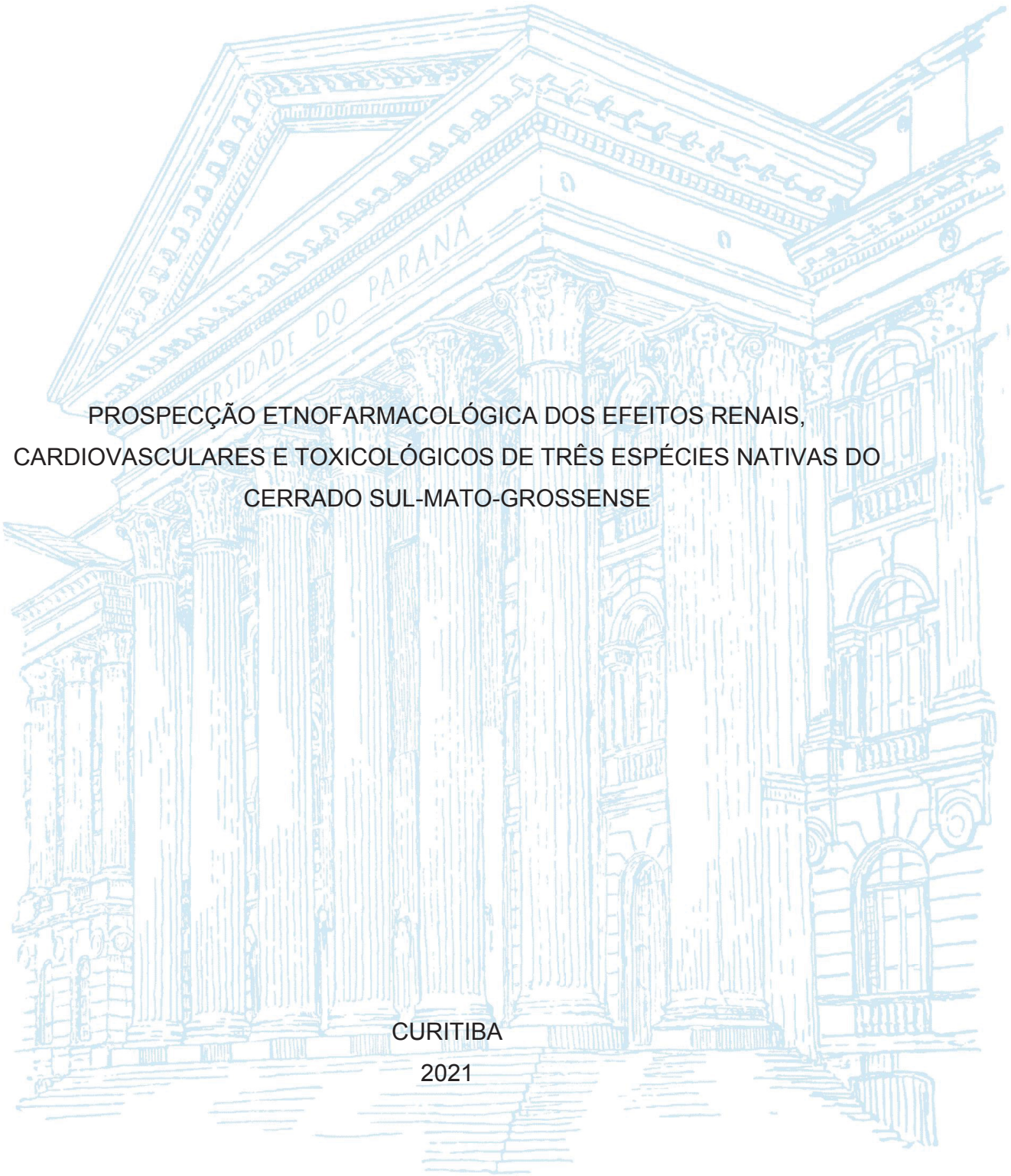
UNIVERSIDADE FEDERAL DO PARANÁ

SARA EMÍLIA LIMA TOLOUEI



PROSPECÇÃO ETNOFARMACOLÓGICA DOS EFEITOS RENAIS, CARDIOVASCULARES E TOXICOLÓGICOS DE TRÊS ESPÉCIES NATIVAS DO CERRADO SUL-MATO-GROSSENSE

CURITIBA

2021

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PROSPECÇÃO ETNOFARMACOLÓGICA DOS EFEITOS RENAIS, CARDIOVASCULARES E TOXICOLÓGICOS DE TRÊS ESPÉCIES NATIVAS DO CERRADO SUL-MATO-GROSSENSE

Tese apresentada ao curso de Pós-Graduação em Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Farmacologia.

Orientador: Prof. Dr. Arquimedes Gasparotto Júnior.
Coorientador: Prof. Dr. Paulo Roberto Dalsenter.

CURITIBA

2021

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ATA DE SESSÃO PÚBLICA DE DEFESA DE DOUTORADO PARA A OBTENÇÃO DO GRAU DE DOUTOR EM FARMACOLOGIA

No dia dezenove de fevereiro de dois mil e vinte e um às 13:30 horas, na sala Google Meet (link ainda não disponível) , Defesa remota pelo Teams. , foram instaladas as atividades pertinentes ao rito de defesa de tese da doutoranda **SARA EMILIA LIMA TOLOUEI**, intitulada**: Prospecção etnofarmacológica dos efeitos renais, cardiovasculares e toxicológicos de três espécies nativas do cerrado sul-mato-grossense**. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: ARQUIMEDES GASPAROTTO JUNIOR (UNIVERSIDADE FEDERAL DA GRANDE DOURADOS), ADRIANO TARGA DIAS SANTOS (INSTITUT DE RECERCA BIOMÈDICA DE LLEIDA), ALEXANDRA ACCO (UNIVERSIDADE FEDERAL DO PARANÁ), EMERSON LUIZ BOTELHO LOURENÇO (UNIVERSIDADE PARANAENSE). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de doutor está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, ARQUIMEDES GASPAROTTO JUNIOR, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

CURITIBA, 19 de Fevereiro de 2021.

Assinatura Eletrônica
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ARQUIMEDES GASPAROTTO JUNIOR
Presidente da Banca Examinadora

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ADRIANO TARGA DIAS SANTOS
Avaliador Externo (INSTITUT DE RECERCA BIOMÈDICA DE LLEIDA)

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ALEXANDRA ACCO
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

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EMERSON LUIZ BOTELHO LOURENÇO
Avaliador Externo (UNIVERSIDADE PARANAENSE)

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **SARA EMILIA LIMA TOLOUEI** intitulada: **Prospecção etnofarmacológica dos efeitos renais, cardiovasculares e toxicológicos de três espécies nativas do cerrado sul-mato-grossense**, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 19 de Fevereiro de 2021.

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NOTA EXPLICATIVA

Esta tese está apresentada em formato alternativo – artigos publicados – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, constando de uma introdução, revisão de literatura, objetivos do trabalho e quatro artigos científicos abordando os experimentos realizados, com resultados, discussões e considerações finais. Ademais, as conclusões gerais bem como as referências citadas na introdução e na revisão de literatura deste trabalho estão apresentadas no final deste documento.

Dedicatória

Dedico esta tese aos meus pais,
Maria Lúcia e Daryoush.

AGRADECIMENTOS

A Deus, por me abençoar com a chance de vir a Curitiba realizar o doutorado em uma instituição que tanto me encanta!

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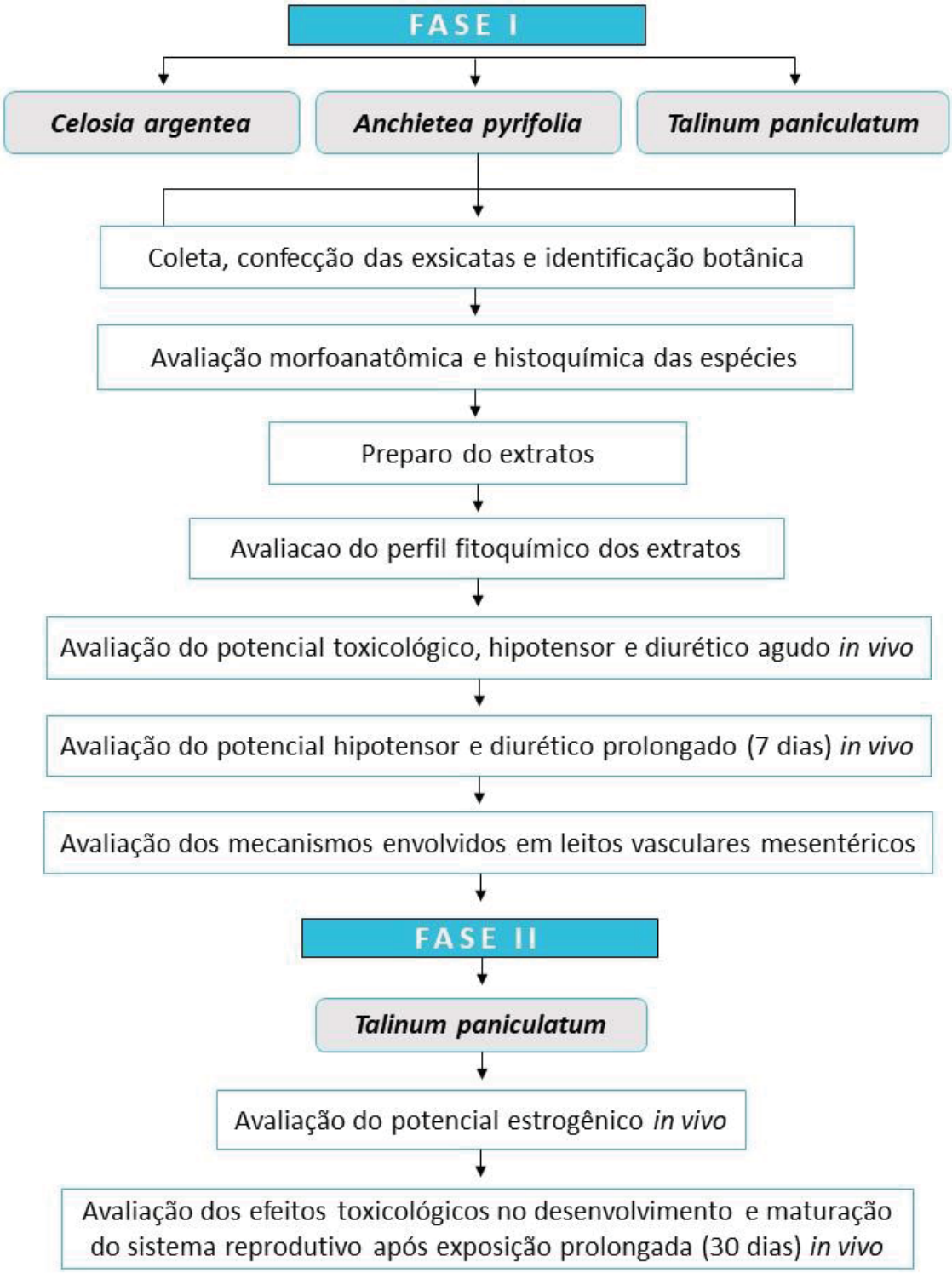
Minha profunda gratidão!

"Where there is love, nothing is too much trouble, and there is always time."

Abdu'l-Bahá

RESUMO GRÁFICO



RESUMO

O uso de plantas medicinais na prevenção e tratamento de doenças é uma prática milenar, que se mantém até o presente momento graças às informações passadas de geração a geração. Esse conhecimento etnobotânico se tornou uma ferramenta de grande valia na seleção de novas espécies a serem investigadas farmacologicamente, especialmente na cardiologia, já que as doenças cardiovasculares (DCVs) afetam quase um terço da população brasileira e são as principais causas de morte no mundo. Dentre as DCVs, a hipertensão arterial sistêmica (HAS) é a doença crônica não transmissível mais frequente e o fator de risco mais importante para a ocorrência de outras DCVs. Por se tratar de uma doença crônica cujo tratamento deve ser prolongado, a continuidade ao tratamento convencional é baixa, visto que alguns efeitos adversos acarretam importante redução à adesão ao tratamento. Devido a isso, a busca por terapias à base de produtos naturais é intensa, o que reforça a importância de estudos que avaliem os efeitos farmacológicos e toxicológicos, visando o controle e/ou auxílio no tratamento da HAS. Desta forma, o presente estudo teve como objetivo investigar os efeitos farmacológicos e toxicológicos de três espécies do cerrado sul-mato-grossense "prescritas" por curandeiros e raizeiros no tratamento de HAS. *Celosia argentea* L., *Anchietea pyrifolia* (Mart.) G.Don e *Talinum paniculatum* (Jacq.) Gaertn. foram as espécies vegetais selecionadas a partir de um estudo etnobotânico realizado na região da Grande Dourados – MS. Primeiramente, partes aéreas de *C. argentea*, *A. pyrifolia* e *T. paniculatum* foram coletadas, devidamente identificadas e submetidas a análises de controle de qualidade através de avaliações morfológicas e histoquímicas. Na sequência, infusões (100 g/1000 mL água filtrada) das três espécies foram preparadas, filtradas e tratadas com etanol (3:1; v/v). O sobrenadante etanólico (ES) de cada preparação foi removido e aplicado em todas as análises do presente estudo, estabelecendo-se os seguintes extratos: sobrenadante etanólico de *C. argentea* (ESCA), sobrenadante etanólico de *A. pyrifolia* (ESAP), e sobrenadante etanólico de *T. paniculatum* (ESTP). ESCA, ESAP e ESTP foram então caracterizados quimicamente e submetidos a avaliações farmacológicas e toxicológicas a partir de ensaios pré-clínicos em *Rattus norvegicus* da linhagem Wistar. Inicialmente, as possíveis atividades hipotensoras e diuréticas agudas (30, 100 and 300 mg/kg v.o.) e o potencial toxicológico agudo (30, 300 and 2000 mg/kg v.o.) das três espécies foram avaliadas. ESAP e ESTP foram então submetidos à avaliação do potencial diurético e hipotensor após exposição prolongada de 7 dias. Ademais, a resposta vasodilatadora de cada espécie em leitos vasculares mesentéricos (MVBs) e seu envolvimento com as vias óxido nítrico/GMPc, prostaglandina/AMPc e canais de potássio foram investigadas. Posteriormente, *T. paniculatum* foi escolhida como a espécie mais promissora e, desta forma, ESTP foi investigada quanto seu potencial estrogênico (30, 100 and 300 mg/kg v.o.), bem como seus possíveis efeitos toxicológicos no desenvolvimento e maturação do sistema reprodutivo após a exposição (30 and 300 mg/kg v.o.) por 30 dias. A partir da análise morfoanatômica, observou-se que *C. argentea* possui folhas anfiestomáticas com estômatos anomocíticos e anisocíticos. Tricomas glandulares multicelulares e unisseriados foram observados. O mesofilo caracterizou-se como isobilateral, a nervura central apresentou formato biconvexo e o pecíolo plano-convexo com duas costelas conspícuas na face adaxial. O caule apresentou formato irregular. Compostos fenólicos

foram encontrados na epiderme, na cabeça do tricoma glandular e no xilema. Cristais de oxalato de cálcio do tipo prismáticos e triangulares foram observados. Na avaliação fitoquímica de ESCA, determinou-se a presença majoritária de compostos fenólicos, megastigmanes e saponinas triterpenóides. ESCA não causou efeitos tóxicos em ratas nem excreção urinária aumentada em ratos após administração aguda. No entanto, ESCA aumentou significativamente a eliminação renal de potássio e cloreto, especialmente no final de 24 horas após a administração. A dose intermediária (100 mg/kg) de ESCA foi capaz de promover hipotensão aguda significativa e bradicardia. Além disso, seus efeitos cardiovasculares parecem estar envolvidos com a ativação dos canais de potássio dependentes de voltagem nos MVBs. *A. pyrifolia* apresentou folha hipoestomática com estômatos paracíticos bem como a presença de tricomas tectores cônicos. Em seção transversal, a folha apresentou mesofilo dorsiventral, nervura central com formato biconvexo e pecíolo de formato arredondado. O caule apresentou formato circular. Compostos fenólicos foram encontrados nas células epidérmicas da folha e caule. Grãos de amido foram encontrados na folha e caule. Cristais de oxalato de cálcio do tipo areia cristalina e prismáticos foram observados. ESAP demonstrou ter flavonóides O-glicosilados, ácidos clorogênicos e outros derivados do ácido fenilpropanóico como compostos bioativos majoritários. ESAP não causou efeitos tóxicos em ratas nem excreção urinária aumentada em ratos após administração aguda. No entanto, ESAP reduziu significativamente a eliminação renal de sódio, potássio e cloreto após exposição prolongada. A maior dose (300 mg/kg) de ESAP foi capaz de promover hipotensão aguda significativa, sem afetar os níveis da pressão arterial após uso prolongado. Além disso, os efeitos cardiovasculares desta espécie parecem estar envolvidos com a ativação dos canais de potássio ativados por cálcio em vasos de resistência. Por fim, a investigação morfoanatômica de *T. paniculatum* demonstrou a presença de folhas anfiestomáticas com estômatos paralelocíticos. Foi observado mesofilo dorsiventral com a presença de células mucilaginosas. A nervura central e o pecíolo apresentaram formato côncavo-convexo, com pequenas projeções neste último. O caule apresentou formato irregular e a presença grãos de amido e compostos fenólicos. Cristais de oxalato de cálcio do tipo drusas e prismáticos foram observados. ESTP demonstrou a presença de ácidos clorogênicos, aminoácidos, nucleosídeos, flavonas O-glicosiladas e ácidos orgânicos como metabólitos majoritários. Após exposição aguda, a administração de ESTP não promoveu sinais de toxicidade nem alterações no volume de urina ou eliminação de eletrólitos. Por outro lado, o tratamento prolongado com todas as doses de ESTP aumentou significativamente o volume de urina e a excreção de eletrólitos (Na^+, K^+ e Cl^-) sem afetar a pressão arterial ou a frequência cardíaca. Aparentemente, esses efeitos estão envolvidos com a ativação dos canais de potássio ativados por cálcio de pequena condutância, contribuindo para o aumento do fluxo sanguíneo renal e da taxa de filtração glomerular. Ademais, ESTP não promoveu efeitos estrogênicos em ratas e, quando avaliado em ratos e ratas durante o período puberal, não promoveu sinais diários de toxicidade, perda de peso, nem causou alterações significativas em parâmetros que marcam o início da puberdade ou em parâmetros bioquímicos e histopatológicos, podendo ser considerado seguro em ratas e ratos Wistar nas doses testadas. Em suma, verificamos que *C. argentea*, *A. pyrifolia* e *T. paniculatum* não apresentam atividade diurética após uma única exposição e apenas *C. argentea* e *A. pyrifolia* apresentaram atividade hipotensora aguda. Entretanto, *C. argentea* foi capaz de promover efeito

salurético após exposição aguda e *T. paniculatum* apresentou efeito diurético e salurético após exposição prolongada de 7 dias. Desta forma, concluímos que *T. paniculatum* apresentou melhores efeitos farmacológicos quando comparado às outras duas espécies em estudo, podendo ser apontado como um possível fitoterápico, especialmente quando um efeito diurético sustentado se é esperado.

Palavras-chave: *Celosia argentea, Anchietea pyrifolia, Talinum paniculatum,* etnofarmacologia, medicina popular, plantas diuréticas, hipertensão, toxicologia.

ABSTRACT

The use of medicinal plants in the prevention and treatment of diseases is an ancient practice, which has been maintained up to the present moment thanks to information passed down across generations. This ethnobotanical knowledge has become a valuable tool in the selection of new species to be further investigated pharmacologically, especially in cardiology, since cardiovascular diseases (CVDs) affect almost one third of the Brazilian population and are the main causes of death worldwide. Among CVDs, systemic arterial hypertension (SAH) is the most frequent non-communicable chronic disease and the most important risk factor for the occurrence of other CVDs. As it is a chronic disease whose treatment must be prolonged, the continuity of treatment is low due to the occurrence of some adverse effects that cause an important reduction in medication adherence. Due to this, the search for therapies based on natural products is intense, which reinforces the importance of studies that evaluate the pharmacological and toxicological effects, aiming at the control and/or assistance in the treatment of SAH. Thus, the present study aimed to investigate the pharmacological and toxicological effects of three species of the cerrado sul-mato-grossense "prescribed" by healers for the treatment of SAH. *Celosia argentea* L., *Anchietea pyrifolia* (Mart.) G.Don and *Talinum paniculatum* (Jacq.) Gaertn. were the plant species selected from an ethnobotanical study carried out in the region of Grande Dourados - MS. First, aerial parts of *C. argentea*, *A. pyrifolia* and *T. paniculatum* were collected, properly identified and submitted to quality control analyzes through morphological and histochemical evaluations. Then, infusions (100 g/1000 mL filtered water) of the three species were prepared, filtered and treated with ethanol (3:1; v/v). The ethanolic supernatant (ES) of each preparation was removed and applied in all analyzes of the present study, establishing the following extracts: ethanol soluble fraction of *C. argentea* (ESCA), ethanol soluble fraction of *A. pyrifolia* (ESAP), and ethanol soluble fraction of *T. paniculatum* (ESTP). ESCA, ESAP and ESTP were then chemically characterized and submitted to pharmacological and toxicological evaluations based on pre-clinical trials in Rattus norvegicus (Wistar strain). Initially, the possible acute hypotensive and diuretic activities (30, 100 and 300 mg/kg, orally) and the acute toxicological potential (30, 300 and 2000 mg/kg, orally) of the three species were evaluated. ESAP and ESTP were then subjected to evaluation of the diuretic and hypotensive potential after 7 days of prolonged exposure. In addition, the vasodilator response of each species in mesenteric vascular beds (MVBs) and their involvement with the nitric oxide/cGMP, prostaglandin/cAMP and potassium channels were investigated. Thereafter, *T. paniculatum* was chosen as the most promising species and, therefore, ESTP was investigated for its estrogenic potential (30, 100 and 300 mg/kg, orally), as well as its possible toxicological effects on the development and maturation of the reproductive system after 30 days of exposure (30 and 300 mg/kg, orally). In the morphoanatomical analysis, it was observed that *C. argentea* has amphistomatic leaves with anomocytic and anisocytic stomata. Multicellular and uniseriate glandular trichomes were observed. The mesophyll was characterized as isobilateral, the central rib had a biconvex shape and the

plane-convex petiole with two conspicuous ribs on the adaxial face. The stem had an irregular shape. Phenolic compounds were found in the epidermis, in the head of the glandular trichome and in the xylem. Prismatic and triangular calcium oxalate crystals were observed. In the phytochemical evaluation of ESCA, phenolic compounds, megastigmans and triterpenoid saponins were determined as major compounds. ESCA did not cause toxic effects in rats nor increased urinary excretion in rats after acute administration. However, ESCA significantly increased renal elimination of potassium and chloride, especially at the end of 24 hours after administration. The intermediate dose (100 mg/kg) of ESCA was able to promote significant acute hypotension and bradycardia. In addition, its cardiovascular effects seem to be involved with the activation of voltage-dependent potassium channels in MVBs. *A. pyrifolia* presented hypoestomatic leaves with paracytic stomata as well as the presence of conic trichomes. In cross-section, the leaf presented dorsiventral mesophyll, central rib with biconvex shape and petiole with round shape. The stem had a circular shape. Phenolic compounds were found in the epidermal cells of the leaf and stem. Starch grains were found in the leaf and stem. Crystal sand and prismatic calcium oxalate crystals were observed. ESAP has been shown to have O-glycosylated flavonoids, chlorogenic acids and other phenylpropanoic acid derivatives as major bioactive compounds. ESAP did not cause toxic effects in rats nor increased urinary excretion in rats after acute administration. However, ESAP significantly reduced renal elimination of sodium, potassium and chloride after prolonged exposure. The highest dose (300 mg/kg) of ESAP was able to promote significant acute hypotension, without affecting blood pressure levels after prolonged use. In addition, the cardiovascular effects of this species appear to be involved with the activation of calcium-activated potassium channels in resistance vessels. Finally, the morphoanatomical investigation of *T. paniculatum* demonstrated the presence of amphistomatic leaves with paralelocytic stomata. Dorsiventral mesophyll was observed with the presence of mucilaginous cells. The central rib and the petiole were concave-convex in shape and has short wing-like projection on either side. The stem had an irregular shape and the presence of starch grains and phenolic compounds. Druses and prismatic calcium oxalate crystals were observed. ESTP demonstrated the presence of chlorogenic acids, amino acids, nucleosides, O-glycosylated flavones and organic acids as major metabolites. After acute exposure, the administration of ESTP did not promote signs of toxicity or changes in the volume of urine or elimination of electrolytes. On the other hand, prolonged treatment with all doses of ESTP significantly increased urine volume and electrolyte excretion (Na^+, K^+ and Cl^-) without affecting blood pressure or heart rate. Apparently, these effects are involved with the small-conductance calcium-activated potassium channels, contributing to the increase of renal blood flow and glomerular filtration rate. Furthermore, ESTP did not promote estrogenic effects in female rats and, when evaluated in male and female rats during the pubertal period, no signs of toxicity such as weight loss were observed. Moreover, no significant changes in parameters that indicate the onset of puberty nor significant alterations in biochemical and histopathological parameters were observed. Therefore, ESTP can be considered safe in rats after prolonged exposure. In summary, we found that *C. argentea*, *A. pyrifolia* and *T.*

paniculatum did not show diuretic activity after a single exposure and only *C. argentea* and *A. pyrifolia* showed acute hypotensive activity. However, *C. argentea* was able to promote a saluretic effects after acute exposure and *T. paniculatum* showed diuretic and saluretic effects after 7 days of exposure. Thus, we conclude that *T. paniculatum* presented better pharmacological effects when compared to the other two species investigated, and it can be pointed out as a possible herbal medicine, especially when sustained diuretic effects are expected.

Palavras-chave: *Celosia argentea, Anchietea pyrifolia, Talinum paniculatum,* ethnopharmacology, folk medicine, diuretic plants, hypertension, toxicology.

LISTA DE ABREVIATURAS E SIGLAS

AMPc	Monofosfato cíclico de adenosina
ANVISA	Agência Nacional de Vigilância Sanitária
ARA-2	Antagonistas dos receptores de angiotensina II
AT1R	Receptor de angiotensina 2 tipo 1
AT2R	Receptor de angiotensina 2 tipo 2
AVE	Acidente vascular encefálico
BCC	Bloqueadores dos canais de cálcio
BRA	Bloqueadores dos receptores AT1 da angiotensina II
Cl^-	Cloreto
CMLV	Células musculares lisas vasculares
DAP	Doença arterial periférica
DC	Débito cardíaco
DCVs	Doenças cardiovasculares/ No inglês cardiovascular diseases
DIU	Diuréticos
DL_{50}	Dose letal de 50%
ECA	Enzima conversora de angiotensina
eNOS	Óxido nítrico sintase endotelial
ES	Sobrenadante etanólico
ESAP	Do inglês ethanolic supernatant of *Anchietea pyrifolia/* Sobrenadante etanólico de *Anchietea pyrifolia*
ESCA	Do inglês ethanolic supernatant of *Celosia argentea/* Sobrenadante etanólico de *Celosia argentea*
ESTP	Do inglês ethanolic supernatant of *Talinum paniculatum/* Sobrenadante etanólico de *Talinum paniculatum*
FC	Frequência cardíaca
FDA	Food and Drug Administration
GCs	Enzina guanilato ciclase solúvel
GMPc	Guanosina monofosfato cíclica
HAS	Hipertensão arterial sistêmica

HCTZ	Hidroclorotiazida
IAM	Infarto agudo do miocárdio
IC	Insuficiência cardíaca
IECA	Inibidor da enzima conversora de angiotensina
IR	Insuficiência renal
K^+	Potássio
MAPA	Monitorização ambulatorial da pressão arterial
MRPA	Monitorização residencial de pressão arterial (MRPA)
MS	Mato Grosso do Sul
MS	Ministério da saúde
MVB	Do inglês mesenteric vascular beds/ Leitos vasculares mesentéricos
Na^+	Sódio
NO	Do inglês Nitric oxid/Óxido nítrico
OECD	Do inglês Organization for Economic Co-operation and Development/ Organização para Cooperação e Desenvolvimento Econômico
OMS	Organização Mundial da Saúde
PA	Pressão arterial
PAD	Pressão arterial diastólica
PAM	Pressão arterial média
PAS	Pressão arterial sistólica
PGI_2	Prostaciclina
PH	Ponto de hidrogênio
PKA	Proteína cinase dependente de AMPc
pO_2	Pressão parcial de oxigênio
pCO_2	Pressão parcial de dióxido de carbono
PPS	Do inglês preputial separation/Separação prepucial
RVP	Resistência vascular periférica
SIM	Sistema de Informações de Mortalidade
SINITOX	Sistema Nacional de Informações Tóxico-Farmacológicas
SNA	Sistema nervoso autônomo
SRAA	Sistema renina-angiotensina-aldosterona

SUS	Sistema Único de Saúde
UEPG	Universidade Estadual de Ponta Grossa
UFGD	Universidade Federal da Grande Dourados
UFPR	Universidade Federal do Paraná
VO	Do inglês vaginal opening/Abertura do canal vaginal

SUMÁRIO

1 INTRODUÇÃO

A hipertensão arterial sistêmica (HAS) é uma doença cardiovascular (DCV) de origem multifatorial caracterizada pela elevação sustentada da pressão arterial (PA; ≥ 140 e/ou 90 mm Hg). Caracterizada como a mais frequente das doenças crônicas não transmissíveis em todo o mundo, a HAS acomete cerca de 1,13 bilhões de pessoas de todas as idades, atingindo cerca de 32,5% dos adultos e mais de 60% dos idosos (MANSUR e FAVARATO, 2012; BARROSO et al., 2020). Sendo considerado o principal fator de risco para as DCVs, a HAS possui diagnóstico relativamente simples e ao alcance de todos. Porém, um levantamento de dados realizado em diversos estados do país apontou que cerca de 50% dos pacientes hipertensos aderem ao tratamento farmacológico convencional, e apenas 43,7% a 67,5% dos indivíduos portadores de HAS apresentam níveis pressóricos controlados (BARROSO et al., 2020; NAKAMOTO, 2012).

Complicações relacionadas as DCVs causam um elevado impacto na perda da produtividade do trabalho e da renda familiar, bem como uma elevação em custos socioeconômicos devido à alta frequência de internações (BARROSO et al., 2020). Ademais, a PA elevada é o fator de risco de maior importância para os acidentes vasculares encefálicos, e contribui diretamente para o desenvolvimento do infarto agudo do miocárdio e para a insuficiência renal (CAMPINO et al., 2002).

Obesidade, tabagismo, alcoolismo, sedentarismo, bem como fatores genéticos e hábitos alimentares são considerados os fatores de risco mais importantes para o desenvolvimento da HAS. A PA também pode ser modificada pela variação do volume de sangue ou da sua viscosidade, pela frequência cardíaca e pela elasticidade dos vasos. Ademais, estímulos hormonais e nervosos que regulam a resistência sanguínea estão sob constante influência individual e ambiental (ZAGO E ZANESCO, 2006).

Na tentativa de reduzir a incidência ou minimizar os efeitos da HAS, terapias medicamentosas e não medicamentosas são empregadas rotineiramente. No tratamento farmacológico, inibidores do sistema renina-angiotensina, betabloqueadores, bloqueadores dos canais de cálcio e diuréticos são considerados com a primeira linha de tratamento. Já a prática de atividades físicas, cessação do tabagismo, controle do estresse, perda de peso e controle nutricional, são hábitos de extrema importância que,

quando observados cuidadosamente, previnem a doença ou auxiliam a terapia medicamentosa (BARROSO et al., 2020). Porém, ainda é baixa a adesão dos pacientes aos tratamentos convencionais, fato este que pode ser compreendido pela incidência de efeitos adversos a alguns pacientes ou as dificuldades de acesso ao Sistema Único de Saúde (SUS). Desta forma, o uso de produtos à base de plantas medicinais aparece como uma alternativa viável para aumentar a aderência ao tratamento farmacológico da HAS, evitar consumo irracional de medicamentos sintéticos e, desde que corretamente prescritos, solucionar problemas da atenção primária (BRASIL, 2001).

As plantas medicinais vêm sendo utilizadas há milhares de anos para o tratamento de diversas doenças (SAMUELSSON, 2004). Visto que a busca por medicamentos naturais está se intensificando, as plantas medicinais se tornaram o foco de numerosos estudos de modo a avaliar sua segurança e eficácia, visando o desenvolvimento de novos fitoterápicos (CALIXTO, 2000; MATTA et al., 2011; STONE, 2008). Por essa razão, o cerrado brasileiro tem sido o grande alvo dessas pesquisas, já que é o segundo maior bioma em área, representando 23% da superfície do país (NETO e MORAIS, 2003).

Estudos relacionados ao conhecimento popular das plantas medicinais e sua utilização, bem como um levantamento das espécies mais utilizadas por curandeiros neste bioma, têm sido frequentemente realizados pela comunidade científica (BUENO et al., 2005). A associação do conhecimento popular com a comprovação da segurança e eficácia se tornou uma ferramenta de grande valor para a terapêutica moderna, especialmente para a cardiologia, visto que as DCVs são as principais causas de morte no Brasil e no mundo (MANSUR e FAVARATO, 2012; NETO e MORAIS, 2003).

Contudo, nem sempre o uso de plantas medicinais e/ou fitoterápicos estão isentos de complicações. As plantas medicinais, como qualquer outro produto natural, podem causar toxicidade, e desta forma, os estudos toxicológicos são de extrema importância para avaliar a segurança e os possíveis efeitos adversos que podem surgir durante o tratamento. O potencial de risco e benefícios do uso das plantas medicinais, desde os extratos brutos até as substâncias delas isoladas, devem ser cuidadosamente considerados, de modo que os benefícios do uso superem os efeitos colaterais (VARANDA, 2006).

Uma vez que a HAS é um problema de saúde pública, haja vista sua relevância na morbimortalidade cardiovascular, o desenvolvimento de um fitoterápico à base de plantas reveste-se de grande importância para esta finalidade. Entretanto, estudos pré-clínicos devem ser realizados de modo a comprovar seu potencial farmacológico, pois muitas plantas utilizadas popularmente mostraram-se ineficazes para o tratamento das patologias indicadas. Portanto, realizamos nesta tese um estudo etnofarmacológico de três espécies vegetais utilizadas por “curandeiros” e “raizeiros” do Cerrado sul-mato-grossense no tratamento de doenças renais e cardiovasculares (COELHO et al. 2019). Para isso, *Celosia argentea* L., *Anchietea pyrifolia* (Mart.) G.Don e *Talinum paniculatum* (Jacq.) Gaertn. tiveram suas propriedades fitoquímicas, morfoanatômicas e histoquímicas caracterizadas. Ademais, o potencial farmacológico sobre o sistema renal e cardiovascular bem como o perfil toxicológico das três espécies foram avaliados.

2 REVISÃO DE LITERATURA

2.1 Doenças cardiovasculares

As doenças cardiovasculares (DCVs) são um conjunto de enfermidades que acometem os vasos sanguíneos e o coração. Entre elas estão as cardiopatias congênitas, doenças arteriais coronarianas, doenças cerebrovasculares, doença arterial periférica, valvulopatias, insuficiência cardíaca, embolia pulmonar e trombose venosa (BARROSO et al., 2020).

No Brasil, um levantamento realizado em 2013 demonstrou que as DCVs foram as principais causas de morte, correspondendo a 29,8% de todos os casos registrados (BARROSO et al., 2020). Em 2017, a Organização Mundial da Saúde (OMS) confirmou que aproximadamente 18 milhões de pessoas morreram por DCVs no ano de 2015, representando 31% de todas as mortes a nível global, e estima que em 2030 quase 23,6 milhões de pessoas morrerão também por DCVs (OMS, 2017).

Mundialmente, as DCVs são consideradas como a principal causa de morte e estão associadas a um conjunto de fatores risco, muitos dos quais não podem ser modificados, como hereditariedade, sexo e idade. Entretanto, fatores de risco comportamentais (sedentarismo, estresse, obesidade, elevada ingestão de sódio e álcool) e fisiopatológicos (dislipidemias e diabetes) podem ser prevenidos e modificados através de mudanças nos estilos de vida e, quando necessário, associados a uma terapia medicamentosa (BARROSO et al., 2020; NGUYEN et al., 2019). Dentre as DCVs, merece destaque a hipertensão arterial sistêmica (HAS) - uma condição assintomática e apontada pela OMS como a principal causa indireta de mortes no mundo (OMS, 2017).

Considerada um problema de saúde pública em âmbito global, a HAS está diretamente associada ao surgimento de diversas doenças, principalmente outras DCVs. Estudos epidemiológicos realizados por Ruilope (2009) e Haywood et al. (2009) revelaram que a HAS aumenta o risco de insuficiência renal crônica, doenças arteriais coronarianas, doença vascular encefálica e insuficiência cardíaca congestiva.

2.2 Regulação fisiológica da pressão arterial

A pressão arterial (PA), definida como a pressão exercida pelo sangue sobre as paredes das artérias, é decorrente do débito cardíaco (DC) e da resistência vascular periférica (RVP) (FREIS, 1960). Em um indivíduo normal, a PA geralmente varia de 120 mm Hg para a pressão sistólica e 80 mm Hg para a pressão diastólica. Ao longo do dia, inúmeros fatores, incluindo, o estresse, o medo e a ansiedade podem alterar a PA, desviando-a do valor de referência. Entretanto, mecanismos endógenos de regulação da PA, que ocorrem em curto, médio e longo prazo, restabelecem seus valores normais. Tais mecanismos são complexos, pois dependem da ação integrada de diversos sistemas, como o neural, renal, endócrino e cardiovascular (PAGE, 1987; GUYTON E HALL, 2011).

A curto prazo, os ajustes da PA são modulados predominantemente pelo sistema nervoso autônomo (SNA), sendo o reflexo barorreceptor o mecanismo nervoso mais conhecido (BAGSHAW, 1985). Esse mecanismo homeostático mantém a PA estável em períodos de segundos a minutos, dentro de uma estreita variação dos seus valores de referência, esteja o indivíduo em movimento ou em repouso (NATALI et al., 2015; GHOSH e PANDIT, 2019).

Os quimiorreceptores arteriais também desempenham um importante papel na regulação da PA a curto prazo. Localizados nos corpúsculos carotídeos e aórticos, os quimiorreceptores são formados por células capazes de detectar alterações na concentração hidrogeniônica (pH) do sangue, bem como alterações na pressão parcial de oxigênio (pO_2) e de dióxido de carbono (pCO_2). Portanto, a diminuição de O_2 e o aumento de CO_2 promovem a ativação dos quimiorreceptores arteriais, os quais estimulam os centros vasomotores e consequentemente regulam a PA (GUYTON E HALL, 2011).

Em médio e longo prazo (minutos, horas ou até dias), o ajuste da PA ocorre principalmente pelos rins a partir do sistema renina-angiotensina-aldosterona (SRAA) (GUYTON E HALL, 2011). A renina, também chamada de angiotensinogenase, é uma enzima sintetizada pelas células justaglomerulares dos rins. É liberada em situações em que estas células são estimuladas pela redução da ingesta de sódio, redução do fluxo sanguíneo renal, contração de volume intravascular, estímulo β-adrenérgico nas células justa glomerulares e redução nos níveis de aldosterona no plasma. Quando liberada,

ocorre a ativação do SRAA, que compreende um conjunto de peptídeos, enzimas e receptores envolvidos no controle da PA e no balanço hídrico e eletrolítico do organismo. A renina atuará no angiotensinogênio hepático convertendo-o em angiotensina I. A angiotensina I é então transformada em angiotensina II, um potente vasoconstritor, através da ação da enzima conversora de angiotensina (ECA) presente na circulação e em diferentes tecidos (LARAGH, 1995).

A angiotensina II, considerada o principal peptídeo efetor na vasculatura das células musculares lisas vasculares (CMLV), desempenhará suas funções por meio dos receptores AT1R e AT2R. O receptor AT1R estimula a vasoconstrição, anti-natriurese, antidiurese, liberação hormonal de aldosterona e vasopressina, proliferação celular e fibrose. Em contrapartida, o receptor AT2R tem a finalidade de contrabalançar esses efeitos. Desta forma, a ligação da angiotensina II ao receptor AT1R potencializa a atividade da bomba de Na^+/K^+/ATPase que por sua vez aumenta a resistência vascular periférica (RVP) e a retenção de eletrólitos e água pelos túbulos renais, restabelecendo a PA em níveis normais (GRÖNHAGEN-RISKA e FYHRQUIST, 1980; LARAGH, 1995; GONSALEZ et al., 2018).

Entretanto, este não é o único mecanismo de liberação de angiotensina II. Laragh (1995) demonstrou que a enzima quimase presente no coração tem a capacidade de converter a angiotensina I em II, sem contar com o auxílio da ECA. Além disso, sugeriram também o cérebro, útero, ovário, glândulas salivares, fígado e parede dos vasos sanguíneos como fontes deste peptídeo.

A diurese de pressão também exerce um importante papel na regulação da PA em longo prazo, uma vez que a excreção renal de água e sódio e a pressão arterial média (PAM) estão diretamente relacionadas. De fato, a expansão do volume plasmático influencia diretamente a pressão hidrostática na arteríola aferente renal, aumentando a taxa de filtração glomerular e a excreção de sal e água. Além disso, em condições de estiramento das células musculares que compõem as câmaras atriais, o peptídeo natriurético atrial (ANP) é secretado pelos miócitos, acarretando aumento da filtração glomerular a partir da vasodilatação das arteríolas aferente e vasoconstrição das arteríolas eferentes. Posteriormente, também ocorre diminuição da secreção de renina, com consequente diminuição da liberação de aldosterona, diminuindo assim a

capacidade de reabsorção de sódio e água. Como consequência, haverá um aumento da filtração glomerular, diminuição da volemia e do débito sistólico, promovendo assim a diminuição do débito cardíaco e reestabelecendo a PA para seus valores normais (Guyton e Hall, 2011).

Existem também mecanismos de controle da PA a partir de mediadores endoteliais, como o óxido nítrico (NO) e a prostaciclina (PGI2). O NO é um gás sintetizado por células endoteliais com potente ação vasodilatadora. O aumento do fluxo sanguíneo no leito vascular pode gerar estresse de cisalhamento, que por sua vez, estimula a produção de NO a partir do nitrogênio da guanidina presente na L-arginina, sob ação da enzima NO sintase endotelial (eNOS). Uma vez secretado, o NO difunde-se para a musculatura lisa dos vasos sanguíneos, ativando a enzina guanilato ciclase solúvel (GCs) e formando o monofosfato de guanosina cíclico (GMPc). A formação de GMPc ativará a bomba de cálcio da célula muscular lisa, diminuindo assim o influxo de cálcio seguido de relaxamento da musculatura lisa vascular e vasodilatação (MONCADA, 1997). Além do NO, a PGI2 também participa na regulação do tônus vascular. A ativação dos receptores da PGI2 nas células musculares lisas promove a ativação da adenilato ciclase, aumento de monofosfato cíclico de adenosina (AMPc) e consequente estímulo da proteína cinase dependente de AMPc (PKA) na musculatura lisa vascular. Portanto, esse prostanóide, o qual é formado a partir da prostaciclina sintase, também exerce um importante papel na função renal uma vez que promove potente efeito vasodilatador (BATLOUNI, 2001). Além disso, muitos outros mediadores endoteliais e autacóides locais podem influenciar o tônus vascular e a pressão arterial. Dentre eles destacam-se mediadores vasodilatadores (p.ex. NO, fator de hiperpolarização derivado do endotélio, e a prostaciclina), e vasocontritores (endotelina-1 e tromboxano A2) que atuando de forma sincrônica e complementar, auxiliam na manutenção da homeostase do tônus vascular.

Embora efetivos, os mecanismos fisiológicos de regulação da PA são passíveis de falhas provenientes de causas multifatoriais. Quando recorrentes, tais falhas podem levar ao aumento persistente da PA e consequentemente a ocorrência da HAS - uma doença silenciosa e considerada o principal fator de risco para as DCV (BRASIL, 2001).

2.3 Hipertensão arterial sistêmica (HAS)

2.3.1 Classificação, fisiopatologia e complicações

A HAS é uma DCV de origem multifatorial e altamente complexa. É caracterizada pela elevação sustentada da PA (≥ 140 e/ou 90 mm Hg), ocasionada pelo aumento persistente da resistência vascular e/ou do débito cardíaco. Atualmente, essa patologia atinge prevalências alarmantes no mundo todo (PESSUTO e CARVALHO, 1998). Em 2015, um levantamento realizado em estados norte-americanos demonstrou que HAS estava presente em 69% dos pacientes com primeiro episódio de infarto agudo do miocárdio (IAM), 77% de acidente vascular encefálico (AVE), 75% com insuficiência cardíaca (IC) e 60% com doença arterial periférica (DAP); sendo responsável por 45% dos óbitos por problemas cardíacos e 51% dos óbitos decorrentes de AVE (BARROSO et al., 2020). De acordo com o Sistema de Informações de Mortalidade (SIM) do Ministério da Saúde (MS), a HAS é o principal fator de risco para as DCVs, as quais são responsáveis por 829 óbitos por dia e mais de 302 mil óbitos no Brasil em 2017 (BRASIL, 2019).

A HAS pode ser classificada em primária ou secundária. A HAS primária, que corresponde cerca de 90-95% dos casos, é geralmente ocasionada por fatores genéticos ou pelo estilo de vida, como a ingestão de sódio e álcool em excesso, tabagismo e sobrepeso. Já a HAS secundária, que corresponde a 5-10% dos casos, tem sua origem em causas identificáveis, tais como doenças renais, vasculares, endócrinas ou neurais (GUYTON e HALL, 2011). A severidade da HAS é determinada de acordo com os valores da PA, preferencialmente obtidos em consultas médicas, conforme descrito nas Diretrizes Brasileiras de Hipertensão Arterial publicada em 2020 (Tabela 1).

Complicações relacionadas às DCVs causam um elevado impacto na perda da produtividade do trabalho e da renda familiar, bem como uma elevação em custos socioeconômicos devido à alta frequência de internações (BARROSO et al., 2020). Ademais, a PA elevada é o fator de risco de maior importância para os acidentes vasculares encefálicos, e contribui diretamente para o desenvolvimento do infarto agudo do miocárdio e para a insuficiência renal (IR) (CAMPINO et al., 2002).

Tabela 1. Classificação da PA segundo as Diretrizes Brasileiras de Hipertensão Arterial publicada em 2020.

Classificação	PAS (mHg)	PAD (mmHg)
PA normal	120-129	80-84
Pré-hipertensão	130-139	85-89
HA em estágio I	140-159	90-99
HA em estágio II	160-179	100-109
HA em estágio III	≥ 180	≥ 110

HA: hipertensão arterial; PA: pressão arterial. PAS: pressão arterial sistólica; PAD: pressão arterial diastólica. Fonte: Adaptado de Barroso et al., 2020.

A HAS acomete pessoas de todas as idades, atingindo cerca de 32,5% dos adultos e mais de 65% dos idosos (MANSUR e FAVARATO, 2012; BARROSO et al., 2020). O fato de ser mais prevalente em idosos corrobora estudos prévios que demonstram que o envelhecimento é um dos processos responsáveis pela piora funcional e estrutural de múltiplos sistemas, inclusive o cardiovascular. Como sabemos, um importante fator relacionado ao envelhecimento é o dano oxidativo que ocorre ao longo do tempo. O estresse oxidativo é uma alteração do estado redox celular, considerada a maior via de dano aos componentes celulares, tais como proteínas, ácidos nucléicos, lipídios e DNA, provocando a morte celular (CUI et al., 2012). Além disso, o estresse oxidativo provoca alterações indesejadas no tecido miocárdico, como aumento de arritmias, diminuição da capacidade contrátil, piora da função diastólica, apoptose dos cardiomiócitos, além de estar relacionada com a fisiopatologia de várias doenças do sistema cardiovascular, como a insuficiência cardíaca e a doença arterial coronariana (VICHOVA e MOTOVSKA, 2013).

Além do envelhecimento, outros fatores de risco são importantes no desenvolvimento da HAS, como sobrepeso/obesidade, alcoolismo, tabagismo, fatores socioeconômicos, genéticos, sedentarismo, alimentação rica em sódio e gorduras saturadas. A PA também pode ser modificada pela variação do volume de sangue ou da sua viscosidade, pela frequência cardíaca e pela elasticidade dos vasos. Ademais, estímulos hormonais e nervosos que regulam a resistência sanguínea sofrem influência individual e ambiental (ZAGO e ZANESCO, 2006).

Embora conhecida como “doença silenciosa” por ser inicialmente assintomática, a HAS possui diagnóstico relativamente simples e ao alcance de todos. O diagnóstico da

HAS é feito a partir de medições repetidas da pressão arterial em consultório médico, preferencialmente em mais de uma consulta. Além disso, medidas domiciliares para medição da PA fora do consultório podem ser empregadas quando economicamente e logisticamente viáveis, como a monitorização ambulatorial da pressão arterial (MAPA) e/ou a monitorização residencial de pressão arterial (MRPA). Uma vez diagnosticada, o paciente acometido deve receber acompanhamento médico e tratamento por toda vida (REINERS et al., 2012).

2.3.2 Tratamento farmacológico e não farmacológico

O tratamento da HAS deve ser realizado, primeiramente, a partir de profundas mudanças no estilo de vida do paciente (tratamento não farmacológico) estreitamente associadas a terapias medicamentosas (BARROSO et al., 2020).

O tratamento não farmacológico da HAS envolve uma série de medidas, como controle ponderal, que consiste na perda de peso; aspectos nutricionais, que implicam em hábitos alimentares com restrição no consumo de sódio (2 g/dia), moderação na ingestão de álcool (30 g de álcool/dia) e um aumento na ingestão de ácidos graxos insaturados e monoinsaturados, fibras e oleaginosas; atividades físicas regulares (150 min/semana de atividades físicas moderadas ou 75 min/semana de atividades físicas vigorosas), cessação do tabagismo e controle do estresse (BARROSO et al., 2020). Entretanto, os benefícios das medidas não medicamentosas, por si só, são discretos e por vezes incertos. Desta forma, a associação de medidas não medicamentosas com o tratamento farmacológico é de extrema importância no controle desta enfermidade (FUCHS, 2016).

O tratamento farmacológico da HAS tem como intuito minimizar a morbimortalidade das DCVs através da prescrição de medicamentos que sejam bem tolerados, eficazes quando administrados pela via oral, que possam ser inicialmente utilizados em menores doses efetivas, num menor número de tomadas por dia, e eventualmente, usados em associações. A escolha do medicamento deve, preferencialmente, basear-se em substâncias que cientificamente demonstraram

potencial para diminuir a ocorrência eventos cardiovasculares graves (BARROSO et al., 2020).

Fármacos diuréticos (DIU), inibidores do sistema nervoso simpático (alfa$_2$-agonistas, betabloqueadores e bloqueadores dos receptores alfa1), vasodilatadores diretos, bloqueadores dos canais de cálcio (BCC), inibidores da enzima conversora da angiotensina (IECA) e os bloqueadores dos receptores AT1 da angiotensina II (BRA) são as principais classes de medicamentos utilizados no tratamento inicial e subsequente da HAS (BARROSO et al., 2020).

Os diuréticos, apontados como uma das primeiras escolhas no manejo da HAS, especialmente em estágio inicial, desempenham um importante papel na diminuição do volume extracelular (devido aos seus efeitos natriuréticos) e na resistência vascular periférica. Inicialmente deve-se dar preferência aos diuréticos tiazídicos, como hidroclorotiazida, clortalidona e indapamida, por acarretarem menor desequilíbrio eletrolítico e possuírem maior tempo de ação. Estes agentes agem inibindo um co-transportador de Na/Cl nos túbulos distais dos rins, promovendo assim uma menor reabsorção de sódio e, consequentemente, um aumento de cloro e sódio no líquido tubular. Os diuréticos de alça, como a furosemida e bumetanida, inibem a reabsorção de cloreto de sódio (NaCl) no segmento ascendente espesso da alça de Henle a partir da inibição do co-transportador $Na^+/K^+/2Cl^-$ na membrana luminal. Devem ser preferencialmente aplicados em casos de insuficiência renal e/ou edema periférico e pulmonar. Já os diuréticos poupadores de potássio, como a amilorida e espironolactona, são responsáveis por reduzir significativamente a excreção de potássio através do bloqueio dos canais de sódio no epitélio renal ou através do antagonismo de receptores de aldosterona, respectivamente. São geralmente aplicados em associação com diuréticos tiazídicos ou de alça, visto que previnem a hipopotassemia consequente da administração dos diuréticos supracitados (FUCHS, 2016). Os principais efeitos adversos dos diuréticos são hipopotassemia, hipomagnesemia, hiperuricemia, hiperlipidemia, hiperglicemia, ototoxicidade, hiperuricemia, hipomagnesemia, irregularidade menstruais, ginecomastia masculina, hiperpotassemia e até reações alérgicas (KATZUNG et al., 2014; FUCHS, 2016, BARROSO, 2020).

Fármacos de ação central, também conhecidos como agentes alfa-agonistas de ação central, nem sempre são seletivos e atuam estimulando receptores α2 envolvidos nos mecanismos simpatoinibitórios do centro vasomotor, diminuindo assim a atividade simpática e do reflexo dos barorreceptores, promovendo bradicardia e hipotensão. Ademais, promovem redução dos níveis plasmáticos de renina, uma sutil diminuição no débito cardíaco e na resistência vascular periférica, e não promovem efeitos metabólicos indesejáveis por não interferirem no perfil lipídico nem na resistência periférica à insulina. Inibidores dos receptores imidazolínicos, como a moxonidina e rilmenidina, bem como a clonidina e a metildopa, são os representantes clássicos desse grupo. Os medicamentos desta classe apresentam diversos efeitos adversos, incluindo sedação, sonolência, xerostomia, disfunção erétil e hipotensão postural. Reações adversas mais graves foram documentadas a partir do uso da metildopa e da clonidina, que foram capazes de promover reações autoimunes e hipertensão rebote quando descontinuados abruptamente (FURLAN et al., 2015; KATZUNG et al., 2014; BARROSO et al., 2020).

Os bloqueadores dos receptores beta-adrenérgicos, ou betabloqueadores, são fármacos com perfis farmacológicos distintos, incluindo o propranolol, nadolol, pindolol (beta bloqueadores não seletivos), atenolol, metoprolol, bisoprolol e nebivolol (bloqueadores seletivos dos receptores $beta_1$). De um modo geral, acarretam redução do influxo simpático a partir do centro vasomotor, diminuição do débito cardíaco (efeitos cronotrópicos e inotrópicos negativos) e da secreção de renina. Algumas drogas desta classe também promovem a vasodilatação a partir do bloqueio concomitante com receptores α1 adrenérgicos (p.ex. carvedilol), ou pelo aumento da síntese e liberação de óxido nítrico (p.ex. nebivolol). Broncoespasmo, insônia, depressão, disfunção sexual, distúrbios da condução atrioventricular, vasoconstrição periférica e o surgimento de doenças metabólicas são alguns dos efeitos adversos relatados para esta classe (CORRER e REIS, 2016; NOBRE et al., 2010; GUESSOUS et al., 2012).

Os alfabloqueadores, representados pela doxazosina, prazosina e terazosina, atuam como antagonistas competitivos dos receptores α1 adrenérgicos pós-sinápticos, promovendo redução da resistência vascular periférica, sem alterações significativas no débito cardíaco. De um modo geral, induzem respostas contrarreguladoras (sobretudo ativação reflexa do SNS e do SRAA) que se opõem ao efeito hipotensor, necessitando

do uso em associação com diuréticos e beta-bloqueadores. Ademais, fármacos desta classe podem provocar o fenômeno de tolerância, incontinência urinária e até hipotensão sintomática após a primeira tomada (ZIEGLER et al., 2011; BARROSO, 2020).

Hidralazina e minoxidil são dois importantes vasodilatadores diretos aplicados no tratamento da HAS. São responsáveis pela redução da resistência vascular periférica através do relaxamento da musculatura lisa arterial. Esses efeitos decorrem da abertura de canais de potássio (hidralazina) ou da redução do aporte de cálcio intracelular (minoxidil). As reações adversas comumente relatadas para esta classe são hirsutismo, que ocorre em cerca de 80% dos pacientes que fazem uso do minoxidil, além de cefaléia, taquicardia e reação lupus-like (dose-dependente da hidralazina) (FURLAN et al., 2015).

Agentes bloqueadores dos canais de cálcio promovem uma diminuição do aporte extracelular de cálcio nas arteríolas, e uma consequente redução na resistência vascular periférica. São classificados como diidropiridinas (p.ex. amlodipino, nifedipino, felodipino, nitrendipino, manidipino, lercanidipino, levanlodipino, lacidipino, isradipino, nisoldipino, nimodipino), as quais exercem um efeito vasodilatador predominante, com mínima interferência na frequência e na função sistólica; e como fenilalquilaminas e benzotiazepinas (verapamil e diltiazem, respectivamente), com pronunciados efeitos bradicardizantes e antiarrítmicos (EISENHOFER et al., 2008; BRAVO, 2002; DERNELLIS e PANARETOU, 2002; VOLZKE et al., 2009; LUMACHI et al., 2011). Um estudo desenvolvido por Lubsen et al. (2005) demonstrou a segurança, eficácia e tolerabilidade dos bloqueadores dos canais de cálcio no tratamento da HAS, incluindo pacientes com doença arterial coronariana. Entretanto, reações adversas como cefaleia, rubor facial, hipotensão postural e edema periférico foram relatados (FUCHS, 2014).

Os inibidores da enzima conversora de angiotensina (IECA), como o captopril, enalapril, lisinopril, benazepril e quinapril, pertencem a uma classe de medicamentos bastante eficazes no tratamento da HAS e em outras DCVs, como insuficiência cardíaca, infarto agudo do miocárdio e na prevenção de doença aterosclerótica e do acidente vascular encefálico. Os IECA atuam inibindo a conversão de angiotensina I em angiotensina II, a qual pode aumentar o débito cardíaco (retendo Na^+ e água) e a resistência vascular periférica (devido potente ação vasoconstritora). Além disso, atuam inativando a bradicinina, um autacóide com atividade hipotensora e que aumenta a

liberação de prostaglandinas. As principais reações adversas incluem tosse seca, diarreia, tontura, e hipotensão ortostática no início do tratamento (PREVEDELLO et al., 2009; CORRER e REIS, 2016).

Existem também os bloqueadores dos receptores AT1 da angiotensina II, os quais são prescritos em situações em que o paciente apresenta intolerância aos IECA. São fármacos que antagonizam de maneira específica os receptores AT1, bloqueando assim a ação da angiotensina II, a qual é responsável pela vasoconstrição, proliferação celular e estimulação da liberação de aldosterona (com consequente retenção de sódio e água). São representados pela losartana, valsartana, candersartana, olmesartana e telmisartana, os quais raramente apresentam eventos adversos significativos (FUCHS, 2014; CORRER e REIS, 2016).

Por fim, na última década entrou em evidência no Brasil os inibidores da renina, representado pelo alisquireno. São responsáveis pelo bloqueio da conversão de angiotensinogênio em angiotensina I, com consequentemente diminuição nas concentrações de angiotensina II. Apresentam boa tolerabilidade, mas os estudos que demonstrem seus benefícios sobre a morbimortalidade ainda são escassos (MÜLLER et al., 2008; CORRER e REIS, 2016).

Apesar da vasta variedade de medicamentos disponíveis para o tratamento da HAS, apenas 50% dos pacientes hipertensos aderem aos tratamentos convencionais, e somente 34% dos pacientes tem seus níveis pressóricos controlados. Esta baixa adesão pode ser compreendida pela incidência de efeitos adversos ou a dificuldades de acesso ao Sistema Único de Saúde (SUS) (BARROSO et al., 2020). Desta forma, o uso de produtos à base de plantas medicinais aparece como uma alternativa para aumentar a aderência ao tratamento farmacológico da HAS, evitar consumo irracional de medicamentos sintéticos e, desde que corretamente prescritos, solucionar problemas da atenção primária. Ademais, acredita-se que a inserção de novas opções terapêuticas oriundas de plantas medicinais pode ser facilmente aceita devido ao apelo tradicional enraizado em inúmeras culturas. Portanto, estudos etnofarmacológicos destinados a investigar criteriosamente a eficácia e segurança das plantas medicinais são de extrema importância (SUNTAR, 2020).

2.4 Plantas medicinais e etnomedicina brasileira

Uma planta é considerada medicinal quando parte (semente, raiz, caule, folha, e flor) ou sua totalidade apresenta uma ou mais substâncias biologicamente ativas quando administradas ao homem ou aos animais (ANVISA, 2013). Tais compostos, denominados metabólitos secundários, estão divididos em quatro principais grupos (alcalóides e compostos nitrogenados relacionados, flavonoides, terpenoides, fenilpropanoides e compostos fenólicos) e são sintetizados por vias bioquímicas das plantas para sua defesa contra predadores e patógenos, ou para atrair polinizadores.

As plantas medicinais têm sido amplamente utilizadas pela humanidade, antes mesmo da invenção da escrita, para a prevenção e tratamento de diversas doenças (SAMUELSSON, 2004). Em 1979, a Organização Mundial da Saúde demonstrou que 80% da população mundial faz uso de algum tipo de erva medicinal (OMS, 1979). De acordo com o Ministério da Saúde, a busca por tratamentos à base de plantas medicinais e fitoterápicos aumentou 161% no Brasil entre 2013 e 2015, comprovando que o anseio por alternativas naturais está se intensificando cada vez mais (BRASIL, 2001).

No Brasil, uma série de plantas medicinais é utilizada para fins terapêuticos e alimentícios. Segundo Carneiro et al. (2014), as espécies mais procuradas e usadas no país são: hortelã (*Mentha piperita* L.), capim-limão (*Cymbopogon citratus* (DC.) Stapf), alecrim (*Rosmarinus officinalis* L.), funcho (*Foeniculum vulgare* Mill.), camomila (*Matricaria recutita* L.), manjericão (*Ocimum basilicum* L.), gengibre (*Zingiber officinale* Roscoe), losna (*Artemisia absinthium* L.), alho (*Allium sativum* L.), cebola (*Allium cepa* L.), coentro (*Coriandrum sativum* L.), eucalipto (*Eucalyptus globulus* Labill.), estragão (*Artemisia dracunculus* L.), cravo-da-índia (*Artemisia dracunculus* L.), citronela (*Cymbopogon nardus* L.), romã (*Punica granatum* L.), cânfora (*Cinnamomum camphora* (L.) J.Presl) e mil folhas (*Achillea millefolium* L.). Além de estarem farmacologicamente bem elucidadas, as propriedades terapêuticas das espécies supracitadas já são reconhecidas pela Agência Nacional de Vigilância Sanitária (ANVISA) (CARVALHO et al., 2009; SILVA et al., 2010; BRASIL, 2001).

Inúmeras outras plantas são também utilizadas no Brasil. Isto se deve graças à vasta vegetação brasileira, a qual é detentora de incontáveis espécies com diversidade

e características únicas, correspondendo a aproximadamente 22% de todas as plantas existentes no mundo. Por esta razão, o país está ranqueado como o mais rico em biodiversidade genética a nível global. Ademais, das 350 mil espécies catalogadas no mundo, 55 mil pertencem a vegetação brasileira (FONSECA, 2012). Tal biodiversidade se deve à grande extensão territorial combinada a uma ampla variação climática, possibilitando o país abrigar seis diferentes biomas (floresta amazônica, cerrado, mata atlântica, caatinga, pampa e pantanal) com extraordinária riqueza biológica.

Entretanto, Fonseca (2012) demonstrou que entre os anos de 1992 e 2012, o número de pesquisas acerca das plantas medicinais cresceu apenas 8% por ano, revelando que os estudos de eficácia e segurança de espécies nativas brasileiras ainda são escassos se comparados com a vasta biodiversidade inerente ao país.

2.4.1 Uso de plantas medicinais no Mato Grosso do Sul

O Mato Grosso do Sul (MS) é um estado brasileiro localizado na região Centro-oeste do país. Compreende uma área de 357.145.534 quilômetros quadrados, 79 municípios e uma população estimada de 2.778.986 milhões de habitantes (IBGE, 2019). Em termos de biodiversidade, o MS se destaca graças ao estreito contato entre vários macroecossistemas, como o Cerrado, Floresta amazônica, Floresta atlântica, Floresta Chiquitana e Chaco (POTT et al. 2012; FARINACCIO et al., 2018).

O Cerrado brasileiro é o segundo maior bioma do mundo (2,8 milhões de km_2) e o maior bioma no MS, ocupando 61% do estado. Além disso, o cerrado sul-mato-grossense possui aproximadamente 20% das espécies vegetais registradas no país. Por essa razão, este bioma tem sido alvo de pesquisas em produtos naturais, já que sua flora é considerada uma das mais ricas do mundo (COELHO et al., 2019).

Uma outra característica que destaca o MS é a vasta presença de populações indígenas. Como sabemos, os povos indígenas são os maiores detentores do conhecimento popular de diversas espécies nativas do Brasil. Inclusive, muitas drogas descobertas pelos indígenas, como o curare e a morfina, são de longa data aplicados na medicina ocidental (ELISABETSKY, 1985). Em 2005, um estudo realizado por Bueno et al. demonstrou que o jatobá (*Hymenaea courbaril* L.), figueira (*Ficus inspida* Willd.),

aroeira (*Myracrodruon urundreuva* Allemão), macela (*Achyrocline satureioides* (Lam.) DC.), angico (*Anadenanthera falcata* (Benth.) Speg.), cipo-milombre (*Aristolochia brasiliensis* Mart.), ipê roxo (*Tabebuia avellanedae* Lorentz ex Griseb.), cancorosa (*Maytenus ilicifolia* Mart. ex Reissek), carqueja (*Baccharis trimera* (Less.) DC.), pariparoba (*Pothomorphe umbellata* (L.) Miq.), dentre outras, são as espécies mais utilizadas pelos indígenas sul-mato-grossenses para tratar e prevenir diversas enfermidades.

Recentemente, um estudo etnobotânico realizado por Coelho et al. (2019) catalogou as espécies vegetais mais utilizadas na medicina popular da Grande Dourados. Para isso, curandeiros e raizeiros remanescentes de 13 municípios desta região foram entrevistados e informações como nome da planta, parte utilizada, indicação, método de preparo, doses e vias de administração foram coletadas. Os entrevistados relataram o uso de 71 plantas medicinais pertencentes a 40 famílias botânicas, sendo a família Asteraceae, Lamiaceae, Amaranthaceae e Verbenaceae as mais ricas em espécies. Ademais, apontaram as folhas como as partes mais utilizadas e a infusão como o método mais comum de preparo destas espécies.

Estudos relacionados ao conhecimento popular das plantas medicinais e sua utilização, bem como um levantamento das espécies mais utilizadas por curandeiros, raizeiros e povos indígenas, têm sido frequentemente realizados pela comunidade científica. Tais estudos nos permitem recuperar o conhecimento e a relação dessas populações com as plantas medicinais, além de servirem de base para os estudos etnofarmacológicos (BUENO et al., 2005; COELHO et al., 2019).

A associação do conhecimento popular com a comprovação científica da segurança e eficácia das espécies vegetais se tornou uma ferramenta de grande valor para a terapêutica moderna, especialmente para a cardiologia, já que as DCVs são as principais causas de morte no Brasil e no mundo (MANSUR e FAVARATO, 2012; NETO e MORAIS, 2003). Além disso, tais investigações são fundamentais para garantir que os benefícios do uso superem os efeitos colaterais (VARANDA, 2006).

2.5 Espécies medicinais avaliadas

Baseando-se no estudo etnobotânico realizado por Coelho et al. (2019), três espécies medicinais utilizadas como diuréticas na medicina popular da região da grande Dourados foram investigadas, sendo elas: *Celosia argentea, Anchietea pyrifolia* e *Talinum paniculatum.*

2.5.1 *Celosia argentea* L.

Celosia argentea L. (Amaranthaceae), popularmente conhecida como "crista de galo", é uma espécie representada sob a forma de arbusto de médio e grande porte. É uma planta ornamental cultivada em jardins domésticos, pois apresenta belíssimas flores vermelhas com textura aveludada e folhas verde-escuras (Figura 1).

Figura 1. Folha e arbusto de *Celosia argentea*. Imagem obtida em propriedade particular na cidade de Dourados, Mato Grosso do Sul (Fonte: Arquivo pessoal).

Esta espécie é amplamente distribuída pelo território nacional e mundialmente (GBIF, 2019). A infusão das folhas de *C. argentea* é utilizada na medicina tradicional como diurético (SHAH et al., 1993; COELHO et al., 2019), antimitótico (MORITA et al., 2000), antidiabético (GHULE et al., 2010), anti-hipertensivo, anti-inflamatório e antitumoral (WU et. al., 2011).

Estudos fitoquímicos prévios detectaram a presença de vários grupos de metabólitos secundários, tais como fenóis, flavonóides, alcalóides, saponinas, taninos, terpenos, glicosídeos cardiotônicos, ácidos graxos, aminoácidos, carboidratos e

esteróides (GHORPADE et al., 2012; OKPAKO e AJIBESIN, 2015; TANG et al., 2016). Estudos farmacológicos demonstraram o potencial desta espécie como uma droga eficaz na prevenção da urolitíase (KACHCHHI et al., 2012), cicatrização de feridas por queimadura (GUO et al., 2016) e no tratamento de algumas doenças oculares (TANG et al., 2016). Além disso, Hase e colaboradores (1996) mostraram que *C. argentea* apresenta efeito hepatoprotetor na lesão hepática induzida quimicamente e imunologicamente em ratos.

Em relação ao potencial toxicológico de *C. argentea*, poucos estudos foram conduzidos até o presente momento. Vetrichelvan et al. (2002) demonstrou a baixa toxicidade do extrato hidroalcoólico de *C. argentea* em ratos diabéticos após uma única exposição. Em 2008, Bhujbal et al. constatou a baixa toxicidade aguda em camundongos de uma fração rica em flavonoides obtida desta espécie. Recentemente, Adegbaju e colaboradores (2019) comprovaram a baixa toxicidade do extrato metanólico, acetônico e aquoso das partes aéreas de *C. argentea* em *Artemia salina* após exposição aguda. Entretanto, não há registros na literatura demonstrando o potencial diurético e toxicológico do extrato aquoso de *C. argentea* em humanos ou roedores.

2.5.2 *Anchietea pyrifolia* (Mart.) G. Don

Anchietea pyrifolia (Mart.) G. D. (Violaceae), popularmente conhecida como “suma roxa” ou “cipó suma”, é um arbusto trepador amplamente distribuído no território brasileiro e em alguns países da América do Sul (Figura 2).

Esta espécie foi descrita pela primeira vez por Saint-Hilaire em 1824, juntamente com o gênero *Anchietea*. Várias preparações de *A. pyrifolia* são usadas na medicina popular devido ao seu potencial antirreumático, antibiótico (BRASILEIRO et al., 2006), depurativo e diurético (MESSIAS et al., 2015; COELHO et al., 2019).

Figura 2. Folha e arbusto de *Anchietea pyrifolia*. Imagem obtida em propriedade particular na cidade de Caarapó, Mato Grosso do Sul (Fonte: Arquivo pessoal).

Estudos fitoquímicos prévios demonstraram a presença flavonoides, saponinas e taninos como os principais constituintes de suas folhas (SILVA et al., 2004). Além disso, Lee e colaboradores (2017) demonstraram que o fitol, um álcool diterpeno acíclico, foi identificado como um dos compostos da fração ativa de *A. pyrifolia* capaz de inibir a liberação de histamina.

Em relação a eficácia e segurança desta espécie medicinal, é possível notar que não há relatos na literatura demonstrando os possíveis efeitos diuréticos e toxicológicos do extrato aquoso de *A. pyrifolia* em ensaios clínicos e não-clínicos.

2.5.3 *Talinum paniculatum* (Jacq.) Gaertn

Talinum paniculatum (Jacq.) Gaertn. (Talinaceae), popularmente conhecida como "major gomes" e "erva gorda", é uma espécie amplamente distribuída no território nacional e mundial (COELHO et al., 2019). Considerada uma planta alimentícia não convencional, *T. paniculatum* é amplamente distribuída no Mato Grosso do Sul, principalmente nas áreas de Cerrado. Esta planta é representada em forma de arbustos de pequeno e médio porte (30-60 cm de altura), de cor verde-escura e suas folhas são bastante suculentas, lisas e de textura macia (REIS et al., 2015) (Figura 3).

Figura 3. Folha e arbusto de *Talinum paniculatum*. Imagem obtida em propriedade particular na cidade de Dourados, Mato Grosso do Sul (Fonte: Arquivo Pessoal).

A infusão das folhas de *T. paniculatum* é usada na medicina popular como diurético (LORENZI, 2002; OLIVEIRA et al., 2007), afrodisíaco (WIDIYANI, 2006), tônico reprodutivo (THANAMOOL et al., 2013), para tratar problemas gastrointestinais, infecções de pele e cicatrização de feridas (REIS et al., 2015).

Investigações fitoquímicas demonstraram a presença de taninos, esteroides, saponinas e triterpenos em sua composição (YULIA et al., 2006). Além disso, alguns dados revelaram a presença de fitoesteróis, tais como campesterol, estigmasterol e sitosterol (REIS et al., 2015). Outro estudo identificou três alcalóides quinolizidínicos (javaberina, hexaacetato de javaberina A e hexaacetato de javaberina B) em *T. paniculatum* (JUNG et al., 2006). Tais compostos apresentaram efeito inibitório significativo na produção de TNF-α por células adiposas e macrófagos (SHIMODA et al., 2001).

Estudos farmacológicos prévios demonstraram sua eficácia como suplemento dietético na prevenção do diabetes (SHIMODA et al., 2001), como analgésico (RAMOS et al., 2010) e efeitos estrogênicos em ratas ovariectomizadas (THANAMOOL et al., 2013). Ademais, o extrato de folhas de *T. paniculatum* e suas frações demonstraram atividade contra *Serratia marcescens*, *Staphylococcus aureus*, *Micrococcus luteus*,

Candida albicans e *Escherichia coli* (REIS et al., 2015). Entretanto, estudos de eficácia que validem o uso popular desta espécie como agente diurético ainda não foram realizados.

No que diz respeito à toxicidade de *T. paniculatum*, os estudos ainda são bastante escassos. Reis et al. (2015) demonstrou baixa toxicidade do extrato hidroalcoólico e das frações hexânicas e de acetato de etila das folhas desta espécie em ensaio *in vitro* de citotoxicidade. Contudo, ensaios *in vivo* que avaliem o perfil toxicológico de *T. paniculatum* ainda não foram realizados.

2.6 Toxicidade de produtos naturais

As plantas medicinais são utilizadas mundialmente com a finalidade de prevenir e curar diversas enfermidades. Tais aplicações são advindas sobretudo do conhecimento de indígenas, curandeiros e raizeiros que, após usos empíricos e muita observação, relatam e prescrevem diversas espécies vegetais para fins terapêuticos. Entretanto, as plantas medicinais, embora naturais, não estão isentas de efeitos tóxicos e podem até levar à morte (MENEGATI et al., 2016).

No Brasil, diversas plantas corriqueiramente utilizadas para fins alimentícios e terapêuticos foram cientificamente apontadas como tóxicas. Moura (2008) demonstrou que a infusão das flores da camomila (*Matricaria recutita* L.*),* amplamente utilizada como ansiolítico, bem como a arruda (*Ruta graveolens* L.), o sene (*Cassia angustifolia* M.Vahl), a arnica (*Arnica montana* L.) e a cânfora (*Cinnamomum canphora* var. *nominale* Hats. & Hayata) podem ser abortivas. A espinheira-santa (*Maytenus ilicifolia* Mart. ex Reissek) e o confrei (*Symphitum officinalis* L.), além de abortivos, também apresentaram efeitos hepatotóxicos quando testados em roedores (VEIGA Jr. et al., 2005; MONTANARI e BEVILACQUA, 2002). O boldo (*Peumus boldo* Molina), rotineiramente utilizado para desconfortos gastrintestinais, demonstrou ser um potencial agente causador de irritação renal.

Além dos dados supracitados, algumas espécies interagem significativamente com outros fármacos, como a erva-de-São-João (*Hypericum perforatum* L.), que pode potencializar a atividade de algumas drogas antidepressivas; o dente-de-leão

(*Taraxacum officinales* (L.) Weber ex F.H.Wigg.), que pode potencializar a atividade de diuréticos sintéticos; e a valeriana (*Valeriana officinalis* L.) e o maracujá (*Passiflora edulis* f. flavicarpa O. Deg.), que têm potencial de interação com hipnóticos e ansiolíticos (NEWALL et al., 1996).

Entretanto, muitas espécies ainda são utilizadas indiscriminadamente, embasando-se apenas no conhecimento popular de raizeiros locais. Segundo Ekor (2014), diversos casos de envenenamento por plantas vêm sendo relatados na literatura. Ademais, um número bastante considerável de efeitos adversos relacionados ao uso de espécies vegetais foram transmitidos a OMS em meados dos anos 90. No Brasil, 761 casos de indivíduos intoxicados por plantas foram registrados no Sistema Nacional de Informações Tóxico-Farmacológicas (SINITOX). Por esta razão, a toxicidade dos produtos naturais se tornou um problema de saúde pública alarmante (TRAESEL, 2017).

Com isso, percebemos que o conhecimento empírico, embora fundamental para se iniciar um estudo etnofarmacológico, por si só não garante a segurança de espécies vegetais. Desta forma, os ensaios pré-clínicos toxicológicos são imprescindíveis para garantir um uso seguro e eficaz.

A ANVISA normatiza diversos ensaios pré-clínicos toxicológicos com base nas agências reguladoras internacionais de medicamentos, como a Food and Drug Administration (FDA) e a Organization for Economic Cooperation and Development (OECD) (ANVISA, 2013). Tais ensaios permitem avaliar o potencial toxicológico de determinada substância a nível comportamental, tecidual, visceral e sistêmico. Além disso, os ensaios toxicológicos nos permitem verificar se os efeitos observados são dose-dependentes e se há possibilidade de reversibilidade ou da ocorrência de efeitos toxicológicos tardios (OECD, 2008).

3 OBJETIVOS

3.1 Objetivo geral

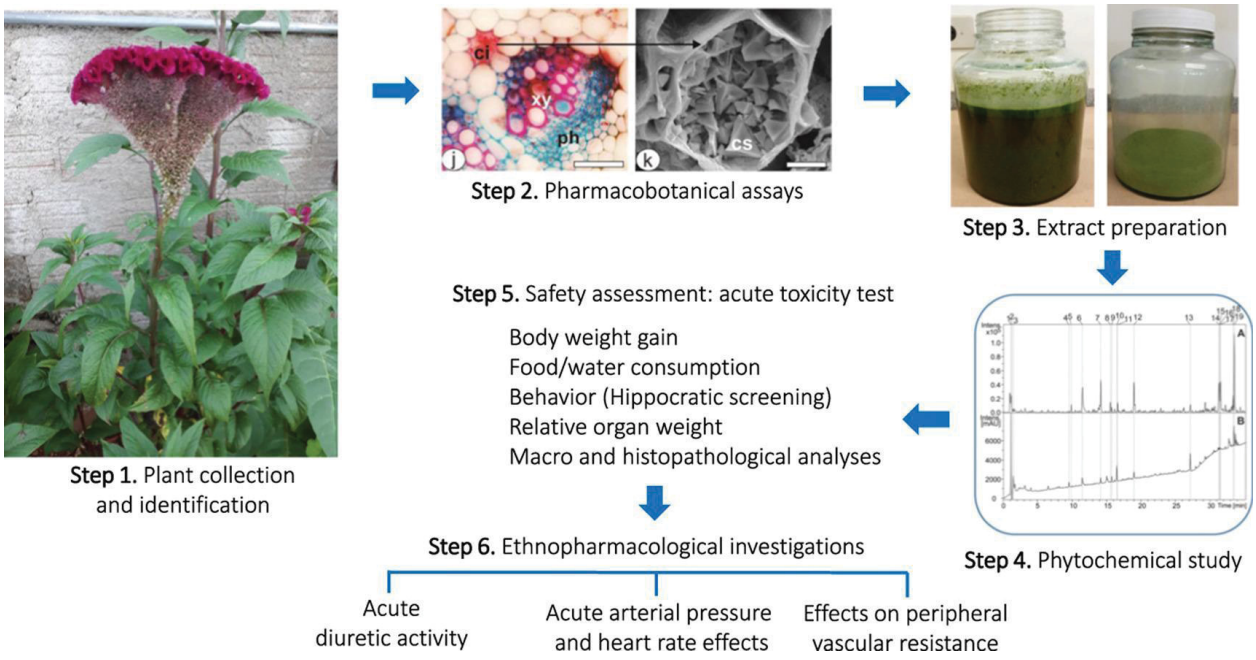
Realizar uma prospecção etnofarmacológicas das atividades cardiovasculares e renais de *Celosia argentea*, *Anchietea pyrifolia* e *Talinum paniculatum*.

3.2 Objetivos específicos

1. Realizar a caracterização botânica das espécies selecionadas, traçando um perfil anatômico e histoquímico das mesmas;
2. Produzir extratos purificados das três espécies e caracterizar o perfil fitoquímico dos mesmos, identificando os principais metabólitos secundários existentes;
3. Avaliar a toxicidade aguda dos extratos purificados obtidos das três espécies selecionadas utilizando como modelo experimental ratas da linhagem Wistar;
4. Avaliar a atividade diurética aguda e prolongada (7 dias) das três espécies utilizando modelo de diurese em ratos;
5. Avaliar a atividade hipotensora aguda e prolongada (7 dias) das três espécies selecionadas em ratos normotensos;
6. Investigar os mecanismos farmacológicos envolvidos na atividade diurética e hipotensora utilizando leito mesentérico isolado e perfundido;
7. Definir a espécie mais promissora e avaliar seu potencial estrogênico em ratas a partir do ensaio uterotrófico;
8. Avaliar os possíveis efeitos toxicológicos da espécie mais promissora no desenvolvimento e maturação do sistema reprodutivo de ratos e ratas após exposição prolongada.

4 ARTIGO CIENTÍFICO 1: *Celosia argentea* L. (Amaranthaceae) a vasodilator species from the Brazilian Cerrado - An ethnopharmacological report.

Graphical abstract



Celosia argentea L. (Amaranthaceae) a vasodilator species from the Brazilian Cerrado - An ethnopharmacological report

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Abstract

Ethnopharmacological relevance: Celosia argentea L. (Amaranthaceae), popularly known as “crista de galo”, is used in folk medicine due to its diuretic and hypotensive effects. However, there are no reports in the literature regarding its pharmacological effects on the cardiovascular system as well as no data proving the safety of this species.

Aim: To perform a detailed ethnopharmacological investigation of the ethanol soluble fraction from *C. argentea* (ESCA) using male and female Wistar rats.

Material and Methods: Firstly, a morpho-anatomical characterization was performed to determine the quality control parameters for the identification of the species under investigation. Then, the ethanol extract was obtained and chemically characterized by LC-DAD-MS. Furthermore, an oral acute toxicity study was performed in female Wistar rats. Finally, the possible diuretic and hypotensive effects of three different doses of ESCA (30, 100 and 300 mg/kg) were evalueted in male Wistar rats. Besides, the vasodilatory response of ESCA in mesenteric vascular beds (MVBs) and its involvement with nitric oxide/cGMP and prostaglandin/cAMP pathways as well as potassium channels were evaluated.

Results: The main secondary metabolites present in ESCA were phenolic compounds, megastigmanes and triterpenoid saponins. ESCA caused no toxic effects in female rats nor increased urinary excretion in male rats after acute administration. However, ESCA significantly increased the renal elimination of potassium and chloride, especially at the end of 24 hours after administration. Intermediary dose (100 mg/kg) of ESCA was able to promote significant acute hypotension and bradycardia. Moreover, its cardiovascular effects appear to be involved with the voltage-dependent K^+ channels activation in MVBs.

Conclusion: This study has brought new scientific evidence of preclinical efficacy of *C. argentea* as a hypotensive agent in normotensive rats. Apparently, these effects are involved with the activation of the voltage-sensitive K^+ channels contributing to the reduction of peripheral vascular resistance and cardiac output.

Keywords: Amaranthaceae, diuretic, hypotensive, potassium channels, vasodilator

Abbreviations: 4-AP, 4-aminopyridine; ACh, acetylcholine chloride; ANOVA, analysis of variance; AP, arterial pressure; Ca^{+2}, calcium; Cl^{-}, chloride; CO_2, carbon dioxide; CVD, cardiovascular disease; DBP, diastolic blood pressure; DAD, diode array detector; EDS, energy disperse system; EDTA, ethylenediaminetetraacetic acid; EI, excretion load; ESCA, ethanol soluble fraction from *Celosia argentea*; FAA, formalin-acetic acid-alcohol; FESEM, field emission scanning electron microscopy; GLB, glibenclamide; HCl, hydrogen chloride; HCTZ, hydrochlorothiazide; HR, heart rate; K^{+}, potassium; KCl, potassium chloride; L-NAME, Nω-Nitro-L-arginine methyl ester; MAP, mean arterial pressure; MVB, mesenteric vascular bed; Na^{+}, sodium; NaCl, sodium chloride**;** OECD, Organisation for Economic Co-operation and Development; pH, potential of hydrogen; Phe, phenylephrine; PP, perfusion pressure; PSS, physiological saline solution; S.E.M., standard error of the mean; SBP, systolic blood pressure; TEA, tetraethylammonium; UEPG, State University of Ponta Grossa; UFGD, Federal University of Grande Dourados, UFPR, Federal University of Paraná.

1. Introduction

Hypertension is a multifactorial cardiovascular disease (CVD) characterized as the most frequent of chronic non-communicable diseases (Mansur and Favarato, 2012), which reaches alarming prevalence worldwide (Pessuto and Carvalho, 1998). It is considered the most important risk factor for stroke and contributes directly to the development of acute myocardial infarction and renal failure (Campino et al., 2002).

Although the diagnosis of hypertension is relatively simple and within everyone's reach, a survey conducted in several Brazilian states indicates that only approximately 50% of individuals adhere to conventional pharmacological treatment (Malachias et al., 2016). Such adherence is still low, a fact that can be understood by the incidence of adverse effects to some patients or challenges of access to medicines by the low income population that uses the Brazilian Unified Health System. In this way, the use of herbal products appears as an alternative to lower costs, minimize undesirable side effects and avoid irrational consumption of synthetic drugs (Brasil, 2006).

Several species are used in Brazilian folk medicine for the treatment of hypertension. One of these plants is *Celosia argentea* L. (Amaranthaceae), popularly known as “crista de galo”. *C. argentea* is an ornamental plant grown in home gardens as it presents fascinating red flowers with velvety texture and dark green leaves. It is used in traditional medicine due to its diuretic (Shah et al., 1993), antimitotic (Morita et al., 2000), antidiabetic (Ghule et al., 2010), antihypertensive, anti-inflammatory and antitumor potentials (Wu et al., 2011).

Previous phytochemical screenings detected the presence of several groups of secondary metabolites such as phenols, flavonoids, alkaloids, saponins, tannins, terpenes, glycosides, fatty acids, amino acids, carbohydrates and steroids (Ghorpade et al., 2012; Okpako and Ajibesin, 2015; Tang et al., 2016). Recent studies have

shown this species as an effective drug in prevention of urolithiasis (Kachchhi et al., 2012), healing burn wounds (Guo et al., 2016) and for treatment of some eye diseases (Tang et al., 2016). Moreover, Hase and collaborators (1996) showed that *C. argentea* presents hepatoprotective effect in both chemically and immunologically induced liver injury in rats.

Despite the infusion of the leaves of *C. argentea* are commonly prescribed by traditional Brazilian healers as diuretic and antihypertensive agents (Shah et al., 1993; Wu et al., 2011) there are no reports in the literature regarding its pharmacological effects on the cardiovascular system as well as no data proving the safety of this species. Therefore, this study aimed to perform a detailed botanical, phytochemical, toxicological, and pharmacological investigation of *C. argentea* (ESCA) using male and female Wistar rats.

2. Materials and Methods

2.1. Drugs

We used xylazine and ketamine hydrochloride from Syntec (São Paulo, SP, Brazil) and heparin from Hipolabor (Belo Horizonte, MG, Brazil). Hydrochlorothiazide, acetylcholine chloride, phenylephrine, indomethacin, Nω-Nitro-L-arginine methyl ester, tetraethylammonium, 4-aminopyridine, glibenclamide, NaCl, KCl, NaHCO3, MgSO4, CaCl2, KH2PO4, dextrose and ethylenediaminetetraacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were obtained in analytical grade.

2.2. Plant material and extract preparation

Leaves of *Celosia argentea* were collected in a Cerrado area (Savana biome) from Dourados, Mato Grosso do Sul - Brazil, at 458 m above sea level (22°12'22.6"S 54°47'43.1"W) in February 2017. A voucher specimen (no. 5636) was authenticated by Dra. Maria do Carmo Vieira and deposited in the herbarium of the Federal University of Grande Dourados (UFGD). The plant name is in accordance with the on-line database published by “The Plant List”, accessed on February 7, 2018.

The extract was prepared in a way that, as much as possible, mimicked the method traditional herbal practitioners use to extract their plant medicines. The infusion was made by pouring 1 L of boiling water (97 °C) on each 100 g of dried and pulverized leaves of *C. argentea*. The mixture obtained was naturally cooled to room temperature for approximately 5 hours. Afterwards, the infusion was treated with 3 volumes of ethanol, originating a precipitate and an ethanol soluble fraction (ESCA). ESCA samples were then lyophilized (ESCA; 6% yield), stored in a freezer at −18 °C and used for the phytochemical analysis and pharmacological studies.

2.3. Pharmacobotanical assays

Freshly collected leaves and stems of *C. argentea* were placed in a solution of formalin–acetic acid–alcohol (FAA) 70 (Johansen, 1940), stored in 70% ethanol for three days and then in 70% ethanol (v/v) (Berlyn and Miksche, 1976). Transverse sections of samples were prepared freehand using razor blades. The sections were placed on glass slides, hydrated and stained using Astra blue and basic fuchsine (Roeser, 1972) to obtain semi-permanent slides. The diaphanisation of the leaves was performed by following a technique described by Kraus and Arduin (1997).

2.3.1. Histochemical tests

For histological analysis, the following standard solutions were tested: phloroglucinol/HCl for lignin (Sass, 1951), potassium dichromate 10% (Gabe, 1968) and ferric chloride 2% (Johansen, 1940) for phenolic components, sudan III for lipophilic compounds (Foster, 1949), and 1% iodine solution for starch (Berlyn and Miksche, 1976). Controls were made in parallel with the tests and semi-permanent slides were prepared as previous described. Photomicrographs were taken using an Olympus CX 31 light microscope that was equipped with a C7070 digital camera.

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

For field emission scanning electron microscopy (FESEM), samples fixed in FAA were passed through a series of ethanol solutions of increasing concentration (80%, 90% and 100%) and then dried in a critical point dryer using liquid CO_2. The totally dried samples were mounted on aluminum stubs using glued carbon tapes and then covered with gold with the aid of a Quorum SC7620 sputter coater. Photomicrographs were generated and analyzed using a Mira 3 Tescan FESEM.

For elemental analysis of calcium oxalate crystals present in the samples, an X-Ray energy dispersive system (EDS) attached to the FESEM was used. This elemental composition was randomly made for the crystals as well as cells devoid of crystals (control) using an EDS machine (Mira 3 Tescan) on the same variable-pressure microscope. This process was carried out at the multi-user laboratory (c-LABMU) of the State University of Ponta Grossa (UEPG).

2.4. Identification of constituents by LC-DAD-MS

ESCA was analysed on a UFLC Prominence Shimadzu coupled to diode array detector (DAD) and a mass spectrometer (MicrOTOF-Q III, Bruker Daltonics, Billerica, MA, USA) with electrospray ionization. A Kinetex C18 chromatographic column (2.6 µm, 150 × 2.1 mm, Phenomenex) was used for the analyses, applying a flow rate of 0.3 mL/min, oven temperature of 50°C, injection volume of 2 µL, and mobile phase was composed of ultrapure water (solvent A) and acetonitrile (solvent B), both added 0.1% formic acid (v/v). The gradient elution profile was the following: 0-2 min: 3% B, 2-25 min: 3-25% B, 25-40 min: 25-80% B and 40-43 min at 80% B. The analyses were done monitoring the wavelength of 240-800 nm and performed in negative and positive ion mode (m/z 120-1200) using nitrogen as nebulizer gas (4 Bar), collision gas, and dry gas (9 L/min). All samples were prepared at the concentration of 1 mg/mL and filtered before injections (Millex®, PTFE 0.22 µm).

The compound identifications from ESCA were performed based on the mass spectrometry (accurate mass and ion fragmentation pathway) and UV data compared with information reported in the literature. Molecular formula of each compound was determined based on the mass errors within ± 5 ppm and mSigma below 30.

2.5. Pharmacological and toxicological studies

2.5.1. Animals

Male and female Wistar rats (8-12 weeks, 178-254 g female; 281-490 g male) from the Federal University of Grande Dourados and Federal University of Paraná, were housed in a temperature- and light controlled room (22 ± 2°C; 12-h light/dark cycle) and had *ad libitum* access to food and water. Before the onset of the experiments, rats were left for ten days to acclimatize to laboratory conditions. All procedures involving animals were performed in accordance with the Ethical

Principles in Animal Research and previously approved by the Ethics Committee in Animal Experimentation from the Federal University of Paraná (protocol: 05/2017) and Federal University of Grande Dourados (protocol: 21/2017).

2.5.2. Safety assessment

2.5.2.1. Acute toxicity test

This study was performed according to the Organization for Economic Co-operation and Development (OECD) guideline 425 (OECD, 2008). Thirty-two female Wistar rats were divided equally into four groups and were fasted prior to treatment (food but not water was withheld overnight). Following the period of fasting, animals were weighed and three doses (30, 300 and 2000 mg/kg) of ESCA or water (1 mL/kg) was administered once by oral gavage and food was withheld for further 2 h. Following administration, animals were closely observed during the first 24 h and thereafter, for 14 consecutive days. Signs of morbidity and mortality, as well as body weight gain, food and water consumption were daily observed. In addition, animals were carefully observed daily for general health and clinical signs of toxicity according to the five parameters of the Hippocratic screening, which aims to measure the activity of a whole organism by analyzing the following parameters: conscious state, activity and coordination of motor system and muscle toning, activities on the central nervous system, corneal and headset reflexes and activities on the autonomic nervous system (Malone and Robichaud, 1962). At the end of the observation period, animals were left under an overnight fasting and had free access to water. On day 15 after administration, animals were euthanized by isoflurane anesthesia (inhalation) followed by exsanguination. Organs (heart, lung, spleen, liver, kidney, uterus and ovaries) were removed, weighed and closely examined for any gross changes. Heart, liver and kidneys were sent to histopathological analysis in order to assess tissue

integrity of the organs as well as infiltration of leukocytes, necrosis, apoptosis, degeneration or any other kind of alterations that could indicate signs of toxicity (OECD, 2008).

2.5.3. Ethnopharmacological investigations

2.5.3.1. Acute diuretic activity

This procedure was performed according to a methodology described by Gasparotto Junior et al., (2009). Male rats, randomly divided into five experimental groups (n = 6), received an oral dose of 5 mL/100 g of physiological saline solution (0.9% NaCl) in order to impose a controlled water and salt balance. Following salinization, rats received a single dose of 30, 100, or 300 mg/kg of ESCA, 25 mg/kg HCTZ (positive control group) or 1 mL/100 g water (negative control group) by oral gavage. Animals were then placed in metabolic cages for 24 h. Urine was collected with the aid of a graduated cylinder and the volume was recorded at 8 and 24 h (expressed as mL/100 g of body weight). Urinary sodium (Na^+), potassium (K^+) and chloride (Cl^-) levels were quantified in an ion selective meter (COBAS INTEGRA 400 plus; Roche®). Excretion load (El) of Na^+, K^+ and Cl^- was obtained by multiplying the concentration of electrolytes (mEq/l) by the urinary flow (mL/min). Results are expressed as µEq/min/100g. 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). At the end of the experimental period, animals were euthanized by isoflurane anestesia (inhalation) followed by exsanguination.

2.5.3.2. Arterial pressure (AP) and heart rate (HR) evaluation

Normotensive male rats were divided into five experimental groups (n = 6) and anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) by intramuscular route (IM). Immediately after anesthesia, a single bolus dosage of heparin (50 IU) was administered subcutaneously. 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 blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and HR. After 15 minutes of stabilization, different groups received a single dose (30, 100, or 300 mg/kg) of ESCA, HCTZ (25 mg/kg) or water (1 mL/100 g of body weight) intraduodenally. After a 15-minute stabilization period, SBP, DBP, MAP, and HR were recorded for 20 min.

2.5.3.3. Effects on peripheral vascular resistance

Male rats were anesthetized with ketamine and xylazine (100 and 20 mg/kg, respectively; i.p.). Mesenteric vascular beds (MVBs) were isolated and prepared using perfusion methods described by McGregor (1965). MVBs (n = 5) were placed in a water-jacketed organ bath and perfused (at 4 mL/min) 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) 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). 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 (30 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. 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 (30 nmol) was directly applied into the perfusion system.

Thus, MVBs with or without functional endothelium were continuously perfused with PSS plus Phe (3 μM). After stabilization, different preparations received bolus injections containing ESCA (0.003, 0.01, 0.03, 0.1, 0.3, and 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. 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, ESCA (0.1, 0.3, and 1 mg) was injected again into the perfusion system. The ability of ESCA to reduce PP in the presence and absence of different inhibitors was evaluated.

2.6. Statistical analysis

The 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. *p*-value of less than 0.05 was considered statistically

significant. Graphs were drawn and statistical analyses were performed using GraphPad Prism software version 6.0.

3. Results

3.1. Pharmacobotanical findings

Leaves of *Celosia argentea* (Figure 1A and B), from the surface view, shows straight anticlinal walls on adaxial and wavy on abaxial epidermises (Figure 1C and D). The leaves are amphistomatic and the stomata (Figure 1E) are anomocytic and anisocytic (Figure 1C and D). Glandular trichomes are present on both the adaxial and abaxial leaf surfaces. These trichomes are multicellular and uniseriate and consist of a stalk formed by 4 cells and an elongated head (Figure 1F).

In cross-section, the leaf presents one-layered epidermis covered by a thin and smooth cuticle. The cells in the adaxial side are bigger than in the abaxial side (Figure 1G). The mesophyll is isobilateral and is formed by 3 layers of palisade and 2 layers of spongy parenchyma (Figure 1G). The veinlets traversing the mesophyll region are represented by small collateral vascular bundles surrounded by parenchymatic sheath. Crystalliferous idioblasts with crystal sand showing triangular shapes are found in the mesophyll (Figure 1H).

In transverse section, the midrib is biconvex in outline (Figure 1I). The epidermis has the same features as described for the leaf blade. Beneath the epidermis, up to 4 layers of angular collenchyma is found in both sides. The ground parenchyma contains several crystalliferous idioblasts (Figure 1I-K) with the same characteristics previously described. The vascular system is represented by 3 free collateral vascular bundles (Figure 1J) arranged in open arc in the ground parenchyma (Figure 1I).

The petiole, in cross-section, is flat-convex in shape with two conspicuous ribs on the adaxial side. A thin and smooth cuticle covers the single-layered epidermis (Figure 1D). About 4 layers of angular collenchyma are found in adaxial side, as interrupted patches all around the petiole and in the edge of the ribs. Between the patches of collenchyma, up to 3 layers of chlorenchyma are observed. The stele is represented by about 10 free collateral vascular bundles of varying sizes arranged in open arc (Figure 1L).

In an incipient secondary structure, the stem is irregular in shape with some ribs (Figure 1M and N). The epidermis is uniseriate and covered by a thin cuticle. On the epidermis, prismatic crystals are found (Figure 1O). Beneath the epidermis, chlorenchyma is present, however, in the ribs it is substituted by angular collenchyma (Figure 1N). A cambial ring that found to move outwards and produce collateral vascular bundles inwards to form the vascular system is observed (Figure 1N). In the secondary growth, solitary or groups of fibers can be seen in the cortex (Figure 1G). The pith is made up of thin-walled parenchymatous cells. Several crystalliferous idioblasts are found in the cortex and in the pith.

The histochemical test showed the presence of the lipophilic compounds in the cuticle (Figure 2D); phenolic compounds in the adaxial epidermal cells (Figure 2A), in the head of the glandular trichome (Figure 2C), and xylem in the midrib (Figure 2B), petiole and stems (Figure 2F); lignified elements were observed in the fibers in the stem cortex (Figure 2G), beyond in the vessel elements in the leaves, petioles (Figure 2E) and stems (Figure 2G).

The EDS spectra of the crystal sand (Figure 3A) and the prismatic crystals on the epidermis (Figure 3B) of *C. argentea* show large peaks of calcium, carbon and oxygen. These results suggest that the chemical composition of these crystals is calcium oxalate. The EDS spectrum of a prismatic crystal (Figure 3A) shows

prominent peaks of calcium (10.26%), carbon (45.12%) and oxygen (44.73%). Whereas, the EDS spectrum of a crystal sand (Figure 3B) shows major peaks of calcium (30.01%), carbon (17.25%) and oxygen (52.74%). The unlabeled peaks in the spectra characterize conductive metal used for coating the samples for FESEM analysis.

3.2. Identification of the constituents by LC-DAD-MS.

ESCA was analyzed by LC-DAD-MS and twenty-nine compounds were detected and identified (Table 1, Figure 4). Peaks **1-4** revealed the deprotonated ions at *m/z* 195.0510 ($C_6H_{12}O_7$), 473.1031 ($C_{17}H_{30}O_{15}$), 191.0197 ($C_6H_8O_7$) and 167.0347 ($C_8H_8O_4$), which were putatively identified as gluconic acid, *O*-pentosyl di-hexoside, citric acid and methylprotocatechuic acid, respectively.

The peak **7** showed a band at λ_{max} 280 nm in the UV spectrum and an ion at *m/z* 195.0663 compatible to $C_{10}H_{12}O_4$. Its product ions *m/z* 180 and 151 were yielded by losses of a methyl radical and a molecule of CO_2 (Heinrich et al., 2013), suggesting dihydroferulic acid. Also, the metabolite **9** revealed the bands at 299 and 322 nm (λ_{max}) relative to the phenylpropanoid caffeic/ferulic acid (Heinrich et al., 2013). However, the product ion *m/z* 193 ($C_{10}H_9O_4^-$) confirmed it as ferulic acid derivative.

Peaks **5**, **8** and **10** showed ions at 257.1394, 241.1445, 221.1191 [M-H]- and with the molecular formulae $C_{13}H_{22}O_5$, $C_{13}H_{22}O_4$, and $C_{13}H_{18}O_3$, respectively. These compounds are suggested megastigmanes, but their structures were not completely characterized due to the absence of mass data reported in the literature. Megastigmanes have been described in *C. argentea* (Tang et al., 2016).

The chromatographic peaks **14-19** showed consecutive losses of sugars in the MS/MS analyses, and presented fragment ions at *m/z* 503 relative to the triterpenoid

aglycone $C_{30}H_{48}O_6$, which is a crucial product ion to determine the aglycone. An essential step for the identification of triterpenoid saponins is the determination of the protonated $[M+H]^+$ and deprotonated ions $[M-H]^-$ to find the molecular formula from the accurate mass. All saponin compounds (**14-19**) exhibited doubly charge adduct ion $[M-2H]^{2-}$, suggesting two sugar chains probably attached to C-3 and C-28 (bidesmosic saponin); these ions are not observed for the saponins with one sugar chain (monodesmosidic saponin). The bidesmosidic triterpenoid saponins have been described in the genus *Celosia* (Li et al., 2010, Jiang et al., 2017).

The consecutive losses of sugars were used to determine them in saponin, such as losses of 176, 162, 146 and 132 *u* that confirmed glucuronic acid, hexose (e.g. glucose), deoxyhexose (e.g. rhamnose or fucose), and pentose (e.g. arabinose or xylose). From the fragment ion *m/z* 503 $[Aglycone-H]^-$, observed for the saponins **14-19**, were yielded the ions at *m/z* 457 and 411 relative to losses of one and two formic acid molecules (46 *u*), corfiming the presence of two carboxyl groups in the aglycones ($C_{30}H_{48}O_6$) (Figure 5). In addition, the product ions at *m/z* 439 ($C_{29}H_{42}O_3$), 421 ($C_{29}H_{40}O_2$) and 411 ($C_{28}H_{42}O_2$) are yielded from losses of water molecules, suggesting the hydroxyl groups on the triterpenoid aglycone moiety. These data are compatible with the aglycone mediagenic acid, which has already been reported in *Celosia* genus (Jiang et al., 2017).

A scheme of the fragmentation pathway from saponin **16** is illustrated in Figure 5, and it was similar for all saponins identified in *C. argentea*. Thus, the fragmentation pathway of triterpenoid saponin **16** suggested a bidesmosic saponin with one glucuronic acid, three deoxyhexoses and one pentose in sugar chains linked to the aglycone medicagenic acid (Figure 5). Saponin **16** was putatively identified as *O*-glucuronyl tri-deoxyhexosyl pentosyl medicagenic acid, which is suggested as a novel saponin.

The triterpenoid saponin **17** revealed fragment ions yielded by losses of 162 *u* (*m/z* 811→649), 146 *u* (*m/z* 1235→1089, *m/z* 1103→957, *m/z* 957→811, *m/z* 649→503), and 132 *u* (*m/z* 1235→1103), indicating the presence of one pentose, three deoxyhexoses and one hexose (Jiang et al., 2017). The fragment ions from aglycone were the same pathway observer for **16**, and they suggest medicagenic acid, an oleanane-type triperpene common in *Celosia* species (Wu et al., 2011, Jiang et al., 2017). So, the compound **17** was identified as *O*-hexosyl tri-deoxyhexosyl pentosyl medicagenic acid, which is suggested as a novel saponin.

Substances **14**, **15**, and **18-19** could be identified by spectral data similarity to the description for saponin **16** and **17**. Thus, they revealed the deprotonated ions *m/z* 1233.5490 ($C_{58}H_{89}O_{28}^-$), 1101.5050 ($C_{53}H_{81}O_{24}^-$), 677.3570 ($C_{36}H_{53}O_{12}^-$) and 809.3935 ($C_{41}H_{61}O_{16}^-$), respectively. Saponins **14**, **15**, and **18-19** were putatively identified as *O*-glucuronyl dideoxyhexosyl dipentosyl medicagenic acid, *O*-glucuronyl di-deoxyhexosyl pentosyl medicagenic acid, *O*-glucuronyl medicagenic acid, and *O*-glucuronyl pentosyl medicagenic acid. Similar saponins have been described in other *Celosia* species, such as celosin J ($C_{58}H_{90}O_{28}$), celosin I ($C_{53}H_{82}O_{24}$), and celosin E ($C_{36}H_{54}O_{12}$) (Jiang et al., 2017).

3.3. Safety data

No deaths were observed as well as no signs of toxicity such as loss weight or changes in water and food consumption (Table 2). Regarding the Hippocratic screening, the behavior of all animals tested was considered normal for the species (data not shown). In relation to the relative organ weight of animals, no statistical differences were observed in all treated-animals when compared to the control (Table 3). Based on the results obtained from the macroscopical and histological observations, no findings suggestive of toxic effects were recorded (Figure 6).

Therefore, since no toxicity was observed, ESCA oral lethal dose (LD_{50}) is above 2000 mg/kg (OECD, 2008).

3.4. Diuretic effects

According to the data obtained from rats treated orally with a single dose of ESCA (30, 100 and 300 mg/kg), no increase in diuresis was observed after 8 and 24 hours when compared to the control group (Table 4). As expected, HCTZ was able to increase diuresis after 8 hours and returned to values similar to the control group 24 hours after drug administration.

Even though ESCA was not able to induce diuresis nor any changes in urine density, all doses of the extract increased pH levels in 8 and 24-hour samples and showed significant differences when compared to the control (Table 4). Regarding the electrolyte contents of urine, animals treated with ESCA 30 and 300 mg/kg showed high amounts of Cl^- in 24-hour urine samples. In 8-hour urine samples, the ESCA highest dose (300 mg/kg) excreted higher contents of K^+ and differed statistically from the control group. Besides that, all doses of the extract were again able to eliminate K^+ in 24-hour samples and therefore differed statistically from the control group (Table 5). As expected, HCTZ was able to increase Na^+, K^+, and Cl^- excretion after 8 hours and returned to values similar to the control group after 24 hours.

3.5. Effects on arterial pressure and heart rate

After a 15-minute stabilization period, and before the administration of drugs, basal SBP, DBP, MAP was 103 ± 3.3, 65 ± 2.1, and 84 ± 2.5 mm Hg whereas HR was 249 ± 8.5 beats per minute (bpm). HCTZ, as expected, reduced basal SBP, DBP and MAP to 88 ± 8.0, 57 ± 2.4 and 71 ± 2.3 mm Hg and HR to 341 ± 33 bpm. The

control group, also as expected, barely changed SBP, DBP, MAP and HR values (104 ± 6.9, 69 ± 3.3, 85 ± 4.2 mm Hg; 331 ± 35 bpm, respectively).

Only the intraduodenal administration of ESCA 100 mg/kg was statistically different from the control since it was able to promote a significant reduction in SBP, DBP, MAP, and HR (85 ± 2.0, 47 ± 4.4, 64 ± 4.0 mm Hg; 248 ± 30 bpm, respectively) (Table 6).

3.6. Effects on peripheral vascular resistance

The continuous perfusion of MVBs with Phe resulted in a sustained increase in the vascular perfusion pressure, which was dose-dependently reduced by ESCA administration into the perfusion apparatus. ESCA was able to induce an expressive dose-dependent vasodilator response in MVBs. The PP reduction values for doses of 0.1, 0.3, and 1 mg were ~ 2, 6 and 18 mm Hg, respectively (Figure 7A).

Treatment with sodium deoxycholate reduced the effects of ACh on MVBs by 98 ± 6% (data no shown), confirming the efficacy of chemically removing the endothelium. The vasodilatory effect of ESCA (0.1 and 0.3 mg) was increased in the absence of endothelium (Figure 7B) or in preparations with intact endothelium perfused with L-NAME (Figure 8A). The vasodilatory effects of ESCA remain unaltered in preparations with intact endothelium perfused with indomethacin (Figure 8B), or L-NAME plus indomethacin (Figure 8C).

The perfusion of MVBs with nutritive solution added of 40 mM KCl abolished the effects of ESCA (Figure 9A). On the other hand, only minor effects were observed after infusion of TEA or GLB (Figure 9B-C). Interestingly, treatment with 4-AP vanished vasorelaxation induced by all doses of ESCA (Figure 9D).

4. Discussion

Medicinal plants have been used for medical treatment of various diseases since the earliest days of humankind. As the search for natural medicines is intensifying, medicinal plants have become the focus of numerous studies in order to evaluate their safety and efficacy (Matta et al., 2011). The use of herbal products appears as an alternative to lower costs, minimize undesirable side effects, avoid irrational consumption of synthetic drugs and, if correctly prescribed, solve primary care problems (Brasil, 2006). In fact, studies related to the popular knowledge on medicinal plants and their use, as well as a survey of the species most used by traditional healers, have been frequently carried out by the scientific community (Oliveira et al., 2011; Bieski et al., 2011; Ribeiro et al., 2017; Tirloni et al., 2017). The association of popular knowledge with proven safety and efficacy has become a valuable tool for modern therapy, especially for cardiology, since cardiovascular diseases (CVDs) are the leading causes of death in Brazil and worldwide (Mansur and Favarato, 2012). For this reason, we proposed to perform, for the first time, a detailed ethnopharmacological investigation of the toxicological, diuretic and hypotensive effects of an important medicinal species from the Brazilian cerrado.

Firstly, in order to expand ethnopharmacological knowledge on the Brazilian Cerrado, a morphoanatomic and microchemical study of *Celosia argentea* was performed. Since some medicinal plants present several popular names, different species may be known by the same common name or even confused by the similar morphological characteristics (American Herbal Pharmacopeia, 2011). For this reason, morpho-anatomical studies are of paramount importance as it serves to provide quality control standards for the identification of the species under investigation and prevent tampering and errors in the use. Since data obtained in the morphoanatomic study ensured reliability of the medicinal species under

investigation, the second stage of this research was designed to assess the phytochemical profile of the ESCA.

In this study, twenty-nine compounds were detected and identified in ESCA by LC-DAD-MS, including phenolic compounds, megastigmanes and triterpenoid saponins. In fact, the phenolics, such as methylprotocatechuic acid and phenylpropanoids derivatives have been described from *C. argentea* (Perveen et al., 2014). Besides, bidesmosidic triterpenoid saponins with the aglycone medicagenic acid have also been described from it (Li et al., 2010, Jiang et al., 2017). According to the number of sugar chains attached on aglycone, saponins are classified as monodesmosidic, bidesmosidic or tridesmosidic. The chemical structures of them are extensively related to their biological properties, which are also related to these different features according to sugar chains attached on aglycone (Lacaille-Dubois and Wagner, 1996). In the cardiovascular system, several saponins have demonstrated significant effects. The extract from *Bacopa monnieri* L. showed several saponins with high amounts of monodesmosidic triterpenoid saponins, such as bacoside A3 and bacopaside II. Such compound was able to reduce blood pressure by NO production from endothelium. Also, saponins showed similar vasodilator effects on mesenteric arteries (Kamkaew et al., 2011) and induced reduction of blood pressure and inhibition of human renin ‘in vitro’ (Hiwatashi et al., 2010).

It is well known that medicinal plants, like other conventional drugs, have the same potential to cause toxic effects. For this reason, toxicological assessments are of high relevance to evaluate the safety and possible adverse effects that may arise when consumed for therapeutic purposes. Hence, the acute toxicity of ESCA was evaluated after one single administration. For this purpose, female rats were used since they are to a certain extent more sensitive than male rats (Lipnick et al., 1995;

OECD, 2008). According to Raina et al. (2015), the evaluation of parameters such as water and food consumption, body weight gain and organ weight are important indicators of pathological and physiological status of animals. Moreover, Hippocratic screening suggests a general estimation of the pharmacological and toxicological nature of an unknown substance. In this study, since ESCA did not promote any changes in behavior, body weight gain as well as no toxic effects in macroscopic and histological analyses after acute exposure, this plant extract can be considered safe in rats at all doses tested.

As safety data provided valuable information on the toxicological profile of ESCA, we began the ethnopharmacological investigation of this species by testing its diuretic potential. Despite its ethnobotanical indication, no significant diuretic effects were observed in male rats treated with ESCA. In fact, we noted that the saluretic effect was limited. ESCA only increased renal excretion of Cl^- after 8 hours and K^+ after 24 hours. Effective diuretic drugs are known to increase the renal excretion of NaCl and water. In addition, increased renal excretion of K^+ is more involved with side effects, including cramps and arrhythmias, than with the desired therapeutic effects (Roush et al., 2014). Despite of a considerable number of species used in folk medicine as diuretic agents, many do not have scientifically proven saluretic effects (Wright et al., 2007). Ivy and Bailey (2014) suggest that such effect might be related to high water intake, which is responsible for increasing renal hydrostatic pressure caused by the expansion of plasma volume, without necessarily presenting a significant saluretic response.

Although ESCA did not present significant diuretic activity, we analyzed its acute effects on blood pressure since many hypotensive drugs do not present diuretic response. In fact, our results show that ESCA promotes a hypotensive and bradycardic effect in rats treated with 100 mg/kg after a single administration. This

result demonstrates that the cardiovascular effect of ESCA is not dose-dependent since the intermediate dose was effective and showed a significant difference when compared to the control group. As initially described, our work was performed based on the way traditional healers use this plant in folk medicine, assuming that, as a crude extract, several secondary metabolites were present. Thus, we believe that the effects presented here are due to a coordinated and quantitatively balanced action of different chemical compounds (Wagner, 2006). For this reason ESCA-highest dose (300 mg/kg) was not effective. This may occur due to a change in the concentration of different secondary metabolites, where increasing the concentration of molecules with antagonistic effects prevented the response observed with the intermediate dose.

Considering that one of the main determinants of levels of arterial pressure is peripheral vascular resistance, we chose to investigate whether ESCA would be capable of causing vasodilatory effects on MVBs. In fact, we have shown that the hypotensive effects induced by ESCA may be due to a reduction in peripheral vascular resistance, since we have identified important vasodilatory effects on MVBs. Moreover, it has been shown that the hypotensive effects of ESCA do not seem to depend on endothelium or by prostaglandin/cAMP or nitric oxide/cGMP pathways activation, since prior administration of L-NAME or indomethacin did not affect its vasodilator effects. Afterwards, we decided to investigate whether ESCA was inducing vasodilator effects in an endothelial-independent manner. In an attempt to promote a blockade of K^+ currents across cellular membranes, some MVBs were perfused with 40 mM KCl. Furthermore we also used specific K^+ channel blockers, such as TEA, GLB and 4-AP. Since the perfusion of MBVs with high-KCl solution abolished the vasodilatory effects of ESCA, and the previous treatment with 4-AP completely vanished the vasodilatory response induced by all tested doses, we

suggest that these effects may be involved with the activation of the voltage-sensitive K^+ channels in MVBs. In fact, this might explain the bradycardic effects observed in rats, since voltage-sensitive K^+ channels are important for repolarizing cardiac pacemakers. Thus, we can conjecture that the hypotensive effects of ESCA may be due to a coordinated action between the reduction of peripheral vascular resistance and cardiac output.

5. Conclusion

This study has brought new scientific evidence of preclinical efficacy of *Celosia argentea* as a hypotensive agent in normotensive rats. Apparently, these effects are involved with the activation of the voltage-sensitive K^+ channels contributing to the reduction of peripheral vascular resistance and cardiac output.

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Conflict of interest

Authors declare there are no conflicts of interest.

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Legend to figures

Figure 1. Morpho-anatomy of *Celosia argentea* [c, d, f, g, i, j, l, m, n: Light microscopy; e, h, k, o: FESEM. a. Plant in habit; b. Leaves; c, d, e, f. Leaf in surface view; g, h. Leaf in cross-section; i, j. Midrib in cross-section; l. Petiole in cross-section; m, n. Stem in cross-section [ab- abaxial side, ad- adaxial side, ca- cambia, ci- crystalliferous idioblast, co- collenchyma, cs- crystal sand, cx- cortex, gt- glandular trichome, pc- prismatic crystal, ph- phloem, pi- pith, pp- palisade parenchyma, sp- spongy parenchyma, st- stomata, vb- vascular bundle, xy- xylem]. Scale bar: a = 10 cm; b = 5 cm; l, m = 500 µm; n = 300 µm; i = 200 µm; c, d, f, g, j = 50 µm; k = 10 µm; h = 20 µm; e = 5µm; o = 2µm.

Figure 2. Histochemistry of *Celosia argentea* [a- potassium dichromate solution (10%), b, c, f- ferric chloride solution, d- sudan III, e, g- phloroglucinol/HCl. Transverse sections - a-c – leaf; d, e – petiole; f, g, h – stem [co- collenchyma, ct- cuticle, ep- epidermis, fi- fiber, gt- glandular trichome, pc-phenolic compounds, ph- phloem, xy- xylem]. Scale bar: f = 500 µm, c, d, g = 200 µm; b, f = 100 µm; a, e = 50 µm.

Figure 3. X-ray energy dispersive elemental analysis of isolate crystals. **A**. Prismatic crystal, **B**. Crystal sand.

Figure 4. Total ion (negative ion mode) and UV (at 220-330 nm) chromatograms from the ethanol soluble fraction of the aqueous extract (ESCA) from *Celosia argentea*.

Figure 5. Fragmentation pathway suggested for the saponin **16**.

Figure 6. Histopathological assessment of the organs of rats control and treated with ESCA 2 g/kg in the acute toxicity test. HE (40X).

Figure 7. Vasorelaxant effect of ESCA does not depend on endothelium mediators in the MVBs of rats. MVBs were perfused with PSS containing Phe (3 μM) on intact (A) or denuded endothelium (B) and the vasorelaxant effect of ESCA was evaluated. The results show the mean ± S.E.M. of 5 preparations. In the graphic A [a] indicates $p < 0.05$ compared with the control (vehicle) group. [b] indicates $p < 0.05$ compared with the respective previous dose. In graphic B [a] indicates $p < 0.05$ compared with the respective previous dose. [b] indicates $p < 0.05$ compared with the effects of ESCA on intact endothelium. End - and End +: denuded and intact endothelium, respectively. MVBs: mesenteric vascular beds; Phe: phenylephrine.

Figure 8. Vasorelaxant effect of ESCA does not depend on nitric oxide or prostaglandins in the MVBs of rats. MVBs were perfused with PSS containing Phe (3 μM) plus L-NAME (A), indomethacin (B), or L-NAME plus indomethacin (C) on intact endothelium, and the vasorelaxant effect was evaluated. The results show the mean ± S.E.M. of 5 preparations. [a] indicates $p < 0.05$ compared with the respective previous dose. [b] indicates $p < 0.05$ compared with the effects of ESCA on the respective vehicle group. INDO: indomethacin; L-NAME: N^G-nitro-L-arginine methyl ester; MVBs: mesenteric vascular beds; Phe: phenylephrine.

Figure 9. Vasorelaxant effect of ESCA depends on voltage-dependent K^+ channels in the MVBs of rats. MVBs were perfused with PSS containing Phe (3 μM) plus KCl (A), or TEA (B), or GLB (C), or 4-AP (D) on intact endothelium, and the vasorelaxant effect of ESCA was evaluated. The results show the mean ± S.E.M. of 5 preparations. [a] indicates $p < 0.05$ compared with the respective previous dose. [b] indicates $p < 0.05$ compared with the effects of ESCA on the respective vehicle group. 4-AP: 4-aminopyridine; GLB: glibenclamide; MVBs: mesenteric vascular beds; Phe: phenylephrine; TEA: tetraethylammonium.

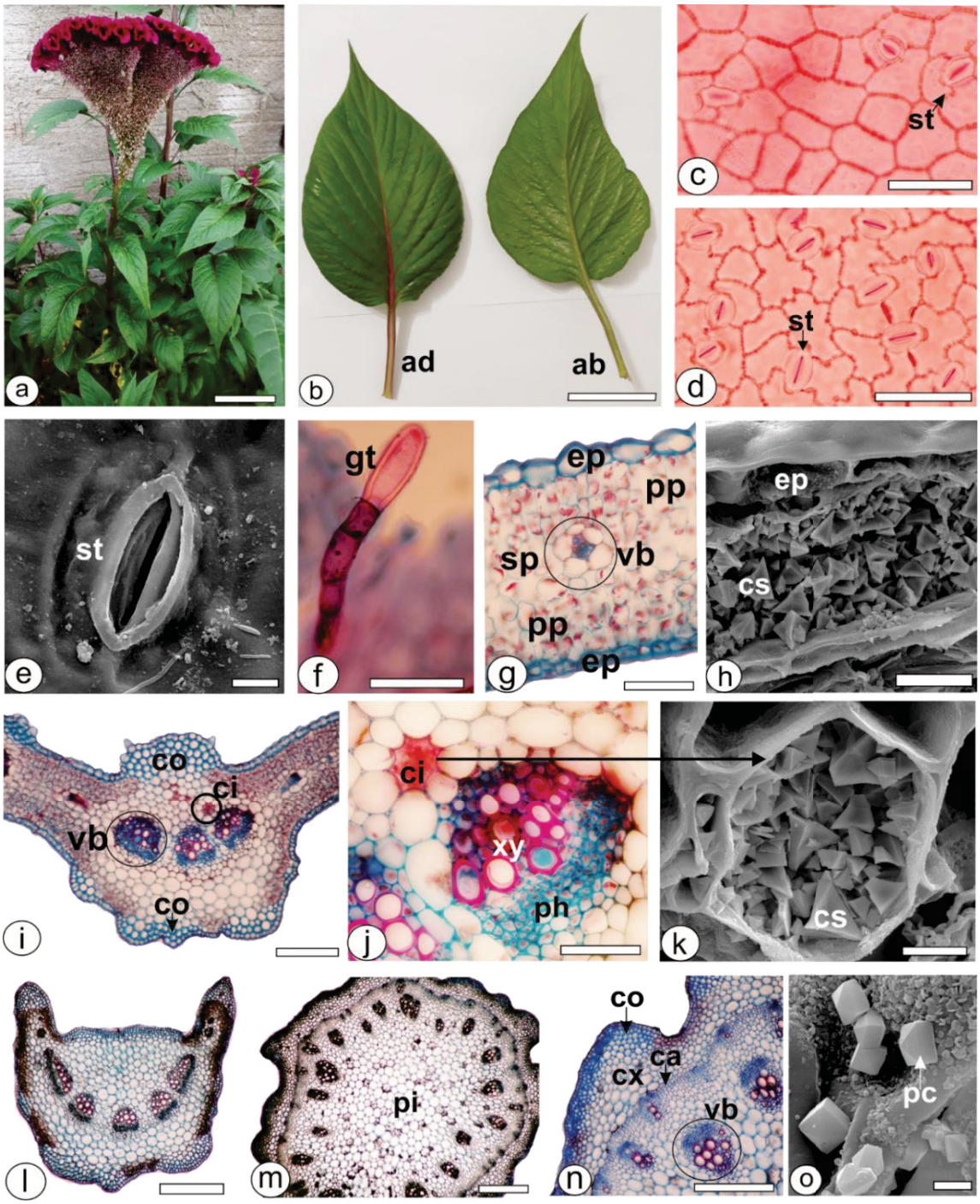

Figure 1

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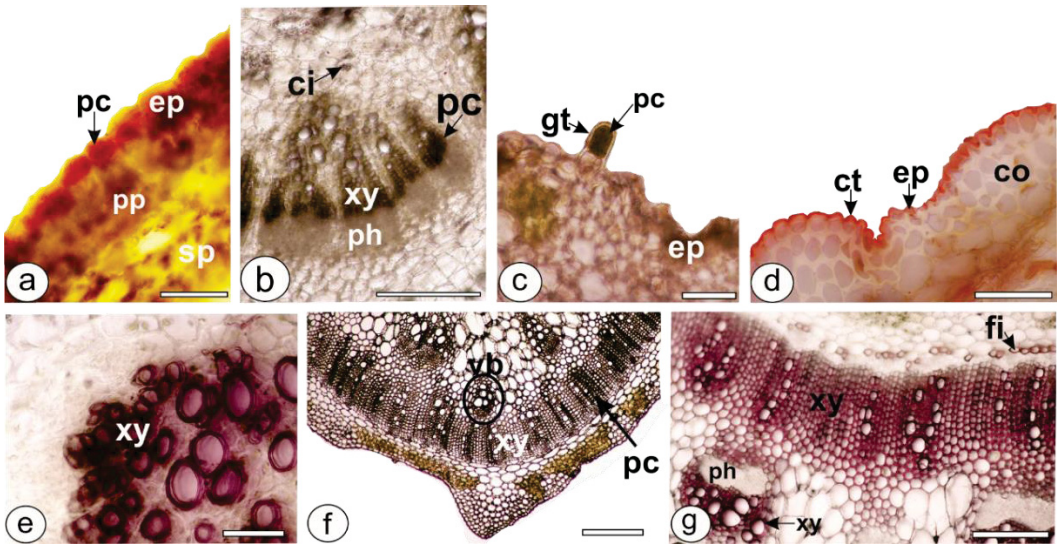

Figure 2

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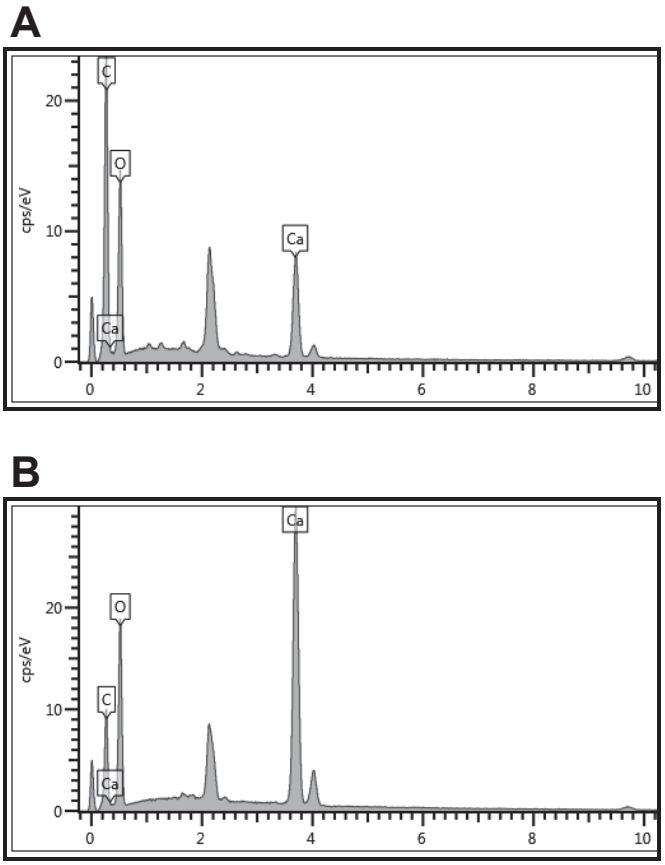

Figure 3

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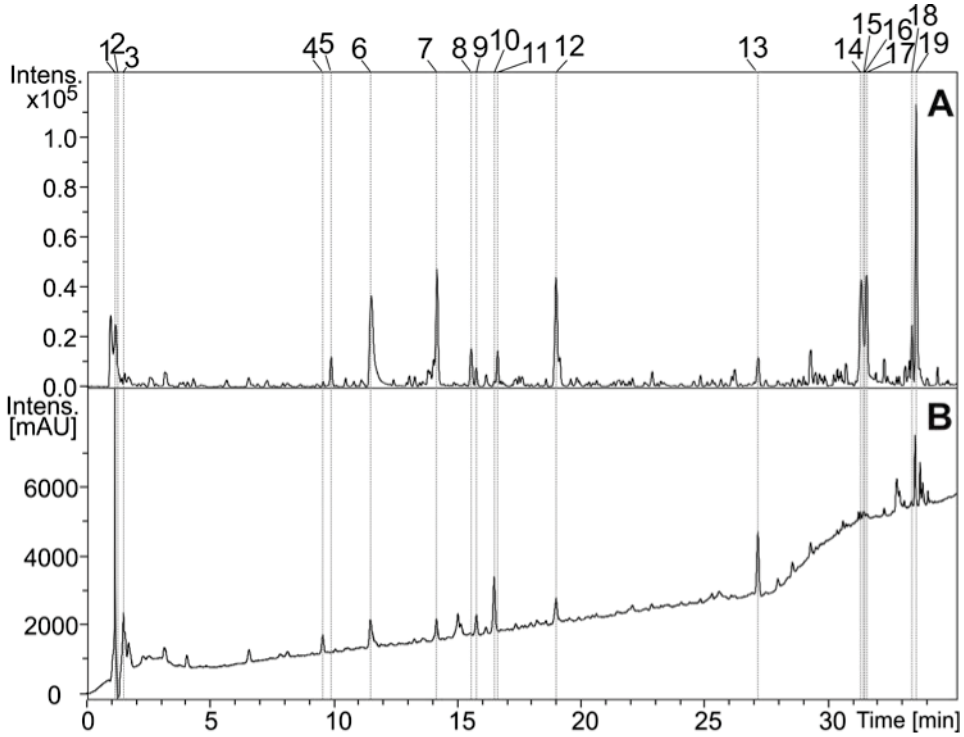

Figure 4

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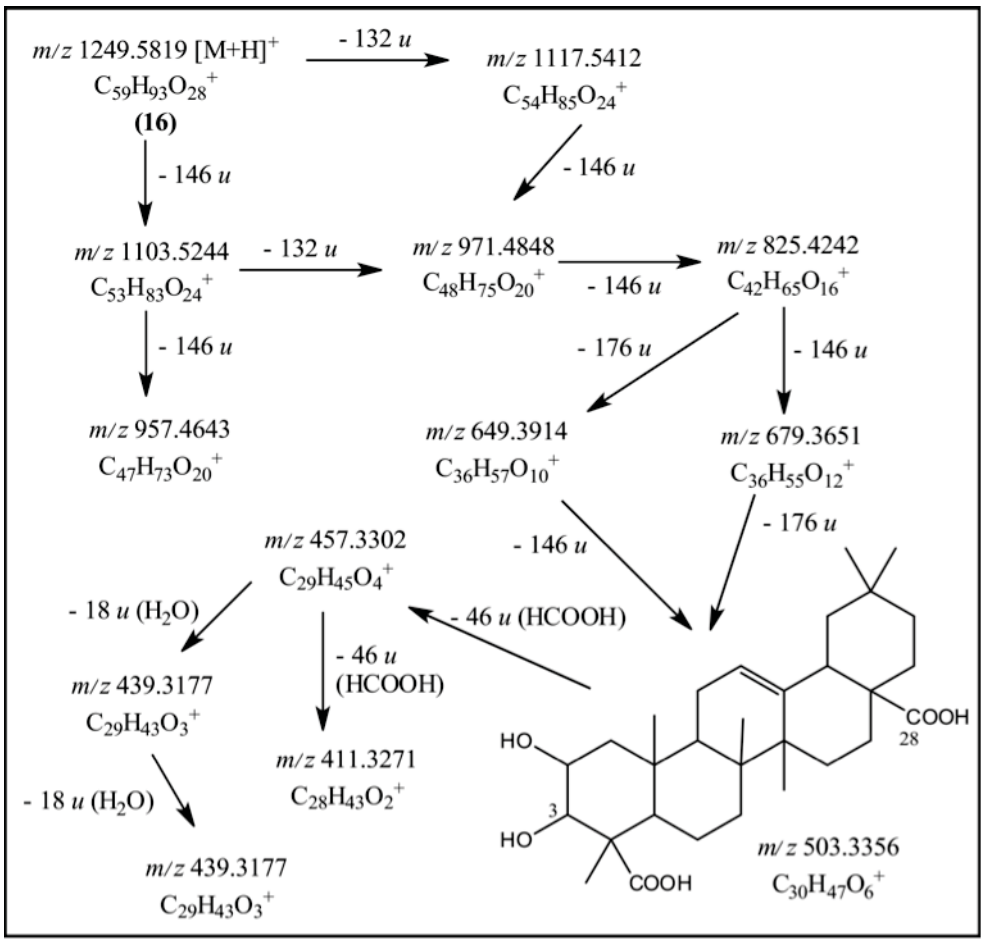

Figure 5

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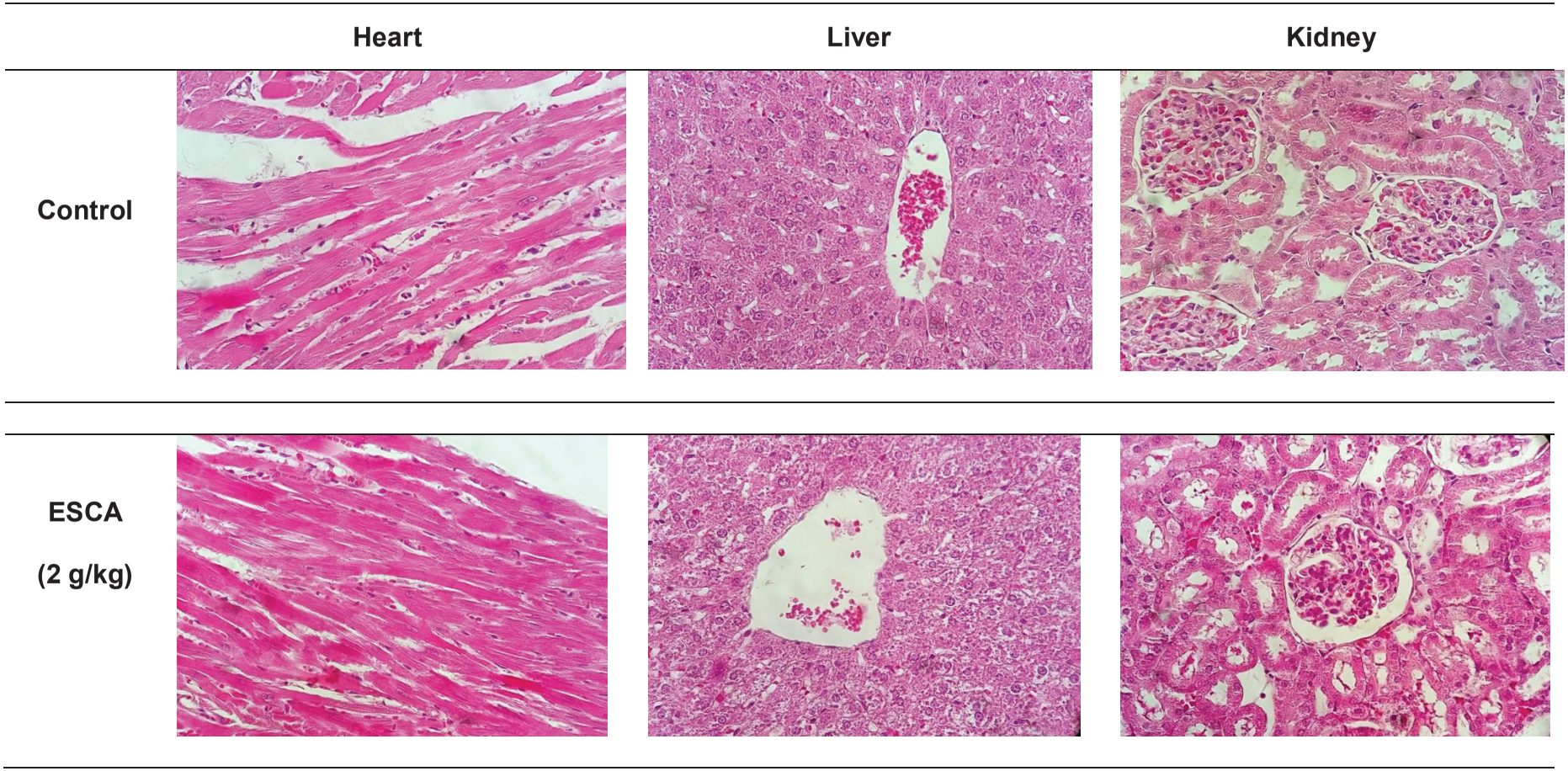

Figure 6

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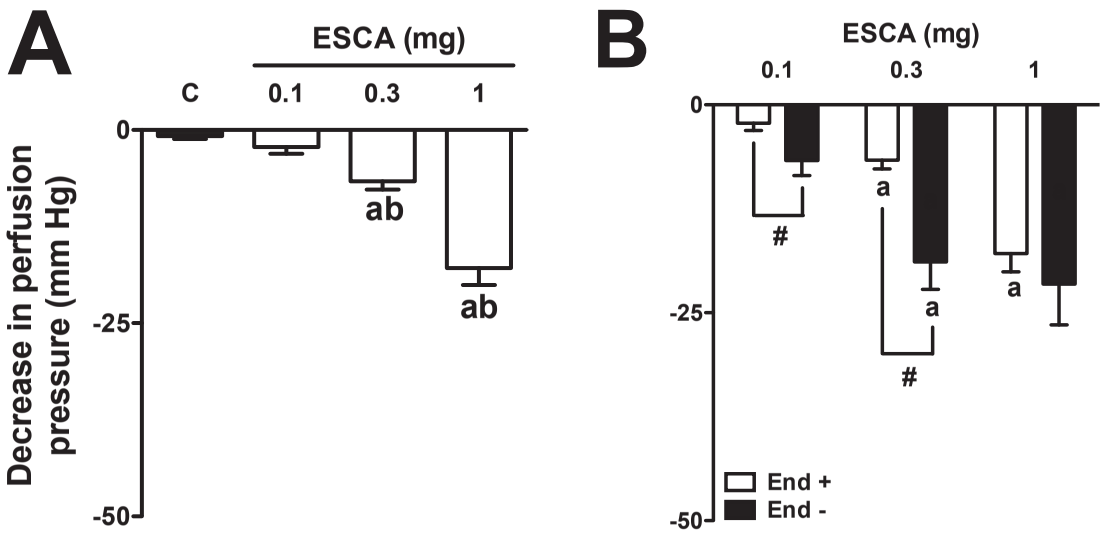

Figure 7

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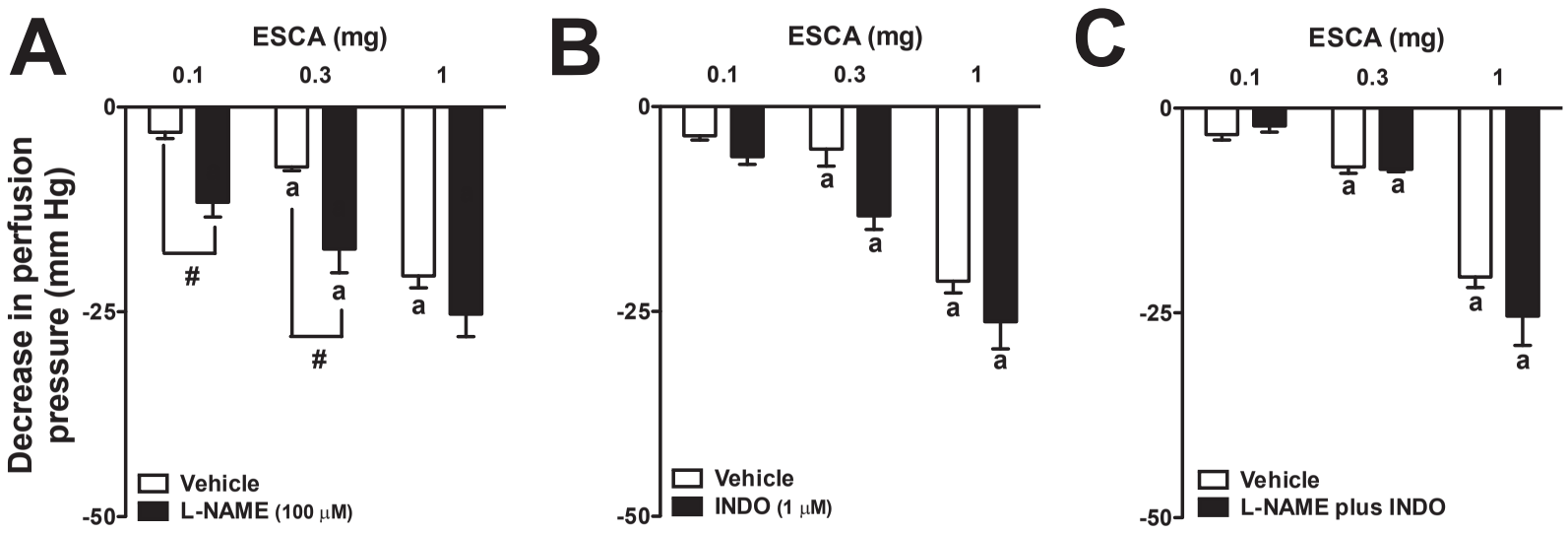

Figure 8

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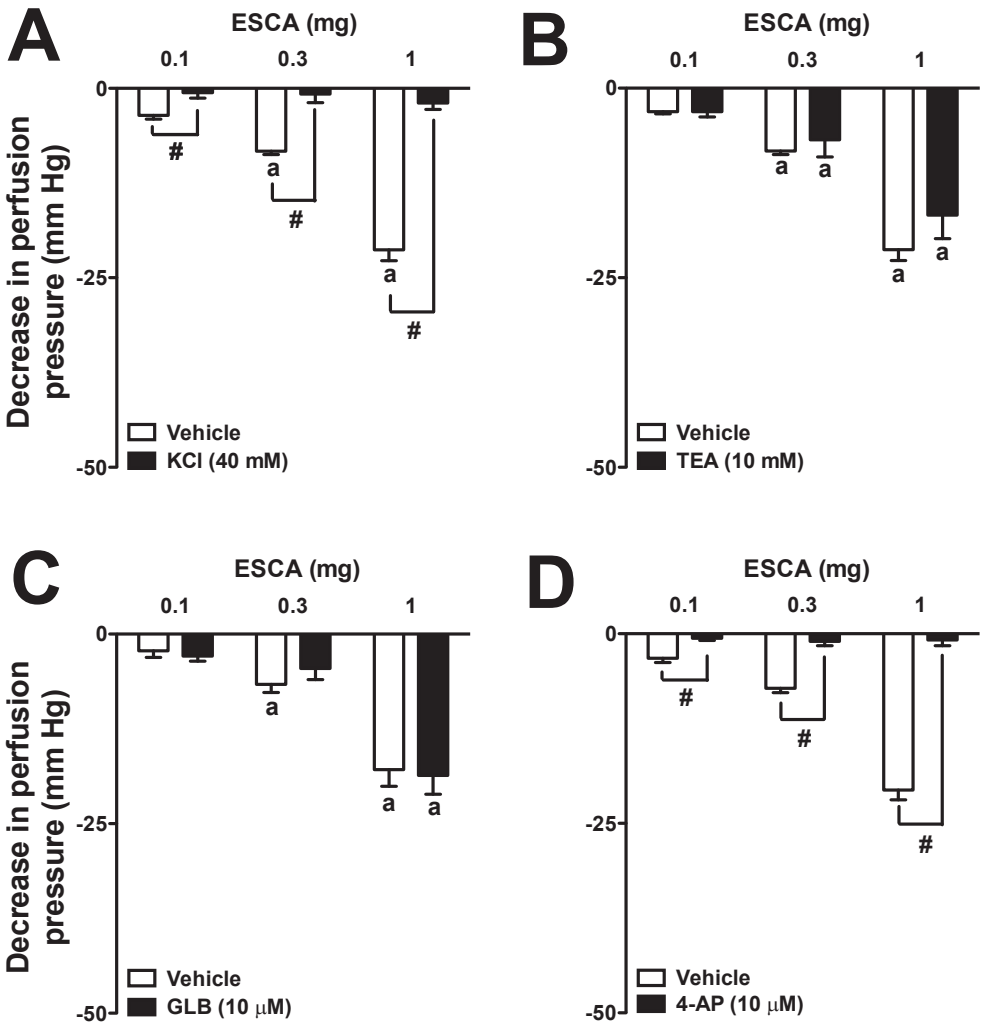

Figure 9

Tolouei et al.

Table 1. Identification of the constituents of the ethanol soluble fraction from *Celosia argentea* (ESCA) by LC-DAD-MS/MS.

Peak	RT (min)	Compound	UV (nm)	MF	Negative mode (*m/z*)		Positive mode (*m/z*)	
					MS $[M-H]^-$	MS/MS	MS $[M+H]^+$	MS/MS
1	1.1	Gluconic acid (isomer)	-	$C_6H_{12}O_7$	195.0510	-	197.0656	-
2	1.2	*O*-pentosyl di-hexoside	-	$C_{17}H_{30}O_{15}$	473.1031	173	475.1657	-
3	1.5	Citric acid	-	$C_6H_8O_7$	191.0197	-	193.0443	-
4	9.6	Methylprotocatechuic acid	260, 290	$C_8H_8O_4$	167.0347	-	169.0482	-
5	9.9	Unknown (megastigmane derivative)	-	$C_{13}H_{22}O_5$	257.1394	155	259.1540	-
6	11.5	Unknown	255, 290	$C_{18}H_{14}O_{10}$	389.0514	183, 165	391.1481	185, 167
		Unknown (Phenolic derivative)	258	$C_8H_8O_5$	183.0299	165	185.0444	-
7	14.2	Dihydroferulic acid	280	$C_{10}H_{12}O_4$	195.0663	193, 180, 151	219.0615[a]	-
8	15.6	Unknown (megastigmane derivative)	-	$C_{13}H_{22}O_4$	241.1445	197	243.1591	-
9	15.8	Ferulic acid derivative	299, 322	$C_{19}H_{30}O_{10}$	417.1768	399, 193, 151	441.1699[a]	-
10	16.5	Unknown (megastigmane derivative)	246	$C_{13}H_{18}O_3$	221.1191	-	223.1312	
11	16.6	Unknown	-	$C_{19}H_{30}O_{10}$	417.1786	-	-	-
12	19.0	Syringaldehyde		$C_9H_{10}O_4$	181.0516	153	183.0642	-
13	27.4	Peptide	310	$C_{45}H_{49}N_3O_6$	726.3573	-	728.3656	563, 287
14	31.3	*O*-glucuronyl dideoxyhexosyl dipentosyl Medicagenic acid	-	$C_{58}H_{90}O_{28}$	1233.5490	677, 193	1235.5651	971, 957, 825, 679, 649, 503, 457, 439, 421 411
15	31.4	*O*-glucuronyl di-deoxyhexosyl pentosyl Medicagenic acid	-	$C_{53}H_{82}O_{24}$	1101.5050	677, 193	1103.5261	971, 957, 825, 679, 649, 503, 457, 411
16	31.5	*O*-glucuronyl tri-deoxyhexosyl pentosyl Medicagenic acid	-	$C_{59}H_{92}O_{28}$	1247.5683	925, 823, 339, 175	1249.5819	1117, 1103, 971, 957, 825, 679, 649, 503, 457, 439, 421, 411
17	31.6	*O*-hexosyl tri-deoxyhexosyl pentosyl Medicagenic acid	-	$C_{57}H_{86}O_{29}$	1233.5180	1101, 809, 325, 307, 193	1235.5340	1103, 1089, 957, 811, 793, 765, 649, 503, 457, 439, 421, 411
18	33.4	*O*-glucuronyl Medicagenic acid	-	$C_{36}H_{54}O_{12}$	677.3570	501, 193	679.3718	503, 457, 439, 421, 411
19	33.6	*O*-glucuronyl pentosyl Medicagenic acid	-	$C_{41}H_{62}O_{16}$	809.3935	677, 501, 193	811.4097	503, 457, 439, 421, 411, 393

RT: retention time; MF: molecular formula; [a]: $[M+Na]^+$

Table 2.

Body weight gain, food and water consumption of rats treated orally with ethanol soluble fraction from *Celosia argentea* (ESCA)

Parameter	Control	ESCA (30 mg/kg)	ESCA (300 mg/kg)	ESCA (2 g/kg)
Initial weight (g)	212.75 ± 13.00	209.62 ± 19.89	213.25 ± 23.21	207.625 ± 19.27
Final weight (g)	223.37 ± 20.23	233.00 ± 30.58	232.12 ± 26.06	226.75 ± 24.74
Body weight gain (g)	10.62 ± 9.84	23.37 ± 12.28[a]	18.87 ± 7.19	19.12 ± 6.66
Body weight gain (%)	4.88 ± 4.28	10.81 ± 5.40[a]	8.85 ± 3.03	9.08 ± 2.47
Food intake (g/day)	77.83 ± 6.83	73.16 ± 9.80	72.83 ± 6.85	66.66 ± 22.48
Water intake (mL/day)	126.66 ± 21.36	129.16 ± 22.00	134.16 ± 14.28	128.33 ± 37.63

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Values are expressed as mean ± S.E.M. (n = 8) in comparison to the control group (a: $p \leq 0.05$).

Table 3.

Relative organ weight of rats treated orally with ethanol soluble fraction from *Celosia argentea* (ESCA)

Parameter	Control	ESCA (30 mg/kg)	ESCA (300 mg/kg)	ESCA (2 g/kg)
Heart (%)	0.37 ± 0.02	0.37 ± 0.03	0.35 ± 0.02	0.37 ± 0.02
Lung (%)	0.76 ± 0.20	0.75 ± 0.15	0.81 ± 0.17	0.76 ± 0.25
Liver (%)	4.52 ± 0.23	4.48 ± 0.25	4.44 ± 0.23	4.34 ± 0.28
Spleen (%)	0.24 ± 0.30	0.24 ± 0.02	0.25 ± 0.03	0.26 ± 0.04
Right kidney (%)	0.39 ± 0.40	0.41 ± 0.03	0.40 ± 0.02	0.41 ± 0.04
Left kidney (%)	0.37 ± 0.20	0.38 ± 0.03	0.37 ± 0.01	0.38 ± 0.03
Right ovary (%)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
Left ovary (%)	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Uterus (%)	0.26 ± 0.07	0.26 ± 0.14	0.25 ± 0.12	0.30 ± 0.17

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Values are expressed as mean ± S.E.M. (n = 8) in comparison to the control group (a: $p \leq 0.05$).

Table 4.

Effect of acute oral administration of ethanol soluble fraction obtained from *Celosia argentea* (ESCA) on the urinary volume, pH and density in 8 and 24-hour urine samples

Group	Urine volume (ml/100g/8h)	Urine volume (ml/100g/24h)	pH (8 h)	pH (24 h)	Density (8 h)	Density (24 h)
Control	3.61 ± 0.61	7.22 ± 0.89	7.33 ± 0.04	9.65 ± 0.10	1018.66 ± 0.49	1034.66 ± 1.52
HCTZ (25 mg/kg)	5.59 ± 0.42[a]	8.47 ± 0.61	8.60 ± 0.08[a]	9.50 ± 0.16	1016.33 ± 0.95	1031.66 ± 1.81
ESCA (30 mg/kg)	3.58 ± 0.41	6.44 ± 0.59	9.75 ± 0.22[a]	10.95 ± 0.22[a]	1017.00 ± 1.69	1032.66 ± 2.40
ESCA (100 mg/kg)	3.24 ± 0.39	5.93 ± 0.91	8.65 ± 0.12[a]	10.85 ± 0.13[a]	1020.66 ± 1.76	1036.16 ± 2.15
ESCA (300 mg/kg)	3.61 ± 0.48	6.48 ± 0.77	8.42 ± 0.30[a]	10.68 ± 0.11[a]	1022.00 ± 2.28	1036.80 ± 2.33

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Values are expressed as mean ± S.E.M. (n = 6) in comparison to the control group (a: $p \leq 0.05$). HCTZ: hydrochlorothiazide.

Table 5.

Effect of acute oral administration of ethanol soluble fraction obtained from *Celosia argentea* (ESCA) on urinary electrolyte excretion in 8 and 24-hour urine samples

Group	$EINa^+$ (µEq/min/100g) (8 h)	$EINa^+$ (µEq/min/100g) (24 h)	EIK^+ (µEq/min/100g) (8 h)	EIK^+ (µEq/min/100g) (24 h)	$EICl^-$ (µEq/min/100g) (8 h)	$EICl^-$ (µEq/min/100g) (24 h)
Control	1.03 ± 0.19	2.06 ± 0.29	0.46 ± 0.09	0.92 ± 0.14	1.18 ± 0.22	2.36 ± 0.36
HCTZ (25 mg/kg)	1.88 ± 0.17[a]	2.30 ± 0.19	0.75 ± 0.05[a]	0.83 ± 0.06	1.99 ± 0.23[a]	2.75 ± 0.28
ESCA (30 mg/kg)	0.91 ± 0.10	1.77 ± 0.07	0.46 ± 0.03	2.42 ± 0.23[a]	0.99 ± 0.09	3.43 ± 0.17[a]
ESCA (100 mg/kg)	1.10 ± 0.16	1.60 ± 0.13	0.48 ± 0.05	1.77 ± 0.24[a]	1.33 ± 0.19	2.98 ± 0.35
ESCA (300 mg/kg)	1.19 ± 0.06	1.99 ± 0.07	0.77 ± 0.03[a]	2.84 ± 0.09[a]	1.58 ± 0.12	4.22 ± 0.26[a]

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Values are expressed as mean ± S.E.M. (n = 6) in comparison to the control group (a: $p \leq 0.05$). HCTZ: hydrochlorothiazide.

Table 6.

Effect of acute oral administration of ethanol soluble fraction obtained from *Celosia argentea* (ESCA) on arterial pressure and heart rate

Group	SBP (mm Hg)	DBP (mm Hg)	MAP (mm Hg)	HR (bpm)
Control	104 ± 6.9	69 ± 3.3	85 ± 4.2	331 ± 35
HCTZ (25 mg/kg)	88 ± 8.0[a]	57 ± 2.4[a]	71 ± 2.3	341 ± 33
ESCA (30 mg/kg)	106 ± 5.8	73 ± 3.3	90 ± 3.7	302 ± 32
ESCA (100 mg/kg)	85 ± 2.0[a]	47 ± 4.4[a]	64 ± 4.0[a]	248 ± 30[a]
ESCA (300 mg/kg)	93 ± 4.2	62 ± 3.3	77 ± 5.7	327 ± 46

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Values are expressed as mean ± S.E.M. (n = 6) in comparison to the control group (a: $p \leq 0.05$). HCTZ: hydrochlorothiazide.

5 ARTIGO CIENTÍFICO 2: *Anchietea pyrifolia* (Mart.) G.Don as a cardiovascular-endowed species.

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Abstract:

Background: Anchietea pyrifolia (Mart.) G.Don (Violaceae), popularly known as “suma roxa” or “cipó suma”, is a native plant widely distributed in the Brazilian Cerrado. In folk medicine, several preparations of *A. pyrifolia* are used as anti-rheumatism, antibiotic, depurative and diuretic. However, there are no reports in the literature proving its pharmacological effects, as well as no data demonstrating the safety of this species.

Purpose: To perform a detailed botanical, phytochemical, toxicological, and pharmacological investigation of *A. pyrifolia* in Wistar rats and investigate the molecular mechanisms adjacent to their pharmacological effects.

Methods: At first, a morpho-anatomical characterization of *A. pyrifolia* leaves and stems was performed. Then, a purified infusion (ESAP) was obtained and chemically characterized by LC-DAD-MS. Furthermore, an oral acute toxicity study was performed, and the acute and prolonged diuretic and hypotensive effects of three different doses of ESAP (30, 100 and 300 mg/kg) were also evaluated in Wistar rats. Finally, the vasodilatory response of ESAP in mesenteric vascular beds (MVBs) and its involvement with nitric oxide/cGMP and prostaglandin/cAMP pathways as well as potassium channels were investigated.

Results: The main secondary metabolites identified from ESAP were O-glycosylated flavonoids, chlorogenic acids and others phenylpropanoic acid derivatives. ESAP caused no toxic effects in female rats nor increased urinary excretion in male rats after acute administration. However, ESAP significantly reduced renal elimination of sodium, potassium and chloride after prolonged exposure. High-dose (300 mg/kg) of ESAP was able to promote significant acute hypotension, without affecting blood pressure levels after prolonged use. Moreover, its cardiovascular effects appear to

be involved with the calcium-activated potassium channels activation in resistance blood vessels.

Conclusion: This study turns the lights on the preclinical safety and efficacy of *A. pyrifolia* as a hypotensive agent. Apparently, these effects are involved with the opening of the calcium-activated potassium channels in smooth muscle from resistance vessels contributing to reduce global peripheral resistance and blood pressure.

Keywords: Diuretic; hypotensive; mesenteric vascular bed; potassium channels; Violaceae

Abbreviations: 4-AP, 4-aminopyridine; ACh, acetylcholine chloride; ANOVA, analysis of variance; AP, arterial pressure; Cl^-, chloride; CVD, cardiovascular disease; DBP, diastolic blood pressure; EDS, energy disperse system; EDTA, ethylenediaminetetraacetic acid; EI, excretion load; ESAP, ethanol soluble fraction from *Anchietea salutaris*; FESEM, field emission scanning electron microscopy; GLB, glibenclamide; HCl, hydrogen chloride; HCTZ, hydrochlorothiazide; HR, heart rate; K^+, potassium; KCl, potassium chloride; L-NAME, Nω-Nitro-L-arginine methyl este, MAP, mean arterial pressur; MVB, mesenteric vascular bed; Na^+, sodium; NaCl, sodium chloride**;** OECD, The Organisation for Economic Co-operation and Development; pH, potential of hydrogen; Phe, phenylephrine; PP, perfusion pressure; PSS, physiological saline solution; S.E.M., standard error of the mean, SAH, systemic arterial hypertension; SBP, systolic blood pressure; TEA, tetraethylammonium; UEPG, State University of Ponta Grossa; UFGD, Federal University of Grande Dourados; UFPR, Federal University of Paraná.

1. Introduction

Hypertension is a clinical condition characterized by sustained blood pressure elevation (≥ 140 and/or 90 mm Hg). Known as the most frequent non-communicable chronic diseases in the world, hypertension affects people of all ages, reaching about 32.5% of adults and over 60% of the Brazilian elderly.[1,2]

In an attempt to reduce the incidence and minimize the effects of hypertension, drug and non-drug therapies are routinely employed. In pharmacological treatment, renin-angiotensin system inhibitors, beta-blockers, calcium channel blockers and diuretics are used as first-line agents.[2] However, patients' adherence to conventional treatments is still low and about one-third of the population present controlled blood pressure levels. This fact can be understood by the incidence of adverse effects to some patients or difficulties of access to medicines by the low-income population that uses the Brazilian Unified Health System. Thus, even though some classes of antihypertensive drugs are distributed free-of-charge to the population by the government, these drugs are not always available.[3]

Therefore, the use of herbal products appears as an alternative to minimize unwanted side effects, avoid irrational consumption of synthetic drugs and, if properly prescribed, solve problems in primary care.[2] The search for natural medicines is intensifying and, for this reason, medicinal plants have become the focus of numerous studies in order to evaluate their safety and efficacy, aiming the development of new herbal medicines.[4] For this reason, the Brazilian cerrado has been the main target of this research, since it is the second largest biome in the area, representing around 23% of the country's surface.[5]

Anchietea pyrifolia (Mart.) G.Don (Violaceae) syn. *Anchietea salutaris* A.St.-Hil., popularly known as “suma roxa” or “cipó suma”, is a native plant widely distributed in the Brazilian Cerrado. This species was first described by Saint-Hilaire in 1824 together with the genus *Anchietea.*[6] Several preparations of *A. pyrifolia* are used in folk medicine as anti-rheumatism, antibiotic,[7] depurative and diuretic.[8] Previous phytochemical studies presented flavonoids, saponins and tannins as the main constituents from its leaves.[9] Moreover, a recent research has shown that phytol, a diterpene alcohol generally found in plants, was identified as one of the compounds in the active fraction o *A. pyrifolia* that was able to inhibit the histamine release.[10]

Although leaves infusion of *A. pyrifolia* is used in traditional medicine as it presents diuretic effects, there are no reports in the literature related to its pharmacological effects, as well as no data demonstrating the safety of this species. Therefore, we performed a detailed botanical, phytochemical, toxicological, and pharmacological investigation of *A. pyrifolia* in Wistar rats after acute and prolonged exposure. Furthermore, we investigated the molecular mechanisms adjacent to their pharmacological effects using isolated mesenteric vascular beds (MVBs).

2. Materials and Methods

2.1. Drugs and solvents

The following drugs were used: xylazine, ketamine hydrochloride (Syntec, São Paulo, SP, Brazil), heparin (Hipolabor, Belo Horizonte, MG, Brazil), 4-aminopyridine, acetylcholine, dextrose, ethylenediaminetetraacetic acid, hydrochlorothiazide, indomethacin, Nω-Nitro-L-arginine methyl ester, phenylephrine, sodium

deoxycholate, tetraethylammonium, NaCl, KCl, NaHCO3, MgSO4, CaCl2, and KH2PO4 (Sigma-Aldrich, St. Louis, MO, USA). All other reagents were obtained in analytical grade.

2.2. Plant material and extract preparation

Anchietea pyrifolia (Mart.) G.Don leaves were collected in the municipality of Caarapó, Mato Grosso do Sul - Brazil, at 458 m above sea level (-22.618107, -54.839976), in February 2017. A voucher specimen was authenticated by Dra. Maria do Carmo Vieira and deposited in the Herbarium DDMS of the Federal University of Grande Dourados (UFGD) under number 2211. The plant name is in accordance with the on-line database published by “The Plant List”, accessed on August 13, 2018.

Leaves were sanitized with tap water and dried in a circulating air oven at 48°C. The infusion was made by pouring 1 L of boiling water (97 °C) on dried and pulverized leaves of *A. pyrifolia* (60 g). The mixture obtained was naturally cooled to room temperature for approximately 5 hours. Subsequently, the plant material was filtered and the infusion was treated with 3 volumes of ethanol, originating a precipitate and an ethanol soluble fraction (ESAP). ESAP samples were then lyophilized, stored in a freezer at −18 °C and used for the phytochemical screening and pharmacological studies.

2.3. Pharmacobotanical assays

2.3.1. Anatomical study

Leaves and stems of *A. pyrifolia* were fixed in formalin–acetic acid–alcohol (FAA) solution[11] for 3 days, washed in distilled water and then stored in 70% ethanol

(v/v).[12] The fixed samples of mature leaves and stems from the third node were hydrated and cut by hand using razor blades. The sections were double-stained with basic fuchsin and astra blue.[13] For detachment of epidermis, fixed leaves were washed in distilled water, immersed in a solution of commercial bleach (5.25% sodium hypochlorite) until translucent. Then, the samples were immersed briefly in a diluted acetic acid solution, washed with water and stained in 1% safranin in ethanol 50%. Slides were mounted in glycerin 50% solution and sealed with transparent nail polish.[14]

The histochemical analysis was performed in sections of fresh leaves and stems with razor blades for the detection of lignin, lipids, starch, and phenolic compounds. Standard solution of phloroglucinol/HCl was used to identify lignified tissues[15], Sudan III to detect the occurrence of lipophilic compounds[16], iodine solution (iodine potassium iodide) to stain starch,[12] ferric chloride,[11] and potassium dichromate[17] to detect the presence of phenolics. Blank sections were used for comparative analysis. The prepared specimens were observed and photomicrographs were prepared using an Olympus CX31 microscope equipped with Olympus C-7070 digital camera.

2.3.2. Field Emission Scanning Electron Microscopy (FESEM) and Energy-dispersive X-ray Spectroscopy (EDS) analyses

The plant material fixed in FAA was sectioned and passed through a series of ethanol solutions (80%, 90% and 100%). The fully dried sections were mounted on aluminum stubs with double-sided adhesive tapes and then coated with gold using a Quorum SC7620 sputter coater. The sections were analyzed and imaged using a Mira 3 Tescan FESEM (Oxford Instruments, Oxford, UK) in high vacuum mode at 15

kV accelerating voltage. Qualitative and quantitative X-ray microanalyses were made for certain crystals using an EDS detector attached to the FESEM. The FESEM and EDS analyses were performed at the multi-user laboratory (c-LABMU) in the State University of Ponta Grossa.

2.4. LC-DAD-MS analyses

ESAP was analyzed by LC-DAD-MS using a UFLC Shimadzu Prominence coupled to diode array detector and mass spectrometry (MicroOTOF-Q III Bruker Daltonics). The analyses were performed on Kinetex column chromatographic C18 (2.6 µ, 100A, 150 × 2.1 mm, Phenomenex), applying the same parameters described by Dembogurski.[18] ESAP was prepared with methanol and deionized water (7:3, v/v) at concentration 1 mg/mL, filtered on Millex filters (0.22mm×13 mm PTFE, Millipore) and 2 µL were injected in the chromatographic system. The MS analyses were performed in negative and positive ion modes, and the constituents were identified based on the spectral data (UV, MS and MS/MS) compared to data reported in the literature.

2.5. Safety and efficacy assessments

2.5.1. Animals

All procedures involving animals were performed in accordance with the Ethical Principles in Animal Research and previously approved by the Ethics Committee in Animal Experimentation from the Federal University of Paraná (protocol: 05/2017) and Federal University of Grande Dourados (protocol: 21/2017). Male and female Wistar rats, at the age of 8-12 weeks, were housed under standard and controlled conditions (22 ± 2°C; 12-h light/dark cycle, 50 ± 20% of relative

humidity) and had free access to water and commercial food. Before the onset of the experiments, animals were left to acclimatize to new laboratory conditions for ten days.

2.5.2. Study design

Animals were randomly divided into four groups (*n* = 8) for the acute toxicity test and into five groups (*n* = 6) for the diuretic and blood pressure investigations. Rats were treated with a single dose (acute toxicity and acute diuretic activity) or once a day, for seven days (prolonged investigations), with ESAP (30, 100, and 300 mg/kg), hydrochlorothiazide (HCTZ; 25 mg/kg) or vehicle (filtered water 1 mL/100 g) by oral gavage.

2.5.3. Safety assessment

2.5.3.1. Acute toxicity test

ESAP acute oral toxicity was evaluated in female rats according to the protocol 425 established by the Organization for Economic Co-operation and Development (OECD) in 2008.[19] After a 12-hour period of fasting (only food was withheld overnight), three different doses (30, 300 and 2000 mg/kg) of ESAP were administered to rats (*n* = 8) by oral gavage. Vehicle (filtered water) was administered to the control group (1 mL/100 g). Food was only given to rats 1 hour after treatment. Animals were closely observed during the first 24 hours for any signs of death or toxicity and thereafter, for 14 consecutive days. Body weight gain, food and water consumption were daily observed and registered. In order to observe possible changes in behavior induced by ESAP, rats were carefully observed for general health and clinical signs of toxicity according to the five parameters of the Hippocratic

screening described by Malone and Robichaub in 1962. The parameters observed were: conscious state, activity and coordination of motor system and muscle toning, activities on the central nervous system, corneal and headset reflexes and activities on the autonomic nervous system.[20]

The night before euthanasia, animals were left under overnight fasting and had free access to water. On day 15 after treatment, animals were euthanized by isoflurane anesthesia (inhalation) followed by exsanguination. Vital (heart, lung, spleen, liver, kidney) and reproductive (uterus and ovaries) organs were removed, weighed, macroscopically examined and the relative organ weight calculated. Heart, liver and kidneys samples were sent to histopathological analyses that were performed by two veterinary pathologists from UFGD.

2.5.4. Pharmacological investigations

2.5.4.1. Acute diuretic activity

This procedure was performed according to a methodology described by Gasparotto Junior.[21] Fasted male rats were divided into five groups (n = 6) and received a single dose of 5-mL/100 g of physiological saline solution (0.9% NaCl) to impose controlled water and salt balance. Afterward, each group received a single dose of ESAP (30, 100, and 300 mg/kg), hydrochlorothiazide (HCTZ; 25 mg/kg) or vehicle (filtered water 1 mL/100 g) by oral gavage. Immediately after treatments, animals were placed in metabolic cages for 24 hours with free access to commercial feed and filtered water. Urine was collected with the aid of a graduated cylinder and the volume was recorded at 8 and 24 hours (expressed as mL/100 g of body weight). Urinary sodium (Na^+), potassium (K^+) and chloride (Cl^-) levels were quantified in an ion selective meter (COBAS INTEGRA 400 plus; Roche®). Excretion load (El) of Na^+,

K^+ and Cl^- was obtained by multiplying the concentration of electrolytes (mEq/l) by the urinary flow (mL/min). Results are expressed as µEq/min/100g. pH was determined on fresh urine samples using a digital pH meter (Q400MT; Quimis Instruments, Brazil). Density was estimated by a handheld refractometer (NO107; Nova Instruments, Brazil).

2.5.4.2. Prolonged diuretic activity

Three different doses of ESAP (30, 100 and 300 mg/ kg), hydrochlorothiazide (HCTZ; 25 mg/kg) or vehicle (filtered water 1 mL/100 g) were daily administered (oral gavage) to different groups of rats (*n* = 6) for 7 days. All animals were individually placed in metabolic cages during the experimental period. The total amount of urine was collected every 24 hours and the following parameters were analyzed: pH, density, volume and electrolyte concentrations (Na^+, K^+ and Cl^-).

2.5.4.3. Arterial pressure and heart rate evaluation

For measurement after acute treatment thirty normotensive male rats were divided into five experimental groups (*n* = 6) and anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) through intramuscular route (IM). After anesthesia, a bolus injection of heparin (50 IU) was administered subcutaneously. Tracheotomy was performed in order to allow animals to spontaneously breathe. The left carotid artery was then 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 blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR). After this procedure, different groups of rats received ESAP (30, 100 or 300 mg/kg),

HCTZ (25 mg/kg) or water (1 mL/100 g of body weight) intraduodenally. Changes in SBP, DBP, MAP, and HR were recorded for 20 minutes after treatments.

For measurement after prolonged treatment, different groups of normotensive rats (*n* = 6) received three different doses of ESAP (30, 100 and 300 mg/ kg), hydrochlorothiazide (HCTZ; 25 mg/kg) or vehicle (filtered water 1 mL/100 g) by oral route for 7 days. After the treatments the rats were anesthetized and prepared for the measurement of the hemodynamic parameters as previously described. Changes in SBP, DBP, MAP, and HR were recorded for 5 minutes after the hemodynamic stabilization period (15 minutes). At the end of the experiments, blood samples were collected directly from left carotid artery in order to analyze serum levels of urea, creatinine, Na+ and K+. Then, the animals were euthanized with an overdose of isoflurane anesthetic (inhalation, 2-3 times of the anesthesic dose).

2.5.4.4. Effects on peripheral vascular resistance

Firstly, normotensive male rats were anesthetized with ketamine and xylazine (100 and 20 mg/kg, respectively, by intraperitoneal route). MVBs were isolated and prepared according to previous methods described by McGregor in 1965.[22] MVBs (*n* = 5) were placed in a water-jacketed organ bath and perfused (at 4 mL/min) 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) 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). To check the endothelial viability of preparations, different MVBs were continuously perfused with

PSS plus Phe (3 μM) to induce a 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, in order to confirm the 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.

MVBs with or without functional endothelium were continuously perfused with PSS plus Phe (3 μM). After the stabilization period, different preparations received bolus injections containing ESAP (0.1, 0.3, and 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 a minimal interval of 3 min between doses. 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 5 mM; a non-selective calcium-sensitive [KCa] K^+ channel blocker) [43], 4-aminopyridine (4-AP 10 μM; a voltage-dependent [KV] K^+ channels blocker), and glibenclamide (GLB 10 μM; a selective Kir6.1 ATP-sensitive [KATP] K^+ channels blocker). After 15 minutes of continuous perfusion, ESAP (0.1, 0.3, and 1 mg) was injected again into the perfusion system. The ability of ESAP to reduce PP in the presence and absence of different inhibitors was evaluated.

2.6. Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test, or by Student's t-test when applicable. The results are expressed as mean ± standard error of the mean (S.E.M.) and *p*-value of less than 0.05 was considered statistically significant. Graphs were drawn and statistical analyses were performed using GraphPad Prism Mac 6.0h.

3. Results

3.1. Anatomical profile and histochemical characterization

Anatomically, the leaves of *Anchietea pyrifolia* (Mart.) G.Don (Figure 1A and B) from the surface view show wavy anticlinal walls on both epidermises (Figure 1C and D). Epidermal cells have thickened walls, prominently raised above on leaf surface (Figure 1H). The leaves are hypostomatic and the stomata are paracytic (Figure 1D). Hypostomatic leaves are frequently observed in members of Violaceae family.[23] Non-glandular trichomes are present on both leaf surfaces. These trichomes are unicellular, conic (Figure 1E) and show thick walls and rugose cuticle (Figure 1M). They measure up to 40 μm in length (Figure 1E and M). Crystal sand showing bipyramidal shapes were observed externally on the adaxial leaf surface (Figure 1F and G).

The leaf, in cross-section, presents one-layered epidermis covered by thin and slightly striate cuticle (Figure 1I). The cells in the upper surface are bigger than in the lower surface and anticlinally elongated (Figure 1I and J). The mesophyll is dorsiventral and is formed by 1-2 layers of the palisade and about 4 layers of spongy parenchyma (Figure 1I and J). The veinlets traversing the mesophyll region are represented by small collateral vascular bundles enclosed by parenchymatic sheath (Figure 1I).

The midrib, in transection, has biconvex shape (Figure 1J). The epidermis is uniseriate and covered by slightly thick and striate cuticle. Crystal sand with the same features as previous described for the leaf blade is found on the epidermis. Non-glandular trichomes are also observed. Beneath the epidermis, up to 4 layers of angular collenchyma is found in the adaxial side and 1 layer in the abaxial side. In the adaxial side, chlorenchyma is continuous. Some crystalliferous idioblasts containing prismatic crystals or druses and tiny starch grains are found in the ground parenchyma. The vascular system is represented by unique and central collateral vascular bundle arranged in open arc (Figure 1J).

In cross-section, the petiole is rounded in shape with slightly raise on the adaxial side. A thin and striated cuticle covers the single-layered epidermis that also shows crystal sand on the outer surface. Beneath the epidermis, a continuous angular collenchyma formed by up to 3 layers is found. The stele is represented by one collateral vascular bundle in open arc. Cambia can be observed. Starch grains and crystalliferous idioblasts (Figure 2F), as described in the midrib are found in the ground parenchyma.

The stem is circular in shape. The epidermis has the same features described for leaves, showing non-glandular trichomes (Figure 1M) and crystal sand on outer surface that seem to come from stomata (Figure 1N). Beneath the epidermis, 3-layered angular collenchyma occurs as an uninterrupted ring. Crystalliferous idioblasts containing druses and prisms (Figure 1P) are found in the cortex. An endodermis bounds the internal part of the cortex (Figure 1O). The vascular cylinder shows cambia that produces inward xylem and outward phloem, and perivascular fibers caps abutting the phloem (Figure 1I and O). The pith is made up of thin-walled parenchymatous cells and some of them contain phenolic compounds (Figure 2J).

Phenolic compounds are present in epidermal cells in the leaf blade (Figures 1I, 2A and B), midrib (Figures 1J and 2C), petiole (Figure 1K) and stem, beyond in the cells near the minor vascular bundles in the leaves (Figure 1I), gathered in the ground parenchyma (Figure 1J) and in the phloem (Figure 2D) of midrib and petiole (Figure 1K). Tanniniferous cells were found in the collenchymatous tissue in the petiole of species of genera *Rinorea* and *Fusispermum*, both belong to Violaceae family.[23]

Lipophilic compounds are observed in the cuticle of leaves, petioles (Figure 2F) and stems (Figure 2K); lignified elements were seen in the fibers of petioles (Figure 2E) and stems (Figure 2H), beyond in the vessel elements of leaves, petioles (Figure 2E) and stems (Figure 2H), and in the cells of perimedullary region (Figure 2I); tiny, isolated or aggregated starch grains are found in the ground parenchyma of midrib, petiole, stem and in the perimedullary region of stem (Figure 2G).

The EDS spectra of the crystals present in *A. pyrifolia* show large peaks of calcium, carbon and oxygen (Figure 3). These results confirm that the chemical composition of these crystals is calcium oxalate. The EDS spectrum of bipyramidal crystal sand on the epidermis (Figure 3A) shows prominent peaks of calcium (53.67%), carbon (24.43%) and oxygen (21.90%). Whereas, the EDS spectrum of a prismatic crystal (Figure 3B) shows major peaks of calcium (40.33%), carbon (14.55%) and oxygen (45.12%) and of a druse (Figure 3C) presents major peaks of calcium (47.49%), carbon (12.26%) and oxygen (40.25%). The unlabeled peaks in the spectra characterize conductive metal used for covering the samples for FESEM analysis. Hoyos-Gómez[23] in 2015 studied 10 species of the Violaceae (*Rinorea* and *Fusispermum* genus) and found calcium oxalate crystals, as rhombic crystals and druses, in the collenchyma tissue.

3.2. Identification of the constituents from ESAP by LC-DAD-MS

ESAP extract was analyzed by LC-DAD-MS and nineteen compounds could be detected and identified (Table 1, Figure 4). The compounds **1** and **2** revealed intense ions at *m/z* 341.1074 and 191.0201 $[M-H]^-$, characterizing the molecular formula $C_{12}H_{22}O_{11}$ and $C_6H_8O_7$, which are compatible with di-hexoside and citric acid. The compounds **4**, **7**, and **12** showed UV spectra similar to caffeic acid (λ_{max} ≈ 299 and 325 nm) [18]. From the deprotonated ions of **7** and **12** (*m/z* 353.0856 and 353.0856, $C_{16}H_{18}O_9$), the fragment ions *m/z* 191 and 179 were observed, and they correspond to quinic and caffeic acid molecules, respectively. In addition, the fragment ion at *m/z* 173 from compound **12** is relative to a dehydrated quinic acid molecule, which characterizes the esterification of position 4 of quinic acid by the caffeoyl substituent. The relative intensities of these fragment ions were also applied to identify the isomers, as described by Clifford and collaborators in 2003.[24] Thus, the compounds **7** and **12** were identified as 5-*O*-caffeoylquinic acid and 4-*O*-caffeoylquinic acid. While the compound **4** (*m/z* 371.0614 $[M-H]^-$) revealed the fragment ion *m/z* 209 corresponding to glucaric acid, and thus this metabolite was putatively identified as *O*-caffeoyl glucarate.[25]

The compounds **6**, **8**, **9**, **10**, and **13** revealed the bands at ≈299 and 310 nm, suggesting coumaroyl group.[18] The metabolites **6** and **8** showed the same deprotonated (*m/z* 355.0661 and 355.0651) and protonated ions (*m/z* 357.0815 and 357.0804), which correspond to molecular formula $C_{15}H_{16}O_{10}$. They showed the fragment ion m/z 209 $[M-H\text{-coumaroyl}]^-$ and suggested the compounds *O*-coumaroyl glucarate, as described by Lorenz and collaborators in 2012.[25] However, the esterification position was not determined due to the absence of fragmentation

pathway in the literature for these isomers. The peaks **9**, **10** and **13** showed the same deprotonated ions (m/z 353) and the molecular formula $C_{16}H_{18}O_8$. The fragment ions *m/z* 163, 191 and 173 were observed, which are yielded by the losses of quinic acid, coumaric acid and subsequent a water molecule, respectively. The identification of isomers was performed based on the data published by Clifford.[24] The metabolites **9**, **10** and **13** were identified as 3-*O*-*p*-coumaroylquinic acid, 5-*O*-*p*-coumaroylquinic acid, and 4-*O*-*p*-coumaroylquinic acid, respectively.

The metabolites **17** and **18** exhibited UV spectra compatible with flavonol (λ_{max} ≈ 260 and 355 nm) and flavone (λ_{max} ≈ 265 and 340 nm), respectively. They yielded the fragment ions *m/z* 300 $[Aglycone-H]^{-\bullet}$ and 285 $[Aglycone-H]^-$ by the losses of a deoxyhexose (146 *u*) and a hexose (162 *u*). All the data confirmed that **17** and **18** are quercetin *O*-deoxyhexosyl-hexoside and luteolin *O*-deoxyhexosyl-hexoside, respectively, and they revealed similar spectral data reported in the literature.[26]

3.3. Safety evaluation

Neither deaths nor changes in behavior were observed in animals after acute exposure to three different doses (30, 300 and 2000 mg/kg) of ESAP (data not shown). Animals did not present any significant changes in food and water consumption as well as in body weight gain when compared to the control (Table 2). However, the relative body weight gain of animals treated with the intermediate dose (ESAP 300 mg/kg) differed statistically when compared to the control. Regarding the relative organ weight, no significant difference was observed among animals treated with all doses of ESAP when compared to the control (Table 2). Furthermore, no gross signs of toxicity were observed in heart, lungs, liver, spleen, kidneys, oravies and uterus (data not shown) as well as no histopathological changes in heart, liver

and kidney samples (Figure 5). Therefore, ESAP median lethal dose (LD_{50}) can be considered higher than 2000 mg/kg.

3.4. Diuretic effects

3.4.1. Acute diuretic activity

No increase in diuresis was observed after acute administration of three different doses (30, 100 and 300 mg/kg) of ESAP in 8 and 24-hour samples. HCTZ, as expected, increased diuresis in 8-hour samples and returned to values similar to the control 24 hours after drug administration (Table 3). Although ESAP did not induce diuresis, the highest dose (300 mg/kg) increased pH levels in 8 and 24-hour samples and differed statistically from the control group. Besides, the lowest (30 mg/kg) and the highest dose (300 mg/kg) promoted a significant decrease in urine density in 8 and 24-hour samples (Table 3). Regarding the urinary electrolyte analysis, HCTZ, as expected, increased Na^+, K^+, and Cl^- excretion after 8 hours and returned to values similar to the control group after 24 hours. None of ESAP doses increased the amounts of Na^+, K^+ and Cl^- in 8 or 24-hour urine samples (Table 4).

3.4.2. Prolonged diuretic activity

Prolonged treatment with ESAP (30, 100 and 300 mg/kg) did not show significant increase diuresis after 7 days of treatment. Only ESAP lowest dose (30 mg/kg) was able to significantly increase diuresis on the 7^{th} day after administration as it statistically differed from the control (Table 5). However, daily oral administration of ESAP (30, 100 and 300 mg/kg) significantly decreased urine elimination of Na^+, K^+, and Cl^- on days 3 and 7 after treatments (Table 5). HCTZ, as expected, increased

urine volumes and electrolyte excretion on days 3 and 7 after treatments (Table 5). pH and density values were not altered by any treatment (data not shown).

3.5. Acute administration of ESAP reduces SBP and DBP values in normotensive male rats

Basal SBP, DBP, MAP and HR recorded after the 15-minute stabilization period and before drug administration were 103.21 ± 3.31 mm Hg, 65.12 ± 2.13 mm Hg, 84.25 ± 2.53 mm Hg and 249.16 ± 8.50 beats per minute (bpm), respectively. The positive control (HCTZ), as expected, significantly reduced basal SBP, DBP, and MAP and HR to 88.35 ± 3.09 mm Hg, 57.71 ± 3.42 mm Hg, and 71.04 ± 2.36 mm Hg, whereas the control group (vehicle) barely changed these values, as follows: 104.53 ± 8.64 mm Hg, 66.85 ± 3.52 mm Hg, and 81.19 ± 5.86 mm Hg. Only the acute administration of ESAP highest dose (300 mg/kg) was able to significantly reduce SBP and DBP values to 87.64 ± 2.54 and 51.72 ± 3.72, respectively (Table 6).

Prolonged administration of different doses of ESAP did not change arterial blood pressure and heart rate values in normotensive rats when compared to animals from the negative control group. On the other hand, HCTZ was able to significantly reduce SBP, DBP, and MAP levels (Table 6). Regarding serum urea, creatinine, K^+ and Na^+ levels, only ESAP highest dose (300 mg/kg) significantly decreased urea values when compared to the control (Table 7).

3.6. Effects on peripheral vascular resistance

The continuous perfusion of MVBs with Phe promoted a sustained increase in the vascular perfusion pressure. Such perfusion was dose-dependently reduced after ESAP administration into the perfusion apparatus. Also, an expressive dose-

dependant vasodilator response in MVBs was induced by ESAP, followed by a reduction in PP values for doses of 0.1, 0.3, and 1 mg to 1.46 ± 0.84, 5.62 ± 1.98, and 12.44 ± 2.19 mm Hg, respectively (Figure 6A).

Treatment with sodium deoxycholate reduced the effects of ACh on MVBs by ~80% (data no shown), confirming the efficacy of chemically removing the endothelium. The vasodilatory effect of ESAP (all doses) was significantly increased in the absence of endothelium (Figure 6B) or in preparations with intact endothelium perfused with L-NAME (Figure 7A). The vasodilatory effects of ESAP remained unaltered in preparations with intact endothelium perfused with indomethacin (Figure 7B), or L-NAME plus indomethacin (Figure 7C).

The perfusion of MVBs with nutritive solution added of 40 mM KCl abolished the effects of ESAP (Figure 8A). On the other hand, only minor effects were observed after infusion of GLB or 4-AP (Figure 8C and 8D). Interestingly, treatment with TEA vanished vasorelaxation induced by all doses of ESAP (Figure 8B).

4. Discussion

In Brazil, the Cerrado represents about 23% of the land surface and possesses a huge diversity of species. For this reason, this area has become the target of numerous scientific studies in order to discover new molecules with powerful biological effects.[27] *Anchietea pyrifolia* (Mart.) G.Don is a Cerrado species widely used for medicinal purposes in Brazil, mainly for its possible depurative and diuretic activities.[8] In the present study, we have shown that this species may actually present an acute hypotensive effect in normotensive rats, and that may be due to the activation of potassium channels on resistance arterioles.

In the first stage of this work, we performed a pharmacognostic study using *A. pyrifolia* leaves and stems aiming to expand our ethnopharmacological knowledge on the Brazilian Cerrado. Thus, we present the main pharmacognostic characteristics of this species, offering a unique pattern for its morphological and microchemical characterization. In fact, this gives us confidence about the identification of the species studied and provides important information for the quality control of the plant drug. Morpho-anatomical studies are the most accurate possible means of identifying plant species to prevent tampering and ensure quality standard of the species under study.[28, 29]

In the meantime, a detailed characterization of the main secondary metabolites from ESAP was performed by LC-DAD-MS and 19 compounds were observed. The chemistry composition of *A. pyrifolia* is underexplored, and non-polar metabolites have been reported, such as triterpenoids and fatty acid derivatives.[30] However, in our study *O*-glycosylated flavonoids, chlorogenic acids and other phenylpropanoid acid derivatives were identified from ESAP. These compounds have been not described from *A. pyrifolia* and they probably are related to pharmacological properties, since the aqueous extraction prevails polar metabolites extraction.

Medicinal plants, as other allopathic drugs, have the same potential to cause toxic effects. Thus, toxicological assessments are of utmost importance to evaluate the safety and possible adverse effects that may arise when consumed for medicinal purposes. For this reason, the next stage of this research was destined to investigate de possible toxic effects of this species after acute exposure. In this experiment, female Wistar rats were used, as they are considered more sensitive than male rats.[19] As known, mortality is a clear sign of toxicity. However, other characteristics may indicate more subtle toxic effects such as body weight loss during treatment.[31] In

the acute toxicity test, no deaths nor weight loss were observed in animals treated with single doses of ESAP. Daily observations of changes in behavior were performed according to the Hippocratic screening, which estimates the pharmacological and toxicological nature of the test substance.[20] Besides, such parameters are fundamental for evaluating animal general health status.[32] According to data obtained in the safety assessment, animals showed no changes in behavior, in food and water consumption, as well as no gross nor histopathological changes in organs examined. Therefore, ESAP can be considered safe in rats at all doses tested.

Since the acute toxicity test provided valuable information on the toxicological profile of ESAP, we then performed an ethnopharmacological investigation of this species in male Wistar rats. First, the diuretic potential of this species was accessed by performing acute and prolonged tests. Despite its clear indication as a diuretic agent by Brazilian popular healers, no diuretic effects were observed in rats after acute administration and only ESAP lowest dose (30 mg/kg) increased diuresis on 7th day of treatment. Although ESAP 30 mg/kg values on cumulative urine were statistically different from the control group, this volume is still low as it significantly differed from HCTZ, demonstrating a not so potent diuretic effect. In addition, the small variations found in the urinary pH after ESAP treatments can be attributed to the extract itself, which, because of its alkaline pH, may have slightly raised pH since this effect did not remain after prolonged treatment. In addition, the small changes in urinary density observed after acute treatments were also considered to be incidental and of no significant clinical relevance. On the other hand, prolonged treatment with all doses of ESAP decreased electrolyte (Na^+, K^+ and Cl^-) excretion on days 3 and 7 after administration. As known, effective diuretic substances are those that are able

to increase renal excretion of water and electrolytes[33] and the obtained data did not suggest any effectiveness of *A. pyrifolia* in this aspect. We believe that the belief in a possible diuretic effect may be due to a large amount of water ingested with the infusion obtained from this species. Thus, ESAP popular indication for diuretic therapy might be related to the increase in renal hydrostatic pressure that is caused by the expansion of plasma volume after high water ingestion (i.e. pressure diuresis).[34] Thus, many species prescribed in traditional medicines for such purpose have shown low or even none diuretic potential.[35]

Many first-line hypotensive drugs do not present diuretic effects.[36] For this reason, even though ESAP did not present significant diuretic activity, the following step of this research was dedicated to investigate the acute and prolonged effects of ESAP on blood pressure. Our results have shown that the hypotensive effects induced by ESAP were only observed after acute exposure, disappearing completely after prolonged treatment. We believe that this activity may have a close relationship with the renal effects. It is possible that acute hypotension induced by ESAP has activated counter-regulators mechanisms in order to counteract the reduction of blood pressure. It is known that the activation of the sympathetic autonomic nervous system in response to acute hypotension may increase renin release by renal juxtaglomerular cells. This activation increases aldosterone release and renal NaCl reabsorption. Besides, angiotensin II itself is able to increase the reabsorption of different electrolytes - especially sodium - through the proximal tubule of the nephron.[37] Thus, salt and water retention induced by the fall in acute blood pressure may have led to a lower renal elimination of electrolytes and contributed to the long-term stabilization of blood pressure. In fact, this is a classic mechanism of resistance

to prolonged treatment with some vasodilator drugs, including hydralazine and minoxidil.[38]

In the last step of this work, we investigated the possibility of ESAP causing vasodilatory effects on MVBs, since the main determinant of arterial pressure is peripheral vascular resistance.[39] Initially, after confirming the vasodilatory effect induced by ESAP, we investigated whether the reduction in perfusion pressure had any relation to the vasodilatory endothelial mediators. The main endothelial mediators responsible for inducing vasodilator response and maintaining vascular tone in the microcirculation are nitric oxide (NO), prostacyclin (PGI2) and the endothelium-derived hyperpolarizing factor (EDHF).[40] Although these mediators indicated possible targets for ESAP, the chemical removal of the endothelium did not prevent the vasodilator response induced by this extract. In addition, the use of inhibitors of prostaglandins (indomethacin) or NO synthesis (L-NAME) also elicited no effect on ESAP-induce vasodilation. Surprisingly, the chemical removal of the endothelium intensified the vasodilatory response of ESAP. This pattern of response, in addition to indicating that the vasodilatory effect possibly comes from the smooth muscle cell, also shows that the removal of some endothelial mediators - possibly vasoconstrictors - intensifies the ESAP-response.[41]

Ion channels in the plasma membrane and endoplasmic reticulum of vascular smooth muscle cells importantly contribute to the regulation of intracellular calcium concentration, the primary determinant of vascular tone. Ion channels provide the main source of activator calcium that determines vascular tone, and strongly contribute to setting and regulating membrane potential and open-state-probability of voltage gated calcium channels, the primary source of calcium in resistance artery. Among the channels that directly influence to the regulation of vascular membrane

potential stand out the KV, KATP, and KCa channels, which also contribute to pressure-induced myogenic tone in resistance arteries. In fact, the modulation of the function of these ion channels by vasoconstrictors and vasodilators, strongly influences the functional regulation of tissue blood flow.[42] In our study, the use of KV and KATP channel blockers did not interfere with the vasodilator response induced by ESAP. However, the previous perfusion of PSS with TEA vanished the vasorelaxant effects of ESAP. Despite the fact that TEA (in high doses) is considered a non-selective potassium channel blocker, small doses of this inhibitor present relative selectivity to KCa.[43] Thus, if we consider the inhibition ilicited by TEA, and the exclusion of the participation of KV and KATP channels presented by the use of 4-AP and GLB, it is possible to conjecture that the vasodilator and hypotensive effects induced by ESAP in normotensive rats is dependent on opening of KCa channels in vascular smooth muscle.

Conclusion

This study turns the lights on the preclinical safety and efficacy of *Anchietea salutaris* as a hypotensive agent. Apparently, these effects are involved with the opening of the calcium-activated potassium channels in smooth muscle from resistence vessels contributing to reduce global peripheral resistance and blood pressure.

Author's contributions

SELT: performed experiments related to toxicity, diuresis, blood pressure, heart rate and mechanisms involved. Also data analysis, discussion and wrote the manuscript. RACP, CAST, MIS, LPG and AOS: performed experiments related to

blood pressure, heart rate and mechanisms involved; data analysis and discussed the results. AAMM: plant collection, extract preparation, and performed experiments related to the diuretic potential of the species. VPA and JMB: performed the anatomical and micro chemical analysis. RICS and ACS: performed all work related to histopathological analysis. VSS and DBS: performed the phytochemical analysis. PRD: project co-advisor. AGJ: project advisor, conceived and planned the experiments, discussed data and wrote the manuscript. All authors read and approved the final manuscript.

Author disclosure statement

No competing financial interests exist.

Conflict of interest

Authors declare there are no conflicts of interest.

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Figure legends

Figure 1. Morpho-anatomy of *Anchietea pyrifolia [c, d, i, j, k, l, o: light microscopy; e, f, g, m, n, p: FE*SEM]. A. Plant in habit; B. Leaves; C-H, M, N. Surface view [C-H. Leaf blade; M, N. Stem]; I-L, O, P. Cross-section [I. Leaf blade; J. Midrib; K. Petiole; L, O, P. Stem]. [ab- abaxial side, ad- adaxial side, ca- cambia, co- collenchyma, cs- crystal sand, cx- cortex, ep- epidermis, fi- fiber, gp- ground parenchyma, nt- non-glandular trichome, pr- prismatic crystal, pc- phenolic compounds, ph- phloem, pp- palisade parenchyma, sp- spongy parenchyma, st- stomata, vb- vascular bundle, xy- xylem]. Scale bar: a = 10 cm; b = 2 cm; j, k = 200 µm; l = 100 µm; c, d, h, i, o = 50 µm; e, m = 10 µm; f, n, p = 5µm; g = 2µm.

Figure 2. Histochemistry of *Anchietea pyrifolia* [a, c, j- ferric chloride solution; b, d- potassium dichromate solution (10%); f, k- sudan III, e, h, i- phloroglucinol/HCl]. Frontal of view – A; Transverse sections – B-D – leaf; E, F – petiole; G-K– stem [co- collenchyma, ct- cuticle, cx- cortex, en- endodermis, ep- epidermis, fi- fiber, nt- non-glandular trichome, pc- phenolic compounds, ph- phloem, pi- pith, sg- starch grains, xy- xylem]. Scale bar = 50 µm.

Figure 3. EDS spectrum of crystals of *Anchietea pyrifolia*. Bipyramidal crystal sand on the epidermis (A), prismatic crystal (B) and druse (C) into the tissues.

Figure 4. Base peak chromatogram (negative ion mode) of the extract from *Anchietea pyrifolia.*

Figure 5. Histopathological assessment of heart, liver and kidney from rats orally treated with the vehicle (control) or with the highest dosage of ESAP (2000 mg/kg) in the acute toxicity test. HE (40 X).

Figure 6. Vasorelaxant effect of ESAP does not depend on endothelium mediators in the MVBs of rats. MVBs were perfused with PSS containing Phe (3 μM) on intact (A) or denuded endothelium (B) and the vasorelaxant effect of ESAP was evaluated. The results show the mean ± S.E.M. of 5 preparations. In the graphic A [a] indicates $p < 0.05$ compared with the control (vehicle) group. [b] indicates $p < 0.05$ compared with the respective previous dose. In graphic B [a] indicates $p < 0.05$ compared with the respective previous dose. [#] indicates $p < 0.05$ compared with the effects of ESAP on intact endothelium. End - and End +: denuded and intact endothelium, respectively. MVBs: mesenteric vascular beds; Phe: phenylephrine.

Figure 7. Vasorelaxant effect of ESAP does not depend on nitric oxide or prostaglandins in the MVBs of rats. MVBs were perfused with PSS containing Phe (3 μM) plus L-NAME (A), indomethacin (B), or L-NAME plus indomethacin (C) on intact endothelium, and the vasorelaxant effect was evaluated. The results show the mean ± S.E.M. of 5 preparations. [a] indicates $p < 0.05$ compared with the respective previous dose. [#] indicates $p < 0.05$ compared with the effects of ESAP on the respective vehicle group. INDO: indomethacin; L-NAME: N^G-nitro-L-arginine methyl ester; MVBs: mesenteric vascular beds; Phe: phenylephrine.

Figure 8. Vasorelaxant effect of ESAP depends on calcium-activated potassium channels in the MVBs of rats. MVBs were perfused with PSS containing Phe (3 μM) plus KCl (A), or TEA (B), or GLB (C), or 4-AP (D) on intact endothelium, and the vasorelaxant effect of ESAP was evaluated. The results show the mean ± S.E.M. of 5 preparations. [a] indicates $p < 0.05$ compared with the respective previous dose. [#] indicates $p < 0.05$ compared with the effects of ESAP on the respective vehicle group. 4-AP: 4-aminopyridine; GLB: glibenclamide; MVBs: mesenteric vascular beds; Phe: phenylephrine; TEA: tetraethylammonium.

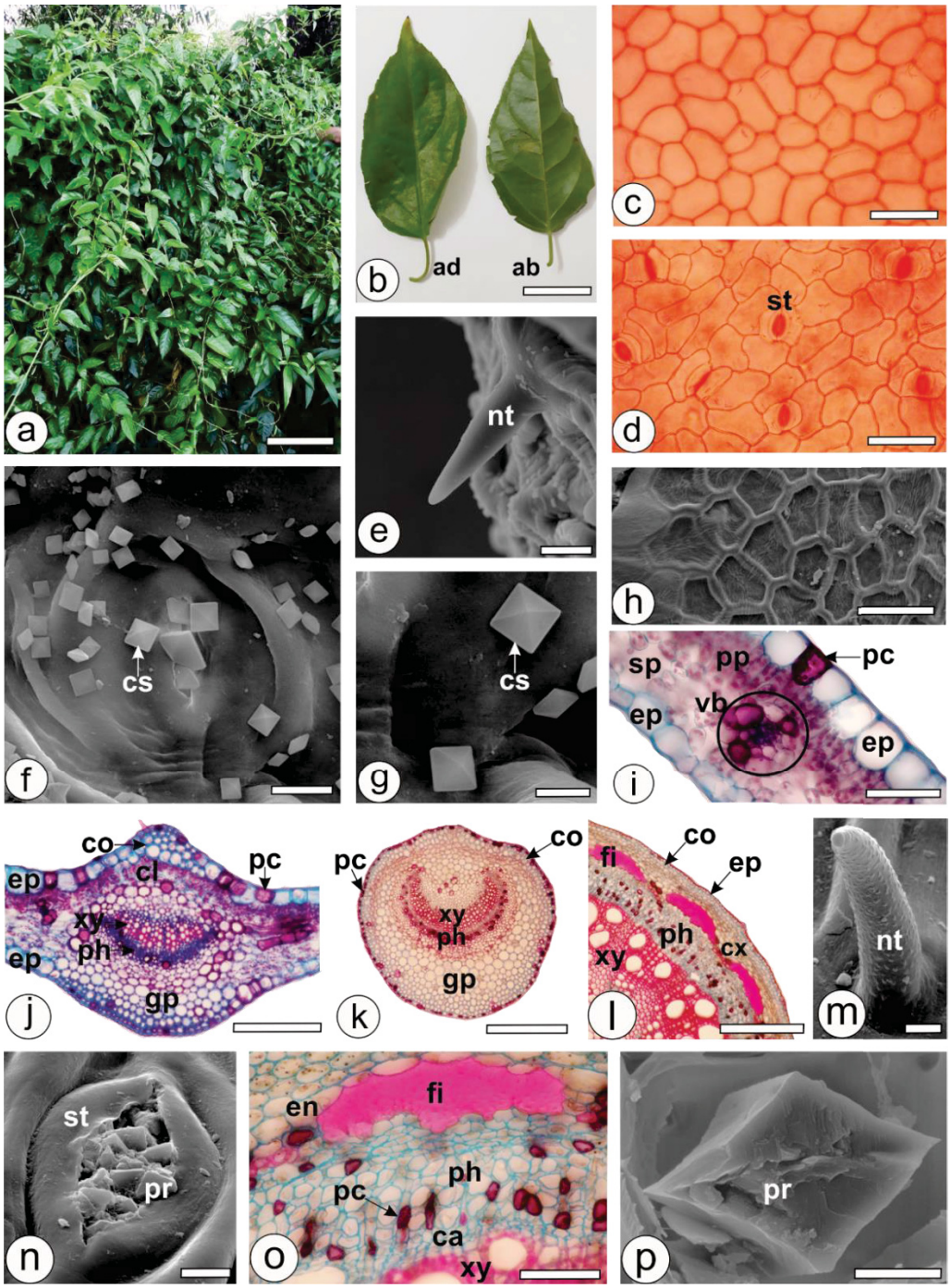

Figure 1

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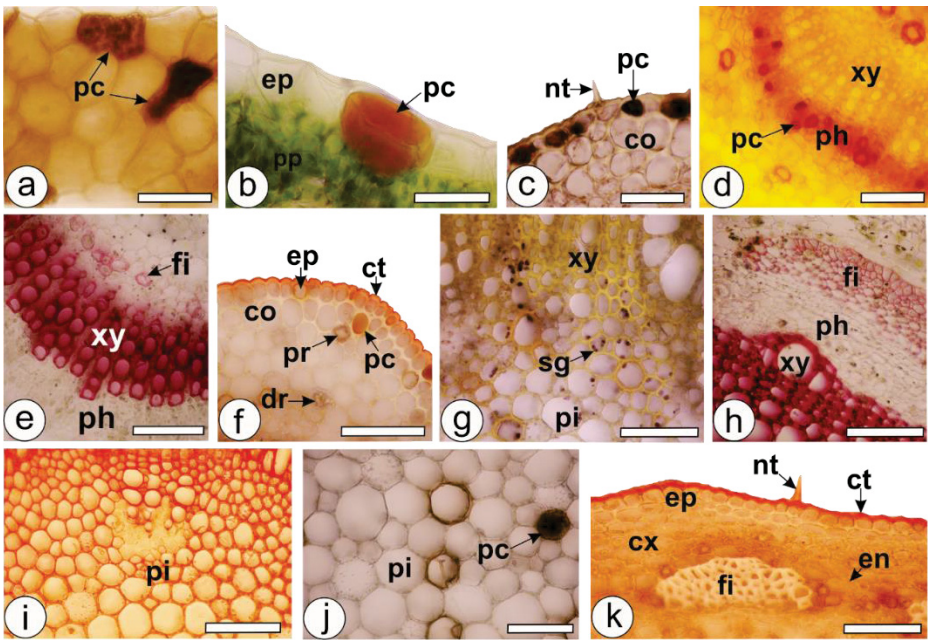

Figure 2

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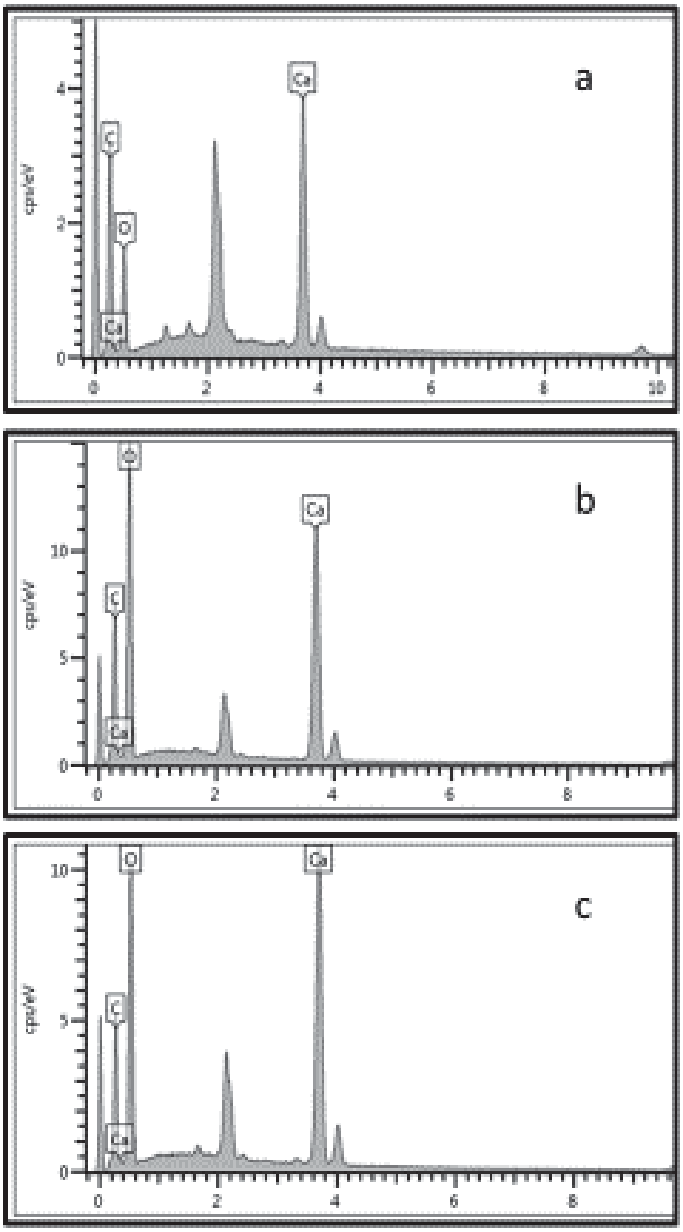

Figure 3

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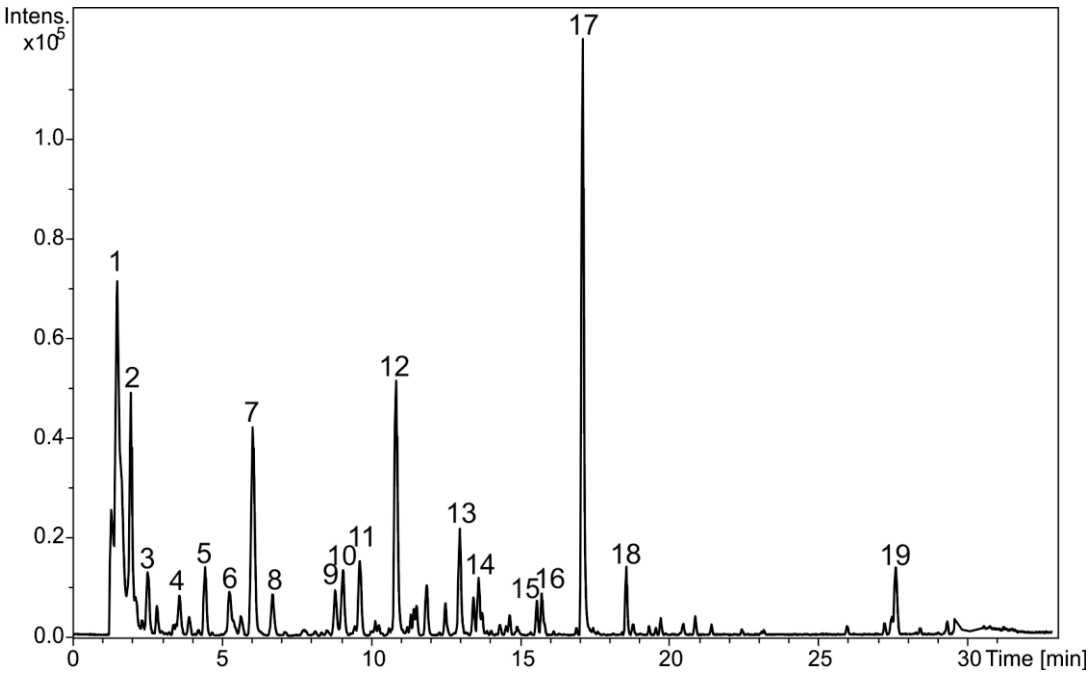

Figure 4

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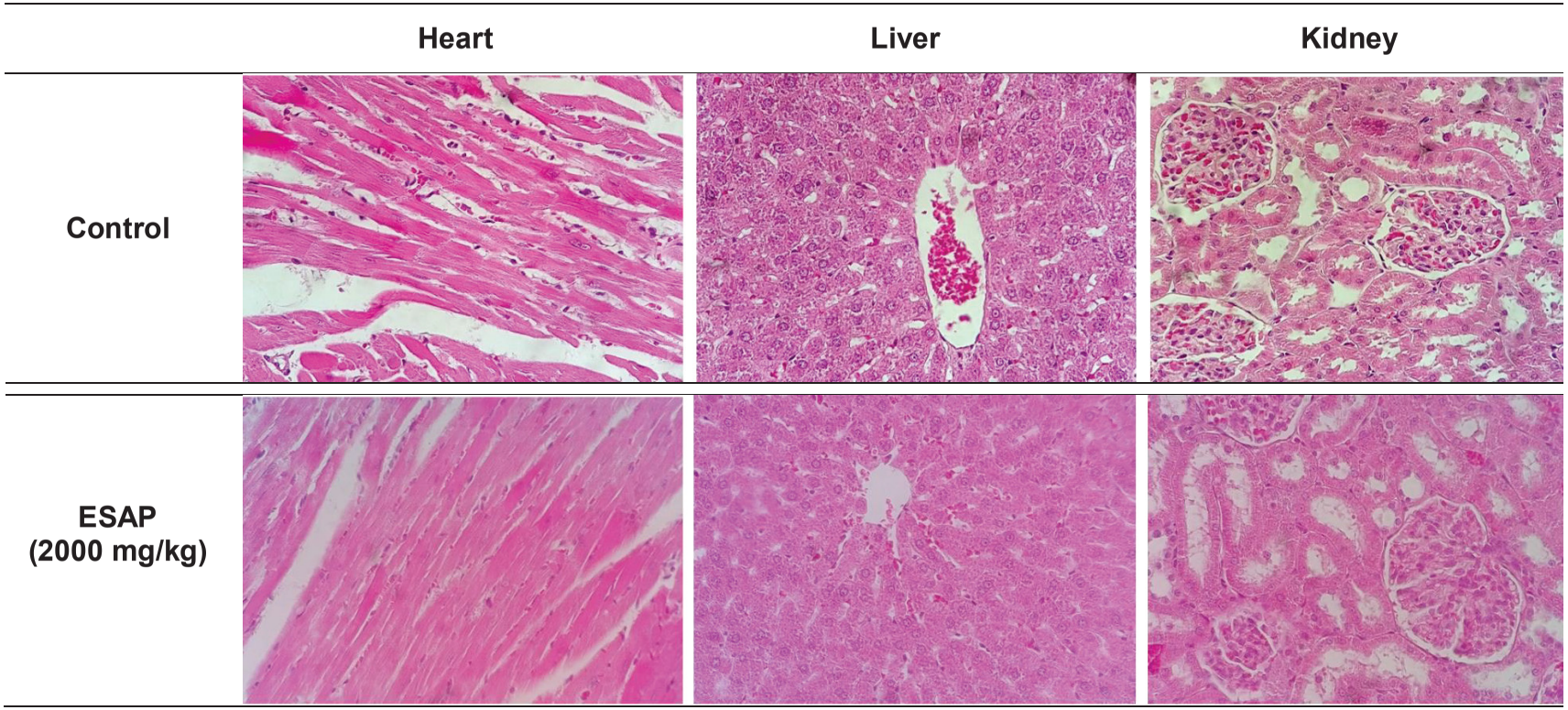

Figure 5

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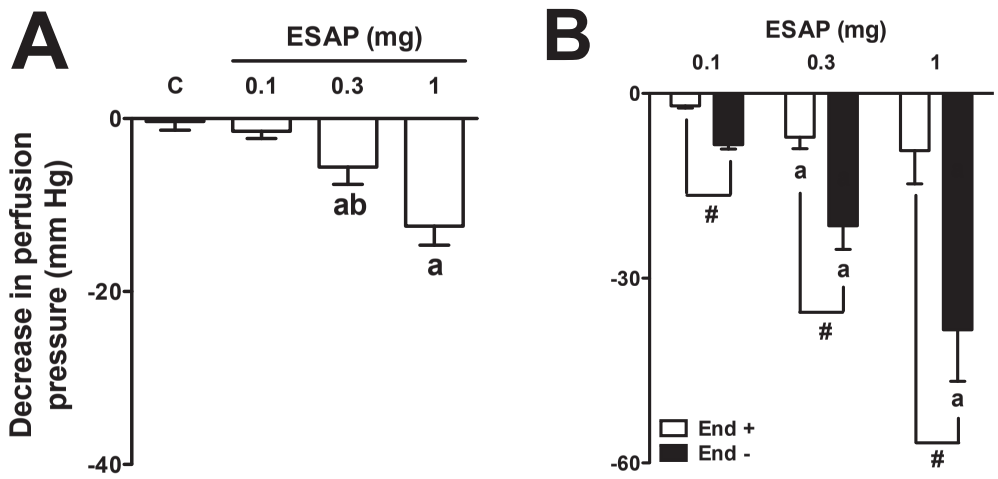

Figure 6

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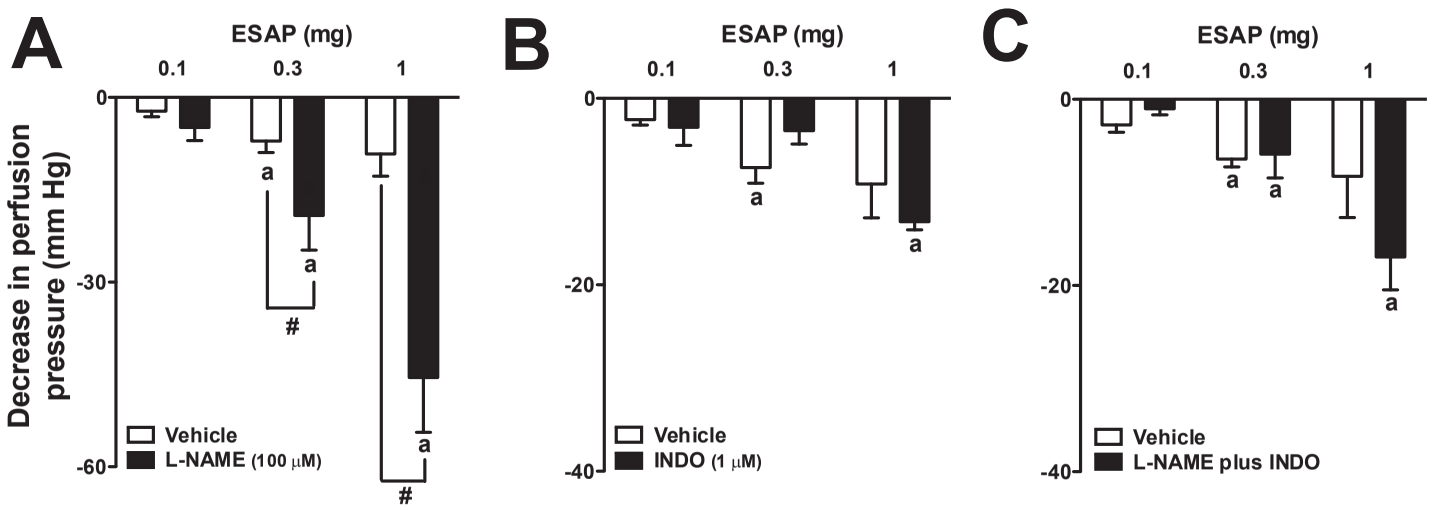

Figure 7

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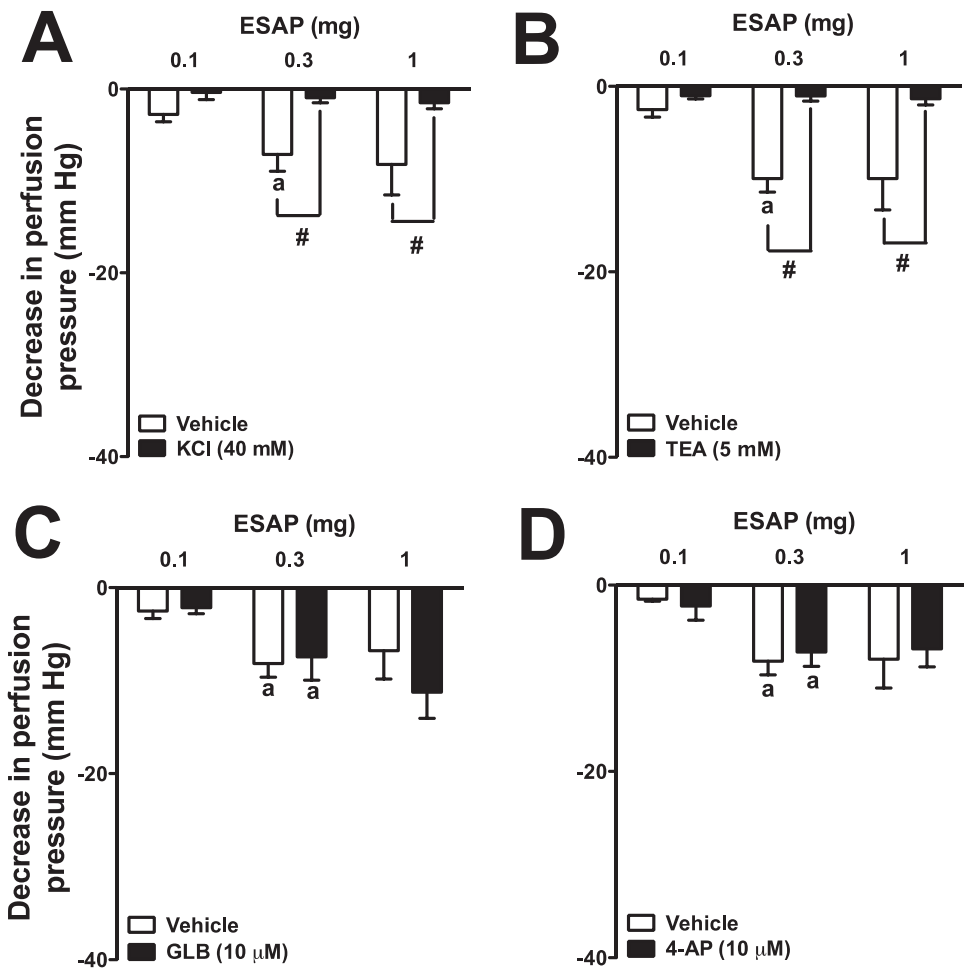

Figure 8

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Table 1. Identification of the constituents from *Anchietea pyrifolia* extract by LC-DAD-MS.

Peak	RT (min)	Compound	MF	UV (nm)	Negative mode (*m/z*)		Positive mode (*m/z*)	
					MS [M-H]-	MS/MS	MS [M-H]-	MS/MS
1	1.4	di-hexoside	$C_{12}H_{22}O_{11}$	-	341.1074	179	343.1266	-
2	1.9	Citric acid	$C_6H_8O_7$	-	191.0201	-	193.0360	-
3	2.5	NI	$C_{10}H_{13}N_5O_5$	256	282.0833	-	284.1009	-
4	3.5	O-caffeoyl glucarate	$C_{15}H_{16}O_{11}$	299, 325	371.0614	209	373.0774	-
5	4.3	NI	$C_{14}H_{26}O_{10}$	-	353.1440	-	-	-
6	5.2	O-coumaroyl glucarate	$C_{15}H_{16}O_{10}$	299, 310	355.0661	209, 191	357.0815	-
7	6.0	5-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	299, 322	353.0856	191, 179	355.1043	163
8	6.7	O-coumaroyl glucarate	$C_{15}H_{16}O_{10}$	299, 312	355.0651	209, 191	357.0804	-
9	8.8	3-*O*-*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	299, 310	337.0913	163	339.1101	-
10	9.0	5-*O*-*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	299, 310	337.0916	191, 163	339.1100	-
11	9.5	NI	$C_{15}H_{28}O_{10}$	-	367.1594	-	369.1753	-
12	10.8	4-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	299, 326	353.0856	191, 173	355.1043	163
13	12.9	4-*O*-*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	299, 310	337.0922	173	339.1094	-
14	13.6	NI	$C_{20}H_{32}O_{10}$	-	431.1907	205	-	-
15	15.5	NI	$C_{17}H_{30}O_{10}$	-	393.1752	-	-	-
16	15.7	NI	$C_{20}H_{34}O_{10}$	-	433.2070	-	-	-
17	17.0	Quercetin *O*-deoxyhexosyl-hexoside	$C_{27}H_{30}O_{16}$	260, 355	609.1445	300, 271, 255, 243, 179, 151	611.1633	303
18	18.5	Luteolin *O*-deoxyhexosyl-hexoside	$C_{27}H_{30}O_{15}$	265, 340	593.1498	285, 255, 227	595.1687	287
19	27.6	Fatty acid derivative	$C_{27}H_{30}O_{16}$	-	227.1285	183	-	-

RT: retention time; MF: molecular formula; NI: non-identified.

Table 2. Body weight (bw) gain, relative organ weight, food and water intake of rats treated orally with ethanol soluble fraction from *Anchietea pyrifolia* (ESAP).

Parameter	Control	ESAP 30 mg/kg	ESAP 300 mg/kg	ESAP 2000 mg/kg
Initial weight (g)	212.75 ± 13.00	209.00 ± 15.42	208.25 ± 15.44	215.25 ± 19.56
Final weight (g)	224.62 ± 20.16	226.75 ± 20.26	229.87 ± 18.34	236.75 ± 24.58
Body weight gain (g)	11.88 ± 3.14	17.75 ± 3.24	21.63 ± 2.57	21.50 ± 2.57
Body weight gain (%)	5.45 ± 1.35	8.44 ± 1.43	10.39 ± 1.20[a]	9.89 ± 1.06
Food intake (g/day)	76.62 ± 0.86	72.81 ± 3.35	74.77 ± 1.18	76.92 ± 2.90
Water intake (mL/day)	130.40 ± 3.48	136.3 ± 2.49	141.90 ± 3.64	137.1 ± 2.80
Relative organ weight				
Heart (g/100g bw)	0.37± 0.00	0.36 ± 0.01	0.38 ± 0.01	0.38 ± 0.00
Lung (g/100g bw)	0.76 ± 0.07	0.74 ± 0.06	0.71 ± 0.03	0.72 ± 0.08
Liver (g/100g bw)	4.52 ± 0.08	4.41 ± 0.17	4.42 ± 0.21	4.33 ± 0.17
Spleen (g/100g bw)	0.24 ± 0.01	0.26 ± 0.01	0.24 ± 0.00	0.27 ± 0.01
Right Kidney (g/100g bw)	0.39 ± 0.01	0.39 ± 0.01	0.39 ± 0.01	0.38 ± 0.01
Left Kidney (g/100g bw)	0.37 ± 0.00	0.38 ± 0.00	0.38 ± 0.01	0.37 ± 0.01
Right Ovary (g/100g bw)	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
Left Ovary (g/100g bw)	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Uterus (g/100g bw)	0.26 ± 0.02	0.29 ± 0.06	0.23 ± 0.02	0.24 ± 0.03

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Values are expressed as mean ± S.E.M. (n = 8) in comparison to the control group (a: $p \leq 0.05$).

Table 3. Effect of acute oral administration of ethanol soluble fraction obtained from *Anchietea pyrifolia* (ESAP) on the urinary volume, pH and density in 8 and 24-hour urine samples.

Group	Urine volume (mL/100g/8h)	Urine volume (mL/100g/24h)	pH (8 h)	pH (24 h)	Density (8 h)	Density (24 h)
Control	3.61 ± 0.61	7.22 ± 0.90	7.33 ± 0.04	9.65 ± 0.10	1018.6 ± 0.49	1034.6 ± 1.52
HCTZ (25 mg/kg)	6.92 ± 0.14[a]	8.93 ± 0.29	8.51 ± 0.07	8.95 ± 0.14	1017.5 ± 0.42	1030.8 ± 0.30
ESAP (30 mg/kg)	4.55 ± 0.46	7.50 ± 0.99	8.26 ± 0.66	10.1 ± 0.26	1013.5 ± 1.31[a]	1026.6 ± 1.42[a]
ESAP (100 mg/kg)	3.51 ± 0.47	6.15 ± 0.67	7.56 ± 0.11	10.2 ± 0.22	1020.6 ± 0.98	1037.5 ± 1.45
ESAP (300 mg/kg)	3.98 ± 0.45	6.97 ± 0.70	10.1 ± 0.12[a]	10.7 ± 0.10[a]	1012.6 ± 1.33[a]	1030.6 ± 1.33

For ESAP treatments, statistical analysis was performed using one-way ANOVA followed by Dunnett's test. HCTZ treatments were compared with the control using Student's t-test. Values are expressed as mean ± S.E.M. (*n* = 6). [a]p≤ 0.05 when compared with the control group. HCTZ: hydrochlorothiazide.

Table 4. Effect of acute oral administration of ethanol soluble fraction obtained from *Anchietea pyrifolia* (ESAP) on urinary electrolyte excretion in urine 8 and 24 hours.

Group	EI_{Na+} (µEq/min/100g)	EI_{K+} (µEq/min/100g)	EI_{Cl-} (µEq/min/100g)	[b]Saluretic índex		
				Na^+	K^+	Cl^-
Urine 8 hours						
Control	1.03 ± 0.19	0.46 ± 0.09	1.18 ± 0.12	-	-	-
HCTZ (25 mg/kg)	1.96 ± 0.05[a]	0.85 ± 0.05[a]	2.04 ± 0.07[a]	1.90	1.85	1.72
ESAP (30 mg/kg)	0.91 ± 0.10	0.46 ± 0.03	0.99 ± 0.09	0.88	1.00	0.83
ESAP (100 mg/kg)	1.10 ± 0.16	0.48 ± 0.05	1.33 ± 0.19	1.06	1.04	1.12
ESAP (300 mg/kg)	1.19 ± 0.06	0.77 ± 0.03	1.58 ± 0.12	1.15	1.67	1.33
Urine 24 hours						
Control	2.06 ± 0.29	0.92 ± 0.14	2.36 ± 0.36	-	-	-
HCTZ (25 mg/kg)	2.57 ± 0.07	1.06 ± 0.05	2.78 ± 0.06	1.24	1.15	1.17
ESAP (30 mg/kg)	1.77 ± 0.07	0.42 ± 0.23	2.43 ± 0.17	0.85	0.45	1.02
ESAP (100 mg/kg)	1.60 ± 0.13	0.77 ± 0.24	2.98 ± 0.35	0.77	0.83	1.26
ESAP (300 mg/kg)	1.99 ± 0.07	0.84 ± 0.09	2.22 ± 0.26	0.96	0.91	0.94

For ESAP treatments, statistical analysis was performed using one-way ANOVA followed by Dunnett's test. HCTZ treatments were compared with the control using Student's t-test. Values are expressed as mean ± S.E.M. (*n* = 6). [a]p≤ 0.05 when compared with the control group. [b]Saluretic index= µEq/min/100g problem group / µEq/min/100g control group. EI: Excreted load; HCTZ: hydrochlorothiazide.

Table 5. Effects of prolonged oral administration of ethanol soluble fraction obtained from *Anchietea pyrifolia* (ESAP) on cumulative urine volume, Na^+, K^+ and Cl^- excretion.

Group	Cumulative urine volume (mL/100 g)	EI_{Na+} (µEq/min/100g)	EI_{K+} (µEq/min/100g)	EI_{Cl-} (µEq/min/100g)
Day 1				
Control	2.91 ± 0.38	1.10 ± 0.17	1.31 ± 0.21	1.61 ± 0.21
HCTZ (25 mg/kg)	2.84 ± 0.70	0.92 ± 0.22	1.12 ± 0.32	1.21 ± 0.32
ESAP (30 mg/kg)	4.24 ± 0.42	0.44 ± 0.10	0.62 ± 0.19	0.73 ± 0.01
ESAP (100 mg/kg)	3.04 ± 0.15	0.31 ± 0.20	0.51 ± 0.17	0.62 ± 0.10
ESAP (300 mg/kg)	2.87 ± 0.26	0.37 ± 0.15	0.55 ± 0.20	0.63 ± 0.15
Day 3				
Control	5.69 ± 0.61	3.30 ± 0.34	4.82 ± 0.47	5.41 ± 0.59
HCTZ (25 mg/kg)	9.65 ± 1.28[a]	6.21 ± 0.24[a]	10.33 ± 0.51[a]	9.92 ± 0.32[a]
ESAP (30 mg/kg)	7.75 ± 0.84	0.91 ± 0.10[a]	1.23 ± 0.13[a]	1.49 ± 0.13[a]
ESAP (100 mg/kg)	6.17 ± 0.39	0.78 ± 0.20[a]	1.10 ± 0.12[a]	1.21 ± 0.11[a]
ESAP (300 mg/kg)	6.36 ± 0.37	0.92 ± 0.17[a]	1.27 ± 0.10[a]	1.55 ± 0.21[a]
Day 7				
Control	7.95 ± 0.36	7.22 ± 0.74	11.2 ± 1.33	13.1 ± 1.58
HCTZ (25 mg/kg)	17.66 ± 2.19[a]	10.62 ± 0.45[a]	16.0 ± 0.79[a]	16.9 ± 0.75[a]
ESAP (30 mg/kg)	12.68 ± 0.84[a]	1.43 ± 0.10[a]	2.04 ± 0.11[a]	2.44 ± 0.10[a]
ESAP (100 mg/kg)	10.09 ± 0.45	1.21 ± 0.12[a]	1.76 ± 0.20[a]	2.12 ± 0.15[a]
ESAP (300 mg/kg)	9.07 ± 0.34	1.16 ± 0.15[a]	1.72 ± 0.16[a]	2.07 ± 0.21[a]

For ESAP treatments, statistical analysis was performed using one-way ANOVA followed by Dunnett's test. HCTZ treatments were compared with the control using Student's t-test. Values are expressed as mean ± S.E.M. (*n* = 6). [a]p≤ 0.05 when compared with the control group. EI: Excreted load; HCTZ: hydrochlorothiazide.

Table 6. Effect of acute and prolonged oral administration of ethanol soluble fraction obtained from *Anchietea pyrifolia* (ESAP) on arterial pressure and heart rate of male Wistar rats.

Group	SBP (mm Hg)	DBP (mm Hg)	MAP (mm Hg)	HR (bpm)
Acute treatment				
Control	104.53 ± 8.64	66.85 ± 3.52	81.19 ± 5.86	231.92 ± 32.28
HCTZ (25 mg/kg)	88.35 ± 3.09[a]	57.71 ± 3.42[a]	71.36 ± 2.36[a]	250.95 ± 33.19
ESAP (30 mg/kg)	105.60 ± 9.41	66.94 ± 6.47	86.92 ± 7.52	222.24 ± 32.87
ESAP (100 mg/kg)	111.77 ± 7.51	79.17 ± 5.98	95.41 ± 7.51	333.36 ± 27.91
ESAP (300 mg/kg)	87.64 ± 2.54[a]	51.72 ± 3.72[a]	69.95 ± 3.94	274.27 ± 48.19
Prolonged treatment				
Control	125.26 ± 8.97	69.93 ± 6.46	95.33 ± 8.06	322.52 ± 28.68
HCTZ (25 mg/kg)	87.35 ± 9.31[a]	51.21 ± 2.71[a]	65.12 ± 3.55[a]	250.75 ± 42.34
ESAP (30 mg/kg)	107.71 ± 4.52	65.94 ± 3.84	87.76 ± 3.24	251.50 ± 20.62
ESAP (100 mg/kg)	94.96 ± 4.92	60.02 ± 3.62	78.46 ± 4.75	256.18 ± 15.69
ESAP (300 mg/kg)	114.56 ± 8.03	62.63 ± 6.71	88.42 ± 6.49	224.21 ± 27.24

For ESAP treatments, statistical analysis was performed using one-way ANOVA followed by Dunnett's test. HCTZ treatments were compared with the control using Student's t-test. Values are expressed as mean ± S.E.M. (*n* = 6). [a]p≤ 0.05 when compared with the control group. HCTZ: hydrochlorothiazide.

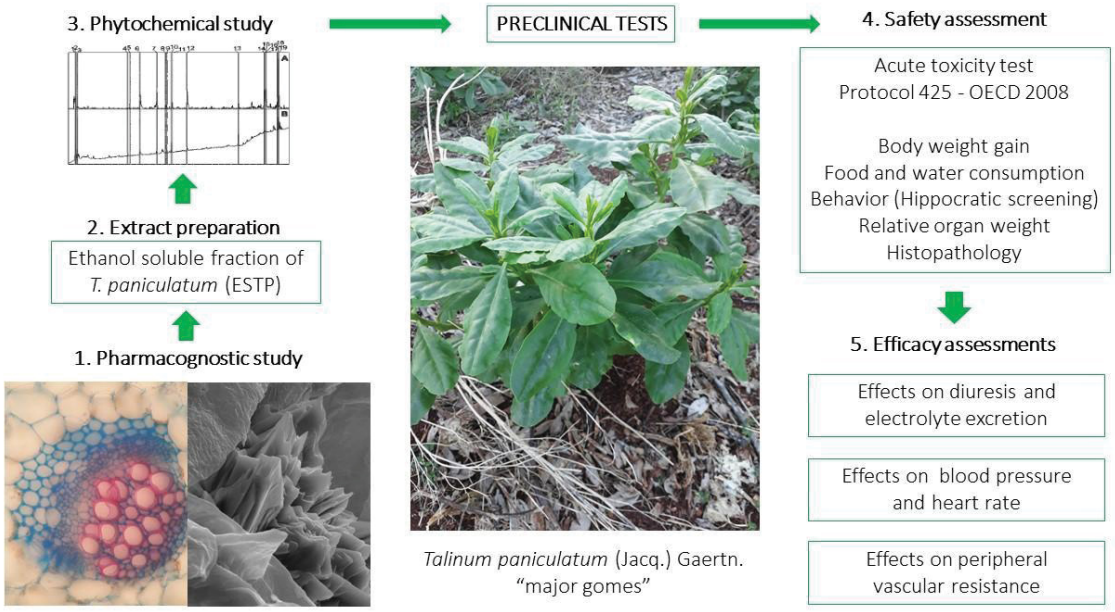
Table 7. Effects of prolonged oral administration of ethanol soluble fraction obtained from *Anchietea pyrifolia* (ESAP) on the biochemical parameters of male Wistar rats after 7 days of treatment.

Parameters	Control	ESAP 30 mg/kg	ESAP 100 mg/kg	ESAP 300 mg/kg	HCTZ 25 mg/kg
Urea (mg/dL)	50.83 ± 2.12	50.77 ± 1.86	50.15 ± 2.35	37.55 ± 4.85[a]	48.52 ± 3.22
Creatinine (mg/dL)	0.22 ± 0.01	0.23 ± 0.01	0.26 ± 0.03	0.20 ± 0.01	0.21 ± 0.02
Sodium (mmol/L)	121.33 ± 2.58	126.02 ± 2.09	127.27 ± 4.89	114.97 ± 4.95	119.52 ± 3.99
Potassium (mmol/L)	5.64 ± 0.22	17.85 ± 12.55	5.87 ± 0.37	4.98 ± 0.24	5.25 ± 0.31

For ESAP treatments, statistical analysis was performed using one-way ANOVA followed by Dunnett's test. HCTZ treatments were compared with the control using Student's t-test. Values are expressed as mean ± S.E.M. (*n* = 6). [a]p≤ 0.05 when compared with the control group. HCTZ: hydrochlorothiazide.

6 ARTIGO CIENTÍFICO 3: Ethnopharmacological approaches to *Talinum paniculatum* (Jacq.) Gaertn.-Exploring cardiorenal effects from the Brazilian Cerrado.

Graphical abstract



Ethnopharmacological approaches to *Talinum paniculatum* (Jacq.) Gaertn. - exploring cardiorenal effects from the Brazilian Cerrado

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Abstract:

Ethnopharmacological relevance: *Talinum paniculatum* (Jacq.) Gaertn. (Talinaceae), popularly known as “major gomes” and “erva gorda”, is a non-conventional food plant extensively distributed throughout the Brazilian territory. In Brazilian folk medicine, this species is used as aphrodisiac, to treat gastrointestinal problems, and as a cardioprotective agent. However, there are no reports in the literature proving its cardiovascular effects.

Aim: To perform a whole-ethnopharmacological investigation of the cardiorenal properties of the ethanol soluble fraction from *T. paniculatum* (ESTP) in Wistar rats.

Material and Methods: First, plant samples were collected, properly identified and a morpho-anatomical characterization was carried out to provide quality control parameters. Then, ESTP was obtained and its chemical profile was determined by LC-DAD-MS. In addition, an acute toxicity assay was conducted in female Wistar rats in order to observe any toxic effects after one single administration. Finally, the diuretic and hypotensive potential of ESTP (30, 100 and 300 mg/kg) were investigated in male rats followed by the evaluation of its possible effects on peripheral vascular resistance.

Results: Chemical compounds identified from ESTP were chlorogenic acids, amino acids, nucleosides, *O*-glycosylated flavones and organic acids. No signs of toxicity as well as no changes in urine volume or electrolyte elimination were observed after ESTP acute treatment. On the other hand, prolonged treatment with all doses of ESTP significantly increased urine volume and electrolyte excretion (Na^+, K^+ and Cl^-) without affecting blood pressure or heart rate. Apparently, these effects are involved with the activation of the small conductance calcium-activated potassium channels contributing to the increase of renal blood flow and glomerular filtration rate.

Conclusion: Data presented show important information about the ethnomedicinal properties of *T. paniculatum*. In addition, the study presents the ESTP as a possible herbal medicine, especially when a sustained diuretic effect is required.

Keywords: Diuretic; mesenteric vascular bed; potassium channels; Talinaceae.

Abbreviations: 4-AP, 4-aminopyridine; ACh, acetylcholine chloride; ANOVA, analysis of variance; AP, arterial pressure; Ca^{+2}, calcium; Cl^{-}, chloride; $CaCl_2$, calcium chloride; CO_2, carbon dioxide; CVD, cardiovascular disease; DBP, diastolic blood pressure; DAD, diode array detector; EDS, energy disperse system; EDTA, ethylenediaminetetraacetic acid; EI, excretion load; ESTP, ethanol soluble fraction from *Talinum paniculatum*; FAA, formalin-acetic acid-alcohol; FESEM, field emission scanning electron microscopy; GFR, glomerular filtration rate; GLB, glibenclamide; HCl, hydrogen chloride; HCTZ, hydrochlorothiazide; HR, heart rate; K^{+}, potassium; KCl, potassium chloride; KH_2PO_4, Monopotassium phosphate; L-NAME, Nω-Nitro-L-arginine methyl ester; MAP, mean arterial pressure; $MgSO_4$, magnesium sulfate; MVB, mesenteric vascular bed; Na^{+}, sodium; NaCl, sodium chloride; $NaHCO_3$, sodium bicarbonate; OECD, Organisation for Economic Co-operation and Development; pH, potential of hydrogen; Phe, phenylephrine; PP, perfusion pressure; PSS, physiological saline solution; S.E.M., standard error of the mean; SBP, systolic blood pressure; SK KCa, small conductance calcium-activated potassium channels; UEPG, State University of Ponta Grossa; UFGD, Federal University of Grande Dourados, UFPR, Federal University of Paraná.

1. Introduction

Brazil is considered one of the most diverse countries when it comes to biodiversity. Classified as the fifth largest country in the world, it is composed of nine different ecosystems with an average of 46 thousand plants species (Carvalho et al., 2017). Such biodiversity has contributed to a better acceptance and greater demand for natural products to preventing and treating diseases. However, the number of studies on the efficacy of these compounds is still low when compared to other countries (WHO, 2014).

Talinum paniculatum (Jacq.) Gaertn. (Talinaceae), popularly known as “major gomes” and “erva gorda”, is a weedy species widely used in traditional medicine and as a food source. Considered a non-conventional food plant, *T. paniculatum* is a shrub species extensively distributed throughout the Brazilian territory, mainly in the Biome Cerrado. This plant is small in size (30-60 cm height), dark green colored and its leaves are quite succulent, smooth and soft textured (Dos Reis et al., 2015).

T. paniculatum leaves are extensively used by remaining healers from the region of Grande Dourados, Mato Grosso do Sul, Brazil, in the treatment of different cardiovascular disorders (Coelho et al., 2018). Moreover, the species is used in folk medicine as aphrodisiac (Widiyani, 2006), reproductive tonic (Thanamool et al., 2013), to treat gastrointestinal problems, skin infections, and wound healing (Dos Reis et al., 2015). Previous pharmacological studies have demonstrated its use as a dieting supplement, for diabetes prevention (Shimoda et al., 2001) besides its antinociceptive (Ramos et al., 2010) and estrogenic effects in ovariectomized rats (Thanamool et al., 2013). In addition, *T. paniculatum* leaves extract and its fractions have demonstrated outstanding activity against *Serratia marcescens, Staphylococcus aureus*, *Micrococcus luteus, Candida albicans* and *Escherichia coli* (Dos Reis et al., 2015).

Phytochemical investigations revealed tannins, steroids, saponins and triterpenes in its chemical composition (Yulia et al., 2006). Moreover, some data revealed the presence of campesterol, stigmasterol and sitosterol as major compounds (Dos Reis et al., 2015). Another study has shown three quinolizidine alkaloids (javaberine, javaberine A hexaacetate and javaberine B hexaacetate) isolated from *T. paniculatum* (Jung et al., 2006). Such compounds showed significant inhibitory effect on TNF-α production by fat cells and macrophages (Shimoda et al., 2001).

Although *T. paniculatum* leaf extracts is popularly used in Brazil as a cardioprotective agent, there is a lack of scientific evidence proving this ethnobotanical indication. Thus, we collected *T. paniculatum* leaves and conducted a morpho-anatomical characterization to provide quality control parameters. Then, an ethanol soluble fraction (ESTP) was obtained and its constituents were identified by LC-DAD-MS. In addition, an acute toxicity assay was performed in female Wistar rats in order to observe any toxic effects after one single administration. Finally, the diuretic and hypotensive potential of ESTP were investigated in male Wistar rats followed by the evaluation of its possible effects on peripheral vascular resistance.

2. Materials and Methods

2.1. Drugs and solvents

Heparin was obtained from Hipolabor (Belo Horizonte, MG, Brazil), xylazine and ketamine hydrochloride from Syntec (São Paulo, SP, Brazil). Hydrochlorothiazide, acetylcholine chloride, phenylephrine, indomethacin, Nω-Nitro-L-arginine methyl ester, tetraethylammonium, 4-aminopyridine, glibenclamide, NaCl, KCl, NaHCO3, MgSO4, CaCl2, KH2PO4, dextrose and ethylenediaminetetraacetic

acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were obtained in analytical grade.

2.2. Plant material

Talinum paniculatum leaves were collected at February 2017 in a private property that belongs to a Cerrado area in Dourados, Mato Grosso do Sul - Brazil, at 458 m above sea level (22°12'22.6"S 54°47'43.1"W). A voucher specimen (no. 5539) was authenticated by Dr. Maria do Carmo Vieira and deposited in the Herbarium of the Federal University of Grande Dourados (UFGD). The plant name is in accordance with the on-line database published by “The Plant List”, accessed on July 11, 2018.

2.3. Botanical characterization

Freshly collected leaves and stems of *T. paniculatum* were fixed in formalin-acetic acid-alcohol (FAA) solution (Johansen, 1940) for three days and then stored in 70% ethanol (v/v) (Berlyn and Miksche, 1976). Transverse sections of the samples were prepared freehand using razor blades. The sections were placed on glass slides, hydrated and double-stained with basic fuchsin and Astra blue stains (Roeser, 1962) or with toluidine blue stain (O’Brien et al., 1964).

2.4. Histochemical analysis

Histochemical analyses were carried out using cross-sections of fixed leaves and stems obtained by the same method used in the anatomical assay. The following standard solutions were evaluated: phloroglucinol/HCl for lignin (Sass, 1951), ferric chloride for phenolic compounds (Johansen, 1940) and 1% iodine solution for starch (Berlyn and Miksche, 1976). Controls were made in parallel with the tests, and semi-

permanent slides were prepared as described above. Photomicrographs were prepared using digital camera (C7070) attached to a light microscope (Olympus CX 31).

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

For field emission scanning electron microscopy (FESEM), samples fixed in FAA were dehydrated using increasing concentrations of ethanol. These samples were then critical point dried (Balzers CPD-030) using liquid CO2. The completely dried samples were mounted on aluminum stubs using glued carbon tapes and then coated with gold using a Quorum SC7620 sputter coater. Photomicrographs were generated and analyzed using a Mira 3 Tescan FESEM.

An X-ray energy dispersive system (EDS), attached to the FESEM, was used for elemental analysis of calcium oxalate crystals present in the preparations of leaves and stems. This elemental microanalysis was randomly performed for the crystals as well as cells devoid of crystals (control). The morphotypes of the crystals were determined based on the work performed by Silva et al. (2014).

2.6. Extract preparation

T. paniculatum leaves were sanitized using potable water and then air-dried in an oven at 40°C for 7 days. Dried leaves were powdered by mechanical mill. The infusion was made by pouring 1 L of boiling water (97 °C) on each 100 g of dried and pulverized leaves and the extraction occurred until room temperature was reached (~ 5 hours). Then, the infusion was treated with 3 volumes of ethanol, originating a precipitate and an ethanol soluble fraction (ESTP; 3.42% yield). ESTP samples were lyophilized and stored in a freezer at -18 ◦C until further analyses.

2.7. LC-DAD-MS analyses

ESTP was analyzed on UFLC Shimadzu Proeminence chromatography, which was coupled to a diode array detector (DAD) and a mass spectrometer MicrOTOF-Q III (Bruker Daltonics). The mass spectrometer has electrospray ion source and analyzers quadrupole – time-of-flight. A Kinetex C18 chromatographic column (Phenomenex, 2.6 µ, 100A, 150 × 2.1 mm,) was used in the analyses, applying the flow 0.3 mL/min and volume injection of 3 µL. All the LC and MS parameters were the same described by Dembogurski et al. (2018). The sample was solubilized in methanol and water (7:3, v/v) at concentration 1 mg/mL and analyzed in negative and positive ion mode.

2.8. Pharmacological studies

2.8.1. Animals

Wistar rats (8-12 weeks) of both sexes were obtained from the Federal University of Grande Dourados (UFGD) and Federal University of Paraná (UFPR). Animals were housed in a temperature- and light controlled room (12-h light/dark cycle; 22 ± 2°C) with free access to food and water. Before the onset of all experiments, animals were left for ten days to acclimatize to laboratory conditions. All procedures involving animals were previously approved by the Ethics Committee in Animal Experimentation from UFPR (protocol: 05/2017) and UFGD (protocol: 21/2017).

2.8.2. Toxicological assessment

Oral acute toxicity was evaluated in accordance with the protocol 425 described by the Organization for Economic Co-operation and Development (OECD)

in 2008. Three single doses of ESTP (30, 300 and 2000 mg/kg) or water (1 mL/kg) were administered to fasted female rats (n = 8) by oral gavage. Following administration, animals were closely observed during the first 24 hours and daily for 14 consecutive days. Mortality, body weight gain, food and water consumption were daily observed and recorded. In addition, animal behaviour was individually observed according to the five parameters of the Hippocratic screening, which aims to analyze conscious state, activity and coordination of motor system and muscle toning, activities on the central nervous system, corneal and headset reflexes and activities on the autonomic nervous system (Malone and Robichaud, 1962). On day 15 after administration, overnight fasted rats were euthanized by isoflurane anesthesia (inhalation) followed by exsanguination. Heart, lung, spleen, liver, kidneys, uterus and ovaries were removed, weighed, macroscopically examined and the relative organ weight calculated. Heart, liver and kidney samples were fixed in 10% buffered formalin. After fixation, organ samples were dehydrated with increasing absolute ethanol concentrations, diaphanized in xylene and embedded in paraffin wax. Samples were sectioned and stained with hematoxylin/eosin (H & E). Analyses were performed under light microscopy (40X).

2.8.3. Acute diuretic activity

The diuretic activity was assessed according to methods previously described with some modifications (Gasparotto Junior et al., 2009). Thirty fasted (12 hours) male rats, randomly divided into five groups (n = 6), received an oral dose of saline solution (0.9% NaCl; 5 mL/100 g) in order to impose body uniformity of salt and water. Following salinization, animals received a single oral dose of ESTP (30, 100 or 300 mg/kg), hydrochlorothiazide (HCTZ, 25 mg/kg) or vehicle (1 mL/100 g) by gavage. Immediately after treatment, all rats were placed in metabolic cages for 8

hours with free access to food (1 hour after treatment) and water. Urine was collected with the aid of a graduated cylinder and the volume was recorded at 8 hours (expressed as mL/100 g of body weight). Density and pH were determined on fresh urine samples using a handheld refractometer (NO107; Nova Instruments, Brazil) and a digital pH meter (Q400MT; Quimis Instruments, Brazil), respectively. Electrolyte (Na^+, K^+ and Cl^-) levels were quantified in an ion selective meter (COBAS INTEGRA 400 plus; Roche®) and the excretion load (El) of Na^+, K^+ and Cl^- were obtained by multiplying the concentration of electrolytes (mEq/l) by the urinary flow (mL/min). Results are expressed as µEq/min/100g.

2.8.4. Prolonged diuretic activity

For the prolonged diuretic activity, thirty male rats (n = 6) received daily ESTP (30, 100 and 300 mg/ kg), HCTZ (25 mg/kg) or vehicle (1 mL/100 g) for 7 days. Immediately after the first treatment, animals were individually placed in metabolic cages and the total amount of urine was collected every 24 hour on days 1, 3 and 7. Density, pH, volume and electrolyte concentrations (Na^+, K^+ and Cl^-) were determined. At the end of the experiments, animals were euthanized by isoflurane anesthesia (inhalation) followed by exsanguination.

2.8.5. Effects on blood pressure and heart rate

For this experiment, thirty normotensive rats (n = 6) received oral doses of ESTP (30, 100, or 300 mg/kg), HCTZ (25 mg/kg) or water (1 mL/100 g) once daily for 7 days. At the end of the experimental period, rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) by intramuscular route (IM). Immediately after anesthesia, a single bolus dosage of heparin (50 IU) was administered subcutaneously. 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 blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR). Changes in SBP, SDP, MAP and HR were recorded for 20 minutes. Blood samples were collected directly from left carotid artery and serum urea, creatinine, Na^+ and K^+ levels were quantified. Then, animals were euthanized with an overdose of isoflurane anesthesia (inhalation, 2-3 times of the anesthetic dose).

2.8.6. Effects on peripheral vascular resistance

Male rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) via intraperitoneal route. Mesenteric vascular beds (MVBs) were isolated and prepared using methods previously described by McGregor (1965). MVBs (n = 5) were placed in a water-jacketed organ bath and perfused (at 4 mL/min) 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) 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 a 45-minute equilibration period, integrity was checked by a *bolus* injection of KCl (120 mmol). In order 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 (30 nmol) was performed, and the PP reduction was measured. Some preparations were perfused with PSS containing sodium deoxycholate (1.8 mg/mL) for 30 seconds with the aim of chemically removing the endothelium of MVBs. 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 (30 nmol) was directly applied into the perfusion system.

Therefore, MVBs with or without functional endothelium were continuously perfused with PSS plus Phe (3 μM). After stabilization, different preparations received bolus injections containing ESTP (0.003, 0.01, 0.03, 0.1, 0.3, and 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 minutes between doses. Afterwards, different MVBs were perfused with PSS containing Phe (3 μM) plus L-NAME (100 μM; a non-selective NO synthase inhibitor), indomethacin (1 μM; a non-selective cyclooxygenase inhibitor), KCl (40 mM), apamin (0.1 μM; a potent blocker of small conductance Ca^{2+}-activated K^+ [SK KCa] channels) (Bond et al., 2005), 4-aminopyridine (4-AP 10 μM; a voltage-dependent K^+ channels blocker), and glibenclamide (GLB 10 μM; a selective Kir6.1 ATP-sensitive K^+ channels blocker), used alone or combined. After 15 minutes of continuous perfusion, ESTP (0.1, 0.3, and 1 mg) was injected again into the perfusion system and its ability to reduce PP in the presence and absence of different inhibitors was evaluated.

2.9. Statistical analysis

The difference between groups was assessed by analysis of variance (ANOVA), followed by Dunnett's test, or by Student's t-test when applicable. *p*-value of less than 0.05 was considered statistically significant. Results are expressed as mean ± standard error of the mean (S.E.M.). Graphs were drawn and statistical analyses were performed using GraphPad Prism software version 6.0.

3. Results

3.1. Anatomical profile and histochemical characterization

In *Talinum paniculatum* (Figure 1A), the leaves (Figure 1B) present both epidermises with wavy anticlinal walls (Figure 1C and D) and covered externally by slightly striate cuticle (Figure 1D). Parallelocytic stomata are present on both abaxial (Figure 1C) and adaxial (Figure 1D) epidermises, characterizing the leaf as amphistomatic. These characteristics are common in *Talinum* genus (Ogburn and Edwards, 2009). However, *Talinum caffrum* (Thunb.) Eckl. & Zeyh. presented brachyparallelocytic stomata besides parallelocytic type.

In the present study, crystals show prismatic shapes (Figure 1F and G) located externally on the adaxial leaf surface (Figure 1F and G). This feature was not previously reported for *Talinum* species.

In cross-section, leaf has a unilayered epidermis with cells varying from tabular to round shapes and covered with a thin cuticle (Figure 1I). Mesophyll is dorsiventral, consisting of three layers of palisade parenchyma with atypical cells and about six layers of spongy parenchyma. Minor collateral vascular bundles traverse the spongy tissue and are encircled by a parenchymatous sheath (Figure 1H). Abundant mucilaginous cells (Figure 1H), druses (Figure 1I) and prismatic crystals (Figure 1J) are found in the mesophyll. Mucilaginous cells and druses were reported for species of *Talinum*, such as *T. caffrum*, *T. portulacifolium* (Forssk.) Asch. ex Schweinf. and *T. fruticosum* (L.) Juss., yet prismatic crystals were not mentioned for these species (Ogburn and Edwards, 2009).

In transverse section, the midrib is concave-convex in outline. The palisade parenchyma cells become gradually shorter toward the middle region (Figure 1H). The ground parenchyma also contains mucilaginous cells, prismatic crystals and

druses. The vascular system of the midrib is represented by one collateral vascular bundle (Figure 1H).

The petiole, in cross-section, is slightly concave–convex in shape and has a short wing-like projection on either side (Figure 1K). The epidermis has the same features as described for the leaf blade. A major portion of the petiole is made up of parenchymatous ground tissue. The stele is represented by about 10 free collateral vascular bundles in open arc (Figure 1I). Many druses and agglomerate of prismatic crystals (Figure 1M) are distributed in the petiole.

In an incipient secondary structure, the stem is irregular in shape (Figure 2A). The epidermis is uniseriate, presents stomata and covered by a striate cuticle (Figure 2B). The cortex is formed by 2-3 layers of angular collenchyma (Figure 2C) and several layers of parenchymatic cells. Crystals can also be seen in the cortex. The vascular system is represented by about 25 bundles (Figure 2A). The vascular bundles have phloem toward the periphery, and xylem facing the pith, separated by intrafascicular cambia (Figure 2D). Perivascular fiber caps are adjoined to the phloem (Figure 2D and E). Scalariform vessel elements have thickened walls (Figure 2F).

The pith occupies a large portion of the stem and is made up of thin-walled parenchymatous cells. Druses (Figure 2G) and prismatic crystals are found (Figure 2H), as well as starch grains are observed in the pith. Starch grains are isolated or in groups, measuring about 10 μm in diameter (Figure 2I and J) and react with 1% iodine solution in the histochemical test (Fig 2I). Phenolic compounds are found in the cortex and especially in the vascular bundles of the stem (Figure 2A). Even though cells containing tannins had been found in the cortex, pith and phloem in *T. caffrum*, *T. portulacifolium* and *T. fruticosum*, they were not present in *T. paniculatum* (Ogburn and Edwards, 2009).

The EDS spectrum of Figure 3 is representative (druses) for all types of crystals in that the peaks for Ca and O varied only slightly among the spectra. This EDS spectrum shows major peaks of calcium (47.33%), carbon (13.77%) and oxygen (38.90%). The major unlabeled peaks represent gold element used in the sputter coating. The features highlighted in this study help *T. paniculatum* identification as well as its distinction among the species of the genus.

3.2. Identification of the constituents by LC-DAD-MS.

ESTP was analyzed by LC-DAD-MS and nineteen compounds were identified (Table 1, Figure 4), including nucleosides, organic acids, chlorogenic acids, amino acids and *O*-glycosylated flavones. The metabolites were identified by UV, high resolution MS and MS/MS data, and confirmed from the comparison of published spectral data. The chromatographic peak **1** revealed intense ions at *m/z* 191.0542 and 209.0324 $[M-H]^-$ were putatively identified as the co-elution of quinic acid and hexaric acid, while the peak **2** (*m/z* 191.0201 $[M-H]^-$) was compatible to citric acid.

The peaks **3** and **4** exhibited intense ions *m/z* 245.0775 and 284.1005 $[M+H]^+$, confirming the molecular formula $C_9H_{12}N_2O_6$ and $C_{10}H_{13}N_5O_5$. These metabolites showed losses of a pentose (132 *u*), yielding the product ions at *m/z* 113 and 152. The spectral data of **3** and **4** were compatible with the nucleosides *N*-pentosyl uracil (uridine) and *N*-pentosyl guanine (guanosine), as reported by Zhu et al. (2017). In addition, **5** and **14** showed an absorption at ≈ 260 nm, while **6** revealed an UV spectrum similar to tryptophan chromophore. They presented the molecular formula $C_9H_{11}NO_2$ (**5**), $C_{11}H_{12}N_2O_2$ (**6**) and $C_{11}H_{13}NO_3$ (**14**), but only compound **14** exhibited a loss of 42 *u*, suggesting the acetyl group. Thus, **5**, **6** and **14** were putatively identified as phenylalanine, tryptophan and *N*-acetyl phenylalanine (Zhu et al., 2017).The metabolites **8-9**, **11-13** and **15** showed UV spectra (λ_{max} ≈ 299 and 310 nm)

compatible to coumaric acid chromophore (Dembogurski et al., 2018). All these compounds revealed the same molecular formula $C_{16}H_{18}O_8$ (*m/z* 337 $[M-H]^-$). They yielded the fragment ions at *m/z* 191 and 173, which are relative to quinic acid and subsequently loss of a water molecule. In addition, the fragment ion *m/z* 163 was also observed and confirmed the coumaroyl group. The relative intensities of these fragment ions were used to determine the esterified position of quinic acid, as reported by Clifford et al. (2003). Thus, **8**, **13**, and **15** were identified as 3-*O*-*p*-coumaroylquinic acid, 4-*O*-*p*-coumaroylquinic acid and 5-*O*-*p*-coumaroylquinic acid. The compounds **9** and **11-12** also were isomers, like as *cis* isomers, but the esterified position could not to be determined and they were identified as *O*-*p*-coumaroylquinic acid. Similarly, the metabolites **7** and **10** showed bands at the wavelength 299 and 325 nm, which are compatible to caffeoyl substituents (Dembogurski et al., 2018). The fragmentation pathway was similar, while the product ion *m/z* 179 is relative to caffeic acid. The spectral data were compared with the hierarchical key for identification of chlorogenic acids described by Clifford et al. (2003), so **7** and **10** were identified as 5-*O*-caffeoylquinic acid 4-*O*-caffeoylquinic acid.

For **16-18**, bands at the wavelength ≈ 265 and 335 nm were observed indicating flavone aglycones (Dembogurski et al., 2018). The losses of 308 *u* (146 + 162 *u*) confirmed the sugars deoxyhexose and hexose. The losses of methyl radical indicated the methoxyl substituents in the aglycones, such as *m/z* 284 and 298 $[Aglycone-H-^{\bullet}CH_3]^-$ from **17** and **18**, respectively, like as reported by Xu et al. (2018). The flavonoids **16**, **17** and **18** were identified as *O*-deoxyhexosyl-hexosyl luteolin, *O*-deoxyhexosyl-hexosyl *O*-methyl-luteolin, and *O*-deoxyhexosyl-hexosyl di-*O*-methyl luteolin.

3.3. Toxicological findings

No changes in behavior nor deaths were recorded during the 14-day observation period. Animals treated with all doses of ESTP presented a significant increase in body weight gain as well as in relative organ weight when compared to the control (Table 2). Besides, rats treated with ESTP intermediate dose (300 mg/kg) demonstrated a significant increase in food and water consumption (Table 2). Regarding the relative organ weight of animals, there was a significant difference in the weight of uterus from rats treated with ESTP 300 mg/kg when compared to the control (Table 2). No gross changes were observed in heart, lung, liver, spleen, kidneys, ovaries and uterus (data not shown) as well as no histopathological changes in heart, liver and kidneys (Supplemental Figure S1 A-F).

3.4. ESTP does not induce acute diuretic effects

ESTP did not increase diuresis 8 hours after treatment when compared to the control. On the other hand, the highest dose of ESTP (300 mg/kg) significantly increased urine density (Table 3). Regarding the urinary electrolyte profile, no significant changes were observed in Na^+, K^+ and Cl^- excretion in animals treated with all doses of ESTP (Table 4). HCTZ, as expected, was able to significantly increase diuresis and electrolyte contents (Na^+, K^+, and Cl^-) in urine samples (Tables 3 and 4). The urinary pH was not altered by any of the treatments.

3.5. ESTP induces sustained diuretic effects after prolonged treatment

The values obtained for urinary volume and renal elimination of electrolytes after prolonged treatment with ESTP or HCTZ are shown in Table 5. All ESTP doses (30, 100 and 300 mg/kg) increased diuresis on day 1, 3 and 7 after treatments and were statistically different from the control group. Regarding the electrolyte content, ESTP 30 and 100 mg/kg were able to increase Na^+ excretion on day 3 and 7 after

administration. ESTP 100 and 300 mg/kg increased K^+ and Cl^- excretion on day 3 and all doses were able to increase K^+ and Cl^- excretion on day 7. Furthermore, on day 7, ESTP 30 and 100 mg/kg significantly decreased pH values when compared to the control (data not shown). As expected, HCTZ was able to significantly increase diuresis and electrolyte contents (Na^+, K^+, and Cl^-) in urine samples on days 3 and 7. No changes were observed in urine density (data not shown).

3.6. ESTP does not promote changes in blood pressure and heart rate

SBP, DBP, MAP and HR values for rats treated orally with the vehicle alone were 125.26 ± 8.97, 69.93 ± 6.46, 95.33 ± 8.06 mm Hg and 322.52 ± 28.68 bpm, respectively. HCTZ, as expected, reduced basal SBP, DBP and MAP from 113 ± 3.30, 65.13 ± 2.11, and 84.23 ± 2.51 mm Hg to 87.35 ± 9.31, 51.21 ± 2.71, and 65.12 ± 3.55 mm Hg, respectively. Prolonged administration of different doses of ESTP (30, 100 and 300 mg/kg) was not able to significantly change blood pressure and heart rate when compared to the control (Table 6). Besides, no changes in serum urea, creatinine, K^+ and Na^+ levels were observed after prolonged treatment with ESTP or HCTZ (data no shown).

3.6. Effects on peripheral vascular resistance

ESTP-administrations induced an expressive dose-dependent vasodilator response in MVBs. The PP reduction values for doses of 0.1, 0.3, and 1 mg were ~ 1.46 ± 0.84, 5.62 ± 1.98, and 10.18 ± 4.29 mm Hg, respectively (Figure 5A). The vasodilatory effects induced by ESTP on preparations with intact endothelium were significantly increased after chemical removal of the endothelium. The vasodilator effects obtained at doses of 0.1, 0.3, and 1 mg were increased by approximately 330, 270, and 460% (Figure 5B). Similarly, the vasodilatory effects of ESTP remained

unaltered or were increased (especially in higher doses) in preparations with intact endothelium perfused with L-NAME (Figure 6A), indomethacin (Figure 6B), or L-NAME plus indomethacin (Figure 6C).

The perfusion of MVBs with nutritive solution added of 40 mM KCl abolished the effects of ESTP (Figure 7A). On the other hand, only minor effects were observed after infusion of GLB or 4-AP (Figure 7C and 8D). Interestingly, treatment with apamin vanished vasorelaxation induced by all doses of ESTP (Figure 7B).

4. Discussion

Talinum paniculatum is widely distributed over the Brazilian territory, mainly in the Biome Cerrado. Despite its use in traditional medicine for several purposes (Dos Reis et al., 2015; Coelho et al., 2018), detailed ethnopharmacological data on this species are still scarce. For this reason, we performed a detailed morpho-anatomical, phytochemical, toxicological, and pharmacological study of *T. paniculatum* leaves.

After plant collection in a Cerrado area of Mato Grosso do Sul state (Brazil) and proper identification, a detailed morphoanatomical and microchemical study of *T. paniculatum* leaves was carried out. Some medicinal plants have a wide variety of popular names, and can often be confused with other species due to similarity in common names as well as in their physical characteristics (American Herbal Pharmacopeia, 2011). For this reason, in order to guarantee quality control standards for the identification of the species under investigation and prevent tampering and errors in the use, morpho-anatomical surveys are applied. Besides, such investigations enable us to broaden our knowledge on the Brazilian Cerrado and its rich biodiversity.

Medicinal plants have been used since the early days for the treatment of several diseases as well as in religious practices (Desmarchelier et al., 1996). The

use of plant species in folk medicine has become increasingly popular due to the belief that whatever is natural is free of toxic effects. However, plants have the same potential to cause harm to human and animal health as other conventional drugs (El Hilaly et al., 2004). For this reason, the acute toxicological potential of ESTP was evaluated in female rats, as they are to a certain extent more sensitive than male rats (OECD, 2008). Parameters such as behavior, body weight gain, relative organ weight, food and water consumption were observed and recorded. Animals under toxic effects tend to exhibit behavioral changes, reduction in feed and water consumption and as a consequence, weight loss. Besides, organ weight is an important parameter as it indicates the physiological and pathological condition of animals (Raina et al., 2015). In our study, ESTP-treated rats presented a significant increase in relative body weight gain and no significant decrease in food and water consumption. No deaths nor changes in behavior were observed during the 14-day observation period. Regarding the relative organ weight, only animals treated with ESTP intermediate dose (300 mg/kg) presented a significant decrease in uterus relative weight when compared to the control. However, animals from this group did not present any changes in behavior, food consumption or body weight gain. Moreover, since the control of the estrous cycle was not performed, variations in reproductive organ weights were expected. Consequently, no biological meaning was attributed to this difference (Menegati et al., 2016). Therefore, ESTP can be considered safe in rats and its median lethal dose (LD50) is above 2000 mg/kg.

As all doses of ESTP demonstrated to be safe in rats after acute exposure, we next began the efficacy studies by first testing its diuretic potential. Despite acute administration of ESTP did not increase diuresis nor electrolyte excretion in 8-hour urine samples, we found that all doses of ESTP promoted a sustained diuretic effect for 7 days. Besides its potential as a diuretic agent, ESTP has demonstrated to be

effective in increasing renal excretion of Na^+, K^+ and Cl^- on days 3 and 7 after administration. According to previous studies (Aquino et al., 2017) renal excretion of electrolytes is usually decreased at the beginning of a prolonged treatment and tends to re-establish itself in approximately 3 days due to hemodynamic regulatory mechanisms that lead to dehydration. Such understanding corroborates with the results obtained in this work since sustained renal excretion of Na^+ and Cl^- are the main characteristics of effective diuretic agents (Roush et al., 2014). In addition, another important advantage is the absence of significant changes in serum urea, creatinine, K^+ and Na^+ levels after prolonged treatment with ESTP or HCTZ. Drugs that affect serum electrolyte balance are representative of significant toxicity. Thus, the absence of these changes points to the relative safety of the ESTP. These data also corroborate with serum urea and creatinine levels, indicating that ESTP does not significantly affect renal clearance. Taken together, these results provided important information about the low renal toxicity of ESTP (Risch and Hess, 2013).

As ESTP presented a significant diuretic activity after prolonged exposure, the following stage of this work was destined to evaluate the possible effects on blood pressure and heart rate. Curiously, prolonged treatment with ESTP did not promote any significant changes in blood pressure and heart rate. It is still premature to state whether the absence of a hypotensive response is due to long-term regulatory blood pressure mechanisms. It is likely that an important activation of the renin-angiotensin-aldosterone system due to significant diuretic activity may have contributed negatively to this process (Hoorn and Ellison, 2017). In addition, other local regulatory mechanisms may also be involved, including vasoconstrictor endothelial mediators such as endothelin and some prostanoids (Triggle et al., 2012). It is worth noting that some hypotensive agents - including diuretics - tend to intensify their hypotensive response within 4 weeks after initiation of therapy, and many of them do

not significantly reduce blood pressure of normotensive individuals (Oparil et al., 2018). Further studies after chronic treatment in different models of hypertension may clarify these doubts and show the real role of *T. paniculatum* as an antihypertensive agent.

One of the main aspects that positively influence the glomerular filtration rate (GFR) and, consequently, the maintenance of diuresis, is the renal blood flow. Undoubtedly, central to the physiologic maintenance of GFR is the differential basal tone of the afferent and efferent arterioles. The filtration rate is dependent on the higher hydrostatic pressure created by vasodilation of the afferent arteriole and by vasoconstriction of the output or efferent arteriole. It is known that the dilation of the afferent arterioles allows the increase of the hydrostatic pressure in the glomerular capillaries increasing the diuretic response (Palsson and Waikar, 2018). In fact, many diuretics including furosemide and HCTZ have part of their diuretic effects attributed to vasodilation on afferent arteriole (Duarte and Cooper-DeHoff, 2010). Thus, taking into account the physiological role of renal blood flow in maintaining diuresis, we decided to investigate whether ESTP could exert some vasodilatory effect in resistance vessels, using MVBs as an experimental tool. In fact, we observed that ESTP induces an important vasodilatory response on MVBs without the dependence of endothelial mediators. Surprisingly, the chemical removal of the endothelium intensified the vasodilatory response of ESTP. This pattern of response, in addition to indicating that the vasodilatory effect possibly comes from the smooth muscle cell, also shows that the removal of some endothelial mediators - possibly vasoconstrictors - intensifies the ESTP-response (Triggle et al., 2012). In addition, we also show that vasodilator activity induced by ESTP in normotensive rats is dependent on opening of SK KCa channels in vascular smooth muscle, since apamin - a potent blocker of SK KCa channels - vanished vasodilation induced by all doses

of ESTP (Bond et al., 2005; Matschke et al., 2018). The data presented allow us to speculate that the arterial vasodilator effects induced by ESTP may have contributed directly to the diuretic response. We believe that the effects observed in MVBs can be extrapolated to the renal vessels; where the consequent increase in glomerular hydrostatic pressure due to dilation of the pre-capillary arterioles may have increased the glomerular filtration rate and contributed to the diuretic response induced by *T. paniculatum.*

In the present study, leaves of *T. paniculatum* were extracted by infusion and after LC-DAD-MS analysis, a variety of amino acids, nucleosides, chlorogenic acids, organic acids and *O*-glycosylated flavones were identified. Previous studies reported that several flavonols and their glycosylated derivatives are highly effective in inducing diuretic and cardioprotective effects (Nijveldt et al., 2001; Wright et al., 2007; Gasparotto Junior et al., 2012; Boeing at al., 2017). Moreover, chlorogenic acids derivatives have showed important antioxidant, antihypertensive, hypolipidemic, and cardioprotective properties in animal models (Zhao et al., 2012; Naveed et al., 2018). Thus, our results suggest that the diuretic effects of ESTP may be due to a coordinated action of its secondary metabolites acting in an integrated and synergistic way.

In summary, the data presented show important information upon the ethnomedicinal properties of *T. paniculatum*. Thus, the study presents the ESTP as a possible herbal medicine, especially when a sustained diuretic effect is required.

5. Conclusion

This study has shown that *Talinum paniculatum* presents an important diuretic effect after prolonged exposure. Apparently, these effects are involved with the activation of the SK KCa channels contributing to the increase of renal blood flow and glomerular filtration rate.

Author's contributions

SELT: performed experiments related to toxicity, diuresis, blood pressure, heart rate and mechanisms involved. Also data analysis, discussion and wrote the manuscript. RACP, CAST, MIS, LPG and AOS: performed experiments related to blood pressure, heart rate and mechanisms involved; data analysis and discussed the results. AAMM and ELBL: plant collection, extract preparation, and performed experiments related to the diuretic potential of the species. VPA and JMB: performed the anatomical and micro chemical analysis. RICS and ACS: performed all work related to histopathological analysis. DBS: performed the phytochemical analysis. PRD: project co-advisor. AGJ: project advisor, conceived and planned the experiments, discussed data and wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

Authors declare there are no conflicts of interest.

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Appendix A: Supplementary information

Supplementary data associated with this article can be found in the online version.

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Legend to figures

Figure 1. Morpho-anatomy of leaves of *Talinum paniculatum* [c, d, h, k, l: light microscopy; e, f, g, i, j, m: FESEM]. (a) - Aspect of aerial parts and habit; (b) – Leaves (ad: adaxial side; ab: abaxial side); (c-g) - Leaf epidermis in surface view (c, e: abaxial side; d, f, g: adaxial); (h, k, l) - Transverse section of leaves (h: midrib; k, l: petiole). [ct, cuticle; dr, druse; ep, epidermis; gp: ground parenchyma, mu: mucilaginous cell; pp, palisade parenchyma; pr, prismatic crystal; sp, spongy parenchyma; st, stomata; vb, vascular bundle]. Scale bars: a = 10 cm, b = 2 cm, k = 500 µm, h = 300 µm, c, d, l = 50 µm, i, m = 10 µm, f, g, j = 5 µm.

Figure 2. Stem anatomy of *Talinum paniculatum* [a, c, d, e, i: light microscopy; b, f, g, h, j: FESEM]. (a, c–f, i, j) transverse sections; (b) frontal view; (g and h) view of crystals. [co: collenchyma, cx: cortex, dr: druse, ep: epidermis, es: stomata, fc: fascicular cambium, fi: fibers, ic: interfascicular cambium, pc: phenolic compounds, ph: phloem, pi: pith, pr: prismatic crystal, sg: starch grains, ve: vessel element, xy: xylem]. Scale bar: a = 500 µm, d = 200 µm, b, c, e, h, i, j = 50 µm, f = 10 µm, g = 5 µm.

Figure 3. EDS spectrum of crystals (druse) of *Talinum paniculatum.*

Figure 4. Base peak chromatogram in negative ion mode from *Talinum paniculatum* extract.

Figure 5. Endothelium-independent vasodilatory effects induced by ESTP in MVBs. MVBs were perfused with PSS containing Phe (3 μM) on intact (A) or denuded endothelium (B) and the vasorelaxant effect of ESTP was evaluated. The results show the mean ± S.E.M. of 5 preparations. In the graphic A [a] indicates $p < 0.05$ compared with the control (vehicle) group. [b] indicates $p < 0.05$ compared with the respective previous dose. In graphic B [a] indicates $p < 0.05$ compared with the respective previous dose. [#] indicates $p < 0.05$ compared with the effects of ESTP on intact endothelium. End - and End +: denuded and intact endothelium, respectively. MVBs: mesenteric vascular beds; Phe: phenylephrine.

Figure 6. Vasorelaxant effect of ESTP does not depend on nitric oxide or prostaglandins. MVBs were perfused with PSS containing Phe (3 μM) plus L-NAME (A), indomethacin (B), or L-NAME plus indomethacin (C) on intact endothelium, and the vasorelaxant effect was evaluated. The results show the mean ± S.E.M. of 5 preparations. [a] indicates $p < 0.05$ compared with the respective previous dose. [#] indicates $p < 0.05$ compared with the effects of ESAS on the respective vehicle group. INDO: indomethacin; L-NAME: N^G-nitro-L-arginine methyl ester; MVBs: mesenteric vascular beds; Phe: phenylephrine.

Figure 7. Vasorelaxant effect of ESAS depends on small-conductance calcium-activated potassium channels. MVBs were perfused with PSS containing Phe (3 μM) plus KCl (A), or apamin (B), or GLB (C), or 4-AP (D) on intact endothelium, and the vasorelaxant effect of ESAS was evaluated. The results show the mean ± S.E.M. of 5 preparations. [a] indicates $p < 0.05$ compared with the respective previous dose. [#] indicates $p < 0.05$ compared with the effects of ESAS on the respective vehicle group. 4-AP: 4-aminopyridine; GLB: glibenclamide; MVBs: mesenteric vascular beds; Phe: phenylephrine.

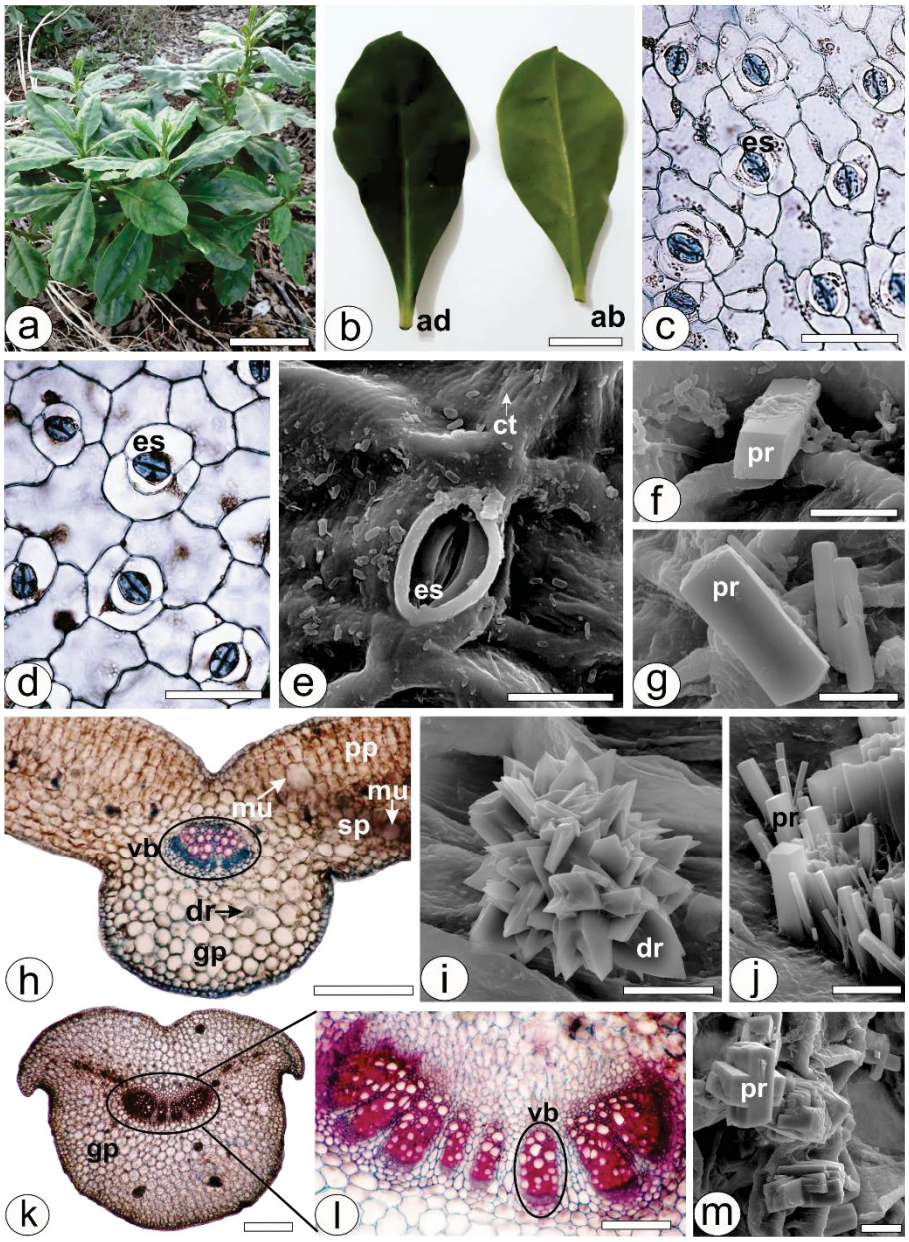

Figure 1

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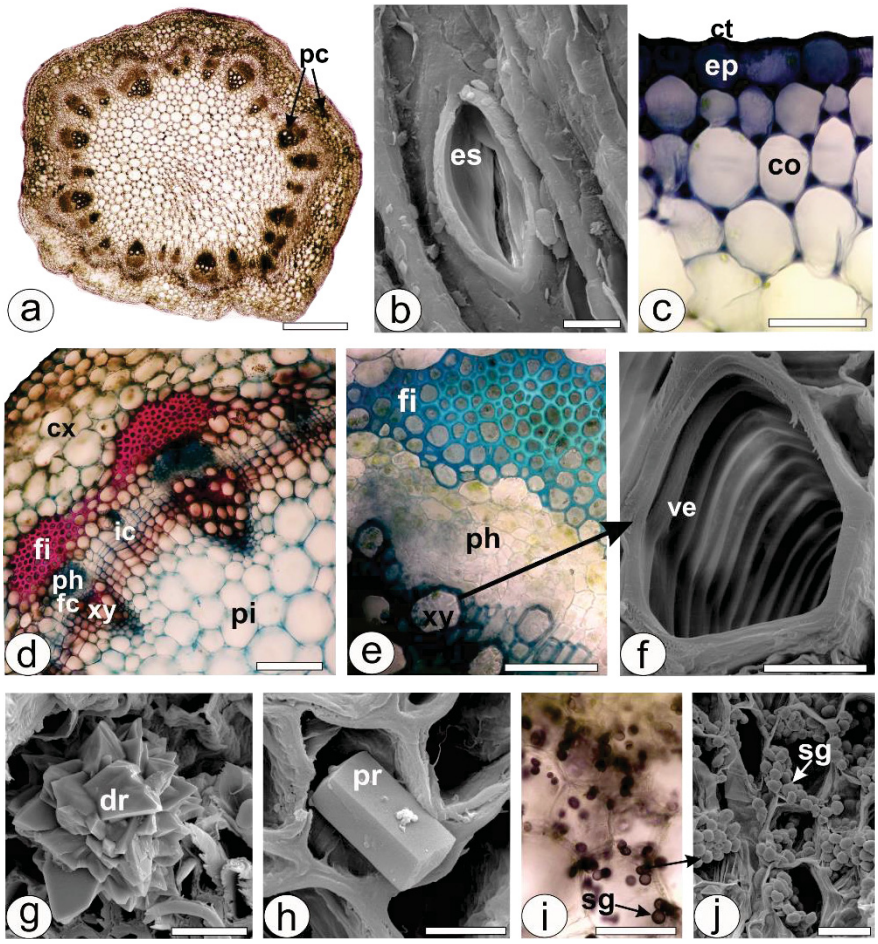

Figure 2

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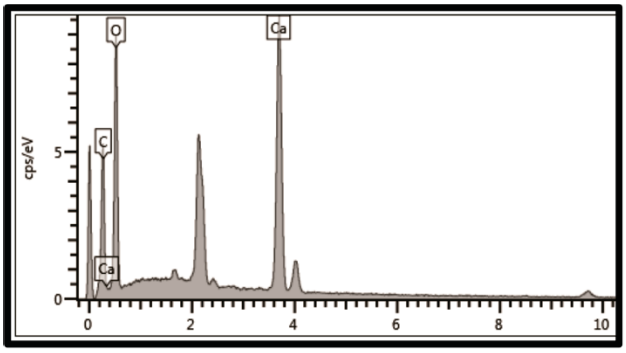

Figure 3

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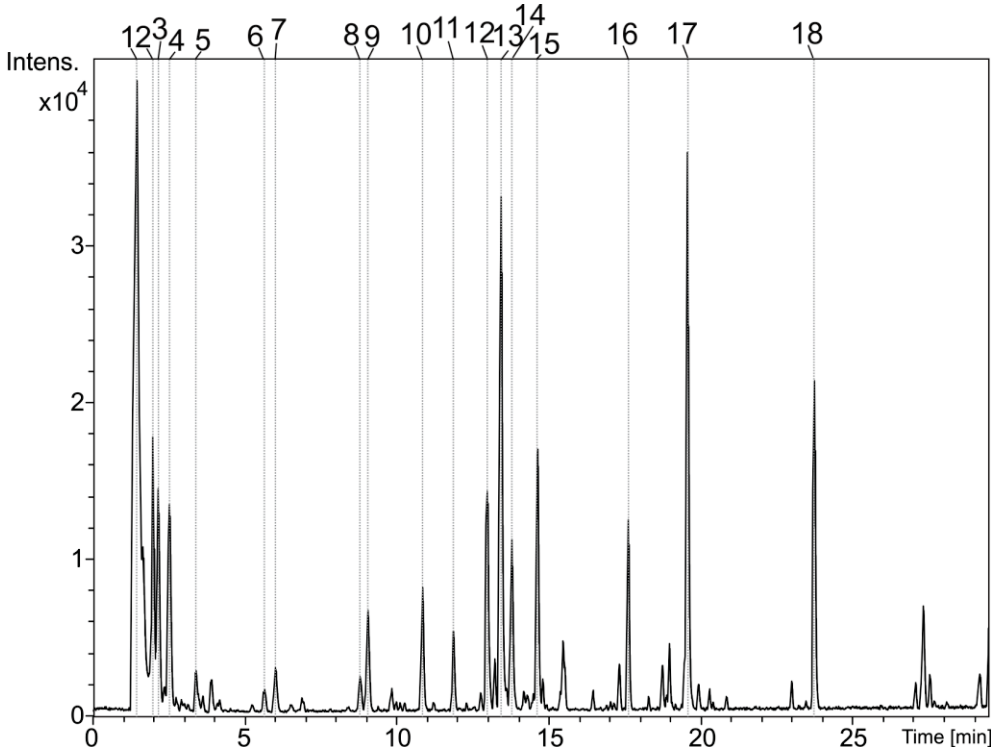

Figure 4

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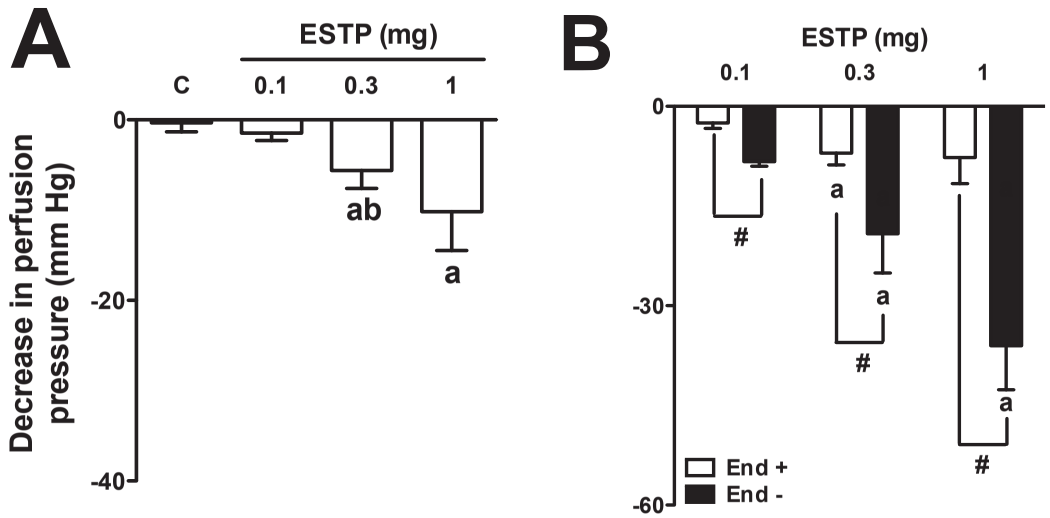

Figure 5

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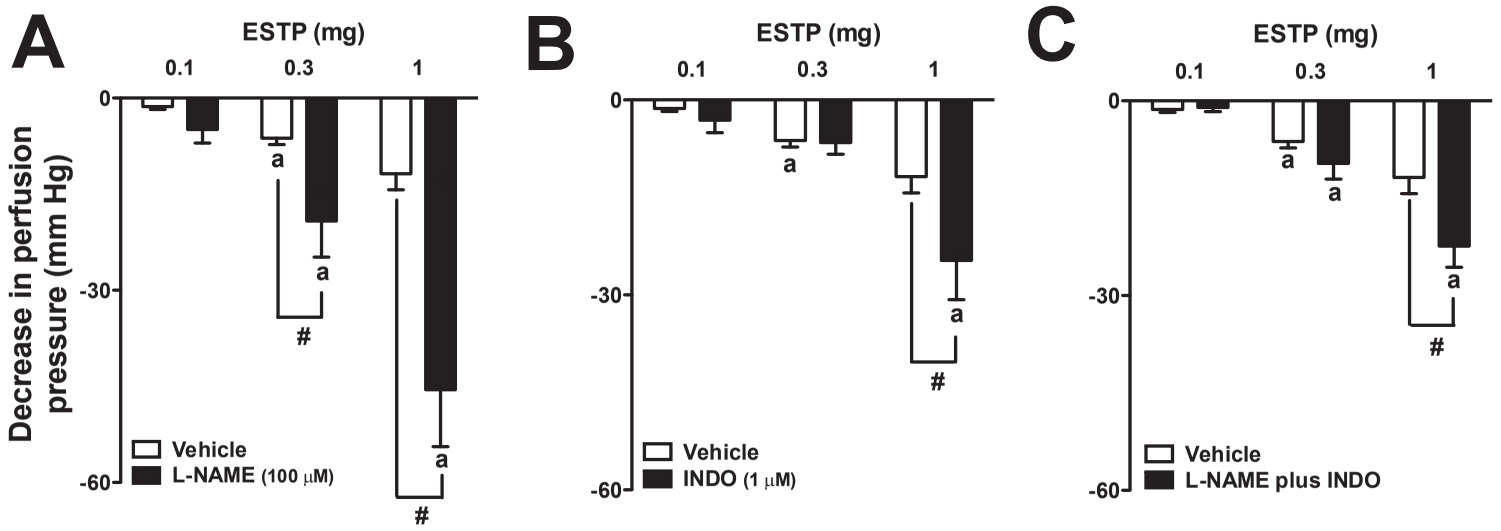

Figure 6

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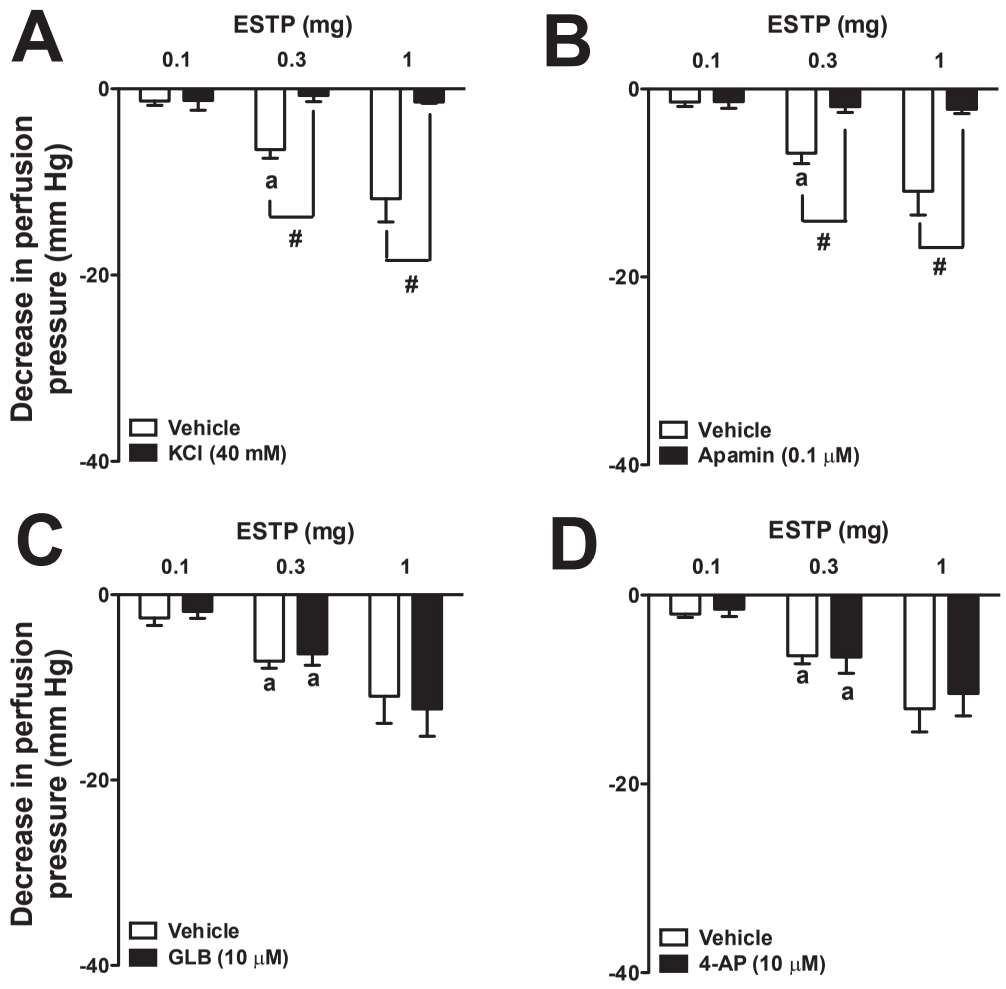

Figure 7

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Table 1. Metabolites identified from *Talinum paniculatum* extract by LC-DAD-MS.

Peak	RT (min)	Compound	MF	UV (nm)	Negative mode (*m/z*)		Positive mode (*m/z*)	
					MS [M-H]-	MS/MS	MS [M-H]-	MS/MS
1	1.4	Quinic acid	$C_7H_{12}O_6$	-	191.0542	-	193.0701	-
		Hexaric acid	$C_6H_{10}O_8$	-	209.0324	-	-	
2	1.9	Citric acid	$C_6H_8O_7$	-	191.0201	-	193.0360	-
3	2.1	N-pentosyl uracil (uridine)	$C_9H_{12}N_2O_6$	262	243.0617	-	245.0775	113
4	2.5	N-pentosyl guanine (guanosine)	$C_{10}H_{13}N_5O_5$	254, 277^{sh}	282.0851	150	284.1005	152
5	3.3	Phenylalanine	$C_9H_{11}NO_2$	255	164.0720	-	166.0865	-
6	5.6	Tryptophan	$C_{11}H_{12}N_2O_2$	260^{sh}, 285	203.0825	-	205.0973	-
7	6.0	5-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	299, 325	353.0879	191, 179	355.1043	163
8	8.8	3-*O*-*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	299, 310	337.0942	163	339.1075	-
9	9.0	*O*-*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	299, 310	337.0938	191, 163	339.1093	-
10	10.8	4-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	299, 325	353.0895	191, 173	355.1041	163
11	11.9	*O*-*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	299, 308	337.0938	191, 173	339.1094	147
12	13.0	*O*-*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	299, 310	337.0942	173, 163	339.1093	147
13	13.4	4-*O*-*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	299, 310	337.0947	173	339.1089	-
14	13.8	*N*-acetyl phenylalanine	$C_{11}H_{13}NO_3$	260	206.0828	164	208.1434	-
15	14.6	5-*O*-*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	299, 310	337.0944	191	339.1073	-
16	17.6	*O*-deoxyhexosyl-hexosyl luteolin	$C_{27}H_{30}O_{15}$	265, 335	593.1544	284, 255, 227	595.1694	449, 287
17	19.6	*O*-deoxyhexosyl-hexosyl *O*-methyl luteolin	$C_{28}H_{32}O_{15}$	265, 332	607.1691	299, 284	609.1783	301, 286
18	23.7	di-*O*-hexosyl di-O-methyl luteolin	$C_{28}H_{32}O_{15}$	265, 332	621.1838	313, 298	623.1952	315, 300

RT: retention time; MF: molecular formula; NI: non-identified; ^{sh}: shoulder. MF were established based on error up to 7 ppm and mSigma below 30 ppm.

Table 2. Effect of acute oral administration of ethanol soluble fraction obtained from *Talinum paniculatum* (ESTP) on body weight gain (g), relative body weight gain (%), relative organ weight (g/100g body weight), food (g/day) and water (mL/day) consumption of female rats in the acute toxicity test

Parameter	Control	ESTP 30 mg/kg	ESTP 300 mg/kg	ESTP 2000 mg/kg
Body weight gain	10.62 ± 3.47	24.87 ± 3.85[a]	22.11 ± 1.65[a]	21.00 ± 2.65
Relative body weight gain	4.88 ± 1.51	11.05 ± 1.41[a]	10.04 ± 0.69[a]	9.96 ± 1.19[a]
Food intake	76.12 ± 0.72	80.48 ± 1.98	85.24 ± 1.81[a]	73.36 ± 3.93
Water intake	130.4 ± 3.55	140.4 ± 4.97	147.29 ± 5.76[a]	141.4 ± 6.53
Heart	0.37 ± 0.00	0.37 ± 0.00	0.36 ± 0.00	0.38 ± 0.01
Lung	0.76 ± 0.07	0.76 ± 0.05	0.75 ± 0.06	0.76 ± 0.04
Liver	4.52 ± 0.08	4.76 ± 0.14	4.48 ± 0.15	4.29 ± 0.16
Spleen	0.24 ± 0.01	0.24 ± 0.00	0.25 ± 0.01	0.25 ± 0.00
Right Kidney	0.39 ± 0.01	0.40 ± 0.01	0.41 ± 0.01	0.40 ± 0.01
Left Kidney	0.37 ± 0.00	0.40 ± 0.01	0.40 ± 0.01	0.40 ± 0.01
Right Ovary	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Left Ovary	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Uterus	0.26 ± 0.02	0.21 ± 0.01	0.19 ± 0.01[a]	0.22 ± 0.01

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Values are expressed as mean ± S.E.M. (n = 8) in comparison to the control group (a: $p \leq 0.05$).

Table 3. Effect of acute oral administration of ethanol soluble fraction obtained from *Talinum paniculatum* (ESTP) on the urinary volume, pH and density in urine samples 8 hours after treatment.

Group	Urine volume (ml/100g/8h)	pH (8 h)	Density (8 h)
Control	5.09 ± 0.69	10.14 ± 0.32	1022 ± 3.71
HCTZ (25 mg/kg)	6.92 ± 0.14[a]	8.51 ± 0.17	1017 ± 0.42
ESTP (30 mg/kg)	4.30 ± 0.43	9.95 ± 0.47	1026 ± 2.21
ESTP (100 mg/kg)	3.67 ± 0.21	10.30 ± 0.61	1028 ± 1.50
ESTP (300 mg/kg)	4.05 ± 0.41	10.37 ± 0.60	1031 ± 1.73[a]

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Values are expressed as mean ± S.E.M. (n = 6) in comparison to the control group (a: $p \leq 0.05$). HCTZ: hydrochlorothiazide.

Table 4. Effect of acute oral administration of ethanol soluble fraction obtained from *Talinum paniculatum* (ESTP) on urinary electrolyte excretion in urine samples 8 hours after treatment

Group	EI_{Na+} (µEq/min/100g)	EI_{K+} (µEq/min/100g)	EI_{Cl-} (µEq/min/100g)	[b]Saluretic índex		
				Na^+	K^+	Cl^-
Control	1.10 ± 0.16	1.10 ± 0.20	1.70 ± 0.24	-	-	-
HCTZ (25 mg/kg)	1.96 ± 0.05[a]	2.85 ± 0.05[a]	2.04 ± 0.07[a]	1.78	2.59	1.20
ESTP (30 mg/kg)	1.03 ± 0.12	1.08 ± 0.10	1.63 ± 0.14	0.93	0.98	0.95
ESTP (100 mg/kg)	1.00 ± 0.17	1.10 ± 0.07	1.68 ± 0.16	0.90	1.00	0.98
ESTP (300 mg/kg)	1.12 ± 0.03	1.39 ± 0.08	2.03 ± 0.09	1.01	1.26	1.19

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. HCTZ treatments were compared with the control using Student's t-test. Values are expressed as mean ± S.E.M. (*n* = 6). [a]p≤ 0.05 when compared with the control group. [b]Saluretic index= µEq/min/100g problem group / µEq/min/100g control group. EI: Excreted load; HCTZ: hydrochlorothiazide.

Table 5. Effects of prolonged oral administration of ethanol soluble fraction obtained from *Talinum paniculatum* (ESTP) on cumulative urine volume, Na^+, K^+ and Cl^- excretion.

Group	Cumulative urine volume (mL/100 g)	EI_{Na+} (µEq/min/100g)	EI_{K+} (µEq/min/100g)	EI_{Cl-} (µEq/min/100g)
Day 1				
Control	4.11 ± 0.30	0.30 ± 0.03	0.60 ± 0.06	0.70 ± 0.08
HCTZ (25 mg/kg)	5.84 ± 0.58	0.30 ± 0.07	0.40 ± 0.10	0.40 ± 0.11
ESTP (30 mg/kg)	10.22 ± 0.94[a]	0.74 ± 0.05	1.35 ± 0.07	1.52 ± 0.11
ESTP (100 mg/kg)	10.10 ± 0.72[a]	0.77 ± 0.09	1.48 ± 0.15	1.66 ± 0.19
ESTP (300 mg/kg)	9.15 ± 0.60[a]	0.78 ± 0.04	1.53 ± 0.09	1.72 ± 0.10
Day 3				
Control	7.38 ± 0.40	0.50 ± 0.24	1.10 ± 0.48	1.20 ± 0.53
HCTZ (25 mg/kg)	15.50 ± 1.62[a]	3.20 ± 0.24[a]	4.30 ± 0.50[a]	4.90 ± 0.30[a]
ESTP (30 mg/kg)	17.49 ± 1.58[a]	1.85 ± 0.35[a]	3.37 ± 0.68	3.90 ± 0.81
ESTP (100 mg/kg)	19.89 ± 1.63[a]	2.37 ± 0.07[a]	4.49 ± 0.17[a]	5.14 ± 0.20[a]
ESTP (300 mg/kg)	17.37 ± 0.88[a]	2.21 ± 0.11	4.49 ± 0.32[a]	5.06 ± 0.26[a]
Day 7				
Control	14.10 ± 0.98	2.50 ± 0.18	4.60 ± 0.36	4.80 ± 0.47
HCTZ (25 mg/kg)	33.16 ± 3.31[a]	7.60 ± 0.45[a]	11.0 ± 0.79[a]	13.90 ± 0.75[a]
ESTP (30 mg/kg)	25.73 ± 2.22[a]	4.08 ± 0.37[a]	7.56 ± 0.73[a]	8.52 ± 1.02[a]
ESTP (100 mg/kg)	30.17 ± 2.54[a]	4.66 ± 0.10[a]	8.66 ± 0.31[a]	9.62 ± 0.24[a]
ESTP (300 mg/kg)	25.89 ± 1.08[a]	4.68 ± 0.28	9.44 ± 0.80[a]	10.24 ± 0.72[a]

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. HCTZ treatments were compared with the control using Student's t-test. Values are expressed as mean ± S.E.M. (*n* = 6). [a]p≤ 0.05 when compared with the control group. EI: Excreted load; HCTZ: hydrochlorothiazide.

Table 6. Effect of prolonged oral administration of ethanol soluble fraction obtained from *Talinum paniculatum* (ESTP) on arterial pressure and heart rate of male Wistar rats.

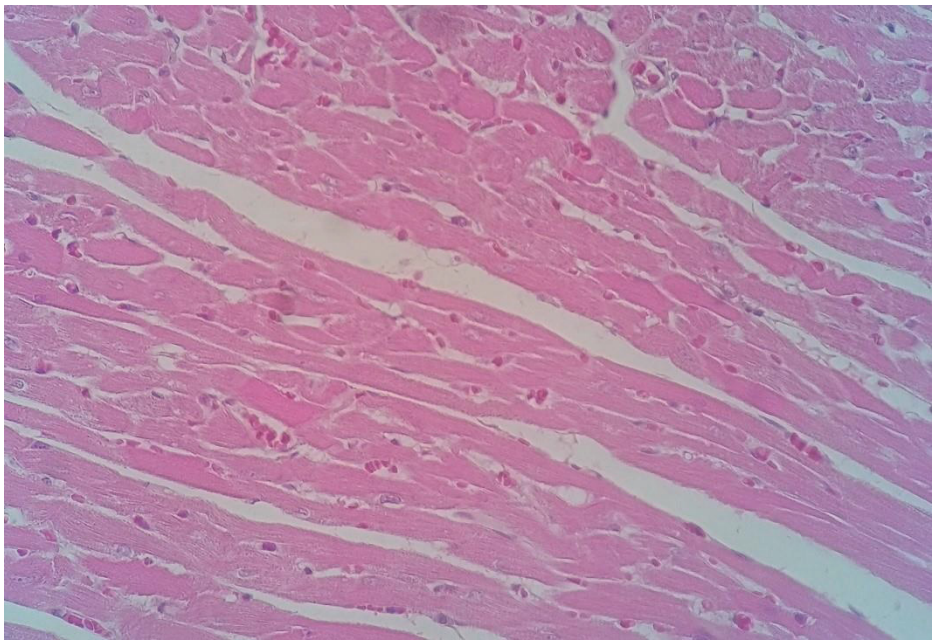
Group	SBP (mm Hg)	DBP (mm Hg)	MAP (mm Hg)	HR (bpm)
Control	125.26 ± 8.97	69.93 ± 6.46	95.33 ± 8.06	322.52 ± 28.68
HCTZ (25 mg/kg)	87.35 ± 9.31[a]	51.21 ± 2.71[a]	65.12 ± 3.55[a]	250.75 ± 42.34
ESTP (30 mg/kg)	119.54 ± 18.41	68.09 ± 10.60	91.41 ± 14.13	290.75 ± 35.58
ESTP (100 mg/kg)	125.74 ± 11.36	74.00 ± 5.73	99.05 ± 7.66	367.50 ± 37.35
ESTP (300 mg/kg)	123.07 ± 10.01	70.63 ± 8.45	96.84 ± 9.16	326.83 ± 25.66

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. HCTZ treatments were compared with the control using Student's t-test. Values are expressed as mean ± S.E.M. (*n* = 6). [a]p ≤ 0.05 when compared with the control group. HCTZ: hydrochlorothiazide.

SUPPLEMENTAL INFORMATION

Appendix A

(A)



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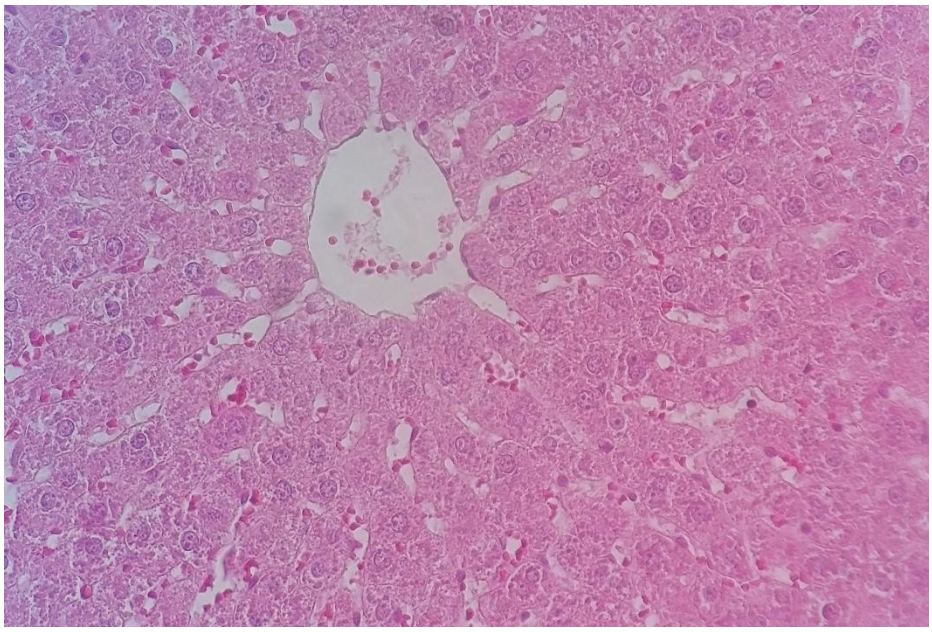

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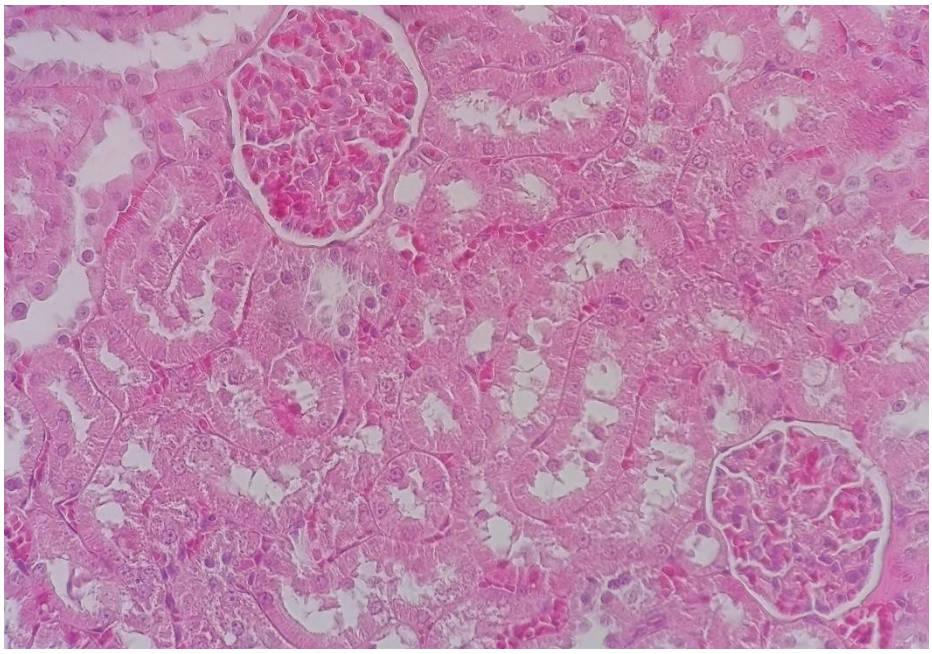

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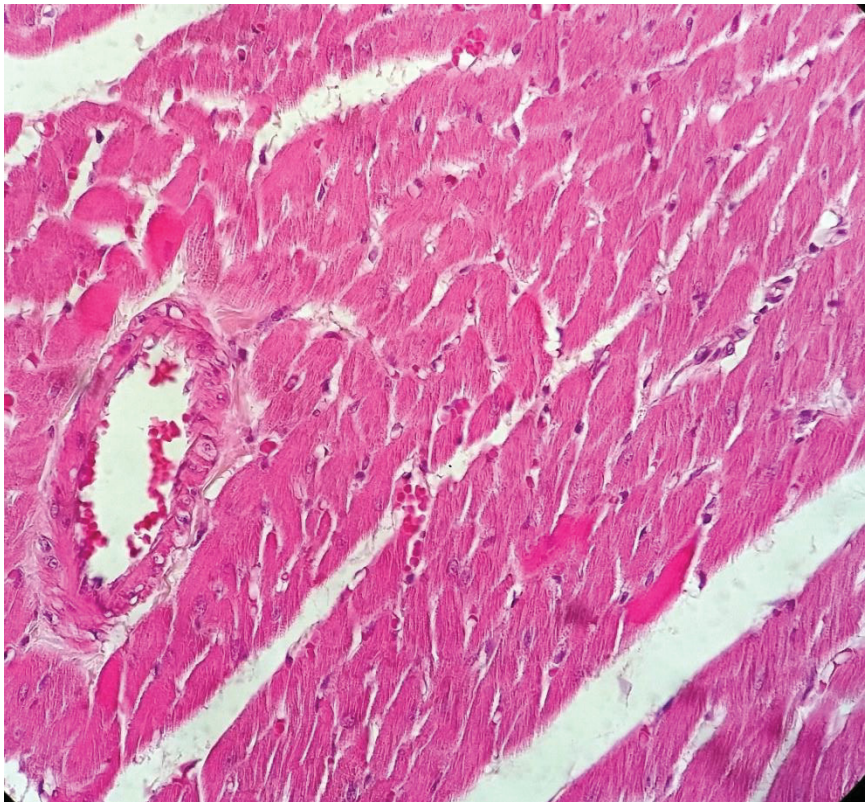

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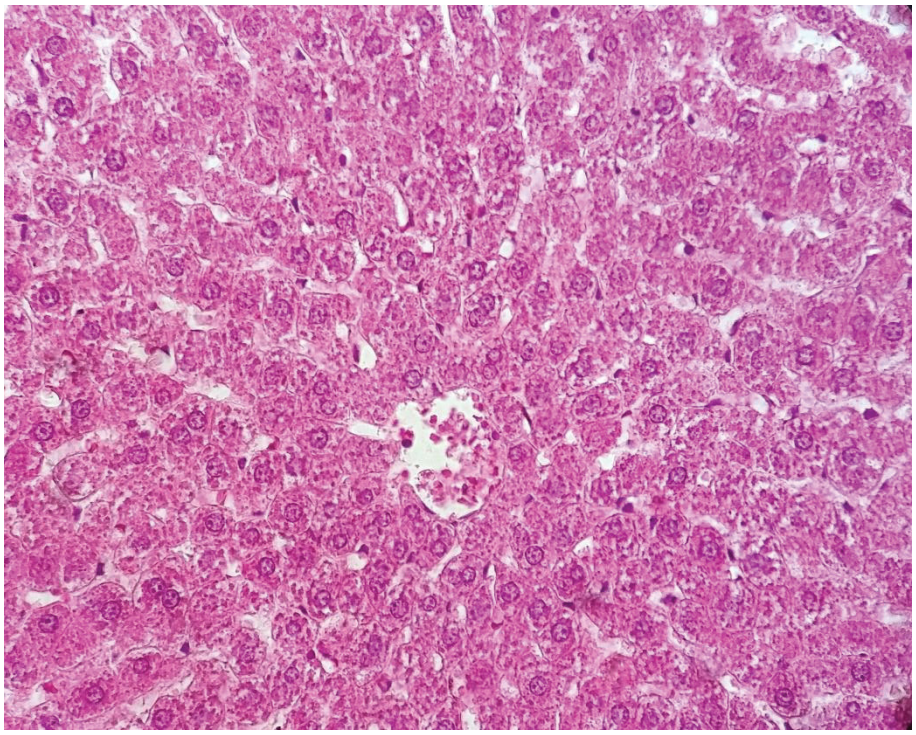

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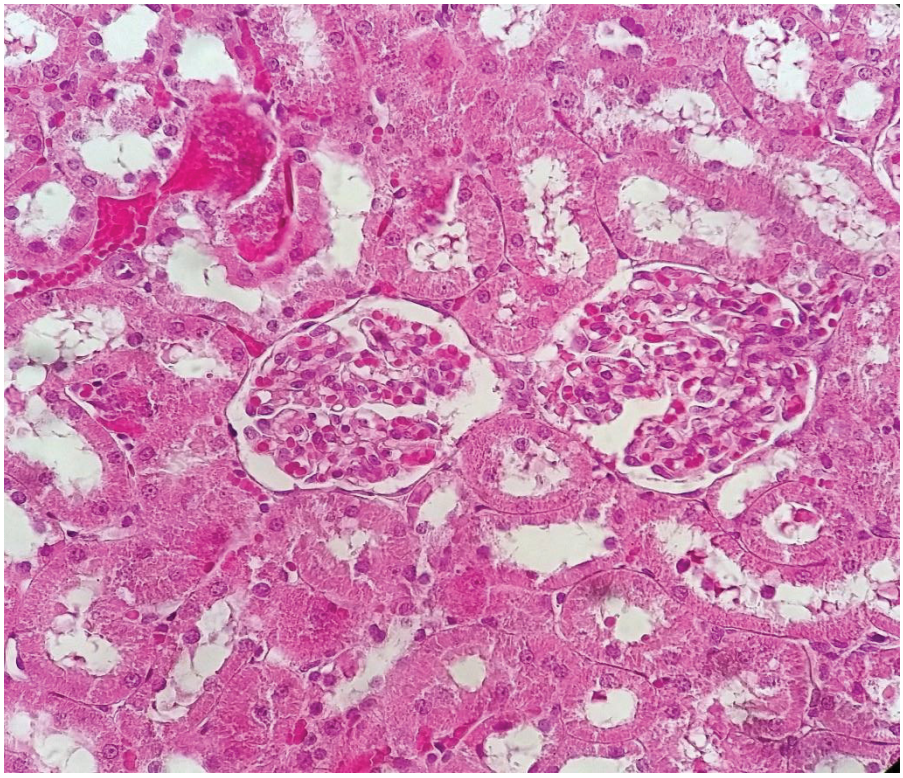
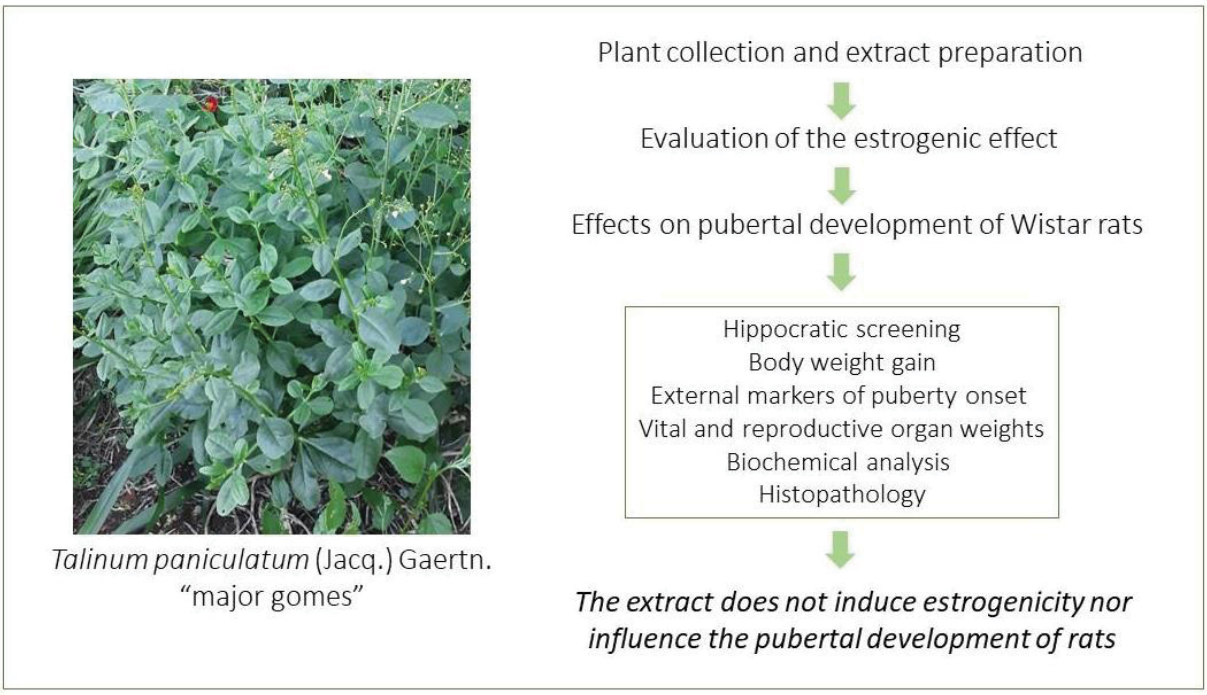


Supplemental Figures

Figure S1. Histopathological assessment of organs from female rats orally treated with the vehicle (control) and ESTP 2000 mg/kg in the acute toxicity test. (A) heart, (B) liver and (C) kidney from the control group; (D) heart, (E) liver and (F) kidney from animals treated with ESTP 2000 mg/kg. HE (40 X).

7 ARTIGO CIENTÍFICO 4: Effects of *Talinum paniculatum* (Jacq.) Gaertn. leaf extract on general toxicity and pubertal development of rats.

Graphical abstract



Effects of *Talinum paniculatum* (Jacq.) Gaertn. extract on pubertal development of male and female Wistar rats

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Abstract:

Ethnopharmacological relevance: Talinum paniculatum (Jacq.) Gaertn. (Talinaceae), popularly known as “major gomes”, is a Cerrado plant used in traditional medicine as diuretic, aphrodisiac, to treat some kinds of infections and gastrointestinal problems. Recent pharmacological studies have demonstrated its diuretic effects after prolonged exposure. However, no studies have been performed on the safety as well as on its effects during puberty after prolonged treatment.

Aim: To investigate the effects of the ethanol soluble fraction of *T. paniculatum* leaves (ESTP) on the reproductive system as well as its toxicological potential in male and female Wistar rats.

Material and Methods: For this purpose, the uterotrophic and the pubertal assays were performed. In the uterotrophic test, female immature rats were treated for three consecutive days with three doses (30, 100 and 300 mg/kg) of ESTP. Uterus without luminal fluid were weighed and the relative weight calculated. For the pubertal assay, male and female immature rats were submitted to a 30-day treatment with two doses (30 and 300 mg/kg) of ESTP and the following parameters were observed: daily signs of toxicity, body weight, food and water intake, the onsets of vaginal opening (VO) and preputial separation (PPS), relative organ weight, and biochemical and histopathological analyses.

Results: Our data revealed that ESTP did not promote estrogenic effects in female rats. In the pubertal test, no daily signs of toxicity nor weight loss were observed. Moreover, ESTP did not affect the onset of VO and PPS and did not cause significant changes in biochemical parameters as well as in organ weight and histopathological analyses of animals.

Conclusion: ESTP can be considered safe and does not accelerate nor delay the onset of puberty in female and male Wistar rats at the doses tested.

Keywords: Reproductive toxicity, safety, Talinaceae, toxicology, uterotrophic.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; DEHP, di (2-ethylhexyl) phthalate; EDCs, endocrine disrupting chemicals; ESTP, ethanol soluble fraction from *Talinum paniculatum*; GGT, gamma-glutamyl transpeptidase; HPA, hypothalamic-pituitary-adrenal; HPG, hypothalamic-pituitary-gonadal; UFGD, Universidade Federal da Grande Dourados; UFPR, Universidade Federal do Paraná; OECD, Organization for Economic Co-operation and Development; PND, postnatal day; PPS, preputial separation; VO, vaginal opening.

1. Introduction

Talinum paniculatum (Jacq.) Gaertn. (Talinaceae) is a weedy species from the Brazilian Cerrado popularly known as “major gomes”. Folk medicine in Mato Grosso do Sul State uses the infusion of *T. paniculatum* leaves to treat several cardiovascular and urinary tract diseases (Coelho et al., 2019). Bioactive compounds of *T. paniculatum leaves* include tannins, triterpenes, steroids, saponins (Yulia et al., 2006) and phytosterols (Dos Reis et al., 2015). In addition, a recent study performed with the ethanol soluble fraction from *T. paniculatum* showed the presence of chlorogenic acids, amino acids, nucleosides, O-glycosylated flavones and organic acids in its composition (Tolouei et al., 2019).

Previous pharmacological studies have proved its potential as antidiabetic (Shimoda et al., 2001), antinociceptive (Ramos et al., 2010) and antimicrobial agents and to treat gastrointestinal disorders (Dos Reis et al., 2015). Moreover, it has also demonstrated its renal effects as it promoted an important diuretic effect after prolonged exposure and no signs of toxicity after a single exposure in Wistar rats (Tolouei et al., 2019). These findings demonstrate an important pharmacological action of the species, showing its great potential in becoming raw material for the development of new diuretic agents. However, no studies have been performed to detect (potential) endocrine and toxicological effects of *T. paniculatum* leaves in rodents.

Medicinal plants and plant-derived compounds can display a wide range of toxicological effects, including hepatic, renal, cardiac, pulmonary, reproductive, and endocrine toxicity. Some of these effects may not be manifested following acute exposures, but may be evident after long-term use. Also, sensitive populations, such as infants and children may be more susceptible to adverse effects induced by drugs,

including medicinal herbs (Mone et al., 2004; Fenton, 2006; Beszterda and Frański, 2018). Effects on the endocrine system are of particular concern, because hormones have essential roles in the homeostatic control of all organ systems and also in tissues differentiation and growth during development. As known, endocrine disrupting chemicals (EDCs) can cause a wide range of negative effects, including developmental, reproductive, neurological, and immunological changes in animals and humans. These substances may disrupt the hypothalamic-pituitary-adrenal (HPA) and the hypothalamic-pituitary-gonadal (HPG) axis as they may act via hormone receptors as well as in the production, storage, release, transport, metabolism, and also in the elimination of hormones, promoting hormonal imbalance (Kavlock et al., 1996; OMS, 2012).

EDCs are considered ubiquitous as they can be found in man-made products such as plasticizers, toiletries, toys, cosmetics, drugs and pesticides as well as in natural sources such as hormones and plant bioactive compounds (Monneret, 2017). For instance, many plants and their extracts contain phytoestrogens that can interact with estrogen receptors and change the development and function of the endocrine and reproductive systems, which are particularly sensitive to hormonal imbalances. Industrial and natural occurring endocrine disruptors have become a major concern worldwide as they can affect the reproductive development, leading to reproductive disorders and infertility (Kabir et al., 2015). *In vivo* male and female pubertal and uterotrophic bioassays are among the recommended screening tests to identify substances with potential to interact with the endocrine system. Based on that, we aimed to investigate the possible effects of *T. paniculatum* leaf extract on general toxicity and on the development and maturation of the male and female rat reproductive system following repeated-dose toxicity protocols as well as its estrogenic potential in the uterotrophic assay using immature female rats.

2. Materials and Methods

2.1. Plant material and extract preparation

Talinum paniculatum aerial parts were collected in February 2017 in a Cerrado area in Dourados, Mato Grosso do Sul State - Brazil, at 458 m above sea level (22°12'22.6"S 54°47'43.1"W). A voucher specimen was then prepared, authenticated by Dr. Maria do Carmo Vieira and deposited in the Herbarium of the Federal University of Grande Dourados (UFGD) under the number 5539.

For the extract preparation, *T. paniculatum* leaves were carefully separated from the stems, washed in running water and then air-dried in an oven at 40°C for 7 days. Afterwards, dried leaves were ground into fine powder and the infusion was made by pouring 1 L of boiling water (97 °C) on each 100 g of ground leaves. The extraction occurred until room temperature was reached (~ 5 hours) and then, the infusion was treated with 3 volumes of ethanol, originating a precipitate and an ethanol soluble fraction (ESTP; 3.42% yield). ESTP samples were freeze-dried and stored in a freezer at -18 ◦C for further experiments. The chemical characterization of the ESTP used in this study was recently described by Tolouei et al. (2019).

2.2. Animals

Forty male and eighty female Wistar rats - postnatal day (PNP) 21 - were obtained from the animal facility of the Federal University of Paraná (UFPR). Animals were housed under standard conditions of temperature (22 ± 3°C), light (12-h light/dark cycle), humidity (50-60%) and had *ad libitum* access to filtered water and food pellets. All procedures involving animals were previously approved by the Ethics Committee in Animal Experimentation from UFPR (protocol: 05/2017).

2.3. Uterotrophic bioassay

This test was performed in accordance with the protocol 440 described by the Organization for Economic Co-operation and Development (OECD) in 2007. Forty immature female rats (PND 21) were randomized and divided into five experimental groups, as follows: negative control (vehicle, 2 ml/kg); ESTP 30 mg/kg; ESTP 100 mg/kg; ESTP 300 mg/kg and 17α-ethinylestradiol (3 µg/kg, positive controle). After one day of acclimation, rats were treated for three consecutive days by oral gavage (PND 22-24) and doses were daily calculated according to each animal body weight. Twenty-four hours after the last treatment (PND 25), rats were individually weighed and humanely euthanized by isoflurane anesthesia (inhalation) followed by decapitation. Necropsy was performed and uterine horns were carefully detached from the body wall and transferred to a petri dish. Uterus without luminal fluid was weighed and the relative weight calculated.

2.4 Pubertal bioassay

Both male and female pubertal assays were conducted simultaneously and were performed in accordance with protocols established by Marty et al., (1999) and Marty et al., (2001), with some modifications.

Immature male and female rats at 21 days of age were randomized and divided into eight experimental groups (n = 10). Animals were treated by oral gavage from PND 23 to PND 53 with two doses of ESTP (30 and 300 mg/kg), vehicle (filtered water, 5 ml/kg), di (2-ethylhexyl) phthalate (DEHP 750 mg/kg, male positive control) or 17α-ethinylestradiol (3 µg/kg, female positive control). The ESTP doses were based on our previous study showing ethnopharmacological properties of *T. paniculatum* (Tolouei et al., 2019). The DEHP-dose was based on previous studies

performed by Kita et al., (2016) and Venturelli et al., (2019), while the selected 17α-ethinylestradiol dose was chosen on the basis of recent uterotrophic assays conducted by us (unpublished).

Throughout the experimental period, all animals were carefully observed once daily for any clinical signs of toxicity according to the Hippocratic screening, as follows: general activity, reflexes, response to tail touch and grip, straightening, strength to grab, tremors, straub tail reaction, sedation, lacrimation, cyanosis, salivation and piloerection (Malone and Robichaud, 1962). Body weights were measured daily for dose calculation and statistical analysis at 23, 29, 36, 43 and 50 days of age. In addition, food and water consumption were evaluated on days 23, 29, 36, 43 and 50 after birth.

Vaginal opening (VO) and preputial separation (PPS), external markers of puberty onset, were examined from PND 30 and PND 35 onwards in female and male rats, respectively. On the day VO and PPS were achieved, weight and age of animals were recorded.

After the last treatment (PND 54), overnight fasted animals were first weighed and then euthanized by isoflurane anesthesia (inhalation) followed by decapitation. Blood samples were collected for further analysis. On necropsy, vital (heart, spleen, liver and kidneys) and reproductive organs (uterus, ovaries, testes, epididymis, prostate, glans penis, levator ani muscle, bulbourethral glands and seminal vesicles) were removed, carefully cleaned, weighed, and macroscopically analyzed for any gross changes. Absolute (g) and relative (%) organ weights were calculated based on animal body weights determined right before euthanasia. Thyroid was also removed but as it was still attached to the trachea, absolute and relative weights were not calculated. Thyroid separation was only performed before histopathological evaluation.

After necropsy, thyroid, heart, spleen, liver, kidneys, uterus, ovaries, testes and epididymis samples were sent to histopathological analysis that was performed by two veterinary pathologists from the Universidade Federal da Grande Dourados (UFGD).

2.4.1. Biochemical analysis

Analysis were performed using commercial kits from Bioclin brand (Belo Horizonte, Minas Gerais, Brazil) in a Mindray BS-200® automated biochemical analyzer, and the following parameters were evaluated: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, gamma-glutamyl transpeptidase (GGT), total protein, albumin, blood urea nitrogen (BUN), creatinine, calcium, total cholesterol, triglycerides, glucose, direct bilirubin, total bilirubin, indirect bilirubin and uric acid.

2.4.2. Histopathology

Vital (heart, liver, spleen and kidney) and reproductive organs (ovary, uterus, testis, and epididymis), and the thyroid gland attached to the trachea were removed and cut into 5 mm fragments. Fragments of thyroid gland and all vital and female reproductive organs were fixed in 10% buffered formalin. Male reproductive organs were fixed in Bouin's solution for 6 hours. Thereafter, fragments were washed in tap water and immersed in 70% ethanol, which was changed every 24 hours for 7 days. Then, fragments were cleaved, dehydrated with increasing absolute ethanol concentrations, diaphanized in xylol and embedded in paraffin. All sections were cut at a thickness of 4 μm and stained with hematoxylin and eosin (HE) for evaluation under light microscopy (40 X). The parameters analyzed were based on the presence or absence of reversible and/or irreversible cellular lesions.

2.5. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test, or by Student's t-test when applicable. Results are expressed as mean ± standard error of the mean (S.E.M.) and the level of statistical significance was 5% ($p < 0.05$). Graphs were drawn and statistical analyses were performed using GraphPad Prism software version 6.0.

3. Results

3.1. ESTP does not induce estrogenic effects in immature female rats

In the uterotrophic assay, female rats exposed to ESTP 30, 100 and 300 mg/kg did not present any significant differences regarding the relative empty uterus weight when compared to the control. As expected, animals from the positive control (17α-ethinylestradiol 3 μg/kg) presented a significant increase in relative uterus weight when compared to the control (Figure 1).

3.2. ESTP does not cause death nor visible signs of toxicity in rats

No deaths nor clinical signs of toxicity, according to the Hippocratic screening, were observed in male and female rats treated orally from PND 33 to PND 53 with ESTP 30 and 300 mg/kg as well as in animals treated with the vehicle, DEHP (750 mg/kg, male negative control) or 17α-ethinylestradiol (3 μg/kg, female positive control). The behavior of animals was recorded daily and no changes were observed (data not shown).

3.3. ESTP does not cause animal weight loss nor changes in food and water consumption

Our data shows that all animals submitted to this research gained weight along the experimental period (Table 1). When statistical analyses were performed, it is possible to observe that animals treated with ESTP 30 and 300 mg/kg gained weight along the 30-day treatment (Figure 2). Thus, no significant differences were observed in relation to final body weight of animals treated with both doses of ESTP when compared to the control (Table 1). On the other hand, animals treated with 17α-ethinylestradiol (female positive control) and DEHP (male positive control) presented a significant decrease in body weight gain when compared to the control (Table 1; Figure 2). Regarding food and water consumption, no significant differences were observed in groups treated with ESTP 30 and 300 mg/kg when compared to the control (Table 1).

3.4. ESTP does not affect the onset of VO and PPS

ESTP (30 and 300 mg/kg) did not cause any significant differences in the onset of VO nor in PPS when compared to the control (Figure 2). As expected, the positive control for males (DEHP, 750 mg/kg) delayed the age of PPS as it was significantly different from the negative control group (Figure 2). In females, VO occurred earlier in animals treated with the positive control (17α-ethinylestradiol, 3 µg/kg), but this change was not statistically different from control.

3.5. ESTP does not induce significant changes in relative organ weight

No significant changes were observed in relative organ weights from female and male rats treated orally with ESTP 30 and 300 mg/kg when compared to the control (Table 2). However, animals treated with DEHP 750 mg/kg (positive control for male rats) showed significant differences in heart, liver, spleen, testicles,

epididymis, empty seminal vesicle, and levator ani muscle relative weights when compared to the control (Table 2).

3.6. Biochemical analysis

The effects of a 30-day administration of ESTP from *Talinum paniculatum* on biochemical parameters of female and male rats in the pubertal assay are presented in Table 3. No significant differences were observed in levels of GGT, total protein, albumin, total cholesterol, triglycerides, glucose, direct bilirubin, indirect bilirubin and uric acid from females treated with two doses of ESTP (30 and 300 mg/kg) when compared to the control. However, it is possible to observe a significant decrease in levels of ALT in females treated with both doses of ESTP (30 and 300 mg/kg) when compared to the control. Also, a subtle but significant increase in levels of calcium was observed in females treated with ESTP 30 mg/kg when compared to the control. Significant differences were observed in levels of AST, ALT, alkaline phosphatase, BUN, creatinine and total bilirubin from female rats from the positive control (17α-ethinylestradiol) when compared to the negative control group.

Regarding the biochemical parameters evaluated in male rats, only creatinine was significantly different in groups treated with ESTP (30 and 300 mg/kg) when compared to the control. Besides, alkaline phosphatase and creatinine were significantly different in animals from the positive control group (DEHP) when compared to the negative control (Table 3).

3.7. Histopathology

No signs of toxicity were observed in the thyroid, liver, ovary, uterus, testis and epididymis of all ESTP-treated rats submitted to the pubertal assay (Figures 3 and 4). In addition, no toxic effects were observed in the heart, spleen and kidney of treated

animals (Supplementary material 1). However, all male rats from the positive control group presented a pronounced diffuse degeneration of the germinal epithelium of the seminiferous tubules (indicated by arrowheads) where only remaining Sertoli cells (arrow) are observed (Figure 5). In the epididymis of these animals, no degenerative changes were found, only eosinophilic amorphous material and cellular debris in the epididymal light-duct (arrow) (Figure 5).

4. Discussion

This study aimed to investigate possible effects of *T. paniculatum* on the reproductive or endocrine systems of Wistar rats as well as any kinds of tissue and/or systemic toxicity outcomes. For this reason, the first step of this work consisted in the evaluation of possible estrogenic effects of the ethanol soluble fraction from *T. paniculatum* leaves (ESTP) in female rats through the uterotrophic assay. In this test, different doses of ESTP did not cause estrogenic effects in immature female rats treated for three consecutive days. This is in contrast to a study performed by Thanamool et al. (2013) in which *T. paniculatum* leaf extract promoted estrogenic effects in ovariectomized adult rats. Although both studies were performed with the same part of the same plant species, animals from this study were treated for 42 consecutive days whereas ours were treated for only three days. Thus, another contradiction between results must be related to a variation of the major secondary metabolites produced by each sample. We collected samples of *T. paniculatum* leaves in the Brazilian Cerrado in February 2017, whereas the samples used by Thanamool et al. (2013) were collected in Thailand in November 2013. According to Harborne (1993), environmental and biological factors may influence the amount of secondary metabolites produced by plants. For this reason, we believe such differences may be attributed to factors such climate, temperature, water availability,

altitude, UV radiation, nutrients among others (Gouvea et al., 2012). Furthermore, as the dose that presented estrogenic effect in Thanamool´s study was three times higher (1000 mg/kg) than the highest dose used in our study (300 mg/kg), it is also believed that this effect may have occurred in a dose-dependent manner.

In the second stage of this work, we investigated the effects of ESTP on the development and maturation of the reproductive system through the pubertal assay. Our data show that male and female rats treated orally with two doses of ESTP gained weight along the 30-day treatment. Thus, weekly evaluation of feed and water consumption have shown that animals treated with test substance had similar intakes when compared to the control. Such parameters are of great importance as reduced body weight and feed and water consumption are clear signs of general toxicity (OECD, 2008). Besides, pubertal exposure to endocrine disrupting substances might lead to metabolic disorders such as exacerbated weight gain and even obesity (Grun and Blumberg, 2006; Darbre, 2017).

When it comes to pubertal development of male and female rats, it is well known that PPS and VO are external signs of such development. According to Stoker et al. (2013), substances with toxic and endocrine disrupting potentials can affect the onsets of such signs. In this study, both doses of ESTP did not advance nor delay the onset of VO nor PPS in animals tested. However, treatment of male rats with a high-DEHP dose (750 mg/kg/day) significantly delayed the age at PPS, which is in accordance with the antiandrogenic mode of action of this compound. On the other hand, although 17α-ethinylestradiol (3 µg/kg) was able to increase the uterine weight in the uterotrophic assay, it was unable to change the age at VO or other estrogen sensitive parameters in female rats treated throughout puberty. These results may indicate that higher estrogen oral doses are needed to change reproductive

parameters in older animals, perhaps because of increased biotransformation (Fernandez et al., 2011; Shang-Fu et al., 2019).

Regarding the relative organ weight of animals exposed to different ESTP-doses, no significant differences were observed in vital nor in the reproductive organs of males and females when compared to the control group. However, some significant differences were observed in the relative organ weights of the male positive control group compared to the control group. As known, organ weight is an essential endpoint for identification of potentially toxic chemicals (Piao, Liu and Xie, 2013). Besides, relative reproductive organ weights are sensitive measures of detrimental effects of substances on the reproductive and endocrine systems and serve as crucial bases in toxicological investigations (Kwak et al., 2017).

We also evaluated the biochemical parameters of male and female rats submitted to the pubertal assay aiming to investigate target organs and a possible systemic toxicity. These parameters are essential as they provide information on major harmful effects in tissues, mainly in kidney and liver (Menegati et al., 2016). In this study, female rats treated with ESTP in all tested doses presented a significant decrease in levels of ALT when compared to the control. As known, ALT ratio is one of the most reliable indicators of liver damage. Therefore, the observed decrease of ALT activity in ESTP treated animals is possibly not related to any liver injury, especially considering that the measured values are within the normal range for the species (Dantas et al. 2006). In relation to the male rats, only creatinine was significantly increased in groups treated with both doses of ESTP when compared to the control. Blood measurement of creatinine is an important indicator of renal health (Ikegawa et al., 2018). However, as creatinine values obtained in male rats treated with ESTP-highest dose (300 mg/kg) was within the normal range for the species (Dantas et al., 2006), we did not identify any relevant clinical impairment derived from

this change. Also, further histopathological assessments revealed the absence of tissue damage in kidneys from both groups.

Finally, a detailed histopathological analysis was also performed in vital and reproductive organs in order to investigate possible pathological changes or signs of toxicity in animals exposed to ESTP. Corroborating the data already presented, no treatment-related effects were noted. Thus, the data obtained extend previous ethnopharmacological knowledge and provide important safety data for the prolonged use of *T. paniculatum*.

5. Conclusion

Our data demonstrate that ESTP does not play a direct effect on estrogen- and androgen-dependent organs as it did not delay nor accelerate the onset of puberty of male and female rats. Besides, ESTP did not cause any signs of toxicity after prolonged exposure and can be considered safe in Wistar rats at all doses tested.

Conflict of interest

Authors declare there are no conflicts of interest.

Funding

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Appendix A: Supplementary information

Supplementary data associated with this article can be found in the online version.

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Legend to Figures

Figure 1. Relative empty uterus weight (%) of female rats treated orally from PND 22 to PND 24 with ESTP 30, 100 and 300 mg/kg. The negative control group received the vehicle (filtered water) alone and the positive control group received 17α-ethinylestradiol (3 µg/kg). Bars represent the mean ± S.E.M (n = 8). * Represents significantly different from control ($p < 0.05$) by ANOVA followed by Dunnett's test.

Figure 2. Mean body weight gains of female (A) and male (B) rats during the pubertal assay. Body weights were daily monitored during the 30-day treatment period and body weight gains were analyzed statistically for the intervals 23-29, 29-36, 36-43 and 43-50 days of age. Animals were treated orally from PND 23 to PND 53 with ESTP 30 and 300 mg/kg. The negative control group received the vehicle (filtered water) alone and the positive control group received 17α-ethinylestradiol (3 µg/kg) for females and di (2-ethylhexyl) phthalate (DEHP, 750 mg/kg) for males. Bars represent the mean ± S.E.M (n = 10). * Represents significantly different from control ($p < 0.05$) by ANOVA followed by Dunnett's test.

Figure 3. Vaginal opening (A) and preputial separation (B) of female and male rats treated orally from PND 23 to PND 53 with ESTP 30 and 300 mg/kg. The negative control group received the vehicle (filtered water) alone and the positive control group received 17α-ethinylestradiol (3 µg/kg) for females and di (2-ethylhexyl) phthalate

(DEHP, 750 mg/kg) for males. Bars represent the mean ± S.E.M (n = 10). * Represents significantly different from control ($p < 0.05$) by ANOVA followed by Dunnett's test.

Figure 4. Histopathological assessment of thyroid, ovary, uterus and liver from female rats treated orally with the vehicle (2 ml/kg, negative control), ESTP 30 mg/kg, ESTP 300 mg/kg, and 17α-ethinylestradiol (3 µg/kg, positive control) in the pubertal assay. HE (40 X).

Figure 5. Histopathological assessment of thyroid, testis, epididymis and liver from male rats treated orally with the vehicle (2 ml/kg, negative control), ESTP 30 mg/kg, ESTP 300 mg/kg, and di (2-ethylhexyl) phthalate (DEHP 750 mg/kg, positive control) in the pubertal assay. Arrows and arrowheads indicate significant changes. HE (40 X).

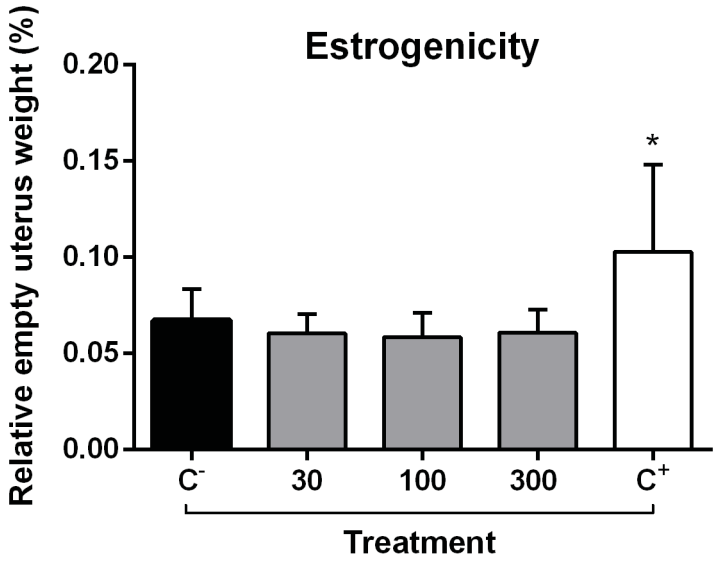

Figure 1

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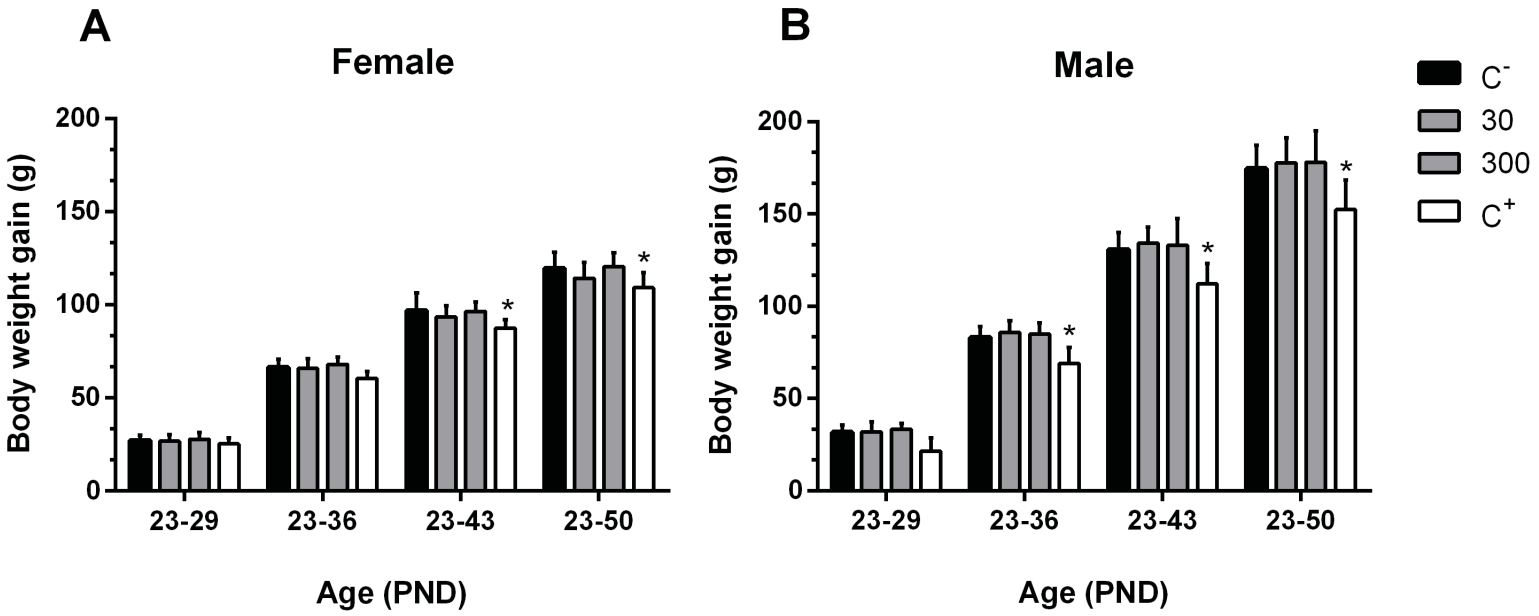

Figure 2

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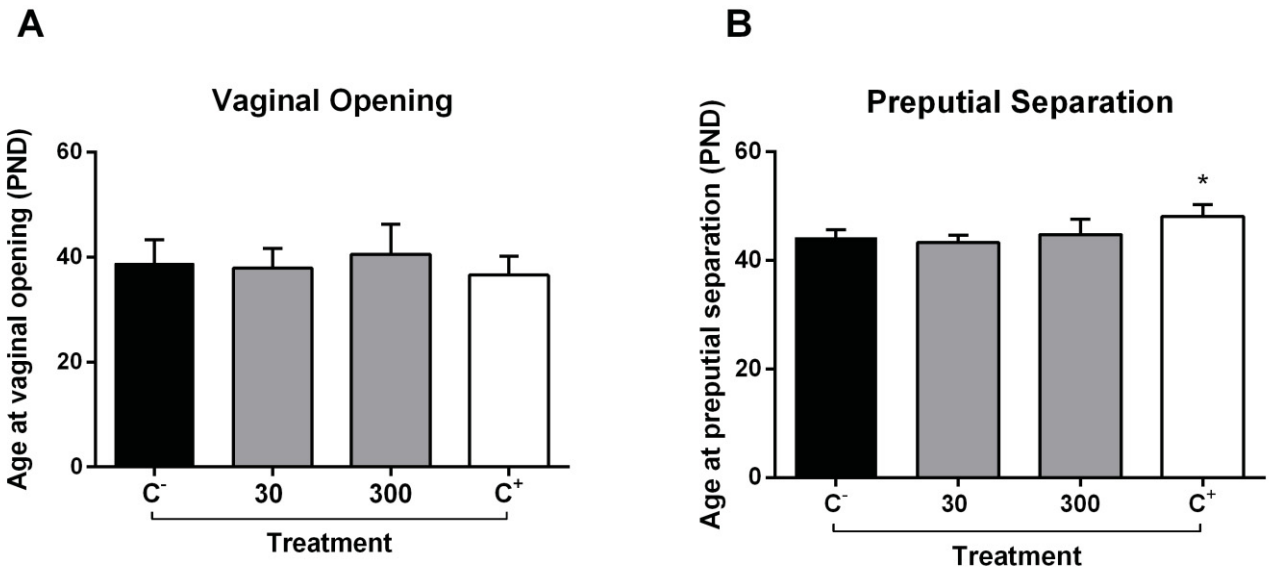

Figure 3

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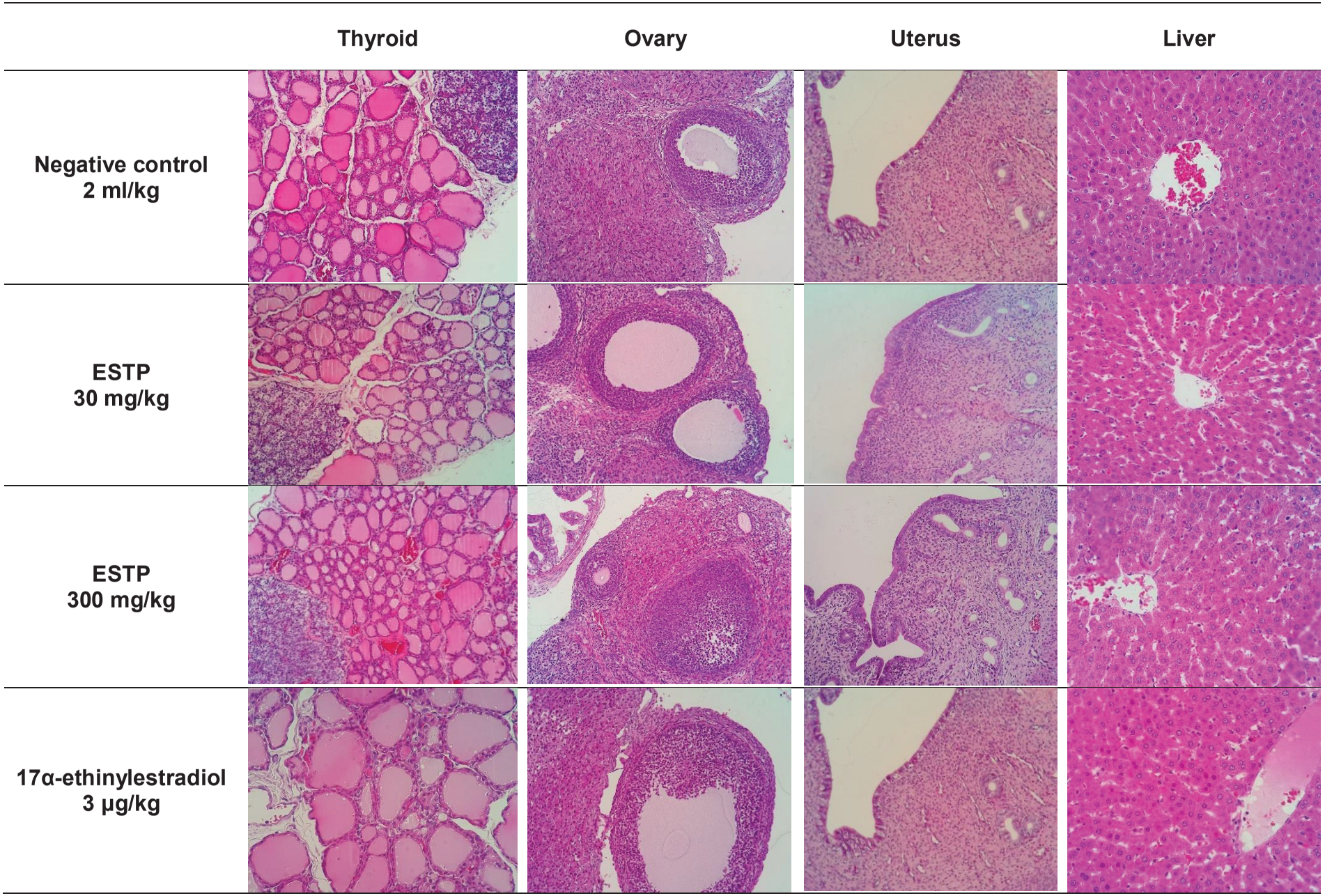

Figure 4

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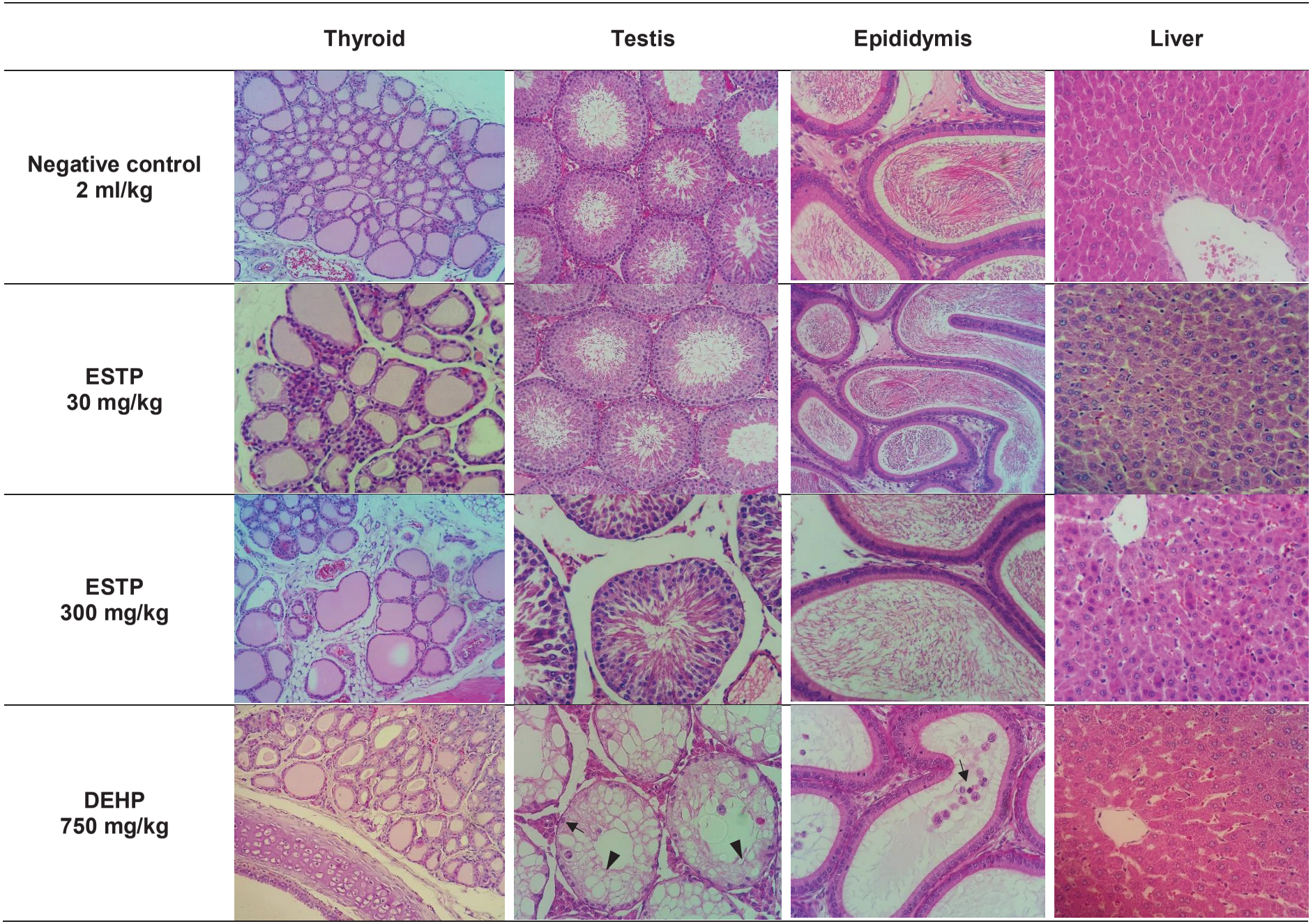

Figure 5

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Table 1. Effects of a 30-day administration of ESTP from *Talinum paniculatum* on body weight gain (g), food (g/day) and water (mL/day) consumption of female and male rats in the pubertal assay.

	Experimental groups			
Parameters	**Negative Control**	**ESTP 30 mg/kg**	**ESTP 300 mg/kg**	**Positive Control**
Female				
Initial body weight	61 ± 5.65	62 ± 6.84	61.80 ± 4.98	62.30 ± 4.32
Final body weight	189.70 ± 11.54	185 ± 12.56	190.30 ± 6.97	178.30 ± 10.13
Body weight gain	128.70 ± 7.70	123 ± 8.74	128.50 ± 8.92	116 ± 8.85*
Food consumption	159.40 ± 41.14	220.40 ± 78.11	157 ± 40.79	133 ± 31.40
Water consumption	255 ± 64.42	274 ± 59.83	277 ± 73.10	249 ± 63.28
Male				
Initial body weight	62.90 ± 3.60	65.10 ± 5.35	63 ± 4.71	63.10 ± 4.30
Final body weight	258.40 ± 17.84	256.10 ± 15.90	258.66 ± 17.63	237.20 ± 18.64
Body weight gain	195.50 ± 15.27	191 ± 12.78	195.66 ± 16.14	174.10 ± 15.89*
Food consumption	197.40 ± 63.85	197.40 ± 61.89	173.20 ± 52.06	162.20 ± 57.54
Water consumption	320 ± 93.54	302 ± 96.28	295 ± 87.60	320 ± 113.96

Note: The negative control group received the vehicle (filtered water). The positive control was treated with 17α-ethinylestradiol (3 µg/kg) for female rats and with di (2-ethylhexyl) phthalate (DEHP, 750 mg/kg) for male rats. Data are shown as mean ± S.E.M (n = 8). * Represents significantly different from negative control ($p < 0.05$) by ANOVA followed by Dunnett's test.

	Experimental groups			
Parameters	**Negative Control**	**ESTP 30 mg/kg**	**ESTP 300 mg/kg**	**Positive Control**
Female				
Heart	0.33 ± 0.02	0.34 ± 0.02	0.33 ± 0.01	0.34 ± 0.02
Lung	0.57 ± 0.11	0.50 ± 0.06	0.57 ± 0.11	0.53 ± 0.08
Liver	3.43 ± 0.30	3.58 ± 0.14	3.58 ± 0.24	3.69 ± 0.31
Spleen	0.26 ± 0.02	0.27 ± 0.03	0.26 ± 0.02	0.28 ± 0.02
Right kidney	0.39 ± 0.03	0.41 ± 0.03	0.39 ± 0.02	0.40 ± 0.01
Left kidney	0.39 ± 0.03	0.40 ± 0.03	0.38 ± 0.01	0.39 ± 0.02
Right ovary	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Left ovary	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Uterus	0.20 ± 0.14	0.18 ± 0.05	0.22 ± 0.09	0.22 ± 0.15
Male				
Heart	0.33 ± 0.02	0.33 ± 0.02	0.34 ± 0.01	0.35 ± 0.02*
Lung	0.54 ± 0.09	0.50 ± 0.16	0.49 ± 0.09	0.54 ± 0.07
Liver	3.65 ± 0.30	3.47 ± 0.19	3.60 ± 0.24	5.10 ± 0.46*
Spleen	0.27 ± 0.02	0.25 ± 0.02	0.26 ± 0.01	0.31 ± 0.02*
Right kidney	0.39 ± 0.02	0.39 ± 0.02	0.04 ± 0.02	0.41 ± 0.03
Left kidney	0.39 ± 0.01	0.39 ± 0.03	0.39 ± 0.02	0.40 ± 0.01
Right testicle	0.62 ± 0.05	0.61 ± 0.03	0.61 ± 0.04	0.22 ± 0.02*
Left testicle	0.61 ± 0.06	0.61 ± 0.03	0.60 ± 0.04	0.22 ± 0.01*
Right epididymis	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.02	0.05 ± 0.00*
Left epididymis	0.09 ± 0.01	0.09 ± 0.00	0.09 ± 0.00	0.05 ± 0.00*
Prostate	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.03 ± 0.00
Seminal vesicle	0.09 ± 0.01	0.08 ± 0.00	0.09 ± 0.01	0.07 ± 0.01*
Glans penis	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Levator ani muscle	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.02	0.15 ± 0.00*
Bulbourethral glands	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00

Table 2. Effects of a 30-day administration of ESTP from *Talinum paniculatum* on relative organ weight (g/100g body weight) of female and male rats in the pubertal assay.

Note: The negative control group received the vehicle (filtered water). The positive control was treated with 17α-ethinylestradiol (3 µg/kg) for female rats and with di (2-ethylhexyl) phthalate (DEHP, 750 mg/kg) for male rats. Data are shown as mean ± S.E.M (n = 8). * Represents significantly different from negative control ($p < 0.05$) by ANOVA followed by Dunnett's test.

	Experimental groups							
	Female				Male			
Parameters	**Negative control**	**ESTP 30 mg/kg**	**ESTP 300 mg/kg**	**Positive control**	**Negative control**	**ESTP 30 mg/kg**	**ESTP 300 mg/kg**	**Positive control**
AST (U/L)	105 ± 79.1	146 ± 31.9	167 ± 54.1	210 ± 84.5*	173 ± 56.5	165 ± 64.7	160 ± 41.7	168 ± 84.9
ALT (U/L)	190 ± 54.7	46.2 ± 21.3*	41.2 ± 6.87*	115 ± 92.6*	109 ± 87.4	105 ± 83.6	83.8 ± 51.5	98.8 ± 60.1
A. phosphatase (U/L)	68.0 ± 10.0	78.0 ± 8.00	82.0 ± 24.0	119 ± 48.0*	132 ± 44.6	124 ± 24.3	141 ± 37.9	172 ± 25.0*
GGT (U/L)	2.22 ± 3.02	2.77 ± 1.71	1.92 ± 1.53	3.25 ± 1.58	2.17 ± 1.48	3.14 ± 0.970	2.63 ± 1.56	3.9 ± 2.21
T. protein (g/dL)	5.88 ± 0.220	5.93 ± 0.300	6.17 ± 0.380	5.94 ± 0.320	5.74 ± 0.420	5.98 ± 0.160	6.05 ± 0.270	5.98 ± 0.460
Albumin (g/dL)	2.83 ± 0.160	2.80 ± 0.150	2.90 ± 0.140	2.78 ± 0.120	2.81 ± 0.260	2.74 ± 0.170	2.85 ± 0.200	2.56 ± 0.850
BUN (mg/dL)	49.8 ± 7.21	53.9 ± 5.97	51.0 ± 4.37	60.1 ± 4.62*	57.9 ± 7.25	56.7 ± 6.99	52.2 ± 7.75	58.1 ± 7.25
Creatinine (mg/dL)	0.680 ± 0.04	0.630 ± 0.060	0.620 ± 0.060	0.600 ± 0.080*	0.490 ± 0.110	0.610 ± 0.050*	0.570 ± 0.060*	0.580 ± 0.040*
Calcium (mg/dL)	11.32 ± 0.430	11.91 ± 0.460*	11.1 ± 0.280	11.6 ± 0.450	11.7 ± 0.600	11.7 ± 0.700	11.5 ± 0.880	12.0 ± 0.450
T. cholesterol (mg/dL)	81.4 ± 16.7	83.6 ± 9.32	84.3 ± 13.6	81.6 ± 10.1	74.3 ± 11.1	78.9 ± 10.2	71.2 ± 6.78	82.2 ± 21.8
Triglycerides (mg/dL)	84.6 ± 37.8	92.9 ± 30.0	80.3 ± 25.9	85.4 ± 35.8	95.5 ± 29.8	76.3 ± 40.2	99.2 ± 47.9	79.9 ± 23.6
Glucose (mg/dL)	103 ± 47.7	107 ± 52.6	124 ± 19.9	83.5 ± 38.1	83.5 ± 60.3	76.5 ± 29.9	97.3 ± 36.7	102 ± 29.8
D. bilirubin (mg/dL)	0.050 ± 0.040	0.080 ± 0.040	0.080 ± 0.060	0.050 ± 0.020	0.030± 0.020	0.050 ± 0.020	0.040 ± 0.030	0.030 ± 0.010
T. bilirubin (mg/dL)	0.190 ± 0.020	0.190 ± 0.020	0.180 ± 0.030	0.220 ± 0.010*	0.180 ± 0.000	0.200 ± 0.020	0.170 ± 0.020	0.170 ± 0.030
I. bilirubin (mg/dL)	0.130 ± 0.040	0.110 ± 0.050	0.090 ± 0.040	0.170 ± 0.030	0.150 ± 0.010	0.150 ± 0.040	0.120 ± 0.050	0.140 ± 0.030
Uric acid (mg/dL)	2.76 ± 0.980	2.37 ± 0.670	3.20 ± 1.260	2.75 ± 0.450	2.50 ± 1.20	2.56 ± 0.900	2.98 ± 1.44	2.67 ± 0.490

Table 3. Effects of a 30-day administration of ESTP from *Talinum paniculatum* on biochemical parameters of female and male rats in the pubertal assay.

Note: The negative control group received the vehicle (filtered water). The positive control was treated with 17α-ethinylestradiol (3 µg/kg) for female rats and with di (2-ethylhexyl) phthalate (DEHP, 750 mg/kg) for male rats. Data are shown as mean ± S.E.M (n = 8). * Represents significantly different from negative control ($p < 0.05$) by ANOVA followed by Dunnett's test. AST: aspartate aminotransferase, ALT: alanine aminotransferase, A. phosphatase: alkaline phosphatase, GGT: gamma-glutamyl transpeptidase , T. protein: total protein, BUN: blood urea nitrogen, T. cholesterol: total cholesterol, D. bilirubin: direct bilirubin, T. bilirubin: total bilirubin, I. bilirubin: indirect bilirubin.

SUPPLEMENTAL INFORMATION

Appendix A

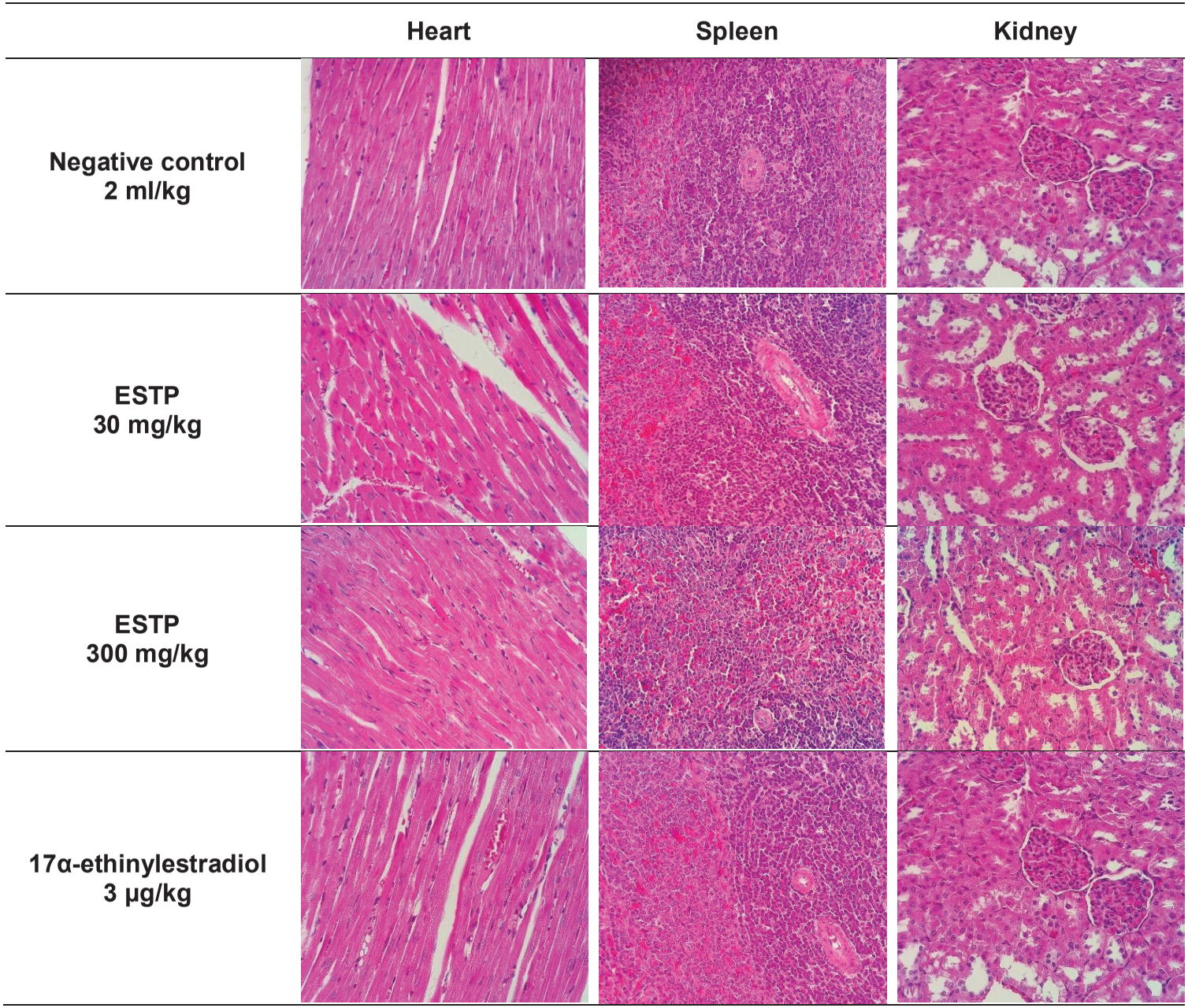


Figure S1. Histopathological assessment of heart, spleen and kidney from female rats treated orally with the vehicle (2 ml/kg, negative control), ESTP 30 mg/kg, ESTP 300 mg/kg and 17α-ethinylestradiol (3 µg/kg, positive control) in the pubertal assay. HE (40 X).

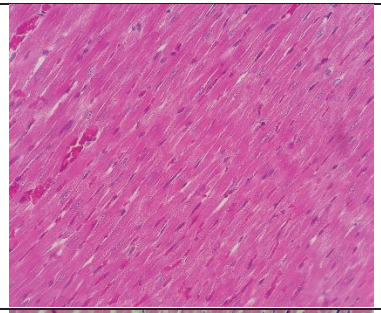
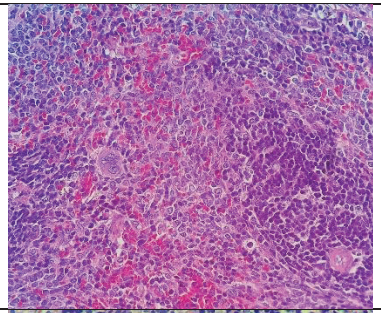
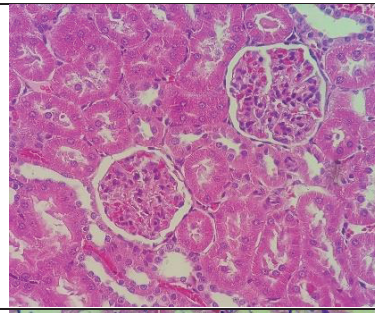
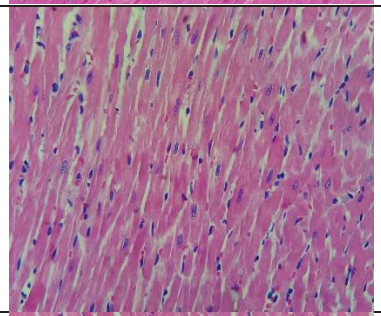
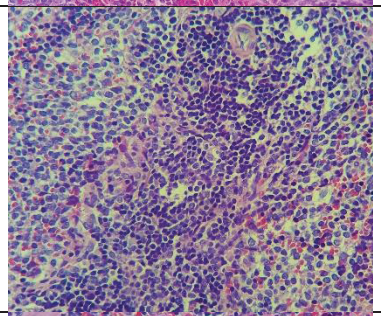
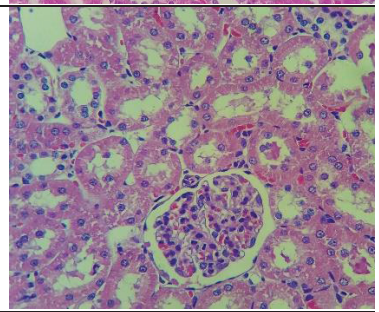
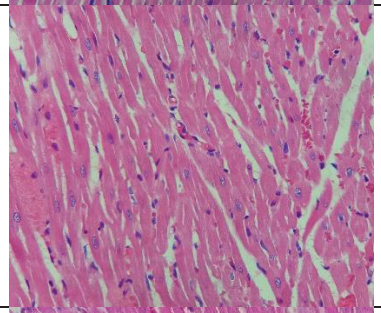
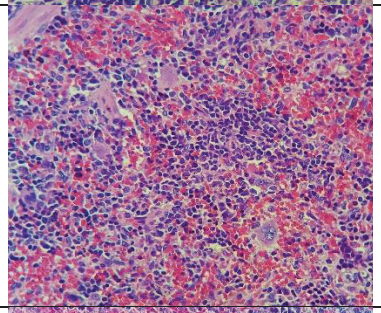
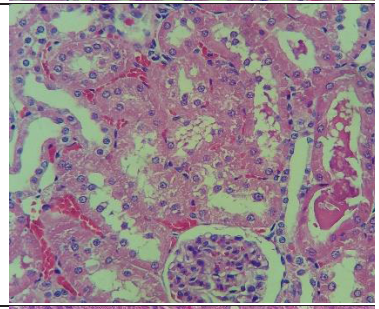
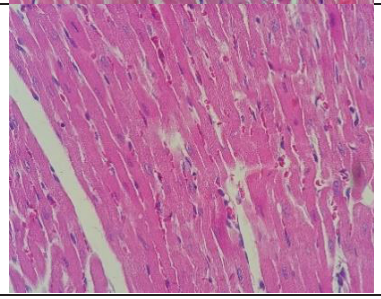
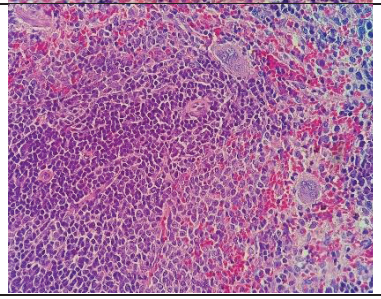
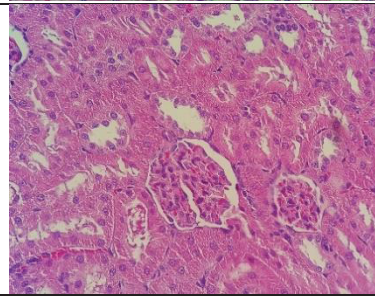
	Heart	Spleen	Kidney
Negative control 2 ml/kg			
ESTP 30 mg/kg			
ESTP 300 mg/kg			
DEHP 750 mg/kg			

Figure S2. Histopathological assessment of heart, spleen and kidney from male rats treated orally with the vehicle (2 ml/kg, negative control), ESTP 30 mg/kg, ESTP 300 mg/kg and di (2-ethylhexyl) phthalate (DEHP 750 mg/kg, positive control) in the pubertal assay. HE (40 X).

8 CONCLUSÕES

A partir dos resultados obtidos no presente estudo, podemos concluir que:

- ✓ O conjunto de características morfoanatômicas destacadas neste estudo fornecem importantes padrões de controle de qualidade para a identificação de *Celosia argentea* L., *Anchietea pyrifolia* (Mart.) G.Don e *Talinum paniculatum* (Jacq.) Gaertn.
- ✓ Na avaliação fitoquímica, determinou-se a presença majoritária de compostos fenólicos, megastigmanes e saponinas triterpenóides em ESCA; flavonóides O-glicosilados, ácidos clorogênicos e outros derivados do ácido fenilpropanóico em ESAP; e ácidos clorogênicos, aminoácidos, nucleosídeos, flavonas O-glicosiladas e ácidos orgânicos em ESTP.
- ✓ O ESCA, ESAP e ESTP não promoveram efeitos tóxicos após tratamento agudo;
- ✓ A DL_{50} de ESCA, ESAP e ESTP podem ser consideradas maior que 2000 mg/kg;
- ✓ O ESCA não promoveu efeitos diuréticos significativos em ratos após tratamento agudo, embora tenha aumentado significativamente a eliminação renal de potássio e cloreto, especialmente no final de 24 horas após a administração. No entanto, a dose intermediária (100 mg/kg) foi capaz de promover bradicardia e hipotensão aguda significativa, os quais parecem estar envolvidos com a ativação dos canais de potássio dependentes de voltagem nos MVBs.
- ✓ O ESAP não promoveu efeitos diuréticos significativos em ratos após tratamento agudo e reduziu significativamente a eliminação renal de sódio, potássio e cloreto após tratamento prolongado. A maior dose utilizada (300 mg/kg) foi capaz de promover hipotensão aguda significativa, sem afetar os níveis da pressão arterial após o tratamento por 7 dias. Ademais, os efeitos cardiovasculares desta espécie parecem estar envolvidos com a ativação dos canais de potássio ativados por cálcio em artérias de resistência.
- ✓ O ESTP não promoveu efeitos diuréticos nem alterações significativas na eliminação de eletrólitos em ratos após tratamento agudo. Por outro lado, todas as doses de ESTP aumentaram significativamente o volume de urina e a excreção

renal de eletrólitos (Na^+, K^+ e Cl^-) durante o tratamento por 7 dias, sem afetar a pressão arterial ou a frequência cardíaca dos animais. Tais efeitos parecem estar envolvidos com a ativação dos canais de potássio ativados por cálcio de pequena condutância, contribuindo para o aumento do fluxo sanguíneo renal e da taxa de filtração glomerular.

- ✓ O ESTP não promoveu efeitos estrogênicos em ratas e, quando avaliado em ratos de ambos os sexos expostos por 30 dias consecutivos durante o período puberal, não promoveu alterações significativas em parâmetros que marcam o início da puberdade ou em parâmetros bioquímicos e histopatológicos, podendo ser considerado seguro em ratas e ratos Wistar nas doses e períodos testados;
- ✓ *T. paniculatum* apresentou melhores efeitos farmacológicos quando comparado às outras duas espécies em estudo, podendo ser apontado como um possível candidato a fitoterápico, especialmente quando um efeito diurético sustentado se é esperado.

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7 ANEXOS

7.1 Parecer de aprovação do Comitê de ética da UFPR

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Comissão de Ética no Uso de Animais
(CEUA)

Nº 1076

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.167522/2017-79

APROVADO/APPROVAL: 13/06/2017 – R.O. 05/2017

TÍTULO: Prospecção etnofarmacológica dos efeitos reno e cardioprotetores de espécies nativas do cerrado sul-mato-grossense

TITLE: Ethnopharmacological prospection of the effects reno and cardioprotectors of native species of the cerrado sul-mato-grossense

AUTORES/AUTHORS: Paulo Roberto Dalsenter, Arquimedes Gasparotto Júnior, Sara Emilia Lima Tolouei

DEPARTAMENTO/DEPARTMENT: Farmacologia

Profa. Dra. Katya Naliwaiko
Coordenadora da CEUA

7.2 Parecer de aprovação do Comitê de ética da UFGD

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, 14 de novembro de 2017.

CERTIFICADO

Certificamos que a proposta intitulada "***Prospecção etnofarmacológica dos efeitos reno e cardioprotetores de espécies nativas do cerrado sul-matogrossense"***, registrada sob o protocolo de nº 21/2017, sob a responsabilidade de *Arquimedes Gasparotto Junior* – 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 aprovada pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 23/06/2017.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*15/11/2017 a 30/06/2021*
Espécie/linhagem/raça	*Rattus norvegicus*
Nº de animais	192
Peso/idade	60 dias
Sexo	132 machos *Wistar* e 60 machos SHR
Origem	Universidade Federal da Grande Dourados-UFGD e Universidade de São Paulo - USP

Melissa Negrão Sepulvida

Melissa Negrão Sepulvida
Coordenadora CEUA