavonoid composition of the two extracts verified that content of the HA extract was two times that of the AQ extract based on the affinity of the compounds to the polar solvent used in the extraction [8]. This finding corroborates the result of two studies on AS freeze-dried extracts [25,26]. The rationale for focusing on the flavonoids in the extracts is the current evidence of the metabolism and transfer of flavonoids to the fetus during pregnancy. The flavanols, or more accurately their metabolites, can reach the fetal tissues, where they could potentially interact with molecules in the developmental processes [51]. Studies have reported that flavonoids, such as quercetin, induce DNA double-strand breaks and prenatal exposure to these substances slightly increases the incidence of malignancies in DNA repair-deficient mice [51]. This phenomenon is implicated in the development of cancer [21,51,52], and may pose a serious threat to the safe reproductive development. However, the evidence of the effects of AS supplementation in women during pregnancy and lactation are still limited.

Therefore, no consensus has been reached on the safety of AS supplementation during gestation in humans. Furthermore, the use of herbal medications is not strictly regulated, unlike other conventional drugs and, unfortunately, the potentially toxic effects of excessive intake are still largely ignored.

5. Conclusions

We conclude AQ in concentrations (47 $mg \cdot kg^{-1} \cdot day^{-1}$) of AS (35 $mg \cdot kg^{-1} \cdot day^{-1}$) inflorescence extracts during gestation could lead to in vivo toxicity, reflected in a decreased delivery index and neonatal survival. These effects could be related, at least in part, to variations in tissue-specific redox homeostasis and enzymatic activity, especially as the liver and kidney were affected in both dams and pups (Figure 2). It was not possible to determine whether these events could be predominantly attributed to pre- or early post-natal treatments with AQ of AS inflorescence extracts in pregnant or breastfeeding rats, respectively. This represents the greatest limitation of our study, which deserves further investigation.

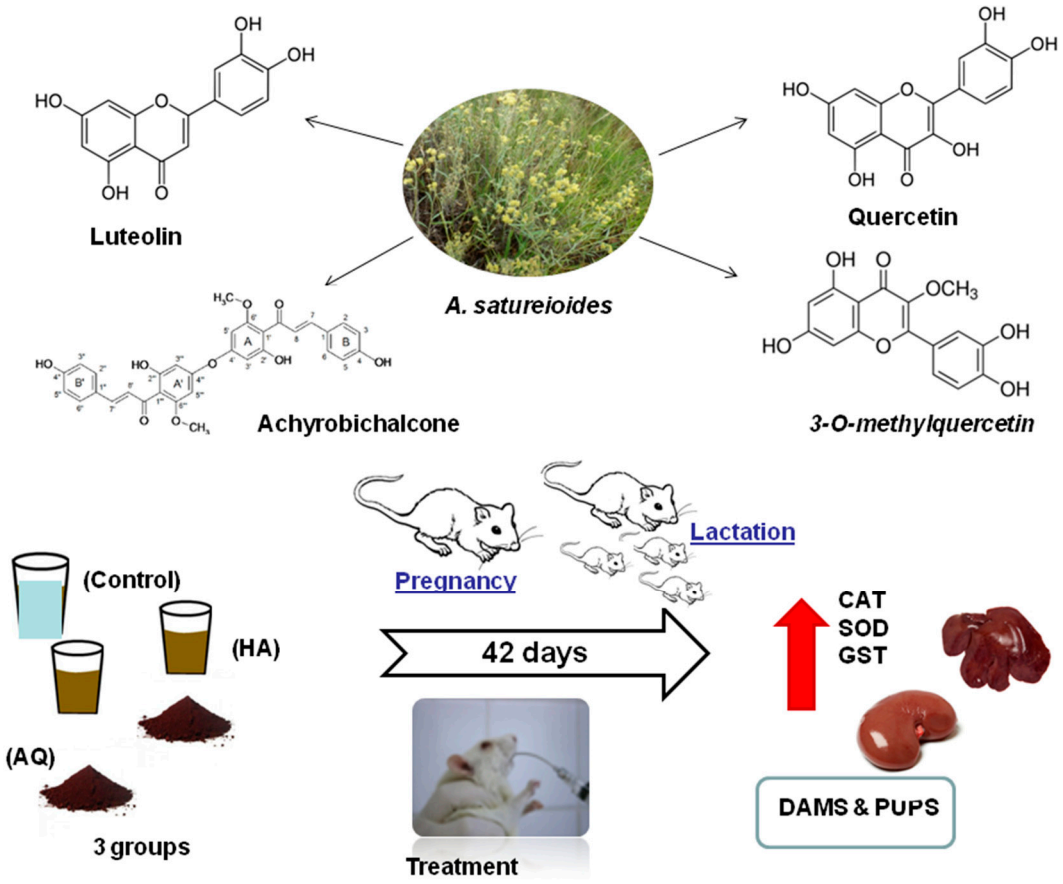

Figure 2. Pregnant and breastfeeding rats supplemented with *Achyrocline satureioides* inflorescence extracts showed tissue-specific changes in enzymatic activity and lower neonatal survival.

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CAPÍTULO III

Oral Administration with *Achyrocline Satureioides* Hydroalcoholic Extract Reduces the Levels of Blood Glucose by Inhibiting Digestive Glycosidases in Maltose/ Starch-supplemented and Streptozotocin-induced Diabetes Mellitus *in Vivo* Models

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Abstract

Background: The aim of the present study was to investigate the effects of an *A. satureioides* hydroalcoholic extract (AS) on blood glucose.

Methods: Oral administration of AS produced promising anti-hyperglycemic activity in rats; this was comparable to that of the positive control, acarbose. This effect was observed in rats ingesting high levels of maltose and starch, and also in a rat streptozotocin model of DM. In order to investigate the mechanism of action of AS, we determined its potential inhibitory effects on maltase and pancreatic α-amylase.

Results: The level of AS associated with 50% enzyme inhibition (IC_{50}) was 185.21 µg/mL for maltase and 265.72 µg/mL for pancreatic α-amylase. Further investigation of AS constituents showed that the flavonoid, achyrobichalcone, produced the greatest enzyme inhibition (IC_{50} values of 4.74 µM for α-amylase and 6.71 µM for maltase), which was consistent with our molecular modelling results with AS main components.

Conclusion: These findings suggest a potential application of AS as a nutritherapeutic agent in DM

Keywords: *A. satureioides*; glycosidase inhibitor; diabetes mellitus.

Introduction

Diabetes mellitus (DM) is a chronic disease that is characterised by hyperglycaemia and associated systemic complications. In most cases, these symptoms emerge as a result of reduced insulin production by the pancreas and/or a reduced effect of insulin on its target organs due to insulin resistance. Approximately 415 million adults aged 20-79 worldwide live with DM and it is estimated that a further 193 million have undiagnosed DM, while 318 million have impaired glucose tolerance and are at risk for DM[1]. Future projections indicate that by 2035, over 1 billion people will be living with DM or will have a high risk of developing this condition[2]. In 2014, DM was the direct cause of 5 million deaths, equivalent to the number attributed to the human immunodeficiency virus[3], and was estimated to cost between 673 billion and 1,197 billion USD in healthcare spending[1].

DM is much more heterogeneous than the present subdivision into types 1 and 2 assumes; these types, and gestational diabetes, probably represent extremes on a continuum of disorders. Type 1 DM is characterised by a deficiency in insulin production and requires daily administration of insulin; the associated symptoms include polyuria, polydipsia, constant hunger, weight loss, vision changes and fatigue[4]. The vast majority of these patients develop serious chronic macro- and microvascular complications that are organ specific; these affect the eyes, kidneys, nerves, heart, liver and blood vessels[5]. Currently, more than half a million children aged 14 and under worldwide live with type 1 DM[1]. Type 2 DM is the most common form of this disorder and although it usually occurs in adults, it is increasingly prevalent in children and adolescents[1]. In this condition, pancreatic insulin secretion may be normal, but cellular glucose uptake is limited by a reduced number of insulin receptors and this leads to elevated blood glucose levels[6]. Many people with type 2 DM remain unaware of their condition for a long time because the symptoms are usually less marked than those of type 1 DM and may take years to be recognised[1]. These symptoms include polyuria, polydipsia, weight loss and blurred vision[1].

Glycosidases catalyse the hydrolysis of glycosidic linkages between disaccharides, oligosaccharides, polysaccharides and glycoconjugates. These enzymes play an essential role in the digestion of carbohydrates[7]. To generate glucose from starchy foods, salivary and pancreatic α-amylase and four intestinal mucosal α-glucosidases are employed. α-Amylase belongs to the glycoside hydrolase family and catalyses the hydrolysis of α-1,4-glycosidic bonds in starch[7].

In humans, the digestion of cooked starch is mainly carried out by pancreatic α-amylase, also known as α-glycosidase, which is produced in the pancreas and excreted into the lumen of

the gastrointestinal tract[8]. α-Glucosidase is a general term for all glycosidase enzymes that catalyse the hydrolysis of glycosidic linkages of the α type. The α-glucoside hydrolases are located in the brush-border surface membranes of intestinal cells and these enzymes catalyse the final step in carbohydrate digestion[9]. Their catalysis of the α-glucopyranosidic bond hydrolysis releases an α-D-glucose from the non-reducing end of the sugar.

Oral anti-hyperglycaemic drugs such as acarbose (AC) and miglitol control blood glucose levels after food intake and have been successfully used in clinics to treat DM[10], either alone or in combination with other oral antidiabetic drugs and/or insulin[11]. These compounds inhibit gastrointestinal α-glucosidases at the brush-border membranes of intestinal cells and in the gut lumen and thus delay the release and absorption of monosaccharides[7]. However, the clinical use of oral anti-hyperglycaemic drugs has been associated with some gastrointestinal side effects[12]. Moreover, the production of AC involves laborious, costly and time-consuming fermentation of strains of the bacterial genus, *Actinoplanes*. For these reasons, many scientists have been searching for new effective and safe α-glucosidase inhibitors to use in patients with DM[13,14].

Studies of alternatives to synthetic chemicals have identified some natural native plant extracts that are very promising as potential therapeutic agents for the treatment and/or prevention of type 2 DM[15]. These extracts contain a large range of bioactive compounds, a number of which may inhibit the activity of digestive glycosidases[16,17,18,19] without causing adverse reactions. Based on the relevant scientific literature worldwide, 411 natural products were identified that were isolated from medicinal plants and showed α-glucosidase inhibitory activity[10]. Structurally, these inhibitors incorporate terpene, alkaloid, quinine, flavonoid, phenol, phenylpropanoid and steride frameworks that are rich in organic acid, ester, alcohol and allyl functional groups. A majority of the reported compounds contained flavonoid, terpene and phenylpropanoid ring structures[10].

Achyrocline satureioides (Asteraceae) is a plant that is native to Latin American countries. Aqueous extracts of *A. satureioides* are frequently used in folk medicine as a treatment for several human ailments, many of which are related to gastrointestinal dysfunction[20], and as hypoglycaemic agents[21]. Previous phytochemical studies of *A. satureioides* have demonstrated that it exhibits anti-diabetic activity and attribute this activity to its flavonoid constituents, such as quercetin, and its dibenzofurans, such as achyrofuran[19,21-23]. Current studies of anti-diabetic activity are also recognising the importance of chalcones[24], which are intermediates in the biosynthesis of flavonoids and isoflavonoids. Recently, a specific

chalcone was isolated from hydroalcoholic extracts of *A. satureioides* and named achyrobichalcone[25]. The aim of the present study was to evaluate the anti-hyperglycaemic potential of *A. satureioides* hydroalcoholic extract (AS) in diabetic and non-diabetic *in vivo* models and to evaluate its inhibition of digestive glycosidase activities.

Methods

Plant material. A. satureioides inflorescences were purchased from the Centro de Pesquisas Químicas, Biológicas e Agronômicas (CPQBA; Universidade de Campinas, Brazil). The plant samples were collected and dried at room temperature in May 2013 and identified as Cultivar CPQBA/2, registered in the Ministério Agricultura, Pecuária e Abastecimento (MAPA-Brazil) as number 22975.

Chemicals. The following compounds were used: methanol (J.T. Baker, USA), acetonitrile (Tedia, Brazil) and phosphoric acid (Merck) were high-performance liquid chromatography grade. The standards for quercetin, luteolin, 3-O-methylquercetin and streptozotocin (STZ) were purchased from Sigma-Aldrich, Alfa Aesar (Germany) and Extrasynthese (France), respectively. AC, α-amylase from porcine pancreas, α-glucosidase from *Saccharomyces cerevisiae*, 2-chloro-4-nitrophenyl-α-D-maltotrioside and 4-nitrophenyl α-D-glucopyranoside were acquired from Sigma-Aldrich. All other chemicals and reagents were of analytical grade.

Preparation of AS. The inflorescences were macerated in 80% (*v/v*) ethanol at a plant:solvent ratio of 7.5:100 (*w/v*). The extraction lasted for 8 days, with occasional stirring[26]. The resultant extract was filtered, frozen at -80°C, and subsequently dried in a freeze-dryer (Edwards Modulyo 4K; Irvine, USA) at a temperature of -60°C and pressure of -10^{-2} Bar.

Flavonoid levels. Approximately 20 mg AS was dissolved in 20 mL 80.0% ethanol and maintained in an ultrasound bath (Unique, São Paulo, Brazil) for 10 min. This solution was appropriately diluted with a methanol:16 mM phosphoric acid solution (1:1, *v/v*), filtered through a 0.45-µm membrane filter (Millipore-HVHP, MA, USA), and evaluated in triplicate. Liquid chromatography (LC) analysis of AS was carried out as described previously[27]. A Shimadzu LC-10A system was employed, equipped with an LC-10 AD pump and a CBM-10A system controller; the system was controlled at 30 ± 1°C and the programmed injection volume was 20 µL. The specificity of the method was evaluated using a Shimadzu LC-20A system, equipped with an LC-20 AT pump, a CBM-20A system controller, an SIL-20A autosampler and an SPD-M20A diode array detector. The limits of detection and quantitation were determined using the equations described in the International Council for Harmonisation guidelines. The

results were expressed as the mean flavonoid level (g) per 100 g extract, for three analyses.

Molecular docking. Protein structures obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB; http://www.rcsb.org/pdb/home/home.do) were employed in the molecular docking calculations and are listed in **Table 1**. 3-O-methylquercetin, luteolin and quercetin structures were retrieved from the ZINC database. The achyrobichalcone structure was drawn on MarvinSketch from ChemAxon.

Docking calculations were performed on the FORECASTER Suite, including the FITTED docking software, from Molecular Forecaster Inc. (Laval; Québec, Canada). The ligands present in the crystal structures were used by the software to create a binding site, which was then used in the subsequent docking calculations. Docking pose scores were calculated and compared. The proposed binding mode was determined as the best ranked scoring function, representing the conformations with the most favourable free binding energy (ΔG). The proposed binding pose images were created on BIOVIA Discovery Studio.

Calculation of pharmacokinetic parameters. Molinspiration online toolkit (http://www.molinspiration.com/cgi-bin/properties) was used to analyse the ligands. The octanol-water partition coefficient, the number of hydrogen bond donors and acceptors, the molecular weight (MW), topological polar surface area (TPSA), number of rotatable bonds and violations of Lipinski's rule of five[28] were taken into account; absorption (%ABS) was calculated using the following equation: %ABS = 109 - [0.345 × TPSA], as described previously[29].

In vitro assay of α-amylase and maltase activities. The enzymatic activities of yeast maltase and porcine pancreatic α-amylase were assessed in a final volume of 50 µL in 384-well microplates. For determination of maltase activity, each sample was pre-incubated at 37°C for 5 min with potassium phosphate buffer (50 mM, pH 7.0) before initiating the reaction by adding 4-nitrophenyl α-D-glucopyranoside (1 mM). For determination of α-amylase activity, each sample was pre-incubated with HEPES buffer (50 mM, pH 7.0) containing $CaCl_2$ (5 mM) and NaCl (100 mM) for 5 min at 37°C prior to starting the reaction by the addition of 2-chloro-4-nitrophenyl-α-D-maltotrioside (1.4 mM)[13]. AC was used as a positive control. The absorbance of p-nitrophenol, the product of the enzymatic hydrolysis, was continuously monitored at 405 nm in a FlexStation 3 Benchtop Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA).

The IC_{50} values for AS and AC were calculated using Sigmaplot software 12.0 after performing the assays described above in the presence of at least 8 inhibitor concentrations, by

adjusting the residual activity of inhibitor concentration data and the 4-parameter log equation: app. Res. = min + (max - min) / (1 + ([I]/IC_{50}) ^ (-Coef.Hill).

Testing for reversible/irreversible binding mode: the enzymes were incubated at concentrations that were 100-fold higher than those employed in the above assays, and the sample concentration was equivalent to 10-fold the IC_{50}[30]. After a 30-min pre-incubation, the reaction was initiated by a 100-fold dilution in reaction buffer containing the appropriate substrate. The progress curve was compared to that generated by the enzyme in the absence of inhibitor[31].

Animals. One hundred 90-day-old male Wistar rats (250 ± 50 g) were obtained from the Animal House of Universidade Federal Rio Grande do Sul. The animals were housed in groups of five in each polypropylene cage. Food and water were available *ad libitum*, except when otherwise stated, and the animals were maintained in a temperature-controlled room at 22 ± 1°C under a 12-h light–dark cycle (7:00 a.m. to 7:00 p.m.). All experiments were approved by the Institutional Animal Care and Use Committee at the Federal University of Rio Grande do Sul (IACUC #25449).

Hypoglycaemic effect of AS in control rats. Following a week of acclimatisation, rats were separated into 8 groups, with 5 rats/group. Each group received the following treatments by oral gavage for 14 days: 1 mL/kg water (CTL); 2 g/kg soluble starch (SS); 2 g/kg SS + 14.28 mg/kg AC; 2 g/kg SS + 265.72 mg/kg AS (based on the concentration that caused 50% inhibition of enzyme activity [IC_{50}] for α-amylase); 2 g/kg maltose (MA); 2 g/kg MA + 14.28 mg/kg AC; 2 g/kg MA + 185.21 mg/kg AS (based on the IC_{50} for maltase); or 265.72 mg/kg AS. AS was prepared in water and each animal received a single gavage daily. Blood glucose levels were determined as described by Butler[32] in 10-h fasted rats at time 0, at 30, 60 and 120 min after the first administration of these treatments (acute effects), and similarly at weekly intervals until the end of the study (7- and 14-day effects) (**Figure 1**). Glucose responses were calculated from the area under the curve using the trapezoidal rule.

STZ model of DM. A single intraperitoneal (i.p.) dose of 60 mg/kg STZ was administered, as described by Wu and Huan[33] (**Figure 2**). An elevation of blood glucose level of ≥ 250 mg/dL (the threshold suggested by Wayhs et al.[34]) in the STZ-treated rats was confirmed using a glucometer (Accu Chek Advantage®; Roche, Grenzach-Wyhlen, Germany) after 48 h, indicating their hyperglycaemic status. Non-diabetic CTL rats received i.p. injections of saline (1 mL/kg) and were also subjected to blood glucose measurement. The STZ animals and 1 non-diabetic CTL animal were then administered the following treatments by oral gavage for 21

days: CTL (1 mL/kg water); 14.28 mg/kg AC; or 265.72 mg/kg AS. AS was prepared in water. Blood glucose levels were determined using the method described by Butler[32] on the 1st, 7th, 14th and 21st day of the study. The animals were then euthanized by decapitation and blood was collected for analysis of fructosamine and glycated haemoglobin.

AS toxicity evaluation. In both experiments, the animals were euthanized by decapitation and blood samples and plasma were collected for analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. In the STZ model, creatinine was also determined as an indicator of renal damage[33]. Rat body weights were also determined daily throughout the experimental period.

Statistical analysis. Data were expressed as the mean ± the standard error of the mean. Variance analysis of repeated measures, via Generalised Linear Models, GZLM/GHG, the SPSS program. The statistical significance of differences between the *in vivo* study groups were calculated by one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

Results

AS composition. Liquid chromatographic separation (**Figure 1**, supplementary material) was used to analyse the compounds present in AS. The main constituents of this extract were flavonoids, comprising 12.4 g/100 g of freeze-dried AS. This was made up of mean (± standard deviation for 3 extractions) levels of 3-O-methylquercetin (6.23 ± 0.5 g/100 g AS), quercetin (2.77 ± 0.6 g/100 g AS), luteolin (1.80 ± 0.01 g/100 g AS) and achyrobichalcone (1.60 ± 0.8 g/100 g AS). Quercetin (22.44%) and luteolin (14.5%) thus corresponded to 36.9% of the total AS flavonoid level.

Molecular docking analyses. A molecular docking method was applied to identify the co-crystallised ligands in the enzyme structures; this information was then utilised to study targeted docking with the selected compounds. The resulting lowest energy and root-mean square deviation values were compared (**Table 1**).

Prediction of bioavailability. The results of our analyses using the Molinspiration online property calculation toolkit are presented in **Table 2**. The compounds tested in the present study generally conformed to Lipinski's rule, with only achyrobichalcone showing one violation (MW > 500; **Table 2**).

In vitro inhibition of α-amylase and maltase activities. AS inhibited the activities of α-

amylase (**Figure 3A**) and maltase (**Figure 3B**) in a concentration-dependent manner. As a measure of the inhibitory potency, the IC_{50} was calculated. The IC_{50} of AS was 265.72 μg mL^{-1} for α-amylase and 185.21 μg mL^{-1} for maltase. As a positive control, AC had an IC_{50} of 2.7 μg mL^{-1} for α-amylase and 516.1 μg mL^{-1} for maltase.

The inhibitory potencies of the flavonoids present in AS against α-amylase and maltase were also analysed separately, as shown in **Figure 3**. These results showed that achyrobichalcone had an IC_{50} value of 4.74 μM for α-amylase (**Figure 3E**) and 6.71 μM for maltase (**Figure 3F**).

Determination of enzyme binding model. We tested whether AS and its constituent flavonoids bound to α-amylase and maltase in a rapidly reversible, slowly reversible, or irreversible manner. Our experiment demonstrated that AS acted as a reversible inhibitor of these enzymes, (**Figure 4)** producing linear progress curves with a slope similar to that of the control (enzyme incubated in the absence of AS) (**Figure 4A** α-amylase **e 4B** maltase).

Investigation of AS toxicity. In order to explore the impact of AS treatment on hepatic tissue damage, plasma activities of alanine transaminase (ALT) and aspartate transaminase (AST) were determined spectrophotometrically. No significant changes were observed in these activities following the administration of SS (**Figures 2A1** and **2A2** supplementary material) or MA (**Figures 2B1** and **2B2** supplementary material).

Acute effect of AS on blood glucose. Rat blood glucose levels were determined on the first day of oral administration of water (CTL), SS, or MA, with or without AS or AC, which was used as the positive control. An additional group received AS only. As expected, rats that received SS showed elevated blood glucose level 30 min after supplementation, as compared to the CTL group ($p < 0.001$). Animals that received SS + AS or SS + AC showed lower blood glucose levels 30 min after supplementation, as compared to the SS animals ($p < 0.001$ and $p < 0.01$, respectively). However, comparison of these two treatments showed that the SS + AS group displayed a stronger suppressive effect than the SS + AC group at 30-min post starch load ($p < 0.01$), and the blood sugar levels in the SS + AS group were lower than those of the CTL group ($p < 0.001$) (**Figure 5A**).

The effect of AS and AC on the hyperglycaemia induced by MA is shown in **Figure 5B**. These results showed that rats treated with MA, MA + AC, or MA + AS showed increased blood glucose levels after 30 and 60 min, as compared to the CTL rats ($p < 0.01$, $p < 0.001$, $p < 0.001$, respectively). However, 30 min after MA loading, the MA + AS group had lower glucose levels than the MA + AC group ($p < 0.01$); the MA + AS group did not differ from the CTL

group at 30 or 60 min. No difference was observed between the MA and MA + AC groups at 30, 60, or 120 min after MA administration.

Long-term effect of AS on blood glucose. Following the acute study, the animals continued to be supplemented daily with the same treatments and on the 7th and 14th days, their blood glucose levels were measured at the indicated time-points after administration (**Figures 6A1** and **6A2**). Animals treated with AC or AS for 7 days showed significant differences ($p < 0.001$, $p < 0.01$, respectively) in blood glucose levels, as compared to CTL rats, 30 min after SS administration (**Figure 6A1**). Treatment with AS produced a more effective attenuation of SS-induced hyperglycaemia, as compared with the AC-treated rats. Animals treated with SS + AS had lower blood glucose levels than the SS animals at 30 and 120 min ($p < 0.001$ and $p < 0.01$, respectively). At 14 days, rats receiving AS showed no significant differences in baseline glucose levels, as compared with CTL rats; however, the AS + SS group showed an effective reduction in glucose blood levels 120 min after administration, as compared to the SS + CTL rats ($p < 0.01$; **Figure 6B1**).

Diabetes induction. In the present study, rats received an i.p. injection of STZ (60 mg/kg), followed by blood glucose determination after 21 days (**Figure 7A**). The results showed significantly higher blood glucose levels in STZ-treated rats, as compared to controls ($p < 0.0001$), suggesting effective induction of a DM-like condition. A group of rats that received daily treatment with AS (265.72 mg/kg) for these 21 days showed significantly lower glucose levels than did the STZ + AC group. Furthermore, the CTL + AS group also showed significantly lower blood glucose levels than the CTL + AC (14.28 mg/kg) rats ($p < 0.004$).

The serum levels of fructosamine and glycated haemoglobin in this model are shown in **Figures 7B** and **7C**, respectively. The STZ-mediated increase in serum fructosamine was prevented by AS ($p < 0.01$), but not by the positive control, AC (**Figure 7B**). We observed (**Figure 7C**) a significant increase in this parameter in STZ-treated rats, as compared to controls ($p < 0.001$). Glycated hemoglobin was significantly lower in the experimental group that received 21-day treatment with STZ + AS, as compared to those who received STZ ($p < 0.001$) or STZ + AC ($p < 0.01$).

Determination of AS toxicity. No significant change was observed in the serum AST or ALT activities in rats treated with STZ, STZ + AC, or STZ + AS (**Figures 3A** and **3B** supplementary). There were no statistically significant differences between the creatinine levels of these study groups (**Figure 3C** supplementary).

Discussion

A molecular docking method was applied to identify the co-crystallised ligands in the enzyme structures; this information was then utilised to study targeted docking with the selected compounds. Overall, quercetin exhibited lower interaction energy values than the other ligands investigated. The lowest energy position of each ligand was selected as the putative α-amylase binding mode and these are depicted in **Figure 8**.

Lipinski's rule of five[28] is based on the idea that most successful orally administered drugs have a MW ≤ 500, an octanol-water partition coefficient ≤ 5, five or fewer hydrogen bond donor sites, and 10 or fewer hydrogen bond acceptor sites. Molecules violating more than one of these rules may have low bioavailability. The compounds tested in the present study generally conformed to Lipinski's rule, with only achyrobichalcone showing one violation (MW > 500).

Our results showed that AS inhibited the activities of α-amylase and maltase in a concentration-dependent manner. When comparing AS with AC a positive control we observed that AC was a relatively potent α-amylase inhibitor. However, the inhibitory effect of AS on maltase was 2.7 times higher than that of AC. In addition, the AS-mediated inhibition was considerably greater than that previously reported for some other plant extracts with anti-hyperglycaemic activity; these had IC_{50} values for α-amylase of approximately 500-7500 $\mu g/mL^{-1}$ [34,35].

The inhibition of glucosidases, which are key glycosidases involved in glucose digestion, can delay the release of D-glucose from foods; this suppresses postprandial hyperglycaemia[7]. This inhibitory potency against α-amylase might reflect the presence of flavonoids such as luteolin, which corresponded to 1.8% of AS and is known to inhibit glucose transporters in small intestinal epithelial cells[36]. Scientific evidence has demonstrated a strong relationship between individual flavonoid levels in natural extracts and intestinal glucosidase inhibition[37,38]. In part, this inhibition may be responsible for the hypoglycaemic activities of these extracts.

The inhibitory potencies of the flavonoids present in AS against α-amylase and maltase were also analysed separately. Luteolin had an IC_{50} of 5.66 nM for α-amylase and 7.19 μM for maltase, corroborating previous studies[36]. Our results suggest that achyrobichalcone showed the most promising potential for anti-hyperglycaemic activity, which was consistent with previous studies that demonstrated this activity of chalcones[39].

Our experiment demonstrated that AS acted as a reversible inhibitor of these enzymes, producing linear progress curves with a slope similar to that of the control (enzyme incubated in

the absence of AS). Reversible inhibitors act by forming an inhibitor-enzyme complex that is in equilibrium, where the enzyme shows a defined degree of inhibition that is determined by the concentrations of reagents in the reaction (enzyme, inhibitor and substrate). This type of inhibitor has several advantages over the other mechanisms because reversible inhibition is not permanent, and reducing the inhibitor dose can therefore easily attenuate undesirable effects.

Many plant extracts are hepatotoxic[15] therefore the investigation of AS toxicity is parameter very important. ALT and AST plasma activities are biomarkers of hepatic damage. Moreover, during the treatments employed by the present study, no animals died. The rat body weight gains did not vary appreciably between the study groups. These findings indicated that AS did not produce significant acute toxic effects in rats.

The present study employed SS and MA as carbohydrate sources to simulate glycaemia and the attenuation of this by AS suggested that it may inhibit α-glucosidase activity. The effects of 7- or 14-day treatments with MA and AS or AC on blood glucose levels are showed that animals treated with MA for 7 days had significantly higher blood glucose levels than CTL rats at 30, 60 and 120 min. These 7-day results indicated that the MA + AS group displayed a stronger suppression of blood glucose at 30 and 60 min, as compared to the MA and MA + AC groups ($p < 0.001$). After 14 days, a strong reduction in postprandial glycaemia was observed in MA + AS-treated animals at 30, 60 and 120 min after administration.

These results suggest that AS could provide an attractive therapeutic approach for reducing the postprandial hyperglycaemia resulting from ingestion of simple (MA) and complex (SS) carbohydrates. A positive correlation between pancreatic α-amylase activity and postprandial glucose levels has been identified previously, and the suppression of postprandial hyperglycaemia is highly relevant to the treatment of type 2 DM[38]. This selective effect against the glycosidases may also form the basis of a particularly effective therapy for postprandial hyperglycemia.

The rats receiving AS had no observable adverse effects during the supplementation period (data not shown). In contrast, those receiving AC had episodes of flatulence and diarrhea, corroborating data reported previously[12]. Excessive inhibition of pancreatic α-amylase by α-glucosidase inhibitors such as AC can produce undesirable side effects due to abnormal bacterial fermentation of undigested carbohydrates in the colon[40]. The optimal treatment would produce a mild inhibitory effect on α-amylase, thus preventing this abnormal colonic fermentation[41]. The identification of the active constituent(s) of AS could therefore lead to the development of a new α-glucosidase inhibitor that could provide effective management of

postprandial hyperglycaemia, with minimal side effects.

Several chemicals have been used to induce diabetes experimentally. STZ provides the preferred means of generating animal models of human type 1 DM because it induces similar clinical features. For this reason, STZ-treated animals have been used to study diabetogenic mechanisms and for preclinical evaluation of novel antidiabetic therapies[42]. STZ induces selective destruction of pancreatic β-cells, insulin deficiency, hyperglycaemia, polydipsia and polyuria. A group of rats that received daily treatment with AS (265.72 mg/kg) for these 21 days showed significantly lower glucose levels than did the STZ + AC group, confirming the effect of AS in an animal model of type 1 DM.

Measurement of serum fructosamine levels provides an alternative approach to assessing blood glucose control. The fructosamine test determines the fraction of total serum protein that has undergone glycation, providing a good indicator of glycaemic control over recent weeks.. This biochemical parameter confirmed that AS improved glucose control in this model. Glycated haemoglobin provides an alternative measure of plasma glucose levels over the previous month.

The significant increase ($p < 0.001$) the glycated haemoglobin in STZ-treated rats, as compared to controls suggesting an effective induction of a DM-like situation. Glycated hemoglobin was significantly lower in the experimental group that received 21-day treatment with STZ + AS, as compared to those who received STZ ($p < 0.001$) or STZ + AC ($p < 0.01$), indicating that AS reduced blood glucose levels to close to those observed in the control group. These results indicated that AS could also contribute to blood glucose management in patients with type 1 DM.

STZ accumulates preferentially in pancreatic β-cells via the glucose transporter 2, resulting in β-cell cytotoxicity[43]. Hyperglycaemia leads to an increased uptake of glucose by β-cells via this transporter. Excess metabolism of glucose by glycolysis and glucose auto-oxidation generates free radicals, including reactive oxygen species. This increases oxidative stress in β-cells, favoring necrosis and apoptosis. ALT and AST represent good markers of oxidative damage in the liver, while measurement of blood creatinine provides an important indicator of renal health because this is an easily measured by-product of muscle metabolism that is excreted unchanged by the kidneys. Analysis of these parameters is important because many plant extracts can be hepatotoxic. No significant change was observed in the serum AST or ALT activities and creatinine levels between the of these study groups, indicating that its consumption in the concentrations and period used in the study did not show hepatotoxic effect.

In conclusion, the findings of the present study indicate that the AS exerted a promising anti-hyperglycaemic activity *in vivo* by lowering the levels of blood glucose in both diabetic and non-diabetic rat models exposed to high concentrations of MA and SS. This effect is likely due to the inhibition of α-amylase and maltase by this extract, preventing the release and absorption of glucose from these dietary carbohydrates.

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Disclosure

The authors declare that they have no conflicts of interest.

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Table 1. Summary of the docking experiments

Target Name	PDB Code	Docking Results	3-O-Methylquercetin	Achyrobichalcone	Luteolin	Quercetin
α-Glicosidase,	4JT5	ΔG[a]	15.66	24.52	9.65	4.95
NAG-complexed		RMSD[b]	7.48	28.25	9	6.33
Isomaltase,	3AXH	ΔG[a]	-3.61	n	-12.78	-20.62
isomaltose-complexed		RMSD[b]	5.33	n	5	4.39
Maltase-glucoamylase,	3TOP	ΔG[a]	-13.33	-16.11	-25.93	-35.63
acarbose-complexed		RMSD[b]	6.03	5.58	4.53	5.33
α-Glucosidase,	3WY2	ΔG[a]	23.38	n	-5.49	-6.58
glucose-complexed		RMSD[b]	5.24	n	5.83	4.44
α-Amylase	1UA7	ΔG[a]	-9.52	-19.94	-23.47	-35.75
acarbose-complexed		RMSD[b]	11.01	11.45	11.40	11.68
α-Amylase,	4E20	ΔG[a]	-11.11	-26.36	-29.04	-34.43

acarbose-complexed		RMSD[b]	13.50	9.59	13.19	11.49
α-Amylase,	4W93	ΔG[a]	-15.02	-31.60	-25.12	-24.03
montbretin A-complexed		RMSD[b]	7.78	10.35	7.70	5.50
α-Amylase,	1VAH	ΔG[a]	-11.74	-11.65	-22.47	-25.18
r-nitrophenyl-a-D-maltoside		RMSD[b]	27.16	38.76	26.18	27.95
α-Amylase,	3L2L	ΔG[a]	1.02	-1.05	-10.39	-16.46
dextrin-complexed		RMSD[b]	4.79	43.44	5.38	5.01

[a]Binding free energy (kcal/mol); [b]Root-mean square deviation (Å)

Table 2. Physicochemical parameters relating to oral bioavailability

Ligand	%ABS[a]	TPSA (Å)[b]	MW[c]	miLogP[d]	HBd[e]	HBa[f]	n-ROTB[g]	Lipinski violation
Rule	-	-	< 500	≤ 5	< 5	< 10	≤ 10	≤1
3-O-Methylquercetin	67.48	120.36	316.26	1.96	4	7	2	0
Achyrobichalcone	57.80	148.41	**552.53**	2.62	2	9	**10**	**1**
Luteolin	70.66	111.12	286.24	1.97	4	6	1	0
Quercetin	66.12	124.29	302.24	0.53	4	7	1	0

[a]Percentage of absorption; [b]topological polar surface area; [c]molecular weight; [d]logarithm of the partition coefficient between n-octanol and water; [e]number of hydrogen bond donors; [f]number of hydrogen bond acceptors; [g]number of rotatable bonds.

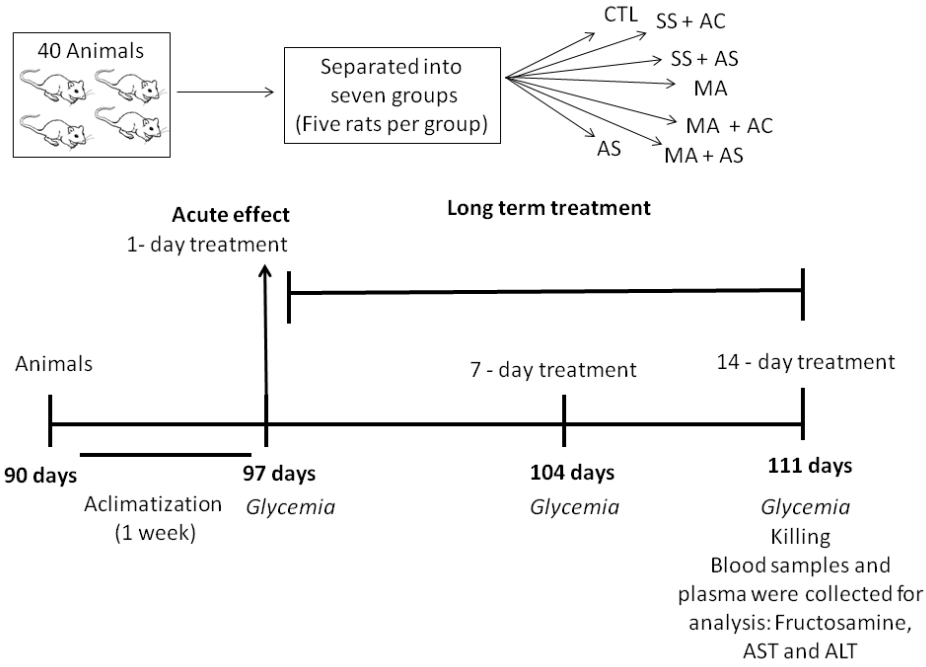

Figure 1. *In vivo* study design timeline showing the acute and long-term treatments of normal rats with the *A. satureioides* hydroalcoholic extract (AS) performed in the current study.

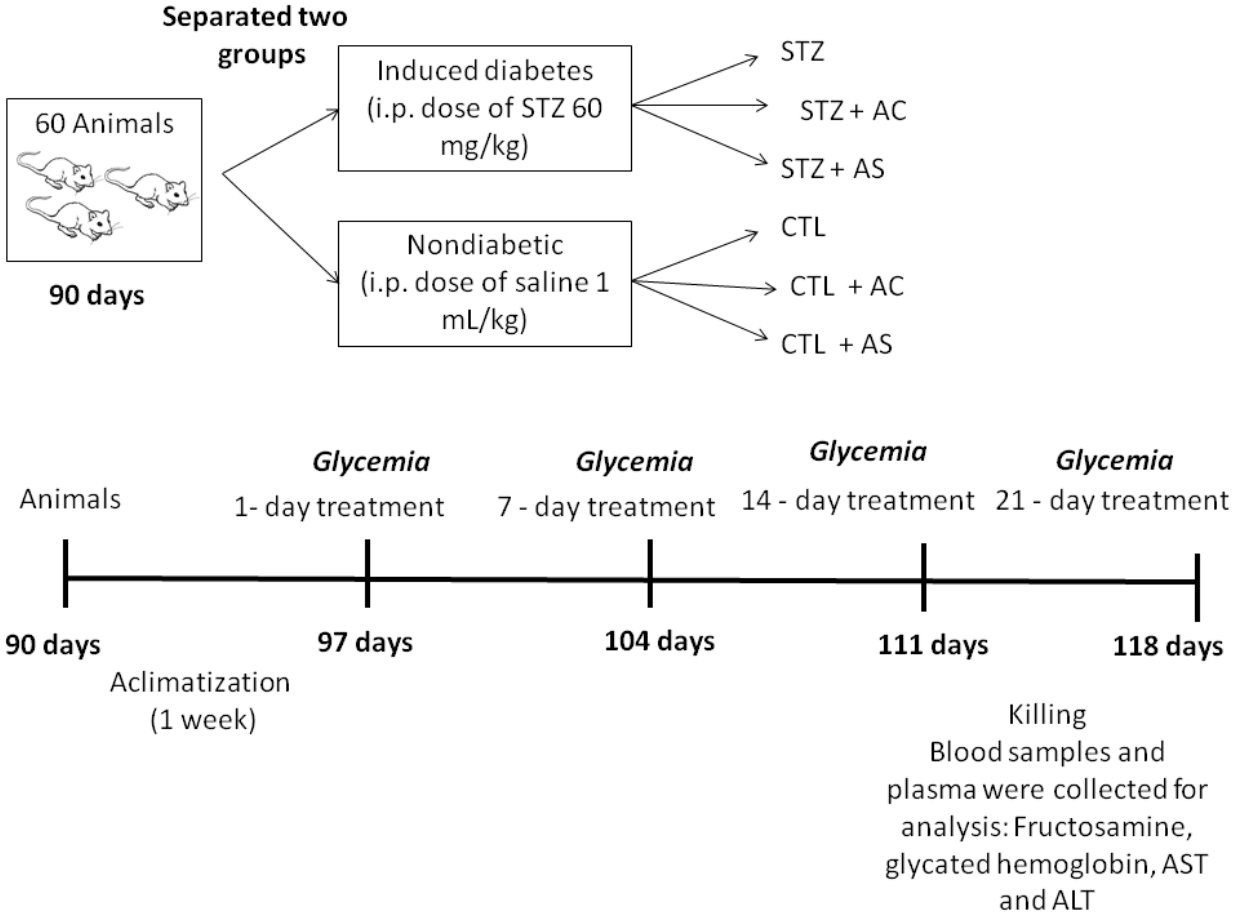

Figure 2. *In vivo* study design timeline showing the control and STZ-induced diabetic rat treatments with *A. satureioides* hydroalcoholic extract (AS) for 21 days.

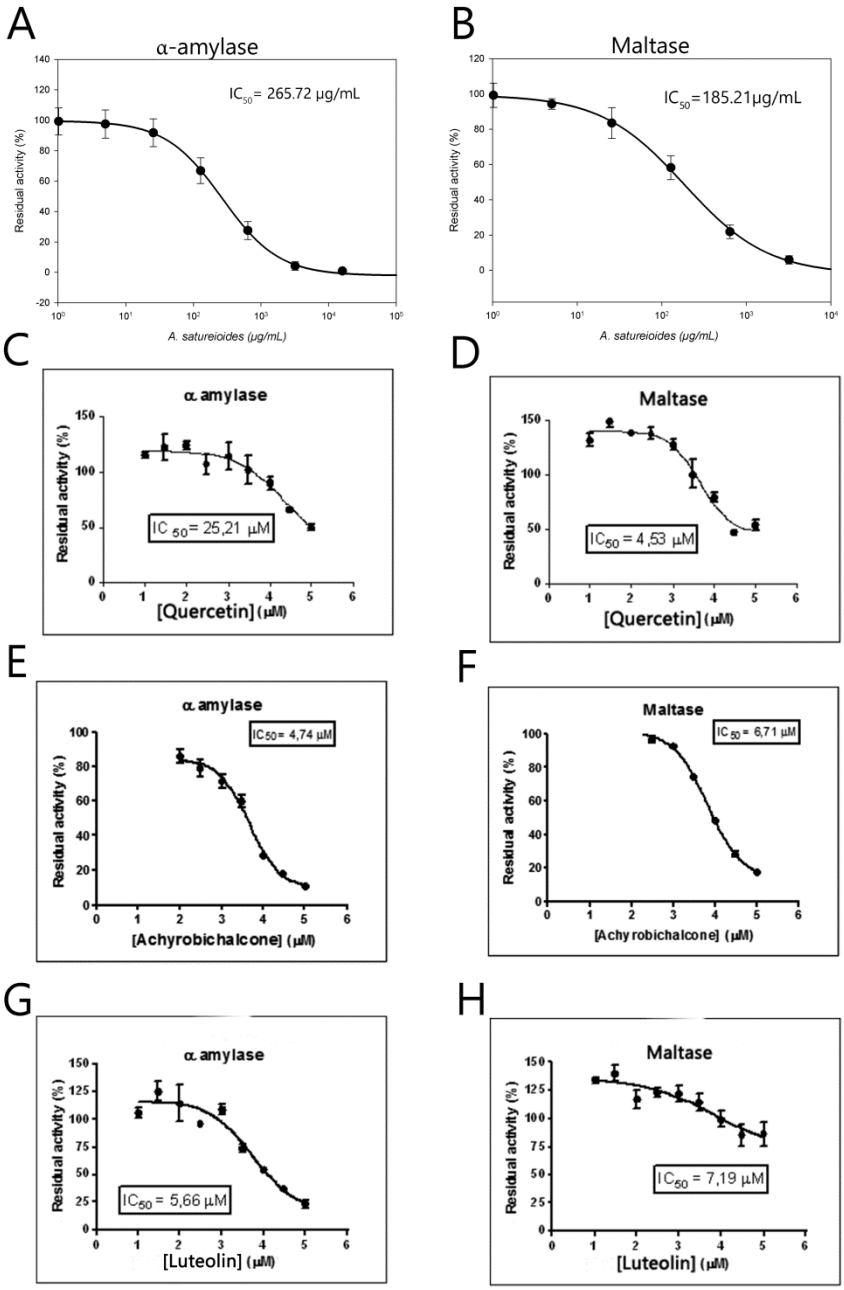

Figure 3. *In vitro* assays of pancreatic α-amylase and maltase activities. The effects of *A. satureioides* hydroalcoholic extract (AS) on (A) pancreatic α-amylase (IC_{50} = 265.72 µg/mL) and (B) maltase (IC_{50} = 185.21 µg/mL), of quercetin on (C) pancreatic α-amylase (IC_{50} = 25.21 µM) and (D) maltase (IC_{50} = 4.53 µM), of achyrobichalcone on (E) pancreatic α-amylase (IC_{50} = 4.74 µM) and (F) maltase (IC_{50} = 6.71 µM), and of luteolin on (G) pancreatic α-amylase (IC_{50} = 5.66 µM) and (H) maltase (IC_{50} = 7.19 µM) are shown.

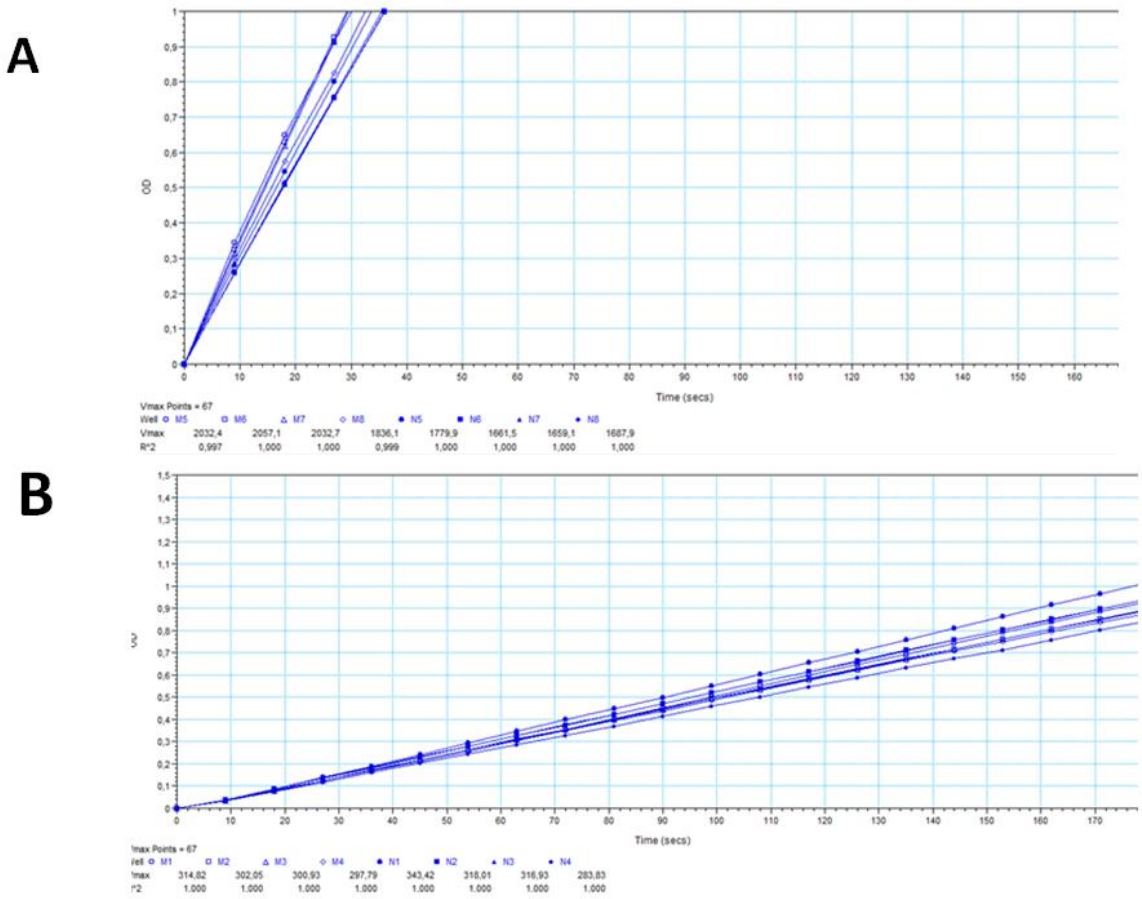

Figure 4. Determination of enzyme binding model. Producing linear progress curves with a slope similar to that of the control (enzyme incubated in the absence of AS) (A) α-amylase e (B) maltase.

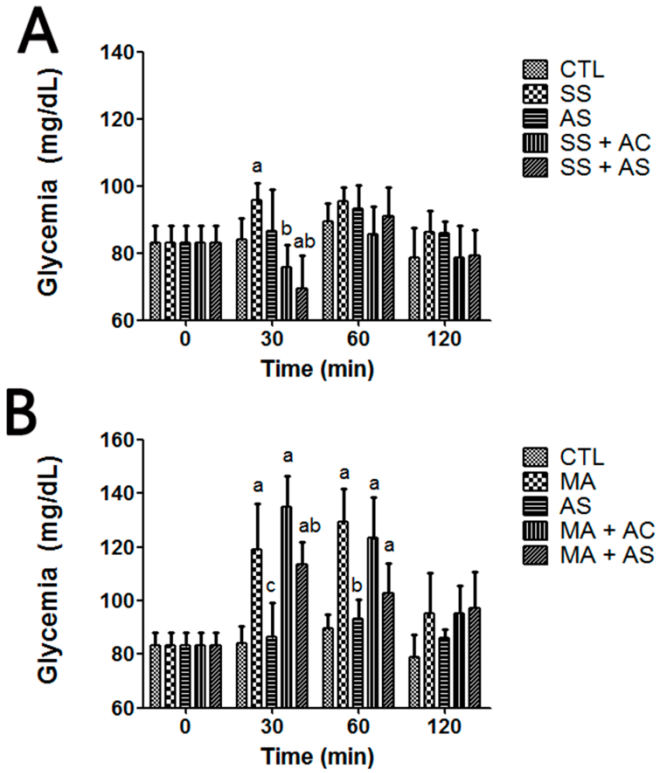

Figure 5. Effect of the *A. satureioides* hydroalcoholic extract (AS) and acarbose (AC) on blood glucose levels in normal rats supplemented with (A) soluble starch (SS) or (B) maltose (MA). Data represent the mean ± S.D.; $^{a}p < 0.05$, as compared to the control group, $^{b}p < 0.05$, as compared to the SS or MA group, and $^{c}p < 0.05$, as compared to the MA + AS group (ANOVA followed by the Tukey test).

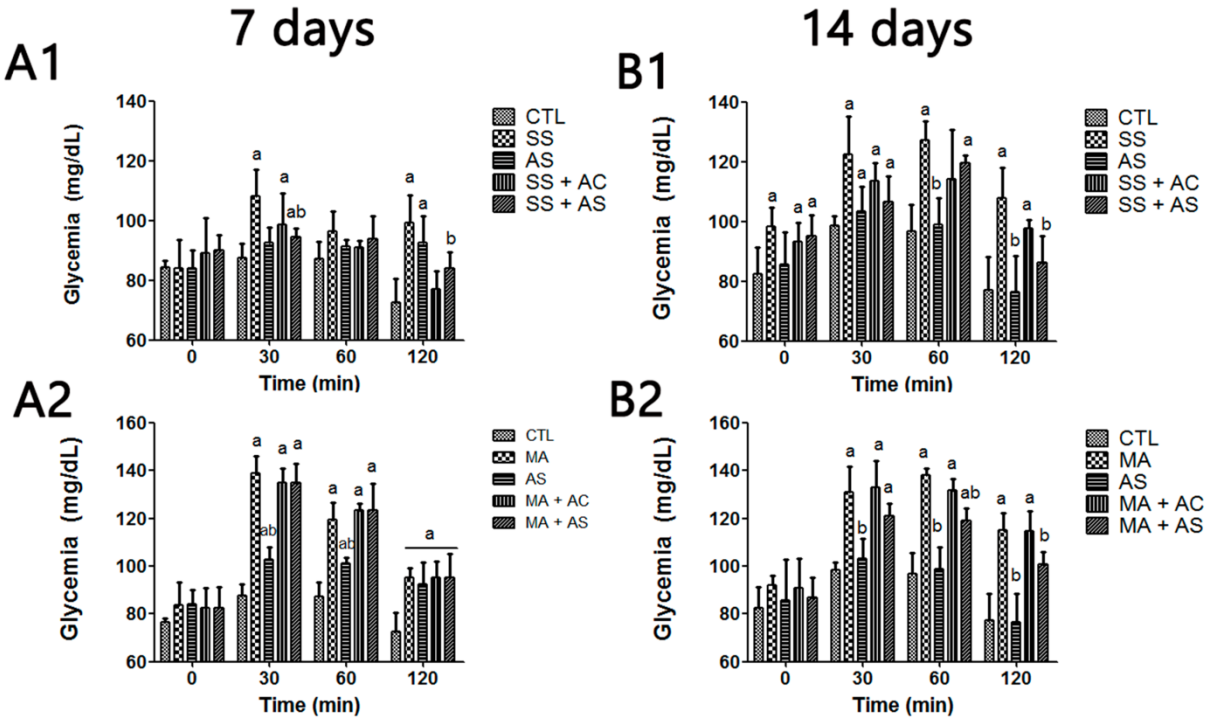

Figure 6. Effect of the *A. satureioides* hydroalcoholic extract (AS) or acarbose (AC) on blood glucose levels in normal rats supplemented with soluble starch (SS) for 7 days (A1) or 14 days (B1) or with maltose (MA) for 7 days (A2) or 14 days (B2). Data represent the mean ± S.D.; $^{a}p < 0.05$, as compared to the control (CTL) group; $^{b}p < 0.05$, as compared to the SS or MA group (ANOVA followed by the Tukey test)

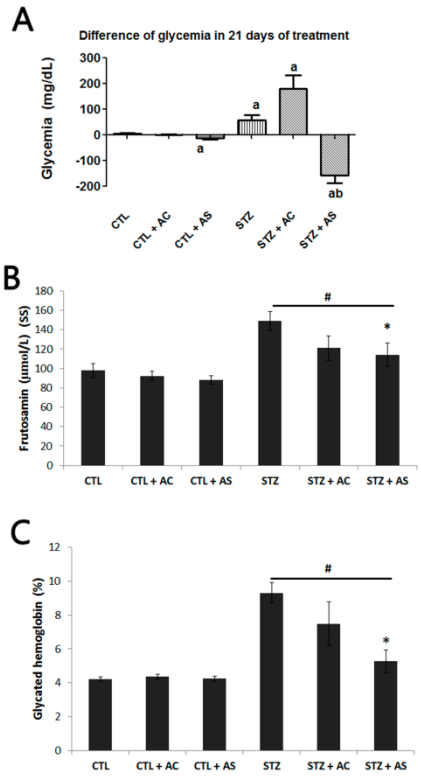

Figure 7. Plasma glucose (A), fructosamine (B) and glycated haemoglobin (C) levels are shown in streptozotocin (STZ)-induced diabetic rats and controls treated with *A. satureioides* hydroalcoholic extract (AS) or acarbose (AC) and soluble starch (SS) over 21 days. Data represent the mean ± S.D.; $^{a}p < 0.001$, as compared to the control group; $^{b}p < 0.05$, as compared to the STZ and STZ + AC group (ANOVA followed by the Tukey test).

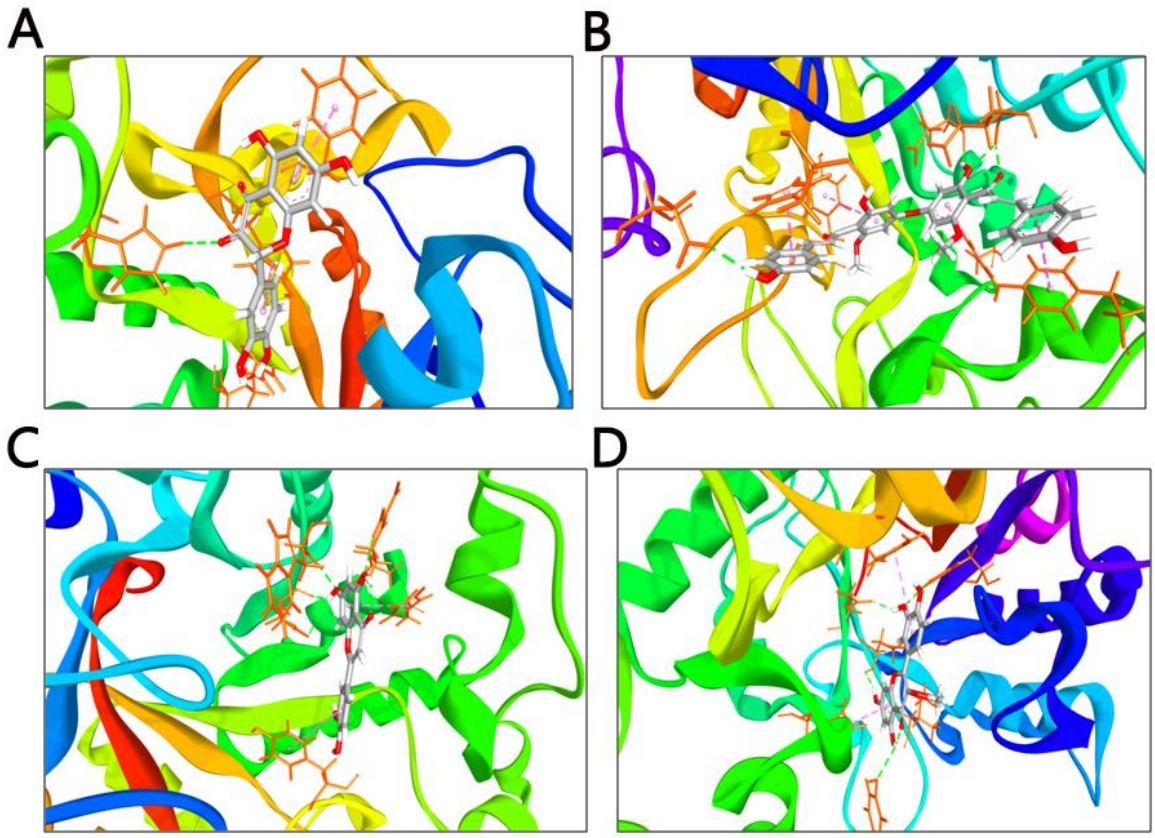

Figure 8. Molecular docking for α-amylase, showing the best-ranked for quercetin (A, PDB ID:1UA7), achyrobichalcone (B, PDB ID:4W93), luteolin (C, PDB ID:4E2O) and 3-O-methylquercetin (D, PDB ID:4W93). Potential hydrogen bonds were dotted in green and π-interactions were dotted in pink. Residues are shown as lines and coloured orange. Ligands are presented as grey sticks, oxygens are in red, and hydrogens are in white.

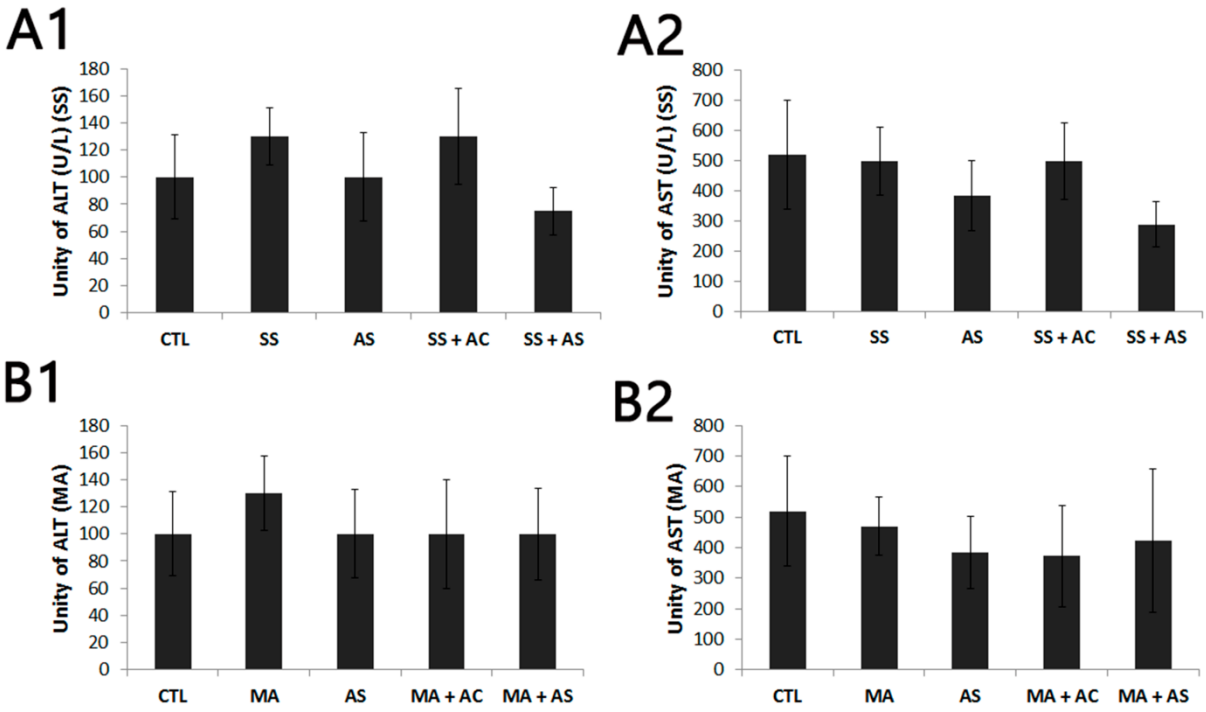

Figure 2 supplementary. Alanine aminotransferase (ALT) activity in plasma from normal rats supplemented with *A. satureioides* hydroalcoholic extract (AS) or acarbose (AC) and soluble starch (SS, A1) or maltose (MA, B1) for 14 days. Aspartate aminotransferase (AST) activity in plasma from normal rats supplemented with AS or AC and SS (A2) or MA (B2) for 14 days. Data represent the mean ± S.

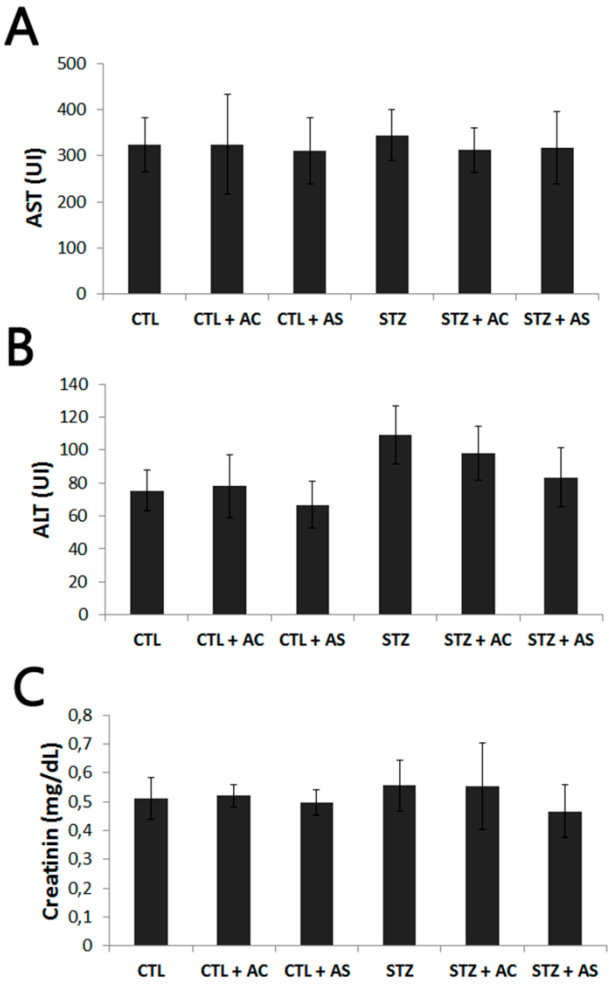

Figure 3 supplementary. Plasma aspartate aminotransferase activity (AST), (A), alanine aminotransferase activity (ALT), (B) and creatinine levels (C) in streptozotocin (STZ)-induced diabetic rats and controls treated with *A. satureioides* hydroalcoholic extract (AS) or acarbose (AC) and soluble starch (SS) for 21 days. Data represent the mean ± S.D.

PARTE 3

DISCUSSÃO

O capítulo I descreve a etapa inicial do trabalho que teve como foco a caracterização de extrato aquoso e hidroalcoolico das inflorescências de *A. satureioides*. Estes resultados foram descritos e discutidos no formato de manuscrito publicado no periódico *Evidence Based Alternative and Complementary Medicine.*

Corroborando com os trabalhos de (CARINI et al., 2015) (BIDONE et al., 2014) quatro flavonoides foram encontrados em ambos os extratos: 3-*O*-metilquercetina (3*O*MQ), quercetina (QCT), luteolina (LUT) e achyrobichalcona (ACB). Estando a LUT a ACB presentes em menores quantidades. O reduzido percentual da ACB no extrato já era esperado, visto que este comportamento foi observado em trabalhos anteriores (DE SOUZA, 2002; PETROVICK, 2005; HOLZSCHUH *et al.*, 2010; BICA *et al.*, 2013). Além destes, outros flavonoides e polifenóis também foram relatados em outros trabalhos com extratos das inflorescências de *A. satureioides* como: ácido cafeico, álcool 3,4-dihidroxibenzílico-4-glucósideo, ácido protocatecúico, galangina, galangina-3-metil éter, (FERRARO ET AI., 2008, BROUSSALIS ET AI., 1989).

A identificação de características de composição química de extratos de *A. satureioides* contrastando especificamente as de espécies intimamente relacionadas tem sido objeto de estudo (CORTADI et al. 2004) tendo em vista que plantas de habitat naturais continuam a apresentar variação na sua composição pelo solo.

Vários autores tem estudado as propriedades terapêuticas do extrato e estas tem sido atribuídas ao alto teor de polifenóis e flavonoides (CASERO et al., 2013)(ARREDONDO et al., 2004). Muitos desses estudos tem sido projetados, levando em consideração a forma de administração do uso tradicional, através do preparo de infusões.

A inclusão de *A. satureioides* na Farmacopeia também constituiu reconhecimento deste uso tradicional muito difundido em toda a região sul do Brasil (BALESTRIN et al., 2016). Parte dos benefícios atribuídos as infusões no tratamento de doenças do trato gastrointestinal, tem sido atribuídos à atividade antibacteriana de *A. satureioides* (CASERO et al., 2013), (CASERO et al., 2015).

Em nosso trabalho verificamos que o extrato hidroalcoólico liofilizado exibiu um amplo espectro de atividade antimicrobiana (100 a 200 mg/mL) contra bactérias patogênicas intestinais.

Corroborando com outros estudos que também observaram atividade antibacteriana testando extrato hidroalcoólicos de *A. satureioides*. (MOTA, 2011; GIROLOMETTO, 2009 e WIEST et al., 2009). Os mesmos autores, ao comparar a atividade antibacteriana de extrato hidroalcoólico e decocto, observaram uma atividade antibacteriana muito superior no extrato hidroalcoólico, o que pode estar relacionado à perda de óleos essenciais voláteis, que na extração por decocção está associada à utilização de temperaturas de ebulição, em comparação com a extração por hidroalcoolatura que é a frio (PASSOS, 2009).

Observamos que os efeitos antibacterianos do extrato foram superiores aos exercidos pela amoxicilina (antibiótico usado como controle positivo), quando testados contra *B. cereus* e *S. aureus*. Uma hipótese é que tais efeitos são atribuíveis aos compostos lipofílicos no extrato interagindo com a parte hidrofóbica da membrana bacteriana, afetando assim a anisotropia da membrana e a organização dipolar.

Atualmente, a resistência aos antimicrobianos é um problema global de importância crescente que põe em perigo a eficácia dos antibióticos, que transformaram a medicina e salvaram milhões de vidas (BROWN; WRIGHT, 2016). Os tratamentos para infecções resistentes custam ao sistema de saúde americano US$ 34 bilhões anualmente (IDSA, 2016).

Durante séculos, preparações contendo flavonoides como o principal constituinte fisiologicamente ativo foram usadas para tratar doenças (CUSHNIE; LAMB, 2005). Dessa forma, o uso de produtos naturais derivados de plantas apresenta-se como uma alternativa terapêutica potencial aos antibióticos e a triagem de compostos químicos provenientes de produtos naturais para a atividade antimicrobiana representa uma estratégia alternativa para o desenvolvimento de novos fármacos.

Alguns pesquisadores relataram sinergia entre flavonoides naturais e outros agentes antibacterianos contra cepas resistentes de bactérias (CUSHNIE; LAMB, 2005). Vários estudos tem demonstrado que as chalconas são muito mais eficazes contra cepas resistentes de bactérias do que flavonas ou flavanonas e que os grupos hidroxilo na posição 2' são importantes para a atividade de inibição do *S. aureus*. Além disso, nem a fluoração nem a cloração na posição 4' do anel B de chalcones podem afetar significativamente nas propriedades antibacterianas (ALCARAZ et al., 2000).

Evidências indicam que entre os flavonoides analisados nos extratos de *A. satureioides*, achyrobichalcona poderia ser responsável pela atividade antibacteriana. No entanto, outros análogos estruturais desta mesma classe de flavonoides teriam de ser sintetizados e examinados antes que o efeito da atividade antibacteriana possa ser avaliado corretamente. Por exemplo, os

grupos metoxila diminuem drasticamente a atividade antibacteriana dos flavonoides (ALCARAZ et al., 2000) e esses dados podem explicar a falta de inibição observada na 3-O-metil-quercetina, o principal composto fenólico no extrato liofilizado.

Estudos demonstram que as propriedades antibacterianas de flavonoides, como a quercetina, podem desempenhar um papel na inibição da síntese de ácido nucleico (MORI et al., 1987). Além disso, as chalconas podem exercer efeitos antibacterianos alterando a permeabilidade das membranas celulares, prejudicando a função da membrana ou inibindo o metabolismo energético (SATO et al., 1997).

Embora existam relativamente poucos estudos sobre os mecanismos subjacentes à atividade antibacteriana induzida por flavonoides, numerosos estudos da literatura indicam que diferentes produtos naturais e fitoquímicos podem atingir diferentes componentes e funções da célula bacteriana.

A microbiota intestinal é considerada de natureza simbiótica e está envolvida em vários processos, incluindo a quebra e absorção de nutrientes, a produção de vitaminas e hormônios e a prevenção da colonização por agentes patogênicos. A incapacidade de alcançar ou manter este equilíbrio entre um hospedeiro e sua microbiota leva à disbiose, o que tem consequências negativas para a saúde intestinal e sistêmica (BISCHOFF, et al., 2014).

Nossos resultados mostram que a abundância e a diversidade da microbiota intestinal em ratos wistar suplementados com o extrato liofilizado preparado a partir de uma solução extrativa hidroalcoólica não foram significativamente diferentes das do controle. Este é um resultado importante porque os estudos mostraram que várias doenças podem estar associadas à alteração da barreira e ao aumento da permeabilidade do epitélio ocasionando mudanças na população da microbiota ou a reduções na diversidade da microbiota (BISCHOFF, 2011).

Infusões de *A. satureioides* são comumente utilizadas na medicina tradicional durante a gravidez para alívio de problemas estomacais e/ou desordens gastrointestinais muitos de seus usos tem despertado o interesse em pesquisas cientificas (JOHN; SHANTAKUMARI, 2015)

Tendo em vista que estudos sobre o efeito do uso indiscriminado de infusões de plantas durante a gravidez são limitados e que estudos recentes relataram que a suplementação crônica de flavonoides também pode exercer efeitos pró-oxidantes, toxicidade *in vivo* além de elevar as taxas de mortalidade entre indivíduos saudáveis, o capítulo II deste trabalho se destinou a investigar o efeito da suplementação com extratos de *Achyrocline satureioides* durante a gravidez e a lactação em parâmetros redox de ratas Wistar e seus filhotes. Neste estudo, suplementamos ratas grávidas e lactantes com extratos de *Achyrocline satureioides* em doses

equivalentes ao consumo de 150 mL de chá por dia de acordo com o rendimento de extração (47mg/kg/dia para extrato aquoso e 35mg/kg/dia para extrato hidroalcoólico). As doses utilizadas foram equivalentes às doses médias ingeridas em bebidas de chá por mulheres grávidas (JOHN; SHANTAKUMARI, 2015).

Podem ser obtidas doses equivalentes aplicando fatores de incerteza de 10 vezes por cada espécie e diferenças entre espécies (DAY, 2001). Nessas doses e condições, os pontos finais de toxicidade reprodutiva e de desenvolvimento foram observados com sinais clínicos relacionados ao tratamento de toxicidade materna e quanto ao índice de nascimentos no parto. Os efeitos relacionados ao tratamento incluíram um ligeiro atraso na taxa de conclusão da abertura dos olhos em ambos os grupos tratados com extrato. Uma provável hipótese que explica esses resultados é a de que os flavonoides são metabolizados de forma rápida pelo fígado por metilação, sulfonação, glucuronidação ou uma combinação desses processos, o que provavelmente modula a biodisponibilidade celular desses compostos (DANKOVIC, et al., 2015)

Observamos também que o extrato aquoso de *Achyrocline satureioides* foi capaz de afetar a relação entre o número de implantações e o número de nascimentos fornecendo evidências que podem justificar a necessidade de controle na suplementação com extratos de *Achyrocline satureioides* na gestação.

Neste trabalho demonstrou-se pela primeira vez que a suplementação diária de *Achyrocline satureioides* em doses equivalentes a duas xícaras de chá em ratas tem toxicidade subclínica durante a gestação e lactação em ratas, com efeitos adversos sobre o número de nascimentos dos filhotes, muito embora observou-se que tanto nas mães como nos filhotes em tecidos como fígado e rim houve um aumento das enzimas com atividade antioxidante. Contudo, são necessárias mais investigações para verificar os seus efeitos sobre a saúde global.

Em estudos de toxicidade onde avaliou-se o efeito de infusões preparadas com extratos liofilizados de *Achyrocline satureioides* sobre a viabilidade de plaquetas humanas de indivíduos saudáveis observou-se uma diminuição no número de células viáveis dependente da dose e as concentrações citotóxicas de 50% (CC_{50}) foram entre 588 μg /mL e 653 μg /mL (CARIDDI et al., 2015).

Nossos resultados mostraram que os extratos de *Achyrocline satureioides* podem induzir sintomas de toxicidade em ratas grávidas na relação entre o número de implantações e o índice de nascimento dos filhotes, no entanto, o mecanismo exato ainda não foi completamente esclarecido.

Diferentes dos resultados obtidos em outros estudos que não relataram efeitos negativos sobre fertilidade, peso fetal ou desenvolvimento pré-natal quando os ratos foram suplementados com extrato de chá verde (LOGSDON et al., 2012), acredita-se que extratos aquosos de *Achyrocline satureioides* podem conter compostos capazes de modificar os parâmetros oxidativos nas mães e apresentar um impacto negativo na relação entre o número de implantações e o índice de nascimento dos filhotes e sobrevivência neonatal.

Os níveis de atividade enzimática são considerados um fator importante que protege os órgãos contra o efeito deletério de potenciais tóxicos (SCHNORR et al., 2011). Nossos resultados mostraram que a atividade de GST, CAT e SOD em fígados maternos foi significativamente aumentada. Alterações na atividade de GST provavelmente alteraram o estado redox e as defesas antioxidantes do tecido (SAHU; GREY, 1996). Finalmente, a GST é também um parâmetro endógeno para o controle das vias de sinalização em cascata e as alterações na sua atividade podem alterar o equilíbrio regulatório de inúmeras vias de quinase (TOWNSEND et al., 2003).

Esses resultados corroboram os de outro estudo que relataram atividade hepatoprotetora do extratos de *Achyrocline satureioides* (KADARIAN et al., 2002) tendo em vista que os principais componentes antioxidantes naturais no extrato são flavonoides (RETTA et al., 2012).

Observando-se a existência de trabalhos que descrevem que *A. satureioides* possui atividade hipoglicêmica e visando avaliar o mecanismo pelo qual isso acontece, também levando em consideração os diversos trabalhos que mencionam a participação dos flavonoides e de metabólitos secundários na inibição das glicosidases, aliado ao fato de *A. satureioides* ser uma planta medicinal com uma vasta história de usos etnofarmacológicos, optou-se por realizar uma pesquisa *in vivo* para confirmação desta temática. Este trabalho é apresentado no Capítulo III e foi submetido à publicação no periódico *Journal of Diabetes.*.

As glicosidases desempenham um papel crucial na digestão de polissacarídeos dietéticos e os seus inibidores podem atuar como fármacos anti-hiperglicêmicos. A administração oral de um extrato hidroalcoólico de *A. satureioides* produziu atividade anti-hiperglicêmica promissora em ratos, quando comparada ao fármaco acarbose (controle positivo). Este efeito foi observado em ratos que ingeriram níveis elevados de maltose e amido, e também num modelo de estreptozotocina de rato de DM.

A Organização Mundial da Saúde (OMS) estima que esta enfermidade será a sétima principal causa de morte em 2030 (IDF, 2015). No DM tipo II a secreção de insulina pelo pâncreas pode ser normal, mas a entrada de glicose nas células está comprometida. A Diabetes

mellitus é uma desordem metabólica progressiva do metabolismo da glicose que eventualmente leva a alterações micro e macro-vasculares, causando complicações secundárias de difícil controle (COPELAND, 2013). Portanto, prevenir a manutenção do quadro de hiperglicemia é de máxima importância para impedir a progressão da doença.

A acarbose é um inibidor de glicosidase que se demonstrou ser capaz de controlar o pico hiperglicêmico pós-prandial (CHEN; JOSSE, 2004), seguro e bem tolerado. No entanto os inibidores de glicosidase atualmente empregados no tratamento da diabetes possuem como efeitos adversos flatulência, desconforto abdominal e diarréia. Uma das vantagens da utilização de extrato de *A. satureioides*, é que o mesmo, não apresentou efeitos colaterais nos animais durante o período de suplementação.

Para investigar o mecanismo de ação de *A. satureioides*, determinamos seus efeitos inibitórios sobre maltase e α-amilase pancreática. O nível de *A. satureioides*, associado a 50% de inibição enzimática (IC_{50}) foi de 185,21 μg/mL para maltase e 265,72 μg / mL para α-amilase pancreática. Uma investigação mais aprofundada dos constituintes de *A. satureioides*, mostrou que o flavonoide, achyrobichalcona, produziu a maior inibição da enzima (IC_{50} de 4,74 μM para α-amilase e 6,71 μM para maltase), o que foi consistente com os nossos resultados de modelagem molecular.

Estes achados corroboram com outros trabalhos que demonstram que derivados sulfonamídicos de chalconas apresentam atividades inibitórias satisfatórias na faixa de micro molar para glicosidases (SEO et al., 2005) além de sugerir uma potencial aplicação dos extratos de *A. satureioides* como agente terapêutico em DM.

De modo geral, os resultados indicam que tanto *A. satureioides* quanto os seus flavonoides configuram-se como promissores agentes hipoglicêmicos, embora mais estudos aprofundados devam ser realizados, para classificá-los como novos agentes terapêuticos em DM.

CONCLUSÕES

Com base nos resultados apresentados neste trabalho, observou-se evidências de atividades antioxidante, antimicrobiana de extratos de *A. satureioides* contra patógenos intestinais, bem como também de efeito hipoglicemiante tanto em modelos *in vivo* como *in vitro*, estes efeitos reforçam a importância de pesquisas futuras sobre seu potencial uso como agente co-adjuvante terapêutico para doenças intestinais derivadas de bactérias que podem apresentar altas taxas de resistência a antibióticos e ou doenças como diabetes mellitus. Também conclui-se que um dos mecanismos pelos quais extratos de *A. satureioides* exercem efeito hipoglicemiante é através da inibição das glicosidases intestinais.

PERSPECTIVAS

Com base nos resultados observados neste trabalho, as perspectivas futuras são:

- Avaliar se os efeitos antimicrobianos do extrato são atribuíveis aos compostos lipofílicos que interagem com a parte hidrofóbica da membrana bacteriana, afetando assim a anisotropia da membrana e a organização dipolar.

- Avaliar efeitos da suplementação de *A. satureioides* durante a gestação e lactação das mães e dos filhotes, quando estes atingirem a vida adulta, realizando ensaios comportamentais e análises histológicas nos tecidos.

- Realizar mais análises complementares para identificar *in vivo*, a capacidade de cada flavonoide na inibição das glicosidases.

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