UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
FACULDADE DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Investigação etnofarmacológica dos efeitos cardiovasculares e renais de três espécies medicinais utilizadas no Pantanal sul-mato-grossense

CLEIDE ADRIANE SIGNOR TIRLONI

Dourados-MS

2018

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Ao décimo primeiro dia do mês de maio do ano de dois mil e dezoito (11/05/2018), às 13h, em sessão pública, realizou-se, no auditório da Faculdade de Ciências da Saúde, Universidade Federal Grande Dourados, a Defesa de Tese de Doutorado intitulada "**Investigação Etnofarmacológica dos efeitos cardiovasculares e renais de três espécies medicinais utilizadas no Pantanal sul-mato-grossense**" apresentada pela doutoranda CLEIDE ADRIANE SIGNOR TIRLONI, do Programa de Pós-Graduação Doutorado em Ciências da Saúde, à Banca Examinadora constituída pelos professores **Dr. Arquimedes Gasparotto Junior** (Presidente/orientador), **Dra. Sara Santos Bernardes** (membro titular/programa), **Dr. Marcio Eduardo de Barros** (membro titular/programa), **Dr. Saulo Euclides Silva Filho** (membro titular/UFGD), e **Dr. Emerson Luiz Botelho Lourenço** (membro titular/externo). Iniciada sessão, a presidência deu a conhecer à candidata e aos integrantes da Banca as normas a serem observadas na apresentação da Tese. Após a candidata ter apresentado a sua Tese, no tempo previsto de 40 até 50 minutos, os componentes da Banca Examinadora fizeram suas arguições, que foram intercaladas pela defesa do candidato, no tempo previsto de até 240 minutos. Terminadas as arguições, a Banca Examinadora, em sessão secreta, passou ao julgamento, tendo sido a candidata considerada **APROVADA**, fazendo *jus* ao título de **DOUTORA EM CIÊNCIAS DA SAÚDE**. Nada mais havendo a tratar, lavrou-se a presente ata, que vai assinada pelos membros da Banca Examinadora.

Dourados, 11 de maio de 2018.

Dr. Arquimedes Gasparotto Junior _____

Dra. Sara Santos Bernardes _____

Dr. Marcio Eduardo de Barros _____

Dr. Saulo Euclides Silva Filho _____

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota”.

(Madre Tereza de Calcuta)

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SÍMBOLOS

AAPH	2,2-Azobis (2-amidinopropano) dihydrochloride
ACs	Anidrase carbônicas
AceFr	Do inglês acetate fraction/fração acetato de etila
AqueFr	Do inglês aqueous fraction/ fração aquosa
AQPs	Aquaporinas
AMPc/cAMP	Adenosina monofosfato cíclica/ no inglês cyclic adenosine monophosphate
ANVISA	Agência Nacional de Vigilância Sanitária
AVE	Acidente vascular encefálico
AT1	Receptor de angiotensina II tipo AT1
AT2	Recepetor de angiotensina II tipo AT2
ATP	Adenosina trifosfato
ButFr	Do inglês buthanolic fraction/ fração butanólica
CO_2	Dióxido de carbono
Cl^-	Cloreto
ChloroFr	Do inglês chloroform fraction/ fração clorofórmica
CMLVs	Células musculares lisas dos vasos
DAP	Doença arterial periférica
DL50	Dose letal de 50%
DRC	Doença renal crônica
DCVs	Doenças cardiovasculares
ECA	Enzima conversora de angiotensina
EETs	Ácidos epoxieicosatrienóicos
eONS/eNOS	Enzima óxido nítrico sintase endotelial/ no inglês nitric oxide syntase
ERRO/ROS	Espécies reativas de oxigênio/ no inglês oxigen-reactive species
ESAH	Do inglês ethanolic supernatant of the *Acanthospermum hispidum*/ Sobrenadante etanólico de *Acanthospermum hispidum*
ESLD	Do inglês ethanolic supernatant of the *Luehea divaricata*/ Sobrenadante etanólico de *Luehea divaricata*
ESTE	Do inglês ethanolic supernatant of the *Talisia esculenta*/ Sobrenadante etanólico de *Talisia esculenta*

FCDP	Fator de crescimento derivado de plaquetas
FHDE	Fatores hiperpolarizantes derivados de endotélio
GCs	Guanilato ciclases
GMPc/cGMP	Guanosina monofosfato cíclica/ no inglês cyclic guanosine monophosphate
GTP	Guanosina trifosfato
H^+	Hidrogênio
HA	Hipertensão arterial
HAS	Hipertensão arterial sistêmica
HCO_3^-	Bicarbonato
HCTZ	Hidroclorotiazida
H_2S	Sulfureto de hidrogênio
IAM	Infarto agudo do miocárdio
IC	Insuficiência cardíaca
K^+	Potássio
LDL	Lipoproteína de baixa densidade
MS	Ministério da Saúde
Na^+	Sódio
NASA	National Aeronautics and Space Administration (Administração Nacional da Aeronautica e Espaço).
OECD	Organização para Cooperação e Desenvolvimento Econômico
ONU	Organiação das Nações Unidas
OMS/WHO	Organização Mundial da Saúde/ no inglês World Health Organization
O_2^-	Ânion superóxido
OH^-	Íon hidroxil
ON	Óxido nítrico
$ONOO^-$	Peroxinitrito
PA	Pressão arterial
PAD	Pressão arterial diastólica
PAM	Pressão arterial média
PAS	Pressão arterial sistólica
pH	Potencial hidrogeniônico
PGI_2	Prostaciclinas
PNPIC	Política Nacional de Práticas Integrativas e Complementares

PNPMF	Política Nacional de Plantas Medicinais e Fitoterápicos
RAA	Renina/angiotensina/aldosterona
SBC	Sociedade Brasileira de Cardiologia
SiBBr	Sistema de Informação sobre a Biodiversidade Brasileira
SRAA	Sistema renina-angiotensina-aldosterona
SUS	Sistema Único de Saúde
UNESCO	Do inglês United Nations Educational, Scientific and Cultural Organization/ Organização das Nações Unidas para a Educação, a Ciência e a Cultura
V2	Receptor da vasopressina do tipo V2

Investigação etnofarmacológica dos efeitos cardiovasculares e renais de três espécies medicinais utilizadas no Pantanal sul-mato-grossense

RESUMO

As doenças cardiovasculares estão entre as principais causas de mortes no mundo e as plantas medicinais podem ser alternativas viáveis para o desenvolvimento de novos medicamentos para o controle destas doenças. O objetivo deste trabalho foi investigar as espécies medicinais *Acanthospermum hispidum*, *Luehea divaricata* e *Talisia esculenta*, utilizadas popularmente na região do Pantanal sul-mato-grossense, para o tratamento de doenças que afetam o sistema cardiovascular e renal. Primeiramente realizamos uma avaliação morfológica e histoquímica para identificar adequadamente as três espécies em estudo. Após devidamente identificadas, foram coletadas as partes aéreas da *A. hispidum* e as folhas da *L. divaricata* e *T. esculenta*. Infusões (100 g/1000 mL água filtrada) foram preparadas com as três espécies, os infusos foram filtrados e tratados com etanol, o sobrenadante etanólico (ES) foi utilizado. Os extratos purificados da *A. hispidum* (ESAH), *L. divaricata* (ESLD) e *T. esculenta* (ESTE) foram analisados fitoquímicamente. Todos os extratos apresentaram compostos fenólicos. Estudos de toxicidade foram conduzidos com ratos da linhagem Wistar (machos e fêmeas). Diferentes grupos de animais receberam 5, 50, 300 e 2000 mg/kg (dose única, v.o.) dos extratos ou água filtrada (5 mL/100g). No 15° dia o experimento foi encerrado e amostras de sangue e tecidos foram obtidas para análises bioquímicas e histológicas. Nenhum extrato promoveu sinais de toxicidade, então estudos farmacológicos foram conduzidos. Primeiramente verificamos os possíveis efeitos diuréticos em diferentes grupos de ratos após tratamento com diferentes doses dos três extratos (30, 100 e 300 mg/kg, v.o.). Após 1, 2, 4, 6, 8 e 24 horas (tratamento agudo) ou diariamente por sete dias (tratamento prolongado) o volume urinário foi determinado. Adicionalmente, nas amostras de urina resultante, o sódio, o potássio, o cloreto, o pH e a densidade foram mensurados. Baseado nos resultados da diurese aguda, somente o ESLD foi avaliado por sete dias. Em um segundo momento, analisamos os efeitos dos três extratos sobre a pressão arterial siastólica (PAS), pressão arterial diastólica (PAD), pressão arterial média (PAM) e taxa cardíaca (batimentos por minuto- BPM) em ratos normotensos. Para isto, os animais foram anestesiados com cetamina/xilasina (100/20 mg/kg, i.m.) e um cateter de polietileno foi introduzido na artéria carotídea direita. Em seguida, realizamos a medida da pressão arterial (PA). Os extratos, nas mesmas doses da diurese, foram administrados diretamente no duodeno dos animais. Hidroclorotiazida (HCTZ 25 mg/kg) e

água filtrada (5 mL/kg) foram utilizados como controle. O ESAH não apresentou atividade diurética, entretanto apresentou atividade hipotensora (reduziu a PAS e a PAM na dose de 30 mg/kg). A partir destes dados, investigamos o papel do óxido nítrico (ON), das prostaciclinas (PGI_2) e o envolvimento dos canais de potássio (K^+) nesta atividade. Diferentes grupos de ratos foram infundidos continuamente pela veia femoral com L-NAME (7 mg/kg/min., inibidor não seletivo da óxido nítrico sintase), indometacina (3 mg/kg/min., inibidor não seletivo das cicloxigenases), azul de metileno (150 nmol/kg/min., inibidor da glanilato ciclase) ou TEA (400 µmol/kg/min., inibidor não seletivo dos canais de K^+ sensíveis ao Ca^+). Em seguida, administrou-se o ESAH (30 mg/kg, i.d.). Constatamos que a via do ON estava envolvida na atividade hipotensora do ESAH, como também os canais de K^+ sensíveis ao Ca^+. O ESLD apresentou atividade diurética, salurética e hipotensora nos tratamentos agudos e as duas primeiras atividades se mantiveram por sete dias. A partir destes resultados realizamos o fracionamento deste extrato com solventes de diferentes polaridades. O ESLD (15 g.) foi solubilizado em água (500 mL) e fracionado sequencialmente com clorofórmio, butanol e acetato de etila gerando as frações ChloroFr, ButFr e AceFr, respectivamente. O resíduo aquoso gerou a fração aquosa (AqueFr). Estas frações foram avaliadas quanto aos efeitos diuréticos e hipotensores agudos. As frações AceFr (6 mg/kg), AqueFr (128 mg/kg) e ButFr (65 mg/kg) apresentaram atividade diurética após 24 horas, as duas últimas frações foram também hipotensoras, sendo que a ButFr apresentou também atividade salurética. Então, investigamos o envolvimento do ON e das PGI_2 na atividade diurética da ButFr. Diferentes grupos de animais receberam, uma hora antes, L-NAME (60 mg/kg, v.o.) e indometacina (5 mg/kg, v.o). Em seguida, foram tratados com ButFr (65 mg/kg, v.o.). O volume urinário foi mensurado após 8 e 24 horas. As duas vias estavam envolvidas com a atividade diurética desta fração. Avaliamos também se o efeito diurético, salurético e hipotensor da ButFr (65 mg/kg, v.o) se manteriam após tratamento prolongado (diariamente por sete dias), o que também se confirmou. Apoiados nestes dados, analisamos esta fração em órgãos isolados. Para isto, ratos foram anestesiados e seus leitos mesentéricos e seus rins foram retirados e perfundidos com solução adequada, em seguida a PA destes órgãos foi analisada. Após a estabilização da PA, os órgãos receberam diferentes concentrações da ButFr. As concentrações mais efetivas da ButFr (0,01; 0,03 e 0,1 mg) foram analisadas quanto ao envolvimento do ON e das PGI_2 nas atividades diuréticas e hipotensoras apresentadas. Retiramos novos órgãos e perfundimos com os inibidores L-NAME (100 µmol) e indometacina (1 µmol), em seguida administramos as diferentes concentrações da ButFr. Observamos que as duas vias estavam envolvidas. E por fim, como a AqueFr (128 mg/kg)

apresentou uma atividade hipotensora nos estudos anteriores, traçamos um perfil fitoquímico da mesma e identificamos a isovitexina como seu principal metabólito. Analisamos o efeito da AqueFr e a isovitexina em leitos mesentéricos isolados de ratos e os mecanismos envolvidos na hipotensão. Pesquisamos o envolvimento do ON, PGI_2 e dos canais de K^+ (sensíveis ao Ca^+, à voltagem e ao ATP). Leitos mesentéricos foram isolados, perfundidos com os inibidores L-NAME (100 µmol), indometacina (1 µmol), KCl (40 mM), TEA (1 mM), 4-aminopiridina (10 µmol- inibidor dos canais de K^+ sensíveis à voltagem) e glibenclamida (10 µmol- inibidor seletivo dos canais de K^+ sensíveis ao ATP- Kir6.1) e administramos diferentes concentrações da AqueFr (0,01- 0,1 mg) e da isovitexina (100- 1000 nmol). Ambos os tratamentos apresentaram atividade hipotensora pela via do ON e por estimular os canais de K^+ sensíveis ao Ca^+ e ao ATP. E finalmente, o ESTE não apresentou atividade diurética significativa e nem atividade hipotensora. Avaliamos se ESTE apresentava atividade antioxidante *in vitro* através de sua capacidade de eliminar os radicais livres DPPH e de impedir a hemólise oxidativa ocasionada em eritrócitos humanos pelo 2,2-Azobis (2-amidinopropano) dihydrochloride (AAPH). Apesar de o ESTE não apresentar atividade diurética e hipotensora, demonstrou ter uma interessante atividade antioxidante. Desta forma, concluímos que a *A. hispidum* não foi diurética, porém apresentou atividade hipotensora, a *L. divaricata* apresentou atividade diurética, salurética e hipotensora e a *T. esculenta* não foi diurética nem hipotensora, entretanto apresentou atividade antioxidante. Os efeitos diuréticos e saluréticos da *L. divaricata* se mantiveram por sete dias. Suas frações AqueFr e ButFr também apresentaram atividade diurética e hipotensora, seus mecanismos de ação foram elucidados em órgãos isolados sendo que atuam pela via do ON e das PGI_2, agindo em diferentes canais de K^+. A *L. divaricata* apresentou melhores efeitos farmacológicos do que as outras duas espécies. Seus efeitos diuréticos e hipotensores corroboram o uso popular no Pantanal sul-mato-grossense podendo ser uma espécie potencial para o desenvolvimento de novos fármacos.

Palavras-chave: *Acanthospermum hispidum, Luehea divaricata, Talisia esculenta*, plantas diuréticas, plantas hipotensoras

Ethnopharmacological investigation of the cardiovascular and renal effects of three medicinal species of Pantanal sul-mato-grossense

ABSTRACT

Cardiovascular diseases are among the leading causes of death worldwide. Medicinal plants can be viable alternatives to the development of new drugs to control these diseases. The aim of this work was to investigate *Acanthospermum hispidum*, *Luehea divaricata* and *Talisia esculenta*, which are medicinal plants popularly used for the treatment of diseases affecting the cardiovascular and renal systems in the Pantanal region of Mato Grosso do Sul state, Brazil. First, we performed a morphological and histochemical evaluation to adequately identify the three species under study. After being properly identified, the aerial parts of *A. hispidum* and leaves of *L. divaricata* and *T. esculenta* were collected. By the infusion method (100 g/1000 mL filtered water) we prepared the crude extract with the aerial parts of *A. hispidum* and with the leaves of the *L. divaricata* e *T. esculenta*. Ethanol (PA) was used to purify the extract and the ethanolic supernatant (ES) was used for the tests. The phytochemical profile of the ESAH, ESLD e ESTE was revealed and all extracts presented phenolic compounds. Male and female Wistar rats were used for the pharmacological and acute toxicity studies. By the acute toxicity test, different groups of male and female rats received 5, 50, 300 e 2000 mg/kg (single dose, v.o.) of the three extracts or filtered water (5 mL/100g). On the 15^{th} day, blood and tissue samples were obtained for biochemical and histological analyzes. Extracts promoted no signs of toxicity and, therefore, pharmacological studies were conducted. First, we analyzed the diuretic effects in different groups of rats (30, 100 and 300 mg/kg, v.o.). After 4-24 hours (acute treatment) or daily for seven days (prolonged treatment) the urinary volume was determined. Furthermore, sodium, potassium, chloride, pH and density were measured in urine samples. In a second moment, we analyzed the effects of the three extracts on systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) in normotensive rats. For this, animals were anesthetized with ketamine/xilasin (100/20 mg/kg, i.m.). A polyethylene catheter was introduced into the right carotid artery, and blood pressure (BP) was then measured. The extracts, at the same doses of diuresis, were administered directly into the duodenum of animals. Hydrochlorothiazide (HCTZ 25 mg/kg) and filtered water (5 mL/kg) were used as controls. ESAH did not induce diuresis, however, it presented hypotensive effect (reduced SBP and MAP at the dose of 30 mg/kg). Based on these results, we analyzed the role of nitric oxide (NO), prostacyclin (PGI_2) and K-channel involvement in this activity. Different

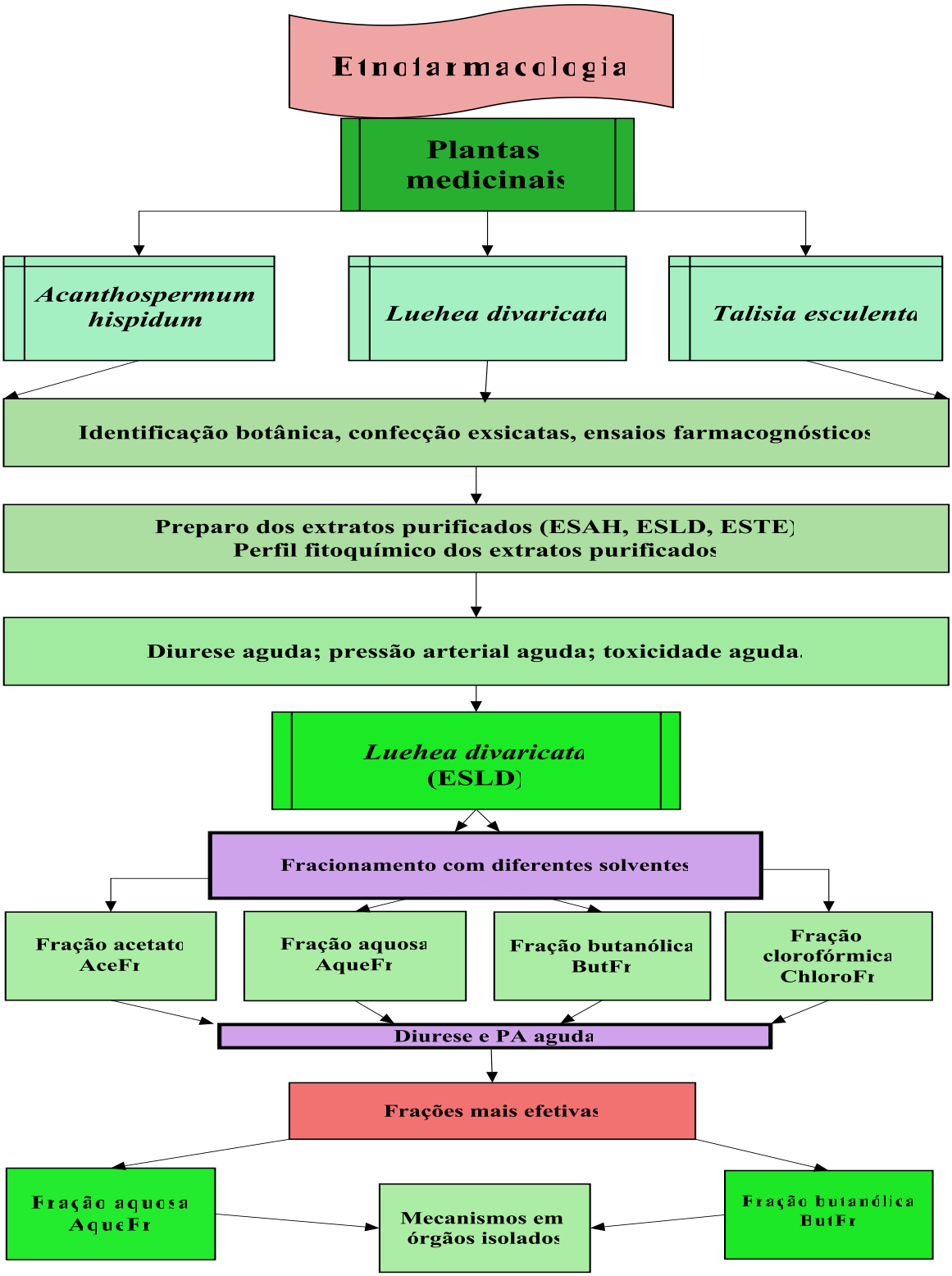
groups of rats were continuously infused through the femoral vein with L-NAME (7 mg/kg/min, NO synthase inhibitor), indomethacin (3 mg/kg/min., a non-selective cyclooxygenase inhibitor), methylene blue (150 nmol/kg/min, glanilate cyclase inhibitor) or TEA (400 μmol/kg/min., a non-selective calcium-sensitive K^+ channel bloker (KCa)). ESAH (30 mg/kg, i.d.) was then administered. We observed that the ON pathway was involved in the ESAH hypotensive activity, as well as the Ca^+ sensitive K^+ channels. ESTE showed no significant diuretic or hypotensive activity. We then evaluated if ESTE presented *in vitro* antioxidant activity through its ability to eliminate free radical DPPH and to prevent oxidative hemolysis caused in human erythrocytes by AAPH. Although ESTE presented no diuretic and hypotensive activity, it has been shown to have an interesting antioxidant activity. Finally, based on the initial results where ESLD presented diuretic, saluretic and hypotensive effects, and the first two activities were maintained for seven days, we performed the fractionation of this extract with solvents with different polarities. The ESLD (15 g.) was solubilized in water (500 mL) and sequentially fractionated with chloroform, butanol and ethyl acetate resulting in fractions ChloroFr, ButFr and AceFr, respectively. The aqueous residue generated the aqueous fraction (AqueFr). These fractions were evaluated for diuretic and acute hypotensive effects. The fractions Acefr (6 mg/kg), AqueFr (128 mg/kg) and ButFr (65 mg/kg) presented diuretic activity after 24 hours. The last two fractions were also hypotensive, besides the saluretic activity presented by ButFr. From this result, we investigated the involvement of ON and PGI_2 in the diuretic activity of ButFr. Different groups of animals received, one hour earlier, L-NAME (60 mg/kg, v.o.) and indomethacin (5 mg/kg, v.o). They were then treated with ButFr (65 mg/kg, v.o.). Urinary volume was measured after 8 and 24 hours. The two pathways were involved with the diuretic activity of this fraction. We also investigated whether the diuretic, saluric and hypotensive effect (ButFr (65 mg/kg)) would be maintained after prolonged treatment (daily for seven days), which was also confirmed. Based on these data, we decided to analyze this fraction in isolated organs. For this, rats were anesthetized and their mesenteric beds and kidneys were removed and perfused with adequate solution and arterial pressure was measure. After stabilization of the arterial pressure, the organs received different concentrations of ButFr. The most effective concentrations of ButFr (0.01-0.1 mg) were analyzed for ON and PGI_2 involvement in the diuretic and hypotensive activities presented. We perfused new beds and kidneys with the inhibitors L-NAME (100 μmol) and indomethacin (1 μmol) and then administered different concentrations of ButFr. Two paths were involved. Finally, since AqueFr (128 mg/kg) presented a hypotensive activity in previous studies, we elucidated its phytochemical profile and identified isovitexin as its main

metabolite. We investigated AqueFr and isovitexin in mesenteric beds isolated from rats and the mechanisms involved in hypotension. We verified the involvement of ON, PGI2 and K^+ channels (sensitive to Ca^+, voltage and ATP). We isolated the beds, perfused with the inhibitors L-NAME (100 μmol), indomethacin (1 μmol), KCl (40 mM, K^+), TEA (1 mM), 4-aminopyridine (10 μmol- a voltage-dependent (KV) K^+ channels bloker) and glibenclamide (10 μmol- a seletive Kir6.1 ATP-sensive K^+ channels bloker) and administered different concentrations of AqueFr (0.01-0.1 mg) and isovitexin (100-1000 nmol). Both treatments presented hypotensive activity via NO and stimulated K^+ channels sensitive to Ca^+ and ATP. Thus, we conclude that although *A. hispidum* was not diuretic, it had hypotensive activity, *T. esculenta* was neither diuretic nor hypotensive but presented antioxidant activity and *L. divaricata* presented diuretic, salinetic and hypotensive activity. The diuretic and saluretic effects of *L. divaricata* were maintained for seven days. Its fractions (AqueFr and ButFr) presented diuretic and hypotensive activity and the mechanisms of action were elucidated in isolated organs, acting through the ON and PGI_2 pathways acting on different K^+-channels. *L. divaricata* presented better pharmacological effects than the other two species and its diuretic and hypotensive effects corroborate the popular use in Pantanal. Therefore, it can be considered a potential species for the development of new drugs.

***Keywords*:** *Acanthospermum hispidum*, *Luehea divaricata*, *Talisia esculenta*, diuretics plants, hypotensive plants.

SUMÁRIO

RESUMO GRÁFICO



1 INTRODUÇÃO

Apesar da farmacologia moderna ter sua principal base na industrialização de produtos sintéticos, a Organização Mundial da Saúde (OMS) vem recomendando aos países membros da Organização das Nações Unidas (ONU) formular e desenvolver políticas para o uso de medicina complementar e alternativa. No Brasil, seguindo esta tendência mundial, o Ministério da Saúde (MS) lançou no ano de 2006 duas importantes políticas para o setor de plantas medicinais e fitoterápicos, a portaria N° 931 que aprovou a Política Nacional de Práticas Integrativas e Complementares (PNPIC) no Sistema Único de Saúde (SUS) e o Decreto N° 5.813 que aprovou a Política Nacional de Plantas Medicinais e Fitoterápicos (PNPMF). Estas duas políticas apresentam entre suas diretrizes o incentivo à pesquisa e o desenvolvimento de meios práticos para o uso de plantas medicinais e fitoterápicos com o objetivo de disponibilizar à população esta alternativa terapêutica com qualidade, segurança e eficácia, priorizando a biodiversidade do país e promovendo um maior acesso a tratamentos seguros e eficazes (BRASIL, 2006 a,b).

Neste sentido, as pesquisas que levam em conta o conhecimento das populações no Brasil e ao redor do mundo são de fato uma importante ferramenta para investigar novas plantas medicinais, que tenham potencial farmacológico e que possam ser utilizadas para o desenvolvimento de novos fitoterápicos ou fármacos oriundos de produtos naturais. Newman e Cragg (2012) demonstraram que em 30 anos, de 1981 a 2010, apenas 79 substâncias anti-hipertensivas foram produzidas ao redor do mundo com base em produtos naturais. Em nosso país apesar dos inúmeros relatos de diversas plantas utilizadas como diuréticas, anti-hipertensivas, cardiotônicas, e para afecções renais, nenhum medicamento oriundo de plantas medicinais foi desenvolvido neste período para tratar as doenças do sistema cardiovascular e renal (BIESKI et al., 2012; BOLSON et al., 2012; 2015; LORENZI, 1982; NETO, 2003; OLIVEIRA et al., 2011).

A HA é considerada o principal fator de risco para as doenças cardiovasculares (DCVs) (SBC, 2106). As DCVS são as principais causas de mortes no mundo, sendo que 80% destas mortes ocorrem em países de baixa e média renda. Estima-se que até 2030 mais de 23,6 milhões de pessoas morram devido as DCVs. O custo destas doenças, ao redor do mundo, pode chegar a 47 trilhões de dólares nos próximos 25 anos. Neste contexto, a hipertensão arterial sistêmica (HAS) se destaca entre os fatores de risco das DCVs. De fato, o número de hipertensos saltou de 600 milhões em 1980 para 1 bilhão em 2008 (LASLETT et al., 2012; WHO,2017).

Atualmente, a terapia medicamentosa para o tratamento da HAS está bem estabelecida, mas apesar disso, o controle da pressão arterial (PA) tem sido ineficaz no Brasil e em vários países do mundo (SBC, 2016; WHO, 2016). Muitos são os motivos para esta ineficácia, tais como: a natureza assintomática da HAS, a falta de hábito em medir a Pressão arterial (PA), o desconhecimento da própria doença, a falta de conhecimento quanto à gravidade da doença, a crença de que o uso contínuo de medicamentos faz mal à saúde; o custo elevado dos tratamentos, a quantidade necessária de fármacos (dois ou mais) para o controle da PA e a falta de acesso às terapias e as consultas médicas. Geralmente os anti-hipertensivos apresentam diversos efeitos colaterais, tais como: boca seca, tontura, desconforto gastrointestinais, distúrbios visuais, distúrbios sexuais, enxaqueca, tosse e até transtornos emocionais. De fato, estes efeitos colaterais diminuem muito a qualidade de vida dos pacientes levando ao abandono das terapias (KROUSEL WOOD et al., 2009; KLARENBACH et al., 2010; LEWIS et al., 2012; SULTANA, 2017).

Neste contexto acredita-se que a adesão às terapias alternativas e complementares será maior, especialmente àquelas terapias provenientes de plantas medicinais, que tradicionalmente são bem aceitas e podem ter reduzidos efeitos colaterias. Estudos que visam investigar criteriosamente plantas medicinais oriundas do conhecimento popular são necessários e devem ser contínuos para que este conhecimento não seja perdido ao longo do tempo. Além disso, espera-se que as espécies medicinais uma vez identificadas e estudadas possam ter seu uso padronizado e seguro. Assim, nesse trabalho, propomos avaliar três espécies utilizadas no Pantanal brasileiro para tratamento de doenças que afetam o sistema cardiovascular e renal, sendo elas: *Acanthospermum hispidum*, *Luehea divaricata* e *Talisia esculenta*. Realizamos um estudo criterioso de identificação e caracterização fitoquímica, morfoanatômica e histoquímica das três espécies, avaliamos o seu potencial farmacológico sobre o sistema cardiovascular e renal e investigamos a toxicidade aguda.

2 REVISÃO DE LITERATURA

2.1 Mecanismos fisiológicos da regulação da PA e o papel central do rim na regulação a médio e longo prazo

Em condições fisiológicas normais a PA varia constantemente, porém raramente desvia dos valores de referência (120 mm Hg para a pressão sistólica e 80 mm Hg para a pressão diastólica). Isto é possível porque o organismo possui mecanismos de controle que

conseguem restabelecer a pressão para a normalidade mediante situações que a alteram. Estes mecanismos de controle ocorrem em curto prazo (reações em segundos) desempenhado pelos barorreflexos, quimioreflexos e sistema nervoso central, e em médio e longo prazo (minutos, horas ou dias) devido à ação principalmente dos rins (HALL, 2016).

Os rins exercem um papel fundamental para o controle da PA através do sistema renina-angiotensina-aldosterona (SRAA), este sistema regula o tônus vascular, o volume de fluidos corporais, o balanço eletrolítico, a secreção hormonal e a atividade neuronal (GONZALEZ et al., 2005; WANG et al., 2007). A ativação deste sistema ocorre quando a baixa pressão sanguínea faz com que o fluxo de sangue para os rins se reduza abaixo do normal alterando as concentrações de sódio nos néfrons. Isto faz com que células justaglomerulares secretem renina, a renina por sua vez catalisa a conversão do angiotensinogênio em angiotensina I, que é convertida à angiotensina II pela enzima conversora de angiotensina (ECA). A angiotensina II é o principal peptídeo efetor na vasculatura das células musculares lisas vasculares (CMLV), ao se ligar nos receptores AT1 desencadeia a vasoconstrição, liberação da aldosterona, que por sua vez aumenta a atividade da bomba de Na^+/K^+/ATPase propiciando uma importante reabsorção de eletrólitos e água pelos túbulos renais, consequentemente reestabelecendo os níveis pressóricos (HALL, 2016; NGUYEN DINH CAT; SAVOIA et al., 2011; TOUYZ, 2011).

Apesar dos sistemas fisiológicos realizarem os ajustes da PA, em determinadas situações estes sistemas podem falhar, as causas destes desajustes são multiforias e, quando a PA se mantem constantemente acima dos níveis pressóricos normais ocorre à HAS. A HAS é considerada um dos principais fatores de risco das doenças cardiovasculares (DCVs) (WHO, 2017).

2.2 Doenças cardiovasculares

As DCVs incluem o conjunto de doenças do coração, as doenças vasculares cerebrais e periféricas e as suas relações com a função renal, neuroquímica e hormonal. Entre estas doenças estão as que se originam da aterosclerose como a doença isquêmica cardíaca, a doença da artéria coronária, o acidente vascular encefático (AVE), as doenças vasculares periféricas e a HAS. Outras causas de DCVs são as doenças cardíacas congênitas como cardiomiopatias, arritmias cardíacas, e as doenças cardíacas reumáticas (LASLETT et al., 2012).

Segundo a OMS as DCVs são responsáveis pela morte anual de 17,7 milhões de pessoas ao redor do mundo, chegando a 31% do total global de mortes. Destas mortes, mais de 3 milhões ocorrem prematuramente em pessoas com menos de 60 anos de idade. O percentual de mortes prematuras em países com alta renda é de apenas 4%, enquanto que em países de baixa renda chega a 42%. De fato, estima-se que 80% das DCVs ocorram em países em desenvolvimento (WHO, 2011).

Esta grande diferença percentual entre os países, envolvem sem dúvida os fatores comportamentais das populações e os programas em atenção primária de saúde de seus governos. Os fatores de risco comportamentais das DCVs incluem o uso excessivo de sal, uso de tabaco, inatividade física, uso excessivo do álcool, o diabetes, o sobrepeso, a obesidade e o colesterol elevado. Em 2016, a OMS lançou a campanha mundial "Global Hearts Initiative" que está apoiando os governos de todo o mundo para expandir os esforços de prevenção e controle destas doenças através de três orientações técnicas: controlar o uso do tabaco, reduzir o consumo de sódio e fortalecer os cuidados primários de saúde. Outros órgãos e associações em diferentes locais do mundo tais como a Associação Americana de Cardiologia, a Federação Mundial de Cardiologia e a Sociedade Europeia de Cardiologia estabeleceram como alvos até 2025 diminuir em 10% o consumo do álcool, em 15% a ingestão de ácidos graxos saturados, reduzir em 10% o sedentarismo e "frear" o aumento da obesidade (LASLETT et al., 2012; WHO, 2011;2016). Todos estes fatores comportamentais levam à HAS que é um dos principais fatores de risco das DCVs.

2.2.1 Hipertensão arterial sistêmica

A HAS é uma condição clínica multifatorial caracterizada pela elevação da pressão arterial sustentada, com níveis pressóricos ≥ 140 e/ou 90 mm Hg. Frequentemente a HAS está associada com distúrbios metabólicos e alterações funcionais e/ou estruturais de órgãos alvos, sendo agravada pela presença de outros fatores de risco como a dislipidemia, obesidade abdominal, intolerância à glicose e diabetes melito. A HAS pode levar a eventos como morte súbita, AVE, infarto agudo do miocárdio (IAM), insuficiência cardíaca (IC), doença arterial periférica (DAP) e doença renal crônica (DRC) (SBC, 2016).

A OMS estima que 1,1 bilhão de pessoas ao redor do mundo possuam a PA elevada e apenas 1 a cada 5 destas pessoas a controlam (WHO, 2017). Dados norte-americanos de 2015 revelaram que a HAS estava presente em 69% dos pacientes com IAM, 77 % dos pacientes com AVE, 75% dos pacientes com IC e em 60 % daqueles com DAP. A HAS foi responsável

por 45% das mortes por patologias cardíacas e por 51% das mortes decorrentes de AVE. No Brasil a HAS atinge 32,5% da população adulta e mais de 68% da população idosa, contribuindo com 50% das mortes por DCVs. Suas complicações cardíacas, renais e vasculares cerebrais tiveram impacto elevado na renda familiar, estimado em 4,18 bilhões de dólares entre os anos de 2006 e 2015 (MOZAFFARIAN et al., 2015; SBC, 2016).

A HAS pode ser classificada em dois tipos: primária (essencial) e secundária. A hipertensão secundária, que afeta de 5-10% dos indivíduos hipertensos, é acarretada, sobretudo, por causas conhecidas e detectáveis, como algumas doenças endócrinas, vasculares e do parênquima renal. Por outro lado, a HAS primária se instala devido a fatores poligênicos, como idade, dieta, estilo de vida, atividade neuro-humoral, dentre outros. A maioria dos indivíduos hipertensos (90-95 %) apresentam HAS sem uma causa orgânica detectável. De fato, esse tipo de hipertensão é mais difícil de ser identificada, retardando o início dos tratamentos (AL DISI et al., 2015).

Fatores como consumo excessivo de sal, excesso de peso, obesidade, tabagismo, ingestão excessiva de álcool e sedentarismo contribuem significativamente para o desenvolvimento da HAS. Alguns fatores socioeconômicos também estão relacionados ao aumento da PA. No Brasil, adultos com menor escolaridade apresentam maior prevalência (31%), enquanto em indivíduos de nível superior (18,2%) e com ensino médio completo (16,7%) a prevalência é menor (BRIASOULIS et al., 2012; ZHAO et al., 2011).

Os fatores de risco que aumentam a PA e que não fazem parte do estilo de vida dos indivíduos contribuem apenas em 30% com o perfil da PA, portanto, mudanças comportamentais são fundamentais para que se tenha um controle efetivo da patologia (RIED; FAKLER, 2014). A Sociedade Brasileira de Cardiologia (SBC) aconselha como primeira medida de controle da PA, através de sua 7° Diretriz Brasileira de Hipertensão Arterial, lançada em setembro de 2016, um controle “agressivo” não medicamentoso, sobretudo através da mudança de estilo de vida, tais como: diminuição do peso corporal através da prática de exercícios físicos, diminuição do consumo de sódio e álcool, abolição do fumo, controle de estresse, ingestão de alimentos saudáveis como fibras, oleaginosas, laticínios com baixo teor de gordura e ricos em vitamina D, consumo de chá verde e café em pequenas quantidades, e o consumo de alho. A SBC não faz referência a medicamentos fitoterápicos, pois apesar dos inúmeros estudos científicos de diversas plantas medicinais que atuam no controle da PA, não existe no mercado farmacêutico brasileiro nenhum fitoterápico para este fim.

De fato, apesar de existirem inúmeras plantas medicinais utilizadas pela medicina tradicional para diminuírem a PA (SULTANA, 2017), especialmente aquelas que apresentam atividades diuréticas (WRIGHT et al., 2007), ainda é muito restrito o número de produtos naturais indicados pela classe médica e utilizados na terapêutica convencional. Apesar disso, as pessoas utilizam as plantas medicinais, mesmo sem a indicação médica, como adjuvante em suas terapias, Agbabiaka et al. (2017) demonstraram que diversos pacientes utilizam produtos naturais derivados de plantas associado com os seus medicamentos anti-hipertensivos e diuréticos convencionais.

Sabe-se que quando os órgãos internacionais e nacionais incentivam seus pesquisadores a investigarem, na medicina tradicional de suas populações, novas plantas medicinais com potencial farmacêutico, não é com o objetivo de substituir as medicações existentes e sim aumentar as opções terapêuticas. Realmente, o arsenal medicamentoso para o controle da PA é bem amplo. A SBC recomenda o uso de diuréticos como os tiazídicos ou similares (clortalidona, hidroclorotiazida e indapaminda); de agentes de ação central (metildopa, clonidina, moxonidina), de beta-bloqueadores (carvedilol e nebivolol); de alfa-bloqueadores (doxazosina e prazosina); dos vasodilatadores diretos (hidralazina, nitroprussiato e minoxidil); de inibidores da ECA (captopril, enalapril, lisinopril, etc.), dentre outros. A SBC recomenda que os medicamentos para a HA sejam utilizados isoladamente ou em associação, e devem ter as seguintes características: ser eficaz por via oral, ter demonstrado a capacidade em reduzir a morbidade cardiovascular, ser bem tolerado, poder ser utilizado em um menor número de tomadas diária, ser iniciado com as menores doses efetivas, poder ser usado em associação, ser utilizado por um período mínimo de quatro semanas antes de modificações e ter aquedado controle de qualidade em sua produção (SBC, 2016).

Uma das principais estratégias para o tratamento medicamentoso da HAS se refere aos medicamentos diuréticos. Geralmente é a primeira escolha medicamentosa, aliados a mudança de estilo de vida, especialmente no estágio inicial da hipertensão (PSATY et al., 1997; SBC, 2016). Os diuréticos exercem suas atividades principalmente pela redução do volume extracelular e por seus efeitos natriuréticos. Este efeito pode ser decorrente, dentre outros, da inibição do co-transportador de $Na^+/K^+/2Cl^-$ na alça de henle (diuréticos de alça), do bloqueio do co-transportador Na^+/Cl^- no túbulo contorcido distal (tiazídicos), do antagonismo dos receptores da aldosterona, do bloqueio dos canais de Na^+ no epitélio renal e/ou por alteração no fluxo sanguíneo renal (LAURENT, S., 2017). Durante o tratamento prolongado o efeito hipotensor é mantido devido a uma redução da resistência periférica total, que pode ser

acompanhada da normalização do débito cardíaco, e do volume extracelular. Estudos demonstram que as drogas diuréticas modulam canais de K^+ e o efeito natriurético prolongado pode reduzir secundariamente a concentração intracelular do Ca^+ resultando em vasodilatação e consequentemente hipotensão (CHADWICK et al., 2017; HERAN et al., 2012; ROUSH; BUDDHARAJU; ERNST, 2013; WARGO; BANTA, 2009).

Apesar da grande variedade de medicamentos para o tratamento da HAS apenas 34% dos pacientes tem a sua PA controlada (AUGUST, 2004; SUSALIT et al., 2011; WANG, XIONG, 2012). Sem dúvida, novas opções terapêuticas, com menores efeitos colaterais, provenientes de plantas medicinais serão muito bem aceitas pelas populações pelo forte apelo cultural e tradicional que elas exercem. Sendo assim, estudos criteriosos que investiguem o potencial farmacológico, a eficácia e a segurança das plantas medicinais são de fundamental importância.

2.3 Etnofarmacologia como ferramenta de pesquisa de novos fármacos

O uso plantas medicinais pelo homem como alternativa para suas doenças data de milhares de anos. Os primeiros registros são oriundos da Mesopotamia há 2600 anos antes de Cristo. Entre as espécies relatadas nessa época estão o *Cedrus sp.* (espécies de cedro), *Cupressus sempervirens* (cipreste italiano), *Glycyrrhiza glabra* (alcaçuz) e a *Commiphora sp*. (mirra). Existem descrições sobre o uso da *Papaver somniferum* (papoula) e da *Cannabis sativa* (maconha) há mais de 4 mil anos (DUTRA et al., 2016; GURIB FAKIM, 2006).

Notavelmente, ainda nos dias atuais, as populações ao redor do mundo, especialmente aquelas que vivem em países em desenvolvimento relatam o uso de plantas medicinais como terapia alternativa de suas doenças crônicas e muitas vezes como única fonte de terapia viável. Segundo a OMS, em países da África, em torno de 80% de suas populações fazem uso de plantas medicinais ou produtos derivados de plantas medicinais (WHO, 2014).

Por outro lado, a pesquisa de constituintes ativos das plantas medicinais somente começou no século XIX. Entre 1806 e 1869 foram isolados importantes constituintes farmacológicos tais como: os alcaloides morfina, codeína e a papaverina da *Papaver somnniferum*, a atropina da *Atropa beladona*, a digoxina da *Digitalis lanata,* a cafeína da *Coffea arábica* e a salicina da *Salix alba* (DUTRA et al., 2016).

Ainda hoje as plantas medicinais exercem um importante papel na medicina moderna e são utilizadas para a síntese de compostos farmacêuticos mais complexos. Estima-se que

50% dos medicamentos disponíveis na terapia atual derivaram de fontes naturais como plantas e microrganismos, e em algumas áreas terapêuticas como a oncológica, antimicrobiana e imunossupressora, este índice chega a 60% (CALIXTO, 2005; MISHRA et al., 2011; PAN et al., 2013).

Nas últimas décadas vários medicamentos desenvolvidos pela indústria farmacêutica foram derivados de plantas medicinais, tais como o fitohormônio diosgenina derivado da *Dioscorea* spp., a reserpina, um alcaloide anti-hipertensivo derivado de espécies de *Rauwolfia*, a pilocarpina, derivada da *Pilocarpus spp*., além de importantes agentes antitumorais como a vimblastina e vincristina, derivados da espécie *Catharanthus roseus*, o taxol derivado da *Taxus brevifolia*, e a podofilotoxina derivado do *Podophyllum peltatum* (GURIB FAKIM, 2006).

O interesse em produtos naturais derivados de plantas medicinais tem aumentado significativamente ao redor do mundo, especialmente em países desenvolvidos como os Estados Unidos e alguns países da Europa. O mercado mundial de agentes fitoderivados movimentou 27 bilhões de dólares em 2014, com maior representatividade na Europa (30%), Asia (27%) e Estados Unidos (18%) (DUTRA et al., 2016). Alguns dos fatores que contribuem para este crescimento envolvem a preferência por terapias naturais, a crença de que os fitoterápicos não fazem mal ou produzem menos efeitos colaterais, a busca por medicinas alternativas, a preferência por terapias preventivas, a simplicidade para o registro em alguns países, e o alto custo dos medicamentos sintéticos (CALIXTO, 2005).

Com uma extensa e diversificada flora, por séculos as plantas medicinais têm sido largamente utilizadas no Brasil, especialmente por populações nativas, que tem dificuldade ao acesso à grandes centros urbanos e tem como prática o uso de plantas medicinais como primeira ou única opção terapêutica. O Brasil é um país privilegiado com a maior biodiversidade do planeta, contém mais de 46 mil espécies de plantas descritas, que correspondem a aproximadamente 22% de todas as plantas existentes no mundo (CALIXTO, 2005).

Muitas classes de princípios ativos têm sido isoladas de plantas medicinais brasileiras e um grande número de pesquisadores brasileiros com conhecimentos em farmacologia, quimiotaxonomia, química de produtos naturais e outras áreas relacionadas tem se esforçado para o desenvolvimento de novos fitoterápicos a partir de plantas nativas do país. Porém, apesar de todos os esforços, poucos produtos foram desenvolvidos exclusivamente no país. Um exemplo foi o desenvolvimento do Acheflan®, primeiro anti-inflamatório produzido no país, com tecnologia totalmente brasileira. Este medicamento foi produzido a partir do extrato

de uma planta da mata Atlântica a *Cordia verbenaceae,* utilizada popularmente para tratar de afecções localizadas de pele. O produto sob a forma farmacêutica de creme foi liberado pela Agência Nacional de Vigilância Sanitária (ANVISA) sob a classificação de fitomedicamento por ser um fármaco que possui em sua composição somente substâncias ativas extraídas de plantas, ou seja, sem a mistura de princípios ativos sintéticos (CALIXTO, 2005; VALLI et al., 2013).

Apesar da grande representatividade do Brasil na flora mundial, a forte tradição popular no uso de plantas medicinais, e todos os esforços dos pesquisadores brasileiros, o mercado destes medicamentos ainda é tímido no país. A venda mundial desta classe de medicamentos em 2014 em países desenvolvidos foi significativamente maior quando comparado com o Brasil. De fato, neste ano o país vendeu apenas 261 milhões de dólares, o que representou apenas 5% da venda mundial. Apesar do Acheflan® estar entre os 20 fitoterápicos mais vendidos no país, os outros 19 produtos são oriundos de plantas de outros países, especialmente da Europa e África, demonstrando uma discrepância entre os esforços dos pesquisadores brasileiros na descoberta de novas plantas com potencial terapêutico, e o número de produtos inovadores desenvolvidos no país (DUTRA et al., 2016).

De fato, o processo para o desenvolvimento de um novo medicamento é demorado. Em média são gastos de 10 a 15 anos para realizar todos os ensaios farmacológicos e toxicológicos pré-clínicos e clínicos, e o custo pode alcançar 800 milhões de dólares (PAN et al., 2010; 2013). O processo de seleção de espécies vegetais para a pesquisa científica não é algo simples de ser feito, trata-se de um processo que pode ser determinado por meio de diversos tipos de abordagens, no entanto, três delas se destacam: a) abordagem randômica, b) abordagem quimiotaxonômica ou filogenética e c) abordagem etnofarmacológica (GURIB FAKIM, 2006; GYLLENHAAL et al., 2012; MACIEL, 2002).

A abordagem randômica é caracterizada pela escolha ao acaso de plantas medicinais para estudos fitoquímicos e farmacológicos. A probabilidade de encontrar plantas de interesse pelo método de randomização é muito baixa, somente 1 em cada 10 mil plantas pode ser útil às pesquisas (SIQUEIRA et al., 2012). A abordagem quimiotaxonômica, por outro lado, baseia-se na seleção de uma família ou gênero pela presença de um ou mais compostos químicos conhecidos de ao menos uma espécie do grupo. É o caso das espécies do gênero Vernonia, ricas em lactonas sesquiterpênicas, triterpenos, diterpenos, esteroides e flavonoides. As lactonas sesquiterpênicas constituem os marcadores para identificação do gênero e o principal ativo responsável pelas atividades antimicrobianas, antineoplásicas e antimaláricas descritas (ALIYU et al., 2015; PRATHEESHKUMAR et al., 2011).

O caminho apontado, entretanto, como o de maior probabilidade para a descoberta de novas substâncias bioativas envolve a seleção etnofarmacológica. Neste caso a espécie é selecionada com base na indicação por grupos populacionais a partir do conhecimento terapêutico construído localmente (GYLLENHAAL et al., 2012; MACIEL, 2002). O conhecimento tradicional e as práticas populares são fundamentais para aumentar a taxa de sucesso de descoberta de novas drogas, pois promovem a economia de tempo, de dinheiro e a probabilidade de toxicidade é muito menor. Mais de 74% dos compostos derivados de plantas medicinais foram oriundos de pesquisas etnofarmacológica (GURIB FAKIM, 2006; SIQUEIRA et al., 2012).

São inúmeros os exemplos de sucesso neste tipo de pesquisa. Bussmann et al. (2010) desenvolveram um estudo com 141 espécies vegetais do norte do Peru utilizadas tradicionalmente por curandeiros da região, nos quais os resultados apresentados confirmaram o potencial antimicrobiano da maioria dos extratos alcoólicos e aquosos testados. Kaileh et al. (2007), a partir de conhecimento popular de uma comunidade da Palestina, selecionaram 34 plantas medicinais usadas pela população local e extraíram quatro potentes espécies citotóxicas. Em um estudo conduzido no México, 75% das plantas pesquisadas para a atividade antibacteriana foram efetivas, corroborando o conhecimento popular daquela região (CANALES et al., 2005). Um dos medicamentos antitumorais mais vendidos do mercado americano, o Taxol® (paclitaxel), foi obtido a partir do conhecimento popular de americanos nativos do norte dos EUA. Entre os anos de 1960 a 1981 pesquisadores do Instituto Nacional do Câncer e do Departamento de Agricultura dos Estados Unidos investigaram 115 mil extratos obtidos de 15 mil espécies relatadas como antitumorais pela população local (WEAVER, 2014).

Pesquisas etnofarmacológicas são muito importantes para a constante busca por plantas com potenciais farmacológicos que possam colaborar com as populações no tratamento de suas doenças. De fato, apesar da grande diversidade de plantas medicinais no mundo, apenas 1% tem sido estudada quanto ao seu potencial farmacêutico. Das mais de 46 mil espécies no Brasil apenas 0,4% foi completamente investigada (GURIB FAKIM, 2006).

2.3.1 Metabólitos secundários de plantas medicinais

As plantas medicinais são fontes riquíssimas de compostos ativos que podem ser utilizados de forma integral, como é o caso dos fitoterápicos, bem como podem servir de fonte para a obtenção de novos compostos isolados ou modificados quimicamente para a produção

de medicamentos sintéticos. A descoberta de uma nova molécula ativa com base em plantas medicinais inicia-se pelo processo de extração do material vegetal. Os extratos são os produtos de uma interação entre uma substância vegetal e um solvente apropriado, o qual é evaporado até que se obtenha a consistência desejada (CECHINEL FILHO, 1998). A água é o solvente mais utilizado popularmente, geralmente as plantas medicinais são preparadas pelo método de infusão (chás) (BIESKI et al., 2012; BOLSON et al., 2015).

As plantas apresentam compostos ativos em todas as suas estruturas anatômicas como folhas, raízes, sementes, frutos e cascas sendo que as concentrações são distintas em cada estrutura. Devido ao custo de se utilizar todas as partes de uma planta, ou mesmo a inviabilidade de se obter algumas partes em algumas espécies, por exemplo, raízes de grandes árvores, o caminho mais viável é utilizar a parte empregada pela população. Outro ponto importante a ser lembrado é que os fatores ambientais como o tipo de solo, o clima, a luminosidade e a época da coleta, são fatores influenciadores na produção e concentração de compostos ativos das plantas (CECHINEL FILHO, 1998).

Os componentes ativos conhecidos como metabólitos secundários são compostos químicos produzidos por vias bioquímicas secundárias das plantas para a sua proteção contra herbívoros, radicais livres e raios ultravioleta. São produzidos também mediante ao ataque de patógenos como bactérias, fungos e vírus ou em situações de privação de nutrientes. Enquanto o metabolismo primário relaciona-se a uma série de processos fundamentais envolvidos na manutenção e desenvolvimento do vegetal, o metabolismo secundário proporciona o aumento da capacidade de sobrevivência e de superação dos desafios locais na interação da planta com o seu meio. Os compostos químicos originados pelo metabolismo secundário são específicos e determinados pelos diferentes gêneros e espécies. Em geral a energia gasta para sua síntese é muito maior do que a necessária para sintetizar metabólitos primários (KENNEDY; WIGHTMAN et al., 2011).

É vastíssima a diversidade de compostos fitoquímicos provenientes de plantas medicinais. Mais de 5 mil compostos foram obtidos de 3 mil plantas medicinais utilizadas pelas comunidades chinesas (ZHANG et al., 2017). Nos EUA, desde a década de 90, vem se extraindo compostos de plantas medicinais baseados no conhecimento popular e foram produzidos mais de 39 mil extratos naturais contendo esta diversidade de compostos fitoquímicos (SITHRANGA BOOPATHY; KATHIRESAN, 2010).

As principais classes de metabólitos secundários extraídos das plantas medicinais são: alcaloides, terpenoides, polifenóis, glicosídeos, óleos voláteis, ácidos orgânicos, ácidos graxos, proteínas, enzimas, sais minerais, oligoelementos, polissacarídeos, dentre vários

outros (MACIEL, 2002; PAN et al., 2013). É estimado que o número de metabólitos secundários exceda 200 mil, e a classe dos terpenos e alcaloides são consideradas umas das mais representativas, com mais de 30 e 20 mil compostos, respectivamente (MATSUURA et al., 2017).

A classe dos polifenóis é também muito representativa. São encontrados em uma grande diversidade de plantas e variam desde compostos simples com baixo peso molecular até compostos mais complexos como os flavonoides e taninos. Os flavonoides representam o maior e mais diversificado grupo de compostos polifenólicos, com mais de 6 mil estruturas identificadas. Os flavonoides podem ser divididos em mais de 10 diferentes subclasses, como as flavonas, isoflavonas, flavononas, e flavonols (SULTANA, 2017). Os flavonoides são também encontrados abundantemente em alimentos como frutas e verduras, e estudos tem sugerido que o seu consumo tem sido benéfico para vários tipos de doenças, inclusive as DCVs (KENNEDY; WIGHTMAN, 2011; MARTENS; MITHOFER, 2005).

Vários benefícios cardiovasculares dos flavonoides e de plantas ricas nesses compostos são descritos na literatura científica, tais como: ação ateroprotetora e estabilizadora de placas ateroscleróticas, antioxidante, inibidora da enzima conversora de angiotensina (ECA), vasodilatadora, anti-hipertensiva, antiplaquetária e antagonista dos receptores da angiotensina II (APPLOVA et al., 2017; BALASURIYA et al. 2012; DE ALMEIDA et al., 2018; HUANG et al., 2017; HOU et al., 2017; NAVARRO et al., 2018; PALOZI et al., 2017; PIRES et al., 2017; SHANKAR et al., 2017; SHAFAEI et al., 2016; TIRLONI et al., 2017; 2018; YANG et al., 2015).

2.3.2 Mecanismos moleculares e celulares das plantas medicinais na PA

A HAS pode ser causada ou catalizada pela alteração em três conjuntos de vasos sanguíneos, sendo eles as grandes artérias de condução (como a aorta), as pequenas artérias de resistência e a microcirculação (arteríolas e capilares). As alterações na reatividade vascular podem ser acarretadas devido a desregulação da enzima óxido nítrico sintase endotelial (ONSe) e enzimas pró-oxidantes, aos níveis basais de Ca^{+} aumentado devido a hiperatividade dos canais de Ca^{+} transmembrana, e/ou a existência de hiperplasia/hipertrofia das células do músculo liso vascular (remodelamento vascular). De fato, todos estes fatores isolados ou associados podem levar à um aumento da vasoconstrição e consequentemente a HAS (FOLKOW, 1990).

As CMLVs participam ativamente na patogênese da HAS. A sua proliferação contribui para aumentar a resistência periférica e diminuir o diâmetro das artérias (OPARIL; ZAMAN; CALHOUN, 2003). A angiotensina II regula a expressão e o crescimento das CMLVs (GU et al., 2017), enquanto o óxido nítrico (ON) e os nucleotídeos monofosfato cíclico de guanosina (GMPc) e o 3',5'- monofosfato cíclico de adenosina (AMPc) diminuem a sua produção (PILZ; CASTEEL, 2003). Algumas espécies de plantas medicinais já tiveram a capacidade de reduzir a produção das CMLVs comprovada. Entre essas destacam-se a *Allium sativum,* a *Camelia sinensis* e a *Salviae miltiorrhizae* (CHO et al., 2013; LUDWIG et al., 2004; RIED; FAKLER, 2014).

As células endoteliais são outro importante fator de controle do tônus vascular, e desempenham um papel significativo na homeostase ao longo da rede vascular. A função endotelial é regulada e mantida por diversos receptores de superfície celular, que induzem uma variedade de substâncias vasoativas que regulam o tônus vascular das CMLVs. As células endoteliais estimulam a produção de substâncias vasodilatadoras como o ON, as prostaciclinas (PGI_2) e os fatores hiperpolarizantes derivado de endotélio (FHDE), e vasoconstritoras como a endotelina, tromboxano A_2, fator de crescimento derivado de plaquetas (FCDP) e o ânion superóxido (O_2^-). Este estímulo pode ocorrer devido a ação de substâncias endógenas como a bradicinina e a acetilcolina, ou por estímulos mecânicos como o estresse de cisalhamento. Quando ocorre o desequilíbrio entre as substâncias vasodilatadoras e vasoconstritoras, é provável que se estabeleça um quadro de HAS (AL DISI et al., 2015; IGLARZ et al., 2007; TIRAPELLI et al., 2009).

O ON é frequentemente reconhecido como um importante fator vascular. Exerce um papel relevante na regulação da PA devido a sua potência vasodilatadora, bem como a sua capacidade de inibir a agregação de plaquetas nos vasos e inibir o crescimento das CMLVs. O ON é sintetizado na célula endotelial a partir da L-arginina através da ação da ONSe. Após a sua produção ele migra para as CMLVs ativando a guanilato ciclase (GCs) a qual converte a guanosina trifosfato (GTP) a GMPc. O GMPc modula os níveis de cálcio intracelular, promovendo a sua diminuição e consequentemente a vasodilatação. De fato, a vasodilatação é uma resposta final da hiperpolarização das membranas das CMLVs que ocorre devido a abertura dos canais de K^+ sensíveis ao Ca^{++}, à voltagem e ao ATP (FRANCIS et al., 2010).

Nos últimos anos várias espécies medicinais foram apresentadas como potenciais representantes capazes de aumentar os níveis de ON. O *Allium sativum, Andrographis paniculata, Camellia sinensis, Hibiscus sabdariffa, Cymbopogon citratus,* e *Crataegus* spp.

são algumas das várias espécies que já tiveram a sua capacidade vasodilatadora comprovada por aumentar os níveis de ON (AL DISI et al., 2015).

Assim como o ON as PGI_2 representam um importante agente local para a regulação do tônus vascular. Quando liberadas pelas células endoteliais as PGI_2 promovem a ativação de receptores acoplados à proteína G nas CMLVs. A ativação destes receptores aumenta a concentração de AMPc, que assim como o GMPc, diminui a concentração de cálcio intracelular promovendo a vasodilatação (YUHKI et al., 2010). De forma semelhante ao que ocorreu com o ON, a *Tropaeolum majus* e a *Centaurea cyanus* são espécies que já tiveram a sua capacidade em aumentar a PGI_2 comprovada (GASPAROTTO JUNIOR et al., 2012; KLIMAS et al., 2007).

O equilíbrio entre antioxidantes e pró-oxidantes no organismo também é importante para a manutenção adequada da PA. Quando ocorre um desequilíbrio entre oxidantes e antioxidantes pode ocorrer vários distúrbios no organismo como aterosclerose e outras complicações vasculares, incluindo a HAS (MONTEZANO et al., 2014). As espécies reativas de oxigênio (ERO) como o ânion superóxido (O_2^-) e o íon hidroxil (OH^-) tem um papel importante em promover o estresse oxidativo celular. O estresse oxidativo, por reduzir a viabilidade do ON, leva a uma importante disfunção endotelial. Assim que o ON é produzido este se liga as moléculas de O_2^- formando o peroxinitrito ($ONOO^-$), que por si só é um oxidante. O $ONOO^-$ além de diminuir a vasodilatação por diminuir a viabilidade do ON, também pode causar danos diretos nas células endoteliais (DRUMMOND et al., 2011). As ERO também são responsáveis pela oxidação da lipoproteína de baixa densidade (LDL) a qual resulta em inflamação e aumento da proliferação da CMLVs contribuindo com o aumento da PA (SLEVIN et al., 2012). Diversas espécies medicinais já foram descritas na literatura por diminuírem a PA devido a suas propriedades antioxidantes (AL DISI et al., 2015).

Outros fatores não menos importantes para a manutenção do tônus vascular são os FHDE como o íon K^+, o sulfeto de hidrogênio (H_2S), e os ácidos epoxieicosatrienóicos (EETs) que por aumentarem a GMPc e induzirem uma hiperpolarização das CMLVs devido a ativação dos canais de K^+ dependentes de Ca^+ promovem a vasodilatação (FRANCIS et al., 2010).

Algumas espécies medicinais também podem exercer seus efeitos diminuindo a pressão arterial por apresentar efeitos diuréticos ao atuarem sobre as bombas de Na^+/K^+/ATPase. Como a água corporal se difunde livremente entre espaços intra e extra-celulares em resposta à diferentes concentrações de solutos, sendo o Na^+ o soluto mais

abundante no meio extracelular, enquanto que o K^+ o mais abundante no meio intracelular, esta bomba é responsável por manter este equilíbrio. A inibição desta bomba pode levar a redução da reabsorção de Na^+ e K^+ tendo como consequência a eliminação dos mesmos juntamente com a água pelos rins. As espécies *Trapaeolum majus* e *Petroselinum crispum* são exemplos de espécies que inibem a bomba de Na^+/K^+/ATPase (GASPAROTTO JUNIOR et al., 2012; KREYDIYYEH et al., 2002).

Outras plantas podem exercer seus efeitos atuando na anidrase carbônica (AC) renal. As ACs são enzimas capazes de catalisar a hidratação reversível do dióxido de carbono (CO_2) em bicarbonato (HCO_3^-) e H^+. Estas enzimas exercem um papel crucial no transporte de CO_2 e HCO_3^-, na manutenção do pH sanguíneo e no transporte de eletrólitos. Estudos tem demonstrado que a inibição destas enzimas pode levar à vasodilatação renal e consequentemente aumento da diurese. Um estudo mostrou que a espécie *Amaranthus spinosus* pode exercer efeito diurético em ratos por inibir a AC (AMUTHAN et al., 2012).

Algumas espécies podem exercer atividades diuréticas atribuídas aos seus efeitos osmóticos. Na verdade, no processo de obtenção dos extratos vegetais podem ser extraídas de algumas espécies uma grande quantidade de solutos, como por exemplo, o Na^+ e o K^+, que em grande quantidade no organismo é eliminado através dos rins juntamente com uma grande quantidade de água. Um exemplo de espécie com este perfil é a *Carica papaya* que exerce seu efeito diurético por conter no extrato de suas sementes alta quantidade de sal (SRIPANIDKULCHAI et al., 2001).

O SRAA nos rins também contribui de forma significativa para o controle da PA. Estudos vêm sendo realizados ao longo de vários anos demonstrando a eficácia de algumas espécies de plantas medicinais que atuam no SRAA, *Allium sativum, Andrographis paniculata, Camellia sinensis, Salviae miltiorrhizae* e *Trapaeolum majus* (AL DISI et al., 2015; GASPAROTTO JUNIOR et al., 2012) são exemplos de espécies que já tiveram sua capacidade em reduzir a atividade da ECA e/ou bloquear os receptores AT1 da angiotensina II comprovados. Também nos rins algumas proteínas promovem a diminuição da PA por aumentar a diurese, exemplo disso são as aquaporinas (AQPs), que são proteínas responsáveis por regular o transporte de água pelas membranas celulares estabelecendo o balanço hídrico no corpo. Já foram identificadas 7 tipos de AQPs sendo que a isoforma AQP2 é a mais atuante em regular o balanço hídrico no ducto coletor. A permeabilidade a água no ducto coletor é regulada pela vasopressina (hormônio antidiurético) via AMPc. Quando o receptor V2 da vasopressina é ativado ocorre o aumento da permeabilidade à água nos ductos coletores via AQP2, sendo então a água reabsorvida. Quando os níveis de vasopressina diminuem, a

ação das AQP2 também é reduzida, eliminando maiores quantidades de água através da urina. Curiosamente, Kang et al. (2004) demonstraram que o efeito diurético da espécie *Salvia miltiorrhiza* deve-se a uma atuação da espécie nas AQP2.

Como podemos observar vários estudos têm demonstrado a ação de diferentes plantas medicinais e seus extratos em um ou em mais dos mecanismos supracitados. Assim, o seu potencial no controle da PA é enorme e deve ser seriamente investigado. A Figura 1 representa os principais alvos de ação das plantas medicinais sobre os mecanismos de controle da PA.

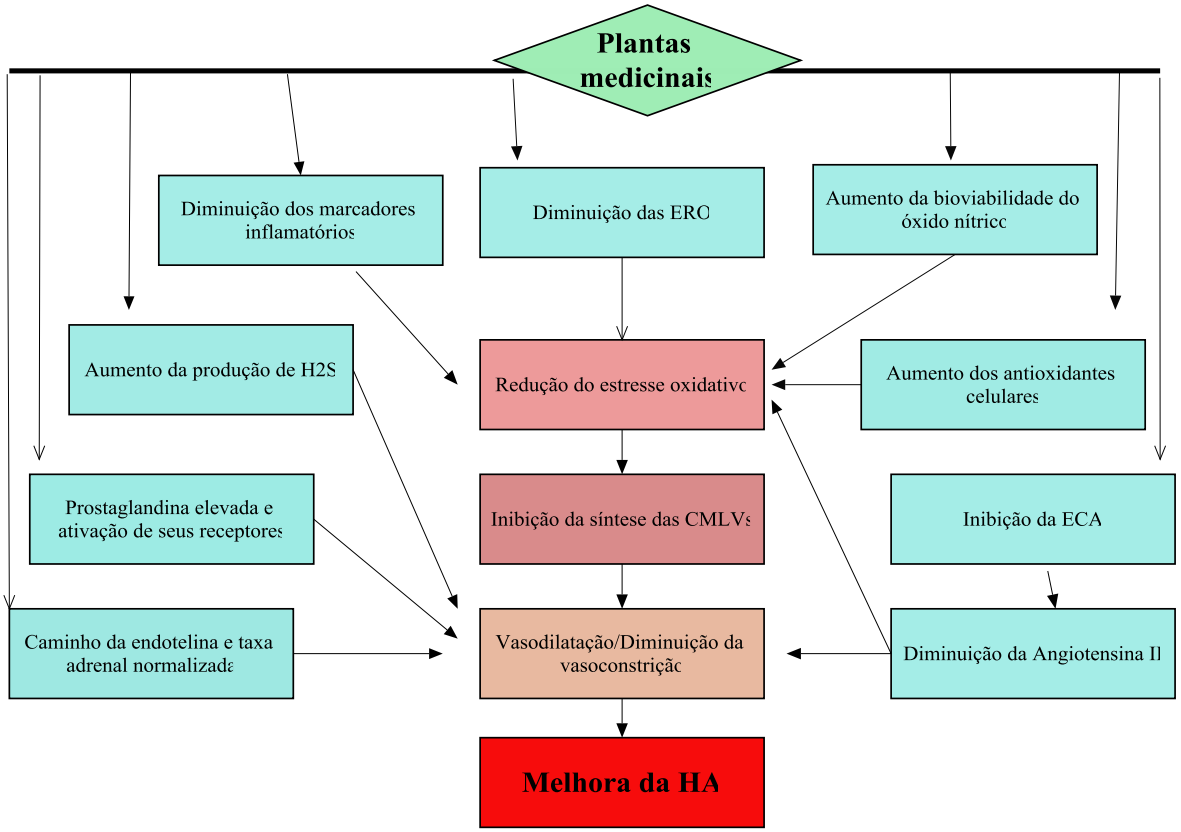


Figura 1- Alvos de atuação das plantas medicinais no controle da pressão arterial (Fonte: adaptado de Al Disi et al., 2015).

2.4 Bioma Pantanal e o conhecimento etnofarmacológico

O Pantanal é considerado umas das maiores áreas úmidas do planeta e foi declarado reserva da biosfera e patrimônio mundial natural pela Organização das Nações Unidas para a Educação, a Ciência e a Cultura (UNESCO). Contém milhões de hectares e está localizado no Brasil, Bolívia e Paraguai (Figura 2). No Brasil possui um território de 150.355 km^2

representando 1,76% do território nacional. Está presente nos estados do Mato Grosso e Mato Grosso do Sul. No Mato Grosso do Sul é onde está o maior percentual do seu território (64%), ocupando 25% do território total do estado (BRASIL, 2010; IBGE, 2004; IBAMA, 2008).

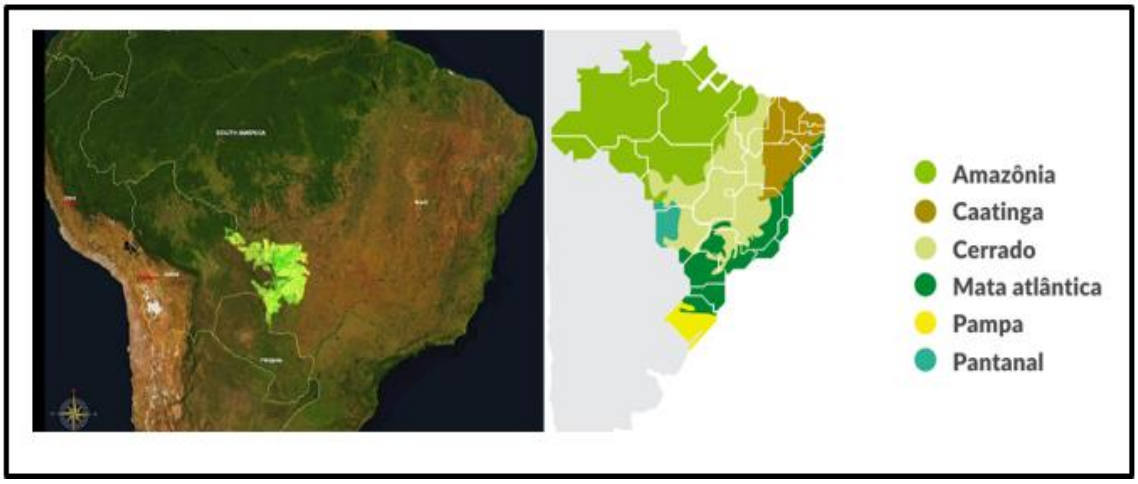


Figura 2: Distribuição geográfica do bioma Pantanal. Imagens aéreas da National Aeronautics and Space Administration (NASA) e mapa gráfico do Sistema de Informação Sobre a Biodiversidade Brasileira (SIBBr).

Segundo a Embrapa Pantanal quase duas mil espécies de plantas já foram identificadas no bioma e classificadas de acordo com seu potencial. Mas este número é ainda considerado muito pequeno pois muitas espécies ainda não foram criteriosamente estudas principalmente pela dificuldade de acesso nas áreas mais remotas (BRASIL, 2018; OLIVEIRA et al., 2011). Assim como a fauna e flora pantaneira são admiráveis, há de se destacar a rica presença das comunidades tradicionais como as indígenas, quilombolas, os coletores de iscas ao longo do rio Paraguai, comunidade amolar e Paraguai-mirim, dentre outras. Pelo fato das comunidades pantaneiras serem relativamente isoladas, suas populações desenvolveram uma íntima relação com as plantas medicinais, utilizando-as como fonte de tratamento para diversas de suas afecções, e preservando um riquíssimo conhecimento etnofarmacológico. O uso de plantas medicinais como propostas terapêuticas nessas populações tem sido reportado de longa data. Desde as primeiras colonizações da região, especialmente durante a guerra entre o Brasil e Paraguai nos anos de 1864 a 1870, atividades extrativistas têm sido intensificadas e passaram a fazer parte do estilo de vida dos pantaneiros, seja para fins medicinais ou para lhes trazer conforto em suas necessidades diárias (BIESKI et al., 2012; OLIVEIRA et al., 2011).

Atualmente o conhecimento do uso correto destas espécies se dá em grupos específicos da população conhecidos como raizeiros (geralmente pessoas com pouca educação formal, que obtem o conhecimento transmitido por seus pais ou de outras pessoas

com conhecimento empírico), benzedeiras (geralmente mulheres que tratam doenças distintas através da oração e ervas) e outros, geralmente idosos, pessoas que receberam informações medicinais de seus antepassados. Como esses grupos são restritos e muitas vezes ameaçados por mudanças ambientais, que alteram seu estilo de vida e cultura, a recuperação desse conhecimento etnofarmacológico é fundamental para resgatar tradições que podem ser perdidas (GUARIM NETO, 2006; OLIVEIRA et al., 2011).

Dado a importância deste conhecimento três espécies de plantas medicinais utilizadas nesta região do país foram investigadas neste trabalho. A abordagem escolhida foi a etnofarmacológica onde avaliamos plantas que são empregadas pelas populações pantaneiras para o tratamento de diferentes doenças cardiovasculares e renais, e que até então, não tinham sido investigadas de forma criteriosa quanto estas atividades e quanto a segurança de seu uso. Estas espécies são a *Acanthospermum hispidum*, *Luehea divaricata* e *Talisia esculenta.*

2.4.1 Espécies medicinais avaliadas

2.4.1.1 *Acanthospermum hispidum* DC.

A *A. hispidum* (Fig. 3) é uma espécie pertencente à família Asteraceae. É uma planta rasteira, que mede aproximadamente 60 cm de altura, conhecida popularmente como carrapicho, espinho-de-cigano e chifre-de-carneiro (LORENZI, 1982).

Figura 3. Partes aéreas de *Acanthospermum hispidum* DC. Foto obtida no horto de plantas medicinais da UFGD (Fonte: C.A.S.Tirloni).

É largamente distribuída em todo o território nacional e em vários países ao redor do mundo (GBIF, 2017; TROPICOS, 2017) (Fig. 4).

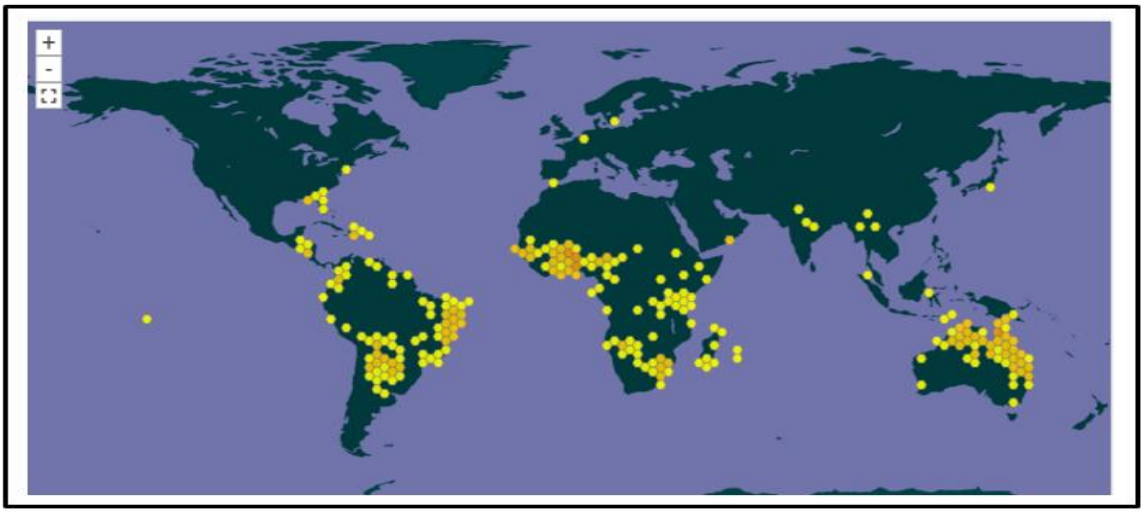


Figura 4: Distribuição geográfica da *Acanthospermum hispidum* DC ao redor do mundo (Fonte: www.gbif.org).

Esta espécie apesar de ser considerada uma erva daninha em áreas agricultáveis, é utilizada para diversos fins medicinais em todo o Brasil. No Pantanal seu infuso é utilizado para induzir a diurese, distúrbios renais, cólicas, tosse e corrimento (BIESKI et al., 2012). Já em outras regiões do país, a planta é utilizada como antitumoral, para tratar hipertensão, asma, bronquite, dores intestinais, verminose e febre (ARAÚJO et al., 2008; DE MELO et al., 2011; LEMOS et al., 2016).

Diversos estudos, utilizando diferentes extratos obtidos das folhas, flores e caule da *A. hispidum* evidenciaram efeitos antitumoral, antioxidante, antimicrobiano, antiplasmódico antidiarreico e anti-hipertensivo (AGUNU et al., 2005; BERO et al., 2009; KOUKOUIKILA KOUSSOUNDA et al., 2013; MOTHANA et al., 2009; PALOZI et al., 2017; SILVA et al., 2012).

Alguns metabólitos secundários já foram identificados nesta espécie, incluindo lactonas sesquiterpênicas, poliois, saponinas, alcaloides, flavonoides, derivados do ácido clorogênico e óleos voláteis (ALVA et al., 2012; CARTAGENA et al., 2000; ODEBIYI; SOFOWORA, 1978; NAIR et al., 1976; SULTANBAWA et al., 1978).

Poucos estudos foram conduzidos quanto a sua toxicidade. Na década de 70 Ali e Adam (1978) realizaram um estudo com as sementes desta espécie coletadas no Sudão

(África) e demonstraram efeitos tóxicos em ratos e em cabras. Na década de 90 foi demonstrado efeito teratogênico em ratas da linhagem Wistar (LEMONICA et al., 1994).

2.4.1.2 *Luehea divaricata* Mart.

A *Luehea divaricata* (Fig. 5) é uma espécie nativa pertencente à família Malvaceae. É uma árvore de médio e grande porte medindo entre 15 e 25 metros, conhecida no Brasil pelos nomes populares de açoita-cavalo, pau-de-canga, ivatingui, ibatingui e caiboti (LORENZI, 1998).

Figura 5. Folhas e árvore da *Luehea divaricata* Mart. Imagem obtida no horto de plantas medicinais da UFGD (Fonte: C.A.S. Tirloni).

Esta espécie encontra-se distribuída principalmente nos países da América do Sul como Paraguai, Argentina, Bolívia e Brasil. No Brasil encontra-se largamente distribuída em todo o território nacional. Tem pouca ocorrência em outros continentes, encontrando-se apenas em pequenos pontos da Oceania e América Central (GBIF, 2017; TROPICOS, 2017) (Fig. 6).

Popularmente esta espécie é utilizada para bronquite, disenteria, leucorréia, tumores e reumatismo, tratamento de problemas de pele e lavagem vaginal e como depurativa (LORENZI, 1998; TANAKA et al., 2005; CALIXTO JUNIOR et al., 2016). Na região do Pantanal sul-mato-grossense suas folhas são utilizadas como diuréticas e para “afecções nos rins”, além de outros empregos como relaxante muscular, “problemas de coluna”, “limpeza do sangue”, ácido úrico, gripe, pneumonia, hemorroidas, tosse e tumores (BIESKI et al., 2012).

Estudos farmacológicos comprovaram seu efeito antifúngico, antimicrobiano, antinoceptivo antiulcerogênico, anti-inflamatório e analgésico, antioxidante e anticolinesterásico e neuroprotetor (ARANTES et al., 2014; COURTES et al., 2015; DE SOUZA et al., 2004; MULLER, 2006; ROSA *et al.*, 2014; SIQUEIRA, 2006; ZACCHINO et al., 1998).

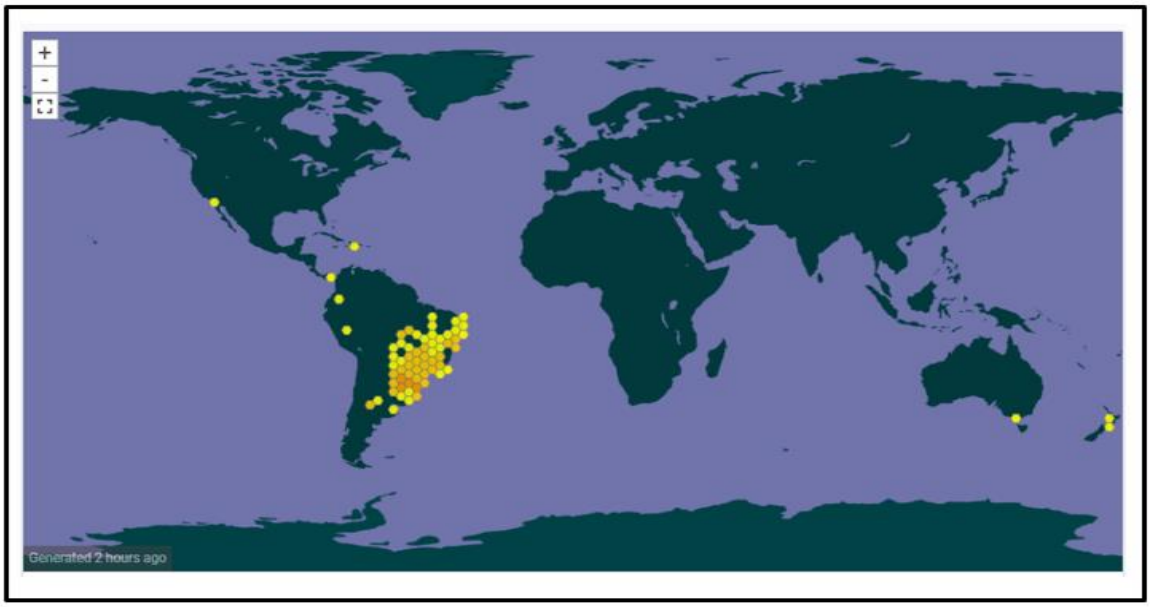


Figura 6. Distribuição geográfica de *Luehea divaricata* Mart. ao redor do mundo (Fonte: www.gbif.org).

Quanto à composição fitoquímica desta espécie alguns estudos já foram conduzidos. Em suas folhas foram descritos a presença de triterpenos, flavonoides, fitoesteroides compostos fenólicos, ácido rosmarinico, saponinas, taninos condensados, alcaloides e óleos essenciais (ARANTES et al., 2014; CALIXTO JUNIOR et al., 2016; COURTES et al., 2015; TANAKA, 2005).

Quanto a toxicidade da *L. divaricata* poucos estudos foram conduzidos. Alguns dados disponíveis sugerem baixo potencial genotóxico e mutagênico, e a ausência de toxicidade em camundongos também já foi reportada (BIGHETTI, 2004; FELICIO *et al.*, 2011; VARGAS; GUIDOBONO; HENRIQUES, 1991).

2.4.1.3 *Talisia esculenta* (A. ST. Hil) Radlk

A espécie *Talisia esculenta* pertence à família Sapindaceae. Geralmente são árvores de grande porte medindo mais de 15 metros de altura, muito frondosas (Fig. 7). É conhecida

popularmente como pitomba, pitombeira ou olho-de-boi, este último nome se dá pelo aspecto de seus frutos, que são adocicados e muito apreciados especialmente no litoral do nordeste brasileiro (LORENZI, 1998; NETO, 2003; SILVA et al., 1994).

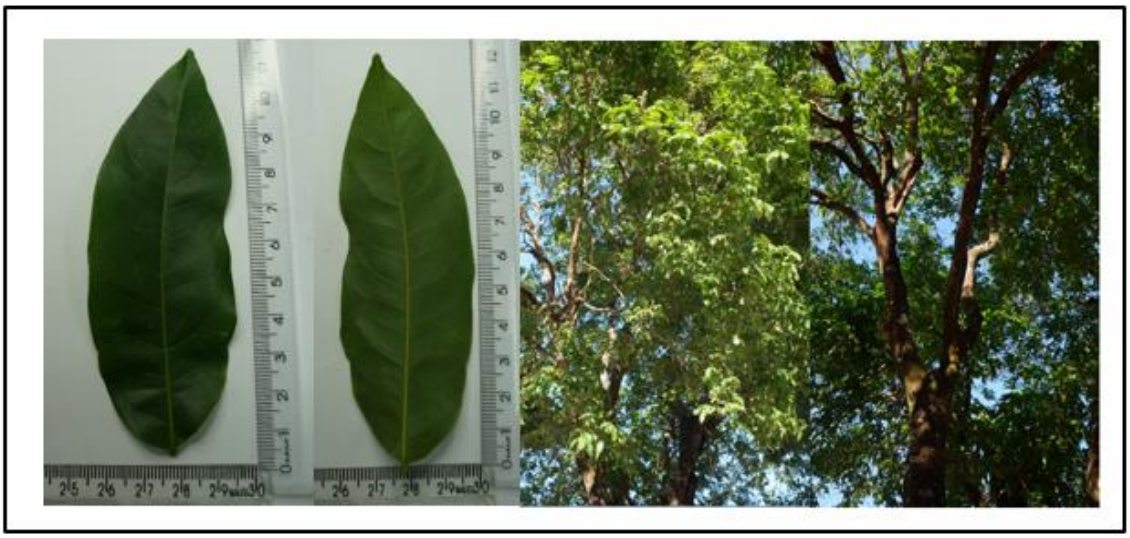


Figura 7. Folhas e árvore da *Talisia esculenta* (A. ST. Hil) Radlk. Imagem obtida no campus da UFGD (Fonte: C.A.S.Tirloni).

Esta espécie distribui-se apenas na América do Sul, em países como a Bolívia, Paraguai, Colômbia e Brasil (GBIF, 2017; TROPICOS, 2017) (Fig.8). É utilizada popularmente para diversos problemas de saúde, incluindo diarreia, disenteria, reumatismo, febre e como diurético (DE LIMA et al., 2006; NETO, 2003). Na região do Pantanal sul-mato-grossense é empregada para o tratamento de "problemas renais", dor, reumatismo, desidratação e "dores nas cadeiras" (BIESKI et al., 2012; NETO, 2006).

Poucos estudos foram conduzidos para avaliar a sua eficácia. Até então foram demonstradas as suas atividades antifúngica, antibacteriana, antioxidante, antimutagênica, antiproliferativa e inseticida (DE LIMA et al., 2006; DE SOUZA et al., 2016; FREIRE, M. et al., 2001, 2009; MACEDO et al., 2011; NERI NUMA et al., 2014; OLIVEIRA et al., 2007; PINHEIRO et al., 2009).

Alguns estudos demostraram a presença de alguns metabólitos secundários nos frutos desta espécie, tais como, catequinas, flavonoides, óleos voláteis e ácidos orgânicos (KUBOTA et al., 2009; NERI NUMA et al., 2014; DE SOUZA et al., 2016). Freire et al. (2001, 2003, 2009 e 2012) vêm ao longo dos anos isolando das sementes desta espécie proteínas do tipo lectina. Além disso, Riet Correa et al. (2014) evidenciaram a toxicidade do consumo "in natura" dos frutos e das folhas dessa espécie por bovinos e ovinos.

Figura 8. Distribuição geográfica da *Talisia esculenta* (A. ST. Hil) Radlk ao redor do mundo (Fonte: www.gbif.org).

3. OBJETIVOS

3.1 Objetivo geral

Investigar os possíveis efeitos cardiovasculares e renais dos extratos aquosos purificados com etanol obtidos das partes aéreas de *Acanthospermum hispidum* e das folhas de *Luehea divaricata* e *Talisia esculenta* utilizadas popularmente no Pantanal sul-mato-grossense e avaliar os prováveis mecanismos moleculares envolvidos nestas atividades.

3.2 Objetivos específicos

1. Realizar a caracterização botânica das três espécies em estudo, traçando um perfil anatômico e histoquímico das mesmas;
2. Produzir extratos purificados das três espécies e realizar o perfil fitoquímico dos mesmos, identificando os principais metabólitos secundários existentes;
3. Avaliar a toxicidade aguda em ratos Wistar dos três extratos purificados obtidos das três espécies;
4. Mensurar a atividade hipotensora aguda em ratos normotensos dos três extratos;
5. Analisar a atividade diurética aguda (dose única) dos três extratos e prolongada (doses repetidas) do extrato mais efetivo utilizando modelo de diurese em ratos;
6. Investigar se o tratamento prolongado com a espécie mais efetiva é capaz de interferir nas concentrações séricas de aldosterona, vasopressina e ECA;
7. Verificar as prováveis alterações na hemodinâmica renal através da investigação do envolvimento das prostaglandinas e óxido nítrico das espécies que apreentarem atividade diurética;
8. Determinar o envolvimento da via do óxido nítrico/ guanilato ciclase na ação hipotensora das espécies que apresentarem atividade hipotensora;
9. Determinar o envolvimento da via da prostaciclina/ adenilato ciclase na ação hipotensora das espécies que apresentarem atividade hipotensora;
10. Após a definição da espécie mais promissora fracionar o extrato purificado com solventes de diferentes polaridades e analisar as frações quanto suas atividades no sistema cardiovascular e renal.
11. Traçar o perfil fitoquímico da fração mais efetiva;

12. Investigar os mecanismos envolvidos na atividade diurética e hipotensora da (s) fração (ões) mais efetiva (s) em leito mesentério isolado e rins isolados de ratos.

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5 APÊNDICES

5.1 Artigo 1: Ethnopharmacological investigation of the cardiorenal properties of a native species from the region of Pantanal, state of Mato Grosso do Sul, Brazil

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Ethnopharmacological investigations of the cardio-renal properties of a native species from the region of Pantanal, state of Mato Grosso do Sul, Brazil

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ABSTRACT

Ethnopharmacological relevance: *Acanthospermum hispidum* DC. is an important medicinal herb that belongs to family Asteraceae, popularly used as a diuretic and hypotensive in the region of Pantanal, state of Mato Grosso do Sul, Brazil. Despite the relevance of this species throughout the country, there are no detailed studies about its possible ethnobotanical indication.

Aim: To carry out a detailed ethnopharmacological investigation of the cardio-renal properties of *A. hispidum*. Materials and methods: First, a detailed morpho-anatomical study with the purpose of characterizing and providing quality control parameters for the species was carried out. Then, purified aqueous extract (ESAH) was obtained and a detailed phytochemical investigation about its main secondary metabolites was performed. In addition, a thorough acute toxicological study was conducted to evaluate the actual toxic effects of this preparation. Finally, the possible diuretic and hypotensive effects of ESAH on male Wistar rats (30, 100, 300 mg/kg; intraduodenally) were evaluated, and using pharmacological antagonists or inhibitors, the involvement of prostaglandin/cAMP and nitric oxide/cGMP pathway and potassium channels in ESAH-induced hypotension was investigated.Results: The analyses performed by liquid chromatography–mass spectrometry showed that the main secondary metabolites present in ESAH were phenolic compounds, such as caffeoylquinic acids (chlorogenic acid), dicaffeoylquinic acids and glycosylated flavonoids (quercetin glucoside and galactoside). ESAH did not induce any acute toxic effects and did not affect the urinary volume or renal excretion of electrolytes in Wistar rats. On the other hand, intraduodenal administration of ESAH induces a significant acute hypotensive effect. Previous treatment with N-(G)-nitro-L-arginine methyl ester, methylene blue, or tetraethylammonium fully avoided the hypotensive effect of ESAH. All other parameters were not affected by treatment with ESAH. Conclusion: Data obtained in this study allow us to suggest that ESAH obtained from *A. hispidum* presents an important acute hypotensive effect, which appears to be dependent on the nitric oxide/cGMP pathway. This study presents new evidences about the therapeutic potential of this species when acute hypotensive response is required.

Abbreviations: AP, arterial pressure; AU, arbitrary units; BW, body weight; Ca^{++}, calcium; CGAs, chlorogenic acid; Cl^{-}, chloride; DBP, diastolic blood pressure; ESAH, ethanol soluble fraction from *Acanthospermum hispidum*; EtOH, ethanol; HCD, higher-energy collisional dissociation; HCTZ, hydrochlorothiazide; HR, heart rate; K^{+}, potassium; K_{Ca},

calciumsensitive potassium channels; LM, light microscopy; L-NAME, N(G)-nitro-L-arginine methyl ester; Na^+, sodium; MeOH, methanol; MS, state of Mato Grosso do Sul; SBP, systolic blood pressure; TEA, tetraethylammonium; UPLC, ultra-performance liquid chromatography.

1. Introduction

Although Brazil has approximately 20% of all plant species on the planet, the vast majority of these species has never been scientifically investigated, and their use is mainly based on the popular culture of different populations. For centuries, medicinal plants have been widely used in Brazil, especially by different native populations, which due to the difficulty of access to large urban centers, have in this practice the first and often the only therapeutic alternative (Dutra et al., 2016).

Pantanal, a region of million hectares in mid-western Brazil, eastern Bolivia and eastern Paraguay, is one of the most immensurable, primitive and biologically rich environments of the world. Diversified flora, fauna and a large freshwater system form these ecological sub-regions, one of the world's greatest natural wonders (Hamilton et al., 1996).

In Brazil, 64.64 percent of Pantanal is located in the state of Mato Grosso do Sul (MS). Different indigenous and riverine populations are among the different populations that live in this ecosystem, which preserve very important knowledge about the benefits of several medicinal species native to the region (Oliveira et al., 2011). This popular knowledge is primordial for the discovery of new species with pharmacological activity but has been lost and underutilized over time due to rural migration, lack of interest by young individuals and technological development (Bieski et al., 2012).

Acanthospermum hispidum DC., a medicinal herb belonging to family Asteraceae and popularly known as "carrapicho", "espinho-decigano", and “chifre-de-carneiro. In the Pantanal region of MS, the infusion of its aerial parts is popularly used as antispasmodic, antitussive, diuretic agent, and for the treatment of vaginal discharge (Bieski et al., 2012; Oliveira et al., 2011). In other regions of the country, the plant is used to treat asthma, bronchitis, fever, hypertension, intestinal pain and worms (Araújo et al., 2008).

Chemically, it is known that *A. hispidum* can produce different secondary metabolites, including sesquiterpene lactones (Cartagena et al., 2000), polyols, saponins, alkaloids, polyphenols (Nair et al., 1976; Odebiyi and Sofowora, 1978; Sultanbawa et al., 1978) and volatile oils (Alva et al., 2012). The plant has been scientifically investigated for its

antimicrobial (Arena et al., 2011; Fleischer et al., 2003), antiviral (Summerfield et al., 1997), antidiarrheal (Agunu et al., 2005), antitumor (Mothana et al., 2009), and antiparasitic activity (Ganfon et al., 2012; Koukouikila-Koussounda et al., 2013).

Few toxicological studies have been conducted with this species. Data published in the last century have shown that mice treated with the shoots of *A. hispidum* collected in Sudan presented several toxic effects like hepatic steatosis and necrosis, reduction of renal glomerular tubules, congestion and hemorrhage in heart, lungs and spleen, as well as catarrhal enteritis (Ali and Adam, 1978). In addition, teratogenic effects have also been reported for female Wistar rats treated with the aqueous extract obtained from this species (Lemonica and Alvarenga, 1994).

Thus, considering the extensive use of this species in the region of Pantanal, MS; the aim of this study was to perform a detailed ethnopharmacological investigation of the cardio-renal properties of *A. hispidum*. First, a detailed morpho-anatomical study with the purpose of characterizing and providing quality control parameters for the species was carried out. Then, purified aqueous extract was obtained and a detailed phytochemical investigation about its main secondary metabolites was performed. In addition, a thorough acute toxicological study was conducted to evaluate the actual toxic effects of this preparation. Finally, the possible diuretic and hypotensive effects of this extract and the molecular mechanisms involved in these activities were evaluated.

2. **Materials and methods**

2.1. Drugs

Atropine, glibenclamide, hydrochlorothiazide, N(G)-nitro-L-arginine methyl ester (L-NAME), methylene blue, propranolol, and tetraethylammonium (TEA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride was obtained from Merck (Darmstadt, Germany). All other reagents were obtained in analytical grade.

2.2. Plant material

Aerial parts of *A. hispidum* were collected from the botanical garden of the Federal University of Grande Dourados (UFGD; Dourados, Brazil) at 458 m above sea level (S 22°11′42.7′′ and W 54°56′10.2′′), in October 2015. A voucher specimen was authenticated by Dra. Maria do Carmo Vieira (no. DDMS 5219) and deposited at the UFGD plant facility.

2.3. Botanical characterization

Adult leaf and stem fragments of *A. hispidum* were fixed in FAA (Johansen, 1940) and stored in 70% ethanol (v/v) (Berlyn et al., 1976). Scanning electron microscopy was performed on leaf and stem fragments that had been dehydrated in ethanol series and coated with gold by Quorum – SC7620. Electron micrographs were taken with VEGA3 TESCAN scanning microscope.

For light microscopy, semi-permanent slides were prepared freehand with the material sectioned in the transversal and longitudinal directions. Slides were hydrated and stained with basic fuchsine and astra blue (Roeser, 1972). Samples were clarified in frontal view of the epidermis, using technique described by Berlyn et al. (1976). Results were photographed with a digital camera (C7070) attached to a light microscope (Olympus® CX 31). Both scanning electron microscopy and light microscopy were conducted at the State University of Ponta Grossa, Paraná, Brazil.

2.3.1. Histochemical analysis

Free-hand cross-sections and longitudinal sections were prepared with fresh *A. hispidum* leaves and the following standard solutions: ferric chloride (Johansen, 1940) and Hoepfner-Vorsatz test, modified by Reeve (1951) to test phenolic substances; Sudan III for testing lipophilic compounds (Foster, 1949) and hydrochloric phloroglucin to reveal lignified elements (Sass, 1958).

2.4. Phytochemical study

2.4.1. Preparation of the purified aqueous extract

Aerial parts of *A. hispidum* were air-dried in an oven (40 °C, 7 days, 66.1% humidity) cut and ground into a powder using mechanical milling. Extracts were obtained by infusion similarly to that popularly used in the region of Pantanal (Oliveira et al., 2011). About 1 L of boiling water was poured over 60 g of dried aerial parts and extraction occurred until room temperature was reached (~ 6 h), with sealed container. Then, the infusion was treated with 3 volumes of EtOH, which gave rise to a precipitate and an ethanol soluble fraction (ESAH; 8% yield). ESAH was freeze-dried and maintained at −20 °C until analyses.

2.4.2. Sample analysis (liquid chromatography-mass spectrometry)

ESAH was analyzed by ultra-performance liquid chromatography (UPLC™ Waters), coupled to high-resolution mass spectrometer, LTQ Orbitrap XL (Thermo Scientific). The reversed-phase chromatography was developed on HSS T3 C18 column, with 100 × 2.1 mm and 1.7 μm of particle size (Waters). The column temperature was held at 60 °C and the flow rate was 0.4 mL/min. The sample was prepared at 2 mg/ mL in MeOH-H_2O (1:1, v/v), with injections of 5 μL. The separation was driven by binary solvent gradient using ultra-pure water and acetonitrile (JT Baker) containing 0.1% (v/v) of 96% formic acid (Tedia), increasing the acetonitrile content from 0% to 10%, in 6 min, then to 80% in 14 min. The solvent system returned to the initial condition in 15 min and was re-equilibrated for 2 min.

Detection was provided by mass spectrometry operating in negative and positive ionization modes. Sample desolvation was carried out by a capillary temperature of 350 °C, and nitrogen flows of 30 arbitrary units (AU) in the sheath gas and 5 AU in the auxiliary gas. For positive ions, voltages were: source at 5 kV, capillary at 30 V and tube lens at 110 V; for negative ions: source at 3.2 kV, capillary at −46 V and tube lens at −200 V. Fragmentations were developed on C-Trap, by higherenergy collisional dissociation, with normalized collision energy of 35. The resolution was set at 15,000 FWHM and the external mass calibration was performed just before analysis.

2.5. Preclinical efficacy and safety assessment

2.5.1. Animals

All procedures were performed in accordance with Brazilian Animal Welfare Legislation and were approved by the Institutional Ethics Research Committee of UFGD (no. 16/2015). Male and female Wistar rats at the age of 12 weeks were adapt to the new conditions for two weeks. Four animals per cage were kept under standard and controlled conditions (22 ± 2 °C of temperature, 50 ± 20% of relative humidity, 12:12 h light–dark cycle) on sterilized aspenwood granulate bedding. Animals received commercial pelletized diet (Nuvilab CR1, Curitiba, Brazil) and water ad libitum.

2.5.2. Safety assessment

2.5.2.1. Acute oral toxicity

ESAH acute oral toxicity was evaluated in rats according to procedures recommended by the Organization for Economic Co-operation and Development (OECD Organization for Economic Co-Operation and Development, 2001). After the 12-h fasting period, five single doses (5, 50, 300, and 2000 mg/kg) at 10 mL/kg of ESAH were administered to male and female rats (n = 5) through the oral route using gavage. Other five male and five female rats (control group) received distilled water. Food was provided to rats approximately one hour after treatment. Rats were observed for indications of toxicity effect (aggressiveness, convulsion, coma, diarrhea, eyes and ears secretion, food or water refusal, hypo- or hyperlocomotion, injury, mortality, pain, salivation, weakness) within the first eight hours after treatment and daily for a period of 14 days. All animals were daily weighed.

On day 15, all animals were euthanized by decapitation for the collection of blood samples and serum, obtaining through centrifugation (1000×g for 10 min). Hematological analysis was performed in automated analyzer (Sysmex® XN-3000). The parameters analyzed were: red blood cell count (10^6/mL), total leukocyte count (10^3/ mm^3), platelet count (10^3/mm^3), differential leukocyte count (relative number, %), hemoglobin (g/mL) and hematocrit determination (%), and determination of RBC indexes: mean cell volume (MCV, fl), mean cell hemoglobin (MCH, pg) and mean cell hemoglobin concentration (MCHC %). Biochemical analysis was performed in an automated analyzer (Roche® Cobas Integra 400 plus). The parameters were: uric acid (mg/dL); urea (mg/dL); creatinine (mg/dL), sodium (Na^+; mEq/ L), potassium (K^+; mEq/L), alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), alkaline phosphatase (ALP, U/ L), total serum protein (g/dL), albumin (g/dL), globulin (g/dL), triglycerides (mg/dL), total cholesterol (mg/dL), HDL-cholesterol (mg/dL), and amylase (U/L).

Heart, liver, kidney, spleen, testicles, epididymis, lavatory ani muscle, seminal vesicle, prostate, ovary and uterus were removed, macroscopically examined and the relative organ weight was then calculated as (absolute organ weight × 100%)/ body weight of rats on the day of euthanasia. Organ samples were also fixed in 10% formalin, dehydrated with alcohol and xylene, embedded in paraffin wax, sectioned, mounted on glass slides and stained with hematoxylin/ eosin. Two board-certified veterinary pathologist performed necropsy and histopathology evaluation.

2.5.3. Ethnopharmacological approaches

2.5.3.1. Acute diuretic activity

Fifty male rats were fasted overnight (12 h) with free access to water. Before treatments, all animals received oral isotonic saline (0.9% NaCl; 5 mL/100 g) to impose controlled water and salt balance as proposed by Kau et al. (1984) with some modifications (Gasparotto Junior et al., 2009). Then, different groups of rats (n = 5) were orally treated with ESAH (30, 100 or 300 mg/kg), hydrochlorothiazide (HCTZ; 25 mg/kg) or vehicle (filtered water, control group) and immediately placed in metabolic cages. Urine samples were collected in graduated cylinders and volume was recorded every hour for 24 h, and expressed as mL/100 g of body weight.

At the end of experiments, rats were anesthetized with isoflurane and blood samples were collected in conical tubes by cardiac puncture. Serum was obtained by centrifugation (1000×g for 10 min), and stored at −20 °C until analysis. Urinary and serum Na^+, K^+, chloride (Cl^-) and calcium (Ca^{++}) levels were quantified in automated analyzer (Roche® Cobas Integra 400 plus). pH was directly determined on fresh urine samples using Q400MT pH-meter (Quimis Instruments, Diadema, Brazil). Density was estimated using Mettler AE163 (± 0.1 mg) analytical scale of urine volume measured with Nichiryo micropipette. Additionally, excretion load (El) of Na^+, K^+, Cl^- and Ca^{++} was obtained according to the equation $El = U_x \times V$; were U_x means concentration of electrolytes (mEq/L) and V means urinary flow (mL/min). Results were expressed as μEq/min/100g.

2.5.3.2. Effects on arterial pressure and heart rate

Normotensive male rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg), through intramuscular via, supplemented at 45–60-min intervals. Immediately, a bolus injection of heparin (50 IU) was intraperitoneally applied. Tracheotomies were performed to allow spontaneous breathing. The left carotid artery was cannulated and connected to a pressure transducer coupled to PowerLab® recording system, and an application program (Chart, v 4 0.1; all from ADI Instruments; Castle Hill, Australia) recorded the systolic (SBP) and diastolic (DBP) blood pressure, mean arterial pressure (MAP) and heart rate (HR). After this procedure, different groups of rats (n = 5) received ESAH (30, 100 or 300 mg/kg) or hydrochlorothiazide (25 mg/ kg) intraduodenally. The control group

received intraduodenal vehicle at constant volume of 100 μL/100 g of body weight. Changes in arterial pressure (AP) and HR were recorded for 35 min after treatments. Each animal received only one of the substances studied. At the end of experiments, animals were sacrificed with an overdose of isoflurane (2–3 times of the anesthetic dose).

2.5.3.3. Investigation of the molecular mechanisms involved in the hypotensive effects of ESAH

2.5.3.3.1. Investigation of the role of prostaglandins/cAMP and nitric oxide/cGMP pathways, and K^+ channels in the hypotensive response of ESAH.

The right femoral vein of rats was cannulated and connected to an infusion pump (EFF 311, Insight®, Ribeirão Preto, Brazil). Continuous infusion of N(G)-nitro-L-arginine methyl ester (LNAME, a non-selective nitric oxide synthase inhibitor; 7 mg/kg/min), or methylene blue (a guanylyl cyclase inhibitor; 150 nmol/kg/min); or bolus intraperitoneal injection of 2′, 5′- dideoxyadenosine (DDA, a adenylate cyclase inhibitor; 0,1 mg/kg), or indomethacin (a cyclooxygenase inhibitor; 3 mg/kg), or tetraethylammonium (TEA, a non-selective calcium-sensitive potassium channel blocker; 400 μmol/ kg) was performed. Simultaneously, the ability of ESAH (300 mg/kg;
i.d.) to change AP under the above conditions was verified. The total volume injected in animals throughout the infusion period was 1000 μL.

2.6. Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls or by Kruskal-Wallis followed by Dunn's test. Results are expressed as mean ± standard error of the mean (S.E.M.) and p-value less than 0.05 was considered statistically significant. Graphs were drawn and statistical analysis was carried out using the GraphPad Prism software version 5.0 for Mac OS X (GraphPad® Software, San Diego, CA, USA).

3. Results

3.1. Anatomical profile and histochemical characterization

Anatomical profile and histochemical characterization of *Acanthospermum hispidum* are show in Fig. 1. In the present study, the most important characteristics were circular stem (Fig. 1C), biconvex midrib (Fig. 1L) and concave-convex petiole (Fig. 1M), angular collenchyma (Fig. 1F), fibers over the phloem (Fig. 1E, 1G) and small secretory ducts near the phloem in the cortex and in the perimedullary zone of the pith (Fig. 1D, 1E) in the stem; one collateral vascular bundle, small secretory ducts and angular collenchyma on both sides of the midrib (Fig. 1L); about 7 vascular bundles in open arc in the petiole (Fig. 1M); capitate uniseriate glandular trichomes (Fig. 1F, 1I, 1K), multicellular and uniseriate non-capitate glandular trichomes (Fig. 1I) and multicellular and uniseriate non-glandular trichomes (Fig. 1H, 1I) in stems and leaves; amphistomatic leaves (Fig. 1J) with anomocytic stomata, striate cuticle (Fig. 1H), and dorsiventral mesophyll (Fig. 1J, 1K, 1L).

The histochemical test using phloroglucin exposed lignin in fibers and in xylem as seen in Fig. 1G. Lipophilic compounds were evidenced in the capitate glandular trichome (Fig. 1K), cuticle and in secretory ducts. Phenolic components were evidenced in the epidermis, collenchyma, phloem and xylem of stems (Fig. 1E) and in the palisade and spongy parenchyma of leaves (Fig. 1J) with ferric chloride, as also observed in the Hoepfner-Vorsatz test in the epidermis, collenchyma and secretory cells of the capitate glandular trichome (Fig. 1F) and in the epidermis, collenchyma, phloem and, xylem of stems (Fig. 1D).

3.2. Phytochemical profile

The ESAH was analyzed by LC-MS in positive and negative ionization modes. However, the negative ionization gave better results than positive one. Thus, the phytochemical analysis will be discussed on the base of negative ionization mass spectrometry. The main peaks on chromatogram (Fig. 2) were identified as phenolic compounds, consisting of several isomers of chlorogenic acids, such as monocaffeoylquinic acids, appearing in 2.38, 3.28, 3.60 min, producing negative ion at m/z 353.087 with fragments at m/z 191.055, 179.034, 173.045 and 135.044, at different ratios (Table 1). Since the caffeoyl group can attach the quinic acid in different hydroxyl site, many different isomers can be produced. Similarly, isomers of dicaffeoylquinic acids were the main peaks on the chromatogram, appearing at 6.44, 6.71 and 7.11 min, with MS^1 at m/z 515.119 and MS^2 at m/z 353.087, 191.055, 179.035, 173.045 and 135.044 (Table 1), confirming these structures as in

previous findings (Dartora et al., 2011; Carlotto et al., 2015). Glycosylated flavonoids were observed at m/z 463.088 (peaks at 5.99 and 6.10 min) producing MS^2 at m/z 300.027, 271.024, 255.029 and 243.029, consistent with quercetin, therefore identified as quercetin glucoside and galactoside (Souza et al., 2008, 2016).

The peaks at 7.31, 7.57 and 8.56 min had a characteristic ion at m/z 337.238, but these compounds were not completely understood. In the peak at 7.31 min, the ion at m/z 337.23858 appeared as a fragment obtained from an ion at m/z 535.26839, being chlorinated ion $[M+Cl]^-$, as evidenced by the intense isotopologue at m/z 537.266 (~37%). However, the m/z 337.238 also appeared as natural in-source fragment without evidence for Cl^- adduct. The elemental composition of the ion at m/z 337.23858 was calculated as $C_{20}H_{33}O_4$, with mass accuracy of 0.44 ppm (in relation to the theoretical m/z 337.23843). The neutral loss (NL) between m/z 535.26839 and 337.23858 was 198.0298 amu (a.m.u.), which is quite close to value calculated for the loss of hydrochloride-hexosyl residue (198.0295 a.m.u.). Thus, it seems that the compound/fragment at m/z 337.238 has a natural negative charge, becoming neutral when attached by a hexose group, therefore producing the ion at m/z 535.268 as Cl^- adduct. A low abundant ion corroborates with these suggestions, appearing at m/z 545.296, which is consistent with same compound adducted by formate anion $[M +HCOO]^-$. It is not clear that if in the other peaks, the main ion at m/z 337.238 is the entire compound or an in-source fragment.

3.3. Safety evaluation

3.3.1. Acute treatment with ESAH does not affect the behavioral, hematological, or biochemical parameters and does not induce histopathological alterations in Wistar rats

No significant changes in behavior or appearance of rats were observed until the end of 14 days. Daily consumption of food, water and body weight gains throughout the experimental period were similar among animals (both sexes) treated with ESAH (5, 50, 300, and 2000 mg/kg) or vehicle. LD50 was not stipulated because no deaths were observed in animals treated with doses up to 2000 mg/kg.

Results of hematologic and biochemical analyses are shown in Tables 2, 3, respectively. Values remained within normal range to the specie and gender used in this study. There were no ESAH-related changes in the relative weight of organs of all experimental

groups (data not shown). Furthermore, no significant change was observed by necropsy or histopathological analysis in all samples (Fig. 3).

3.4. Ethnopharmacological evidences

3.4.1. ESAH does not induce acute diuretic effects on Wistar rats

Treatment with a single dose of ESAH (30, 100 or 300 mg/kg) did not induce any diuretic effects after 8 or 24 h (Table 4) when compared with control group. As expected, HCTZ-treated group showed a significant increase in urinary volume after 8 h, and returned to values close to the control group 24 h after treatment.

The effects of acute treatment with ESAH (30, 100 and 300 mg/kg) and HCTZ on electrolyte excretion 8 and 24 h after treatments are presented in Tables 5, 6, respectively. Na^+, K^+, Ca^{++}, and Cl^- excretion was not significantly increased in groups treated with ESAH (30, 100 and 300 mg/kg) when compared with control group. Similarly, pH and density were not significantly changed after treatment with ESAH at all doses tested. On the other hand, all animals treated with HCTZ showed high amounts of urine Na^+, K^+ and Cl^-.

3.4.2. Hypotensive effects of ESAH on normotensive rats

After 15 min of stabilization and before the administrations of drugs, basal SBP, DBP, and MAP were 129.4 ± 6.0, 54.6 ± 6.8, and 102.4 ± 4.6 mm Hg, respectively, while HR was 339 ± 28.5 beats per minute (bpm). Intraduodenal administrations of ESAH (30, 100 and 300 mg/kg) were able to induce an important reduction in SBP (~35 mm Hg; Fig. 4A), while only at dose of 30 mg/kg, MAP was significantly reduced (~30 mm Hg; Fig. 4C). Throughout the experimental period, none of the drugs tested were able to alter DBP or HR values (Fig. 4B and D). Data recorded after intraduodenal administration of HCTZ were not statistically different from those obtained for ESAH at dose of 30 mg/kg.

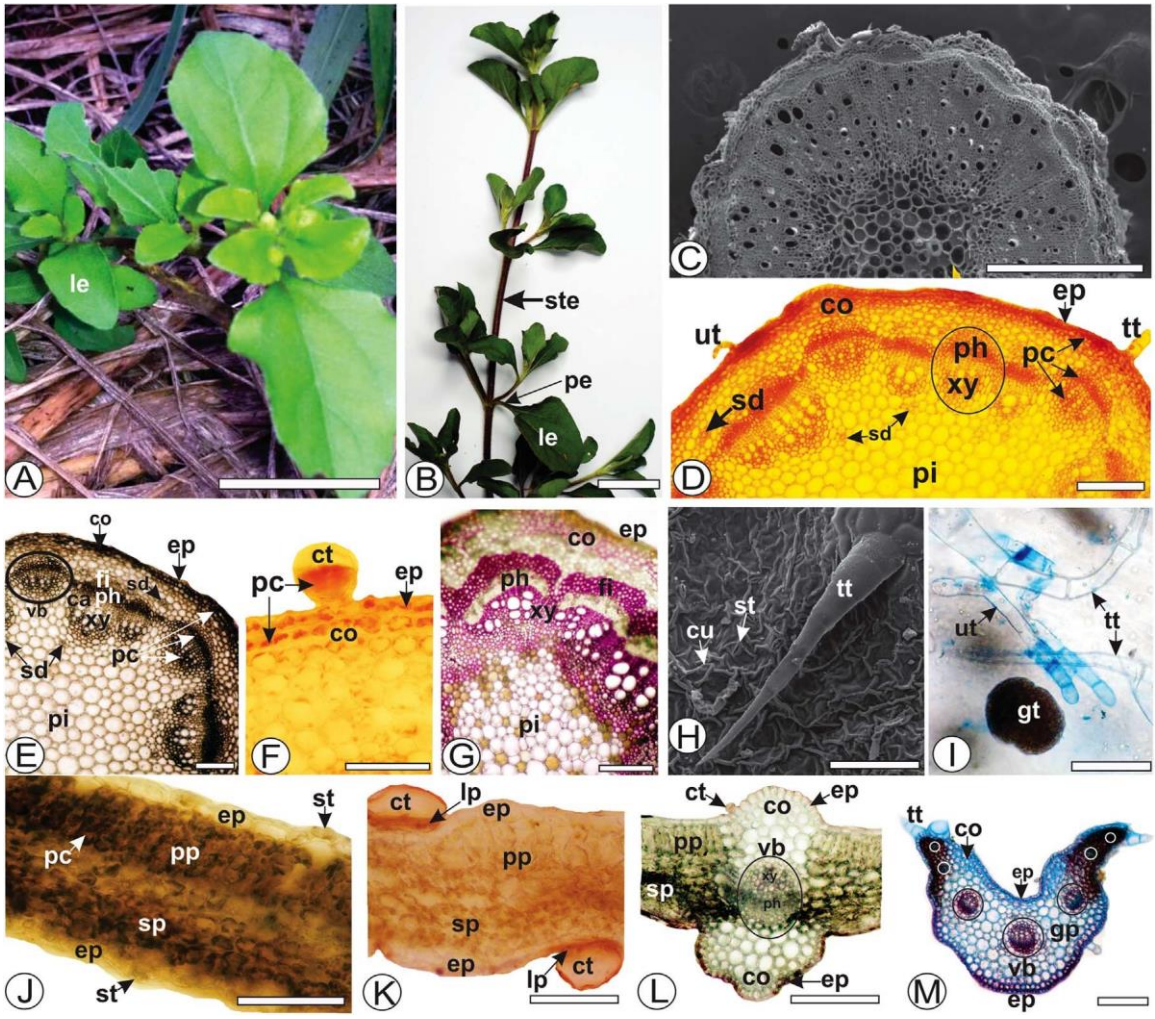

Fig. 1. *Acanthospermum hispidum* DC (Asteraceae). A. Plant in habit; B. General aspect, showing leaves (le), petiole (pe) and stem (ste); C. Stem in cross-section in Scanning electron microscopy; D. Stem in transection in positive reaction with Hoepfner-Vorsatz modified evidencing phenolic compounds and showing collenchyma (co), epidermis (ep), phloem (ph), pith (pi), secretory ducts (sd), non-glandular trichome (tt), uniseriate non-capitate glandular trichome (ut), vascular bundle (vb), and xylem (xy); E. Stem transverse section in reaction with ferric chloride for phenolic compounds (pc), showing cambia (ca), collenchyma (co), epidermis (ep), fibers (fi), phloem (ph), pith (pi), secretory ducts (sd), vascular bundle (vb), and xylem (xy); F. Phenolic compounds (pc) in reaction with Hoepfner-Vorsatz modified test in the epidermis (ep), collenchyma (co) and capitate glandular trichome (gt) in the stem; G. Lignified elements in reaction with hydrochloric phloroglucin in the fibers (fi) and xylem (xy) and showing collenchyma (co), epidermis (ep); phloem (ph), and pith (pi) H. Lower epidermis showing stomatum (st), thick and striate cuticle (cu) and non-glandular trichome (tt); I. Upper epidermis showing capitate glandular trichome (gt), uniseriate non-capitate glandular trichome (ut) and non-glandular trichome (tt); J. Leaf transverse section in reaction with ferric

chloride indicating phenolic compounds in the palisade parenchyma (pp) and spongy parenchyma (sp) and showing upper and lower epidermis (ep) and stomata (st); K. Leaf transverse section showing capitate glandular trichome in reaction with Sudan III, and epidermis (ep), palisade parenchyma (pp), spongy parenchyma (sp) and stomata (st); L. Midrib in cross-section showing capitate glandular trichome (gt), collenchyma (co), epidermis (ep), palisade parenchyma (pp), spongy parenchyma (sp), phloem (ph), vascular bundle (vb), xylem (xy); M. Petiole transverse section showing collenchyma (co), epidermis (ep), ground parenchyma (gp), non-glandular trichome (tt), vascular bundle (vb). Scale bar = A (4 cm), B (2 cm), C (500 µm), M (200 µm), D, E, G, H, L (100 µm), F, I, J, K (50 µm).

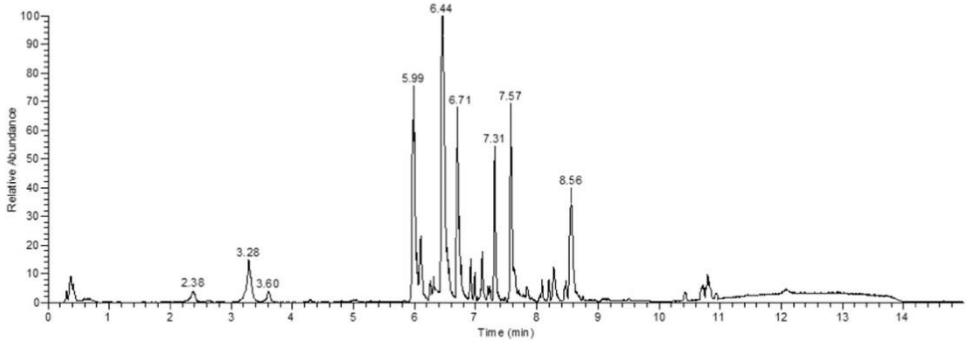

Fig. 2. UPLC analysis of ES obtained from *Acanthospermum hispidum* DC.

Table 1. Phytochemical composition of ESAH obtained by LC-PDA-MS.

Phytochemical composition of ESAH obtained by LC-PDA-MS

Peak	Rt (min)	MS^1 (-)	MS^2 (key fragments)	Tentative identification
1	2.38	353.08773 $[M-H]^-$	191.055, 179.034, 135.045	Chlorogenic acid
2	3.28	353.08745 $[M-H]^-$	191.055	Chlorogenic acid
3	3.60	353.08773 $[M-H]^-$	191.055, 179.034, 173.045, 135.044	Chlorogenic acid
4	5.99	463.08838 $[M-H]^-$	300.027	Quercetin-hexoside
5	6.10	463.08804 $[M-H]^-$	300.027	Quercetin-hexoside
6	6.44	515.11964 $[M-H]^-$	353.087, 335.076, 191.055, 179.034, 173.045, 135.045	Dicaffeoylquinic acid
7	6.71	515.11970 $[M-H]^-$	353.087, 191.055, 179.034,173.045, 135.044	Dicaffeoylquinic acid
8	7.11	515.11951 $[M-H]^-$	353.087, 191.055, 179.034,173.045, 135.044	Dicaffeoylquinic acid
9	7.31	535.26876 $[M+Cl]^-$	337.238	$C_{26}H_{44}O_9Cl$

3.4.2.1. Lack of involvement of prostaglandins/cAMP pathway in the hypotensive response of ESAH

The hypotensive effect of ESAH or HCTZ was not reduced in animals previously treated with indomethacin (Fig. 5A and B), or DDA (Fig. 5C and D). Furthermore, the indomethacin and DDA doses used did not change basal SBP and MAP in all experimental groups.

3.4.2.2. Involvement of the nitric oxide-guanylate cyclase pathway and K^+ channels in the hypotensive response of ESAH.

The continuous infusion of L-NAME increased SBP from 128.3 ± 4.0 mm Hg to 200.7 ± 6.9 mm Hg, and MAP from 109.4 ± 3.2 mm Hg to 166.6 ± 4.2 mm Hg. Under L-NAME infusion, the ability of ESAH (30 mg/kg) to reduce SBP and MAP was entirely inhibited, while the effects of HCTZ were only weakly perceived (Fig. 6A and B). Similarly, the infusion of methylene blue significantly reduced the ability of ESAH to cause hypotension, with irrelevant effects on the hypotensive action of HCTZ (Fig. 6C and D). On the other hand, the hypotensive action of ESAH and HCTZ was completely blocked in all animals pretreated with TEA, a nonspecific K^+ channel blocker (Fig. 6C and D).

4. **Discussion**

The state of Mato Grosso do Sul is the only Brazilian state with three large biomes in its territory (Cerrado, Atlantic Forest, andPantanal) preserving an immense ethno-botanical knowledge coming from different indigenous, mixed-race and riverside populations (Ciconini et al., 2013). Despite the rich biodiversity, many species have not yet been critically studied, especially in the region of Pantanal, where the difficulty of access to more remote areas makes it difficult to characterize the ethnopharmacological properties of many of these species (Oliveira et al., 2011). Thus, in an attempt to broaden ethnopharmacological knowledge from this region, a detailed morphoanatomical and microchemical study of a native species from the region of Pantanal, MS, was performed and the profile of secondary metabolites present in a preparation commonly used by the different populations that inhabit this biome was characterized. In addition, a sequence of toxicological and pharmacological studies were also conducted, showing that the species does not induce any signs of toxicity in

the short term, and have significant hypotensive effects dependent on the activation of the nitric oxide/cGMP pathway and the opening of potassium channels.

One of the most important steps in the performance of ethnopharmacological studies is the correct identification of species directly collected in its habitat. However, species are often found in the chopped or crushed form by local healers, which make it difficult to identify them by comparison with botanical vouchers. In this case, morpho-anatomical and microchemical studies can provide essential information for correct identification, avoiding possible adulteration or improper substitution of the desired medicinal species (Upton et al., 2011). This study presented the main morphological characteristics of *Acanthospermum hispidum* native to MS and the anatomical distribution of different metabolites that may be involved with the various pharmacological properties attributed to this species. These data provided valuable information for the quality control of the plant drug and ensured reliability of the raw material used in phytochemical, pharmacological and toxicological studies.

The second stage of our study was dedicated to characterize in detail the main secondary metabolites present in ESAH. Analyses performed by liquid chromatography–mass spectrometry have shown that the main secondary metabolites present in ESAH were phenolic compounds, such as caffeoylquinic acids (chlorogenic acid), dicaffeoylquinic acids and glycosylated flavonoids (quercetin glucoside and galactoside). It is now known that derivatives of caffeoylquinic or dicaffeoylquinic acids, such as chlorogenic acid (CGAs), have good intestinal absorption and can be readily identified in human plasma (Matsui et al., 2007). In addition, several data have shown that CGAs are effective hypotensive and antihypertensive drugs in controlled clinical trials (Mubarak et al., 2012) and in different pre-clinical hypertension models (Zhao et al., 2012; Suzuki et al., 2006). Mechanically, CGAs can decrease oxidative stress and increase nitric oxide bioavailability, which leads to improved endothelial function and reduce overall peripheral vascular resistance (Jiang et al., 2016; Kim et al., 2013; Zhao et al., 2012). Thus, phytochemical data have provided important evidence that secondary metabolites from *A. hispidum* could be directly related to available ethnobotanical information for the species, especially its effects on blood pressure. One concern that emerged at the beginning of this study was the possible toxicity of the species. Data published in the 1970s showed possible toxic effects of *A. hispidum* collected in some African countries, including lesions in the liver, kidneys, lungs and heart of mice (Ali and

Adam, 1978). In addition, some teratogenic effects in rats have also been reported (Lemonica and Alvarenga, 1994).

Table 2. Effect of acute oral administration of ethanol soluble fraction obtained from *Acanthospermum hispidum* (ESAH) on the hematological parameters of Wistar rats after 14 days of exposition.

Parameters	Control		ESAH (50 mg/kg)		ESAH (300 mg/kg)		ESAH (2 g/kg)	
	Male	Female	Male	Female	Male	Female	Male	Female
RBC (10^6/mL)	8.2 ± 0.1	9.0 ± 0.2	8.3 ± 0.2	9.1 ± 0.1	7.9 ± 0.1	8.6 ± 0.1	8.2 ± 0.2	8.5 ± 0.2
Hemoglobin (g/mL)	15 ± 0.7	15 ± 0.9	14 ± 1.0	15 ± 0.9	14 ± 0.8	15 ± 0.7	15 ± 0.9	14 ± 1.2
Hematocrit (%)	45 ± 0.5	51 ± 1.0	46 ± 1.7	52 ± 1.0	44 ± 0.9	50 ± 0.6	43 ± 0.8	49 ± 0.7
MCV (fl)	55 ± 1.5	59 ± 0.4	56 ± 0.6	58 ± 0.8	56 ± 0.3	59 ± 0.6	53 ± 0.4	55 ± 1.1
MCH (pg)	18 ± 0.2	17 ± 0.2	17 ± 0.1	17 ± 0.2	17 ± 0.1	17 ± 0.1	18 ± 0.1	25 ± 8.4
MCHC (%)	32 ± 0.9	29 ± 0.3	31 ± 0.4	29 ± 0.2	31 ± 0.2	29 ± 0.3	34 ± 0.3	27 ± 2.6
Platelets (10^3/mm^3)	953 ± 89	847 ± 112	987 ± 35	986 ± 35	999 ± 124	975 ± 69	972 ± 58	848 ± 107
WBC (10^3/mm^3)	5 ± 0.9	9 ± 1.4	4 ± 0.7	11 ± 0.8	4 ± 0.1	11 ± 2.0	4 ± 0.3	8 ± 1.1
Neutrophils (%)	21 ± 3.4	25 ± 2.1	30.1 ± 1.9	22 ± 1.5	31 ± 2.5	22 ± 4.1	22 ± 1.0	26 ± 4.9
Lymphocytes (%)	69 ± 3.5	66 ± 3.7	62 ± 1.1	72 ± 2.5	60 ± 2.2	72 ± 3.9	67 ± 2.0	68 ± 4.9
Monocytes (%)	6.5 ± 0.6	4.7 ± 1.3	5.6 ± 1.4	4.2 ± 0.7	5.9 ± 0.7	4.0 ± 0.5	7.5 ± 1.3	4.7 ± 0.6
Eosinophils (%)	1.7 ± 0.2	0.9 ± 0.2	2.1 ± 0.3	0.6 ± 0.1	2.0 ± 0.2	0.8 ± 0.1	2.1 ± 0.4	0.8 ± 0.2
Basophils (%)	0.3 ± 0.1	0.1 ± 0.02	0.2 ± 0.04	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.02	0.6 ± 0.2	0.2 ± 0.02

Values are expressed as mean ± S. E. M. of five rats in each group in comparison to the control using one-way ANOVA followed by Newman-Keuls test

Table 3 Effect of acute oral administration of ethanol soluble fraction obtained from *Acanthospermum hispidum* (ESAH) on the biochemical parameters of Wistar rats after 14 days of exposition.

Parameters	Control		ESAH (50 mg/kg)		ESAH (300 mg/kg)		ESAH (2 g/kg)	
	Male	Female	Male	Female	Male	Female	Male	Female
Total cholesterol (mg/dL)	57.8 ± 3.6	80.4 ± 9.8	58.0 ± 3.5	85.6 ± 3.7	63.1 ± 2.1	80.8 ± 3.8	63.1 ± 2.3	71.2 ± 1.5
HDL cholesterol (mg/dL)	30.4 ± 3.8	66.5 ± 9.7	28.9 ± 1.9	79.0 ± 3.9	33.3 ± 2.2	73.6 ± 3.6	36.5 ± 3.8	68.2 ± 1.9
Triglycerides (mg/dL)	58.2 ± 7.5	67.6 ± 17.5	66.4 ± 4.7	59.0 ± 3.7	51.7 ± 2.9	66.5 ± 6.5	48. 2 ± 5.7	40.1 ± 3.6
Urea (mg/dL)	43.2 ± 1.6	38.4 ± 3.0	40.7 ± 1.9	33.9 ± 1.1	47.1 ± 2.6	30.9 ± 1.3	33.1 ± 1.6	38.6 ± 0.9
Creatinine (mg/dL)	0.31 ± 0.02	0.26 ± 0.01	0.31 ± 0.02	0.26 ± 0.03	0.28 ± 0.02	0.26 ± 0.02	0.26 ± 0.02	0.30 ± 0.02
Sodium (mEq/L)	140.7± 0.8	141.1 ± 0.5	139.9 ± 0.7	141.9 ± 0.3	142.2 ± 0.7	142.1 ± 0.4	140.4 ± 0.7	141.7 ± 0.8
Potassium (mEq/L)	5.1 ± 0.5	5.6 ± 0.6	4.5 ± 0.1	5.2 ± 0.3	5.1 ± 0.3	5.2 ± 0.4	4.9 ± 0.2	6.3 ± 0.3
Uric acid (mg/dL)	1.3 ± 0.4	1.4 ± 0.3	0.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	1.4 ± 0.2	0.9 ± 0.1	1.9 ± 0.3
Total protein (g/dL)	6.3 ± 0.1	5.6 ± 0.2	6.3 ± 0.1	6.0 ± 0.1	6.1 ± 0.1	5.8 ± 0.1	6.3 ± 0.1	5.4 ± 0.1
Albumin (g/dL)	4.2 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.0 ± 0.1	4.1 ± 0.1	4.1 ± 0.1
Globulin (g/dL)	2.1 ± 0.04	1.5 ± 0.2	2.1 ± 0.04	1.8 ± 0.1	2.0 ± 0.2	1.8 ± 0.1	2.2 ± 0.1	1.3 ± 0.03
Amylase (U/L)	2301 ± 257	1576 ± 138	2026 ± 119	1484 ± 51	2106 ± 91	1288 ± 28	1917 ± 25	1378 ± 93
Alkaline phosphatase (U/L)	123 ± 6	95. ± 11	133 ± 11	71 ± 4	142 ± 16	68 ± 6	138 ± 14	57 ± 9.2
AST (U/L)	124.8 ± 8.5	85.8 ± 5.8	93.3 ± 1.8	70.9 ± 2.9	96.5 ± 5.6	77.2 ± 6.9	89.3 ± 5.6	81.2 ± 4.3
ALT (U/L)	47.4 ± 4.4	35.2 ± 3.5	38.9 ± 1.5	30.3 ± 1.9	42.8 ± 2.9	32.4 ± 3.6	37.5 ± 1.7	31.4 ± 4.8

Values are expressed as mean ± S. E. M. of five rats in each group in comparison to the control using one-way ANOVA followed by Newman-Keuls test

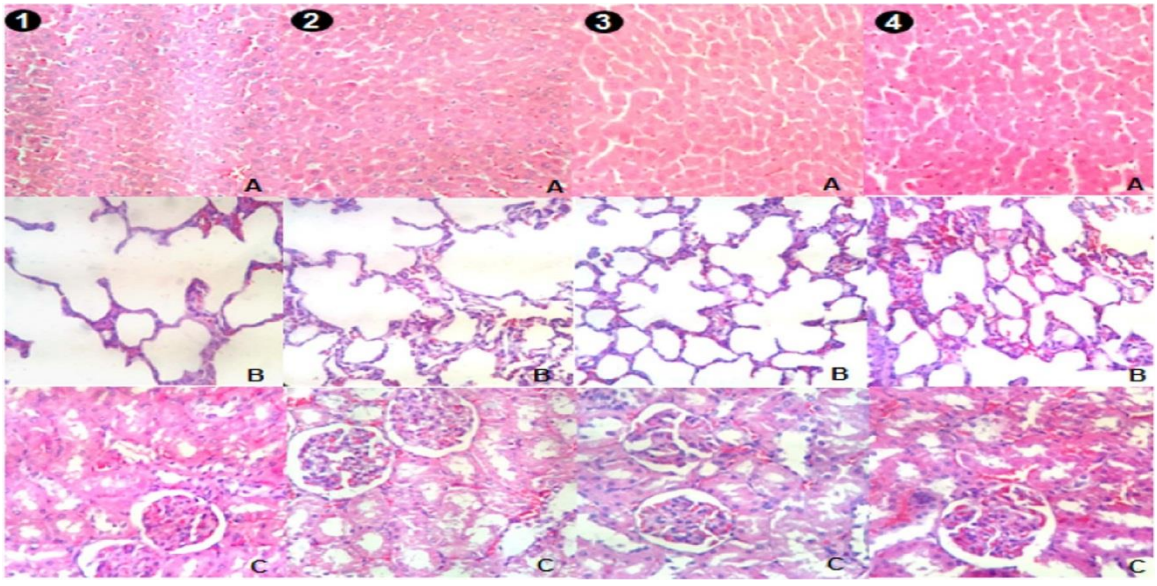

Fig. 3. Photomicrographs of liver (A; 400 X), lung (B; 400 X) and kidney (C; 400 X) histopathology from representative male (1 and 2) and female (3 and 4) rats treated with vehicle (1 and 3) or the highest dosage of ESAH (2000 mg/kg) (2 and 4). Hematoxylin and eosin stain.

Table 4 ffect of acute oral administration of ethanol soluble fraction obtained from *Acanthospermum hispidum* (ESAH) on the urinary volume, pH and density.

Group	Urine volume (ml/100g/8h)	Urine volume (ml/100g/24h)	pH (8h)	pH (24h)	Density (g/mL/8h)	Density (g/mL/24h)
Control	3.2 ± 0.50	8.7 ± 1.00	7.7 ± 0.37	8.1 ± 0.23	1023 ± 1.84	1033 ± 5.78
HCTZ (25 mg/kg)	5.6 ± 0.37[a]	10.0 ± 0.85	7.2 ± 0.28	7.6 ± 0.22	1017 ± 1.13[a]	1027 ± 2.45
ESAH (30 mg/kg)	3.4 ± 0.36	11.0 ± 1.20	8.1 ± 0.08	8.0 ± 0.07	1021 ± 3.64	1032 ± 3.07
ES AH (100 mg/kg)	2.8 ± 0.36	7.8 ± 0.57	7.8 ± 0.17	8.1 ± 0.07	1026 ± 3.66	1035 ± 5.11
ESAH (300 mg/kg)	2.7 ± 0.37	8.3 ± 1.20	7.7 ± 0.11	8.2 ± 0.03	1030 ± 4.69	1044 ± 2.18

Values are expressed as mean ± S. E. M. of ten rats in each group in comparison to the control group using one-way ANOVA followed by Newman-Keuls test. [a] $p \leq 0.05$ when compared with the control. HCTZ: hydrochlorothiazide.

Table 5
Effect of acute oral administration of ethanol soluble fraction obtained from *Acanthospermum hispidum* (ESAH) on urinary electrolyte excretion after 8 h of the treatment.

Group	El_{Na+} (µEq/min/100g)	El_{k+} (µEq/min/100g)	El_{Ca++} (µEq/min/100g)	El_{Cl-} (µEq/min/100g)	Saluretic index[b]			
					Na^+	K^+	Ca^{++}	Cl^-
Control	0.22 ± 0.06	0.10 ± 0.04	0.006 ± 0.0022	0.26 ± 0.06	-	-	-	-
HCTZ (25 mg/kg)	0.41 ± 0.05[a]	0.21 ± 0.04[a]	0.004 ± 0.0008	0.45 ± 0.07[a]	1.86	2.1	0.66	1.73
ESAH (30 mg/kg)	0.21 ± 0.04	0.14 ± 0.03	0.002 ± 0.0005	0.22 ± 0.04	0.95	1.4	0.33	0.85
ESAH (100 mg/kg)	0.17 ± 0.04	0.14 ± 0.03	0.002 ± 0.0003	0.21 ± 0.04	0.77	1.4	0.33	0.81
ESAH (300 mg/kg)	0.17 ± 0.04	0.13 ± 0.02	0.003 ± 0.0011	0.21 ± 0.04	0.77	1.3	0.50	0.81

Values are expressed as mean ± S. E. M. of five rats in each group in comparison with the control group using one-way ANOVA followed by Newman-Keuls test.
[a]$p < 0.05$ when compared with the control.
[b]Saluretic index = mmol/L problem group/mmol/L control group. El: Excreted load; HCTZ: hydrochlorothiazide.

Table 6
Effect of acute oral administration of ethanol soluble fraction obtained from *Acanthospermum hispidum* (ESAH) on urinary electrolyte excretion after 24 h of the treatment.

Group	El_{Na+} (µEq/min/100g)	El_{k+} (µEq/min/100g)	El_{Ca++} (µEq/min/100g)	El_{Cl-} (µEq/min/100g)	Saluretic index[b]			
					Na^+	K^+	Ca^{++}	Cl^-
Control	0.47 ± 0.11	0.46 ± 0.13	0.009 ± 0.0029	0.60 ± 0.14	-	-	-	-
HCTZ (25 mg/kg)	0.48 ± 0.07	0.51 ± 0.11	0.009 ± 0.0024	0.59 ± 0.10	1.02	1.11	1.00	0.98
ESAH (30 mg/kg)	0.47 ± 0.09	0.58 ± 0.13	0.010 ± 0.0040	0.62 ± 0.11	1.00	1.26	1.11	1.03
ESAH (100 mg/kg)	0.39 ± 0.03	0.47 ± 0.12	0.008 ± 0.0030	0.52 ± 0.08	0.83	1.02	0.89	0.86
ESAH (300 mg/kg)	0.60 ± 0.11	0.92 ± 0.29	0.010 ± 0.0038	0.94 ± 0.23	1.28	2.00	1.11	1.57

Values are expressed as mean ± S. E. M. of five rats in each group in comparison with the control using one-way ANOVA followed by Newman-Keuls test.
[a]$p < 0.05$ when compared with the control.
[b]Saluretic index = mmol/L. El: Excreted load; HCTZ: hydrochlorothiazide.

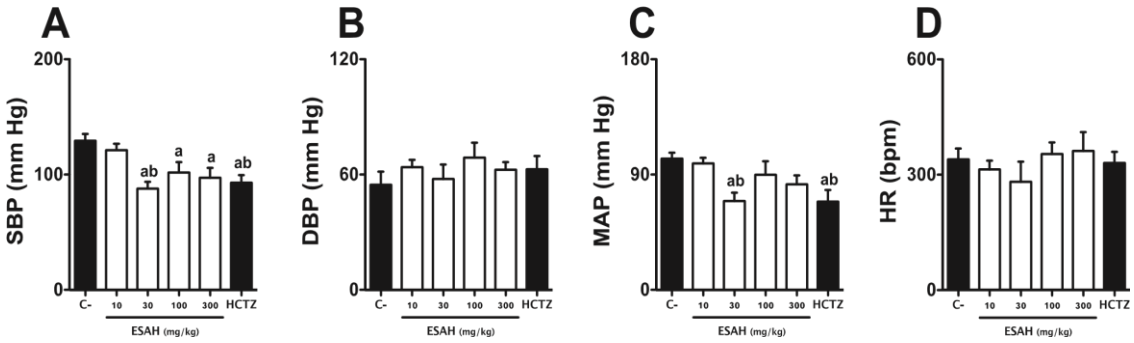

Fig. 4. Hypotensive effects induced by ESAH obtained from *Acanthospermum hispidum*. The ESAH was administered i.d. in anesthetized rats. The letter "C -" indicates the effect measured after administration of vehicle (saline 0.9%, 200 µL) only. The results show the mean ± S.E.M. (n = 5). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Newman–Keuls test. $^{a}p < 0.05$ when compared to respective control group; and $^{b}p < 0.05$ when compared to the dose 10 mg/kg. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; HR: heart rate.

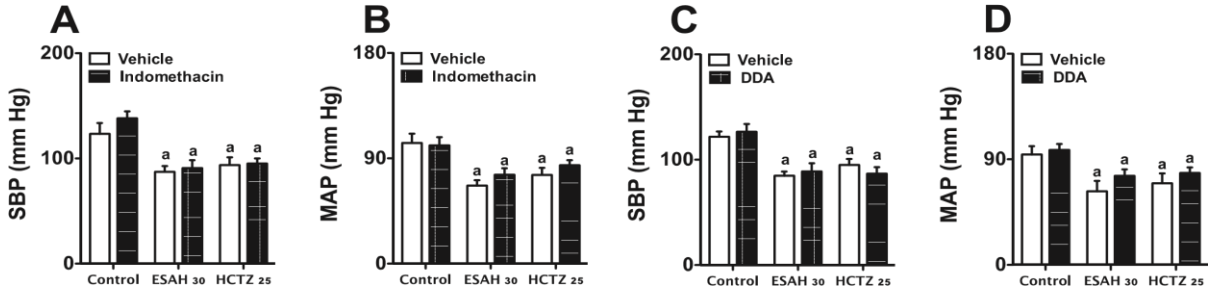

Fig. 5. Absence of involvement of prostaglandins/cAMP pathway in the hypotensive effect induced by ESAH. The animals received ASAH (30 mg/kg; i.d.) in the presence and absence of an intraperitoneal injection of indomethacin (3 mg/kg) (A and B), or DDA (0.1 mg/kg) (C and D). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Newman–Keuls test. $^{a}p < 0.05$ when compared with the respective control group. SBP: systolic blood pressure; MAP: mean arterial pressure.

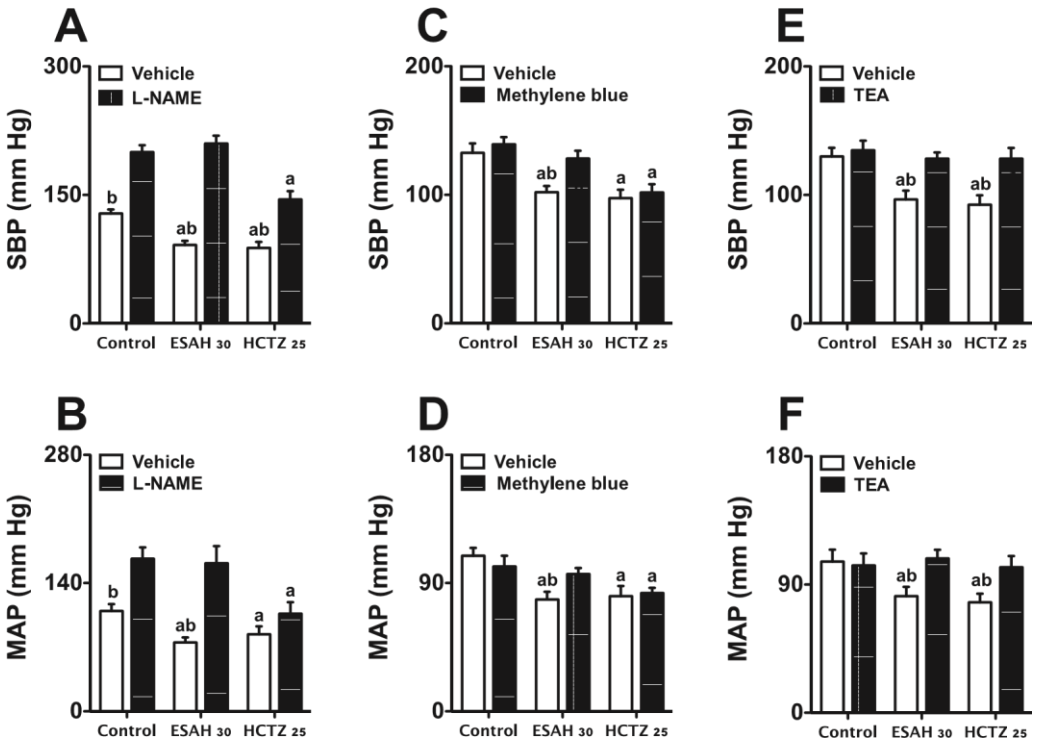

Figure 6. ESAH-induced hypotension depends on the nitric oxide/cGMP pathway and potassium channels activation. The animals were subjected to an intraduodenal administration of ESAH (30 mg/kg) in the presence and absence of continuous infusion of L-NAME (7 mg/kg/min) (A and B), or methylene blue (150 nmol/kg/min) (C and D), or an intraperitoneal injection of tetraethylammonium (400 μmol/kg) (E and F). The results are the mean ± S.E.M. of 5 experiments in each group. Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Newman–Keuls test. $^{a}p < 0.05$ when compared with the respective control group; $^{b}p < 0.05$ when compared with the respective inhibitor. SBP: systolic blood pressure; MAP: mean arterial pressure.

Despite the above information, no detectable toxic effects including detailed hematological, biochemical and histopathological evaluation were observed at oral doses of ESAH of up to 2000 mg/kg. This possible discrepancy may be related to the production characteristics of the different secondary metabolites present in *A. hispidum*, which can be directly influenced by soil, climate, habitat, and luminosity, or even by the method through which the extract was obtained. The fact is that the data obtained at this stage already indicated possible safety of ESAH at all doses used.

Based on the ethnobotanical, phytochemical and toxicological data, we began to investigate the ethnopharmacological properties of the species. First, we investigated the possible acute diuretic activity of ESAH. Despite the clear ethnobotanical indication (Oliveira et al., 2011), no diuretic or natriuretic effect was observed in male Wistar rats.

However, since diuretics, especially thiazides, are routinely used for the treatment of arterial hypertension regardless of their diuretic effects (Blowey, 2016), we decided to investigate the possible hypotensive activity of ESAH. In fact, we found that ESAH can induce a significant acute hypotensive effect after intraduodenal administration, with effectiveness very similar to that obtained with hydrochlorothiazide, a first-line antihypertensive.It is known that blood pressure regulation may involve several endogenous mediators, including prostaglandins, nitric oxide and the endothelium-derived hyperpolarizing factor (EDRF), synergistically acting in a complex hemodynamic and neurohumoral interaction (for review see Feletou and Vanhoutte, 2006).

In our study, it has been shown that the hypotensive effects induced by ESAH appear not to depend on the prostaglandin/cAMP pathway, since prior administration of indomethacin or DDA does not affect ESAH-induced hypotension. On the other hand, as the hypotensive effects induced by ESAH were completely inhibited by L-NAME (a non-selective nitric oxide synthase inhibitor) or methylene blue (a guanylyl cyclase inhibitor), the nitric oxide/cGMP pathway appears to be directly involved. Nitric oxide is able to activate the soluble guanylate cyclase enzyme through its attachment to the iron of the enzymatic heme group. This activation forms cGMP, an important vasodilatory agent. It is currentlythat cGMP can lead to hyperpolarization and relaxation of vascular smooth muscle by the activation of cGMP-dependent protein kinase, which causes phosphorylation of calcium-sensitive potassium channels (K_{Ca}) (Archer et al., 1994; Bolotina et al., 1994).

The data presented here suggest that ESAH may, in some way that has not yet been clarified, influence the increase in endothelial nitric oxide activity and consequently increase cGMP levels. As a final response, the opening of K_{Ca} channels induces hyperpolarization and

consequent vasodilation, explaining, at least in part, the hypotensive effects of ESAH. Indeed, the reduction of the hypotensive response observed after TEA administration belives reinforces this hypothesis, since the hypotensive effect disappears after blockade of K_{Ca} channels.

5. Conclusion

Data obtained in this study allow us to suggest that ESAH obtained from *A. hispidum* presents an important acute hypotensive effect, which appears to be dependent on the nitric oxide/cGMP pathway. This study presents new evidences about the therapeutic potential of this species when acute hypotensive response is required.

Conflict of interest

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

Authors' contributions

All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; CAST, RCAS and RACP conducted the experiments; PCPV was involved with hematological and biochemical analysis; LMS was involved with chemical analysis of extract; JMB and VPA was involved with histochemical and anatomical analysis of the plant; ACS and RICS was involved with pathological analysis and CAST, FARL and AGJ performed data analyses and wrote the manuscript. All authors read and approved the final manuscript.

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5.2 Artigo 2: Ethnopharmacological approaches to kidney disease-prospecting an indigenous species from Brazilian Pantanal

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Ethnopharmacological approaches to kidney disease-prospecting an indigenous species from Brazilian Pantanal

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ABSTRACT

Ethnopharmacological relevance: Although *Luehea divaricata* Mart. (Malvaceae) is popularly used by the population of the Brazilian Pantanal for the treatment of different types of kidney diseases, no study has been carried out to prove this ethnobotanical indication.

Aim: To investigate the possible cardiorenal effects of an herbal preparation obtained from *L. divaricata* leaves. Materials and methods: First, to provide quality control standards, a detailed morphological and microchemical characterization of *L. divaricata* leaves was performed. Then, the purified aqueous extract was obtained from the leaves of this species (ESLD) and a thorough phytochemical characterization was performed. Subsequently, acute oral toxicity test was performed after single administration of different doses (5, 50, 300, 2000 mg/kg) in male and female Wistar rats. Finally, the diuretic, hypotensive and antioxidant properties of ESLD (30, 100, 300 mg/kg) were evaluated after acute and prolonged treatment and the role of angiotensin converting enzyme, aldosterone, vasopressin, and nitric oxide in these effects was investigated.

Results: Analyses performed by liquid chromatography–mass spectrometry showed that the main secondary metabolites present in ESLD were flavonol O-glycosides and flavone C-glycosides. Acute and prolonged treatment with ESLD was able to expressively increase urinary volume and electrolyte excretion. Mean blood pressure and systolic blood pressure were also significantly reduced after acute treatment. Moreover, treatment with ESLD was able to reduce thiobarbituric acid reactive species and increase serum nitrate levels.

Conclusion: The data obtained showed that ESLD has an important diuretic and hypotensive effect, which is probably dependent on the reduction of oxidative stress and increased bioavailability of nitric oxide.

Abbreviations: ACE, angiotensin converting enzyme; AELD, aqueous extract from *Luehea divaricata*; ANOVA, analysis of variance; bpm, beats per minute; BW, body weight; Ca^{+2}, calcium; Cl^{-}, chloride; DBP, diastolic blood pressure; EDS, energy-dispersive X-ray spectroscopy; ESLD, ethanol soluble fraction from *Luehea divaricata*; ESI, electrospray ionization; EtOH, ethanol; FESEM, field emission scanning electron microscopy; HCD, higher-energy collisional dissociation; HCTZ, hydrochlorothiazide; HR, heart rate; HR-MS, high-resolution mass spectrometry; K^{+}, potassium; MAP, mean arterial pressure; Na^{+}, sodium; NO, nitric oxide; OECD, Organization for Economic Co-operation and Development; PDA, photodiode array;SBP, systolic blood pressure; SEM, standard error of the mean; TBARS, thiobarbituric acid reactive species; UHPLC, ultra-high performance liquid chromatography; W/W, weight/weight.

1. Introduction

In Brazil, there is a vast region still untouched by modern life, where different indigenous and riverside populations cohabit with rich flora and fauna. The Pantanal region of the state of Mato Grosso do Sul is an immense wetland area where few ethnopharmacological studies have been conducted (Dutra et al., 2016; Hamilton et al., 1996).

Ethnobotanical studies have shown that the native population of Pantanal still has products obtained from nature as one of the main sources of medicines. Among the species most frequently mentioned in ethnobotanical studies carried out in the region, *Luehea divaricata* Mart. (Malvaceae) stands out (Bieski et al., 2012).

The species, a large tree distributed throughout South America (Lorenzi, 1992) and popularly known as "açoita cavalo" and "caiboti",

is used by native populations of Pantanal to treat uric acid, kidney diseases, throat inflammation, flu, hemorrhoids, pneumonia, muscle aches, cough, and tumors (Bieski et al., 2012). Preliminary phytochemical studies have shown the presence of phenolic compounds such as chlorogenic acid, rosmarinic acid, rutin, vitexin and epicatechin, along with triperpenoids and phytosterols (Tanaka et al., 2005; Arantes et al., 2014; Courtes et al., 2015). Biological studies with different preparations obtained from *L. divaricata* have shown antifungal (Zacchino et al., 1998), antioxidant (Courtes et al., 2015; Arantes et al., 2014), neuroprotective (De Souza et al., 2004), anti-inflammatory, analgesic, immunostimulatory (Rosa et al., 2014), and anticholinesterase (Arantes et al., 2014) effects. Toxicological studies with the specie are very scarce. Felício et al. (2011) evaluated the mutagenic potential of the aqueous extract obtained from *L. divaricata* leaves in somatic cells of Drosophila melanogaster Meig. The frequency of mutations was not significantly higher compared to the negative control group.

Thus, considering the lack of data on the effectiveness of *L. divaricata* on renal function, and the absence of data on the acute toxicity of the infusion obtained from the leaves of this species, we propose to carry out a complete ethnopharmacological study. First, to provide quality control standards, a detailed morphological and microchemical characterization of *L. divaricata* leaves was performed. Then, the purified aqueous extract from leaves of this species was obtained and a thorough phytochemical characterization was performed. Finally, an acute toxicity study of this preparation was performed, and the

molecular mechanisms involved in the diuretic and hypotensive effects on Wistar rats were evaluated.

2. **Materials and methods**

2.1. Drugs and solvents

Hydrochlorothiazide and rutin were obtained from Sigma-Aldrich (St. Louis, MA, USA). Acetonitrile and formic acid were purchased from J.T. Baker (Center Valley, PA, USA) and Tedia (Fairfield, OH, EUA), respectively. All other chemicals and solvents were of analytical grade.

2.2. Plant material

Luehea divaricata leaves were collected in September 2015 from the botanical garden of the Federal University of Grande Dourados (UFGD) (Dourados, Brazil) at 458 m above sea level (S 22°16′46, 9″ and W 54°49′06, 3″). A voucher specimen was authenticated by Dr. Maria do Carmo Vieira under number DDMS 5220 and deposited in the herbarium of UFGD.

2.3. Anatomical analysis

The leaves of *L. divaricata* were cut about 10 cm from the apex and put in containers containing FAA 70 solution, and stored in 70% ethanol. The leaves were segmented by hand in transverse sections and stained using astra blue and/or basic fuchsine. The leaves were also bleached to analyses the epidermis in frontal view (Upton et al., 2011).

2.4. Histochemical study

The following standard solutions were used for histochemical tests: Methylene blue 1% to reveal mucilage; Sudan III for testing lipophilic compounds; Hydrochloric phloroglucin to reveal lignin; Iodine-iodide to test for starch (Upton et al., 2011); Ferric chloride and HoepfnerVorsatz test, modified by Reeve (1951) to test phenolic substances. The photomicrographs were captured by an Olympus CX 31 light microscope equipped with a C 7070 control unit.

2.5. Field Emission Scanning Electron Microscopy (FESEM) and Energy-Dispersive X-ray Spectroscopy (EDS)

For FESEM (Mira 3 Tescan) of the leaf surface and cross-section of the blade and petiole, high vacuum with high accelerating voltage was used (15 kV). This method requires

samples to be previously dehydrated using increasing amounts of ethanol, being subsequently dried in a critical point dryer. Later, samples were submitted to metallization with gold (Quorum, model SC7620).

EDS chemical microanalysis was randomly performed in crystals and cells without crystals (control), with X-ray detector coupled to FESEM under the same FESEM operating conditions that were used to obtain electron micrographs. This procedure was carried out at the multi-user laboratory (C-Labmu), State University of Ponta Grossa (UEPG, Brazil).

2.6. Preparation of the purified aqueous extract

L. divaricata leaves were air-dried in an oven at 40 °C for 7 days. Percentage of the moisture content (64.5%) was determined using the following equation: Mn = ((Wf-Wd)/Wf) × 100, in which: Mn = moisture content (%) of material; Wf = wet weight of the sample; and Wd = weight of the sample after drying. After drying, the leaves were slowly ground with an ultra centrifugal mill operating by impact and shearing effects. Grinding was performed at room temperature. After grinding, plant powders were sieved with a vibratory sieve shaker with 0.5 mm vibration amplitude for 10 min 20 mm diameter sieves (Fritsch) were employed so as to obtain a 100–180 μm granulometric fraction. The extract was obtained by infusing 1 l of boiling water for each 60 g of dried and pulverized plant. Extraction was carried out until room temperature was reached (~5 h). The infusion was treated with 3 volumes of EtOH, which gave rise to a precipitate and an ethanol soluble fraction (ESLD). ESLD was filtered, concentrated and freeze-dried (yield of 2.8% w/w). All preparations were kept in freezer until analyses.

2.7. Phytochemical investigation

ESLD constituents were identified based on ultra-high-performance liquid chromatography (UHPLC) (Acquity-UPLC™, Waters), detected by photodiode array (PDA, Waters) and high-resolution mass spectrometry (HR-MS). Chromatography was developed in a BEH-C18 column, 100 × 2.1 mm with particle size of 1.7 μm. The solvent used was ultra-pure water (MilliQ) and acetonitrile, both containing 0.1% of 96% formic acid. A linear gradient was applied, increasing acetonitrile content from 0% to 10% in 5 min, then to 70% in 13 min, returning to initial condition (acetonitrile at 0%) in 13.5 min. The column was re-

equilibrated at initial condition for 2 min. The column temperature was held at 60 °C with constant flow rate of 400 µl/min. The sample was prepared in MeOH-H_2O (1:1, v/v) at 2 mg/ml and 5 µl was injected. Compounds were detected by PDA 200 − 400 nm and HR-MS.

HR-MS was developed in LTQ Orbitrap XL (Thermo-Scientific), operating both negative and positive polarities. The electrospray (ESI) source temperature was 350 °C and nitrogen was used in sample desolvation at flow rate of 40 arbitrary units (a.u.) in the sheath gas and 5 a.u. in the auxiliary gas. In the positive polarity, ions were detected with voltages at 5 kV in the source, 30 V in the capillary and 110 V in the tube lens; for negative ions, voltages were: 3.5 kV, −45 V and −200 V, respectively. Fragmentation with HCD (higher-energy collisional dissociation) on C-Trap was performed with normalized collision energy of 35%. Resolution was set at 15,000 FWHM and prior to analysis, the instrument was externally calibrated with Pierce™ LTQ ESI Positive Ion (Caffeine, MRFA, Ultramark-1621) and Negative Ion (SDS, sodium taurocholate, Ultramark-1621) calibration solutions (ThermoFischer).

2.8. Safety and efficacy assessment

2.8.1. Animals

Wistar rats of both sexes (250–300 g) were purchased from Federal University of Grande Dourados (UFGD, Brazil) and kept at controlled conditions (temperature: 22 ± 2 °C; luminosity: 12 h light/dark cycle) with free access to food and filtered water. All experimental procedures were approved by Institutional Ethics Committee of UFGD (protocol number 16/2015) and conducted in accordance with the Brazilian Legal Framework on the Scientific Use of Animals.

2.8.2. Toxicological study

To evaluate the acute oral toxicity, different doses of ESLD (single-5, 50, 300, 2000 mg/kg doses) or vehicle (10 ml/kg) were orally administered in male and female Wistar rats (n = 5) according to recommendations of the Organization for Economic Co-operation and Development (OECD) protocol No. 420. Behavioral parameters and clinical signs of toxicity, including mortality rate, were monitored during the first 8 h after ESLD administration and daily for 14 days. At this end point, animals were sacrificed by decapitation and blood

samples were collected. Serum was obtained by centrifugation (1000×g for 10 min). Hematological analysis was performed in a full blood count automated analyzer (Sysmex® XN-3000), and biochemical parameters were determined in automated biochemistry analyzer (Roche® Cobas Integra 400 plus). Reproductive (ovaries, uterus, epididymis, testicles, prostate, levator ani muscle, and seminal vesicles) and vital organs (liver, heart, spleen, and kidneys) were removed to gross pathology and relative weight determination (Absolute organ weight × 100/ Body weight of rat on sacrifice day). The histopathological evaluation of lungs, heart, spleen, kidneys, and liver were also performed according to technique described by Stevens and Wilson (1996).

2.8.3. Pharmacological evaluation

2.8.3.1. Diuretic activity.

Diuretic activity was evaluated according to methods described by Gasparotto Junior et al. (2009). After randomization, different groups of male rats (n = 7) received saline in a volume of 5-ml/100 g body weight (BW) by gavage to impose body uniformity of salt and water. Then, three groups of rats were treated once daily for 7 days with ESLD at doses of 30, 100, and 300 mg/kg. Positive and negative controls groups receive hydrochlorothiazide (25 mg/kg) and vehicle (filtered water; 0.2 ml/100 g), respectively. Immediately after the first treatment, all animals were placed in metabolic cages and urine was collected and measured (expressed as ml/100 g of BW) every hour for the first 8 h, and then every 24 h on days 1, 3, 5 and 7. pH and density were determined on fresh urine samples using digital pH meter (Q400MT; Quimis Instruments, Brazil) and a handheld refractometer (NO107; Nova Instruments, Brazil), respectively. At the end of experiments, blood samples were collected by decapitation. Serum samples were obtained by centrifugation (800g, 10 min) and serum and urinary sodium (Na^+), potassium (K^+), and chloride (Cl^-) levels were determined using ion selective meter (COBAS INTEGRA 400 plus; Roche®). Excretion load (El) was obtained by multiplying the Na^+, K^+, and Cl^- concentration (mEq/l) by the urinary flow (ml/min). Results are expressed as μEq/min/100 g BW.

2.8.3.2. Effects on blood pressure and heart rate.

The hypotensive activity was evaluated after acute and prolonged treatment according to methods described by Gasparotto Junior et al. (2011). For the investigation of the acute hypotensive effects, different groups of male rats (n = 6–7) were intramuscularly anesthetized with an association of ketamine (100 mg/kg) and xylazine (20 mg/kg). Subsequently, the right carotid artery was isolated, cannulated and connected to a pressure transducer coupled to a PowerLab® recording system (Chart, v 4.1; all from ADI Instruments; Castle Hill, Australia). After hemodynamic stabilization (15 min), different groups of male rats received ESLD (30, 100 or 300 mg/kg), HCTZ (25 mg/kg) or vehicle (filtered water; 0.2 ml/100 g) by intraduodenal route. Changes in mean arterial pressure (MAP), systolic (SBP) and diastolic blood pressure (DBP), and heart rate (HR) were recorded for 35 min.

Similarly, to evaluate the prolonged hypotensive activity, different groups of male rats received (by oral route) ESLD (30, 100 or 300 mg/kg), HCTZ (25 mg/kg), or vehicle (filtered water; 0.2 ml/100 g) once daily for 7 days. At the end of experiments, all animals were anesthetized and prepared (as described above) for the measurement of MAP, SBP, DBP, and HR. The different cardiovascular parameters were recorded for 5 min after the hemodynamic stabilization period.

2.8.3.3. Investigation of the molecular mechanisms involved in the cardiorenal activities of ESLD.

After 7 days of oral treatment (male Wistar rats; n = 7) with different doses of ESLD (30, 100, and 300 mg/ kg), HCTZ (25 mg/kg), or vehicle (filtered water; 0.2 ml/100 g), blood samples were collected by decapitation. Then, serum was obtained by centrifugation (800g, 10 min). Serum ACE activity was determined by indirect fluorimetry according to methods described by Santos et al. (1985). Aldosterone and vasopressin levels were measured by Enzyme Linked Immunosorbent Assay (ELISA, Immuno-Biological Laboratories, Inc). Thiobarbituric acid (TBARS) levels were measured using TBARS assay kits (Cayman Chemical, Ann Arbor, Michigan, USA) according to manufacturer's instruction. Finally, the plasma nitrite concentration was determined by enzymatically reducing nitrate according to technique described by Schmidt et al. (1989).

2.9. Statistical analysis

Results are expressed as mean ± standard error of mean (S.E.M) of 5–7 animals per group. Statistical analyses were performed using oneway analysis of variance (ANOVA) followed by Dunnett's test. P-value less than 0.05 was considered statistically significant. Graphs were drawn and statistical analysis was carried out using GraphPad Prism software version 5.0 for Mac OS X (GraphPad® Software, San Diego, CA, USA).

3. **Results**

3.1. Pharmacobotanical investigations

The frontal view of *Luehea divaricata* leaves (Fig. 1A and B) showed straight and thin epidermal cell anticlinal walls (Fig. 1D) on both sides. Stomata are anomocytic and leaves are hypostomatic. *L. divaricata* showed uniseriate and capitate glandular (Fig. 1D and E), and stellate non-glandular trichomes (Fig. 1C, D, L, O, and Q), which appeared in great amount on the abaxial face. In cross-section, the epidermis was uniseriate (Fig. 1G, H, and I) and was covered by a thin and striate cuticle (Fig. 1C and F). Upper epidermal cells were larger than lower epidermal cells (Fig. 1G). The mesophyll was dorsiventral (Fig. 1G) and beneath the epidermis, mucilage idioblasts were observed (Fig. 1G, H, and I). Druses and prismatic crystals were found in the mesophyll (Fig. 1I and J).

The midrib showed oval shape; the vascular system was represented by a single collateral vascular bundle in open arc. Mucilage idioblasts were spread in the ground parenchyma (Fig. 1K). The petiole was almost circular and showed several trichomes. Mucilage idioblasts were spread in the ground parenchyma (Fig. 1Q and S). Starch endodermis was surrounded by the vascular system (Fig. 1L, M, and N), which was represented by a collateral vascular bundle at the center and about 4 dorsal vascular bundles (Fig. 1L). Sclerenchymatous cells and/or bundles were found in the phloem (Fig. 1P and R). Druses and prismatic crystals are observed in the ground parenchyma and near vascular bundles (Fig. 1T).

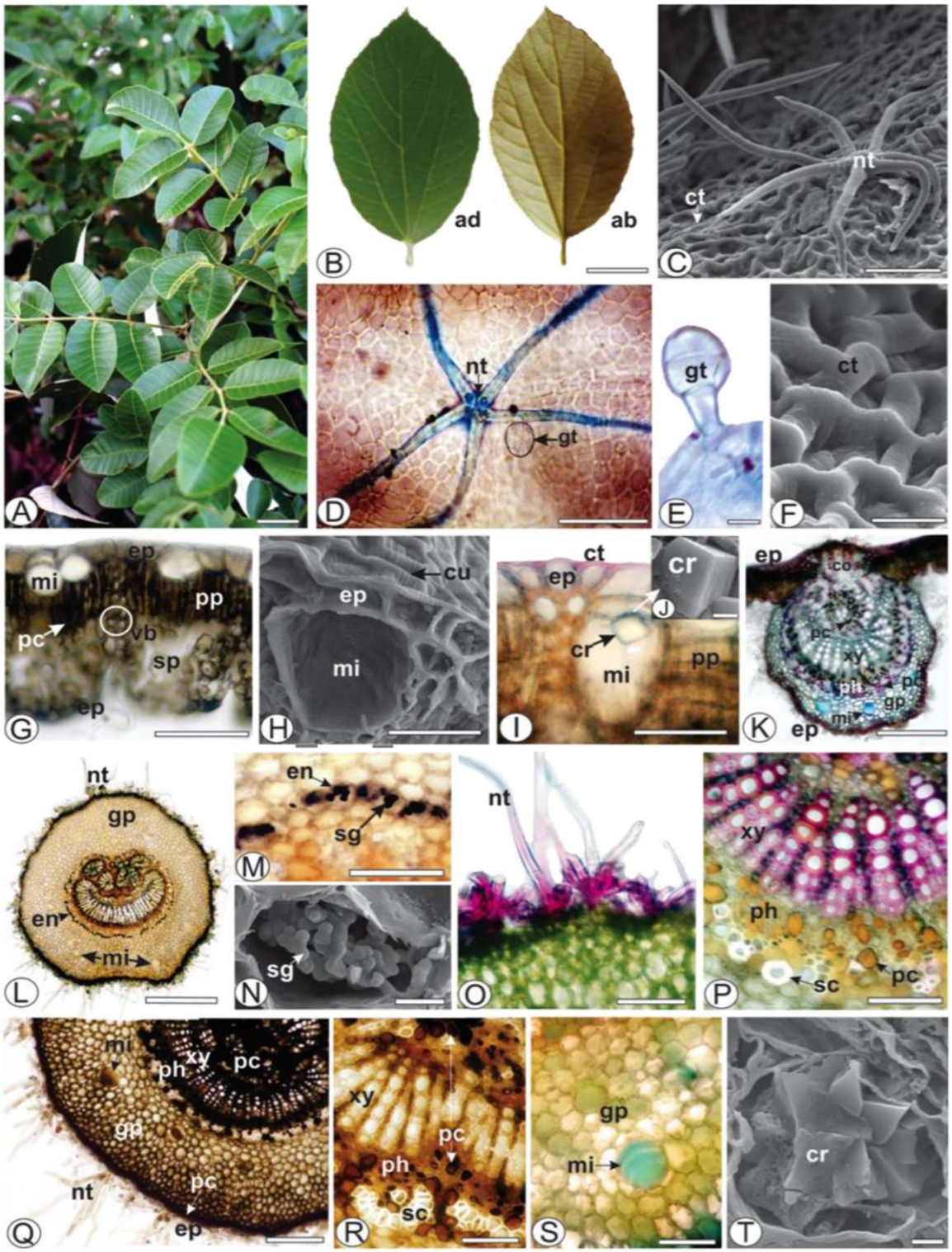

Fig. 1. *Luehea divaricata* Mart. (Malvaceae). A. Plant in habit; B. Leaves on adaxial and abaxial side; C. Upper epidermis; D. Upper epidermis (FESEM); E. Glandular trichome (gt); F. Detail of the cuticle (FESEM); G. Mesophyll in cross-section; H. Detail of mucilage idioblast (FESEM); I. Blade in cross-section; J. Detail of the prismatic crystal (FESEM); K.

Midrib in crosssection; L. Petiole in reaction with Iodine-iodide; M. Detail of the figure L; N. Starch grains (FESEM); O, P. Petiole in reaction with hydrochloric phloroglucin; Q. Petiole in reaction with ferric chloride; R. Petiole in reaction in the Hoepfner-Vorsatz test. S. Petiole in reaction with methylene blue 1%; T. Druse crystal in FESEM. Abaxial side (ab); adaxial side (ad); collenchyma (co); cuticle (ct); epidermis (ep); endodermis (en); glandular trichome (gt); ground parenchyma (gp); leaves (le), mucilage idioblasts (mi), non-glandular trichome (nt); palisade parenchyma (pp); phenolic compounds (pc), phloem (ph); sclerenchymatous cells (sc); spongy parenchyma (sp); starch grains (sg); vascular bundle (vb); xylem (xy). Scale bar = A, B (2 cm), K (400 µm), J, Q (200 µm), C, D, G, L, O, P, R, S (50 µm), H (20 µm), E, F, I, M (10 µm), J, N, T (2 µm).

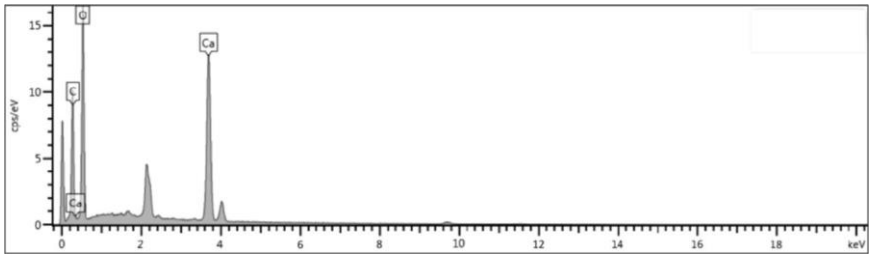

Fig. 2. EDS (energy-dispersive X-ray spectroscopy) spectra of crystals of *Luehea divaricata*.

Lipophilic compounds were evidenced with Sudan III in glandular trichomes and in the cuticle (Fig. 1I). Hydrochloric phloroglucin revealed traces of lignin in non-glandular trichomes (Fig. 1O), sclerenchyma and xylem vessels (Fig. 1P). In the petiole, the iodineiodide solution indicated the presence of starch grains in the endodermis (Fig. 1L, M, and N). Phenolic compounds were evidenced by ferric chloride in the mesophyll, especially in the palisade parenchyma (Fig. 1G) and in the epidermis, phloem and xylem in the petiole (Fig. 1Q and Fig. 1Q and R). Mucilage was found in idioblasts, in the mesophyll (near the epidermis), and in the ground parenchyma in the midrib (Fig. 1K) and petiole (Fig. 1S).

Druses (Fig. 1T) were analyzed for their elemental composition and the spectra showed prominent peaks for calcium (40.54%), carbon, (16.72%) and oxygen (42.74%), as can be seen in Fig. 2, indicating that these crystals were formed by calcium oxalate. Not only the druses but also the prismatic crystals (Fig. 1I and J) showed calcium oxalate as composition.

3.2. Phytochemical characterization

There are few reports on the chemical composition of *L. divaricata*, indicating the presence of phenolic compounds such as chlorogenic acid, rosmarinic acid, rutin, vitexin and epicatechin, as well as triperpenoids and phytosterols (Tanaka et al., 2005; Arantes et al., 2014; Courtes et al., 2015). In our study, the phytochemical composition of ESLD showed mainly the presence of flavonol O-glycosides and flavone C-glycosides (Table 1; Fig. 3). Peak 1, observed at m/z 593.150 $[M-H]^-$, was consistent with vicenin 2, with fragments at m/z 503.120, 473.106, 383.076, 353.065, exhibiting the sequential neutral losses (NL) of 90 and 120 amu (a.m.u.), characteristic of C-glycosides (Prando et al., 2016). Other compounds with similar MS profile were peaks 4 and 6, at m/z 431.098 $[M-H]^-$ and main fragments at m/z 341.066 and 311.056. Based on their chromatographic elution, peaks 4 and 6 were identified as vitexin and isovitexin, respectively (Farsi et al., 2014).

The major compounds observed were identified as flavonol-O-glycosides. Although these compounds are well observed by negative ionization MS, fragments of negative ions are frequently worse than positive ones, lacking essential information on oligosaccharides. Thus, protonated $[M+H]^+$ ions were preferred for the analysis of O-glycosides, since these fragments indicate the monosaccharide sequence. Peak 2 at m/z 757.218 presented fragments at m/z 611.160, 465.102, 303.049 with NL of 146 (x 2) followed by 162 a.m.u., consistent with losses of deoxyhexosyl (x 2) and hexosyl residues, respectively. The most common deoxyhexose attached to flavonols is rhamnose, whereas 2 hexoses are frequently found, galactose or glucose (Souza et al., 2016). Fragment at m/z 303.049 was consistent with quercetin; thus, peak 2 was identified as dirhamnosyl-hexosyl-quercetin, similar to that previously found in Maytenus ilicifolia (Souza et al., 2008). Peaks 3 and 5 appeared at m/z 611.160 with fragments consistent with losses of rhamnosyl and hexosyl residues. These were consistent with rhamnosylhexosyl-quercetin and peak 5 was confirmed as rutin, in comparison with authentic standard. Peaks 7 and 8 (m/z 595.165) were similar to peaks 3 and 5, but containing kaempferol in aglycone moiety (fragment at m/z 287.055).

Chlorogenic acids were not found in ESLD, but 2 low-abundance peaks (9 and 10) were consistent with dicaffeoylquinic acids. They appeared at m/z 515.119 $[M-H]^-$ with main fragments at m/z 353.87 and 191.055 and UV profile characteristic of hydroxycinnamic acids with λmax at 325 nm.

Peak 11 was observed at m/z 581.129 $[M-H]^-$ and fragments 431.098, 413.088, 311.056, 293.045. Fragments at m/z 431.098 and 311.056 were consistent with (iso) vitexin due to the loss of vanilloyl residue, and the main fragments (m/z 413.088 and 311.056) seem to be a dehydrated form of the former fragments, produced after the loss of entire vanillic acid moiety (Wu et al., 2013). Peaks 12 and 13 appeared at m/z 593.129 $[M-H]^-$ being similar to rhamnosyl-hexosyl-kaempferol (m/z 593.151). However, this subtle difference observed in decimals indicated a structure different from those of diglycosides. Fragments 285.039 and 284.032 were consistent with kaempferol (regular and radical ions, respectively) and fragment 307.081, consistent with a pcoumaroyl-hexose residue (Felipe et al., 2014), suggesting tiliroside isomers. The presence of p-coumaric acid linked to kaempferol-hexoside was consistent with the ion observed and, also, with the retention time of peaks, since being more hydrophobic than the monosaccharide residue, compounds containing p-coumaric acid interact stronger with the C18 column.

3.3. Toxicological findings

No significant behavioral change was observed throughout the experimental period. All animals received regular diet and body weight gain was homogeneous (Table 2). Hematological (Table 3) and biochemical (Table 4) analyses did not detectany statistically significant alteration between the animals treated with ESLD or vehicle.

Table 1
Phytochemical composition of ESLD obtained by LC-PDA-MS.

Peak	Rt (min)	MS¹	MS² (key fragments)	Tentative identification
1	5.07	593.15066[a]	[a]503.120, 473.106, 383.076, 353.065	Vicenin 2
2	5.77	755.20423[a] 757.21897[b]	[b]611.160, 465.102, 303.049	Dirhamnosyl-hexosyl-quercetin
3	5.95	609.14621[a] 611.16030[b]	[b]465.103, 303.050	Rhamnosyl-hexosyl-quercetin
4	6.03	431.09815[a]	[a]341.066, 311.056, 283.060	Vitexin
5	6.06	609.14670[a] 611.16072[b]	[b]465.103, 303.050	Rutin
6	6.12	431.09843[a]	[a]341.066, 311.056, 283.061	Isovitexin
7	6.24	593.15164[a] 595.16546[b]	[b]449.108, 287.055	Rhamnosyl-hexosyl-Kaempferol
8	6.39	593.15176[a] 595.16552[b]	[b]449.107, 287.055	Rhamnosyl-hexosyl-Kaempferol
9	6.46	515.11945[a]	[a]353.087, 191.055, 179.034, 173.045, 135.045	Dicafeoylquinic acid
10	6.69	515.11975[a]	[a]353.087, 191.055, 179.034, 173.045, 135.045	Dicafeoylquinic acid
11	7.05	581.12986[a]	[a]431.098, 413.088, 311.056, 293.045	Vanilloyl-vitexin
12	7.32	593.12979[a]	[a]307.081, 285.039, 284.032	Kaempferol-(*p*-coumaroyl)-hexoside
13	7.45	593.12972[a]	[a]307.081 285.039, 284.032	Kaempferol-(*p*-coumaroyl)-hexoside

[a]Spectra recorded in the negative ionization mode. [b]Spectra recorded in the positive ionization mode.

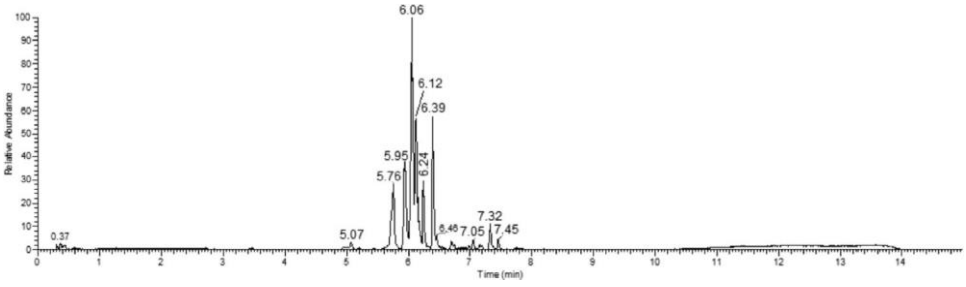

Fig. 3. Analysis of the ESLD from *Luehea divaricata* by UHPLC-HRMS in the negative ionization mode. The analysis was performed in full scan and total ion current (TIC) mode

The appearance and relative weight of reproductive and vital organs were within normality for species and gender (Table 4). Histopathological analyses did not show any signs of lesions or parenchymal alterations that could suggest direct toxic effects

3.4. Pharmacological report

3.4.1. ESLD induces a potent diuretic and saluretic effect after acute treatment

The effects of acute treatment with ESLD and HCTZ on urinary excretion of male rats are presented in Table 5. The urinary volume produced by animals treated with vehicle after 4 and 8 h were 2.8 ± 0.45, and 4.0 ± 0.50 ml/100 g, respectively. Treatment with ESLD at doses of 30 and 100 mg/kg was able to significantly increase urinary volume after 4 (4.9 ± 0.30 and 4.1 ± 0.32, respectively; $p < 0.05$) and 8 h (5.9 ± 0.36 and 5.4 ± 0.39, respectively; $p < 0.05$). Values obtained for animals treated with ESLD (30 and 100 mg/kg) were not different from those obtained for animals receiving HCTZ. Table 6 shows the profile of urinary electrolyte excretion induced by acute treatment with ESLD and HCTZ. After 8 h of treatment, ESLD (at all doses) was able to increase the renal excretion of electrolytes (Na^+, K^+, and Cl^-) by approximately 100 % when compared to animals treated with vehicle alone. Values obtained for the elimination of Na^+, K^+, and Cl^- induced by ESLD were not statistically different from those obtained with HCTZ. pH, density or serum parameters (Na+ and K+) showed no significant differences between experimental groups (data no shown).

3.4.2. ESLD induces long-lasting diuretic effect during 7 days of treatment

Daily oral administration of ESLD at the dose of 30 mg/kg was able to significantly increase urinary volume during 7 days of treatment. When compared to the control group, ESLD was able to increase the daily urinary volume by approximately 40% on days 3, 5, and 7 after treatments (Fig. 5A). The cumulative urinary volume found in animals treated with ESLD was not different from values obtained in animals treated with HCTZ.

The effects of prolonged treatment with ESLD (30, 100, and 300 mg/kg) and HCTZ on electrolyte (Na^+, K^+, and Cl^-) excretion are presented in Fig. 5B, C, and D. Renal Na^+ elimination was significantly increased on days 3, 5 and 7 in animals receiving ESLD at dose of 30 mg/kg. Similarly, HCTZ (25 mg/kg) and ESLD at dose of 100 mg/kg were able to significantly increase Na^+ elimination on days 5 and 7 after the beginning of treatments. Furthermore, urinary Cl^- levels showed a similar pattern of response between animals treated with ESLD at doses of 30 and 100 mg/kg or HCTZ. As compared to animals treated with vehicle, urinary Cl^- excretion was significantly increased from day 5 of treatment. On the other hand, HCTZ was able to increase the renal elimination of potassium during prolonged treatment. The other urinary (pH and density) or serum (Na^+ and K^+) parameters were not altered by any of treatments (data no shown).

Table 2
Effect of acute oral administration of ethanol soluble fraction obtained from *Luehea divaricata* (ESLD) on the body weight (BW) and relative (%) organ weights of male and female Wistar rats exposed for 14 days.

Organ	Control		ESLD (50 mg/kg)		ESLD (300 mg/kg)		ESLD (2 g/kg)	
	Male	Female	Male	Female	Male	Female	Male	Female
BW day 0 (g)	280 ± 6.7	204 ± 8.1	270 ± 10.9	198 ± 8.4	273 ± 12.0	208 ± 6.9	274 ± 6,5	207 ± 6.8
BW day 15 (g)	327 ± 6.1	200 ± 7.2	316 ± 11.2	192 ± 6.3	323 ± 12.1	199 ± 6.1	321 ± 5.2	198 ± 6.2
Liver (%)	3.67 ± 0.08	3.26 ± 0.15	3.34 ± 0.05	3.37 ± 0.07	3.63 ± 0.11	3.18 ± 0.14	3.68 ± 0.17	3.10 ± 0.07
Heart (%)	0.37 ± 0.013	0.28 ± 0.007	0.38 ± 0.010	0.28 ± 0.004	0.41 ± 0.0058	0.29 ± 0.013	0.41 ± 0.007	0.30 ± 0.006
Spleen (%)	0.19 ± 0.005	0.23 ± 0.009	0.20 ± 0.002	0.25 ± 0.011	0.19 ± 0.013	0.22 ± 0.005	0.20 ± 0.008	0.23 ± 0.008
Kidneys (%)	0.40 ± 0.006	0.37 ± 0.008	0.40 ± 0.007	0.37 ± 0.016	0.41 ± 0.011	0.38 ± 0.012	0.41 ± 0.005	0.37 ± 0.011
Ovaries (%)	---	0.031 ± 0.001	---	0.038 ± 0.002	---	0.039 ± 0.002	---	0.034 ± 0.001
Uterus (%)	---	0.21 ± 0.036	---	0.20 ± 0.020	---	0.22 ± 0.032	---	0.22 ± 0.023
Epididymis (%)	0.17 ± 0.006	---	0.16 ± 0.003	---	0.18 ± 0.005	---	0.17 ± 0.007	---
Testicles (%)	0.44 ± 0.010	---	0.45 ± 0.012	---	0.44 ± 0.010	---	0.44 ± 0.005	---
Prostate (%)	0.014 ± 0.001	---	0.016± 0.001	---	0.015 ± 0.001	---	0.014 ± 0.001	---
Levator ani muscle (%)	0.27± 0.012	---	0.27 ± 0.010	---	0.28 ± 0.016	---	0.26 ± 0.013	---
Seminal vesicles (%)	0.21 ± 0.011	---	0.22 ± 0.019	---	0.23 ± 0.005	---	0.21 ± 0.005	---

Values are expressed as mean ± S. E. M. of five rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's test.

Table 3
Effect of acute oral administration of ethanol soluble fraction obtained from *Luehea divaricata* (ESLD) on the hematological parameters of Wistar rats after 14 days of exposition.

Parameters	Control		ESLD (50 mg/kg)		ESLD (300 mg/kg)		ESLD (2 g/kg)	
	Male	Female	Male	Female	Male	Female	Male	Female
RBC (10^6/mL)	8.8 ± 0.2	8.4 ± 0.2	8.2 ± 0.2	8.4 ± 0.2	8.2 ± 0.1	8.9 ± 0.2	8.8 ± 0.5	8.6 ± 0.1
Hemoglobin (g/mL)	15.1 ± 0.4	14.6 ± 0.3	14.6 ± 0.4	14.7 ± 0.4	14.4 ± 0.2	15.4 ± 0.3	15.1 ± 0.7	14.9 ± 0.2
Hematocrit (%)	49.2 ± 1.3	43.7 ± 0.8	45.1 ± 1.2	43.6 ± 1.2	46.1 ± 0.8	45.6 ± 0.7	48.0 ± 2.5	43.6 ± 0.5
MCV (fl)	56.0 ± 0.4	52.3 ± 0.5	54.9 ± 0.3	52.1 ± 0.2	56.0 ± 0.4	51.3 ± 0.4	54.8 ± 0.5	51.0 ± 0.5
MCH (pg)	17.2 ± 0.2	17.5 ± 0.2	17.8 ± 0.1	17.5 ± 0.1	17.5 ± 0.1	17.4 ± 0.2	17.3 ± 0.2	17.4 ± 0.3
MCHC (%)	30.7 ± 0.2	33.5 ± 0.2	30.5 ± 0.2	33.6 ± 0.2	31.2 ± 0.2	33.9 ± 0.2	31.5 ± 0.2	34.1 ± 0.4
Platelets (10^3/mm^3)	801 ± 89	1167 ± 71	750 ± 154	1170 ± 155	810 ± 50	1337 ± 81	798 ± 55	1101 ± 160
WBC (10^3/mm^3)	8.8 ± 0.5	9.6 ± 1.6	5.93 ± 0.8	7.5 ± 1.2	6.79 ± 0.7	8.8 ± 1.2	9.38 ± 1.7	7.8 ± 0.8
Neutrophils (%)	15.3 ± 2.3	15.5 ± 0.8	19.3 ± 1.3	16.8 ± 1.6	14.2 ± 1.8	17.8 ± 1.1	15.8 ± 1.9	16.5 ± 1.8
Lymphocytes (%)	82.3 ± 2.2	77.8 ± 1.3	77.8 ± 1.1	72.4 ± 3.8	81.8 ± 0.7	75.9 ± 1.3	81.7 ± 1.7	75.2 ± 2.3
Monocytes (%)	1.0 ± 0.2	5.1 ± 0.6	0.9 ± 0.2	4.7 ± 0.5	2.1 ± 1.5	5.0 ± 0.9	1.2 ± 0.4	4.6 ± 0.7
Eosinophils (%)	0.9 ± 0.1	1.5 ± 0.3	1.5 ± 0.3	0.9 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	0.5 ± 0.2	3.5 ± 1.9
Basophils (%)	0.2 ± 0.05	0.2 ± 0.04	0.1 ± 0.05	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.02

Values are expressed as mean ± S. E. M. of five rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's test.

3.4.3. Acute administration of ESLD reduces SBP and MAP levels of normotensive rats

Fig. 6 (A, B, C, and D) shows the systemic blood pressure and HR values obtained after acute treatment with ESLD or HCTZ. SBP, DBP, MAP, and HR values for animals treated with vehicle alone were 110 ± 3.5, 58 ± 3.6, 95 ± 2.9 mm Hg, and 263 beats per minute (BPM), respectively. Acute administration of ESLD at doses of 30 and 100 mg/kg was able to reduce SBP levels to 87 ± 5.3 and 81 ± 4.1, respectively ($p < 0.05$), while MAP levels were reduced to 71 ± 6.1 and 69 ± 4.2 mm Hg, respectively ($p < 0.05$). Values obtained with administration of HCTZ were not statistically different from those observed for treatment with ESLD. DBP and HR values did not show statistically significant differences after administration of ESLD or HCTZ.

In a different way, prolonged administration of different doses of ESLD was not able to change blood pressure or heart rate when compared to animals treated with vehicle alone (Fig. 6E, F, G, and H). As expected, HCTZ was able to significantly reduce SBP levels by approximately 27%.

3.4.4. Treatment with ESLD increases NO bioavailability and reduces TBARS

In animals treated with vehicle alone, TBARS levels were 6.2 ± 0.8 mmol/L. Treatment with ESLD at dose of 30 mg/kg was able to reduce TBARS levels to 3.7 ± 0.6 mmol/L (Fig. 7E; $p < 0.05$). In addition, prolonged ESLD administration (30 mg/kg) was able to increase nitrite levels by ~ 45% (Fig. 7D). On the other hand, none of the ESLD doses were able to affect aldosterone and vasopressin concentrations or serum ACE activity (Fig. 7A-C). Treatment with HCTZ did not significantly alter any of the parameters evaluated (Fig. 7A-E).

4. Discussion

The medicinal use of *Luehea divaricata* is widely disseminated throughout Brazil, especially the infusion and decoction of its leaves, barks and roots. Despite its wide use for the most varied pathologies, detailed ethnopharmacological studies aimed at validating the popular use of the species are still very preliminary or inconclusive. In this work, for the first time, a detailed morphological and microchemical study of *L. divaricata* leaves was performed, and using modern spectrometric techniques, the phytochemical profile of ESLD obtained from this species was characterized. In addition, it was demonstrated that ESLD is

capable of inducing an expressive diuretic, hypotensive, and antioxidant effect, without showing any signs of acute toxicity in Wistar rats.

In the first stage of this work, *L. divaricata* leaves were collected and their morphoanatomic and histochemical characterization was carried out. Plant species are defined by their morpho-anatomical features and the microscopic technique is in fact the most accurate possible means of identifying plant species (Upton et al., 2011). *L. divaricata* is not included in any pharmacopoeia and its anatomical characteristics help authentication, and may serve as standards to avoid adulteration or unintentional use of other morphologically similar species.

Table 4
Effect of acute oral administration of ethanol soluble fraction obtained from *Luehea divaricata* (ESLD) on the biochemical parameters of Wistar rats after 14 days of exposition.

Parameters	Control		ESLD (50 mg/kg)		ESLD (300 mg/kg)		ESLD (2 g/kg)	
	Male	Female	Male	Female	Male	Female	Male	Female
Total cholesterol (mg/dL)	87 ± 10.4	46 ± 2.9	85 ± 10.2	45 ± 2.8	107 ± 3.5	45 ± 3.8	105 ± 8.0	51 ± 2.4
HDL cholesterol (mg/dL)	75 ± 6.7	46 ± 2.3	69 ± 4.6	45 ± 2.3	81 ± 1.2	45 ± 3.7	80 ± 4.4	49 ± 2.4
Triglycerides (mg/dL)	47 ± 7.5	33 ± 1.6	67 ± 10.8	28 ± 2.0	74 ± 4.3	30 ± 1.9	70 ± 13.4	30 ± 2.5
Urea (mg/dL)	28 ± 0.8	55 ± 3.1	24 ± 0.6	45 ± 2.3	31 ± 1.4	46 ± 3.4	25 ± 1.0	38 ± 1.8
Creatinine (mg/dL)	0.23 ± 0.01	0.37 ± 0.01	0.20 ± 0.01	0.34 ± 0.01	0.21 ± 0.01	0.31 ± 0.01	0.22 ± 0.01	0.31 ± 0.02
Sodium (mEq/L)	137 ± 2.2	141 ± 0.2	132 ± 1.0	140 ± 0.5	141 ± 0.7	140 ± 0.7	141 ± 0.5	140 ± 1.0
Potassium (mEq/L)	5.8 ± 0.2	6.1 ± 0.3	4.9 ± 0.3	5.9 ± 0.1	5.4 ± 0.1	6.0 ± 0.3	5.8 ± 0.3	6.8 ± 0.8
Uric acid (mg/dL)	1.9 ± 0.2	1.7 ± 0.2	1.5 ± 0.2	2.5 ± 0.2	1.5 ± 0.1	2.1 ± 0.2	1.7 ± 0.2	2.6 ± 0.6
Total protein (g/dL)	5.2 ± 0.4	6.2 ± 0.1	4.6 ± 0.2	6.5 ± 0.1	5.5 ± 0.1	6.3 ± 0.1	5.5 ± 0.2	6.2 ± 0.1
Albumin (g/dL)	3.5 ± 0.2	4.7 ± 0.1	3.2 ± 0.2	4.7 ± 0.1	3.7 ± 0.04	4.6 ± 0.1	3.7 ± 0.1	4.6 ± 0.1
Globulin (g/dL)	1.7 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.7 ± 0.04	1.8 ± 0.1	1.6 ± 0.1
Amylase (U/L)	1942 ± 212	1044 ± 63	1703 ± 47	925 ± 31	2441 ± 71	904 ± 94	2317 ± 138	830 ± 54
Alkaline phosphatase (U/L)	106 ± 15.8	56 ± 16.1	102 ± 15.1	48 ± 4.9	129 ± 14.8	55 ± 5.7	112 ± 18.1	47 ± 8.2
AST (U/L)	44 ± 18.9	98 ± 4.6	85 ± 4.0	84 ± 1.1	103 ± 4.4	98.3 ± 6.7	100 ± 5.1	95 ± 11.9
ALT (U/L)	33 ± 2.9	34 ± 2.6	27 ± 0.9	25 ± 3.2	35 ± 1.9	30 ± 3.6	37 ± 1.4	27 ± 1.8

Values are expressed as mean ± S. E. M. of five rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's test.

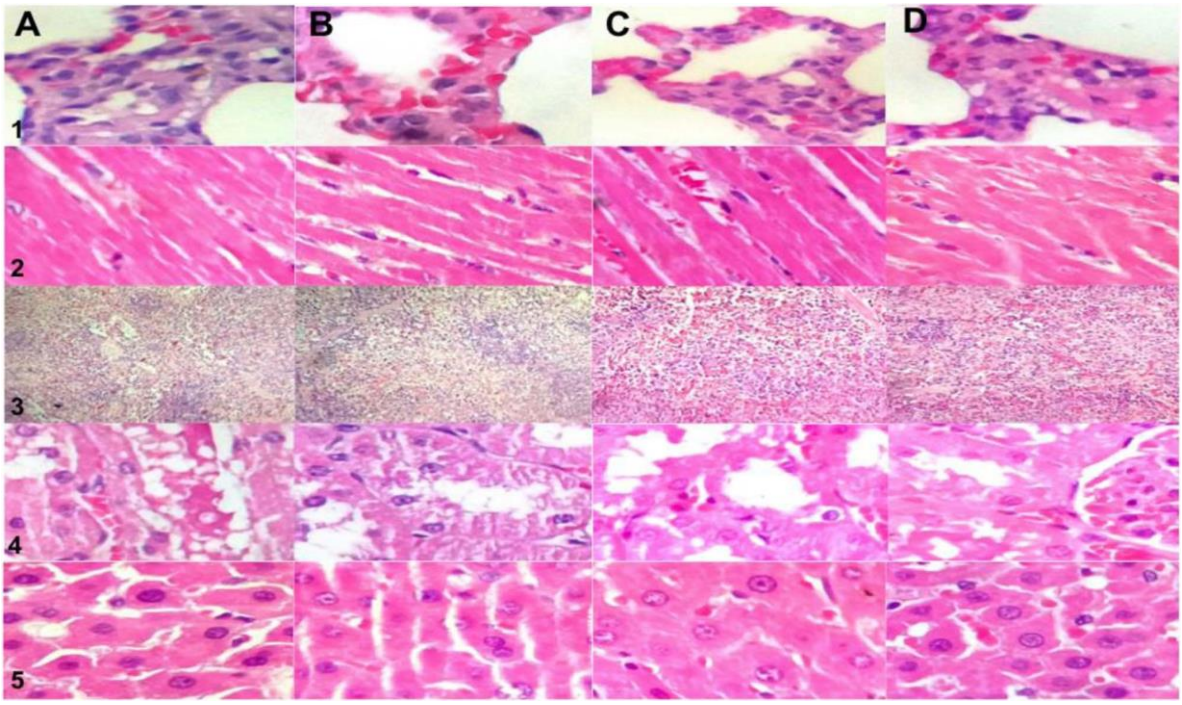

Fig. 4. Photomicrographs of lung (1), heart (2), spleen (3), kidney (4) and liver (5) histopathology from representative male (A and B) and female (C and D) rats treated with vehicle (A and C) or the highest dosage of ESLD (2000 mg/kg) (B and D). Hematoxylin and eosin stain (400 ×).

Simultaneously to the pharmacognostic study, the semi-purified aqueous extract (ESLD) from *L. divaricata* leaves was obtained and by means of spectrometric techniques, the secondary metabolites concentrated in this preparation were characterized in detail. Differently from previously reported data describing the presence of chlorogenic acid, rosmarinic acid, and epicatechin, along with triperpenoids and phytosterols (Tanaka et al., 2005; Arantes et al., 2014; Courtes et al., 2015), a large amount of flavonol O-glycosides and flavone C-glycosides was evidenced. The apparent discrepancy may be due not only to the different extraction and purification processes, but also to the climatic and environmental characteristics of each collection region.

Another interesting aspect concerns the acute toxicity of the ESLD. Acute toxicity studies allow the evaluation of a broad spectrum of clinical signs indicative of toxicity, and in addition to helping to define the median lethal dose (LD50), they allow the selection a low-harm potential dose for subsequent pharmacological studies. In our study, ESLD did not induce any signs of behavioral, metabolic or physiological changes during 14 days of

observation, and allowed us suggesting that even at very high oral doses (2 g/kg), the safety profile of ESLD can be considered satisfactory according to guidelines from international regulatory agencies (OECD, 2001).When we direct our work to efficacy studies, we find that acute and prolonged administrations the ESLD were able to induce an intense diuretic and saluretic response, with effects very similar to those obtained after administration of HCTZ. The response pattern of thiazide diuretics in rats, especially HCTZ, is characterized by a significant increase in renal elimination of water, Na^+, K^+ and Cl^- within the first 8 h after administration. Due to hemodynamic regulatory mechanisms and the resulting dehydration, renal elimination of water and electrolytes is reduced after 24 h, and only partially reestablished after 2 or 3 days (Aquino et al., 2017). The natriuretic effect remains, and together with the vasodilatory response induced by the opening of potassium channels in the vascular smooth muscle, the hypotensive effects of HCTZ remain in the long term (Chan et al., 2012). Similarly, the effects of ESLD on renal excretion of Na^+ and Cl^- disappear at the end of the first 24 h, and are promptly reestablished (after 3 days of treatment) without affecting the renal elimination of K^+, which can be an advantage due to the reduction of adverse effects (such as cramps and cardiac arrhythmias), typical of drugs with high kaliuretic response.

Table 5
Effect of acute oral administration of ethanol soluble fraction obtained from *Luehea divaricata* (ESLD) on the urinary volume, pH, and density after 4 and 8 h of the treatment.

Group	Urine volume (ml/100g/4h)	Urine volume (ml/100g/8h)	pH (4 h)	pH (8 h)	Density (4 h)	Density (8 h)
Control	2.8 ± 0.45	4.0 ± 0.50	7.7 ± 0.37	8.1 ± 0.23	1023 ± 1.84	1033 ± 5.78
HCTZ (25 mg/kg)	5.0 ± 0.30[a]	6.4 ± 0.36[a]	7.2 ± 0.28	7.6 ± 0.22	1017 ± 1.13	1027 ± 2.45
ESLD (30 mg/kg)	4.9 ± 0.30[a]	5.9 ± 0.36[a]	8.1 ± 0.11	7.8 ± 0.18	1022 ± 2.60	1036 ± 2.59
ESLD (100 mg/kg)	4.1 ± 0.32[a]	5.4 ± 0.39[a]	8.0 ± 0.20	7.9 ± 0.15	1028 ± 2.60	1039 ± 4.17
ESLD (300 mg/kg)	3.4 ± 0.32	4.8 ± 0.26	7.9 ± 0.19	7.9 ± 0.15	1026 ± 3.04	1038 ± 2.75

Values are expressed as mean ± S. E. M. of 7 rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's test ([a]$p \leq 0.05$); HCTZ: hydrochlorothiazide.

Table 6
Effect of acute oral administration of ethanol soluble fraction obtained from *Luehea divaricata* (ESLD) on urinary electrolyte excretion after 8 h of the treatment.

Group	El_{Na+} (µEq/min/100g)	El_{K+} (µEq/min/100g)	El_{Cl-} (µEq/min/100g)	Saluretic index[b]		
				Na^+	K^+	Cl^-
Control	0.22 ± 0.06	0.10 ± 0.04	0.26 ± 0.06	-	-	-
HCTZ (25 mg/kg)	0.41 ± 0.05[a]	0.22 ± 0.04[a]	0.45 ± 0.04[a]	1.86	2.2	1.73
ESLD (30 mg/kg)	0.40 ± 0.03[a]	0.21 ± 0.03[a]	0.45 ± 0.03[a]	1.81	2.1	1.73
ESLD (100 mg/kg)	0.38 ± 0.05[a]	0.21 ± 0.03[a]	0.49 ± 0.05[a]	1.72	2.1	1.88
ESLD (300 mg/kg)	0.41 ± 0.03[a]	0.19 ± 0.01[a]	0.47 ± 0.04[a]	1.86	1.9	1.80

Values are expressed as mean ± S. E. M. of 7 rats in each group in comparison with the control using one-way ANOVA followed by Dunnett's test ([a]$p < 0.05$). [b]Saluretic index = mmol/L problem group/mmol/L control group. El: Excreted load; HCTZ: hydrochlorothiazide.

A significant difference between the effects of ESLD and HCTZ is on the hypotensive response. The hypotensive effects induced by ESLD were only observed after acute administration, disappearing completely after 7 days of treatment. It is still premature to say if the hypotensive effect induced by ESLD is directly dependent on its saluretic activity, since even HCTZ maintains its hypotensive effect in the long term through complementary pharmacological mechanisms, such as the opening of potassium channels in the vascular smooth muscle (Chan et al., 2012). Another interesting fact is the inverse dose-rate effect induced by ESLD. The lowest ESLD dose was responsible for the most effective response, different from most highly purified compounds that exhibit a classic dose–response relationship. This can be explained, at least in part, due to the fact that ESLD is a phytocomplex, where antidiuretic metabolites have their concentration increased with increasing dose. In addition, it is possible that with increasing dose, active compounds lose their selectivity, and begin to act in distinct targets where the pharmacological response becomes antagonistic.

In a last step of this work we shown the mechanisms involved in the pharmacological effects induced by ESLD. The results pointed to a probable involvement of NO in diuretic and hypotensive responses.

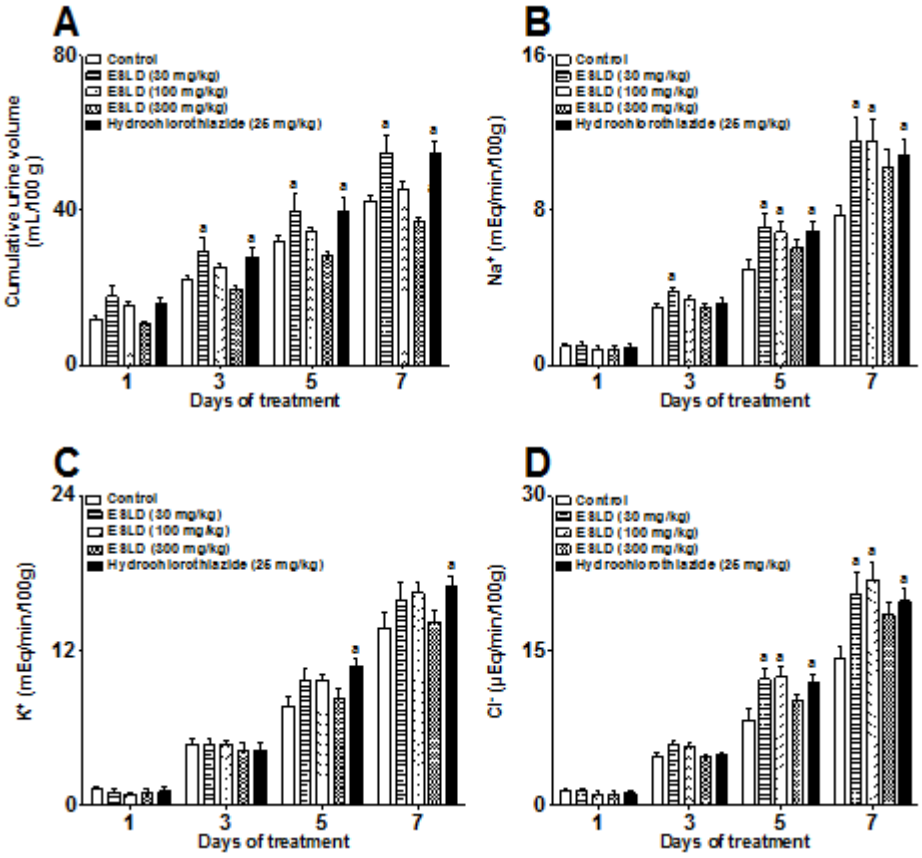

Fig. 5. Effect of prolonged oral treatment with ESLD on cumulative urinary volume (A), Na^+ (B), K^+ (C), and Cl^- (D) excretion. The urine samples were collected for 24 h on days 1, 3, 5 and 7, and cumulative urinary volume and electrolyte levels were analyzed. Each bar represents the mean of 7 animals and the vertical lines show the S.E.M. Statistical analyses were performed by means of two-way analysis of variance (ANOVA) followed by Dunnett's test. $^a p < 0.05$ when compared to respective control group. HCTZ: hydrochlorothiazide.

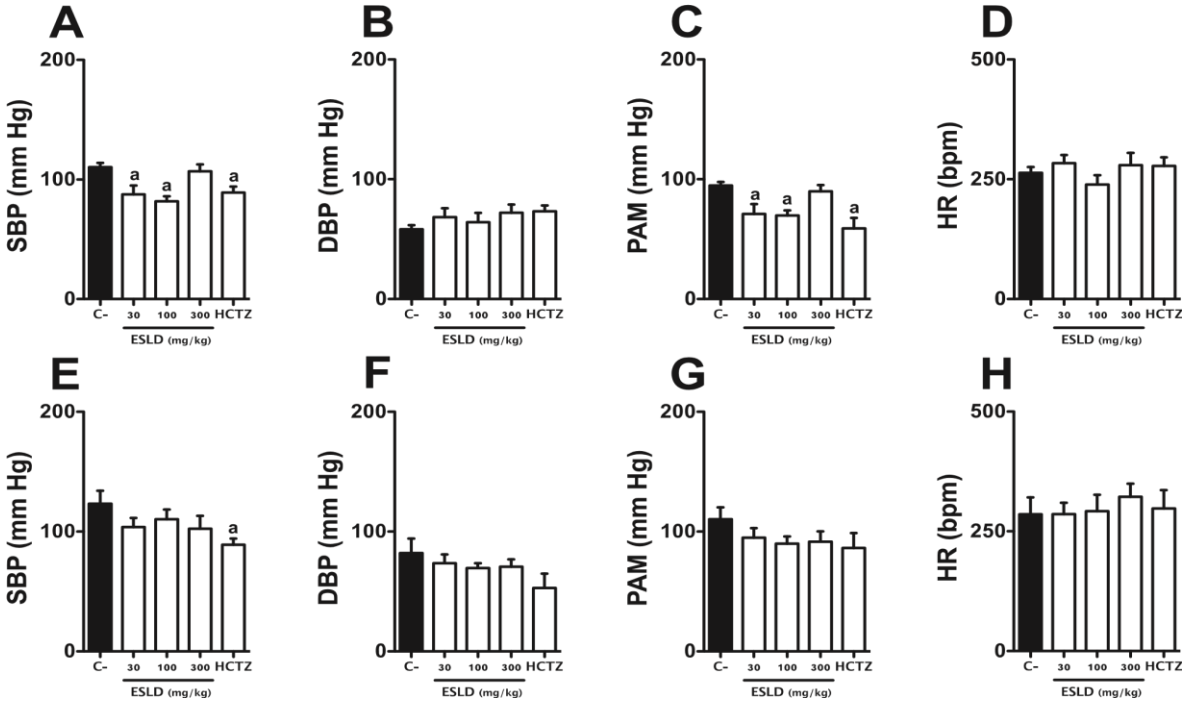

Fig. 6. Acute administration of ESLD obtained from *Luehea divaricata* reduces SBP and MAP levels in normotensive Wistar rats. ESLD was given as a single dose intraduodenally (A, B, C, and D) or orally for 7 days (E, F, G, and H). The letter "C-" indicates the effect measured after administration of vehicle only. The results show the mean ± S.E.M. (n = 6–7). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Dunnett's test. $^{a}p < 0.05$ when compared to respective control group. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; HR: heart rate; HCTZ: hydrochlorothiazide.

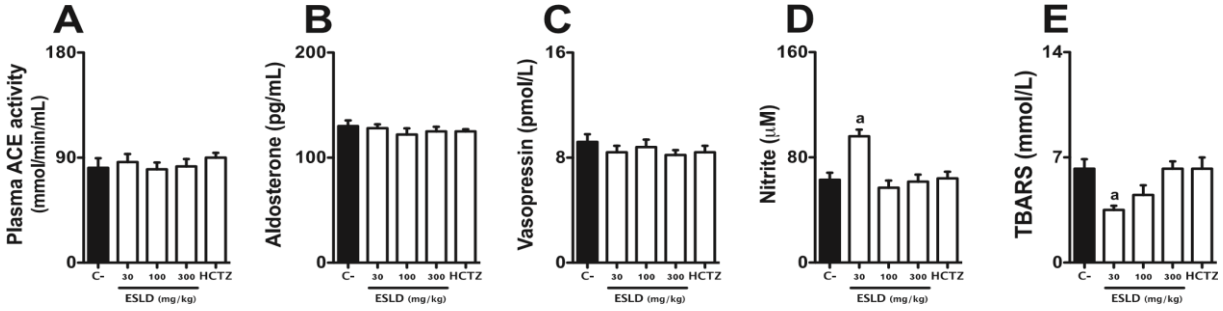

Fig. 7. ESLD- treatment increases NO bioavailability (D) and reduces TBARS levels (E) without affecting concentrations of aldosterone (B), vasopressin (C), or serum ACE activity (A). The serum samples were obtained after 7-days of treatment. The results show the mean ± S.E.M. (n = 7). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Dunnett's test. $^{a}p < 0.05$ when compared to respective control group (C-). HCTZ: hydrochlorothiazide.

Several studies have shown that the effects of NO at renal level are related to its ability to dilate the afferent arteriole and increase renal blood flow, which leads to an increase in glomerular filtration rate and consequently diuresis (Da Silva et al., 2015; Alarcón-Alonso et al., 2012; Gasparotto Junior et al., 2012). Although suggestive, there is still some doubt whether the effect is due to direct release of NO by secondary metabolites present in ESLD, or a consequence of the potent antioxidant activity of flavonol O-glycosides and flavone C-glycosides present in ESLD. The fact is that substances that reduce reactive oxygen species may increase the bioavailability of nitric oxide and consequently induce systemic vasodilator responses (Zizkova et al., 2017). One limitation of our study was that we did not investigate whether the diuretic and hypotensive effects induced by ESLD would be sustained following administration of selective inhibitors of the nitric oxide-cGMP pathway. However, if we consider the pleiotropic effects of many natural products on renal level (Lívero et al., 2017), it is quite possible that the effects presented in this study are due to an integrated activity of different secondary metabolites synergistically acting in multiple pharmacological targets.

5. Conclusion

In this study, a morpho-anatomical and microchemical characterization of *Luehea divaricata* was performed, and the profile of secondary metabolites of a preparation popularly used in the Brazilian Pantanal region (ESLD) was defined. In addition, the study has shown that ESLD has an important diuretic and hypotensive effect, which is probably dependent on the reduction of oxidative stress and increased bioavailability of nitric oxide.

Conflict of interest

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

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5.3 Manuscrito 1- Biological characterization of an edible species from Brazilian Cerrado: from pharmacognostic data to ethnopharmacological investigation

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ABSTRACT

Although *Talisia esculenta* (A. St.-Hil.) Radlk. is a edible species with relevant economic and medicinal uses in Brazil, no study has investigated its effectiveness as a diuretic and hypotensive agent. So, the aim of this study was to present a detailed anatomical and histochemical study for *T. esculenta* and provide important safety and efficacy parameters. After morpho-anatomical and micro-chemical study, a purified aqueous extract (ESTE) was obtained and detailed phytochemical and "*in vitro*" antioxidant investigations were performed. Subsequently, acute oral toxicity test was performed in male and female Wistar rats. Finally, the possible diuretic and hypotensive effects of ESTE on male rats were evaluated. The analyses performed by liquid chromatography–mass spectrometry showed that the main metabolites present in ESTE were phenolic compounds. As expected, this preparation showed significant free radical scavenging activity and reduced lipid peroxidation and oxidative hemolysis "*in vitro*". Additionally, acute treatment was able to increase urinary volume in the first 2 hours after treatment and potassium excretion was significantly increased after 8 hours. Moreover, ESTE did not induce acute toxic effects and did not affect blood pressure or heart rate of Wistar rats. Data obtained provided important anatomical, microchemical and phytochemical data of *T. esculenta* leaves. In addition, it has been shown that ESTE does not present risks if acutely administered in rodents. On the other hand, the use of this species as a diuretic and hypotensive agent should be performed with caution, since administration in rodents did not produce any important renal and/or hemodynamic responses.

KEY WORDS: Antioxidant; diuretic; hypotensive; Sapindaceae; toxicity.

INTRODUCTION

Talisia esculenta (A. St.-Hil.) Radlk. is a large tree belonging to family Sapindaceae, native and cultivated in different regions of Brazil. It is popularly known as "pitombeira" or "pitomba", a word in "Tupi" language that means slap or strong kick. Its fruits (rich in vitamin C) are marketed by different native populations of Brazil, mainly due to their nutritional properties.[1] In several regions of Brazil, its leaves are popularly used by different indigenous and riverside populations due to their possible diuretic, analgesic and antidiarrheal (astringent) activities[1,2].

In recent years, the phytochemical profile of *T. esculenta* seeds and fruits has been gradually characterized, while the chemical characteristics of its leaves remain not fully elucidated. The available data describe the presence of carbohydrate-binding proteins,[3,4] catechins, flavonoids and organic acids,[5,6] especially in the fruits of this species.

Some preliminary biological investigations have been conducted with *T. esculenta* preparations, showing its insecticidal,[7,8] antifungal,[9] antibacterial,[10,11] antioxidant, antiproliferative and antimutagenic properties.[5,6] On the other hand, few toxicological studies have been conducted with this species. Recently, Riet-Correa et al.[12] reported the poisoning of sheep and cattle after the consumption of *T. esculenta* leaves and fruits, drawing attention due to its wide use by Brazilian populations.

Thus, considering the wide use of this species in Brazil, and especially due to the lack of data on its efficacy and safety on the cardiovascular system, a detailed ethnopharmacological investigation with a popular herbal preparation made with *T. esculenta* was performed. First, leaves of this species were collected and a detailed morpho-anatomical and microchemical study was carried out. Then, a purified fraction was obtained by infusion (ESTE) and its phytochemical profile was characterized through ultra-performance liquid chromatography coupled to photodiode-array detection and electrospray ionization tandem mass spectrometry method (UPLC-PAD-MS/MS). Finally, a detailed acute toxicological study was carried out and the diuretic and hypotensive properties of ESTE were investigated, relating its effects to a possible antioxidant activity.

MATERIALS AND METHODS

Drugs

Hydrochlorothiazide (HCTZ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma-Aldrich (St.

Louis, MO, USA). Sodium chloride was obtained from Merck (Darmstadt, Germany). Acetonitrile and formic acid were purchased from J.T. Baker (Center Valley, PA, USA) and Tedia (Fairfield, OH, EUA), respectively. All other chemicals and solvents were of analytical grade.

Plant material

Talisia esculenta leaves were collected in August 2015 from the preservation area near the city of Dourados, Mato Grosso do Sul (S 22°12'218'' and W 54° 45' 05,3''). A voucher was cataloged at the UFGD Herbarium under DDMS number 5221.

Pharmacobotanical assays

T. esculenta leaves and stems (at least 3 samples) obtained from the sixth node and below (median, intercostal and margin regions), as well as 5-15 cm stem fragments from shoots were prepared for pharmacobotanical assays. The plant material was placed in a FAA 70 solution, and stored in 70% ethanol. For the assessment of leaf and stem material, free-hand longitudinal and cross-sections were made. The midrib, interneural regions, and lateral veins were included in leaves. Leaves and stems were stained using toluidine blue, astra blue and basic fuchsine to obtain semi-permanent slides.[13]

Histochemical tests

The following standard solutions were used in histochemical tests: sulphuric acid solution for calcium oxalate crystals; Sudan III for testing lipophilic compounds; Hoepfner-Vorsatz test, modified by Reeve[14] (aqueous 10% sodium nitrate, aqueous 10% acetic acid, aqueous 10% urea and, 2 N NaOH) and ferric chloride to assess phenolic substances.[15] Photomicrographs were captured using an Olympus CX 31 light microscope equipped with a C7070 digital camera.

Preparation of aqueous extracts

T. esculenta leaves were air-dried in an oven at 40° C for 5 days, and then crushed and pulverized. The aqueous extract (AETE) was obtained by infusion in a similar manner to that described by Prando et al.[16] Thus, AETE was treated with 3 volumes of ethanol, which gave rise to a precipitate and an ethanol soluble fraction (ESTE). ESTE was filtered, concentrated and freeze-dried (yield 6.6 %). All preparations were kept in freezer until time of experiments.

Phytochemical investigation

The chemical constituents obtained from *T. esculenta* leaves were analyzed by ultra-high-performance liquid chromatography (Acquity-UPLC™, Waters), with detection provided by photodiode array (PDA, Waters) and high-resolution mass spectrometry (HR-MS/MS). The chromatographic separation was performed in a 100 x 2.1 mm BEH-C18 column with particle size of 1.7 µm. The column temperature was 60 ºC, and a linear gradient of solvents was developed with a mixture of ultra-pure water (Milli-Q) and acetonitrile, both containing 0.1% of formic acid (96%), at constant flow rate of 400 µL/min. The linear acetonitrile increase was from 0 to 10% in 5 min, then to 70% in 13 min. The solvent returned to the initial condition (0% acetonitrile) at 13.5 min, held additional 2 min to re-equilibrate the system. The extract was prepared at 2 mg/ml in MeOH-H_2O (1:1, v/v), with injection of 5 µL. To aid in the identification of compounds, detection was performed by PDA screening (200-400 nm) and HR-MS (*m/z* 150-2000).

Mass spectrometry was carried out in a LTQ Orbitrap XL (Thermo-Scientific), in the negative and positive ionization modes, at atmospheric ionization pressure, with ESI source at 350 ºC. Desolvation was aided by nitrogen stream, with sheath gas at flow rate of 40 arbitrary units (a.u.) and auxiliary gas at 5 a.u. The energies in the positive ionization were 5 kV in the source, 30 V in the capillary and 110 V in the tube lens; and in the negative ionization, energies were 3.5 kV, -45 V and -200 V, respectively. Fragmentation with higher-energy collisional dissociation (HCD) was performed with normalized collision energy of 35%. For higher accuracy, the spectrometer was externally calibrated with Pierce™ LTQ ESI Positive Ion (Caffeine, MRFA, Ultramark-1621) and Negative Ion (SDS, sodium taurocholate, Ultramark-1621) calibration solutions (Thermo-Fischer). In LC-MS mode, resolution was set at 15,000 FWHM.

Experimental approach

Animals

Male and female Wistar rats (250-300 g) were obtained from Federal University of Grande Dourados (UFGD, Brazil) and kept at controlled temperature (22 ± 2 C°) and luminosity conditions (12 hours light/dark cycle) with free access to filtered water and food. All procedures were previously approved by Institutional Ethics Committee of UFGD (protocol number 16/2015) and conducted in accordance with Guidelines for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Health Institute.

Toxicological evaluation

Acute oral toxicity

Acute oral toxicity was determined according to procedures recommended by the Organization for Economic Co-operation and Development (OECD) protocol No. 420.[17] ESTE was administered as a single dose in male and female rats at doses of 5, 50, 300, 2000 mg/kg by oral route (gavage). Control animals received filtered water (10 ml/kg). All animals were directly monitored through the first eight hours and daily for 14 days. Behavioral parameters and clinical signs of toxicity were recorded. At the end of the experimental period, all animals were sacrificed by decapitation and blood samples were obtained. Hematological analysis was performed in an automated hemogram analyzer (Sysmex® XN-3000). Biochemical analyses were performed on serum samples (obtained by centrifugation; 1,000 x g for 10 min) in an automated biochemistry analyzer (Roche® Cobas Integra 400 plus). After euthanasia, liver, heart, spleen, kidneys, ovaries, uterus, epididymis, testicles, prostate, levator ani muscle, and seminal vesicles were removed to gross pathology and their relative weights were determined (Absolute organ weight × 100/ Body weight of rats on the day of sacrifice). Heart, lung, spleen, kidney, and liver samples were collected for histopathological evaluation.[18]

Pharmacological investigation

Diuretic activity

The diuretic activity was evaluated according to methods previously described by Gasparotto Junior et al.[19] Different single ESTE doses (30, 100, or 300 mg/kg) were orally administered in saline-loaded male rats (n = 6). Positive control animals received HCTZ at dose of 25 mg/kg. Negative controls were treated only with vehicle (filtered water; 0.2 ml/100g). Immediately after treatment, rats were placed in metabolic cages and urine was collected for 24 h. Urinary volume was measured and expressed as ml/100 g of body weight. At the end of experiments, under isoflurane anesthesia, blood samples were obtained by cardiac puncture. Serum was obtained by centrifugation (800 *g*, 10 min). Urinary and serum sodium (Na^+), potassium (K^+), chloride (Cl^-) and calcium (Ca^{+2}) levels were quantified in an ion selective meter (COBAS INTEGRA 400 plus; Roche®). pH was determined on fresh urine samples using digital pH meter (Q400MT; Quimis Instruments, Brazil). Density was estimated by handheld refractometer (NO107; Nova Instruments, Brazil). Excretion load (El)

of Na^+, K^+, Cl^- and Ca^{+2} was obtained by multiplying the concentration of electrolytes (mEq/l) by the urinary flow (ml/min). Results are expressed as μEq/min/100g

Hypotensive activity

The acute hypotensive activity was evaluated according to methods previously described by Gasparotto Junior et al.[20] After anesthesia with ketamine (100 mg/kg) and xilazine (20 mg/kg) by intramuscular route, different groups of rats had their right carotid artery cannulated and connected to a pressure transducer coupled to a PowerLab® recording system (Chart, v 4 .1; all from ADI Instruments; Castle Hill, Australia). After 15 minutes to stabilize cardiovascular hemodynamics, different groups of male rats (n = 5) received ESTE (30, 100 or 300 mg/kg), HCTZ (25 mg/kg), or vehicle (filtered water; 0.2 ml/100g) intraduodenally. Changes in systolic blood pressure (SBP), mean arterial pressure (MAP), diastolic blood pressure (DBP), and heart rate (HR) were recorded for 35 min.

Antioxidant potential

Antioxidant potential was evaluated through three classical methods using different EAST concentrations (1000, 300,100 and 30 μg/ml). First, the radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) was performed according to methodology previously described by Gupta and Gupta.[21] Ascorbic acid (AA) was used as antioxidant control. Then, the ability to reduce oxidative hemolysis induced by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was evaluated according to methods described by Valente et al.[22] Assays were performed using an erythrocyte suspension at 2.5% hematocrit. Three independent experiments were performed in triplicate. Finally, lipid peroxidation was determined by measuring malondialdehyde (MDA) formed as described in Campos et al.[23] The calculation for the amount of MDA in sample was obtained by the formula: MDA = Absorbance of samples × (20 × 220.32/Absorbance 1,1,3,3-tetrahydroxipropane standard). Experiments were performed in triplicate and the results were expressed in nmol/ml. In the evaluation of the oxidative hemolysis and lipid peroxidation, AAPH was used as oxidizing agent (positive control; C+). In all experiments, vehicle was used as negative control (C-).

Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test. The half maximal inhibitory concentration (IC 50) values were calculated by

linear regression. P-values less than 0.05 were considered statistically significant. Graphs were drawn and statistical analysis was carried out using the GraphPad Prism software version 5.0 for Mac OS X (GraphPad® Software, San Diego, CA, USA).

RESULTS

Pharmacobotanical characterization

Talisia esculenta leaves were composed (Figure 1A) of the following parts: petiolule, rachis, petiole and leaflet. The venation pattern of leaves was brochidodromus, conspicuous on both sides, and tertiary veins were reticulate (Figure 1B). In frontal view, *T. esculenta* leaves showed epidermal cells with straight to slightly wavy anticlinal walls (Figure 1C and D), which were thin on both sides.

Leaves are hypostomatic and anomocytic stomata are observed (Figure 1D). Stomata measured 25 µm in length on average. The striate cuticle was located around the stomata (Figure 1E) and on subsidiary cells (Figure 1F). The cuticle contains cutin, which is a lipophilic polymer that is deposited on and the top of the outer wall of epidermal cells. Its ornamentation is a taxonomic feature of the epidermis in leaves, appearing as striations, ridges, or papillae[13,24].

With reference to the indumentum, *Talisia* species can be either glabrous or have different types of trichomes (glandular and/or non-glandular). Glandular trichomes were reported for *T. hemidasya* Radlk., *T. morii* Acev.-Rodr., and *T. setigera* Radlk.; however, simple non-glandular trichomes are more common in the genus.[25] In the present study, *T. esculenta* showed a conic non-glandular trichome with rough cuticle (Figure 1F and G). It is located especially on the veins on the abaxial side. Additionally, glandular trichomes were not found.

In cross-section, leaves presented the uniseriate epidermis covered by a thick cuticle and the cells were larger on the adaxial side. The mesophyll was dorsiventral and formed by a layer of palisade parenchyma and about six layers of spongy parenchyma. Small collateral vascular bundles were immersed in the mesophyll (Figure 1H). Phenolic compounds were found in the chlorenchyma, mainly in the palisade parenchyma.

The midrib, in transection, had oval shape (Figure 1I). The epidermis was uniseriate and covered by a striate and thick cuticle, which reacted positively with Sudan III in the histochemical test (Figure 1J). Beneath the epidermis, on both sides, the chlorenchyma was interrupted and about three strata of angular collenchyma were apparent. Several prismatic

calcium oxalate crystals were evident in the collenchyma (Figure 1J) and in the ground parenchyma. They reacted positively with sulphuric acid in the histochemical test.

The vascular system was represented by a medullated vascular cylinder. Phenolic compounds were found in the phloem and near the xylem. They reacted with ferric chloride (Figure 1K) and in the Hoepfner-Vorsatz test (Figure 1L).

In cross-section, the *T. esculenta* petiolule was concave-convex with 2 ribs on the adaxial side (Figure 2A). The epidermis had the same characteristics as described to leaves, including the presence of conic non-glandular trichomes. The vascular system was represented by a medullated vascular cylinder as reported to leaves. Phenolic compounds were found in all parts of the petiole (Figure 2A), especially in the phloem (Figure 2B) and in the epidermis and collenchyma (Figure 2C).

The rachis was rounded and obtusely angled (Figure 2D). It was pubescent as the petiolule and midrib. The vascular system was represented by a collateral vascular cylinder (Figure 2D and E). Phenolic compounds were found mainly in the phloem (Figure 2E) and in the medullary region (Figure 2C and D).

The petiole, in transection, had irregular shape. It had not only the same indumentum but also similar vascular cylinder as observed in the rachis and petiolule. Phenolic compounds had the same distribution as the rachis (Figure 2F).

In transection, the stem had a rounded shape (Figure 2G). The epidermis appeared in a single series with thickened cuticle. There were several cells layers in the cortex. The sclerenchymatous ring surrounded the vascular system. This was typical and formed by phloem outward and xylem inward. The presence of continuous sclerenchymatous ring helped in the *T. esculenta* identification. Phenolic compounds were found in the phloem (Figure 2H) and in the perimedullary region (Figure 2I). The pith was composed of relatively small parenchymatous cells with thin walls (Figure 2H).

Phytochemical profile

Few compounds were identified in *T. esculenta* leaves. They were assigned mainly as flavonol-derivatives with characteristic UV-absorbance profile with λ_{max} at ~255 nm (band B) and ~360 nm (band A). Lower abundant compounds were derivatives of benzoic and cinnamic acids. HR-MS/MS allowed confirming the structures observed with UV-PDA. Thus, peak at 1.90 min had *m/z* 315.072 $[M-H]^-$ was identified as dihydroxybenzoic acid-hexoside. The major peak appeared at 6.02 min (Figure 3), producing ion at *m/z* 609.146 [M-H]- and 611.160 [M+H]+. Fragments obtained at *m/z* 611.160 (Table 1) indicated the neutral losses

(NL) of 146 atomic mass units (a.m.u.), followed by 162 a.m.u., with intense aglycone ion at *m/z* 303.050, characteristic of the removal of rhamnosyl and hexosyl residues, respectively, as observed in the analysis of rutin.[26] Similarly, peak at 6.37 min, *m/z* 595.165 $[M+H]^+$, also exhibit NL of 146 and 162 a.m.u., with aglycone fragment at *m/z* 287.055, characteristic of kaempferol rutinoside (or isomer). Peak at 6.46 min, at *m/z* 447.093 $[M-H]^-$ or 449.108 $[M+H]^+$, was identified as quercetin-rhamnoside.

Dicaffeoylquinic acid was also identified in this extract, appearing at 6.67 min, with negative ion at *m/z* 515.119 and fragments at *m/z* 353.087, 191.055, 179.034, 173.045, and 135.045. The last peak found appeared at 7.14 min, with *m/z* 623.161 $[M-H]^-$ and 625.176 $[M+H]^+$. Fragments from protonated ion appeared at *m/z* 479.118 (-146 a.m.u.) and 317.065 (-162 a.m.u.). This compound is 14 a.m.u higher than rutin and this difference was present in the aglycone moiety, consistent with methyl-quercetin-diglycoside. Similar structure has been described by Chukwumah et al.[27]

Toxicological findings

ESTE obtained from *T. esculenta* does not induce any signs of acute toxicity in Wistar rats

All animals treated with ESTE, regardless of dose, presented a behavior pattern considered normal for the species and genus. Body weight (Table 4) and water and food intake did not show any changes throughout the experimental period (data not shown). Values obtained for blood parameters (biochemical and hematological) from ESTE-treated rats were statistically similar to vehicle-treated animals (Tables 2 and 3). In addition, all organs examined did not show any evidence of changes during macro or microscopic evaluations (Figures 4 and 5). Relative weight of vital or reproductive organs did not show any statistically significant changes among experimental groups (Table 4).

Pharmacological investigation

ESTE from Talisia esculenta induces weak diuretic and kaliuretic response after acute treatment

The urinary volume produced by the different experimental groups after 1, 2, 8 and 24 hours is shown in Table 5. ESTE (100 mg/kg) obtained from *T. esculenta* was able to significantly increase urinary volume only in the first 2 hours after treatment. After 8 hours of experiments, only HCTZ was able to increase urinary excretion significantly, returning to

values similar to the control group after 24 hours. The pH and urinary density values were not altered in any of the experimental groups (data not shown).

The urinary Na^+, K^+, Cl^- and Ca^{+2} concentrations in the different experimental groups are presented in Tables 6 and 7. All ESTE doses tested were able to increases significantly the renal K^+ elimination after 8 hours from the beginning of treatments, returning to values similar to controls after 24 hours. On the other hand, HCTZ was able to significantly increase the urinary Na^+, K^+, and Cl^- excretion in the first 8 hours, returning to non-significant values at the end of the experimental period (24 hours).

Heart rate and baseline blood pressure values did not change after acute administration of ESTE

Baseline values obtained for SBP, DBP, and MAP prior to the administration of any substance and after the stabilization period were 110 ± 4.6, 72 ± 5.8, and 94 ± 4.2 mm Hg, respectively, while HR values were in 282 ± 18.2 bpm. The intraduodenal administration of ESTE, regardless of dose, was not able to induce any statistically significant changes in SBP, DBP, MAP and HR values (Figure 6A-D). On the other hand, HCTZ (25 mg/kg) was able to reduce SBP and MAP values by approximately 20% (Figure 6A and C), without affecting baseline DBP (Figure 6B) or HR (Figure 6D).

ESTE obtained from Talisia esculenta induces significant "in vitro" antioxidant activity

The radical scavenging activity of ESTE against DPPH, and its ability to reduce lipid peroxidation and oxidative hemolysis induced by AAPH are shown in Figure 7. At all concentrations, ESTE was able to induce significant DPPH free radical scavenging activity (Figure 7A). ESTE IC50 and maximum activity (%) were estimated in 108 ± 6.9 μg/ml and 85%, respectively. Similarly, ESTE at concentrations of 100, 300, and 1000 μg/ml was able to expressively reduce AAPH-induced lipid peroxidation. Malondialdehyde concentrations were reduced by approximately 52, 66, and 93%, respectively (Figure 7B).

In AAPH-induced oxidative hemolysis experiments, ESTE was able to prevent the hemolysis of erythrocytes (in all doses tested) after 1 hour of incubation (Figure 7C). On the other hand, after 2 hours of incubation, only concentration of 300 and 1000 μg/ml were able to prevent oxidative hemolysis (Figure 7D), while after 3 or 4 hours, only concentration of 1000 μg/ml presented this protective effect (Figure 7E and F).

DISCUSSION

In this study, we performed a complete morphological, and microchemical characterization of *T. esculenta* leaves, as well as identifying the main secondary metabolites present in a preparation commonly used in Brazil. In addition, we have shown safety aspects and evaluated the efficacy of the ESTE as a cardioprotective agent.

Initially, a detailed macro- and micro-anatomical study was carried out to provide important parameters for the standardization and quality control of the plant drug. Moreover, this was the first study conducted on the anatomical characteristics of this taxon. In fact, *Talisia esculenta* is not included in any pharmacopoeia, and its anatomical features provide substantial information for quality assessment in the herbal industry.

In a second stage, a semi-purified aqueous extract from *T. esculenta* was obtained and a detailed phytochemical profile was determined. Analyses performed by UPLC-PDA-MS/MS showed a reduced spectrum of secondary metabolites, mainly flavonol-derivatives. It is now known that these compounds are among the main metabolites capable of influencing renal hemodynamics and induce diuretic response.[28] However, many of these compounds have different toxicity degrees,[29] and safety data available for *T. esculenta* is very limited. Riet-Correa et al.[12] showed that the ingestion of *T. esculenta* leaves and fruits by cattle and sheep brought a varied spectrum of intoxication, including ruminal bloat, weakness, ataxia, and spastic paresis. Nevertheless, information on the toxicity of this species to humans and non-herbivorous animals remains unknown. This work showed that the acute administration of a wide range of ESTE doses obtained from *T. esculenta* leaves did not induce any signs of acute toxicity in male and female rats. It is believed that the apparent discrepancy between data obtained in herbivorous and rodents may be in part due to the anatomical and biochemical differences in the gastrointestinal system of these species. In addition, the fresh form of this species has different concentration of metabolites that may not be found in significant amounts in ESTE. In fact, the results obtained here amplify the scientific knowledge for the species and provide important safety parameters for the rational use of *T. esculenta*.

The next step of this work was to investigate whether the use of this species as a probable diuretic and hypotensive agent has some scientific evidence. A positive result is that the secondary metabolites found in this preparation are classically recognized to have antioxidant activity, and many of them have been found to be responsible for numerous hemodynamic effects of several natural products.[28,30] If on the one hand, "in vitro" antioxidant effects were quite evident, on the other, a significant response of these compounds after "in

vivo" treatment was not observed. Following intraduodenal administration, ESTE was not able to produce any significant changes in blood pressure or heart rate in Wistar rats. Additionally, the diuretic response was very limited, showing only a slight increase in urinary volume in the first 2 hours after treatments, and completely disappearing after 8 or 24 hours. Furthermore, electrolyte excretion was concentrated only in potassium, which contributes more to adverse effects than to global saluretic response.[31]

Currently, it is known that some diuretics of high efficacy, such as furosemide, have an intense and short diuretic response, which in a way compensatory to dehydration, loses its effect after 8 or 24 hours. However, the saluretic profile of these compounds is very different from the response obtained with ESTE, and involves the extensive elimination of Na^+, K^+, Cl^- ,Ca^{2+}, Mg^+, among others.[32] In fact, even HCTZ, a moderately effective diuretic, lost its diuretic response after 24 hours, and its saluretic pattern was quite different from ESTE. The results of this study allow us suggesting that the secondary metabolites responsible for high "in vitro" efficacy are not substantially absorbed by the duodenum of rodents or are unable to activate or inhibit, in a sustained manner, the molecular pathways involved with the production of urine or renal reabsorption of electrolytes. The aforementioned data reinforce the idea that not always a compound with significant biological "in vitro" activities will have significant "in vivo" effects. "In vitro" studies are fundamental for the screening and initial evaluation of a new candidate for advanced studies, but the extrapolation of data obtained in these studies for a high-complexity living organism must be performed with caution.

In conclusion, the results obtained in this study provided important anatomical and microchemical aspects of aerial vegetative organs and phytochemical data of *Talisia esculenta* leaves. In addition, it was observed that ESTE does not present risks if acutely administered in rodents. On the other hand, the use of this species as a diuretic and hypotensive agent should be carried out with caution, since administration in rodents did not produce renal and/or hemodynamic responses that justify this ethnobotanical indication.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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Table 1.

Phytochemical composition of ESTE obtained by LC-PDA-MS

Peak	Rt (min)	MS^1	MS^2 (key fragments)	Tentative identification
1	1.90	315.07178	153.019, 152.011, 109.029, 108.021	Dihydroxybenzoic acid-hexoside
2	6.02	609.14614 611.16083	301.033, 300.027, 271.024, 255.029 465.103, 303.050	Quercetin-diglycoside
3	6.37	593.15108 595.16521	285.039, 284.032, 255.029 449.108, 287.055	Kaempferol-diglycoside
4	6.46	447.09318	301.033, 300.027, 271.024, 255.029	Quercetin-rhamnoside
5	6.67	515.11930	353.087, 191.055, 179.034, 173.045, 135.045	Dicafeoylquinic acid
6	7.14	623.16171 625.17654	315.049, 314.043, 299.019 479.118, 317.065	Methylquercetin-diglycoside

Table 2.

Effect of acute oral administration of ethanol soluble fraction obtained from *Talisia esculenta* (ESTE) on the hematological parameters of Wistar rats after 14 days of exposition

Parameters	Control		ESTE (50 mg/kg)		ESTE (300 mg/kg)		ESTE (2 g/kg)	
	Male	Female	Male	Female	Male	Female	Male	Female
RBC (10^6/ml)	8.8 ± 0.2	8.0 ± 0.5	8.8 ± 0.2	8.4 ± 0.2	8.6 ± 0.4	8.4 ± 0.04	8.7 ± 0.1	8.4 ± 0.2
Hemoglobin (g/ml)	15.1 ± 0.4	15.2 ± 0.2	15.4 ± 0.4	15.1 ± 0.2	15.0 ± 0.5	15.0 ± 0.1	15.2 ± 0.2	15.2 ± 0.3
Hematocrit (%)	49.2 ± 1.3	51.6 ± 0.8	48.7 ± 1.6	49.4 ± 0.8	48.2 ± 2.0	49.3 ± 0.3	48.0 ± 0.6	47.6 ± 1.3
MCV (fl)	56.0 ± 0.4	58.7 ± 0.4	55.1 ± 0.6	58.5 ± 0.9	55.8 ± 0.7	58.4 ± 0.3	55.3 ± 0.4	56.2 ± 0.8
MCH (pg)	17.2 ± 0.2	17.6 ± 0.1	17.4 ± 0.2	17.9 ± 0.2	17.4 ± 0.2	17.8 ± 0.1	17.5 ± 0.2	17.8 ± 0.2
MCHC (%)	30.7 ± 0.2	29.5 ± 0.6	31.5 ± 0.2	30.6 ± 0.6	31.1 ± 0.2	30.5 ± 0.3	31.8 ± 0.8	31.7 ± 0.5
Platelets (10^3/mm^3)	801 ± 89	904 ± 67	722 ± 36	917 ± 70	702 ± 79	882 ± 52	685 ± 28	1081 ± 49
WBC (10^3/mm^3)	8.8 ± 0.5	5.2 ± 0.4	6.7 ± 0.5	4.8 ± 0.6	7.3 ± 0.8	4.6 ± 0.3	8.7 ± 0.8	5.0 ± 0.4
Neutrophils (%)	15.3 ± 2.3	21.3 ± 1.4	9.8 ± 1.6	26.4 ± 1.4	17.7 ± 1.2	24.0 ± 3.7	16.2 ± 1.5	21.5 ± 2.3
Lymphocytes (%)	82.3 ± 2.1	68.3 ± 1.6	84.3 ± 1.6	63.2 ± 0.9	79.8 ± 1.3	68.6 ± 3.2	79.9 ± 2.2	69.0 ± 3.1
Monocytes (%)	1.0 ± 0.2	6.7 ± 1.8	4.1 ± 2.8	8.7 ± 1.3	0.9 ± 0.1	6.2 ± 1.0	1.1 ± 0.3	7.8 ± 1.2
Eosinophils (%)	0.9 ± 0.1	3.3 ± 2.2	1.0 ± 0.07	1.3 ± 0.2	1.1± 0.1	1.0 ± 0.1	2.1 ± 1.0	1.6 ± 0.3
Basophils (%)	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.04	0.2 ± 0.03	0.4 ± 0.1	0.2 ± 0.04

Values are expressed as mean ± S. E. M. of five rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's test

Table 3.

Effect of acute oral administration of ethanol soluble fraction obtained from *Talisia esculenta* (ESTE) on the biochemical parameters of Wistar rats after 14 days of exposition

Parameters	Control		ESTE (50 mg/kg)		ESTE (300 mg/kg)		ESTE (2 g/kg)	
	Male	Female	Male	Female	Male	Female	Male	Female
Total cholesterol (mg/dL)	87.6 ± 10.4	53.0 ± 4.7	83.8 ± 3.3	59.1 ± 5.5	76.8 ± 8.5	54.7 ± 11.8	77.1 ± 7.6	69.0 ± 4.7
HDL cholesterol (mg/dL)	74.9 ± 6.7	51.2 ± 4.5	73.48 ± 2.3	58.0 ± 3.6	64.7 ± 4.4	52.9 ± 9.4	65.7 ± 5.1	62.3 ± 4.0
Triglycerides (mg/dL)	47.5 ± 7.5	37.8 ± 2.3	58.0 ± 7.3	34.6 ± 5.1	52.0 ± 5.8	30.1 ± 2.8	49.6 ± 3.0	47.5 ± 2.7
Urea (mg/dL)	27.7 ± 0.8	25.3 ± 1.7	29.4 ± 1.6	37.2 ± 1.5	26.8 ± 1.2	35.2 ± 4.2	31.4 ± 1.5	34.0 ± 1.6
Creatinine (mg/dL)	0.22 ± 0.02	0.23 ± 0.01	0.23 ± 0.02	0.30 ± 0.01	0.21 ± 0.01	0.28 ± 0.03	0.20 ± 0.01	0.31 ± 0.03
Sodium (mEq/L)	137 ± 2.2	134 ± 2.1	132 ± 3.2	142 ± 1.4	129 ± 2.9	136 ± 4.1	134 ± 1.4	143 ± 1.2
Potassium (mEq/L)	5.8 ± 0.2	5.2 ± 0.1	5.4 ± 0.4	6.5 ± 0.2	5.7 ± 0.3	5.8 ± 0.1	5.9 ± 0.4	6.8 ± 0.6
Uric acid (mg/dL)	1.9 ± 0.2	2.4 ± 0.2	1.5 ± 0.2	2.4 ± 0.4	2.3 ± 0.2	1.8 ± 0.2	1.4 ± 0.3	2.6 ± 0.4
Total protein (g/dL)	5.2 ± 0.4	4.3 ± 0.2	5.1 ± 0.3	4.7 ± 0.5	4.7 ± 0.2	4.4 ± 0.7	4.2 ± 0.2	5.6 ± 0.3
Albumin (g/dL)	3.5 ± 0.2	3.2 ± 0.1	3.3 ± 0.2	3.5 ± 0.4	3.2 ± 0.1	3.3 ± 0.5	2.8 ± 0.1	4.1 ± 0.3
Globulin (g/dL)	1.7 ± 0.1	1.1 ± 0.1	1.8 ± 0.1	1.2 ± 0.1	1.6 ± 0.1	1.2 ± 0.2	1.4 ± 0.1	1.5 ± 0.1
Amylase (U/L)	1994 ± 213	1308 ± 91	1948 ± 129	1298 ± 81	1785 ± 105	1217 ± 162	1493 ± 199	1366 ± 49
Alkaline phosphatase(U/L)	106 ± 15.8	66 ± 3.9	97 ± 13.5	79 ± 8.7	116 ± 6.2	76 ± 7.1	93 ± 14.2	58 ± 11.7
AST (U/L)	100 ± 7.0	81 ± 6.3	105 ± 9.6	126 ± 26.8	65 ± 3.2	108 ± 28.3	86 ± 3.8	121 ± 16.2
ALT (U/L)	33 ± 2.9	30 ± 2.7	30 ± 2.4	38 ± 4.3	27 ± 1.7	30 ± 4.2	37 ± 2.1	38 ± 5.0

Values are expressed as mean ± S. E. M. of five rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's test

Table 4.

Effect of acute oral administration of ethanol soluble fraction obtained from *Talisia esculenta* (ESTE) on the body weight (BW) and relative (%) organ weights of male and female Wistar rats exposed for 14 days

Organ	Control		ESTE (50 mg/kg)		ESTE (300 mg/kg)		ESTE (2 g/kg)	
	Male	Female	Male	Female	Male	Female	Male	Female
BW day 0 (g)	280 ± 6.7	212 ± 2.8	311 ± 7.3	209 ± 7.4	314 ± 12.4	208 ± 8.7	301 ± 5.6	208 ± 6.5
BW day 15 (g)	327 ± 6.1	223 ± 2.8	343 ± 7.2	219 ± 8.8	337 ± 11.3	213 ± 8.4	327 ± 6.6	210 ± 5.5
Liver (%)	3.7 ± 0.08	3.9 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	3.6 ± 0.1	3.9 ± 0.2	3.5 ± 0.01	3.7 ± 0.2
Heart (%)	0.38 ± 0.013	0.37 ± 0.006	0.35 ± 0.006	0.38 ± 0.014	0.36 ± 0.006	0.39 ± 0.008	0.38 ± 0.005	0.38 ± 0.01
Spleen (%)	0.19 ± 0.005	0.22 ± 0.007	0.21 ± 0.01	0.22 ± 0.008	0.16 ± 0.004	0.23 ± 0.003	0.19 ± 0.010	0.21 ± 0.008
Kidneys (%)	0.40 ± 0.006	0.41 ± 0.01	0.40 ± 0.003	0.43 ± 0.01	0.41 ± 0.02	0.42 ± 0.01	0.41 ± 0.007	0.42 ± 0.004
Ovaries (%)	---	0.023 ± 0.001	---	0.027 ± 0.001	---	0.029 ± 0.001	---	0.028 ± 0.002
Uterus (%)	---	0.18 ± 0.03	---	0.19 ± 0.02	---	0.29 ± 0.03	---	0.19 ± 0.02
Epididymis (%)	0.17 ± 0.006	---	0.18 ± 0.005	---	0.17 ± 0.004	---	0.20 ± 0.007	---
Testicles (%)	0.44 ± 0.0098	---	0.44 ± 0.018	---	0.42 ± 0.014	---	0.46 ± 0.015	---
Prostate (%)	0.015 ± 0.001	---	0.013 ± 0.001	---	0.017 ± 0.002	---	0.014 ± 0.001	---
Levator ani muscle (%)	0.27 ± 0.012	---	0.27 ± 0.013	---	0.29 ± 0.013	---	0.29 ± 0.008	---
Seminal vesicles (%)	0.21 ± 0.011	---	0.27 ± 0.038	---	0.21 ± 0.017	---	0.28 ± 0.016	---

Values are expressed as mean ± S. E. M. of five rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's test.

Table 5.

Effect of acute oral administration of ethanol soluble fraction obtained from *Talisia esculenta* (ESTE) on the urinary volume after 1, 2, 8, and 24 hours of the treatment

Group	Urine volume (ml/100g/1h)	Urine volume (ml/100g/2h)	Urine volume (ml/100g/8h)	Urine volume (ml/100g/24h)
Control	0.8 ± 0.1	1.4 ± 0.2	3.2 ± 0.5	8.7 ± 1.0
HCTZ (25 mg/kg)	1.6 ± 0.2[a]	2.9 ± 0.2[a]	5.6 ± 0.4[a]	10.0 ± 0.8
ESTE (30 mg/kg)	1.2 ± 0.2	2.1 ± 0.3	3.9 ± 0.4	11.0 ± 0.8
ESTE (100 mg/kg)	1.7 ± 0.2[a]	2.3 ± 0.1[a]	4.0 ± 0.3	8.2 ± 0.5
ESTE (300 mg/kg)	0.9 ± 0.1	1.5 ± 0.1	3.4 ± 0.3	9.5 ± 1.0

Values are expressed as mean ± S. E. M. of ten rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's. [a]$p \leq 0.05$ when compared with the control. HCTZ: hydrochlorothiazide.

Table 6.

Effect of acute oral administration of ethanol soluble fraction obtained from *Talisia esculenta* (ESTE) on urinary electrolyte excretion after 8 hours of the treatment

Group	El_{Na+} (µEq/min/100g)	El_{k+} (µEq/min/100g)	El_{Ca++} (µEq/min/100g)	El_{Cl-} (µEq/min/100g)	Saluretic index[b]			
					Na^+	K^+	Ca^{++}	Cl^-
Control	0.22 ± 0.06	0.10 ± 0.04	0.006 ± 0.0022	0.26 ± 0.06	-	-	-	-
HCTZ (25 mg/kg)	0.41 ± 0.05[a]	0.21 ± 0.04[a]	0.004 ± 0.0008	0.45 ± 0.07[a]	1.86	2.1	0.66	1.73
ESTE (30 mg/kg)	0.23 ± 0.06	0.22 ± 0.03[a]	0.005 ± 0.0009	0.25 ± 0.05	1.45	2.2	0.83	0,97
ESTE (100 mg/kg)	0.24 ± 0.06	0.19 ± 0.01[a]	0.006 ± 0.0009	0.27 ± 0.06	1.09	1.9	1.00	1.04
ESTE (300 mg/kg)	0.27 ± 0.08	0.25 ± 0.03[a]	0.007 ± 0.0005	0.34 ± 0.08	1.23	2.5	1.17	1.31

Values are expressed as mean ± S. E. M. of five rats in each group in comparison with the control using one-way ANOVA followed by Dunnett's test ([a]$p < 0.05$). [b]Saluretic index = mmol/L problem group/mmol/L control group. El: Excreted load; HCTZ: hydrochlorothiazide.

Table 7.

Effect of acute oral administration of ethanol soluble fraction obtained from *Talisia esculenta* (ESTE) on urinary electrolyte excretion after 24 hours of the treatment

Group	El_{Na+} (µEq/min/100g)	El_{k+} (µEq/min/100g)	El_{Ca++} (µEq/min/100g)	El_{Cl-} (µEq/min/100g)	Saluretic index[b]			
					Na^+	K^+	Ca^{++}	Cl^-
Control	0.47 ± 0.11	0.46 ± 0.13	0.009 ± 0.003	0.60 ± 0.14	-	-	-	-
HCTZ (25 mg/kg)	0.48 ± 0.07	0.51 ± 0.11	0.009 ± 0.002	0.59 ± 0.10	1.02	1.11	1.00	0.98
ESTE (30 mg/kg)	0.58 ± 0.07	0.75 ± 0.13	0.020 ± 0.004	0.77 ± 0.11	1.23	1.63	2.22	1.28
ESTE (100 mg/kg)	0.49 ± 0.09	0.66 ± 0.16	0.020 ± 0.005	0.71 ± 0.14	1.04	1.44	2.22	1.18
ESTE (300 mg/kg)	0.61 ± 0.11	1.20 ± 0.29	0.030 ± 0.010	0.91 ± 0.18	1.30	2.61	3.33	1.52

Values are expressed as mean ± S. E. M. of five rats in each group in comparison with the control using one-way ANOVA followed by Dunnett's test ([a]$p < 0.05$). [b]Saluretic index = mmol/L problem group/mmol/L control group. El: Excreted load; HCTZ: hydrochlorothiazide.

Legend to figures

Figure 1. *Talisia esculenta* (A. St.-Hil.) Radlk. (Sapindaceae). A. Vegetative organs; B – L - Leaflet. B. Venation pattern on abaxial side; C. Upper epidermis; D. Lower epidermis showing stomata (st) and non-glandular trichome (tt); E. Lower epidermis showing stomatum (st) and cuticle (ct) in Scanning Electron Microscopy (SEM); F. Lower epidermis indicating cuticle (ct), stomatum (st) and conic non-glandular trichome (tt) in SEM; G. Detail of the conic non-glandular trichome in SEM; H. Leaflet transverse section, showing epidermis (ep), spongy parenchyma (sp), palisade parenchyma (pp) and vascular bundle (vb); I. Midrib in cross-section indicating epidermis (ep), collenchyma (co), ground parenchyma (gp), sclerenchymatous sheath (sc), phloem (ph), and xylem (xy). J. Detail of the upper portion of midrib, showing epidermis with cuticle (ct) in reaction with Sudam III and prismatic calcium oxalate crystals (cr); K. Detail of the vascular region, showing sclerenchymatous sheath (sc), phloem (ph), xylem (xy) and phenolic compounds (pc) in reaction with ferric chloride; L. Detail of the vascular region, showing xylem (xy) and phenolic compounds (pc) reacted in the Hoepfner-Vorsatz test. Scale bar = A (5cm), I (200 µm), B, C, D, H, J, K, L (50µm), F (10µm), E, G (5µm).

Figure 2. *Talisia esculenta* (A. St.-Hil.) Radlk. (Sapindaceae). Cross-section. A. General aspect of petiolule; B. Detail of the previous figure, revealing sclerenchymatous sheath (sc), phloem (ph), xylem (xy), and phenolic compounds (pc); C. Epidermis (ep) and collenchyma (co) of petiolule, indicating phenolic compounds (pc); D. General aspect of rachis; E. Detail of the figure D evidencing vascular system: phloem (ph), phenolic compounds (pc), sclerenchymatous sheath (sc), and xylem; F. General aspect of petiole; G. Stem showing epidermis (ep), cortex (cx), sclerenchymatous sheath (sc), phloem (ph), and xylem (xy); H. Detail of the vascular system of the stem indicating sclerenchymatous sheath (sc), phenolic compounds (pc), phloem (ph), and xylem (xy); Pith (pi) of the stem showing phenolic compounds (pc) in the perimedullary region. Scale bar = A, D, F (300µm), H (100µm), B, C, E, G, I (50 µm).

Figure 3. UPLC analysis of ESTE obtained from *Talisia esculenta* (A. St.-Hil.) Radlk.

Figure 4. Photomacrographs of heart (1), lung (2), spleen (3), kidneys (4) and liver (5) of male (A and B) and female (C and D) rats treated with vehicle (A and C) or highest dosage of ESTE (2000 mg/kg) (B and D).

Figure 5. Photomicrographs of heart (1), lung (2), spleen (3), kidney (4) and liver (5) histopathology from representative male (A and B) and female (C and D) rats treated with vehicle (A and C) or the highest dosage of ESTE (2000 mg/kg) (B and D). Hematoxylin and eosin stain (400 X).

Figure 6. ESTE obtained from *Talisia esculenta* (A. St.-Hil.) Radlk. does not affect the values of blood pressure and the heart rate of Wistar rats. The ESTE was administered intraduodenally in anesthetized rats. The letter "C-" indicates the effect measured after administration of vehicle (saline 0.9%, 200 μl) only. The results show the mean ± S.E.M. (n = 5). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Dunnet's test. $^{a}p < 0.05$ when compared to respective control group. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; HR: heart rate.

Figure 7. "In vitro" antioxidative effects of ESTE obtained from *Talisia esculenta* (A. St.-Hil.) Radlk. The antioxidant potential was evaluated through radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) (A), lipid peroxidation (B) and by protective effects against oxidative hemolysis induced by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) after 1 to 4 hours (C-F). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Dunnet's test. $^{a}p < 0.05$ when compared to respective negative control (vehicle). $^{b}p < 0.05$ when compared to respective positive control group (AAPH) or ascorbic acid (AA). MDA: malondialdehyde.

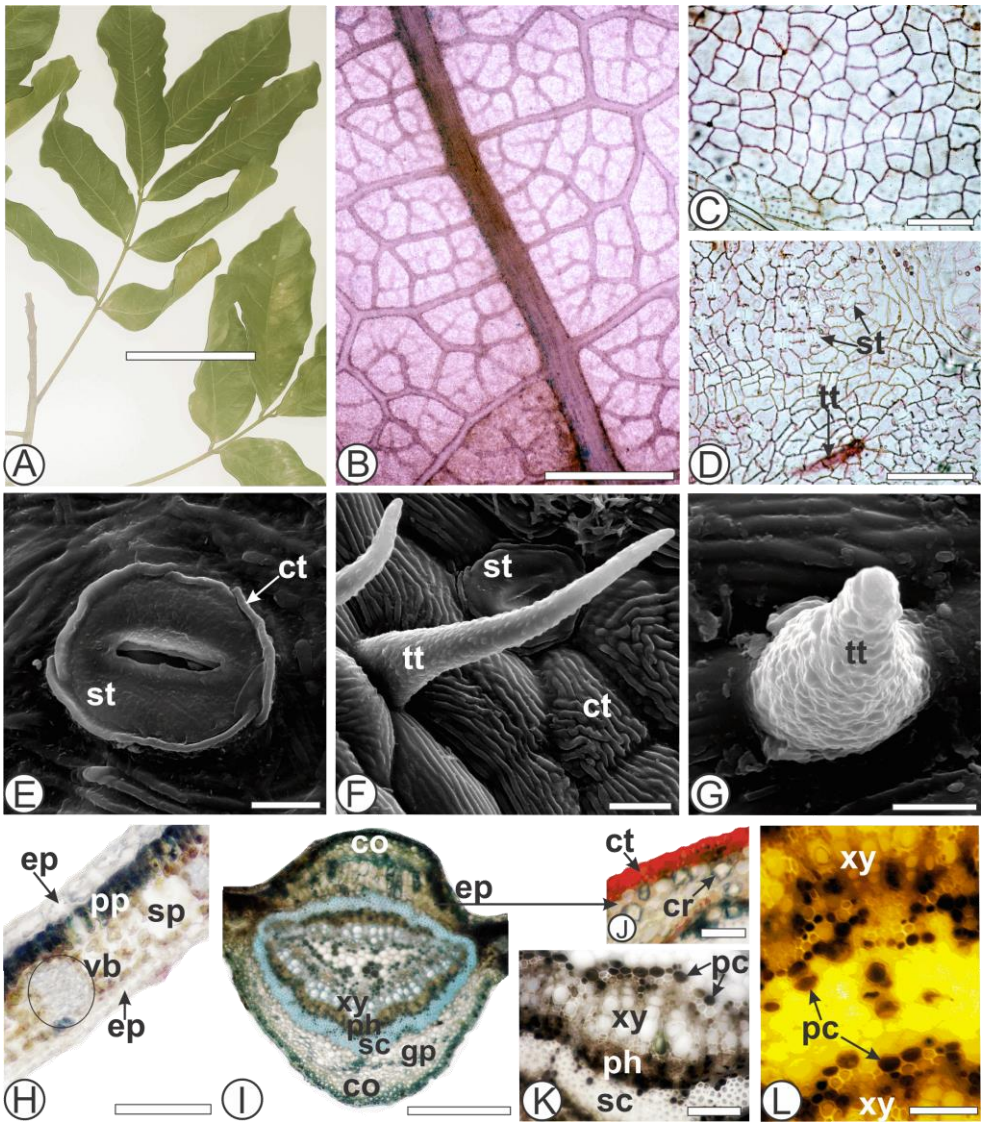

Figure 1

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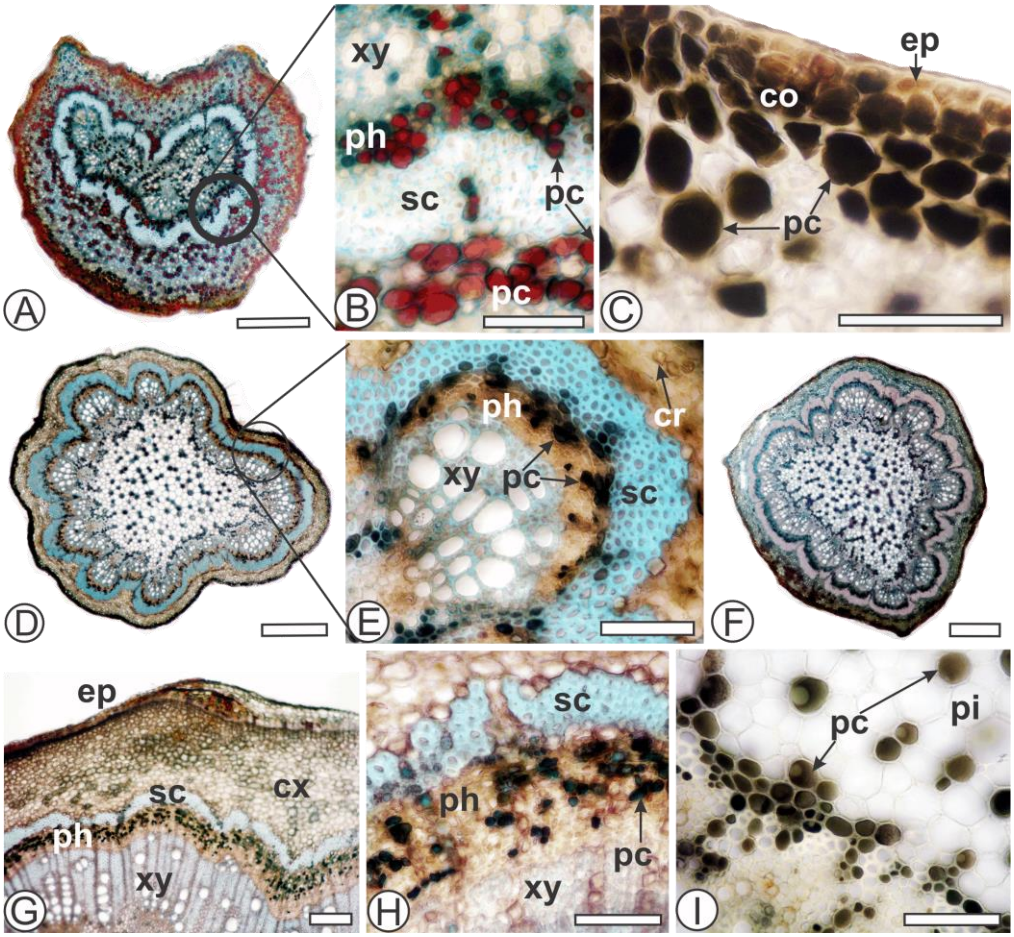

Figure 2

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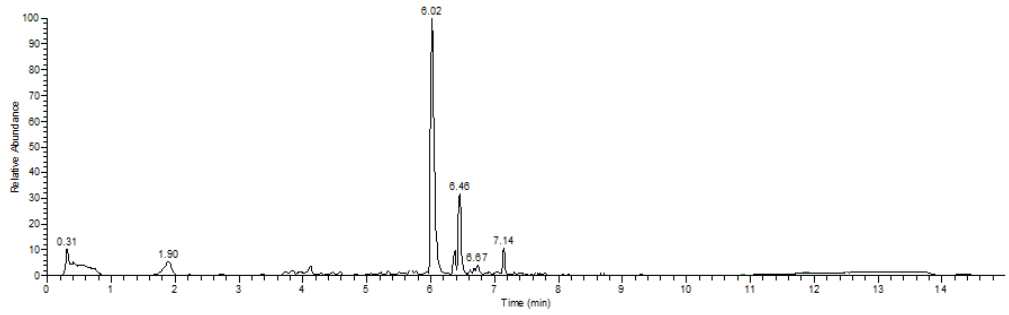

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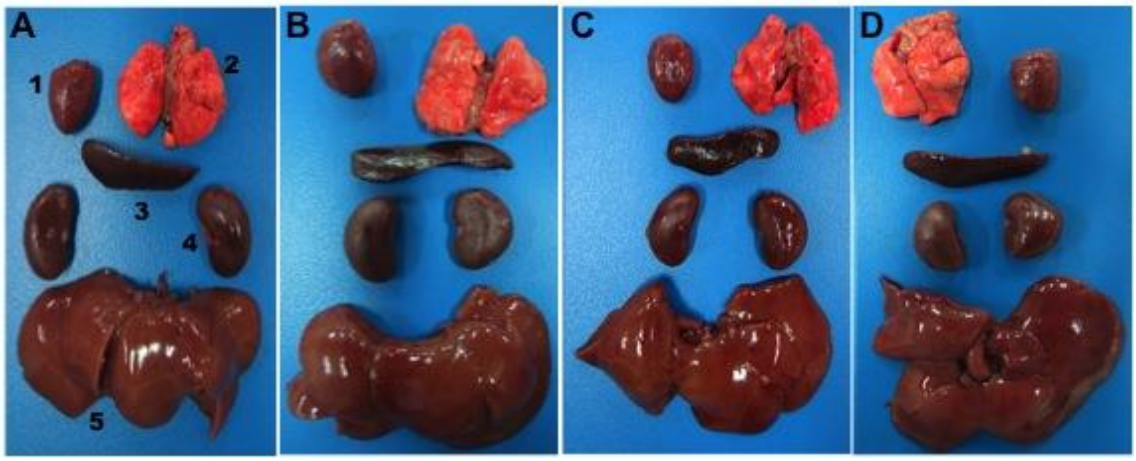

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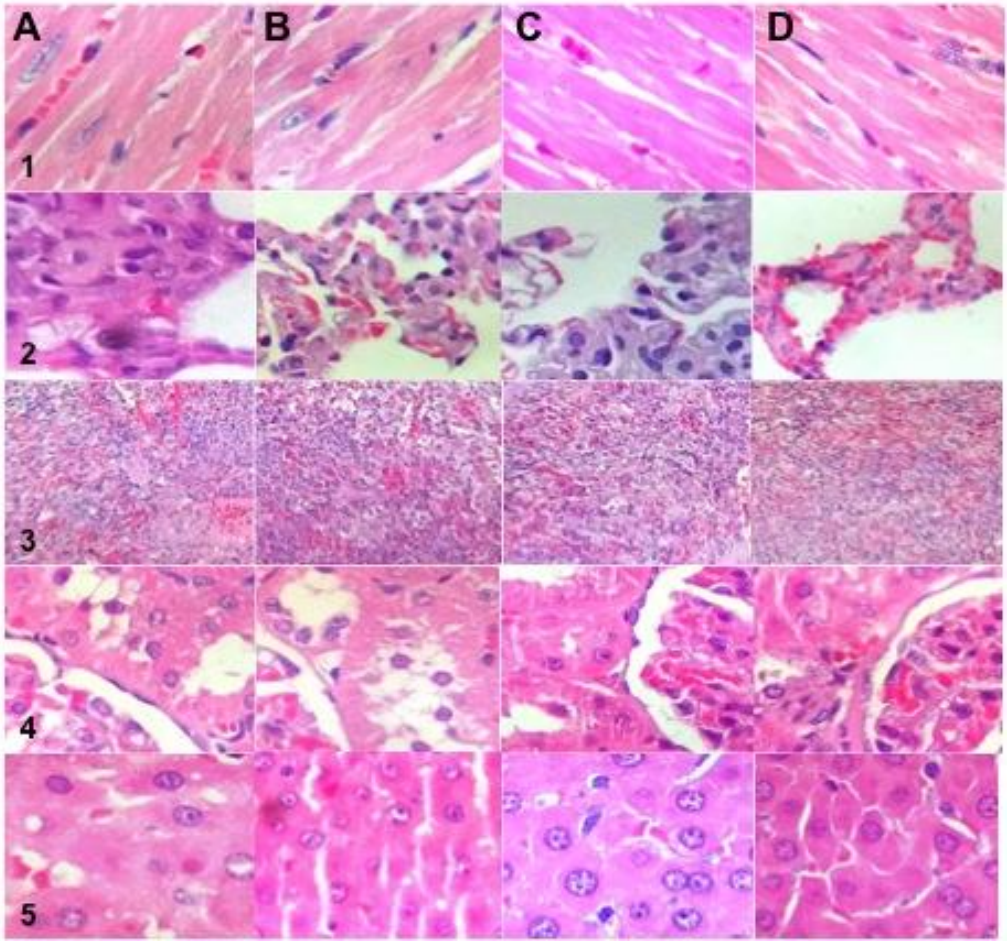

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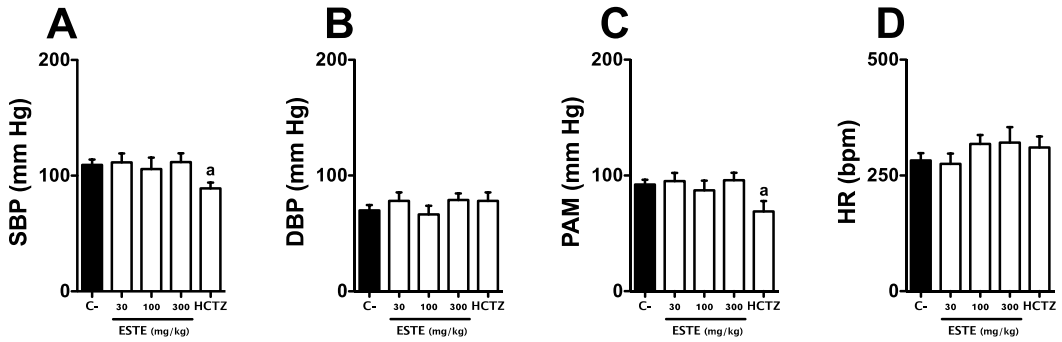

Figure 6

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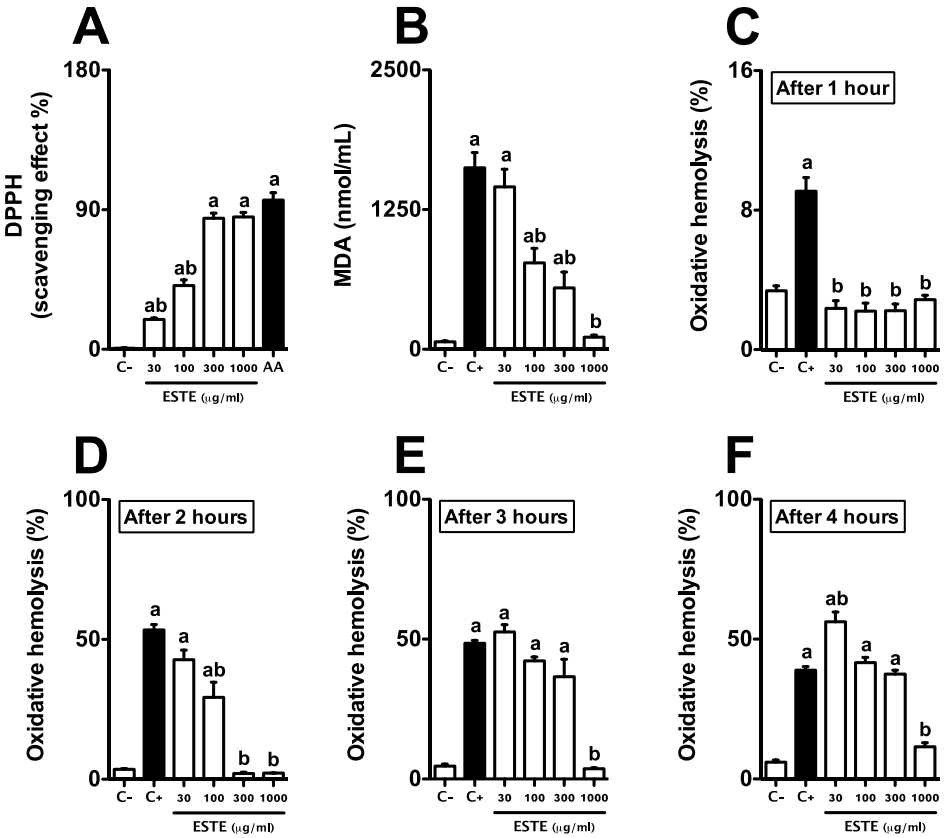

Figure 7

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5.4 Manuscrito 2- Biomonitoring the cardiorenal effects of *Luehea divaricata* Mart.: an ethnoguided approach

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Abstract

Ethnopharmacological relevance: *Luehea divaricata* Mart. (Malvaceae) is an important medicinal species widely used as a diuretic in the Brazilian Pantanal region. It has been recently shown that the ethanolic supernatant obtained from leaves of this species (ESLD) has important hypotensive and diuretic activity. Nevertheless, the secondary metabolites

responsible for this activity, as well as the molecular mechanisms responsible for their pharmacological effects remain unknown.

Aim: To carry out a biomonitoring study to identify possible active metabolites present in different ESLD fractions and investigate their effects on the renal and peripheral arteriolar tone, showing their interrelation with sustained diuretic and hypotensive activities.

Materials and Methods: First, ESLD was obtained from *L. divaricata* leaves and a liquid-liquid fractionation was performed. The resulting fractions were analyzed by liquid chromatography-mass spectrometry. Then, ethyl acetate (AceFr), *n*-butanolic (ButFr) and aqueous (AqueFr) fractions were orally administered in a single dose or for seven days, to male Wistar rats. The doses were previously defined from the yield obtained in each fraction. Hydrochlorothiazide was used as a positive control. Then, blood pressure, heart rate, urinary volume, pH, density, urinary sodium, potassium, chloride, and calcium concentrations were measured. In addition, serum levels of nitrite, thiobarbituric acid reactive species, nitrotyrosine, aldosterone, vasopressin and plasma angiotensin converting enzyme activity were also measured. Finally, the direct effects of ButFr on renal and mesenteric arteriolar tone, as well as the role of nitric oxide and prostaglandins on the renal and hemodynamic effects were also investigated.

Results: Of all fractions tested, only ButFr showed significant diuretic and saluretic effect. AceFr and ButFr fractions also showed acute hypotensive effect, but only ButFr maintained its response after 7 days of treatment. Prolonged treatment with ButFr was able to increase serum nitrite levels and significantly reduce oxidative and nitrosative stress markers. In addition, ButFr induced an important vasodilatory response in the renal and mesenteric arteriolar beds through the release of nitric oxide and prostaglandins. Finally, the diuretic and hypotensive effects induced by ButFr were completely blocked by previous administration of L-NAME or indomethacin, showing the direct involvement of nitric oxide and prostaglandins in these effects.

*Conclusion***:** ButFr obtained from *Luehea divaricata* has important and sustained diuretic and hypotensive effects. Apparently, these effects are due to the release of nitric oxide and prostaglandins, which reduces renal and peripheral arteriolar tone, leading to an increase in the glomerular filtration rate and reduction of the global peripheral resistance. This study presents ButFr as a potential complementary therapy in several situations where diuretic and hypotensive effect is required.

Keywords: Diuretic, hipotensive, nitric oxide, prostaglandins, vasodilatory

Abbreviations:

ACE: angiotensin converting enzyme; AceFr: ethyl acetate fraction; ANOVA: analysis of variance; AqueFr: aqueous fraction; ButFr: *n*-butanolic fraction; Ca^{+2}: calcium; CO_2: carbon dioxide; Cl^-: chloride; $CaCl_2$: calcium chloride; DBP: diastolic blood pressure; EDTA: ethylenediaminetetraacetic acid; EI: excretion load; ELISA: enzyme-linked immunosorbent assay; ESI-MS: electrospray ionization; EtOH: ethanol; HCD: higher-energy collisional dissociation, HCTZ: hydrochlorothiazide; HPLC-MS/MS: high performance liquid chromatography - tandem mass spectrometer; HR: heart rate; K^+: potassium; KCl: potassium chloride; KH_2PO_4: monobasic potassium phosphate; L-NAME: N(G)-nitro-L-arginine methyl ester; LC-MS: liquid chromatography-mass spectrometry; MAP: mean arterial pressure; MeOH: methanol; $MgSO_4$: magnesium sulfate; MVB: mesenteric vascular bed; Na^+: sodium; NaCl: sodium chloride; $NaHCO_3$: sodium bicarbonate; NO: nitric oxide; NT: nitrotyrosine; O_2: oxygen; Phe: phenylephrine; PP: perfusion pressure; SBP: systolic blood pressure; PSS: physiological saline solution; SEM: standard error of the mean; TBARS: thiobarbituric acid reactive species; UV: ultraviolet.

Introduction

Luehea divaricata Mart. (Malvaceae) is a small to medium tree that reaches about 10 meters in height. It is native to Brazil, occurring mainly in the southern states, and in the southern regions of the southeastern and midwestern states. Due to its aggressive behavior, it is indicated for the recovery of degraded areas, and its wood is often used for the making of furniture and internal constructions (Lorenzi, 1992). It is popularly known as “açoita-cavalo”, “caiboti”, or "pau-de-canga", being widely used by the native population of the Brazilian Pantanal region to treat kidney diseases, uric acid disorders, throat inflammation, flu, cough, pneumonia, hemorrhoids, muscle aches, and tumors (Bieski et al., 2012).

Chemically, the extracts obtained from its leaves are characterized by concentrating large amounts of phenolic compounds such as chlorogenic acid, rosmarinic acid, rutin, vitexin and epicatechin, along with triperpenoids and phytosterols (Tanaka et al., 2005; Arantes et al., 2014; Courtes et al., 2015). In addition, a recent study showed the abundant presence of vicenin, dirhamnosyl-hexosyl-quercetin, rhamnosyl-hexosyl-quercetin, vitexin, isovitexin, rhamnosyl-hexosyl-kaempferol, dicafeoylquinic acid, vanilloyl-vitexin, and kaempferol- (p-coumaroyl) -hexoside in the infusion obtained from *L. divaricata* leaves (Tirloni et al., 2017).

Some toxicological studies with *L. divaricata* extracts have been performed. Felício et al. (2011) showed absence of mutagenic potential of the aqueous extract obtained from *L. divaricata* leaves in somatic cells of *Drosophila melanogaster* Meig. Moreover, Tirloni et al. (2017) showed that the ethanolic supernatant obtained from leaves of this species does not have any clinical signs of acute toxicity in Wistar rats.

A series of investigations on the biological activities of different preparations obtained from *L. divaricata* have been conducted. In fact, several studies have shown the antifungal (Zacchino et al., 1998), neuroprotective (De Souza et al. 2004), anticholinesterase (Arantes et al., 2014), antioxidant (Courtes et al., 2015, Arantes et al., 2014), anti-inflammatory, analgesic, and immunostimulatory effects of this species (Rosa et al., 2014). Furthermore, a recent ethnopharmacological survey carried out by our research group have shown that the ethanolic supernatant obtained from *L. divaricata* leaves (ESLD) has important acute and prolonged diuretic, hypotensive and antioxidative effects (Tirloni et al., 2017).

Thus, this study aimed to perform a detailed biomonitoring investigation of the cardiorenal effects of ESLD. First, ESLD fractionation was performed by obtaining three semi-purified fractions. Then, a detailed phytochemical study about its main secondary metabolites was performed through high performance liquid chromatography - tandem mass spectrometer (HPLC-MS/MS). Subsequently, the diuretic and hypotensive effects of the

different fractions obtained from ESLD were evaluated, and the pharmacological mechanisms involved in these activities were shown. Finally, the molecular mechanisms involved in the vasodilator effects of the ESLD-fraction using the isolated perfused kidney and mesenteric vascular beds (MVBs) were investigated.

Materials and methods

Drugs and solvents

The following drugs, salts and solutions were used: xylazine and ketamine hydrochloride (from Syntec, São Paulo, SP, Brazil) and heparin (from Hipolabor, Belo Horizonte, MG, Brazil). Hydrochlorothiazide, rutin, acetylcholine chloride, phenylephrine, indomethacin, Nω-Nitro-L-arginine methyl ester (L-NAME), NaCl, KCl, $NaHCO_3$, $MgSO_4$, $CaCl_2$, KH_2PO_4, dextrose, and ethylenediaminetetraacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were obtained in analytical grade.

Phytochemical study

Plant material and preparation of the purified aqueous extract

Luehea divaricata leaves (5.5 kg) were collected in October 2015 from the botanical garden of the Federal University of Grande Dourados (UFGD) (Dourados, Brazil) at 458 m above sea level (S 22°16'46, 9'' and W 54°49'06, 3''). A voucher specimen was authenticated by Dr. Maria do Carmo Vieira under number DDMS 5220 and deposited in the herbarium of UFGD. Leaves were dried for 5 days in an air circulation oven and then ground, yielding 2.6 kg of dry powder (47.3% of the initial weight). The infusion was prepared by adding 1 liter of boiling water to each 100 g of powder. The infusion was kept in an amber bottle, hermetically sealed until it reached room temperature (approximately 3 hours). Then, the infusion was treated with 3 volumes of EtOH, which gave rise to a precipitate and an ethanol soluble fraction (ESLD). ESLD was filtered, concentrated and freeze-dried (yield 3.8% w/w).

Liquid-liquid fractionation of ESLD

ESLD (15.52 g) was solubilized in 1 liter of distilled water and sequentially partitioned with ethyl acetate (AceFr), and *n*-butanol (ButFr). Semi-purified extracts were

concentrated and lyophilized. The resulting fractions showed the following yields: AceFr (2%), ButFr (21.8%), and AqueFr (42.6%).

Phytochemical analysis – Liquid chromatography-mass spectrometry (LC-MS)

Components from ESLD fractions (AceFr, ButFr, and AqueFr) were analyzed by high performance liquid chromatography (HPLC, 1220 Infinity LC - Agilent). Chromatography was developed in an Ascentis® Express C18 column (Supelco), with 150 x 4.6 mm (L. x I.D.) and 2.7 μm of particle size. Solvents used were ultra-pure water (MilliQ) and acetonitrile (J.T.Baker), both containing 0.1% of formic acid (96% - Tedia). The column temperature was held at 40° C, and a gradient was applied in the separation, increasing the acetonitrile content from 0% to 35% in 10 min, then to 80% in 15 min at flow rate of 800 μl/min. The solvent returned to initial condition (0% acetonitrile) in 16 min and the column was re-equilibrated with 3 more minutes. Samples were prepared in MeOH-H_2O (1:1, v/v) at 1 mg/ml and 5 μl was injected. Compounds were detected by ultraviolet (UV) and mass spectrometry.

Mass spectrometry was carried out by electrospray ionization (ESI-MS) LTQ-XL - Linear Ion Trap (Thermo-Scientific), operating in the negative ionization mode at atmospheric pressure ionization. The source temperature was 350 °C and N_2 was used in sample desolvation with sheath and auxiliary gas at flow rates of 60 and 20 arbitrary units, respectively. Energies used for negative ionization were: electrospray at 3.5 kV, capillary at -20 V and tube lens at -120 V. Fragmentation was obtained by collision induced dissociation with 20-30 normalized energies. Instrument calibration was externally performed with calibration solution (Pierce™) covering *m/z* 100 to 2000. Acquisition was obtained in total ion current mode.

In the quantification analysis, the rutin standard was prepared at 1, 5, 10, 20, 30, 40 and 50 mg/ml, in a water-methanol mixture (1:1, v/v). Similarly, fractions were prepared at 1 mg/ml in water-methanol (1:1, v/v) and the analysis was performed as previously described, with detection obtained by UV at 355 nm.

Pharmacological study

Animals

Fourteen-week-old male Wistar rats weighing 330-350 g, were randomized and housed in plastic cages, with environmental enrichment, at 22 ± 2 ºC under a 12/12 h light dark cycle, 55 ± 10% humidity conditions, and *ad libitum* access to food and water. All

experimental procedures were approved by Institutional Ethics Committee of UFGD (protocol number 16/2015) and conducted in accordance with the Brazilian Legal Framework on the Scientific Use of Animals.

Effects on renal function

Acute and prolonged diuretic activity

For acute treatment, animals were divided into 11 experimental groups (*n* = 8), as follows: control (filtered water, 0.2 ml/100g), hydrochlorothiazide (HCTZ, 25 mg/kg), AceFr (0.6, 2 and 6 mg/kg), ButFr (6.5, 22 and 65 mg/kg), and AqueFr (13, 43 and 128 mg/kg). All fractions-doses were defined from a previous study by Tirloni et al. (2017) using the following equation: ESLD-doses (30, 100, 300 mg/kg) x yield of each fraction (%) / 100.

The diuretic activity was measured according to previously described methods (Gaparotto Junior et al., 2009) with some modifications. Previously, all animals received an oral dose of NaCl (0.9%, 5ml/100g) to impose body hydration uniformity. Treatments were performed by gavage in a single dose. Immediately after treatments, animals were placed in metabolic cages for a period of 24 hours. Urine was collected 8 and 24 hours after treatments. Urinary volume was measured and expressed as ml/100 g of body weight. Urinary sodium (Na^+), potassium (K^+), chloride (Cl^-) and calcium (Ca^{+2}) levels were quantified in an ion selective meter (COBAS INTEGRA 400 plus; Roche®). pH was determined on fresh urine samples using digital pH meter (Q400MT; Quimis Instruments, Brazil). Density was estimated by handheld refractometer (NO107; Nova Instruments, Brazil). Excretion load (El) of Na^+, K^+, Cl^- and Ca^{+2} was obtained by multiplying the concentration of electrolytes (mEq/l) by the urinary flow (ml/min). Results are expressed as μEq/min/100g.

Based on the results obtained with the acute treatment, we chose to evaluate the effects of the best ButFr dose after prolonged treatment. For this purpose, three groups of animals were used (*n* = 8), which received filtered water (control, 0.2 ml/100g), HCTZ (25 mg/kg), or ButFr (65 mg/kg) daily and orally for seven days. Urine was collected every 24 hours and the same parameters of acute treatments were analyzed.

Effects on renal arterial tone

Kidneys from normotensive male rats (*n* = 5), without any previous treatment, were isolated and prepared for arterial perfusion methods described by Bowman (1970). Different preparations were then placed in a water-jacketed organ bath maintained at 37 °C and

perfused with PSS gassed with 95% O_2/5% CO_2 at constant flow rate of 4 ml/min through a peristaltic pump. After setup in the perfusion apparatus, preparation was allowed to equilibrate for 60 min, and its viability was checked by a *bolus* injection of KCl (120 mmol). Then, kidneys were continuously perfused (by renal artery) with physiological saline solution (PSS) plus phenylephrine (Phe, 3 μM) to induce prolonged increase in perfusion pressure (PP). Under these conditions, different preparations received *bolus* injections containing 0.001, 0.003, 0.01, and 0.03 mg of ButFr, and the reduction in PP was measured. Then, after a new equilibration period (60 min), different preparations were perfused with PSS containing 3-μM Phe plus L-NAME (100 μM; a non-selective NO synthase inhibitor), and/or indomethacin (1 μM; a non-selective inhibitor of cyclooxygenases). After 30 min of continuous perfusion, ButFr (0.003, 0.01, and 0.03 mg) was injected again into the perfusion system. The ability of ButFr to reduce PP in the presence and absence of the different inhibitors was evaluated. Changes in the perfusion pressure (mm Hg) were measured by a pressure transducer connected to an acquisition system (PowerLab®), and its application software (Chart, v 7.1; both from ADI Instruments, Castle Hill, Australia). (PSS, composition in mM: NaCl 119; KCl 4.7; $CaCl_2$ 2.4; $MgSO_4$ 1.2; $NaHCO_3$ 25.0; KH_2PO_4 1.2; dextrose 11.1; and EDTA 0.03).

Effects of nitric oxide (NO) and prostaglandin biosynthesis inhibition on the diuretic effects induced by ButFr

Only the best ButFr dose used in the diuretic evaluation was selected for this study. So, after water and salt loading (NaCl 0.9%, 5 ml/100 g), different groups of male rats (n = 6) were orally treated with indomethacin (5 mg/kg) or L-NAME (60 mg/kg). Animals from the control group were pretreated only with vehicle (filtered water). After 60 minutes, different experimental groups received oral doses of ButFr (65 mg/kg), HCTZ (25 mg/kg), or vehicle (filtered water, 0.2 ml/100 g). Urine was collected for 8 hours and its volume was measured. Urinary Na^+, K^+, and Cl^- levels were quantified and the excretion load was estimated.

Effects on hemodynamic parameters

Effects on blood pressure and heart rate

According to methodology described by Gasparotto Junior et al. (2011), normotensive male Wistar rats (*n* = 6-7) were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) intramuscularly administered. Immediately, a *bolus* injection of heparin (15 IU) was

subcutaneously applied. Then, the left carotid artery was isolated, cannulated and connected to a pressure transducer coupled to a PowerLab® recording system, and an application program (Chart, v 4 .1; all from ADI Instruments; Castle Hill, Australia) recorded the systolic (SBP) and diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR). After 15 minutes of stabilization, different groups of rats received filtered water (control, 0.2 ml/100g), hydrochlorothiazide (HCTZ, 25 mg/kg), AceFr (0.6, 2 and 6 mg/kg), ButFr (6.5, 22 and 65 mg/kg), and AqueFr (13, 43 and 128 mg/kg) by intraduodenal route. Changes in SBP, DBP, MAP, and HR were recorded for 35 min.

Based on the results obtained with the acute treatment, we chose to evaluate the effects of the best ButFr dose after prolonged treatment. So, different groups of male rats received (by oral route) ButFr (65 mg/kg), HCTZ (25 mg/kg), or vehicle (filtered water; 0.2 ml/100g) once daily for 7 days. At the end of experiments, all animals were anesthetized and prepared for MAP, SBP, DBP, and HR measurements. Different cardiovascular parameters were recorded for 5 min after the hemodynamic stabilization period (15 min).

Effects on isolated MVBs

MVBs from normotensive male rats (n = 5), without any previous treatment, were isolated and prepared using perfusion methods described by McGregor (1965). MVBs were placed in a water-jacketed organ bath and perfused (at 4 ml/min) with PSS (composition in mM: NaCl 119; KCl 4.7; CaCl2 2.4; MgSO4 1.2; NaHCO3 25.0; KH2PO4 1.2; dextrose 11.1; and EDTA 0.03) at 37 °C and gassed with 95% O_2/5% CO_2. Changes in PP (mm Hg) were detected by a pressure transducer coupled to a PowerLab® recording system, and an application program (Chart, v 4 .1; all from ADI Instruments; Castle Hill, Australia). After equilibration (45 min), its integrity was checked by a bolus injection of KCl (120 mmol). Then, MVBs were continuously perfused with PSS plus Phe (3 μM) to induce prolonged increase in PP. Under these conditions, different preparations received bolus injections containing 0.003, 0.01, 0.03, and 0.1 mg of ButFr, and the reduction in PP was measured. Then, after a new equilibration period (45 min), different preparations were perfused with PSS containing 3-μM Phe plus L-NAME (100 μM), and/or indomethacin (1 μM). After 30 min of continuous perfusion, ButFr (0.01, 0.03, and 0.1 mg) was injected again into the perfusion system. The ability of ButFr to reduce PP in the presence and absence of different inhibitors was evaluated.

Effects of NO and prostaglandin biosynthesis inhibition on hipotensive effects induced by ButFr

As previously described, only the best ButFr dose identified in the hypotensive evaluation was selected for this study. So, different groups of male rats (n = 6) were orally treated with indomethacin (5 mg/kg), L-NAME (60 mg/kg), or vehicle (filtered water; 0.2 ml/100g). After 1 hour, animals were anesthetized and prepared for direct blood pressure and HR measurement as previously described. Then, different groups of rats received intraduodenal ButFr (65 mg/kg), HCTZ (25 mg/kg), or vehicle (filtered water, 0.2 ml/100 g). Changes in MAP, SBP, DBP, and HR were recorded for 35 min.

Serum biochemical parameters

Different groups of rats (n = 6) were daily treated (by oral route) for seven days with ButFr (65 mg/kg), HCTZ (25 mg/kg) or vehicle (filtered water). Then, blood samples were collected from the left carotid artery and the serum was obtained (centrifugation at 1000 *g* for 10 min). Urea, creatinine, ALT, and AST levels were measured using automated biochemical analyzer (Roche® Cobas Integra 400 plus). Na^+, and K^+ levels were quantified using ion selective meter (COBAS INTEGRA 400 plus; Roche®). Nitrotyrosine (NT), vasopressin, and aldosterone levels were measured by enzyme-linked immunosorbent assay (ELISA; BD Biosciences, CA, USA). Plasma nitrite concentrations were enzymatically determined by reducing nitrate according to methods described by Schmidt et al. (1989). Serum angiotensin converting enzyme (ACE) activity was determined by indirect fluorimetry according to methods described by Santos et al. (1985). Finally, thiobarbituric acid reactive substances (TBARS) levels were measured by TBARS assay kit (Cayman Chemical, Ann Arbor, Michigan, USA).

Statistical analysis

Results are expressed as mean ± standard error of mean (S.E.M) of 5-8 animals or preparations per group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test, or student's t-test when applicable. P-values less than 0.05 were considered statistically significant. Graphs were drawn and statistical analysis was carried out using GraphPad Prism software version 5.0 for Mac OS X (GraphPad® Software, San Diego, CA, USA).

Results

Phytochemical analysis

In a previous work, compounds were identified from the crude extract from *Luehea divaricata*, being then treated with ethanol to remove high molecular weight components (Tirloni et al., 2017). Then, liquid/liquid fractionation was performed yielding 3 main fractions, obtained from ethyl acetate (AceFr), *n*-butanol (ButFr), and aqueous (AqueFr) solvents. The main components in all fractions were very similar; however, their abundance varies from fraction to fraction. The main compounds were identified on the basis of their negative $[M-H]^-$ ions and fragments (Table 1). These compounds were identified as: dirhamnosyl-hexosyl-quercetin (*m/z* 755.3, t_R 9.09 min), rhamnosyl-hexosyl-quercetin (*m/z* 609.2, t_R 9.63), vitexin (*m/z* 431.1 , t_R 9.91 min), rutin (*m/z* 609.1, t_R 9.98 min), isovitexin (*m/z* 431.1, t_R 10.12 min), rhamnosyl-hexosyl-kaempferol (*m/z* 593.2, t_R 10.46 min), rhamnosyl-hexosyl-kaempferol (*m/z* 593.1, t_R 10.73 min).

Since the composition of fractions was very similar, rutin quantification (with the authentic standard available) was carried out. A 7-point calibration curve was prepared in triplicate yielding $R^2 = 0.9997$. As observed in Figure 1, the AqueFr fraction was the one with the lowest abundances of all compounds observed, and rutin appeared at 0.5 mg/g of the extract. ButFr yielded the highest rutin concentration, 40.2 mg/g of the extract, and AceFr had 29 mg/g of the extract. Although other components from fractions could not be quantified, all fractions exhibited higher amounts of isovitexin, mainly in AceFr fraction.

ButFr obtained from Luehea divaricata induces important acute diuretic and saluretic effects

The effects of the acute treatment with HCTZ and different fractions obtained from ESLD on urinary volume and urinary electrolytes excretion are shown in Tables 2 and 3. Urinary volume produced by animals treated with vehicle after 8 and 24 h was 4.9 ± 0.48 and 8.6 ± 1.04-ml/100 g, respectively. ButFr at 65 mg/kg was able to significantly increase the urinary volume after 8 (6.6 ± 0.35 ml/100 g) and 24 (12.6 ± 0.40 ml/100 g) hours when compared to control animals. In a similar manner, AceFr (6 mg/kg) and AqueFr (128 mg/kg) also increased the urinary volume after 24 h (AceFr: 13.3 ± 1.06; AqueFr 13.2 ± 1.74 ml/100 g). As expected, HCTZ increased urinary volume in both periods, with estimated values of 7.1 ± 0.29 and 13.4 ± 0.96-ml/100 g at 8 and 24 h, respectively.

Similarly, to data already presented, ButFr (65 mg/kg) increased urinary Na^+ excretion after 8 and 24 hours, while K^+ and Cl^- excretion was increased only after 24 hours of

treatment (Table 3). On the other hand, AceFr (6 mg/kg) was able to increase Cl^- excretion only after 24 hours, whereas HCTZ significantly increased renal Na^+ elimination only in the first 8 hours (Table 3). Urinary Ca^{+2} levels were not altered by any of treatments performed (data not shown).

ButFr maintains its diuretic and saluretic response after prolonged treatment

Figure 2A shows that ButFr (65 mg/kg) increased the urinary volume in the first 24 hours, reducing this effect between the second and fifth day of treatment. On the other hand, the diuretic effect resumed from the sixth day, with increase in urinary volume of approximately 39% and 44% for the sixth and seventh days of treatment, respectively. Interestingly, although HCTZ had an important acute diuretic effect, the diuretic effects after prolonged treatment began to appear only at the third day of treatment, with an estimated daily increase of 65% (Table 2 and Figure 2A).

Similarly to results observed after acute treatment, ButFr (65 mg/kg) was able to increase renal excretion of Na^+, K^+, and Cl^- in the first 24 hours (Figure 2B-D). On the other hand, after the first 24 hours, this effect was suppressed, showing statistical significance only after the sixth day of treatment (Figure 2B-D). Curiously, despite increasing urinary volume, HCTZ was not able to induce significant increase in renal elimination of Na^+, K^+, and Cl^- when compared to control animals. Moreover, none of treatments were able to change pH, urinary density, or Ca^{+2} levels (data no shown).

Diuretic effects induced by ButFr are involved with vasodilation of renal arteries depending on NO and prostaglandin release

The continuous perfusion of kidneys isolated with Phe induced a sustained increase in the vascular perfusion pressure, which was dose-dependently reduced by ButFr into the perfusion apparatus. The estimated reduction in perfusion pressure was ~ 13, 28, and 30 mm Hg, for doses of 0.003, 0.01, and 0.03 mg, respectively (Figure 3A). On the other hand, the effects of ButFr administration were decreased by around 50% in MVB perfused with L-NAME or indomethacin. Interestingly, the co-administration of L-NAME plus indomethacin abolished the vascular effects of ButFr (Figure 3B-D).

L-NAME and indomethacin administration suppresses the diuretic effects induced by ButFr

Data on urinary volume and renal elimination of Na^+, K^+ and Cl^- before and after administration of L-NAME and indomethacin are shown in Table 4. After administration of

L-NAME or indomethacin, 1 h before treatments, the diuretic activity of ButFr was completely inhibited when compared to control. Without pre-treatment, ButFr increased urinary volume by ~ 33 and 55% after 8 and 24 h, respectively. After administration of indomethacin and L-NAME, ButFr had its diuretic activity decreased by ~ 23 and 48%, and ~ 15 and 41%, after 8 and 24 h, respectively. Similarly, L-NAME and indomethacin also reduced the renal elimination of Na^+, K^+ and Cl^- induced by ButFr. On the other hand, HCTZ presented only reduction of urinary volume and renal elimination of Na^+ after treatment with indomethacin.

Acute administration of different fractions obtained from L. divaricata reduces blood pressure in normotensive rats

The effects of acute administration of different fractions obtained from *L. divaricata* on blood pressure and HR of male Wistar rats are shown in Table 5. Data presented have shown that AqueFr (128 mg/kg) and ButFr (65 mg/kg) were able to significantly reduce SBP, DBP, and MAP levels when compared to control animals. In addition, values were very close to those found for animals treated with HCTZ (25 mg/kg). On the other hand, AceFr was not able to change blood pressure when compared to control rats. In addition, none of treatments performed were able to significantly change HR of all experimental animals.

ButFr from L. divaricata maintains its hypotensive effect after 7 days of treatment

The SBP, DBP, and MAP values for vehicle-treated animals were 123 ± 9.8, 81 ± 6.3, and 110 ± 7.9 mm Hg, respectively (Figure 4A-D). Treatment with ButFr (65 mg/kg) or HCTZ (25 mg/kg) was able to significantly reduce PA (ButFr: 95 ± 6.7; HCTZ: 89 ± 5.0 mmHg), PAD (ButFr: 59 ± 3.2; HCTZ: 59 ± 6.3 mm Hg), and MAP (ButFr: 80 ± 4.7; HCTZ: 75 ± 5.5 mm Hg) values when compared to control animals. On the other hand, none of treatments significantly altered HR values when compared to the control group (Figure 4A-D).

Hypotensive responses induced by ButFr are involved in the reduction of peripheral vascular resistance by NO and prostaglandin release

Continuous perfusion of MVBs with Phe resulted in a sustained increase in the vascular perfusion pressure, which was significantly reduced by ButFr (0.01, 0.03, and 0.1 mg) (Figure 5A). Previous infusion with L-NAME reduced part of the effect on MVBs perfusion obtained with ButFr. The peak effect of ButFr at doses of 0.01, 0.03, and 0.1 mg

was decreased from ~ 13, 29 and 30 mm Hg to 7, 8, and 6 mm Hg, respectively (Figure 5C). Similarly, the vasodilatory effect of ButFr at doses of 0.01, 0.03, and 0.1 mg was reduced in ~ 58, 68, and 75% in preparations perfused with indomethacin (Figure 5B). Interestingly, co-administration with L-NAME and indomethacin (Figure 5D) abolished the effect induced by all ButFr-doses.

Previous L-NAME or indomethacin administration abolish the hypotensive effects induced by ButFr

Under L-NAME administration, the ability of ButFr (65 mg/kg) or HCTZ (25 mg/kg) to reduce SBP, DBP and MAP was entirely inhibited (Figure 6A-C). Similarly, previous indomethacin administration significantly reduced the ability of ButFr to cause hypotension, with similar effects on the hypotensive action of HCTZ (Figure 6E-G). As previously reported, HR was not significantly altered by any of treatments performed.

Prolonged treatment with ButFr increases serum nitrite levels and reduces lipid peroxidation and nitrosative stress without affecting plasma ACE activity or serum urea, creatinine, ALT, AST, Na^+, K^+ aldosterone and vasopressin levels

Prolonged treatment with ButFr (65 mg/kg) was able to significantly increase nitrite levels when compared to control animals (Control: 56 ± 5.0 μM; ButFr: 87 ± 5.3 μM) (Figure 7A). Similarly, serum TBARS (Control: 7.7 ± 0.6; ButFr: 4.0 ± 0.6 mmol/l) and NT levels (Control: 0.015 ± 0.0013; ButFr: 0.011 ± 0.0010 μmol/l) were also significantly reduced (Figure 7B-C). On the other hand, treatment with HCTZ was not able to significantly change nitrite, TBARS or NT levels (Figure 7A-C). Similarly, none of treatments were able to change plasma ACE activity or serum aldosterone, vasopressin, urea, creatinine, ALT, AST, Na^+, and K^+ levels (data not shown).

Discussion

This study showed for the first time that ButFr, a semi-purified fraction obtained from *Luehea divaricata*, has important and sustained diuretic and hypotensive activity. These effects appear to be due to its vasodilator effect on the renal and peripheral vascular beds, increasing renal hydrostatic pressure and reducing overall peripheral resistance. In addition, the results showed a direct involvement of NO and prostaglandins in this process, as well as a potent and prolonged antioxidant activity.

In a previous work, it was shown that the ethanolic supernatant of the infusion obtained from *L. divaricata* (ESLD) leaves has an important diuretic and hypotensive effect

(Tirloni et al., 2017). Although data are interesting, the main secondary metabolites involved in this activity and the mechanisms adjacent to these effects remained unknown. Thus, in this study, using three fractions with different polarities obtained from ESLD, we chose to identify through a biomonitoring study the molecular mechanisms that could be involved with the possible diuretic and hypotensive effects. As a starting point, we tried to evaluate if the different fractions obtained from *L. divaricata* have some significant diuretic and saluretic effects on normotensive rats. In fact, only ButFr showed significant diuretic and saluretic response acutely and after 7 days of treatment, suggesting that possibly the active metabolites of ESLD would be concentrated in ButFr. An interesting fact is the reduction of the diuretic and saluretic response after 24 hours, resuming this effect only after the sixth day of treatment. This apparent gap is common after the use of diuretics of high efficacy. It is noteworthy that HCTZ also showed this effect, which is possibly a counter-adjusting response caused by an increase in the NaCl "offer" to the macula densa, which by local release of adenosine, increases the resistance in the afferent arteriole and reduces the glomerular filtration rate (Hoorn and Ellison, 2017). In addition, it is likely that the release of renin by juxtaglomerular cells also has some influence in this process, where through the release of angiotensin II and aldosterone, increases the reabsorption of sodium and water by the different tubular branches. Then, after the gradual return of serum osmolality, the diuretic effects try to return in a more subtle but sustained way (Bowman et al., 2016).

The renal function has an important dependence on the maintenance of perfusion pressure in the renal arterioles. In fact, the systemic blood pressure plays a significant role in this process, but the intricate relationship between afferent arteriole dilation and the increase in efferent arteriole tone has a limiting regulatory effect (Ahmeda and Alzoghaibim, 2016). Several vasodilatory mediators contribute to the dilation of the afferent arteriole and consequently to increased blood flow to glomerular capillaries. Prostaglandins and NO appear to play a prominent role, and the maintenance of vasodilatation induced by these mediators leads to an important increase in the glomerular filtration rate, with a consequent increase in urine output and in the excretion of electrolytes (Satoh et al., 2017; Kovi ć et al., 2016). In fact, several diuretic drugs, including HCTZ, have part of their effect attributed to prostaglandins (Dormans et al., 1996). In addition, important diuretic and saluretic effects from different natural products have a direct contribution to the release of prostaglandins and NO (Lívero et al., 2017). According to the above, the diuretic and natriuretic effects induced by ButFr also appear to depend on the increased hydrostatic pressure of the renal afferent artery, possibly through the release of prostaglandins and NO. Regardless of inhibition of the

diuretic activity following prior administration of L-NAME and indomethacin, data obtained after ButFr administration in isolated and perfused kidney reinforce this hypothesis, since the dilation of renal arteries in the presence of ButFr was also dependent on the release of NO and prostaglandins.

Since many diuretic drugs are also routinely used as antihypertensive agents (Tsioufis and Thomopoulos, 2017), we have evaluated the hypotensive potential of the different fractions obtained from ESLD. Although two fractions (AqueFr and ButFr) have demonstrated hypotensive potential after a single administration, only ButFr showed both diuretic and hypotensive activities. In fact, the hypotensive effect induced by ButFr was sustained after 7 days of treatment, showing that, as occurs in the diuretic response, its effect is sustained after prolonged treatment.

Blood pressure can be defined as the product between cardiac output and peripheral vascular resistance. A determinant factor for peripheral vascular resistance is the tone of pre-capillary arterioles, which adjust to the tissue demand of nutrients and oxygen (Tykocki et al., 2017). Among several, two vasodilator mediators stand out in the maintenance of resistance vessel tones, NO and protaglandins. Released in different physiological conditions, these mediators play a central role in the maintenance of vascular tone, platelet aggregation and renal function (Godo and Shimokawa, 2017). In fact, several antihypertensive drugs have part of their effects attributed to the reduction of peripheral vascular resistance due to the release of these endogenous mediators (Howlett, 2014; Chan et al., 2012). As one of the central axes of this work, we have shown that ButFr is able to reduce arterial pressure and the tonus of resistance vessels apparently through the release of NO and prostaglandins. As HR was not altered after treatments, it is suggested possibly that the cardiac output was not significantly changed, allowing inferring that the hypotensive effects induced by ButFr may be due to the global reduction of the peripheral vascular resistance.

The fact that this study did not identify the way that ButFr acts in mediating the release of NO and prostaglandins is a limitation. However, this study has some strengths. After 7 days of treatment, ButFr was able to significantly reduce oxidative and nitrosative stress, increasing the nitrite concentration, an important marker of NO bioavailability. In fact, it is still premature to say whether the antioxidant effects were a determining factor in the pharmacological effects evidenced. However, if we take into account that the degradation of important endothelial mediators may be due to the increase of reactive oxygen and nitrogen species, it is probable that there will be a direct or indirect participation of oxidative stress reduction in the events presented here. Moreover, although phytochemical data have shown

that ButFr has many secondary metabolites with classic antioxidant properties, especially rutin and other flavonoids (Li et al., 2014), it is doubtful whether the diuretic and hypotensive activities can be attributed to an integrated or independent action of these compounds. Further studies can help answer these questions and check the clinical effects of ButFr, opening the prospect for this compound to be a phytodrug in the future.

Conclusion

This study showed that ButFr obtained from *Luehea divaricata* has important and sustained diuretic and hypotensive effects. Apparently, these effects are due to the release of NO and prostaglandins, which reduce renal and peripheral arteriolar tone, leading to an increase in the glomerular filtration rate and reduction of the global peripheral resistance. This study presents ButFr as a potential complementary therapy in several situations where diuretic and hypotensive effect is required.

Author's contribution

All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; CAST, RACP, MIS, AAMM, LPG, and ELBL conducted the experiments; LMS and GSS was involved with the chemical analysis of extract; AGJ was responsible for data discussion, manuscript correction and was the senior researcher responsible for this work. All authors read and approved the final manuscript.

Conflict of interest

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

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Table 1.

Compounds present in the fractions from *Luehea divaricata* extract

Rt	MS^1	MS^2	Tentative identification	AqueFr	ButFr	AceFr
9.09	755.3	609.2, 463.1, 301.0	Dirhamnosyl-hexosyl-quercetin	+	++	+
9.63	609.2	463.1, 301.0	Rhamnosyl-hexosyl-quercetin	+	++	+
9.91	431.1	341.066, 311.056, 283.060	Vitexin	+	+++	+
9.98	609.1	463.1, 301.0	Rutin	+	++++	++
10.12	431.1	341.066, 311.056, 283.061	Isovitexin	+	+++++	+++++
10.46	593.2	447.1, 285.0	Rhamnosyl-hexosyl-Kaempferol	+	++	+
10.73	593.1	447.1, 285.0	Rhamnosyl-hexosyl-Kaempferol	+	++	+

The symbol + was used to indicate the relative abundances of each compound. AceFr: Ethyl acetate fraction; ButFr: Butanolic fraction; AqueFr: Aqueous fraction.

Table 2.

Effect of acute oral administration of different fractions obtained from ethanolic supernatant of the *Luehea divaricata* (ESLD) on the urinary volume, pH, and density after 8 and 24 hours of the treatment in male Wistar rats

Group	Urine volume (ml/100g/8h)	Urine volume (ml/100g/24h)	pH (8 h)	pH (24 h)	Density (8 h)	Density (24 h)
Control	4.9 ± 0.48	8.6 ± 1.04	7.85 ± 0.20	8.50 ± 0.20	1019 ± 0.96	1038 ± 3.10
HCTZ (25 mg/kg)	7.1 ± 0.29[a]	13.4 ± 0.96[a]	7.87± 0.22	8.16 ± 0.23	1017 ± 1.13	1032 ± 1.17
AceFr (6 mg/kg)	6.0 ± 0.38	13.3 ± 1.06[a]	7.92 ± 0.14	8.52 ± 0.15	1016 ± 2.14	1031 ± 2.33
AceFr (2 mg/kg)	5.8 ± 0.30	11.0 ± 0.63	8.03 ± 0.19	8.88 ± 0.10	1017 ± 2.37	1033 ± 1.86
AceFr (0.6 mg/kg)	6.1 ± 0.27	12.8 ± 3.11	7.80 ± 0.30	8.70 ± 0.20	1013 ± 0.96	1032 ± 2.16
ButFr (65 mg/kg)	6.6 ± 0.35[a]	12.6 ± 0.40[a]	8.10 ± 0.17	8.68 ± 0.10	1016 ± 3.87	1035 ± 4.52
ButFr (22 mg/kg)	6.2 ± 0.54	11.4 ± 0.64	7.56 ± 0.43	8.90 ± 0.12	1014 ± 1.26	1028 ± 2.32
ButFr (6.5 mg/kg)	6.3 ± 0.51	11.7 ± 1.00	7.66 ± 0.20	6.56 ± 0.12	1013 ± 1.36	1038 ± 0.75
AqueFr (128 mg/kg)	4.7 ± 0.26	13.2 ± 1.74[a]	6.90 ± 0.28	8.07 ± 0.12	1022 ± 3.65	1025 ± 4.06
AqueFr (43 mg/kg)	5.7 ± 0.23	11.1 ± 0.51	6.93 ± 0.39	8.75 ± 0.17	1014 ± 1.09	1029 ± 1.61
AqueFr (13 mg/kg)	5.4 ± 0.48	10.4 ± 0.63	8.02 ± 0.14	8.95 ± 0.06	1015 ± 1.24	1028 ± 3.48

Values are expressed as mean ± S. E. M. of 8 rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's test. ([a]$p \leq 0.05$). Control (vehicle); AceFr: Ethyl acetate fraction; ButFr: Butanolic fraction; AqueFr: Aqueous fraction; HCTZ: hydrochlorothiazide.

Table 3.

Effect of acute oral administration of different fractions obtained from ethanolic supernatant of the *Luehea divaricata* (ESLD) on the renal electrolyte excretion after 8 and 24 hours of the treatment in male Wistar rats

Group	$ElNa^+$ (µEq/min/100g) (8 h)	$ElNa^+$ (µEq/min/100g) (24 h)	ElK^+ (µEq/min/100g) (8 h)	ElK^+ (µEq/min/100g) (24 h)	$ElCl^-$ (µEq/min/100g) (8 h)	$ElCl^-$ (µEq/min/100g) (24 h)
Control	1.3 ± 0.17	0.9 ± 0.11	0.6 ± 0.06	1.1 ± 0.11	1.7 ± 0.25	1.4 ± 0.16
HCTZ (25 mg/kg)	2.0 ± 0.18[a]	0.8 ± 0.07	0.7 ± 0.05	0.9 ± 0.09	2.0 ± 0.22	1.2 ± 0.09
AceFr (6 mg/kg)	1.4 ± 0.16	1.1 ± 0.07	0.9 ± 0.11	1.2 ± 0.07	1.8 ± 0.22	1.8 ± 0.08[a]
AceFr (2 mg/kg)	1.4 ± 0.17	0.8 ± 0.05	0.7 ± 0.11	1.2 ± 0.05	1.6 ± 0.12	1.7 ± 0.06
AceFr (0.6 mg/kg)	1.7 ± 0.11	0.7 ± 0.03	0.7 ± 0.01	1.1 ± 0.10	1.9 ± 0.11	1.8 ± 0.03
ButFr (65 mg/kg)	1.8 ± 0.07[a]	1.2 ± 0.09[a]	0.6 ± 0.08	1.6 ± 0.20[a]	2.0 ± 0.08	2.2 ± 0.22[a]
ButFr (22 mg/kg)	1.3 ± 0.18	1.0 ± 0.16	0.5 ± 0.07	1.0 ± 0.04	1.2 ± 0.12	1.3 ± 0.05
ButFr (6.5 mg/kg)	1.3 ± 0.26	0.8 ± 0.07	0.4 ± 0.04	1.2 ± 0.01	1.5 ± 0.14	1.1 ± 0.20
AqueFr (128 mg/kg)	1.4 ± 0.11	0.9 ± 0.05	0.7 ± 0.04	1.0 ± 0.08	1.7 ± 0.10	1.4 ± 0.11
AqueFr (43 mg/kg)	0.9 ± 0.11	0.8 ± 0.03	0.5 ± 0.07	0.9 ± 0.06	1.4 ± 0.13	1.2 ± 0.07
AqueFr (13 mg/kg)	1.1 ± 0.08	0.9 ± 0.05	0.6 ± 0.04	1.1 ± 0.05	1.4 ± 0.07	1.6 ± 0.07

Values are expressed as mean ± S. E. M. of 8 rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's test. ([a]$p \leq 0.05$). Control (vehicle); AceFr: Ethyl acetate fraction; ButFr: Butanolic fraction; AqueFr: Aqueous fraction; EI: Excretion load; HCTZ: hydrochlorothiazide.

Table 4.

Effect of acute oral administration of HCTZ (25 mg/kg) and ButFr (65 mg/kg) from ethanolic supernatant of the *Luehea divaricata* (ESLD) on the urinary volume (ml/100g) and Na^+, K^+, and Cl^- concentration (µEq/min/100g) in the absence and presence of L-NAME and indomethacin

Group	Vehicle (8 h)	Vehicle (24 h)	INDO (8 h)	INDO (24 h)	L-NAME (8 h)	L-NAME (24 h)
Control (urinary volume)	5.1 ± 0.25	8.7 ± 1.05	4.1 ± 0.35	7.1 ± 0.99	6.1 ± 0.23	8.5 ± 0.99
ButFr (urinary volume)	6.8 ± 0.25[a]	13.5 ± 0.30[a]	5.2 ± 0.44	6.9 ± 0.72[b]	6.0 ± 0.33	7.9 ± 0.66[b]
HCTZ (urinary volume)	7.3 ± 0.23[a]	14.1 ± 0.85[a]	4.9 ± 0.75	9.5 ± 0.85[b]	8.5 ± 0.22[a]	15.5 ± 0.40[a]
Control (Na^+)	1.3 ± 0.17	0.8 ± 0.11	0.9 ± 0.14	0.8 ± 0.05	1.2 ± 0.14	0.7 ± 0.07
ButFr (Na^+)	1.8 ± 0.07[a]	1.2 ± 0.09[a]	0.9 ± 0.14[b]	0.7 ± 0.05[b]	1.2 ± 0.05[b]	0.7 ± 0.05[b]
HCTZ (Na^+)	2.0 ± 0.18[a]	0.8 ± 0.07	1.2 ± 0.06[b]	0.7 ± 0.04	1.4 ± 0.14[b]	0.8 ± 0.06
Control (K^+)	0.6 ± 0.06	1.1 ±0.11	0.5 ± 0.06	1.1 ± 0.06	0.4 ± 0.04	0.6 ± 0.08
ButFr (K^+)	0.6 ± 0.08	1.6 ± 0.20[a]	0.6 ± 0.11	0.9 ± 0.08[b]	0.4 ± 0.05	0.6 ± 0.05[b]
HCTZ (K^+)	0.7 ± 0.05	0.9 ± 0.09	0.5 ± 0.04	1.0 ± 0.04	0.5 ± 0.05	0.8 ± 0.06
Control (Cl^-)	1.6 ± 0.22	1.4 ± 0.16	1.0 ± 0.15	1.3 ± 0.08	1.2 ± 0.13	0.9 ± 0.09
ButFr (Cl^-)	2.0 ± 0.08	2.2 ± 0.22[a]	1.4 ± 0.23[b]	1.1 ± 0.09[b]	1.2 ± 0.07[b]	0.9 ± 0.07[b]
HCTZ (Cl^-)	2.0 ± 0.22	1.2 ± 0.09	1.5 ± 0.08	1.3 ± 0.05	1.6 ± 0.14	1.1 ± 0.08

Values are expressed as mean ± S. E. M. of 6 rats in each group in comparison to the respective control ($^a p \leq 0.05$) or with the same group in the absence of the inhibitor ($^b p \leq 0.05$) using one-way ANOVA followed by Dunnett's test. Control (vehicle); ButFr: Butanolic fraction; HCTZ: hydrochlorothiazide; INDO: indomethacin.

Table 5.

Effect of acute oral administration of different fractions obtained from ethanolic supernatant of the *Luehea divaricata* (ESLD) on the arterial pressure and heart rate of male Wistar rats

Group	SBP (mm Hg)	DBP (mm Hg)	MAP (mm Hg)	HR (bpm)
Control	126 ± 6.8	77 ± 3.3	102 ± 4.3	320 ± 44
HCTZ (25 mg/kg)	89 ± 9.2[a]	46 ± 3.2[a]	71 ± 7.7[a]	304 ± 32
AceFr (6 mg/kg)	103 ± 8.0	64 ± 7.3	84 ± 7.0	327 ± 24
AceFr (2 mg/kg)	110 ± 10.7	63 ± 7.1	86 ± 8.2	335 ± 55
AceFr (0.6 mg/kg)	108 ± 11.7	61 ± 8.5	85 ± 8.5	329 ± 28
ButFr (65 mg/kg)	102 ± 5.6[a]	53 ± 4.0[a]	78 ± 3.7[a]	385 ± 20
ButFr (22 mg/kg)	119 ± 3.9	71 ± 3.7	99 ± 6.0	318 ± 31
ButFr (6.5 mg/kg)	122 ± 9.6	59 ± 9.2	91 ± 9.0	300 ± 52
AqueFr (128 mg/kg)	85 ± 2.6[a]	51 ± 4.6[a]	69 ± 3.4[a]	373 ± 19
AqueFr (43 mg/kg)	114 ± 4.1	66 ± 7.4	88 ± 6.6	356 ± 20
AqueFr (13 mg/kg)	111 ± 4.4	66 ± 6.1	92 ± 4.6	382 ± 35

Values are expressed as mean ± S. E. M. of 6-7 rats in each group in comparison to the control using one-way ANOVA followed by Dunnett's test. ([a]$p \leq 0.05$). Control (vehicle); AceFr: Ethyl acetate fraction; ButFr: Butanolic fraction; AqueFr: Aqueous fraction; HCTZ: hydrochlorothiazide. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; HR: heart rate.

Legend to figures

Figure 1. Chromatographic profile of the fractions obtained from *Luehea divaricata* extract: (A) aqueous fraction (AqueFr), (B) *n*-butanol fraction (ButFr), and (C) ethyl acetate fraction (AceFr).

Figure 2. Effect of prolonged oral treatment with ButFr (65 mg/kg) and HCTZ (25 mg/kg) on cumulative urinary volume (A) and Na^+ (B), K^+ (C), and Cl^- (D) excretion. The urine samples were collected every day until 7 days, and cumulative urinary volume and electrolyte levels were analyzed. Each bar represents the mean of seven animals and the vertical lines show the S.E.M. [a]denote the significance levels in comparison with the control group. [b]denote the significance levels in comparison with the HCTZ. (Two-way ANOVA followed by Dunnett's test) ([a or b]$p < 0.05$). HCTZ: hydrochlorothiazide.

Figure 3. ButFr from *Luehea divaricata* promotes vasodilation in the renal arteries depending on nitric oxide and prostaglandins. Effects of ButFr (0.001, 0.003, 0.01, and 0.03 mg) on the perfusion pressure of the isolated kidneys perfused with physiological saline solution containing 3-μM Phe in the absence (A) and presence of INDO (B), L-NAME (C), or INDO plus L-NAME (D). Values are expressed as mean ± S.E.M. of 5 experiments. [a]indicates $p < 0.05$ compared with the perfusion pressure recorded before ButFr-administration (A) or with respective control group (vehicle) (B-D). [b]indicates $p < 0.05$ compared with the previous dose. All experiments were performed in endothelium-intact preparations. C: control (basal perfusion pressure); INDO: indomethacin; Phe: phenylephrine.

Figure 5. Role of nitric oxide and prostaglandins on the vasorelaxant effect of the ButFr from *Luehea divaricata* in the mesenteric vascular bed of rats. Effects of ButFr (0.003, 0.01, 0.03, and 0.1 mg) on endothelium-intact mesenteric vascular beds continuously perfused in the absence (A) and presence indomethacin (B), L-NAME (C), or co-administration of L-NAME and indomethacin (D). Values are expressed as mean ± S.E.M. of 5 experiments. [a]indicates $p < 0.05$ compared with the perfusion pressure recorded before ButFr (A) or with respective control group (vehicle) (B-D). [b]indicates $p < 0.05$ compared with the previous dose. All experiments were pero formed in endothelium-intact preparations. C: control (basal perfusion pressure); INDO: indomethacin; Phe: phenylephrine.

Figure 6. ButFr-induced hypotension depends on the nitric oxide and prostaglandins release. The animals were subjected to an intraduodenal administration of ButFr (65 mg/kg) or HCTZ (25 mg/kg) in the presence and absence of previous administration of L-NAME (60 mg/kg, by oral route, 1 hour prior) or indomethacin (5 mg/kg, by oral route, 1 hour prior) and SBP (A and E), DBP (B and F), MAP (C and G) and HR (D and H) were measured. The results are the mean ± S.E.M. of 6 rats in each group. Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Dunnett's test. [a]$p < 0.05$ when compared with the respective control group. DPB: diastolic blood pressure; HR: heart rate; INDO: indomethacin; MAP: mean arterial pressure; SBP: systolic blood pressure.

Figure 7. Prolonged ButFr- treatment increases nitrite concentration (A), and reduces TBARS (B) and NT (C) levels. The serum samples were obtained after 7-days of treatment with vehicle, ButFr (65 mg/kg), or HCTZ (25 mg/kg). The results show the mean ± S.E.M. (n = 6-7). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Dunnett's test. [a]$p < 0.05$ when compared to control group (C). [b]$p < 0.05$ when

compared to HCTZ. HCTZ: hydrochlorothiazide; NT: nitrotyrosine; TBARS: thiobarbituric acid reactive substances.

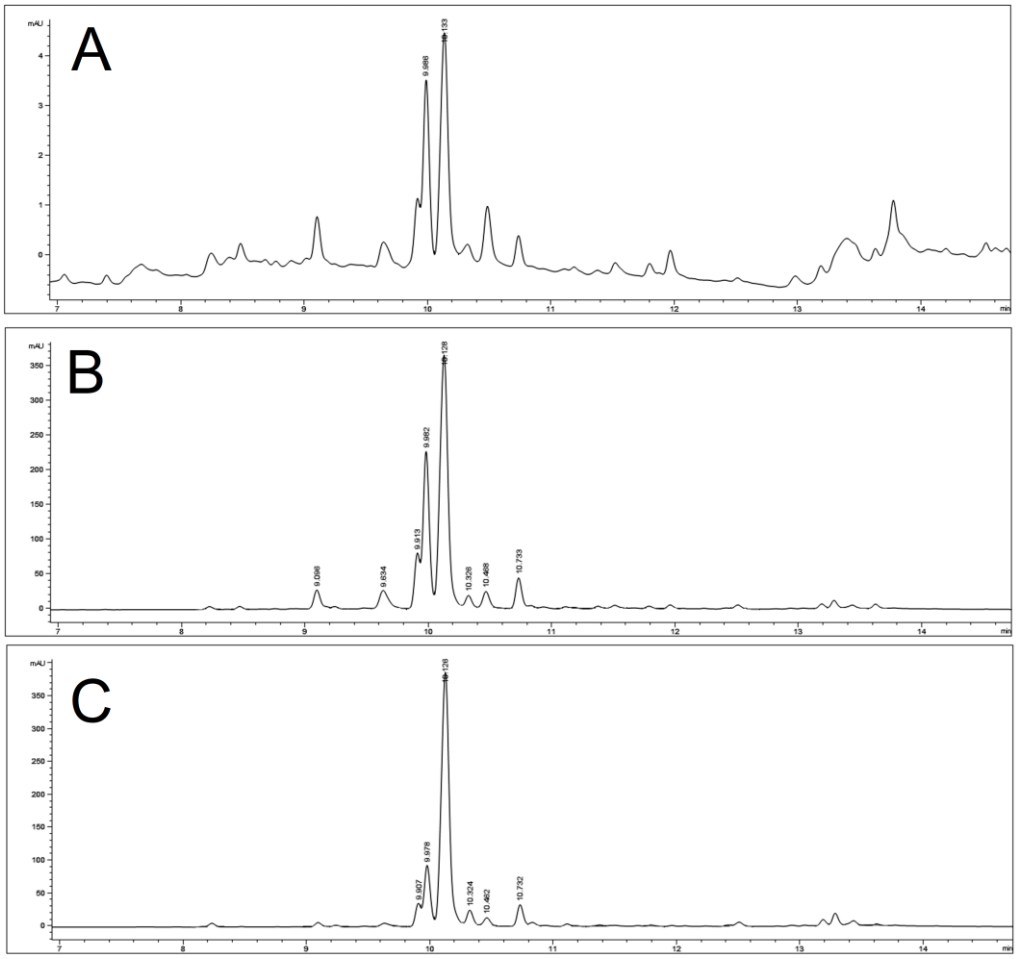

Figure 1

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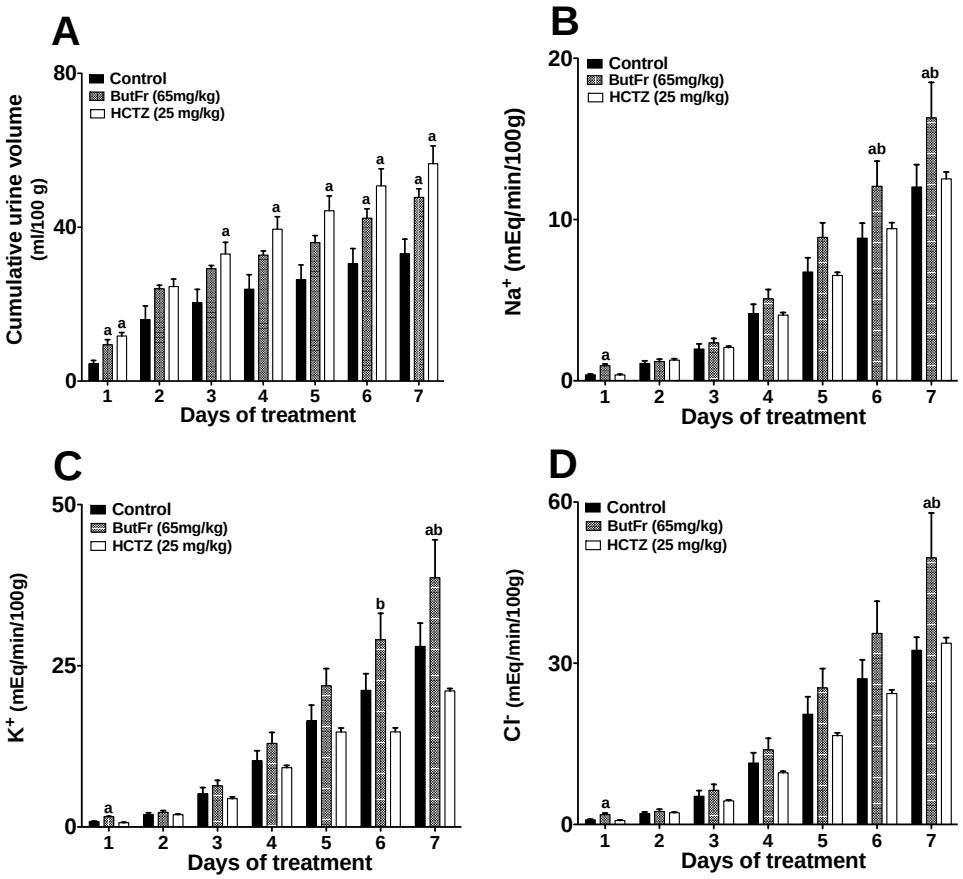

Figure 2

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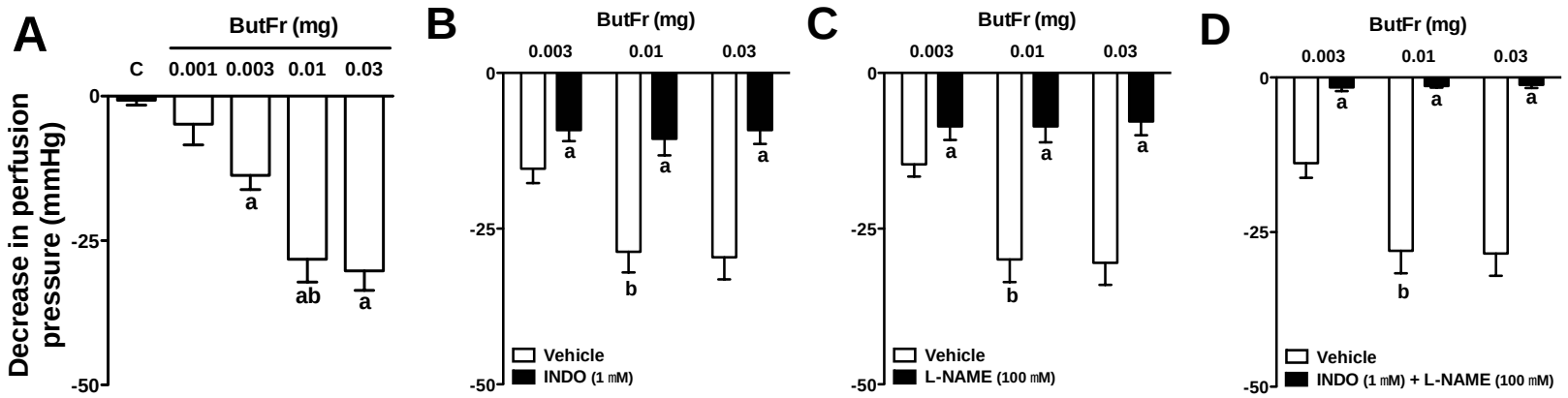

Figure 3

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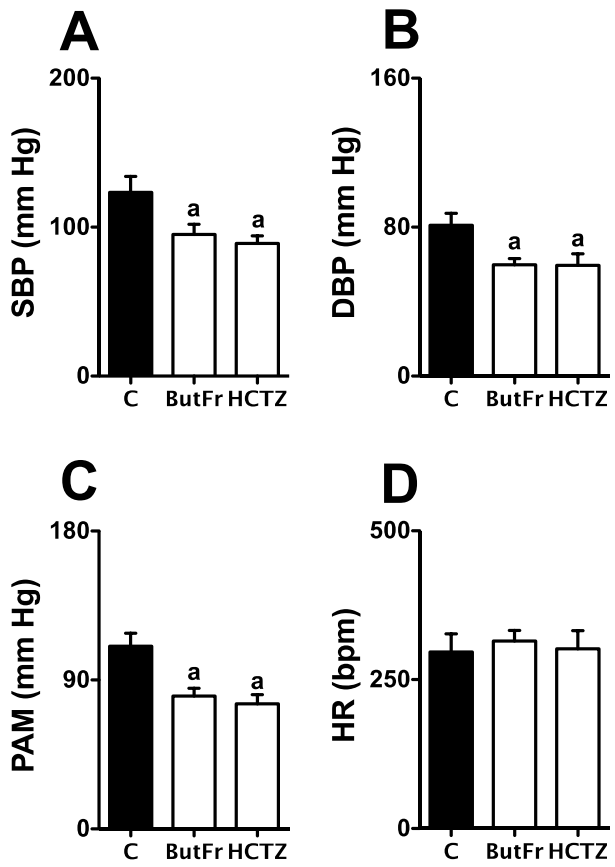

Figure 4

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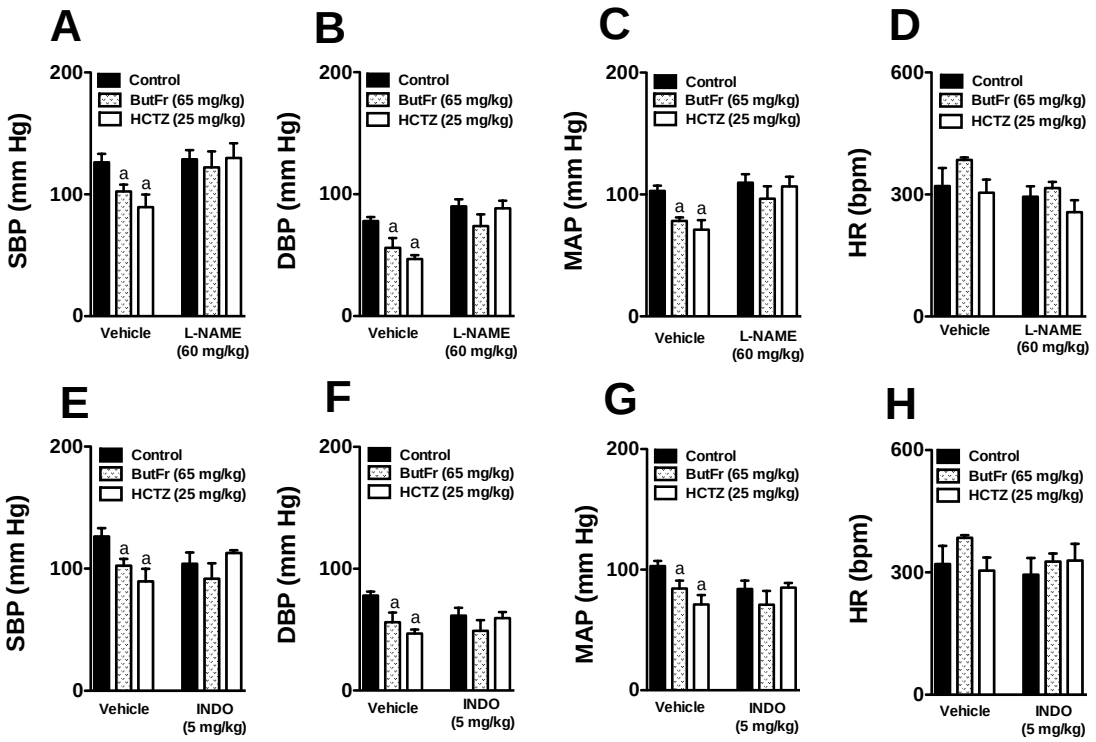

Fig 5

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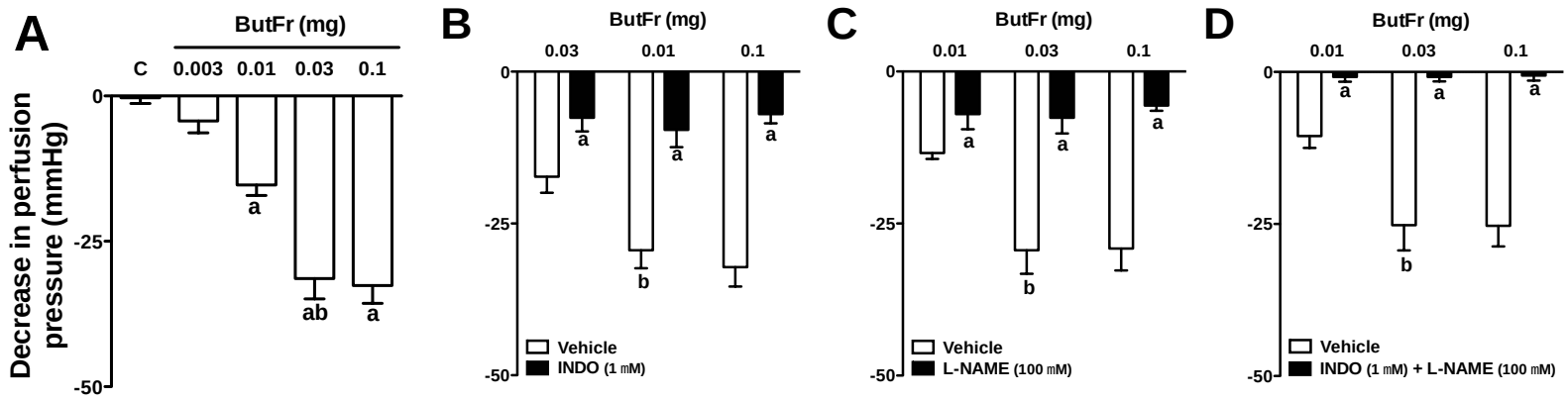

Figure 6

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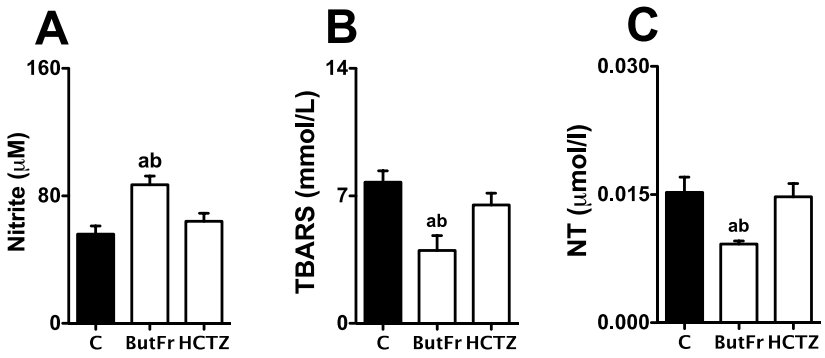

Figure 7

Tirloni et al.

5.5 Manuscrito 3- Influence of *Luehea divaricata* Mart. extracts on peripheral vascular resistance and the role of nitric oxide and both Ca^{+2}-sensitive and Kir6.1 ATP-sensitive K+ channels in the vasodilatory effects of isovitexin on isolated perfused mesenteric beds

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Dear Gasparotto,

We have received your article "Influence of *Luehea divaricata* Mart. extracts on peripheral vascular resistance and the role of nitric oxide and both Ca+2-sensitive and Kir6.1 ATP-sensitive K+ channels in the vasodilatory effects of isovitexin on isolated perfused mesenteric beds" for consideration for publication in Phytomedicine asOriginal Article

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Abstract

Background: Luehea divaricata Mart. (Malvaceae) is an important medicinal species widely used by indigenous and riverside populations of the Brazilian Pantanal region. It has been shown that the several extracts obtained from leaves of this species have important cardioprotective effects. Nevertheless, the secondary metabolites responsible for this activity, as well as the molecular mechanisms responsible for their pharmacological effects remain unknown.

Purpose: To carry out a biomonitoring study to identify possible active metabolites present in different ESLD fractions and evaluate the mechanisms responsible for the vasodilatory effects on isolated perfused mesenteric beds.

Methods: First, ESLD was obtained from *L. divaricata* leaves and a liquid-liquid fractionation was performed. The resulting fractions were analyzed by liquid chromatography-mass spectrometry. Then, the possible vasodilatory effects of ESLD, chloroform, ethyl acetate, *n*-butanolic and aqueous fractions on perfused arterial mesenteric vascular beds were evaluated. Finally, the molecular mechanisms involved in vasodilator responses of the aqueous fraction and its chemical component, isovitexin, on the mesenteric arteriolar tone were also investigated.

Results: In preparations with functional endothelium ESLD, *n*-butanolic, aqueous fraction and isovitexin dose-dependently reduced the perfusion pressure in mesenteric vascular beds. Endothelium removal or inhibition of nitric oxide synthase enzymes by L-NAME reduced the vasodilatory effects induced by aqueous fraction and isovitexin. Perfusion with nutritive solution containing 40 mM KCl abolished the vasodilatory effect of all aqueous fractions and Isovitexin doses. Treatment with glibenclamide, a $K_{ir}6.1$ (ATP-sensitive) potassium channels blocker, or tetraethylammonium, a non-selective K_{Ca} (calcium-activated) potassium channels blocker, reduced by around 70% vasodilation induced by all aqueous fractions and isovitexin doses. In addition, association of tetraethylammonium and glibenclamide, or L-NAME and glibenclamide, fully inhibited aqueous fraction and Isovitexin -induced vasodilation.

*Conclusion***:** This study showed that AqueFr obtained from *Luehea divaricata* and its metabolite - isovitexin - has important vasodilatory effects on MVBs. Apparently, these

effects are dependent on endothelium-NO release and both KCa K+ channels and Kir6.1 ATP-sensitive K+ channels activation in the vascular smooth muscle.

Keywords: Arterial bed; cGMP; Malvaceae; nitric oxide; potassium channels

List of abbreviations

4-AP: 4-aminopyridine; AceFr: ethyl acetate fraction; ANOVA: analysis of variance; AqueFr: aqueous fraction; ButFr: *n*-butanol fraction; $CaCl_{2:}$ calcium chloride; cGMP: cyclic guanosine monophosphate; ChloroFr: chloroform fraction; CO: cardiac output; CO_2: carbon dioxide; EDTA: ethylenediaminetetraacetic acid; ESI-MS: electrospray ionization; ESLD: ethanol soluble fraction from *L. divaricata*; EtOH: ethanol; GLB: glibenclamide; HCD: higher-energy collisional dissociation, HPLC-MS/MS: high performance liquid chromatography - tandem mass spectrometer; K^+: potassium; KCl: potassium chloride; KH_2PO_4: monobasic potassium phosphate; L-NAME: N(G)-nitro-L-arginine methyl ester; LC-MS: liquid chromatography-mass spectrometry; MeOH: methanol; $MgSO_4$: magnesium sulfate; MVB: mesenteric vascular bed; NaCl: sodium chloride; $NaHCO_3$: sodium bicarbonate; NO: nitric oxide; O_2: oxygen; Phe: phenylephrine; PP: perfusion pressure; PSS: physiological saline solution; PVR: peripheral vascular resistance; SEM: standard error of the mean; TEA: tetraethylammonium; UV: ultraviolet.

Introduction

Luehea divaricata Mart. (Malvaceae) is a small to medium-size tree popularly known in Brazil as “açoita-cavalo”, “caiboti”, or "pau-de-canga", (Lorenzi, 1992). The cardiovascular properties of this species have been widely explored by different ethnic groups native to Brazil, including indigenous and riverside populations of the Pantanal region (Bieski et al., 2012). In fact, some benefits on the cardiovascular system have been recently investigated. Some studies have shown that different extracts obtained from *L. divaricata* leaves present important diuretic, hypotensive, and antioxidative effects (Arantes et al., 2014; Courtes et al., 2015; Tirloni et al., 2017) without showing any signal of mutagenic potential or acute toxicity in rodents (Felicio et a., 2011; Tirloni et al., 2017).

Chemically, the extracts obtained from its leaves are characterized by concentrating large amounts of phenolic compounds such as chlorogenic acid derivatives and several flavonoids, including rutin, vicenin, vitexin, isovitexin, quercetin, kaempferol, epicatechin,

and their respective glycosylated conjugates, along with some triperpenoids and phytosterols (Arantes et al., 2014; Courtes et al., 2015; Tanaka et al., 2005; Tirloni et al., 2017).

Although previously mentioned studies clearly indicate that *L. divaricata* extracts present potentially protective effects on the cardiovascular system, the mechanisms involved remain unclear. In this study, the perfused mesenteric arterial bed was used to evaluate the hypothesis that the semi-purified fractions obtained from *L. divaricata* leaves also causes direct relaxation of the arteriolar smooth muscle, an effect that may contribute to the systemic action against hypertension. In addition, the mechanisms involved in the vascular effects of the semi-purified fraction with greater activity, and its metabolite, isovitexin, were also investigated.

Materials and methods

Drugs and solvents

The following drugs, salts and solutions were used: xylazine and ketamine hydrochloride (from Syntec, São Paulo, SP, Brazil) and heparin (from Hipolabor, São Paulo, SP, Brazil). Isovitexin, acetylcholine chloride (ACh), phenylephrine (Phe), indomethacin, tetraethylammonium (TEA), 4-aminopyridine (4-AP), glibenclamide, sodium deoxycholate, Nω-Nitro-L-arginine methyl ester (L-NAME), NaCl, KCl, $NaHCO_3$, $MgSO_4$, $CaCl_2$, KH_2PO_4, dextrose, and ethylenediaminetetraacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were obtained in analytical grade.

Phytochemical study

Plant material and preparation of the purified aqueous extract

Luehea divaricata leaves (5.5 kg) were collected in October 2015 from the botanical garden of the Federal University of Grande Dourados (UFGD) (Dourados, Brazil) at 458 m above sea level (S 22°16’46, 9’’ and W 54°49’06, 3’’). A voucher specimen was authenticated by Dr. Maria do Carmo Vieira under number DDMS 5220 and deposited in the herbarium of UFGD. Leaves were dried for 5 days in an air circulation oven and then ground, yielding 2.6 kg of dry powder (47.3% of the initial weight). The infusion was prepared by adding 1 liter of boiling water to each 100 g of powder. The infusion was kept in an amber bottle, hermetically sealed until it reached room temperature (approximately 3 hours). Then, the infusion was treated with 3 volumes of EtOH, which gave rise to a precipitate and an ethanol soluble fraction (ESLD). ESLD was filtered, concentrated and freeze-dried (yield 3.8% w/w). A detailed phytochemical study of the main secondary metabolites present in

ESLD (from the same batch of dried and pulverized L. divaricata leaves) was recently published by our research group (Tirloni et al., 2017).

Liquid-liquid ESLD fractionation

ESLD (15.52 g) was solubilized in 1 liter of distilled water and sequentially partitioned with chloroform (ChloroFr), ethyl acetate (AceFr), and *n*-butanol (ButFr). Semi-purified extracts were concentrated and lyophilized. The resulting fractions showed the following yields: ChloroFr (yield 1.6% w/w), AceFr (yield 2% w/w), ButFr (yield 21.8% w/w), and AqueFr (yield 42.5% w/w).

Phytochemical analysis – Liquid chromatography-mass spectrometry (LC-MS)

Components from ESLD fractions were analyzed by high performance liquid chromatography (HPLC, 1220 Infinity LC - Agilent). Chromatography was developed in an Ascentis® Express C18 column (Supelco), with 150 x 4.6 mm (L. x I.D.) and 2.7 μm of particle size. Solvents used were ultra-pure water (MilliQ) and acetonitrile (J.T. Baker), both containing 0.1% formic acid (96% - Tedia). The column temperature was held at 40° C, and a gradient was applied in the separation, increasing the acetonitrile content from 0% to 35% in 10 min, then to 80% in 15 min at flow rate of 800 μl/min. The solvent returned to initial condition (0% acetonitrile) in 16 min and the column was re-equilibrated with 3 more minutes. Samples were prepared in MeOH-H_2O (1:1, v/v) at 1 mg/ml and 5 μl were injected. Compounds were detected by ultraviolet (UV) and mass spectrometry.

Mass spectrometry was carried out by electrospray ionization (ESI-MS) LTQ-XL - Linear Ion Trap (Thermo-Scientific), operating in the negative ionization mode at atmospheric pressure ionization. The source temperature was 350 °C and N_2 was used in sample desolvation with sheath and auxiliary gas at flow rates of 60 and 20 arbitrary units, respectively. Energies used for negative ionization were: electrospray at 3.5 kV, capillary at -20 V and tube lens at -120 V. Fragmentation was obtained by collision-induced dissociation with 20-30 normalized energies. Instrument calibration was externally performed with calibration solution (Pierce™) covering *m/z* 100 to 2000. Acquisition was obtained in total ion current mode.

Pharmacological study

Animals

Fourteen-week-old female Wistar rats weighing 250-300 g, were randomized and housed in plastic cages, with environmental enrichment, at 22 ± 2 ºC under 12/12 h light dark cycle, 55 ± 10% humidity conditions, and *ad libitum* access to food and water. All experimental procedures were approved by Institutional Ethics Committee of UFGD (protocol number 16/2015) and conducted in accordance with the Brazilian Legal Standards on Scientific Use of Animals.

Isolation and perfusion of mesenteric vascular beds (MVBs)

Female rats were anesthetized with ketamine and xylazine (100 and 20 mg/kg, respectively; i.p.). MVBs were isolated and prepared using perfusion methods described by McGregor (1965). First, the superior mesenteric artery was cannulated and gently flushed with PSS (composition in mM: NaCl 119; KCl 4.7; $CaCl_2$ 2.4; $MgSO_4$ 1.2; $NaHCO_3$ 25.0; KH_2PO_4 1.2; dextrose 11.1; and EDTA 0.03) plus heparin (250 IU/ml) to prevent blood clotting. After removal of the entire intestine, 10 ml of PSS were perfused through the superior mesenteric artery, and the MVB was separated from the intestine. The four main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused. All other branches of the superior mesenteric vascular bed were tied off. MVBs (n = 5) were placed in a water-jacketed organ bath and perfused (at 4 ml/min) with PSS at 37 °C and gassed with 95% O_2/5% CO_2. Changes in perfusion pressure (PP, mm Hg) were detected by a pressure transducer coupled to a PowerLab® recording system, and an application program (Chart, v 4 .1; all from ADI Instruments; Castle Hill, Australia). After equilibration (45 min), its integrity was checked by a *bolus* injection of KCl (120 mmol). Then, to check the endothelial viability of preparations, different MVBs were continuously perfused with PSS plus Phe (3 μM) to induce prolonged increase in perfusion pressure (PP). Under these conditions, a *bolus* injection containing ACh (1 nmol) was performed, and the PP reduction was measured.

In order to chemically remove the endothelium of MVBs, some preparations were perfused with PSS containing sodium deoxycholate (1.8 mg/ml) for 30 seconds. Then, the system was perfused with regular PSS for additional 40 minutes for stabilization. So, to confirm loss of endothelial responsiveness, preparations were continuously perfused with PSS plus Phe (3 μM), and following sustained PP increase, a dose of ACh (1 nmol) was directly applied into the perfusion system.

Effects of L. divaricata extract and semi-purified fractions on arterial MVBs

MVBs with or without functional endothelium were continuously perfused with PSS plus Phe (3 µM). After stabilization of PP increase, different preparations received bolus injections containing ESLD, ChloroFr, AceFr, ButFr, and AqueFr (0.003, 0.01, 0.03, and 0.1 mg), and the PP reduction was measured. Each next dose was administered only after the return of the perfusion pressure to the same level recorded before the injection, with minimal interval of 3 min between doses.

Investigation of the mechanisms involved in the vascular effects of AqueFr and isovitexin

Effects on endothelial mediators and potassium channels

For these experiments, only the semi-purified fraction with the best activity in MVBs, and its metabolite (isovitexin) were used. So, after recording the first dose-response curve to AqueFr (0.01, 0.03, and 0.1 mg) and isovitexin (30, 100, 300 and 1000 nmol), MVBs were left to equilibrate for an additional period of 30-45 min. Then, different MVBs were perfused with PSS containing Phe (3 µM) plus the following agents, used alone or combined: L-NAME (100 µM; a non-selective NO synthase inhibitor), indomethacin (1 µM; a non-selective cyclooxygenase inhibitor), KCl (40 mM), tetraethylammonium (TEA 1 mM; a non-selective calcium-sensitive [KCa] K^+ channel blocker), 4-aminopyridine (4-AP 10 µM; a voltage-dependent [KV] K^+ channels blocker), and glibenclamide (GLB 10 µM; a selective Kir6.1 ATP-sensitive K^+ channels blocker). After 15 min of continuous perfusion, AqueFr (0.01, 0.03, and 0.1 mg) and isovitexin (30, 100, 300 and 1000 nmol) were injected again into the perfusion system. The ability of AqueFr and isovitexin to reduce PP in the presence and absence of different inhibitors was evaluated.

Effects on intracellular cyclic guanosine monophosphate (cGMP) levels

The intracellular concentration of cyclic guanosine monophosphate (cGMP) was evaluated according to methods described by Estancial et al. (2015). For this, the aortic rings of female rats (2-3 mm; n = 5) were removed and mounted on an organ bath with Krebs-Henseleit solution (composition in mm: 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 11 glucose) at 37 °C and gassed with 95% O_2/5% CO_2. A resting period of 1 h, under tension of 2 grams, was allowed before experiments. Then, the aortic rings were incubated for 15 min with SNP (10 µm), or AqueFr (0.01, 0.03, and 0.1 mg), or isovitexin (30, 100, 300 and 1000 nmol) in the absence and presence of soluble guanylyl

cyclase (sGC) inhibitor ODQ (100 μm, 30 min). Then, tissues were removed, frozen, homogenized in trichloroacetic acid (5% wt/vol), centrifuged (10 min at 4 °C at 1500 g) and the supernatant was collected. Methods for incubating antibodies and measuring intracellular cGMP levels strictly followed the test kit recommendations (Cayman Chemical Cyclic GMP EIA kit, Ann Arbor, MI, USA). All experiments were performed in triplicate.

Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M) of 5 preparations per group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test, or student's t-test when applicable. P-values less than 0.05 were considered statistically significant. Graphs were drawn and statistical analysis was carried out using GraphPad Prism software version 5.0 for Mac OS X (GraphPad® Software, San Diego, CA, USA).

Results

Phytochemical analysis

In a previous work, compounds from the crude *Luehea divaricata* extract were identified, being then treated with ethanol to remove high molecular weight components (Tirloni et al., 2017). Then, liquid/liquid fractionation was performed yielding 4 main fractions, obtained from chloroform (ChloFr), ethyl acetate (AceFr), *n*-butanol (ButFr), and aqueous (AqueFr) solvents. As the fraction with the best vasodilator activity in MVBs was AqueFr, we chose to perform a detailed phytochemical study only in this semi-purified fraction. So, the main compounds were identified on the basis of their negative $[M-H]^-$ ions and fragments. These compounds were identified as: dirhamnosyl-hexosyl-quercetin (*m/z* 755.3, t_R 9.09 min), rhamnosyl-hexosyl-quercetin (*m/z* 609.2, t_R 9.63), vitexin (*m/z* 431.1, t_R 9.91 min), rutin (*m/z* 609.1, t_R 9.98 min), isovitexin (*m/z* 431.1, t_R 10.12 min), rhamnosyl-hexosyl-kaempferol (*m/z* 593.2, t_R 10.46 min), and rhamnosyl-hexosyl-kaempferol (*m/z* 593.1, t_R 10.73 min).

Vasorelaxant effect of semi-purified fractions from L. divaricata on MVBs

The continuous perfusion of MVB_S with Phe resulted in a sustained increase in the vascular perfusion pressure, which was dose-dependently reduced by ESLD, ButFr and AqueFr administration into the perfusion apparatus. Although ESLD, and butanolic fraction

had some vasodilator response, the effects of AqueFr were significantly higher, with values estimated at ~ 18, 46 and 53 mm Hg at doses of 0.01, 0.03, and 0.1 mg (Figure 2C and D), respectively. Moreover, it was found that isovitexin was able to induce an expressive dose-dependent vasodilator response in MVBs, with values similar to those obtained for AqueFr. The PP reduction values for doses of 100, 300 and 1000 nmol were ~ 26, 56 and 66 mm Hg, respectively (Figure 3B). In fact, the tracing of a typical experiment shown in Figure 3C reveals that, when the highest dose was used, the vasodilatory effect of isovitexin reached the same profile as that of ACh. ChloroFr and AceFr did not induce any vasodilator effects on MVBs.

The vascular effect of AqueFr and isovitexin is partially dependent on the endothelial function

Treatment with sodium deoxycholate reduced the effects of ACh on MVBs by 98 ± 6%, confirming the efficacy of chemically removing the endothelium. Similarly, the peak effect of all tested AqueFr and isovitexin doses was decreased by 72 ± 7 and 80 ± 9%, respectively, in preparations without functional endothelium (Figure 4A and B). Similarly, the effects of all AqueFr and isovitexin doses were reduced by 66 ± 8% and 77 ± 8, respectively, in MVBs perfused with L-NAME (Figure 4C and D). On the other hand, the vasodilatory effect of AqueFr or isovitexin remained unchanged in preparations perfused with indomethacin (Figure 4E and F).

AqueFr and isovitexin induce a significant increase in intracellular cGMP levels

The incubation of AqueFr (0.01, 0.03 and 0.1 mg/ml) and isovitexin (100, 300 and 1000 nmol) with the aortic rings of female rats increased the cGMP levels by ~ 30%, 60%, and 100%, respectively, when compared with basal levels, whereas its co-incubation with ODQ (100 μm) completely abolished this effect. The NO-donor SNP increased the cGMP levels by ~ 161%, whereas co-incubation with ODQ completely vanished SNP-mediated increases in cGMP (Figure 5A and B).

The effects of AqueFr and isovitexin on MVBs are dependent on the activation of KCa and Kir6.1 ATP-sensitive K^+ potassium channels

The perfusion of MVBs with nutritive solution added of 40 mM KCl abolished the effects of AqueFr and isovitexin (Figure 6A and B). In addition, the reduction in PP generated by 0.01, 0.03 and 0.1 mg of AqueFr and 100, 300 and 1000 nmol of isovitexin in control preparations were reduced by ~ 78 ± 8% and ~ 70 ± 7% in MVBs perfused with TEA and

GLB, respectively (Figure 6C-F). On the other hand, only minor effects were observed after infusion of 4-AP (Figure 6G and H). Interestingly, simultaneous treatment (co-administration) with TEA and GLB (Figure 7A and B), or L-NAME and GLB (Figure 7C and D), vanished vasorelaxation induced by all AqueFr and isovitexin doses.

Discussion

In this work, through a biomonitoring study, we were able to identify the semi-purified bioactive *Luehea divaricata* fraction and its main active metabolites. In addition, it was shown that AqueFr and isovitexin are able to significantly reduce peripheral vascular resistance (PVR) in MVBs, an effect that may, at least in part, explain their cardioprotective activity. Importantly, the vasodilatory effects induced by AqueFr and isovitexin depend on a coordinated activity involving the release of endothelial NO and the activation of potassium channels in vascular smooth muscle.

Blood pressure can be defined by the product between blood flow and vessel resistance. Considering the circulation as a whole, blood flow is dependent on the cardiac output (CO), while vessel resistance is represented by total PVR. In fact, as CO represents the blood volume ejected from the left ventricle every minute, PVR can be represented by the tone of pre-capillary arterioles (Osborn and Foss, 2017). Thus, when evaluating the effect of new drugs on blood pressure, it is an essential factor to investigate their ability to affect these parameters. As it has recently been shown that the hypotensive effects of *L. divaricata* extract do not depend on the CO reduction (Tirloni et al., 2017), we decided to investigate the capacity of ESLD, its semi-purified fractions, and one of the main active metabolites to affect peripheral vascular resistance using the isolated and perfused mesenteric bed as an experimental tool.

Initially, a biomonitoring screening was performed in order to map the semi-purified fraction with better vasodilator activity. Thus, we showed that AqueFr, rich in glycosylated flavonoids like rutin and especially isovitexin, was able to expressively reduce perfusion pressure in MVBs, showing an effect greater than ESLD or other semi-purified fractions. In addition, isovitexin has vasodilatory effects similar to AqueFr, opening the perspective that this compound may be one of the main active metabolites present in this semi-purified fraction. In fact, several studies have suggested that isovitexin could be potential substitute medicines for many diseases, and may be adjuvants for several health products. Although several studies point out the benefits of this flavonoid, including important antioxidant

properties (He et al., 2016), its effects on the cardiovascular system are quite incipient, and its activity on PVR is totally unknown.

When we decided to investigate the molecular mechanisms responsible for the vasodilatory effects of AqueFr and isovitexin, we considered two key points. The first one concerns the endothelial mediators involved in the control of the vascular tone, and the second refers to potassium channels that hyperpolarize the vascular smooth muscle. The vascular endothelium is currently known to control the arterial tone through the release of various vasodilators or vasoconstrictors in response to different chemical and physical stimuli (Godo and Shimokawa, 2017). NO, prostacyclin, epoxyeicosatrienoic acids derivatives, and endothelium-derived hyperpolarizing factors are among these substances, which apparently play a major role in resistance arteries such as MVBs (Roca et al., 2018). Due to the fact that the endothelial damage reduced the effects of AqueFr and isovitexin doses, it could be concluded that the vascular endothelium plays an important role in the vasodilator response induced by both substances. In addition, since the vasodilator response has been significantly reduced by L-NAME without any interference from the cyclooxygenase inhibition by indomethacin, it is reasonable to state that AqueFr and isovitexin are able to induce vascular relaxation in resistance arteries by endothelium-independent and endothelium-dependent mechanisms, the latter using nitric oxide.

Although our results do not allow us concluding whether AqueFr and isovitexin increase NO production or reduce its inactivation, we have shown that both substances are capable of increasing intracellular cGMP levels, showing that possibly this increase is due to a direct activation of guanylate cyclase by NO. In addition, we have previously demonstrated that *L. divaricata* extracts significantly reduced the *in vivo* generation of reactive oxygen and nitrogen species in rats (Tirloni et al., 2017). In fact, the superoxide anion may rapidly combine with nitric oxide to form peroxynitrite, a reaction that regulates NO-mediated arterial relaxation (Beckman and Koppwnol, 1996). Thus, it is reasonable to speculate that the superoxide-scavenging effect of *L. divaricata* extracts could increase the bioavailability of NO, contributing to the effects of AqueFr and isovitexin on MVBs. On the other hand, although several studies have shown the antioxidant potential of isovitexin, the effects of this compound on the redox state of vascular smooth muscle cells and its contribution to isovitexin-induced vasodilation remains to be investigated.

To explore the role of potassium channels in the vasodilation effects of AqueFr and isovitexin, we evaluated their ability to reduce the perfusion pressure in preparations perfused with high KCl (40 mM), a condition able to induce K+-mediated depolarization and increase

pressure in MVBs, which was accompanied by suppression of K+ currents across cellular membranes (Brayden, 1996). In fact, since potassium current blockade prevents the vasodilatory effects of AqueFr and isovitexin, it is possible that the modulation of K+ efflux could be involved in the vasodilatory response observed in MVBs. This hypothesis was addressed using classical K+ channel blockers TEA and glibenclamide, which caused a partial reduction in the vascular effects of all AqueFr and isovitexin doses when used alone, and completely abolished when administered in combination. These data have shown that the activation of both Kir6.1 ATP-sensitive K+ channels and KCa K+ channels is a crucial step for AqueFr and isovitexin induced vasodilation in MVBs. Taking into account that the downstream targets of the nitric oxide pathway in vessels include opening of K+ channels (Totzeck et al., 2017; Tykocki et al., 2017), and the lack of responses to isovitexin after treatment with glibenclamide in association with L-NAME, it is reasonable to suggest that K+ channels are directly involved in the vasodilatory effects observed in MVBs.

Conclusion

This study showed that AqueFr obtained from *Luehea divaricata* and its metabolite - isovitexin - has important vasodilatory effects on MVBs. Apparently, these effects are dependent on endothelium-NO release and both KCa K+ channels and Kir6.1 ATP-sensitive K+ channels activation in the vascular smooth muscle. This study opens perspectives for the use of AqueFr or isovitexin in situations where PVR reduction is required.

Author's contribution

All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; CAST, RACP, MIS, LPG, AOS, MAM, and ELBL conducted the experiments; LMS was involved with the chemical analysis of extract; AGJ was responsible for data discussion, manuscript correction and was the senior researcher responsible for this work. All authors read and approved the final manuscript.

Conflict of interest

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

Acknowledgements

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Legend to figures

Figure 1. Chromatographic profile of the aqueous fraction obtained from *Luehea divaricata* extract (AqueFr).

Figure 2. Effects of *L. divaricata* extract and its semi-purified fractions on MVBs from female rats. MVBs were perfused with physiologic saline solution (PSS) containing Phe (3 μM) and the vasorelaxant effect of ESLD (A), ChloroFr (B), AceFr (C), and ButFr (D) was evaluated. The results show the mean ± S.E.M. of 5 preparations. [a] indicates $p < 0.05$ compared with the perfusion pressure recorded before the administration of fractions. [b] indicates $p < 0.05$ compared with the previous dose. All experiments were performed in endothelium-intact preparations. C: control (basal perfusion pressure); MVBs: mesenteric vascular beds; Phe: phenylephrine.

Figure 3. AqueFr and isovitexin promotes dose-dependent vasorelaxant effect on MVBs. MVBs were perfused with physiologic saline solution (PSS) containing Phe (3 μM) and the vasorelaxant effect of AqueFr (A) and isovitexin (B) was evaluated. (C) Perfusion pressure recording of acetylcholine and isovitexin injection in the mesenteric vascular beds of rats. The results show the mean ± S.E.M. of 5 preparations. [a]indicates $p < 0.05$ compared with the perfusion pressure recorded before AqueFr or isovitexin administration. [b]indicates $p < 0.05$ compared with the previous dose. All experiments were performed in endothelium-intact preparations. C: control (basal perfusion pressure); MVBs: mesenteric vascular beds; Phe: phenylephrine.

Figure 4. Vasorelaxant effect of AqueFr and isovitexin depends in part on endothelium and nitric oxide in the MVBs of rats. MVBs were perfused with PSS containing Phe (3 μM) on denuded endothelium (A and B) or plus L-NAME (C and D), or indomethacin (E and F) on intact endothelium, and the vasorelaxant effect of AqueFr and isovitexin was evaluated. The results show the mean ± S.E.M. of 5 preparations. [a] indicates $p < 0.05$ compared with the effects of AqueFr or isovitexin on the respective vehicle group. [b] indicates $p < 0.05$ compared with the respective previous dose. End - and End +: denuded and intact endothelium, respectively; INDO: indomethacin; L-NAME: N^G-nitro-L-arginine methyl ester; MVBs: mesenteric vascular beds; Phe: phenylephrine.

Figure 5. AqueFr and isovitexin increase intracellular cGMP levels. Intracellular cGMP levels in the aortic rings of rats incubated with AqueFr (0.01, 0.03, and 0.1 mg/ml) (A), isovitexin (100, 300, and 1000 nmol) (B), or sodium nitroprusside (SNP, 10 μm), in the absence and presence of ODQ (100 μm) are shown. The results show the mean ± S.E.M. of 5 preparations per group. $p < 0.05$ *vs* [a] control or after incubation with [b]ODQ.

Figure 6. Vasorelaxant effect of AqueFr and isovitexin depends on K^+ channels activation in the MVBs of rats. MVBs were perfused with PSS containing Phe (3 μM) plus KCl (A and B), or TEA (C and D), or GLB (E and F), or 4-AP (G and H) on intact endothelium, and the vasorelaxant effect of AqueFr and isovitexin was evaluated. The results show the mean ± S.E.M. of 5 preparations. [a] indicates $p < 0.05$ compared with the effects of AqueFr or isovitexin on the respective vehicle group. [b] indicates $p < 0.05$ compared with the respective previous dose. 4-AP: 4-aminopyridine; GLB: glibenclamide; MVBs: mesenteric vascular beds; Phe: phenylephrine; TEA: tetraethylammonium.

Figure 7. Vasorelaxant effect of AqueFr and isovitexin depends on calcium-sensitive and Kir6.1 ATP-sensitive K^+ channels in the MVBs of rats. MVBs were perfused with PSS containing Phe (3 μM) in the presence of TEA plus GLB (A and B), or L-NAME plus GLB (C and D) on intact endothelium, and the vasorelaxant effect of AqueFr and isovitexin was evaluated. The results show the mean ± S.E.M. of 5 preparations. [a] indicates $p < 0.05$ compared with the effects of AqueFr or isovitexin on the respective vehicle group. [b] indicates $p < 0.05$ compared with the respective previous dose. GLB: glibenclamide; L-NAME: N^G-nitro-L-arginine methyl ester; MVBs: mesenteric vascular beds; Phe: phenylephrine; TEA: tetraethylammonium.

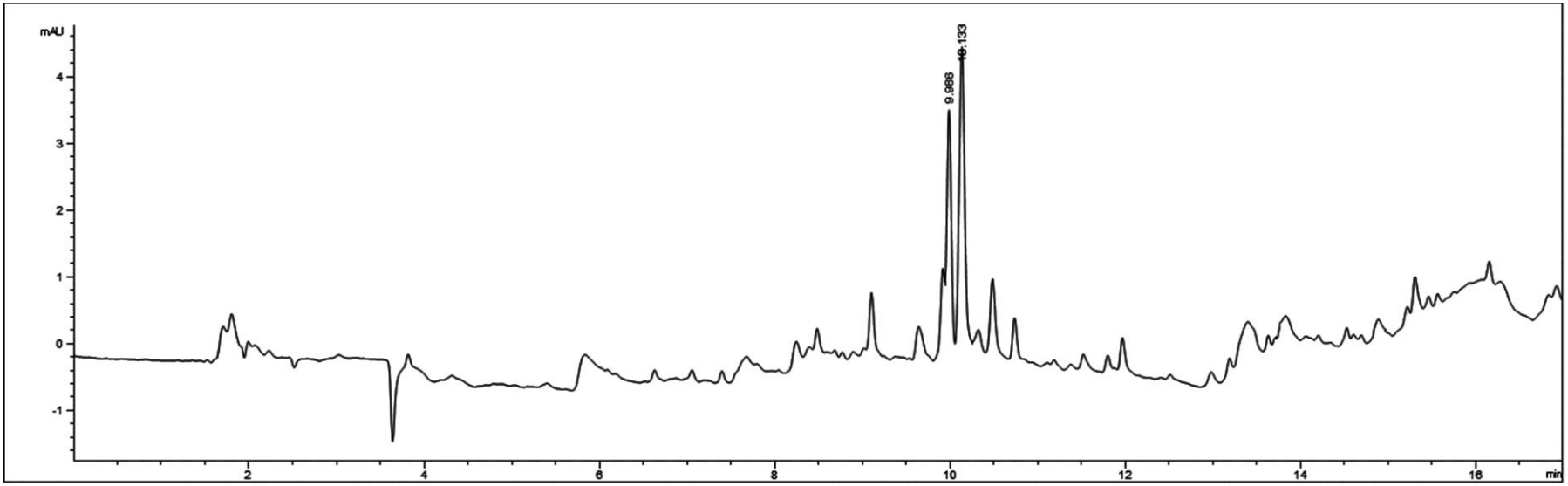

Figure 1

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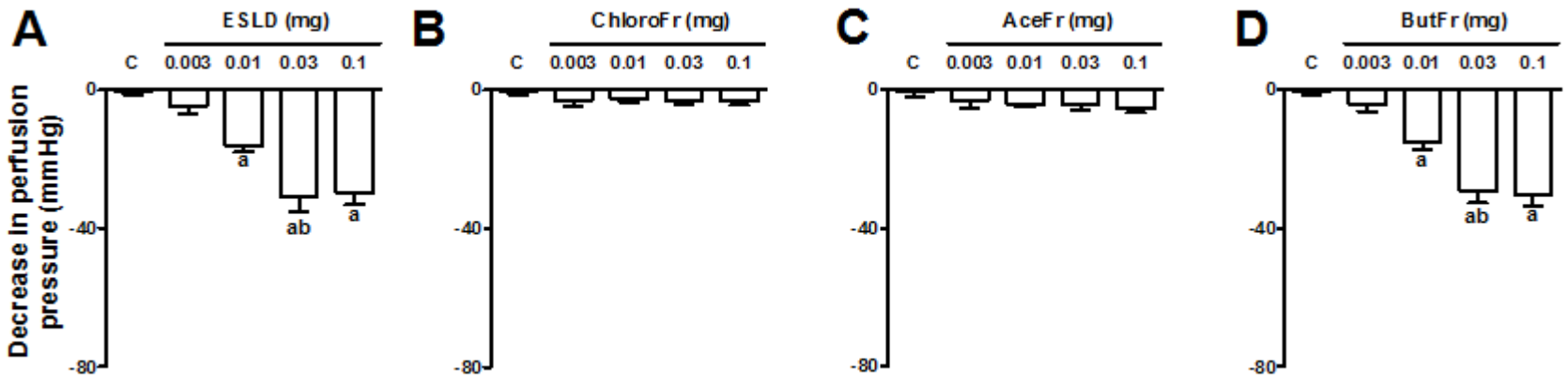

Figure 2

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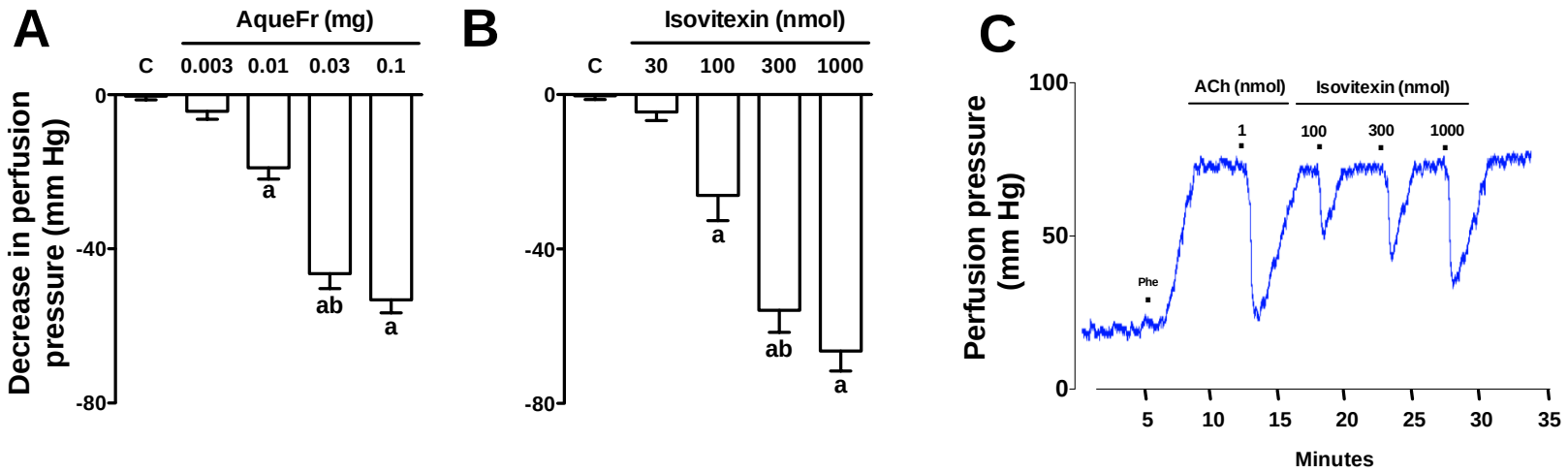

Figure 3

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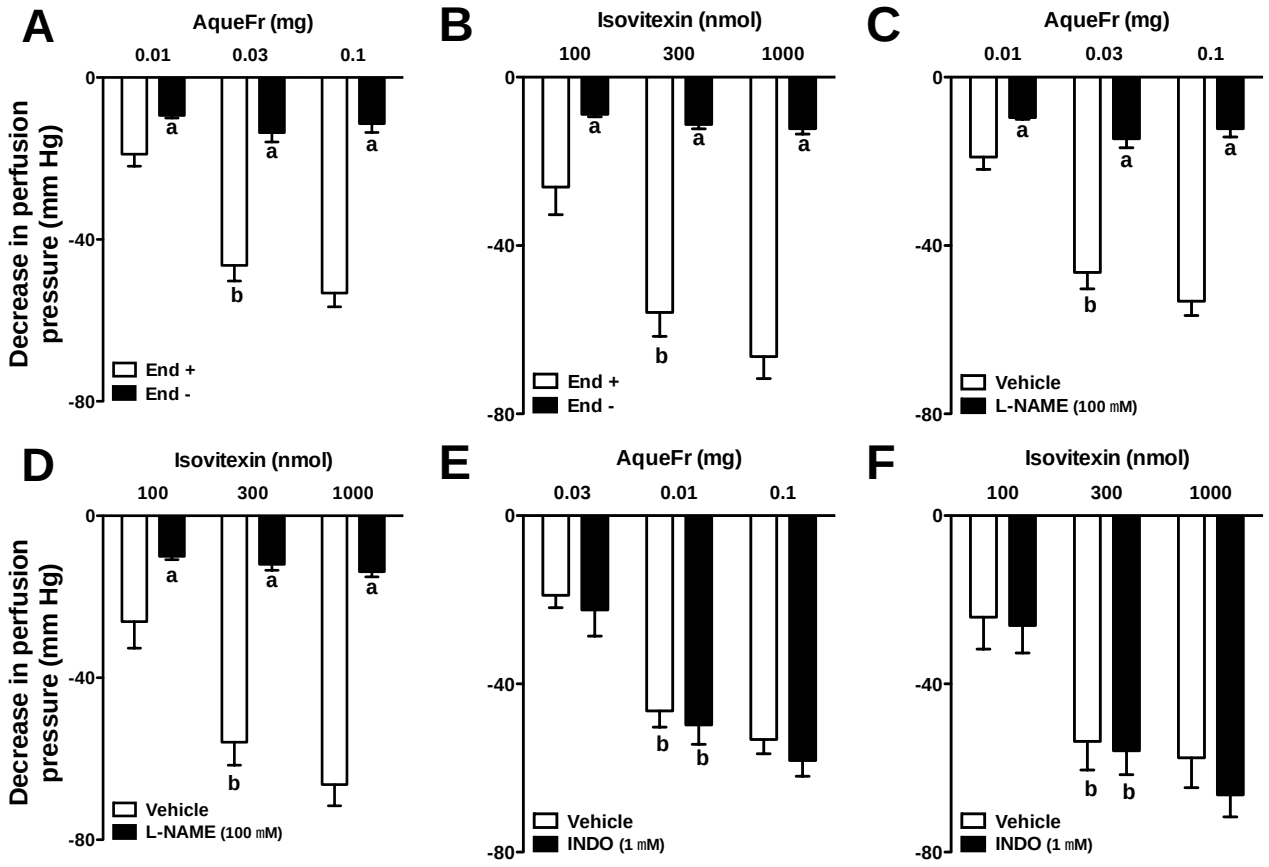

Figure 4

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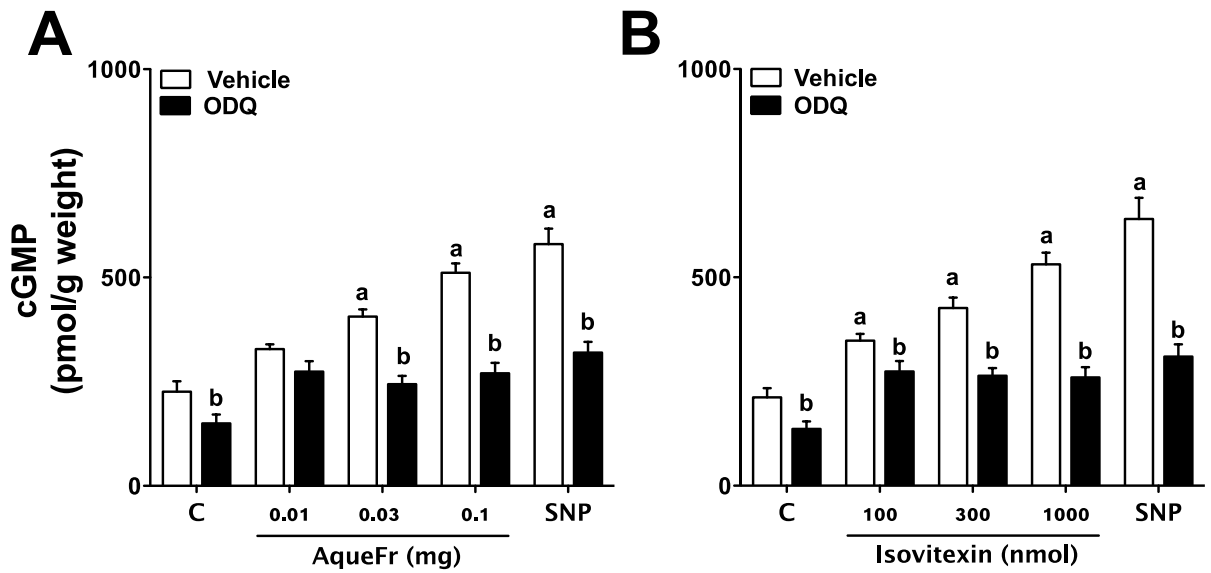

Figure 5

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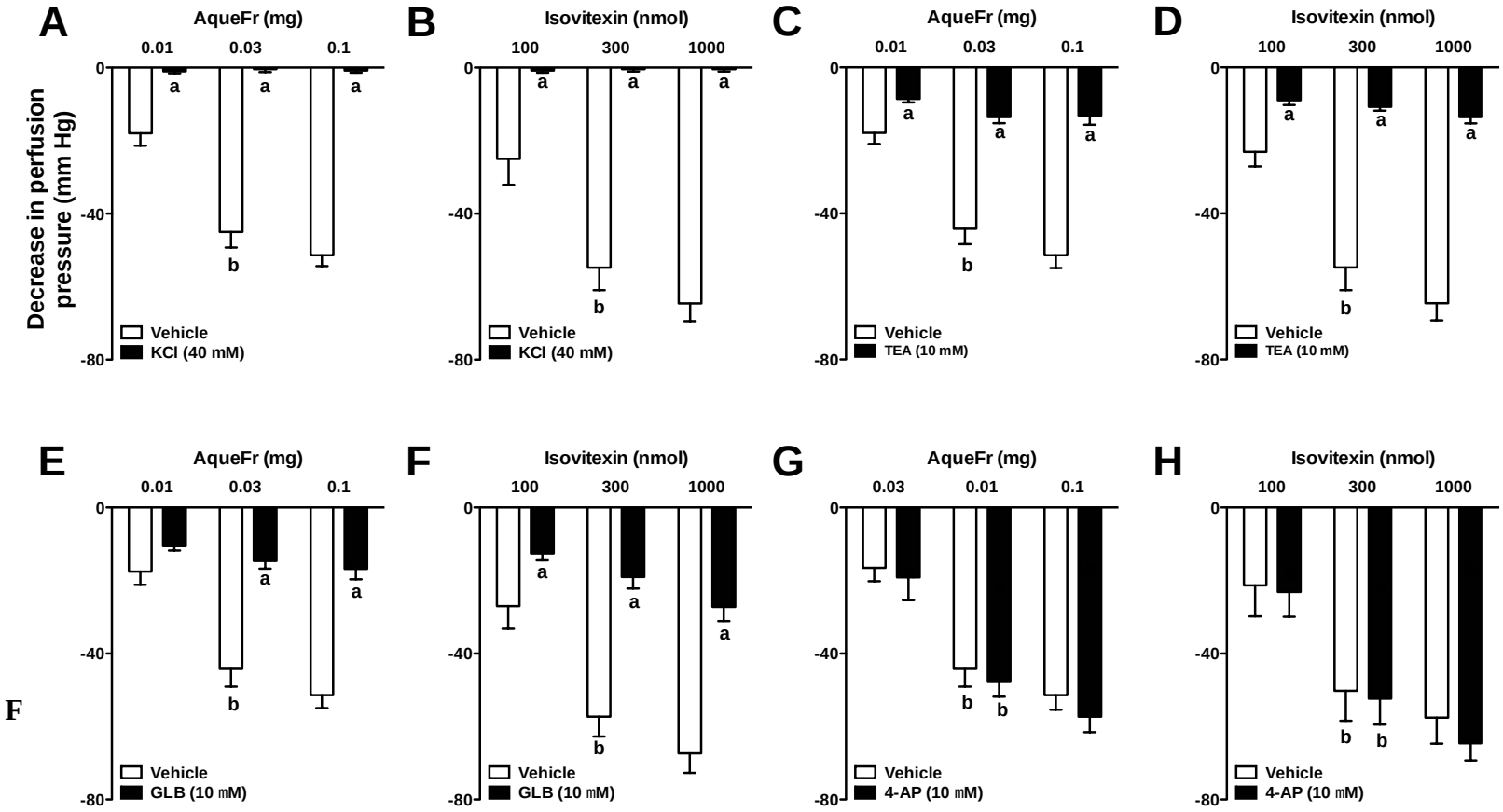

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Figure 6

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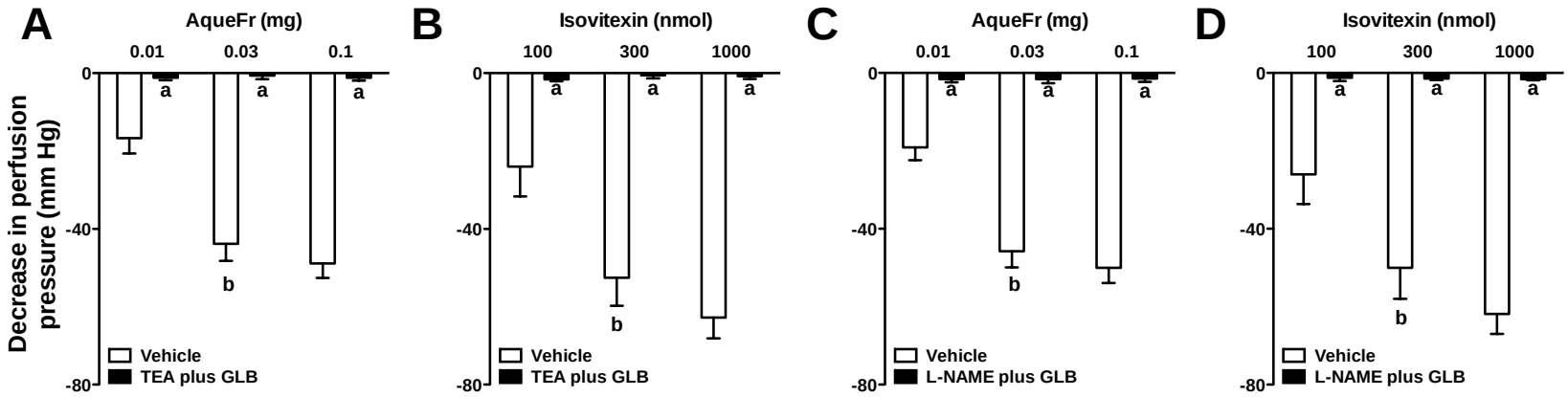

Figure 7

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6 CONCLUSÕES

Considerando todos os resultados podemos concluir que:

I. Nenhuma das três espécies apresentou toxicidade aguda no modelo experimental avaliado;

II. A espécie *A. hispidum* não apresentou atividade diurética no modelo experimental utilizado, porém apresentou atividade hipotensora aguda, efeitos esses que parecem ser dependentes do ON/GMPc e ativação dos canais de K^+ sensíveis ao Ca^+;

III. A *T. esculenta* não foi diurética nem hipotensora, entretanto apresentou atividade antioxidante *in vitro* podendo ser utilizada para outros fins medicinais. O seu uso para fins diuréticos e hipotensores deve ser mais bem avaliado uma vez que não apresentou resposta renal ou hemodinâmica em roedores;

IV. A *L. divaricata* confirmou o conhecimento popular quanto as suas atividades no sistema cardiovascular e renal. No modelo experimental estudado apresentou atividade diurética, salurética e hipotensora. O efeito diurético e salurético se mantiveram após tratamento prolongado;

V. A ButFr obtida da *L. divaricata* também apresentarou efeito diurético e hipotensor em ratos, efeitos estes que se mantiveram após tratamento prolongado;

VI. O efeito diurético e hipotensor *in vivo* da ButFr foi confirmado *ex vivo* em rins e em leitos mesentéricos isolados e perfundidos de ratos. As vias ON/GMPc prostaglandinas/AMPc parecem estar envolvidas com estas atividades;

VII. A AqueFr obtida da *L. divaricata* apresentou atividade diurética e hipotensora em ratos;

VIII. A atividade hipotensora da AqueFr e seu principal metabólito secundário isovitexina foram avaliadas em leitos mesentéricos isoldos de ratos, ambos os tratamentos foram vasodilatadores e a via do ON/GMPc está envolvida com a vasodilatação bem como a ativação de canais de K^+ sensíveis ao Ca^+ e ao ATP.

7 ANEXOS

7.1 PARECER DE APROVAÇÃO DO COMITÊ DE ÉTICA

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS

PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 2 de março de 2016.

CERTIFICADO

Certificamos que o projeto intitulado **"Bioprospecção das atividades renovasculares de espécies nativas do bioma pantaneiro sul-matogrossense",** protocolo nº 16/2015, sob responsabilidade de Cleide Adriane Signor Tirloni – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto n° 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 11 de dezembro de 2015.

Vigência do Projeto	15/03/2016 – 01/11/2018
Espécie/linhagem	Rattus norvegicus/ Wistar
Nº de animais	318
Peso/idade	250g / 3 meses
Sexo	278 Machos e 40 Fêmeas
Origem	Universidade Paranaense-UNIPAR/ Universidade Federal da Grande Dourados-UFGD

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