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Avaliação dos efeitos cardivoasculares e renais do extrato etanólico de *Croton urucurana* em ratos espontaneamente hipertensos

KATIANA SIMÕES LOPES

Dourados - MS
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EPÍGRAFE

You’re the poet in my heart,
never change...
never stop.

Stevie Nicks

LISTA DE ABREVIATURAS E SÍMBOLOS

ACh	Acetilcolina
ADH	Hormônio anti-diurético
ALT	Alanina aminotransferase
Ang-II	Angiotensina II
AST	Aspartato aminotransferase
ATP	Adenosina trifosfato
CVDs	*Cardiovascular diseases* (doenças cardiovasculares)
DC	Débito cardíaco
ECA	Enzima conversora da angiotensina
EDRF	*Endothelium-derived relaxing fator* (fator de relaxamento derivado do endotélio)
ESCU	Extrato *Croton urucurana*
GMPc	Monofosfato cíclico de guanosina
HAS	Hipertensão arterial sistêmica
HCTZ	Hidroclorotiazida
mmHg	Milímetros de mercúrio (unidade de pressão)
NO	Óxido nítrico
NOS	Óxido nítrico sintase
NPS	Nitroprussiato de sódio
OMS	Organização Mundial de Saúde
PAM	Pressão arterial média
Phe	Fenilefrina
RVP	Resistência vascular periférica
SHR	*Sponteanously hypertensive rats* (ratos espontaneamente hipertensos)
SRAA	Sistema renina-angiontesina-aldosterona
SUS	Sistema Único de Saúde

Avaliação dos efeitos cardiovasculares e renais do extrato etanólico de *Croton urucurana* em ratos espontaneamente hipertensos.

RESUMO

A hipertensão arterial sistêmica (HAS) é uma doença multifatorial, assintomática, que afeta mais de 30% da população mundial adulta. Possui altas taxas de morbimortalidade e pode estar relacionada a doenças cerebrovasculares e cardiovasculares. A HAS não tem cura - desta forma, seu tratamento é prolongado e abrangente, dividindo-se em medicamentoso e não-medicamentoso, definido de acordo com a necessidade de cada paciente. Quando prescritos, os fármacos agem para que o organismo mantenha ou retorne ao estado de homeostasia e, consequentemente, que regule os parâmetros de pressão arterial para que permaneçam dentro dos valores de normalidade. No entanto, há o risco de interações medicamentosas e possível resistência à fármacos quando em tratamentos prolongados, justificando-se assim, a necessidade do desenvolvimento de métodos alternativos. Desta forma, este estudo tem por objetivo investigar os efeitos cardiovasculares e renais de compostos derivados da *Croton urucurana*, uma planta de uso popular com potencial anti-hipertensivo, através de seu extrato etanólico (ESCU) administrado em tratamento prolongado (28 dias) à ratos espontaneamente hipertensos (SHR). Após avaliação dos parâmetros bioquímicos, histopatológicos, toxicológicos e também dos mecanismos envolvidos no efeito anti-hipertensivo, chegou-se à conclusão de que, contrária a hipótese inicial, o ESCU é dose-dependente e não age de forma diurética no organismo, mas pela via do óxido nítrico (NO) através da vasodilatação – dado corroborado pela inibição do extrato em leitos mesentéricos a) sem endotélio e; b) com endotélio tratado com inibidor da via de NO (L-NAME). Além disso, afirma-se que ação do ESCU pode ser mediada por canais de K^+ de pequena condutância ativados por Ca^{+2} livre, uma vez que o ESCU foi inibido em testes com leito mesentérico tratado com bloqueadores específicos e inespecíficos de K^+.

Palavras-chave: Anti-hipertensivo. Etnofarmacologia. Vasodilatação. Óxido nítrico.

ABSTRACT

Systemic arterial hypertension is an asymptomatic, multifactorial disease that affects more than 30% of the adult population worldwide. It has high morbidity and mortality rates and may be related to cerebrovascular and cardiovascular diseases. There is no cure for hypertension - therefore, its treatment is prolonged and wide, being divided into drug therapy and non-drug therapy, defined according to the needs of each patient. When prescribed, drugs act so that the body maintains or returns to a state of homeostasis and, consequently, regulates blood pressure parameters so they remain within normal values. However, there is a risk of drug interactions and possible drug resistance when in prolonged treatments, hence justifying the need to develop alternative methods. Thus, this study aims to investigate the cardiovascular and renal effects of compounds derived from *Croton urucurana*, a popular plant with anti-hypertensive potential, through its ethanolic extract (ESCU) administered in prolonged treatment (28 days) to spontaneously hypertensive rats (SHR). After evaluating biochemical, histopathological, toxicological parameters and also the mechanisms involved in the antihypertensive effect, it was concluded that, contrary to the initial hypothesis, ESCU is dose-dependent and does not act as a diuretic in the organism, but via the nitric oxide (NO) pathway through vasodilation – data corroborated by the inhibition of the extract in mesenteric beds a) without endothelium and; b) with endothelium treated with NO pathway inhibitor (L-NAME). Furthermore, it is affirmed that ESCU action can be mediated by free Ca^{2+} activated small conductance K^+ channels, since ESCU was inhibited in tests with mesenteric bed treated with specific and non-specific K^+ blockers.

Keywords: Antihypertensive. Ethnopharmacology. Vasodilation. Nitric oxide.

SUMÁRIO

1 INTRODUÇÃO

Atualmente, a OMS (2021) estima que a hipertensão afeta mais de um bilhão de pessoas mundialmente, onde destes, 17 milhões são brasileiros, com sua prevalência aumentando anualmente. É o principal fator de risco para o desenvolvimento de doenças cardiovasculares e cerebrovasculares devido à disfunção endotelial característica da doença, e também pode causar danos no trato urinário devido à um dos mecanismos de regulação fisiológica (BRASIL, 2006; BARROSO *et al*., 2020). É a maior causa de morte prematura, responsável por cerca de 10.4 milhões de mortes anuais, reflexo do diagnóstico que pode ocorrer tardiamente devido à assintomatologia da doença, e do tratamento prolongado, que por muitas vezes é negligenciado (BRASIL, 2006; UNGER et al., 2020).

O objetivo principal dos tratamentos para hipertensão consiste na redução dos valores médios de pressões sistólica e diastólica, quando nas formas graves da doença, e assim, diminuir os riscos de morbimortalidade a longo prazo (KHATIB *et al*., 2005; UNGER *et al*., 2020). Para tanto, as medidas implementadas podem ser medicamentosas ou não, onde a conduta é tomada pelo médico responsável. Dentre as medidas não medicamentosas, incluem o incentivo de hábitos saudáveis como exercícios físicos, redução do consumo de álcool e cigarros, alimentação apropriada, entre outros. A respeito do uso medicamentoso, é comum que os fármacos sejam combinados entre si, associando, inclusive, compostos naturais para a complementação do tratamento (KHATIB *et al*., 2005; FARIAIS *et al*., 2016).

Para uma grande quantidade da população mundial, as plantas medicinais bem como os tratamentos popularmente utilizados provenientes da medicina tradicional representam a principal, e com frequência, a única fonte de atenção à saúde, principalmente em países subdesenvolvidos (OMS, 2013). Segundo o Ministério da Saúde (2006), o Brasil possui a maior biodiversidade no mundo, onde cerda de 82% da população se utiliza de plantas medicinais nos tratamentos para saúde. No SUS, existem programas governamentais que recomendam o uso de plantas medicinais e da fitoterapia de acordo com os respectivos biomas, portanto, é essencial que novos estudos sejam desenvolvidos para a ampliação de tratamentos seguros e eficazes, visto que apenas uma pequena porção da diversidade em plantas medicinais disponíveis foi investigada fitoquímica e farmacologicamente.

No Mato Grosso do Sul, especificamente na região da Grande Dourados, Coelho *et al.* (2018) catalogou diversas espécies vegetais utilizadas na medicinal tradicional regional em um estudo etnobotânico. Neste âmbito, para doenças cardiovasculares, destaca-se o uso da *Croton*

urucurana, da família Euphorbiaceae, popularmente conhecida como *sangra d'água* ou sangue *de dragão* devido a seiva avermelhada de suas cascas, que possui grande distribuição no país (ALVES *et al.*, 2009). Estudos farmacológicos realizados com *C. urucurana* revelaram variadas propriedades medicinais, como efeitos antimicrobiano e antitumoral (PERES *et al*., 1997), porém seus efeitos anti-hipertensivos e mecanismos de ação ainda precisam de elucidação.

2 REVISÃO DE LITERATURA

2.1 Doenças cardiovasculares

As doenças cardiovasculares (do inglês, *cardiovascular diseases*, ou CVDs) que correspondem a um grupo de doenças cardíacas e vasculares, incluem as doenças coronariana e cerebrovascular, doença arterial periférica, doença cardíaca reumática, cardiopatia congênita, trombose venosa profunda e embolia pulmonar, segundo a OMS (2021).

Fisiologicamente, segundo Kumar *et al.* (2010), as disfunções cardiovasculares resultam de falha dos mecanismos principais, sendo estes: mal funcionamento ou falência da contração do miocárdio; obstrução da passagem de fluxo sanguíneo por vasos sanguíneos ou câmaras cardíacas; fluxo regurgitante e aumento da força de contração cardíaca; fluxo colateral e troca de câmaras cardíacas; distúrbios da condução elétrica em miocárdio e; ruptura muscular ou endotelial por causas externas. São resultado de ricos não modificáveis e genéticos, tais como idade, sexo e raça. Dentre os principais fatores de risco modificáveis, pode-se citar o uso de tabaco, obesidade e sobrepeso, sedentarismo e dieta inadequada como consumo descontrolado de açúcares e sal, alcoolismo, *diabetes mellitus* e hipertensão arterial sistêmica (BISPO *et al*., 2016).

2.2 Hipertensão Arterial Sistêmica (HAS)

Caracterizada como doença crônica, de origem multifatorial, multissistêmica e de alta prevalência, a hipertensão é principal fator de risco para doenças cardiovasculares, aumentando a morbimortalidade destas. O diagnóstico, realizado através de múltiplas avaliações da pressão arterial em consulta médica e em exames externos, identifica o hipertenso quando sua pressão sistólica é $\geq$ 140 mmHg e a diastólica $\geq$ 90 mmHg (UNGER *et al*., 2020; BARROSO *et al*., 2020).

Segundo o Ministério da Saúde (2021), dentre os principais fatores de risco para o desenvolvimento da HAS, destacam-se a idade dos indivíduos (mulheres > 65 anos; homens > 55 anos), tabagismo, doenças como *diabetes mellitus* e dislipidemias, além de fatores genéticos e histórico familiar. Mais de 50% dos pacientes que possuem HAS também possuem fatores adicionais de risco para doenças cardiovasculares, como maus hábitos alimentares e alcoolismo, aumentando proporcionalmente o risco de CVDs em pacientes hipertensos (BRASIL, 2021).

No Brasil, o Sistema Único de Saúde (SUS) conta com diversas estratégias multiprofissionais para incentivar a adesão e o tratamento contínuo da hipertensão. Estima-se que o custo com hospitalizações, procedimentos ambulatoriais para controle da doença passem dos US$523 milhões em 2018, superando os gastos de tratamentos de outras doenças com alta prevalência como o *diabetes mellitus* e a obesidade (BARROSO *et al*., 2020).

2.3 Regulação da pressão arterial

A pressão arterial média (PAM) é resultado da função entre o débito cardíaco (DC) pela resistência vascular periférica (RVP), e é considerada um dos mecanismos mais complexos do organismo. A variação de DC pode ser dependente de volume sanguíneo, habilidade de contração do miocárdio, frequência cardíaca, entre outros; enquanto que a RVP é determinada por fatores endoteliais como mecanismos vasoconstritores e vasodilatadores e também, pelo Sistema Renina-Angiotensina-Aldosterona (SRAA) (SANJULIANI, 2002; KUMAR *et al*., 2010).

2.3.1 Sistema Renina-Angiotensina-Aldosterona (SRAA)

A regulação inicia-se com a excreção da enzima *renina* pelas células justaglomerulares renais como feedback para queda da pressão arterial percebida por mecanorreceptores nas arteríolas eferentes renais. Na circulação, a renina atua sobre a glicoproteína *angiotensinogênio* plasmático liberada pelo fígado, realizando a conversão deste em *angiotensina I*. Na circulação pulmonar, a *enzima conversora da angiotensina* (ECA) converte *angiotensina I* em *angiotensina II*. Por sua vez, a angiotensina II atua na elevação da pressão arterial através: da vasoconstrição pelo aumento da resistência vascular periférica; do volume sanguíneo ao estimular a secreção do hormônio *aldosterona* pelas glândulas adrenais e aumentar a reabsorção de sódio no túbulo renal distal; e ainda, da secreção da *vasopressina* ou *hormônio anti-diurético* (ADH), secretado pela hipófise, que aumenta a sede e diminui o fluxo urinário, aumentando assim o volume sanguíneo e, consequentemente, o débito cardíaco (SANJULIANI, 2002; KUMAR *et al.*, 2010; SILVERTHORN, 2017).

A contratilidade dos vasos sanguíneos é dependente de variados fatores fisiológicos, sendo o principal, a existência de uma parede endotelial íntegra, para que substâncias como a acetilcolina, histamina, trombina, ATP, noradrenalina, angiotensina, entre outros, sejam

capazes de liberar óxido nítrico, um potente vasodilatador que também atua na regulação da pressão arterial (ZAGO; ZANESCO, 2006).

2.3.2 Óxido Nítrico (NO)

O óxido nítrico (NO) é uma molécula gasosa sinalizadora, que previamente a sua elucidação, foi conhecida por *fator de relaxamento derivado do endotélio* (EDRF). Sua biossíntese se dá através da catálise do aminoácido essencial L-arginina pela enzima óxido nítrico sintase (NOS), onde o NO produzido se liga a proteínas intracelulares das células-alvo, sendo multifuncional nos diversos tecidos orgânicos (QUEIROZ; BATISTA, 1999; SILVERTHORN, 2017).

O grupo de isoformas da enzima óxido nítrico sintase (NOSs) são caracterizadas por complexas famílias proteicas com genes distintos, diferenciadas de acordo com localização, forma de ativação e peso molecular. Diferem-se em categorias: NOS constitutiva (c-NOS) dependente de cálcio livre intracelular e calmodulina; e em NOS induzível (i-NOS), dependente de síntese por macrófagos e células ativadas por citocinas mediante estímulo inflamatório. Compreendidas no grupo c-NOS, estão as subcategorias e-NOS (NOS endotelial) e n-NOS (NOS neuronal) (DUSSE; VIEIRA; CARVALHO, 2003).

O NO tem papel essencial para a homeostase vascular e vasoproteção endotelial. Com a e-NOS ancorada à membrana celular endotelial e em contato com células circulantes, em resposta a estímulos e bloqueios de canais de cálcio, há a síntese de NO que se difunde no meio intra e extracelular. No meio intracelular, há o aumento da concentração de monofosfato cíclico de guanosina (GMPc) que resulta em relaxamento celular. Assim, a síntese de óxido nítrico é constante, garantindo vasodilatação discreta e controle da pressão arterial. No coração, a e-NOs está presente no endotélio arterial e venoso dentro do miocárdio. Dentre as principais funções do NO para o sistema cardiovascular, incluem-se a inibição da agregação plaquetária quando NO adentra as hemoglobinas; inibição da adesão de células do sistema imunológico ao endotélio vascular - fator de risco para aterosclerose; tem efeito antioxidativo, ajudando a prevenir doenças tromboembólicas; e ainda, estudos sugerem que quando na hipertensão, o aumento da atividade da eNOS pode ter efeito protetivo à órgãos-alvo da lesão hipertensiva (VIARO; NOBRE; EVORA, 2000; DUSSE; VIEIRA; CARVALHO, 2003).

2.4 Tratamentos disponíveis

O tratamento para hipertensão tem como objetivo a redução da pressão arterial. Dentre as condutas modificáveis e não-farmacológicas incluem-se o controle de peso e adoção de hábitos saudáveis, como melhoria na dieta e a prática de exercícios físicos, redução do consumo alcóolico, de sal e açúcares e abandono do tabagismo. Tais condutas podem também estar combinadas com estratégias farmacológicas (BRASIL, 2006).

Segundo Barroso *et al.* (2020), as práticas farmacológicas variam entre monoterapia ou medicações combinadas, variando para cada paciente visando atender suas necessidades. A associação de fármacos ocorre para o aumento anti-hipertensivo no organismo principalmente por ações sinérgicas, e que ainda, pode reduzir a ocorrência de fatores colaterais, uma vez que a dose de cada fármaco é reduzida na combinação – que são preferenciais quando reduzidas a um único comprimido.

Dentre os principais fármacos utilizados na terapia anti-hipertensiva, podem-se citar: diuréticos, inibidores adrenérgicos, inibidores da ECA, inibidor direto da renina, bloqueadores dos canais de cálcio, bloqueadores do receptor AT_1 e vasodilatadores diretos (BRASIL, 2006; BARROSO *et al.*, 2020).

Um dos principais desafios encontrados para o tratamento da HAS é justamente a adesão ao tratamento prolongado, cujas justificativas circulam entre dosagem e quantidade de medicamentos, seu custo e baixa renda populacional, efeitos colaterais e até mesmo dificuldade para leitura e compreensão de embalagens e receitas médicas. Sem essa adesão, o bom prognóstico para pacientes hipertensos é comprometido (GEWEHR *et al.*, 2018; UNGER *et al.*, 2020).

2.5 Plantas medicinais e fitoterápicos

O emprego de plantas e ervas para tratamento de doenças por humanos é uma ação comum na sociedade desde a antiguidade. Por definição, *plantas medicinais* são plantas que contém em sua anatomia, substâncias farmacológicas com potencial de tratamento e prevenção de doenças humanas; enquanto que os *fitoterápicos* são os medicamentos industrializados oriundos dessas plantas (GADELHA *et al.*, 2013). Visto que a situação socioeconômica é um problema para a adesão de doenças como hipertensão em vários lugares, a implementação da medicina tradicional e uso de plantas medicinais em países subdesenvolvidos ou em

desenvolvimento tem sido base normativa para a manutenção da saúde. Assim, seguindo recomendações da OMS, o Ministério de Saúde nacional aprovou a Política Nacional de Plantas Medicinais e Fitoterápicos em 2006, com diretrizes coordenadoras para o uso racional, seguro e eficaz destes produtos (BRASIL, 2006).

Ainda que haja incentivo à pesquisa de plantas medicinais e ao desenvolvimento de fitoterápicos, a quantidade de estudos científicos de comprovação de sua eficácia ainda é escassa, considerando a biodiversidade nacional e o rico conhecimento tradicional (SILVA; HAHN, 2011). Na UFGD, estudos vem sendo realizados frequentemente de modo a elucidar propriedades anti-hipertensivas de plantas populares como demonstram Tolouei *et al*. (2019), Marques *et al.* (2021), Lorençone *et al.* (2021), entre outros.

Além dos estudos visando a etnofarmacologia, estudos etnobotânicos também são realizados como o de Coelho *et al.* (2018), que agrupou informações da medicina tradicional compartilhadas por curandeiros da região da Grande Dourados e catalogou plantas medicinais utilizadas e sua ação terapêutica, muitos os quais não possuem comprovação cientifica. Através do qual obteve-se a *Croton urucurana* como objeto de estudo, cujas propriedades anti-hipertensivas ainda não foram elucidadas.

2.5.1 *Croton urucurana* Baillon

Croton urucurana (Euphorbiaceae), conhecida popularmente como “sangra d’água” ou “sangue de dragão” recebe esse nome devido a seiva avermelhada presente em suas cascas e tronco (SIMIONATTO et al., 2007). Possui hábito arvorista, ou seja, médio porte, com tricomas estrelados, cujas folhas são em formato de lança e com haste comprida. Suas folhas são dispostas em glomérulos, de coloração amarelo-esverdeada, com florescimento durante longos períodos do ano, de Dezembro à Junho (GUIMARÃES; SECCO, 2010; CAMPOS, 2020). Possui grande distribuição nacional em estados como Bahia, Minas Gerais, Mato Grosso do Sul, Rio de Janeiro e Rio Grande do Sul devido ao clima (SALATINO; SALATINO; NEGRI, 2007; ALVES *et al.*, 2009). A *C. urucurana* é uma espécie protegida pela legislação (Lei n.º 12.651 de maio de 2012), pois se trata de uma espécie presente em matas ciliares, que são áreas de preservação (ALVES *et al.*, 2009).

Estudos fitoquímicos mostraram várias classes de compostos, como diterpenos, que possuem ação anti-inflamatória e antibacteriana; alcalóides que agem como analgésicos;

flavonóides, que possuem várias ações biológicas como anti-inflamatória, antiviral, antibacteriana, antioxidante e vasodilatadora, entre outros (OLIVEIRA; ESPESCHIT; PELUZIO, 2006; SALATINO; SALATINO; NEGRI, 2007; CAMPOS, 2020) enquanto Peres et al. (1998) isolou compostos como catequinas e galocatequinas, que possuem ação antioxidante, além de β-sitosterol e campesterol, que reduzem o colesterol LDL (BREDA, 2010). Desta forma, estudos farmacológicos previamente realizados com casca e seiva de *C. urucurana* demonstraram propriedades antimicrobianas e antitumorais (PERES *et al.*, 1997), atividade antiúlcera (ALVES *et al.*, 2009; CORDEIRO *et al.*, 2012) e atividade antidiarreica (GURGEL *et al.*, 2001), efeitos anti-inflamatórios e antinoceptivos (CORDEIRO *et al*., 2012), cicatrização de feridas e tratamento de câncer (BACANI, 2016), entre outros.

Diante destes compostos previamente elucidados e suas respectivas funções, sugere-se que a *Croton urucurana* tenha potencial anti-hipertensivo que ainda precisa de comprovação científica sobre sua eficácia.

2.6 Modelo experimental animal de hipertensão arterial

O rato de laboratório (Norway) é um dos principais animais escolhidos para a experimentação não apenas por seu tamanho e habitat, mas principalmente por se tratar de um modelo de mamífero funcionalmente caracterizado, suscetíveis a variadas doenças comumente aparente em humanos (como câncer, doenças autoimunes, metabólicas, cardiovasculares) além de possibilitar o estudo de procedimentos cirúrgicos e também, comportamentais (LAPCHIK; MATTARAIA; KO, 2017).

Historicamente, Okamoto e Aoki (1963) detectaram um casal ratos Wistar com hipertensão espontânea e através destes, desenvolveram a linhagem *outbred* conhecida hoje por SHR, do inglês, *spontaneously hypertensive rat.* De acordo com Lapchik; Mattaraia e Ko, a incidência de hipertensão e outras doenças cardiovasculares entre os animais é alta, porém sem apresentarem lesões no sistema renal. Os ratos SHR começam a desenvolver hipertensão com 5 semanas de vida, exibindo pressão arterial média maior que 200 mmHg a partir do 4º mês de idade, associada principalmente ao aumento da resistência periférica total, em especial, em pequenas artérias, arteríolas e capilares – o aumento da resistência vascular periférica neste caso é similar à hipertensão primária no homem (FAZAN JUNIOR; DA SILVA; SALGADO, 2001; LAPCHIK; MATTARAIA; KO, 2017).

3 OBJETIVOS

GERAL

- Investigar e determinar os mecanismos de ação mediadores do efeito anti-hipertensivo do extrato etanólico de *Croton urucurana* em ratos espontaneamente hipertensos (SHR) durante tratamento prolongado.

ESPECÍFICOS

- Coletar folhas da árvore *Croton urucurana* e identificá-las;

- Produzir extrato etanólico de *Croton urucurana* (ESCU) e realizar sua caracterização fitoquímica;

- Investigar a toxicidade aguda em ratas wistar, seguindo protocolo de toxicidade OECD 425;

- Efetuar o tratamento oral prolongado por gavagem (28 dias) em ratos SHR com diferentes doses do ESCU e avaliar as alterações fisiológicas apresentadas pelos animais;

- Mensurar a atividade diurética dos tratamentos semanalmente para avaliação de função renal;

- Realizar eletrocardiograma para avaliação de parâmetros cardíacos;

- Avaliar a pressão arterial sistólica, diastólica, média e frequência cardíaca, de forma direta (invasiva) para verificar alterações pressóricas causadas pelo ESCU;

- Avaliar a reatividade vascular em órgãos isolados (rim e leito mesentérico) sob a ação de substâncias vasoativas (Phe, Ang-II, ACh e NPS) nos grupos tratados e determinar mecanismo de ação do ESCU;

- Mensurar parâmetros bioquímicos séricos e urinários de função renal, hepática e testes hematológicos para elucidação de outros possíveis mecanismos de ação do ESCU;

- Averiguar dados anatômicos histológicos pós-eutanásia dos animais tratamos para comprovação de não-toxicidade do ESCU.

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5 APÊNDICES

5.1 ARTIGO 1

Nitric oxide and Ca^{2+} activated small conductance K^+ channels mediate antihypertensive effects of *Croton urucurana* Baill. in spontaneously hypertensive rats

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Abstract

Ethnopharmacological relevance: Croton urucurana Baill. (Euphorbiaceae), popularly known as ‘sangra d’água’ is a Brazilian species widely used by traditional medicine for the treatment of cardiovascular diseases. However, its cardioprotective effects are still unknown.

Aim: To evaluate the role of the ethanol-soluble fraction from *C. urucurana* leaves (ESCU) against hypertension in spontaneously hypertensive rats.

Materials and Methods: First, plant samples were collected, identified and a morpho-anatomical characterization was carried. ESCU was obtained, and its chemical profile was analyzed by LC-DAD-MS. The acute toxicity of ESCU was evaluated in female Wistar rats. Then, male spontaneously hypertensive rats (six months old) received ESCU (30, 100, 300 mg/kg), hydrochlorothiazide (25 mg/kg), or vehicle once daily for 28 days. On the first and twenty-eighth day of treatment, kidney function was monitored by analyzing urinary parameters. At the end of the 28-day treatment, the electrocardiographic profile, blood pressure, and mesenteric vascular bed reactivity were evaluated. Serum angiotensin-converting enzyme activity, as well as urea, creatinine, sodium, potassium, nitrite, thiobarbituric acid reactive substances, nitrotyrosine, and aldosterone levels, were measured. Relative organ (heart, kidney, and liver) weights and histopathological analysis were performed. Finally, the molecular mechanisms involved in the vasodilatory effects of ESCU in mesenteric vascular beds were also investigated.

Results: The metabolites annotated from ESCU by LC-DAD-MS included mainly phenylpropanoid derivatives, alkaloids, O-glycosylated megastigmanes, glycosylated flavonoids, flavan-3-ols, and others, such as quercetin O-deoxyhexosyl-hexoside, magnoflorine, reticuline, and taspine. No signs of acute toxicity were observed in female Wistar rats. Male spontaneously hypertensive rats from the negative control group presented

significant hypertension, reduction in renal function, alterations in the mesenteric vascular bed reactivity, and electrocardiographic changes typical of ventricular hypertrophy. In addition, a significant increase in malondialdehyde and nitrotyrosine levels, in addition to a reduction in serum nitrite concentration was observed. Oral prolonged ESCU-administration in spontaneously hypertensive rats was able to reverse renal, electrocardiographic, and hemodynamic changes induced by hypertension. Moreover, ESCU-treatment was able to modulate the mesenteric vascular bed reactivity and serum redox state. In mesenteric vascular beds preparations with an intact endothelium, ESCU (0.1, 0.3, and 1 mg) dose-dependently induced vasodilation. Endothelium removal or the inhibition of nitric oxide synthase by N(ω)-nitro-L-arginine methyl ester prevented the vasodilatory effect of ESCU. Perfusion with a physiological saline solution that contained KCl, tetraethylammonium, or apamin abolished the vasodilatory effect of ESCU.

Conclusion: The ESCU is safe after acute administration and presents significant cardioprotective effects in spontaneously hypertensive rats after prolonged treatment. Moreover, the data pointing to a predominant role for nitric oxide and Ca2+ activated small conductance K+ channels in the cardioprotective effects of ESCU.

Keywords: Antihypertensive; antioxidant; cardioprotective; safety; Euphorbiaceae

Abbreviations

ACE: angiotensin-converting enzyme; ACh: acetylcholine; ANOVA: One-way analysis of variance; CaCl2: calcium chloride; DBP: diastolic blood pressure; ECG: electrocardiography; EDTA: ethylenediaminetetraacetic acid; EDS: energy-dispersive X-ray spectroscopy; ELISA: enzyme-linked immunosorbent; ESCU: ethanol-soluble fraction from *C. urucurana* leaves; HCTZ: hydrochlorothiazide; HR: heart rate; KCl: potassium chloride; KH2PO4: potassium dihydrogen phosphate; LC-DAD-MS: liquid chromatography coupled to diode array detector and tandem mass spectrometry; L-NAME: Nω-nitro-L-arginine methyl ester; MAP: mean arterial pressure; MDA: malondialdehyde; MgSO4: magnesium sulfate; MVB: mesenteric vascular bed; NaCl: sodium chloride; NaHCO3: sodium bicarbonate; NC: negative control; NT: nitrotyrosine; Phe: phenylephrine; PP: perfusion pressure; ROS: reactive oxygen species; RW: relative weight; SBP: systolic blood pressure; SEM: scanning electron microscopy; SHR: spontaneously hypertensive rat; SNP: sodium nitroprusside; TBARS: thiobarbituric acid reactive substances; TEA: tetraethylammonium; UFGD: Federal University of Grande Dourados.

1. Introduction

Hypertension is a chronic metabolic disease that affects more than 1 billion people worldwide, being one of the main risk factors for other cardiovascular, cerebrovascular, and renal diseases (Wyss et al., 2020). The treatments already established can vary between non-pharmacological and pharmacological measures, depending on the degree of the disease (Williams et al., 2018). Due to the tolerance and side effects induced by pharmacological treatment, the Brazilian population often uses natural products as an alternative and complementary therapy. Thus, there is an emerging need for ethnopharmacological studies the prove or disprove probable therapeutic effects, supporting the selection and development of new therapies for hypertension (Patwardhan, 2005; Gao et al., 2021).

In folk Brazilian medicine, species from the genus *Croton* have been applied in various traditional preparations of leaves and bark to treat hypertension. In addition, different studies have already shown that species of the genus can positively alter cardiovascular hemodynamics. According to a recent ethnobotanical study carried out in the Grande Dourados region, Mato Grosso do Sul state, Brazil, the species *Croton urucurana* Baill. (family Euphorbiaceae) is widely used for the treatment of different cardiovascular diseases, including hypertension (Coelho et al., 2018).

The species is known as ‘sangra d’água’ or ‘sangue de dragão’ (dragon's blood) and is widely distributed throughout the Brazilian territory (Alves et al., 2008). Pharmacological studies previously performed with *C. urucurana* extracts have demonstrated antimicrobial, antitumor (Peres et al., 1997), and cytotoxic activity against specific leukemic cell lines (Vieira et al., 2017). Antiulcer (Alves et al., 2008; Cordeiro et al., 2012), antidiarrheal (Gurgel et al., 2001), anti-inflammatory, antinociceptive (Cordeiro et al., 2016), and anti-hemorrhagic effects have also been reported (Esmeraldino et al., 2004). Phytochemical studies have shown several classes of compounds such as diterpenes, alkaloids, flavonoids, steroids, and

triterpenoids (Salatino et al., 2007), including gallocatechin, β-sitosterol, and campesterol (Peres et al., 1998).

Although the species is widely used in Brazil for the treatment of different cardiovascular diseases, its probable antihypertensive effects are unknown. Thus, the main objective of this work is to investigate the molecular mechanisms involved in the antihypertensive effects of *C. urucurana* in spontaneously hypertensive rats (SHR).

2. Materials and methods

2.1. Drugs, salts, and solutions

Heparin was purchased from Hipolabor Pharmaceutical (Belo Horizonte, MG, Brazil). Xylazine and ketamine hydrochloride were obtained from Syntec (São Paulo, SP, Brazil). Phenylephrine (Phe), hydrochlorothiazide (HCTZ), sodium nitroprusside (SNP), indomethacin, acetylcholine chloride (ACh), glibenclamide, tetraethylammonium chloride (TEA), Nω-nitro-L-arginine methyl ester (L-NAME), iberiotoxin, charybdotoxin, apamin, sodium deoxycholate, dextrose, NaCl, $NaHCO_3$, KCl, $CaCl_2$, $MgSO_4$, KH_2PO_4, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant collection and identification

Leaves of *Croton urucurana* were collected in the region of Grande Dourados, Mato Grosso do Sul state, Brazil's Midwest, at 430 m above sea level (21°13'15"S 54°48'21"O). A voucher specimen under the number 5536 was authenticated by Dr. Zefa Valdivina Pereira and deposited in the Herbarium of the Federal University of Grande Dourados (UFGD). The

plant name is in accordance with the online database published by "The Plant List", accessed on April 13, 2021 (http://www.theplantlist.org/).

2.3. Anatomical analysis

For the anatomical analysis, the leaves *C. urucurana* were fixed in FAA 70 solution (formalin–acetic acid–alcohol) (Johansen, 1940) for seven days and then were stored in 70% ethanol (v/v) (Berlyn and Miksche, 1976). Were performed transverse free-hand sections and executed double-stained with basic fuchsin and Astra blue (Roeser, 1962).

Epidermical characteristics of leaves were analyzed through diaphanization. The leaves were immersed in a solution of commercial bleach, then were washed in distilled water, passed on acetic acid 5%, washed again in distilled water, and stained in 1% safranin in 50% ethanol.

Histochemical tests were performed with transverse sections of leaves using ferric chloride and potassium dichromate (Johansen, 1940), Sudan III (Sass, 1951), Sudan black B (Pearse, 1985), iodine solution (Berlyn; Miksche, 1976), methylene blue 0.1% (Farmacopeia Brasileira VI, 2019) solution of phloroglucinol/HCl (Foster, 1949), ruthenium red (Gregory; Baas, 1989), Nile blue (Cain, 1947) and Wagner's reagent (Furr; Mahlberg 1981). The results of the anatomical analysis were registered using an Olympus CX31 microscope equipped with an Olympus C-7070 digital camera.

2.3.1. Scanning Electron Microscopy (SEM) and Energy-dispersive X-ray Spectroscopy (EDS)

The leaves were dehydrated in a series of ethanol solutions (80, 90, and 100%). Then, the samples were realized metallization with gold using a Quorum SC7620 sputter coater and the SEM analysis using a Mira 3 (Tescan, Brno-Kohoutovice, Czech Republic) field emission

SEM in high vacuum mode at 15 kV accelerating voltage. X-ray microanalyses of crystals were performed with an EDS detector attached to the SEM.

2.4. Extract preparation

C. urucurana leaves were dried in a circulating air oven for 7 days at 45°C (113°F) and pulverized into fine powder. The extract was prepared by infusion using the methodology described by Bolson et al. (2015). The pulverized material (100 g) was subjected to the extraction process by infusion with 1L of boiling water. The resulting infusion was kept in an amber bottle, hermetically sealed until it reached room temperature (approximately for 5 hours). After filtration, the infusion was treated with ethanol (EtOH: 95%) in the proportion of 1: 3 v/v. The addition of ethanol-induced the formation of a precipitate (polysaccharides and proteins) and an ethanol-soluble fraction (ESCU). The precipitate was phase removed by filtration. The ESCU was concentrated in a rotary evaporator, lyophilized, and stored at -18ºC (64.4ºF).

2.5. Liquid chromatography coupled to diode array detector and tandem mass spectrometry (LC-DAD-MS) analyses

The ethanol-soluble fraction (ESCU) from C. urucurana was prepared at concentration 3 mg/mL in methanol and deionized water (7:3, v/v), filtered on syringe filters (0.22 μm, PTFE, Millex, Millipore®), and 1 μL was injected into the equipment Shimadzu LC-20AD UFLC chromatography coupled with a diode array detector and a mass spectrometer ESI-QTOF (MicroOTOF QIII, Bruker Daltonics, Billerica, MA, USA). The mobile phase applied in the analyses was composed of ultrapure water (Phase A) and acetonitrile (Phase B), both with 0.1% formic acid, and the gradient elution profile was: 0-2 min - 3% B; 2-25 min - 3-25% B; 25-40 min - 25-80% B; 40-43 min - 80% B, followed by column washing and

reconditioning (5 min). A chromatography column Kinetex C18 (2.6 µm, 150 x 2.1 mm, Phenomenex) was applied, the flow rate was 0.3 mL/min, and the oven was maintained at 50 ºC. The MS/MS data were acquired by automatic method (collision energy 30-65 eV) with the mass spectrometer operating in negative and positive ion modes (m/z 120-1200 and) and the UV was monitored between 240-800 nm. Data were processed by Data Analysis software version 4.2 (Bruker) and compounds were annotated based on UV, MS, and MS/MS spectral data, compared with published data, and the confirmation by injection of authentic standards was performed when possible. The molecular formulas were determined based on the accurate mass considering up ±5 ppm and mSigma below 30.

2.6. Ethnopharmacological investigations

2.6.1. Animals

Ten female Wistar rats (84 days of age), 53 male SHRs, and 7 male Wistar-Kyoto rats (180 days of age) obtained from the central vivarium of the UFGD were used in this study. Throughout the experimental period, animals were kept in a temperature and light-controlled room (22 ± 2°C; 12-h light/dark cycle) with ad libitum access to water and food. Before the onset of the experiments, all animals were left for seven days to acclimatize to laboratory conditions. All procedures involving animals were previously approved by the Ethics Committee in Animal Experimentation from the UFGD (protocol no. 18/2019) and were in accordance with the Brazilian Legal Framework on the Scientific Animals Use.

2.6.2. Safety evaluation

2.6.2.1. Acute toxicity

The acute toxicity assay was evaluated in accordance with Palozi et al. (2020). After the acclimation period, 10 female Wistar rats were equally divided into two experimental

groups (n = 5) and submitted to an overnight fasting period prior to treatments. Then, rats were weighed and the limit dose (2000 mg/kg) of ESCU or vehicle (filtered water; 0.2 mL/100 g) was administered orally (via gavage) to one animal per group. As no signs of toxicity nor lethality were observed in animals treated during the first 24 h, the remaining animals were also treated and submitted to a 14-day observation period. Bodyweight gain, mortality, food, and water intake as well as clinical signs of toxicity were daily observed. On the fifteenth day, rats were euthanized by isoflurane anesthesia (inhalation) followed by exsanguination. At necropsy, heart, lung, liver, spleen, kidneys, ovaries, and uterus were removed, weighed, and relative organs weight calculated. Furthermore, some samples were submitted for histopathological analyses.

2.6.3. Cardioprotective investigations

2.6.3.1. Effects on renal function

Renal function was evaluated in accordance with Gasparotto et al. (2009). Thirty- five male SHRs and seven male Wistar-Kyoto rats were randomly divided into six experimental groups (n = 7), as follows: 1) HCTZ (SHRs treated with hydrochlorothiazide; 25 mg/kg); 2) Negative control (NC; SHRs treated with filtered water; 0.2 mL/100 g); 3) ESCU 30 (SHRs treated with ESCU 30 mg/kg); 4) ESCU 100 (SHRs treated with ESCU 100 mg/kg); 5) ESCU 300 (SHRs treated with ESCU 300 mg/kg); and 6) naïve (Wistar-Kyoto rats treated with filtered water; 0.2 mL/100 g). The animals were treated orally, once a day, for 28 days. On days 1 and 28 all animals received orally (gavage) 5 mL/100g of saline solution (NaCl 0.9%) to impose body salt and water uniformity. Then, each animal was placed in individual metabolic cages. Urine samples were collected over a 24-hours period and their volumes were recorded (all results were expressed for mL/100 g of body weight). The pH and density were determined by a digital pH meter (Q400MT; Quimis Instruments, Brazil) and handheld

refractometer (NO107; Nova Instruments, Brazil), respectively. Urinary sodium, potassium, chloride, urea, and creatinine levels were quantified in an automated biochemical analyzer (Cobas Integra 400 plus, Roche).

2.6.3.2. Effects on electrocardiographic profile

On the morning of the twenty-ninth day, all animals underwent electrocardiography (ECG). Initially, all animals were anesthetized (ketamine 100 mg/kg plus xylazine 20 mg/kg; intramuscularly). Using four alligator clips, the electrodes were positioned on the animal's two forelimbs and two hindlimbs. An acclimatization period of 5 min elapsed, and ECG waves were recorded for 5 min. The following data were recorded: Segments (ms): PR, QRS, QT, and QTc; wave amplitudes (mV): P, Q, R, and S. Electrocardiography was recorded using a 12-lead ECG recorder (WinCardio, Micromed, Brasília, Brazil).

2.6.3.3. Effects on blood pressure (BP) and heart rate (HR)

Immediately after ECG recording, all animals received a subcutaneous single bolus injection of heparin (30 IU) prior to the surgical procedure. Then, the left carotid artery was isolated, cannulated, and connected to a pressure transducer coupled to a PowerLab® recording system. Heart rate, systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP) were recorded by an application program (Chart, v 7.1; both from ADI Instruments, Castle Hill, Australia) for 20 minutes. The results presented refer to the average of the last 5 min of recording.

2.6.3.4. Biochemical analysis

After hemodynamic evaluations, blood samples were collected directly from the left carotid artery and serum was obtained (centrifugation at 1000 g for 10 min). Urea, creatinine,

sodium, and potassium levels were measured using an automated biochemical analyzer (Roche® Cobas Integra 400 plus). Nitrotyrosine (NT) and aldosterone levels were measured by enzyme-linked immunosorbent assay (ELISA; BD Biosciences, CA, USA). Plasma nitrite concentrations were enzymatically determined by reducing nitrate according to methods described by Schmidt et al. (1989). The serum angiotensin-converting enzyme (ACE) activity was determined by indirect fluorimetry according to methods described by Santos et al. (1985). Finally, thiobarbituric acid reactive substances (TBARS) levels were measured by a TBARS assay kit (Cayman Chemical, Ann Arbor, Michigan, USA).

2.6.3.5. Effects on vascular reactivity

After the direct blood pressure measurements, the mesenteric vascular beds (MVBs) were isolated, cannulated, and prepared according to methods described by McGregor (1965). MVBs were then coupled in an perfusion system and continuously perfused with PSS (composition in mM: NaCl 119; KCl 4.7; CaCl2 2.4; MgSO4 1.2; NaHCO3 25.0; KH2PO4 1.2; dextrose 11.1; and EDTA 0.03), and gassed with 95% O2/5% CO2 at 37°C. Changes in perfusion pressure (PP, mmHg) were detected by a pressure transducer connected to a PowerLab® recording system and its application software (Chart, v 7.1; both from ADI Instruments, Castle Hill, Australia). After a 30-min stabilization period, tissue integrity was assessed with a bolus injection of KCl (120 mmol). Different doses of Phe were then administered in the MVBs (10, 30, and 100 nmol, 10-30 µL) preparations. After another 30-min stabilization period, tissues were continuously perfused with PSS plus 3 µM Phe to induce a prolonged increase in perfusion pressure. After stabilization of the contractile process, vascular reactivity to acetylcholine (ACh; 10, and 30 pmol) and sodium nitroprusside (SNP; 1, 3, and 10 pmol) was evaluated. At the end of the experiments, animals were euthanized under deep anesthesia in a saturation chamber (isoflurane with > 50% saturation).

2.6.3.6. Histopathology and relative organ weight

After euthanasia, the heart, kidneys, and liver were removed, cleaned, weighed, and longitudinally sectioned. The relative organ weight (RW%) was determined as follows: RW% = absolute organ weight x 100 / body weight. Then, organ fragments were fixed in 10% buffered formalin. The samples were cleaved, dehydrated with increasing absolute ethanol concentrations, diaphanized in xylol, and embedded in paraffin. Sections were then cut at a thickness of 4 μm and stained with hematoxylin and eosin for evaluation under light microscopy (40 X). The parameters analyzed were based on the presence or absence of reversible and/or irreversible cell lesions (Cunha et al., 2009). All images were obtained and evaluated using Motic Images Plus 2.0 software.

2.6.4. Investigation of the molecular mechanisms involved in the vascular effects of ESCU

Eighteen male SHR rats that had not received any previous treatment were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). Then, the MVBs were removed using previously described methods (McGregor, 1965). The MVBs were coupled with a perfusion system and continuously perfused with PSS that was aerated with 95% O2 and 5% CO2 at a constant flow rate of 4 mL/min. After a stabilization time, tissue integrity was verified with a bolus injection of 120 mmol KCl. Responses were recorded 10 s after administration. Changes in perfusion pressure (in mm Hg) were recorded by a pressure transducer that was connected to the PowerLab system and Chart 7.1 software (ADInstruments, Castle Hill, Australia). Then, different preparations with a functional endothelium (n = 6) were continuously perfused with PSS that contained 3 μM phenylephrine. After stabilization of the increase in perfusion pressure, the preparations received bolus injections (200 μL) that contained 0.003, 0.01, 0.03, 0.1, 0.3, and 1 mg ESCU,

and decreases in perfusion pressure were recorded. Each subsequent dose of ESCU was administered only after the perfusion pressure returned to basal levels, with a minimal time interval of 3 min between doses. To chemically remove the endothelium of the MVBs, some preparations were perfused with PSS that contained sodium deoxycholate (1.8 mg/mL) for 30 s via a parallel perfusion line that was near the main cannula and preparation. After the infusion of sodium deoxycholate, the system was perfused with regular PSS for an additional 40 min for stabilization. The preparations were continuously perfused with PSS that contained 3 μM phenylephrine, which induced a sustained increase in perfusion pressure. The reliability of the ability of sodium deoxycholate to promote endothelium removal was verified by the lack of vasodilatation after a bolus injection of 1 nmol acetylcholine. An ESCU dose-response curve (0.1, 0.3, and 1 mg) was generated for preparations without an endothelium. In preparations with an intact endothelium, a separate ESCU dose-response curve (0.1, 0.3, and 1 mg) was generated. A 30-45 min rest period was maintained to preserve the integrity of the preparations. The preparations were perfused with PSS that contained 3 μM phenylephrine plus the following agents, given alone or combined: 40 mM KCl (modified PSS solution containing: 83.5 mM NaCl, 40 mM KCl, 2.4 mM CaCl2, 1.2 mM MgSO4, 25.0 mM NaHCO3, 1.2 mM KH2PO4, 11.1 mM dextrose, and 0.03 mM EDTA), 1 μM indomethacin (nonselective cyclooxygenase inhibitor), 100 μM L-NAME (nonselective nitric oxide synthase inhibitor), 10 mM TEA (nonspecific potassium channel blocker), 10 μM glibenclamide (selective Kir6.1 channel inhibitor), 10 μM 4-aminopyridine (voltage-dependent [KV] K+ channel blocker), 10 nM iberiotoxin (selective high-conductance Ca2+-activated K+ channel [KCa1.1] blocker), 1 nM charybdotoxin (selective high [KCa1.1]- and intermediate [KCa3.1]-conductance Ca2+-activated K+ channel blocker), and 10 nM apamin (selective small-conductance Ca2+-activated K+ channel [KCa2.1, KCa2.2, KCa2.3] blocker). After 15 min of perfusion with one of the solutions, ESCU (0.1, 0.3, and 1mg) was

injected again into the perfusion system. The ability of ESCU to reduce perfusion pressure was compared with the results that were obtained with the control preparations that were perfused only with the vehicle.

2.7. Statistical analysis

Statistical analyzes were performed using one -way analysis of variance (ANOVA) followed by the Tukey's test. Results were expressed as mean ± standard error of the mean (SEM) of 5-7 preparations or animals per group. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Anatomical profile and histochemical characterization

The leaves of *Croton urucurana* (Figure 1a) measure 8-13 × 5-10 cm, have cordate to ovate-lanceolate shape, cordate base, acute to acuminate apex, entire margin, occasionally pubescent on the adaxial side and densely pubescent on abaxial surface, with colleters (minuscule basilaminar glands). The petiole is straight and 6-9 cm in length. Several species have similar morphology to *C. urucurana*, such as *Croton draconoides* Müll. Arg. and *Croton sampatik* Müll. Arg. (Guimarães; Secco, 2010). Consequently, it is common to find adulterants mixed with the medicinal species traded. When the sample is fragmented or powdered, the identification of the species and the detection of the adulteration or substitution is very difficult. In this case, the presence of anatomical markers is very important to support the identification and quality control of herbals (Machado et al., 2021).

Anatomically, in frontal view both epidermal leaf surfaces had wavy anticlinal walls. The leaves were amphistomatic with paracytic stomata (Figure 1b, c). Secretory idioblasts were spread in the epidermis (Figure 1c). They are larger than the other epidermal cells and

projected outside the surface. A great variety of non-glandular trichomes with lignified basal cells were frequently found, especially on the abaxial side (Figure 1d). They are classified as dendritic, dendritic porrect, fasciculate, rosulate, stellate, and stellate porrect. These types of trichomes were also reported by Lucena and Sales (2006) for other *Croton* species. Bipyramidal square-shaped crystals were found externally to the epidermal cells (Figure 1e). This characteristic was unique to *C. urucurana* and not previously reported for any other *Croton*. However, another genus also showed this characteristic (Klider et al., 2021; Lorençone et al., 2021). In the cross-section, the epidermis is uniseriate and covered outwardly by a thick and striated cuticle (Figure 1b, c), thicker and more evident on the adaxial surface and around stomata. Dorsiventral mesophyll was formed by one layer of palisade and several layers of spongy parenchyma (Figure 1f) with collateral bundles. Midrib, in cross-section, was biconvex more rounded on the abaxial side (Figure 1g). The epidermis was unilayered followed by 4-5 layers of subepidermal angular collenchyma on the adaxial side (Figure 1g, h). The vascular system was organized in two arches of collateral bundles with a bundle between the arcs (Figure 1g, j). The different organization was reported by Sa'Haiad et al. (2009) for other *Croton* species. Non-articulated laticifers were present close to the vascular bundles (Figure 1g, j). The petiole had a round shape, unilayered epidermis with non-glandular trichomes previously described for the leaf blade. Beneath the epidermis more than ten layers of angular collenchyma were present. The vascular system was horseshoe-shaped with two accessory bundles (Fig. 1m). Starch grains (Figure 1h, i) and crystalliferous idioblasts containing druses (Fig. 1n) were found dispersed in the ground parenchyma of midrib and petiole.

The histochemical tests reveal various classes of secondary metabolites that support the biological activities of secretion designated in the literature. Lipophilic material reacted with Sudan III and Sudan Black B and was evidenced in cuticles (lamina and petiole) (Figure

1h), in the laticifers (Figure 1l), and in the secretory idioblasts as well. In the laticifers, the latex also reacted positively with Nile blue. These characteristics were found in several species of *Croton* (Sa´-Haiad et al., 2009). Lignified compounds were reacted with phloroglucinol/HCl and found in the xylem (Figure 1j). Phenolic components were highlighted in the mesophyll, especially in the palisade parenchyma, and in the laticifers (Figure 1f, g). Alkaloids were detected with Wagner's reagent in the laticifers (Figure 1k). Starch grains reacted with Lugol solution. Carbohydrates were evidenced with Ruthenium red in the laticifers, whereas mucilage was not found with methylene blue. Lipophilic material, phenolic compounds, alkaloids, and carbohydrates were also detected by Feio et al. (2016) for *C. urucurana* and *Croton echinocarpus* Müll. Arg.

Spectra of the qualitative X-ray microanalyses of the crystals in *C. urucurana* displayed large peaks of calcium, carbon, and oxygen (Figure 2a, b), suggesting that these crystals were composed of calcium oxalate (CaC2O4). The unlabeled peak was gold (Au). To the best of our acknowledge, this is the first study of crystal-chemical composition in *Croton* species. EDS has been performed in microanalyses of the crystals in a different genus (Pauzer et al., 2021; D'Almeida et al., 2021).

3.2. Chemical constituents from ESCU

From LC-DAD-MS analyses, forty compounds were annotated from ESCU, including phenylpropanoid derivatives, flavan-3-ols, C- and O-glycosylated flavonols and flavones, alkaloids, O-glycosylated megastigmanes, a diterpene, and others. All the spectral data are presented in Table 1, and the chromatogram with the annotated compounds is illustrated in Figure 3.

The peaks 7 and 13 showed UV spectra compatible with flavan-3-ol (Markham, 1982), similarly with the MS data. They were confirmed by injection of standards and identified as catechin (7) and epicatechin (13). The compounds 4 and 6 revealed absorption bands at the wavelength ≈ 310 nm in the UV spectra, besides they showed the same production at m/z 152 [gentisic acid-H]-• yielded from losses of a hexosyl and pentosyl substituent (Han et al., 2008), respectively. Thus, they were annotated as O-hexosyl gentisic acid (4) and O-pentosyl gentisic acid (5).

The compounds 5, and 8-12 showed absorption bands in the UV (λmax ≈ 299 and 325 nm) compatible with the chromophore of caffeic/ferulic acid (Alonso-Salces et al., 2009). They presented the fragment ions at m/z 193 [ferulic acid-H]-, which are relative to C10H9O4-. This fragment ion was yielded from different losses of 192 or 162 u, suggesting the substituents hexaric acid and hexose (Kujala et al., 2002). Thus, they were annotated as O-feruloyl hexaric acid (5, and 8-11) and O-feruloyl hexoside (12).

The peaks 21, 23-25, 28, 32 showed UV spectra similar to flavones (λmax≈ 265 and 340 nm), while 22, 27, 29-31, 33-34, and 37-38 revealed as reported to flavonols (λmax≈ 270 and 355 nm) (Suzuki et al., 2011, Markham, 1982). These flavonoids revealed a fragmentation profile compatible with O-glycosylated (losses of the complete sugar) or C-glycosylated (losses of consecutive water molecules and parts of sugars) (Suzuki et al., 2011, Deng et al., 2008). The losses of hexosyl (162 u), deoxyhexosyl (146 u), and coumaroyl groups (146 u) suggested their occurrences in the compounds 22, 26-27, 29, 30-34, 37, and 38. The fragment ions m/z 303, 287, 287, and 317 [aglycone+H]+ suggested the aglycones quercetin (22, 26-27, 29), kaempferol (30-31, 37-38), luteolin (32), and O-methyl quercetin (33-34), which were also confirmed by important fragments yielded from C-ring fissions (Suzuki et al., 2011).

In addition, the flavones 21, 23-25, and 28 revealed consecutive losses of water molecules and the diagnostic fragment ions of C-hexose (losses of 120 and 90 u) and C-pentose (losses of 90 and 60 u) (Deng et al., 2008; Djoukeng et al., 2008). For example, the productions m/z 357 [M-H-90]- and 327 [M-H-120]- yielded from the precursor ion m/z 447 [M-H]- of compound 23. The flavones apigenin di-C-hexoside (21), luteolin C-hexoside (23, 25), apigenin C-pentosyl C-hexoside (24), and apigenin C-hexoside (28) were annotated.

The compounds 14, 17-19, and 36 revealed nitrogens in their molecular formulae, suggesting alkaloids. The alkaloids 17 and 36 showed productions yielded from losses of 45 and 15 u, which are relative to the HN(CH3)2 and •CH3. They were annotated as the aporphinic alkaloids magnoflorine (17) and taspine (36), which are alkaloids already described from C. urucurana (Cordeiro et al., 2016).

The alkaloids 14, 18, and 19 showed similar UV spectral data and their protonated ions were observed in some fragments to suggest the substituents such as aromatic methoxyls and methyl bond to nitrogen. The productions, for example, m/z 329 [M+H-•CH3]+• (14), 299 [M+H-3x•CH3]+• (14), 267 [M+H-4x•CH3-NH3]+ (14), 299 [M+H-NH2CH3]+ (18), and 284 [M+H-NH2CH3-•CH3]+• (18). Besides, other diagnostic productions were observed from 14, 18, and 19, and thus they were annotated as tetrahydropapaverine (14), reticuline (18), and O-methyl-laudanosoline O-hexoside (19) (Iwasa et al., 2005; Zhang et al., 2006).

3.3. Toxicological studies

3.3.1. Acute toxicity

No mortality nor changes in general behavior were observed in Wistar female rats submitted to the 14-day observation period. No significant changes were found in the final body weight, body weight gain as well as in food and water consumption of treated animals when compared to the control. Moreover, no significant differences were observed in the

absolute (g) or relative weight (%) of all organs isolated from animals treated with ESCU when compared to the control (data not shown). The macroscopic examination of vital organs did not reveal any abnormality among both experimental groups. No signs of inflammation, hemorrhage, fluid accumulation, or other changes suggestive of lesions in all tissues studied were observed. Therefore, the median lethal dose (LD_{50}) of the ESCU was stipulated as higher than 2,000 mg/kg.

3.4. Pharmacological assays

3.4.1. Effects on renal function

On the first day of treatment, the animals in the NC group or treated with ESCU at 30 mg/kg showed a significant reduction in the volume of urine, as well as in the urinary levels of chloride, potassium, sodium, and creatinine, when compared with the animals in the naïve group. Acute treatment with HCTZ or ESCU at doses of 100 and 300 mg/kg was able to maintain urine volume and renal excretion of chloride, potassium, sodium, and creatinine in a similar way to that found in naïve rats (Table 2).

The effects of prolonged administration (28 days) with ESCU on the renal function of SHRs are shown in Table 3. Similar to what was observed on the first day of treatment, the animals in the NC group or treated with ESCU at 30 mg/kg showed a significant reduction in renal function, with urine volume as well as urinary creatinine and electrolyte levels statistically lower than those observed in naïve group. The prolonged treatment with HCTZ or ESCU (100 and 300 mg/kg) was able to prevent these changes, maintaining urinary parameters similar to those observed in the naïve animals.

3.4.2. Electrocardiography

All SHRs treated with the vehicle showed a significant prolongation of the QT and QTc intervals when compared to the animals in the naive group. Treatments with HCTZ or ESCU (30, 100, or 300 mg/kg) prevented this change, maintaining values similar to those obtained in rats in the naïve group. All other electrocardiographic parameters did not show any statistically significant changes when compared between the different experimental groups (Table 4).

3.4.3. Blood pressure and HR

The SBP, DBP, MAP, and HR values of the SHRs of the NC group showed a statistically significant increase when compared to the animals of the naïve group. All treatments performed (HCTZ or ESCU) significantly reduced BP values when compared to animals in the NC group. In addition, when the animals were treated with ESCU at doses of 100 and 300 mg/kg, the values for RH were statistically lower than those found in all other experimental groups, as shown in Table 5.

3.4.4. Biochemical analysis

All animals in the NC groups or treated with HCTZ showed a significant increase in MDA and NT levels, in addition to a significant reduction in serum nitrite when compared to the naïve group. The prolonged treatments with the ESCU (30, 100, or 300 mg/kg) were able to reverse this change, maintaining the values of MDA, NT, and serum nitrite in a similar way to that obtained in the naïve animals (Table 6). Aldosterone and serum ACE activity (Table 6), as well as levels of urea, creatinine, sodium, and potassium, were not altered by any of the treatments performed (data not shown).

3.4.5. MVBs reactivity

In MVBs from NC rats, the administration of Phe 10 and 30 nmol were able to induce a vasoconstrictive effect ~ 100% higher than in naïve rats (Table 7). Prolonged treatments with HCTZ or ESCU (30, 100, and 300 mg/kg) were able to prevent this alteration, maintaining the vasoconstrictor response similar to naïve animals. The vasodilator effects induced by ACh and SNP on MVBs obtained from naïve, NC, HCTZ, or ESCU groups (30, 100, and 300 mg/kg) did not show statistically different responses.

3.4.6. Relative organ weight and histopathological analysis

The effects of oral treatment with an ethanol-soluble fraction from *C. urucurana* leaves (30, 100, and 300 mg/kg) and hydrochlorothiazide (HCTZ) on relative organ weight are shown in Table 8. It was observed a significant increase in heart WT% in the NC group when compared with naive animals. The prolonged treatments with the HCTZ or ESCU (30, 100, or 300 mg/kg) were able to reverse this change, maintaining the values of heart WT% in a similar way to that obtained in the naïve animals. The kidneys and liver WT% were not changed in any of the experimental groups. No evidence of fibrillary derangement, fibrosis, inflammation, apoptosis, or necrosis of cardiac, renal, or hepatic cells was identified in any of the experimental groups (data not shown).

3.4.7. Investigation of the molecular mechanisms involved in the vascular effects of ESCU

The administration of ESCU (0.1, 0.3, and 1mg) in the MVBs induced a dose-dependent relaxant effect (Figure 4A). Treatment with sodium deoxycholate attenuated the effects of acetylcholine by 97% ± 2% in the MVBs (data not shown). The vasodilatory effects of ESCU were blocked in preparations without endothelium (Figure 4B) or pretreated with L-NAME (Figure 4C). In preparations with functional endothelium that were perfused with indomethacin, the vasorelaxant effects of ESCU remained unchanged (Figure 4D). Perfusion

of the preparations with a nutritive solution that was added to 40 mM KCl (Figure 5A), TEA (Figure 5B), or apamin (Figure 5D) abolished the effects of ESCU. However, only nonsignificant effects were observed after the infusion of 4-aminopyridine (data not shown), glibenclamide (Figure 5C), charybdotoxin (Figure 5E), and iberiotoxin (Figure 5F).

4. Discussion

In this study, we collected the leaves of *Croton urucurana* in a Cerrado area in the region of Grande Dourados, Mato Grosso do Sul state, Brazil. From the dried leaves, we produce the ESCU, a preparation popularly used in traditional Brazilian medicine for the treatment of different cardiovascular diseases (Coelho et al., 2019). We analyzed the chemical composition of the ESCU by LC-DAD-MS, and several metabolites could be annotated such as O- and C-glycosylated flavonoids, alkaloids, megastigmanes, ferulic acid derivatives, and others. The flavonoid tiliroside (like as the metabolites 37 and 38) has been reported as a chemical marker from *Croton* species, besides we observed a complex flavonoid mixture in *C. urucurana* that has been suggested as a trend of evolution (Oliani et al., 2021).

A preclinical trial was performed to evaluate the acute toxicity of ESCU. The ESCU acute administration did not show acute signs of toxicity, with a median lethal dose above 2,000 mg/kg. As the pre-clinical data indicated relative safety, we chose to investigate the cardioprotective effects of ESCU using male SHRs at 6 months of age. Hypertension in SHRs reaches a plateau between the twentieth and twenty-eighth weeks of life, with no sexual influences (Doggrell and Brown, 1998). Therefore, we chose to use males' rats to verify the structural and functional changes that could be prevented in this gender, considering the profile of the hypertensive disease that affects men worldwide (Lodi et al., 2018). Corroborating our hypothesis, all SHRs that did not receive any treatment showed a

significant reduction in renal function, increase in serum oxidative stress, and hemodynamic and electrocardiographic changes typical of hypertensive disease.

In general, all doses of ESCU (30, 100, and 300 mg/kg) showed a significant cardioprotective effect in the experimental model used. The 28-day treatment normalized renal, electrocardiographic, and hemodynamic parameters affected by hypertension. In some cases, treatment with ESCU at doses of 100 and 300 mg/kg showed a response statistically higher to doses of 30 mg/kg. Although dose-dependent effects have been observed in some cases, non-dose-dependent responses with crude extracts are very common. As the crude extracts have many metabolites, it is possible that antagonistic or synergistic responses can occur between the different compounds. Thus, crude extracts that present cardioprotective response in smaller doses, may have their effects decreased or increased with a progressive increase of the doses (Caesar and Cech, 2019).

In the cardiovascular system, cells from different tissues are frequently under the action of the free radicals and reactive oxygen species (ROS). These molecules are present in a homeostatic state between their production and elimination, i.e., the redox state. In normal situations, the redox state is maintained by the presence of natural antioxidants and antioxidant enzymes. Different cardiovascular pathologies, including hypertension, affect this balance, increasing the formation of free radicals and peroxides that may affect several cell components, including proteins and lipids (Lubrano and Balzan, 2015; Pignatelli et al., 2018). In our study, hypertensive animals that received no treatment showed a significant increase in NT and MDA, two important markers of protein and lipid oxidation. In addition, nitrite levels were significantly reduced, indicating a lower bioavailability of body nitric oxide. Currently, it is known that the hemodynamic changes and oxidative stress induced by hypertension directly contribute to ventricular hypertrophy and to the increase in the relative weight of the heart (Wang et al., 2018; Li et al., 2018). On the other hand, the prolonged treatment with the ESCU

prevented the redox state imbalance, affecting the levels of NT, MDA, and nitrite. The synergistic effect between the reduction of oxidative stress and blood pressure levels can be pointed out as the main responsible for the prevention of cardiac structural changes induced by hypertension.

Considering that ESCU showed important cardioprotective activity in hypertensive rats, we decided to investigate the pharmacological mechanisms through which *C. urucurana* exerts its effects. First, we showed that the ESCU can induce a significant vasodilator response in resistance vessels in hypertensive rats. In addition, these effects were directly dependent on the release of NO by the vascular endothelium. These data could be corroborated by the increase in the bioavailability of NO, identified indirectly by the levels of nitrite (a stable marker of NO metabolism), after prolonged treatment with ESCU. In a second step, we investigate the role of the K^+ channels in the vasodilator response induced by ESCU. This hypothesis was tested using different classic K^+ channel blockers, including three different toxins that block different Ca^{2+} -activated K^+ channels (i.e., charybdotoxin, apamin, and iberiotoxin). The nonspecific K + channel blocker TEA, as well as apamin completely blocked the vascular effects of ESCU. These data suggest the direct participation of Ca^{2+} activated small-conductance K^+ channels in the response to ESCU in mesenteric arteries. As the downstream targets of the nitric oxide pathway in vessels include the opening of K^+ channels, is reasonable to speculate that small-conductance Ca^{2+} -activated K^+ channels participate in the nitric oxide-dependent effects of ESCU.

Two limitations are presented in this study. First, we do not accurately identify which of the secondary metabolites present in the ESCU are the main ones responsible for the cardioprotective activity. Although the hypothesis of a single active molecule is interesting, we believe that the effect is due to a synergistic and coordinated action of different compounds. Finally, we did not evaluate possible additive or synergistic effects of ESCU and

HCTZ. Further studies can assess whether there is a likely beneficial interaction between the prolonged use of ESCU with other classic antihypertensive drugs.

5. Conclusion

In this study we show that a preparation popularly used in Brazil for the treatment of cardiovascular diseases is effective in preventing the evolution of hypertensive disease in SHRs. Apparently, these effects are dependent on the nitric oxide pathway and Ca2+ activated small-conductance K+ channels.

Credit author statement

Conceptualization, funding acquisition, and project administration: AGJ. Methodology, investigation, and data curation: KSL, AAMM, KGTM, BRL, PRTL, GPS, ACS, RICS, FMF, NSC, EMBL, LMK, JMB, and DBS. Writing - original draft: KSL. Supervision and writing - review and editing: AGJ, JMB, and DBS.

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Conflicts of Interest

The authors declare no conflict of interest.

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Table 1. Compounds annotated from ethanol-soluble fraction from *Croton urucurana* leaves (ESCU) by LC-DAD-MS.

Peak	RT (min)	UV (λ_{max})	MF	Negative ion mode (*m/z*)		Positive ion mode (*m/z*)		Compound
				MS $[M-H]^-$	MS/MS	MS $[M+H]^+$	MS/MS	
1	1.2	-	$C_{12}H_{21}O_{11}$	341.1090	191	-	-	Di-*O*-hexoside
2	1.3	-	$C_7H_8O_7$	203.0195	-	-	-	Unknown
3	1.5	-	$C_6H_8O_7$	191.0195	-	-	-	Citric acid
4	4.0	310	$C_{13}H_{16}O_9$	315.0718	152	-	-	*O*-hexosyl gentisic acid
5	6.0	295, 324	$C_{16}H_{18}O_{11}$	385.0737	317, 209, 193, 191, 175, 161, 146	387.0936	342, 300, 266, 177, 149	*O*-Feruloyl hexaric acid
6	6.6	316	$C_{12}H_{14}O_8$	285.0621	152	-	-	*O*-pentosyl gentisic acid
7	9.0	279	$C_{15}H_{14}O_6$	289.0725	247, 229, 221, 203, 187, 173, 159	291.0863	255, 226, 207, 189, 179, 161, 147	Catechin[st]
8	9.2	299, 326	$C_{16}H_{18}O_{11}$	385.0773	193	387.0916	-	*O*-feruloyl hexaric acid
9	10.2	300, 326	$C_{16}H_{18}O_{11}$	385.0782	209, 193, 191, 175	387.0909	177, 149	*O*-feruloyl hexaric acid
10	10.7	292, 326	$C_{16}H_{18}O_{11}$	385.0779	193	387.0922	-	*O*-feruloyl hexaric acid
11	12.0	290, 326	$C_{16}H_{18}O_{11}$	385.0774	193	387.0927	-	*O*-feruloyl hexaric acid
12	12.0	287,328	$C_{23}H_{16}O_4$	355.0993	193, 175, 160	-	-	*O*-feruloyl hexoside
13	12.3	281	$C_{15}H_{14}O_6$	289.0679	221, 201, 187, 175, 161	291.0859	245, 229, 207, 187, 177, 161, 147	Epicatechin[st]
14	13.5	282	$C_{20}H_{25}NO_4$	342.171	-	344.1876	329, 299, 267, 239, 207, 192, 175, 160, 151	Tetrahydropapaverine
15	13.5	-	$C_{19}H_{30}O_8$	385.1869	273, 209, 191	387.2034	207, 189	*O*-glycosylated megastigmane
16	13.5	280	C16H20O10	371.0943	337, 329, 311, 263, 249, 232, 213, 189, 175,161, 157	373.1111	313, 263, 197, 179	Dihydroferulic acid derivative
17	14.1	270, 309	$C_{20}H_{24}NO_4^+$	340.1513	325, 310, 282, 267, 252, 239, 224, 209, 196, 157, 101	342.1716	297, 282, 265, 237, 222, 209, 191, 181, 165, 153	Magnoflorine
18	14.7	283	$C_{19}H_{23}NO_4$	-	-	330.1712	299, 284, 267, 239, 235, 207, 192, 177	Reticuline
19	14.8	282	C24H31NO9	476.1908	449, 314, 299, 284, 266, 232, 203, 162	478.2083	316, 178, 161	*O*-methyl-laudanosoline *O*-hexoside

20	15.4	-	C19H32O8	387.2011	375, 355, 315, 225, 207, 167, 154	411.1993^{Na}	345, 277, 271, 181	O-glycosylated megastigmane
21	16.0	270, 334	$C_{27}H_{30}O_{15}$	593.1454	473, 431, 341, 311, 297, 269	595.1670	415, 397, 379, 337, 323, 313, 283	Apigenin di-*C*-hexoside
22	16.2	268, 350	$C_{27}H_{30}O_{17}$	625.1359	300, 179, 151	627.1562	465, 303, 217	Quercetin di-*O*-hexoside
23	16.3	265, 340	$C_{21}H_{20}O_{11}$	447.0878	357, 339, 327, 311, 297, 285, 269, 255	449.1092	431, 413, 401, 395, 383, 365, 353, 339, 329, 299, 229, 217, 205	Luteolin *C*-hexoside
24	16.6	273, 338	$C_{26}H_{28}O_{14}$	563.1359	545, 504, 485, 473, 443, 425, 413, 383, 353, 325, 311, 297	565.1561	457, 445, 427, 409, 391, 379, 349, 337, 325, 307, 295, 175	Apigenin *C*-pentosyl *C*-hexoside
25	17.9	267, 338	$C_{21}H_{20}O_{11}$	431.0941	341, 323, 311, 283, 191	433.1139	415, 397, 379, 367, 351, 343, 337, 323, 313, 297, 283	Luteolin *C*-hexoside
26	17.8	259, 353	$C_{27}H_{30}O_{16}$	609.1409	300, 271, 255, 243, 179, 151	611.1633	303	Quercetin *O*-deoxyhexosyl-hexoside
27	18.6	259, 354	$C_{27}H_{30}O_{16}$	609.1408	300, 271, 255, 243, 179, 151	611.1619	303	Quercetin *O*-deoxyhexosyl-hexoside
28	18.7	269, 339	$C_{21}H_{20}O_{10}$	431.0939	341, 323, 311, 283, 239	433.1144	397, 379, 361, 349, 337, 323, 313, 295, 283, 271	Apigenin *C*-hexoside
29	18.9	266, 356	$C_{21}H_{20}O_{12}$	463.0838	300, 271, 255, 243, 151	465.1029	303	Quercetin *O*-hexoside
30	19.7	267, 347	$C_{27}H_{30}O_{15}$	593.1461	284, 255, 227	595.1669	449, 287	Kaempferol *O*-deoxyhexosy *O*-hexoside
31	20.7	266, 346	$C_{27}H_{30}O_{15}$	593.1463	285, 284, 255, 227, 211, 151	595.1659	449, 287	Kaempferol *O*-deoxyhexosy *O*-hexoside
32	21.0	267, 338	$C_{21}H_{20}O_{11}$	447.0884	284, 255, 227	449.1079	287	Luteolin *O*-hexoside
33	21.4	270, 350	$C_{28}H_{32}O_{16}$	623.1566	315, 300, 271, 243, 151	625.1769	479, 317	*O*-Methylquercetin *O*-deoxyhexosyl *O*-hexoside
34	21.6	270, 352	$C_{28}H_{32}O_{16}$	623.1588	315, 300, 271, 243, 151	625.1751	479, 317	*O*-Methylquercetin *O*-deoxyhexosyl *O*-hexoside
35	21.8	280	$C_{28}H_{36}O_{13}$	579.2067	417, 402, 387, 357, 341, 300, 181, 166	603.2053^{Na}	441, 373, 293, 237	Syringaresinol *O*-hexoside
36	22.6	290	$C_{20}H_{19}NO_6$	-	-	370.1293	325, 310, 295, 283, 266, 254	Taspine
37	27.7	266, 284, 318, 350	$C_{30}H_{26}O_{13}$	593.1288	447, 284, 255, 227, 151	595.1453	449, 287, 263, 249, 217, 205, 147	Kaempferol *O*-coumaroyl *O*-hexoside

38	28.0	266, 284, 318, 350	$C_{30}H_{26}O_{13}$	593.1299	284, 255, 227, 151	595.1467	287, 237, 215, 147	Kaempferol *O*-coumaroyl *O*-hexoside
39	30.4	-	$C_{40}H_{64}O_{17}$	815.4071	785, 768, 688, 615, 555, 484, 413, 385, 280, 170	817.4197	800, 772, 686, 599, 515, 412, 319, 205, 162	Unknow
40	32.9	-	$C_{20}H_{24}O_5$	343.1541	299, 231, 213, 205	345.1697	251, 233, 205, 187, 159	Diterpene

RT: retention time; MF: molecular formula; NI: non-identified; ^{Na}: $[M+Na]^+$; st: confirmed by injection of authentic standard. All the MF was determined from the errors and mSigma below 5 ppm and 30, respectively.

Table 2. Effects of oral treatment with ethanol-soluble fraction from *Croton urucurana* leaves (ESCU) (30, 100, and 300 mg/kg) and hydrochlorothiazide (HCTZ) on urinary parameters on the 1st day of treatment

Parameter	Naïve	NC	HCTZ	ESCU (30 mg/kg)	ESCU (100 mg/kg)	ESCU (300 mg/kg)
Urinary volume (mL/100g/24h)	4.71 ± 1.42	2.32 ± 0.27[a]	2.99 ± 0.28	2.27 ± 0.37[a]	3.12 ± 0.34	2.94 ± 0.22
pH	7.71 ± 0.26	7.73 ± 0.11	7.72 ± 0.25	7.09 ± 0.23	7.71 ± 0.90	7.50 ± 0.29
Density	1034 ± 5.53	1028 ± 2.09	1029 ± 5.54	1043 ± 2.35	1043 ± 2.36	1046 ± 2.36
Chloride (µmol/100g/24h)	1211.13 ± 112.33	703.11 ± 56.11[a]	1355.88 ± 155.77[b]	752.05 ± 66.06[ac]	1130.44 ± 112.04[bd]	1233.17 ± 119.12[bd]
Potassium (µmol/100g/24h)	867.11 ± 104.14	413.66 ± 66.33[a]	977.11 ± 121.62[b]	412.66 ± 67.11[ac]	977.10 ± 97.32 [bd]	1112.18 ± 102.22[bd]
Sodium (µmol/100g/24h)	1521.66 ± 211.17	902.11 ± 99.55[a]	1482.97 ± 152.10[b]	916.33 ± 93.43[ac]	1616.77 ± 133.21[bd]	1613.57 ± 133.12[bd]
Urea (mg/100g/24h)	114.55 ± 33.41	117.11 ± 45.16	112.29 ± 73.12	115.11 ± 38.51	114.41 ± 50.21	113.77 ± 39.41
Creatinine (mg/100g/24h)	2.18 ± 0.33	1.23 ± 0.22[a]	1.99 ± 0.22[b]	1.19 ± 0.13[ac]	2.21 ± 0.21[bd]	2.15 ± 0.18[bd]

Statistical analyses were performed using one–way ANOVA followed by Tukey's test. Values are expressed as mean ± SEM (standard error of the mean). n = 7 rats per group. [a]p ≤ 0.05 when compared to naïve; [b]p ≤ 0.05 when compared to NC group; [c]p ≤ 0.05 when compared to HCTZ group; [d]p ≤ 0.05 when compared to ESCU 30 mg/kg. ESCU: hypertensive animals that received different doses of ethanol-soluble fraction from *Croton urucurana* leaves; HCTZ: hypertensive animals that were treated with hydrochlorothiazide (25 mg/kg); Naïve: normotensive animals treated with vehicle (water); NC: (negative control; hypertensive rats treated with vehicle).

Table 3. Effects of oral treatment with ethanol-soluble fraction from *Croton urucurana* leaves (ESCU) (30, 100, and 300 mg/kg) and hydrochlorothiazide (HCTZ) on urinary parameters on the 28th day of treatment

Parameter	Naïve	NC	HCTZ	ESCU (30 mg/kg)	ESCU (100 mg/kg)	ESCU (300 mg/kg)
Urinary volume (mL/100g/24h)	4.46 ± 1.16	2.66 ± 0.26[a]	3.67 ± 0.55	3.61 ± 0.53	3.32 ± 0.48	4.25 ± 0.39
pH	8.25 ± 0.19	8.32 ± 0.09	8.24 ± 0.07	8.00 ± 0.17	7.98 ± 0.03	7.98 ± 0.08
Density	1022 ± 5.90	1024 ± 2.34	1029 ± 2.39	1036 ± 2.56	1033 ± 1.36	1032 ± 2.89
Chloride (µmol/100g/24h)	1333.13 ± 122.22	617.18 ± 66.21[a]	1255.99 ± 144.54[b]	556.03 ± 55.77[ac]	1255.34 ± 121.12[bd]	1399.21 ± 121.77[bd]
Potassium (µmol/100g/24h)	917.64 ± 188.17	394.15 ± 43.11[a]	911.15 ± 119.33[b]	385.77 ± 74.12[ac]	1021.12 ± 102.33 [bd]	977.99 ± 98.33[bd]
Sodium (µmol/100g/24h)	1611.58 ± 176.14	761.11 ± 102.33[a]	1533.23 ± 161.12[b]	617.22 ± 88.33[ac]	1733.83 ± 157.44[bd]	1601.66 ± 112.15[bd]
Urea (mg/100g/24h)	121.21 ± 40.41	117.55 ± 44.12	119.11 ± 55.11	120.18 ± 39.54	122.45 ± 38.32	119.88 ± 44.55
Creatinine (mg/100g/24h)	2.14 ± 0.23	1.14 ± 0.33[a]	2.04 ± 0.21[b]	1.21 ± 0.12[ac]	2.19 ± 0.23[bd]	2.20 ± 0.21[bd]

Statistical analyses were performed using one–way ANOVA followed by Tukey's test. Values are expressed as mean ± SEM (standard error of the mean). n = 7 rats per group. [a]p ≤ 0.05 when compared to naïve; [b]p ≤ 0.05 when compared to NC group; [c]p ≤ 0.05 when compared to HCTZ group; [d]p ≤ 0.05 when compared to ESCU 30 mg/kg. ESCU: hypertensive animals that received different doses of ethanol-soluble fraction from *Croton urucurana* leaves; HCTZ: hypertensive animals that were treated with hydrochlorothiazide (25 mg/kg); Naïve: normotensive animals treated with vehicle (water); NC: (negative control; hypertensive rats treated with vehicle).

Table 4. Effects of oral treatment with ethanol-soluble fraction from *Croton urucurana* leaves (ESCU) (30, 100, and 300 mg/kg) and hydrochlorothiazide (HCTZ) on electrocardiographic parameters on the 29th day

Parameter	Naïve	NC	HCTZ (25 mg/kg)	ESCU (30 mg/kg)	ESCU (100 mg/kg)	ESCU (300 mg/kg)
Segments (ms)						
PR	79.80 ± 7.06	79.50 ± 2.44	80.25 ± 2.77	84.13 ± 7.03	84.88 ± 2.16	81.14 ± 5.50
QRS	69.40 ± 2.87	70.13 ± 1.80	71.88 ± 1.80	66.75 ± 5.78	71.00 ± 2.00	72.29 ± 2.34
QT	140.6 ± 10.76	184.6 ± 5.59[a]	147.1 ± 6.04[b]	152.0 ± 12.57[b]	154.8 ± 5.18[b]	150.1 ± 7.02[b]
QTc	271.2 ± 21.53	367.9 ± 14.16[a]	269.4 ± 14.15[b]	285.0 ± 28.49[b]	258.1 ± 10.16[b]	267.0 ± 11.20[b]
Waves (mV)						
P	0.056 ± 0.009	0.069 ± 0.012	0.065 ± 0.007	0.066 ± 0.004	0.063 ± 0.007	0.064 ± 0.013
Q	-0.010 ± 0.006	-0.014 ± 0.005	-0.015 ± 0.003	-0.014 ± 0.004	-0.013 ± 0.006	-0.013 ± 0.006
R	0.30 ± 0.021	0.33 ± 0.036	0.35 ± 0.019	0.33 ± 0.013	0.32 ± 0.037	0.34 ± 0.036
S	-0.076 ± 0.014	-0.073 ± 0.024	-0.079 ± 0.018	-0.078 ± 0.024	-0.068 ± 0.021	-0.074 ± 0.024

Statistical analyses were performed using one–way ANOVA followed by Tukey's test. Values are expressed as mean ± SEM (standard error of the mean). n = 7 rats per group. [a]p ≤ 0.05 when compared to naïve; [b]p ≤ 0.05 when compared to NC group. ESCU: hypertensive animals that received different doses of ethanol-soluble fraction from *Croton urucurana* leaves; HCTZ: hypertensive animals that were treated with hydrochlorothiazide (25 mg/kg); Naïve: normotensive animals treated with vehicle (water); NC: (negative control; hypertensive rats treated with vehicle).

Table 5. Effects of oral treatment with ethanol-soluble fraction from *Croton urucurana* leaves (ESCU) (30, 100, and 300 mg/kg) and hydrochlorothiazide (HCTZ) on blood pressure and heart rate on the 29th day

Parameter	Naïve	NC	HCTZ (25 mg/kg)	ESCU (30 mg/kg)	ESCU (100 mg/kg)	ESCU (300 mg/kg)
DBP	76.4 ± 8.04	92.3 ± 5.97[a]	68.3 ± 4.28[b]	73.5 ± 4.41[b]	68.9 ± 7.61[b]	67.3 ± 5.03[b]
SBP	114 ± 11.3	144 ± 9.44[a]	101 ± 8.89[b]	109 ± 4.65[b]	114 ± 11.8[b]	111 ± 4.40[b]
MAP	86.8 ± 8.69	101.2 ± 7.01[a]	76.5 ± 1.97[b]	81.5 ± 2.79 [b]	81.7 ± 3.34 [b]	86.3 ± 4.98 [b]
HR	237 ± 10.3	464 ± 15.72[a]	492 ± 17.53[a]	453 ± 20.71[a]	360 ± 26.01[abcd]	302 ± 15.541[abcd]

Statistical analyses were performed using one–way ANOVA followed by Tukey's test. Values are expressed as mean ± SEM (standard error of the mean). n = 7 rats per group. [a]p ≤ 0.05 when compared to naïve; [b]p ≤ 0.05 when compared to NC group; [c]p ≤ 0.05 when compared to HCTZ group; [d]p ≤ 0.05 when compared to ESCU 30 mg/kg. DBP: diastolic blood pressure; ESCU: hypertensive animals that received different doses of ethanol-soluble fraction from *Croton urucurana* leaves; HCTZ: hypertensive animals that were treated with hydrochlorothiazide (25 mg/kg); HR: heart hate; MAP: mean arterial pressure; Naïve: normotensive animals treated with vehicle (water); NC: (negative control; hypertensive rats treated with vehicle); SBP: systolic blood pressure.

Table 6. Effects of oral treatment with ethanol-soluble fraction from *Croton urucurana* leaves (ESCU) (30, 100, and 300 mg/kg) and hydrochlorothiazide (HCTZ) on biochemical parameters on the 29th day

Parameter	**Naïve**	**NC**	**HCTZ (25 mg/kg)**	**ESCU (30 mg/kg)**	**ESCU (100 mg/kg)**	**ESCU (300 mg/kg)**
Nitrite (μM)	89.1 ± 6.04	33.2 ± 3.33[a]	37.2 ± 5.33[a]	100.5 ± 4.55[bc]	99.3 ± 6.2[bc]	90.3 ± 5.12[bc]
NT (μmol/L)	0.011 ± 0.003	0.037 ± 0.007[a]	0.027 ± 0.006[a]	0.013 ± 0.005[bc]	0.011 ± 0.004[bc]	0.012 ± 0.005[bc]
MDA (mmol/L)	7.9 ± 2.0	22.3 ± 3.43[a]	18.42± 4.16[a]	10.6 ± 4.18	8.9 ± 2.6[bc]	7.6 ± 3.1[bc]
Aldosterone (pg/mL)	113.9 ± 9.99	116.2 ± 10.88	111.5 ± 10.22	117.4 ± 9.12	119.2 ± 11.10	116.5 ± 8.77
ACE activity (nmol/min/mL)	82.1 ± 4.11	97.3 ± 8.22	90.2 ± 8,73	87.11 ± 7.01	90.11 ± 7.66	88.3 ± 11.10

Statistical analyses were performed using one–way ANOVA followed by Tukey's test. Values are expressed as mean ± SEM (standard error of the mean). n = 7 rats per group. [a]p ≤ 0.05 when compared to naïve; [b]p ≤ 0.05 when compared to NC group; [c]p ≤ 0.05 when compared to HCTZ group; ACE: angiotensin-converting enzyme; ESCU: hypertensive animals that received different doses of ethanol-soluble fraction from *Croton urucurana* leaves; HCTZ: hypertensive animals that were treated with hydrochlorothiazide (25 mg/kg); Naïve: normotensive animals treated with vehicle (water); MDA: malondialdehyde; NC: (negative control; hypertensive rats treated with vehicle); NT: nitrotyrosine.

Table 7. Effects of oral treatment with ethanol-soluble fraction from *Croton urucurana* leaves (ESCU) (30, 100, and 300 mg/kg) and hydrochlorothiazide (HCTZ) on mesenteric vascular reactivity on the 29th day

Drug (dose)	**Naïve**	**NC**	**HCTZ (25 mg/kg)**	**ESCU (30 mg/kg)**	**ESCU (100 mg/kg)**	**ESCU (300 mg/kg)**
Phe (nmol)						
1	2.69 ± 2.55	5.14 ± 2.34	3.62 ± 1.02	3.26 ± 1.71	2.59 ± 1.23	5.82 ± 2.92
3	4.41 ± 1.08	6.43 ± 2.49	7.87 ± 2.13	6.05 ± 3.56	5.68 ± 3.44	7.95 ± 3.97
10	17.36 ± 2.12	37.24 ± 6.10[a]	19.04 ± 3.11[b]	16.50 ± 4.83[b]	19.07 ± 4.83[b]	20.41 ± 5.60[b]
30	73.62 ± 11.98	156.61 ± 43.76[a]	87.64 ± 12.21[b]	66.19 ± 11.88[b]	75.03 ± 10.49[b]	67.47 ± 13.69[b]
ACh (pmol)						
10	10.81 ± 1.39	-10.84 2.93	-13.56 ± 1.64	-11.48 ± 6.99	-12.71 ± 6.82	-12.65 ± 2.85
30	-15.78 ± 6.09	-17.03 4.67	-16.15 ± 2.98	-17.98 ± 5.85	-16.89 ± 13.40	-18.14 ± 5.31
100	-17.52 ± 11.76	-22.89 5.73	-19.78 ± 5.95	-19.45 ± 6.15	-20.81 ± 10.31	-20.84 ± 6.87
300	-28.65 ± 8.46	-30.74 6.76	-29.83 ± 6.89	-28.41 ± 10.04	-29.48 ± 11.53	-27.59 ± 9.38
SNP (pmol)						
10	-7.11 ± 4.40	-11.25 ± 3.89	-9.93 ± 4.95	-8.98 ± 5.65	-9.26 ± 6.18	-7.03 ± 4.13
30	-12.57 ± 6.73	-14.43 ± 1.62	-13.89 ± 4.62	-13.11 ± 3.96	-14.64 ± 6.47	-13.15 ± 3.01
100	-15.01 ± 5.14	-16.50 ± 8.10	-14.53 ± 3.91	-17.08 ± 4.74	-18.08 ± 2.59	-15.49 ± 3.60
300	-17.64 ± 2.48	-19.99 ± 2.59	-20.34 ± 5.01	-21.08 ± 4.01	-19.29 ± 8.37	-20.26 ± 5.18

Statistical analyses were performed using one–way ANOVA followed by Tukey's test. Values are expressed as mean ± SEM (standard error of the mean). n = 7 rats per group. [a]p ≤ 0.05 when compared to naïve; [b]p ≤ 0.05 when compared to NC group; ACh: acetylcholine; ESCU: hypertensive animals that received different doses of ethanol-soluble fraction from *Croton urucurana* leaves; HCTZ: hypertensive animals that were treated with hydrochlorothiazide (25 mg/kg); Naïve: normotensive animals treated with vehicle (water); NC: (negative control; hypertensive rats treated with vehicle); Phe: phenylephrine; SNP: sodium nitroprusside.

Table 8. Effects of oral treatment with ethanol-soluble fraction from *Croton urucurana* leaves (ESCU) (30, 100, and 300 mg/kg) and hydrochlorothiazide (HCTZ) on relative organ weight on the 29th day

Parameter	**Naïve**	**NC**	**HCTZ (25 mg/kg)**	**CU (30 mg/kg)**	**CU (100 mg/kg)**	**CU (300 mg/kg)**
Heart (%)	0.23 ± 0.016	0.39 ± 0.015[a]	0.25 ± 0.0017[b]	0.23 ± 0.018[b]	0.26 ± 0.0019[b]	0.22 ± 0.013[b]
Kidney (%)	0.34 ± 0.024	0.37 ± 0.021	0.34 ± 0.015	0.35 ± 0.016[b]	0.34 ± 0.014	0.35 ± 0.019
Liver (%)	3.3 ± 0.16	3.4 ± 0.17	3.5 ± 0.18	3.4 ± 0.10	3.5 ± 0.12	3.3 ± 0.18

Statistical analyses were performed using one–way ANOVA followed by Tukey's test. Values are expressed as mean ± SEM (standard error of the mean). n = 7 rats per group. [a]p ≤ 0.05 when compared to naïve; [b]p ≤ 0.05 when compared to NC group; ESCU: hypertensive animals that received different doses of ethanol-soluble fraction from *Croton urucurana* leaves; HCTZ: hypertensive animals that were treated with hydrochlorothiazide (25 mg/kg); Naïve: normotensive animals treated with vehicle (water); NC: (negative control; hypertensive rats treated with vehicle).

Figure legends

Figure 1. Microscopy of *Croton urucurana* in cross-section (f, g, h, j, m) and longitudinal section (k, l): light microscopy; b-e, i, n: SEM. (b-e) Frontal view. (g-l). Midrib. (m, n) Petiole. (co, collenchyma; ct, cuticle; cr, bipyramidal square-shaped crystal; dr, druse; ep, epidermis; la, laticifers; ng, non-glandular trichome; ph, phloem; pp, palisade parenchyma; si, secretory idioblasts; sg, starch grains; sp, spongy parenchyma; st, stomata; vb, vascular bundle; xy, xylem). Scale bar: a = 5 cm; g, m = 500 µm; f = 200 µm; d = 100 µm; j, k, l = 50 µm; c, h = 25 µm; e, i, n = 10 µm; b = 5 µm. SEM: scanning electron microscopy.

Figure 2. **SEM image and EDS spectrum of a (a) bipyramidal-shaped crystal on the stomata, (b) druse in the petiole of *Croton urucurana.*** EDS: energy-dispersive X-ray spectroscopy; SEM: scanning electron microscopy.

Figure 3. Base peak chromatograms (BPC) obtained from negative (A) and positive ion modes (B) of the ethanol-soluble fraction from *Croton urucurana* leaves.

Figure 4. ESCU induce dose-dependently vasorelaxation in the mesenteric vascular beds (MVBs) from SHRs by nitric oxide pathway. (**A**) Effects of ESCU on perfusion pressure in isolated MVBs that were perfused with physiological saline solution (PSS) that contained 3 µM phenylephrine (Phe). The data are expressed as the mean ± SEM from six experiments. $^{a}p < 0.05$,

compared with perfusion pressure after PSS administration (control); $^{b}p < 0.05$, compared with previous dose. All of the experiments were performed in endothelium intact MVBs preparations. (**B**) Effects of ESCU on endothelium-intact (End+) and endothelium-denuded (End-) MVBs preparations. Endothelium-intact MVBs preparations that were continuously perfused with L-NAME (**C**) or indomethacin (**D**). The data are expressed as the mean ± SEM from six experiments. $^{a}p < 0.05$, compared with the effects of ESCU on endothelium-intact preparations (B) and the vehicle group (C and D). **ESCU:** ethanol-soluble fraction from *Croton urucurana* leaves; **SHRs**: spontaneously hypertensive rats.

Figure 5. Effect of potassium channel blockers on the vasodilatory effect of ESCU. The effects of ESCU were evaluated in MVBs that were perfused with PSS that contained 3 μM phenylephrine that was added of 40 mM KCl (**A**), tetraethylammonium (TEA) (**B**), glibenclamide (**C**), apamin (**D**), charybdotoxin (**E**), and iberiotoxin (**F**). The data are expressed as the mean ± SEM from six experiments. $^{a}p < 0.05$, compared with the effects of ESCU on the vehicle perfusion. All of the experiments were performed in endothelium intact MVBs. **ESCU:** ethanol-soluble fraction from *Croton urucurana* leaves; **MVBs**: mesenteric vascular beds.

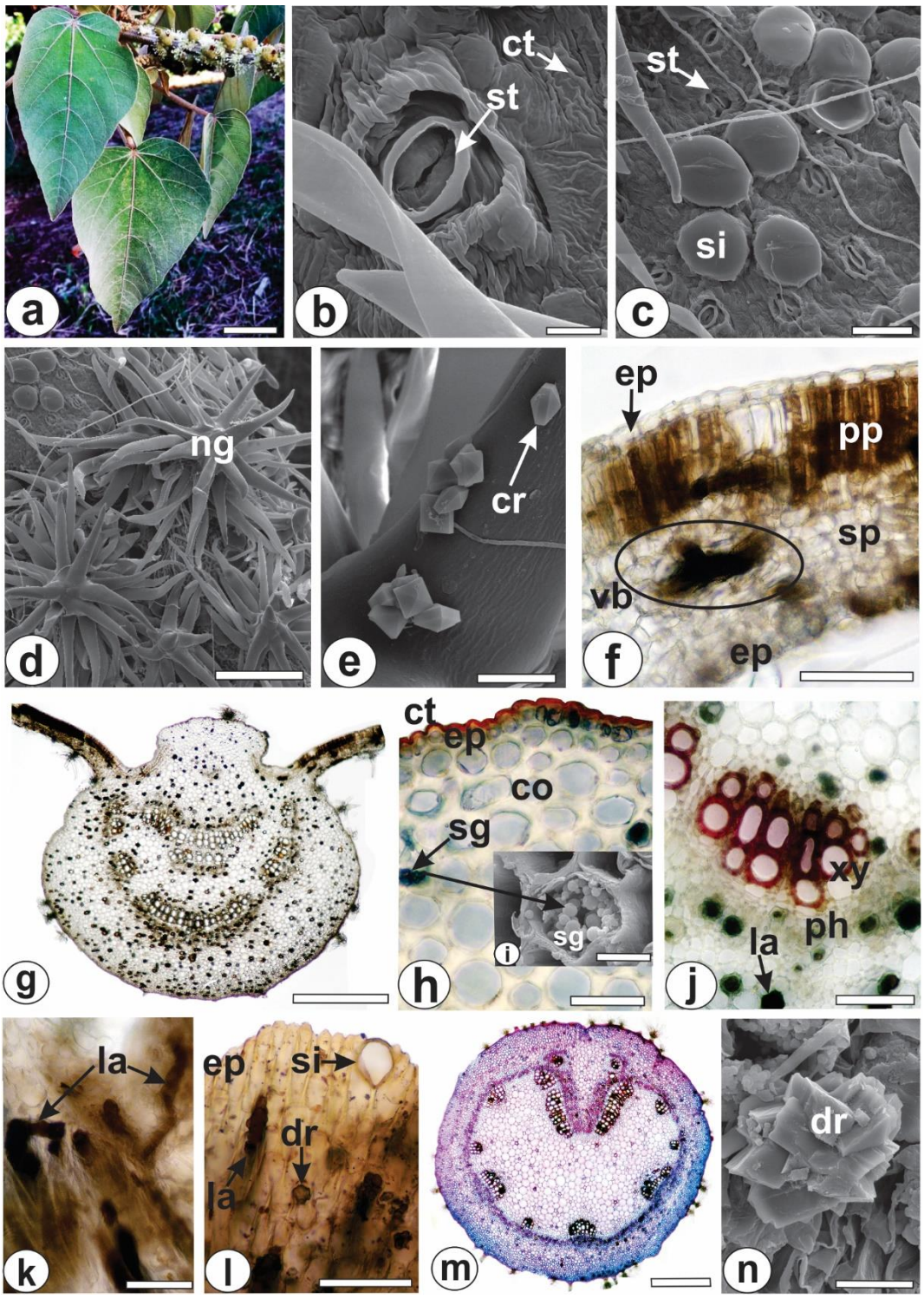

Figure 1

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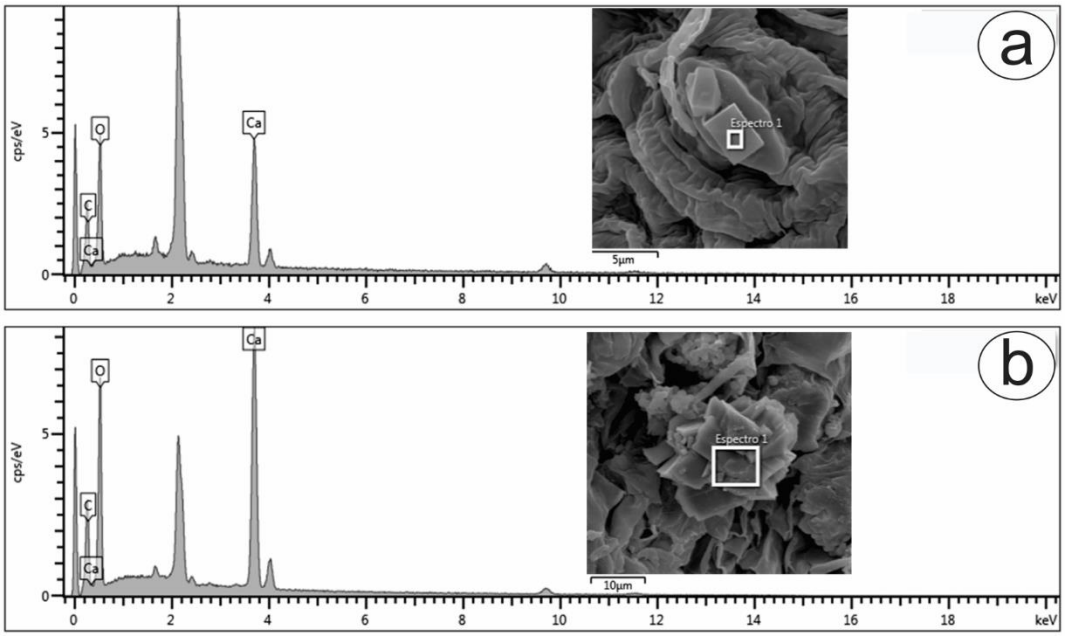

Figure 2

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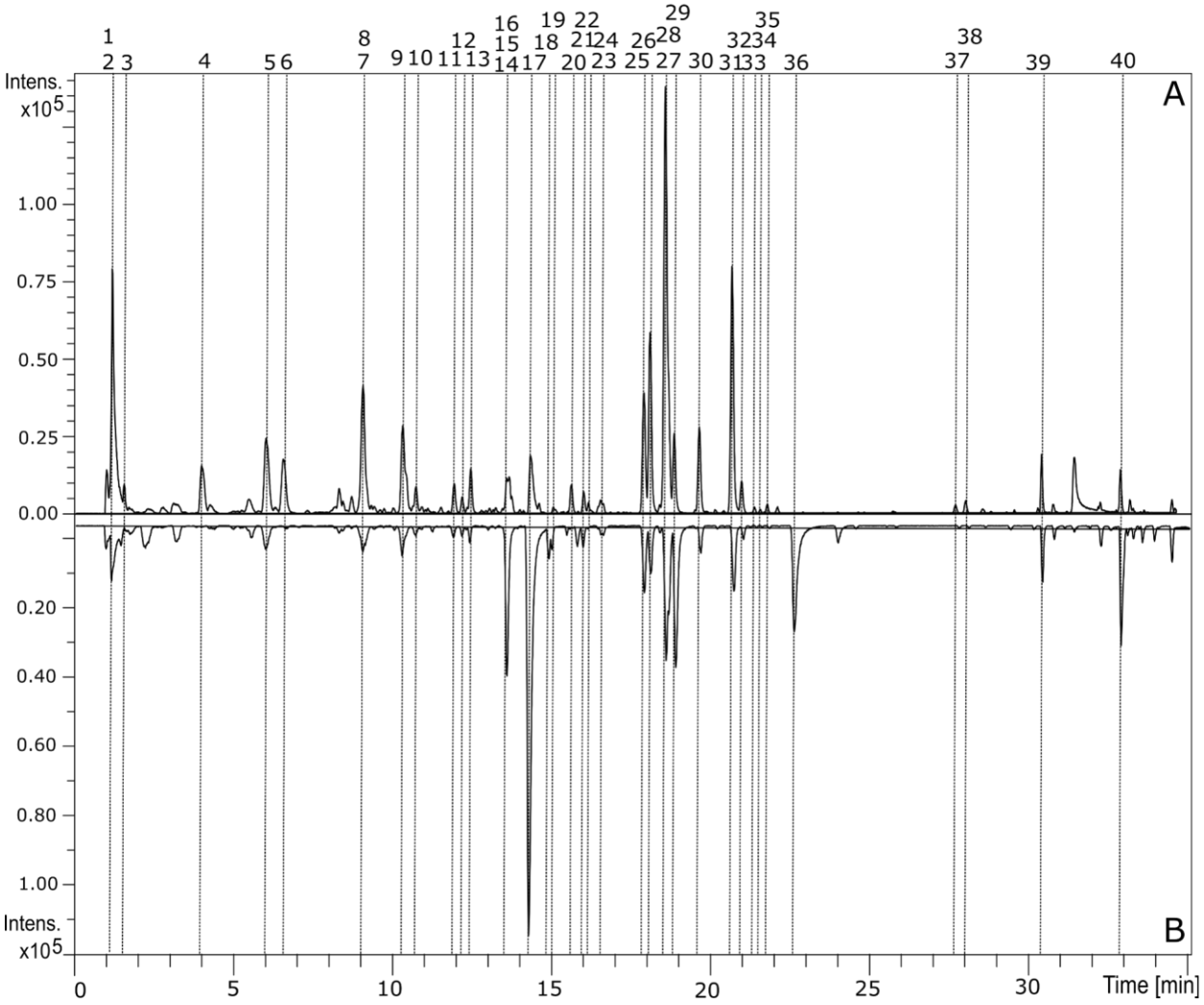

Figure 3

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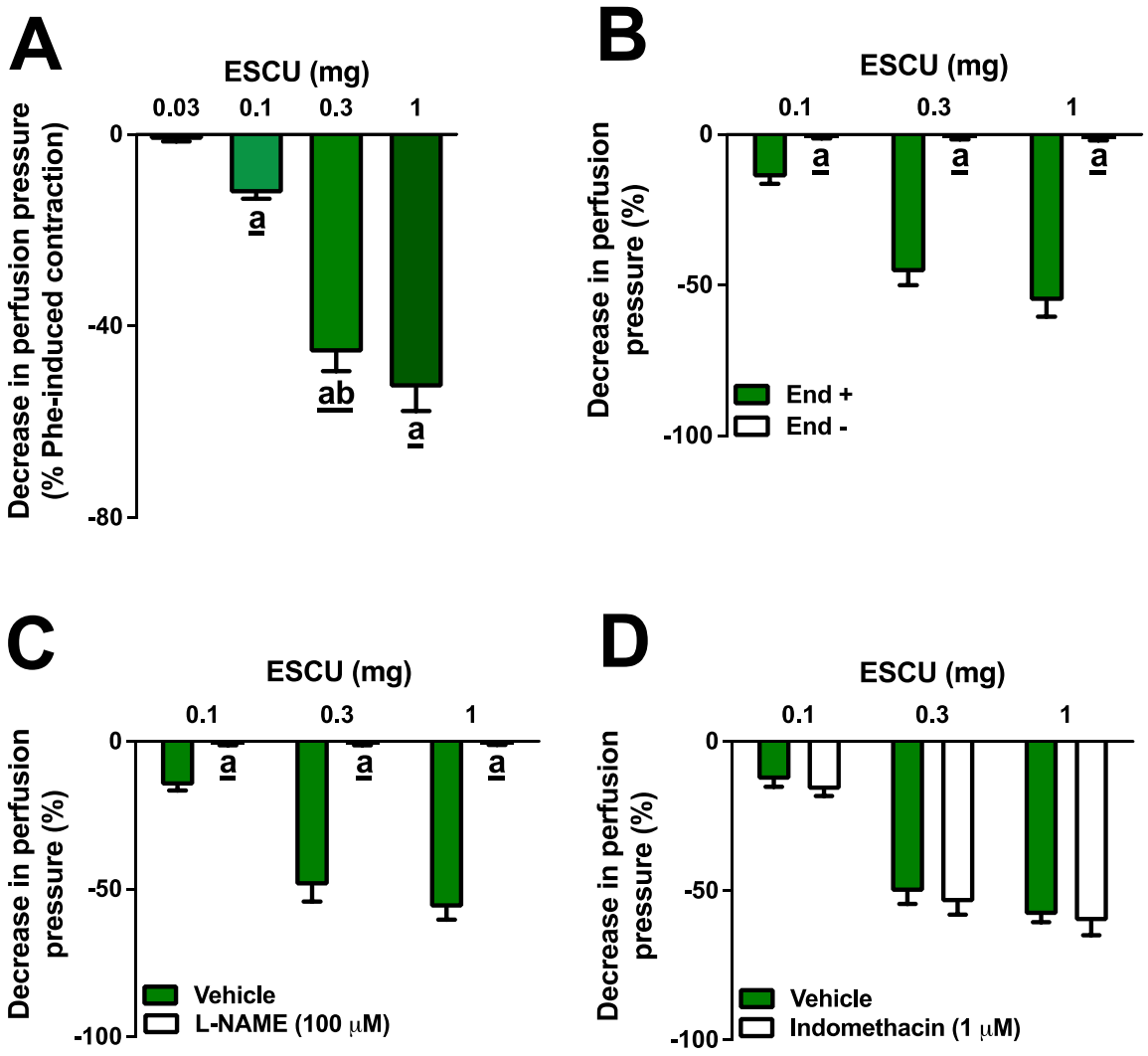

Figure 4

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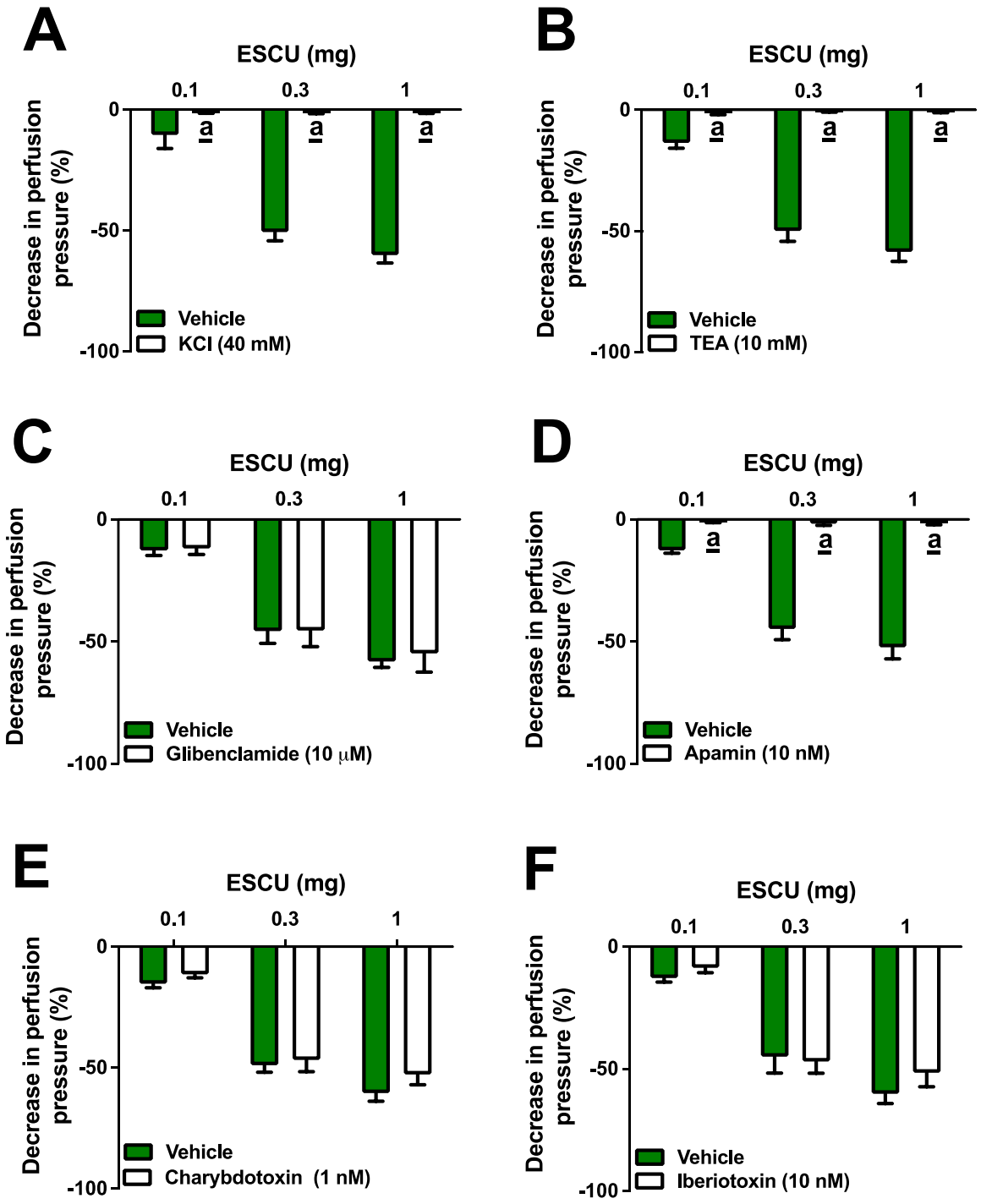

Figure 5

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6 CONCLUSÃO

O estudo demonstrou que o uso do ESCU não possui efeitos tóxicos, considerando sua DL_{50} inferior a 2000 mg/kg quando em tratamento a ratas Wistar. Também apontou significativos efeitos cardioprotetores no modelo animal de hipertensão estudado, onde houve uma regularização de parâmetros hemodinâmicos e renais, através de uma ação vasodilatadora dependente de NO sintetizado no endotélio vascular – dado corroborado pela disponibilidade de NO no organismo dos animais, observado por seu metabólito, nitrito. Há ainda a resposta dos canais de K^+ mediante a ação do ESCU. Bloqueadores destes canais inibiram os efeitos vasculares do extrato em artérias mesentéricas, sugerindo uma função sinérgica ao NO, uma vez que, fisiologicamente, estes canais também estão intrinsicamente intercalados à via do NO. O ESCU não demonstrou ação diurética, dado corroborado pelos valores de diurese dentro dos padrões comparativos aos grupos controle e naive.

Sendo assim, é possível afirmar que o extrato etanólico da *Croton urucurana* tem ação anti-hipertensiva mediada pela via do óxido nítrico.

7 ANEXOS

7.1 Aprovação do Comitê de Ética do Uso de Animais em pesquisa (CEUA)

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, 01 de Setembro de 2020.

CERTIFICADO

Certificamos que a proposta intitulada ***"Avaliação dos efeitos cardiovasculares e renais de nanopartículas lipídicas de curcumina em ratos espontaneamente hipertensos."***, registrada sob o protocolo de nº 31/2019, 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 nº 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 08/11/2019, passa a ser nomeado ***"Avaliação dos efeitos cardiovasculares e renais do extrato etanólico de C. urucurana e S. microglossa em ratos espontaneamente hipertensos"***, tendo como nº de protocolo **31/2019-1.**

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*01/06/2020 a 28/02/2022*
Espécie/linhagem/raça	*Rattus norvegicus – Wistar e SHR*
Nº de animais	60 – 50 SHR e 10 Wistar
Peso/idade	*90 dias – Wistar e 180 dias - SHR*
Sexo	5 fêmeas e 5 machos Wistar e 50 machos SHR
Origem	Biotério Central da UFGD

Melissa Negrão Sepulvida
Coordenadora CEUA

Comissão de Ética no Uso de Animais – CEUA/UFGD – Rua João Rosa Góes, 1761 – Vila Progresso.
Dourados/MS. E-mail: ceua@ufgd.edu.br

7.2 Comprovação de submissão de artigo para a revista Journal of Ethnopharmacology

De: "Journal of Ethnopharmacology" <em@editorialmanager.com>
Assunto: Confirming submission to Journal of Ethnopharmacology
Data: 31 de agosto de 2021 14:57:16 AMT
Para: "Arquimedes Gasparotto Junior" <arquimedesjunior@ufgd.edu.br>
Responder A: "Journal of Ethnopharmacology" <support@elsevier.com>

This is an automated message.

Nitric oxide and Ca2+ activated small conductance K+ channels mediate antihypertensive effects of Croton urucurana Baill. in spontaneously hypertensive rats

Dear Dr Gasparotto Junior,

We have received the above referenced manuscript you submitted to Journal of Ethnopharmacology.

To track the status of your manuscript, please log in as an author at https://www.editorialmanager.com/jethno/, and navigate to the "Submissions Being Processed" folder.

Thank you for submitting your work to this journal.

Kind regards,
Journal of Ethnopharmacology