UNIVERSIDADE FEDERAL DA GRANDE DOURADOS

FACULDADE DE CIÊNCIAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Efeitos cardio e renoprotetores induzidos pela *Cuphea carthagenensis* (Jacq.) J.F. Macbr. em ratas ovariectomizadas e com hipertensão renovascular

MAYSA ISERNHAGEN SCHAEDLER

Dourados – MS

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Área do CNPq: Etnofarmacologia (2.10.06.00-8)

Exame de Defesa apresentado ao Programa de Pós-Graduação em Ciências da Saúde da Faculdade de Ciências da Saúde da Universidade Federal da Grande Dourados (UFGD), para obtenção do título de Mestre em Ciências da Saúde

Área de concentração: Farmacologia

Orientador: Prof. Dr. Arquimedes Gasparotto Junior

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DEDICATÓRIA

Dedico este trabalho ao meu querido avô Walter Isernhagen por me ensinar a importância da gentileza para com o próximo e que devemos trabalhar com felicidade, ética e capricho.

AGRADECIMENTOS

Gostaria de agradecer primeiramente a Deus, pois sem ele nada disso seria possível.

A minha família que me deu forças e incentivo para continuar seguindo meus sonhos e objetivos, mesmo que isso levasse à distância entre nós. Por mostrar que sempre no final por mais difícil que seja tudo vai dar certo.

Ao Gustavo por me dar forças nas horas de desespero, entender meu mau humor e minhas crises de insegurança. Por corrigir meus erros gramaticais e por me acompanhar nos finais de semana de experimentos.

Ao meu orientador Prof. Dr. Arquimedes Gasparotto Junior por me aceitar em seu grupo de pesquisa, pelos ensinamentos e por ter me ajudado em tudo o que precisei. Muito obrigada.

Ao grupo de pesquisa do Laboratório de Farmacologia e Eletrofisiologia Cardiovascular, por me acolherem tão bem e aceitarem participar e me ajudar nessa jornada, sei que sem a ajuda e apoio de vocês tudo isso não seria possível.

Aos meus amigos Ana, Carol, Lucas, Matheus e Jhon, por me acompanharem durante toda a minha jornada acadêmica, fazendo com que todos os meus dias fossem mais leves e felizes.

A todos os meus amigos e todas as pessoas que contribuíram de alguma forma durante esta etapa da minha vida.

EPÍGRAFE

“Perguntaram ao matemático árabe Al-Khwarizmi sobre o ser humano e ele respondeu:

- Se tiver ética, ele é 1

-Se também for inteligente, acrescente 0 e será 10

-Se também for rico, acrescente mais um 0 e será 100

-Se também for belo, acrescente mais um 0 e será 1000

Mas... se perder o 1, que corresponde à ética, então perderá todo o seu valor e restarão apenas zeros.”

(Al-Khwarizmi)

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SÍMBOLOS

ANGII	Angiotensina II
AT_1R	Receptor da Angiotensina II
AVE	Acidente Vascular Encefálico
BH_4	Tetrahidrobiopterina
cGMP	Monofosfato Cíclico de Guanosina
DAP	Doença Arterial Periférica
DC	Doenças Cardiovasculares
DCNTs	Doenças Crônicas não Transmissíveis
DRC	Doença Renal Crônica
E1	Estrona
E2	Estradiol
E3	Estriol
EAR	Estenose da Artéria Renal
ECA	Enzima Conversora de Angiotensina
ENAL	Enalapril
eNOS	Óxido Nítrico Sintase Endotelial
EROs	Espécies Reativas de Oxigênio
ESCC	Ethanol Soluble Fraction of *Cuphea carthagenensis* (Jacq.) J.F. Macbr. ou Fração solúvel em etanol de *C. carthagenensis* (Jacq.) J.F. Macbr.
HARV	Hipertensão Arterial Renovascular
HAS	Hipertensão Arterial Sistêmica
IAM	Infarto Agudo do Miocárdio
IC	Insuficiência Cardíaca
NO	Óxido Nítrico
NOX	NADPH Oxidase
OMS	Organização Mundial da Saúde
OVT	Ovariectomia
PA	Pressão Arterial
1K1C	1-kidney-1-clip ou 1-Rim-1Clipe
2K1C	2-kidney-1-clip ou 2-Rins-1-Clipe
$O_2^{-\bullet}$	Superóxido
$ONOO^-$	Peroxinitrito

Efeitos cardio e renoprotetores induzidos pela *Cuphea carthagenensis* (Jacq.) J.F. Macbr. em ratas ovariectomizadas e com hipertensão renovascular

RESUMO

Devido a grande biodiversidade brasileira, o uso de plantas medicinais para o tratamento de diversas doenças é muito difundido, principalmente quando se trata de doenças crônicas como a hipertensão. Dentre estas plantas, a *Cuphea carthagenensis* (Jacq.) J.F. Macbr. é amplamente utilizada para o tratamento de doenças cardiovasculares, contudo não existem estudos que comprovem sua atividade anti-hipertensiva. Desta forma, este estudo propõe a elucidação da atividade cardio e renoprotetora de uma fração solúvel em etanol obtida da *C. carthagenensis* (ESCC) em ratas Wistar ovariectomizadas submetidas a um modelo de hipertensão renovascular. Quatro semanas após a cirurgia os animais foram divididos em seis grupos, sendo o grupo falsamente operado (SHAM), controle positivo (2K1C+OVT), enalapril (15 mg/kg) e os grupos ESCC (30, 100 e 300 mg/kg); todos por via oral, uma vez ao dia, por 28 dias. A atividade diurética foi determinada nos dias 1, 7, 14, 21 e 28, e no último dia de tratamento foram mensuradas a pressão arterial sistólica, diastólica, média e a frequência cardíaca. Amostras de coração, aorta e rim foram coletados para a avaliação do sistema antioxidante e os níveis séricos de creatinina, ureia, sódio, potássio, nitrosamina, nitrito, espécies reativas ao ácido tiobarbitúrico, aldosterona, vasopressina e a atividade da enzima conversora de angiotensina (ECA) foram também determinados. Além disso, foram avaliadas a reatividade vascular e mecanismos moleculares envolvidos com a vasodilatação induzida pelo ESCC em leito vascular mesentérico isolado. O tratamento prolongado com a *C. carthagenensis* acarretou diminuição da pressão arterial e da frequência cardíaca. Também foi capaz de recuperar a reatividade vascular em todas as doses utilizadas, o que provavelmente está relacionado ao sistema de defesa antioxidante e possível aumento da biodisponibilidade de óxido nítrico. Além disso, observou-se a ativação da via NO/GMPc e a abertura de canais de potássio no leito vascular mesentérico, indicando um potencial mecanismo para os efeitos cardiovasculares da fração solúvel ESCC.

Palavras-chave: Anti-hipertensivo. Antioxidante. Cardioprotetor. Diurético. renoprotetor.

Cardio and renoprotective effects induced by *Cuphea carthagenensis* (Jacq.) J.F. Macbr. in ovariectomized rats and with renovascular hypertension

ABSTRACT

Due to the great Brazilian biodiversity the use of medicinal plants for the treatment of various diseases is very widespread, especially when it comes to chronic diseases such as hypertension. Among these plants are *Cuphea carthagenensis* (Jacq.) J.F. Macbr. is widely used for the treatment of cardiovascular diseases, however there are no studies that prove its antihypertensive activity. Thus, this study proposes the elucidation of cardio and renoprotective activity of a soluble fraction in ethanol obtained from *C. carthagenensis* (ESCC) in ovariectomized Wistar rats submitted to a model of renovascular hypertension. Four weeks after surgery, the animals were divided into six groups: the SHAM group, the positive control group (2K1C + OVT), the enalapril group (15 mg / kg) and the ESCC groups (30, 100 and 300 mg / kg); all orally, once daily for 28 days. Diuretic activity was determined on days 1, 7, 14, 21 and 28, and on the last day of treatment, systolic, diastolic, mean and heart rate were measured. Liver, heart, aorta and kidney samples were collected for evaluation of the tissue antioxidant system, and serum levels of creatinine, urea, sodium, potassium, nitrosamine, nitrite, thiobarbituric acid reactive species, aldosterone, vasopressin and enzyme activity angiotensin converting enzyme (ACE) were also determined. In addition, vascular reactivity and molecular mechanisms involved with ESCC-induced vasodilation in the mesenteric vascular bed were also evaluated. Prolonged treatment with *C. carthagenensis* resulted in a decrease in blood pressure and heart rate. It was also able to recover vascular reactivity at all doses used, which is probably related to the antioxidant defense system and possible increase of NO bioavailability. In addition, the activation of the NO / cGMP pathway and the opening of potassium channels in the mesenteric vascular bed were observed, indicating a potential mechanism for the cardiovascular effects of the ESCC.

Keywords: Antihypertensive. Antioxidant. Cardioprotective. Diuretic. renoprotective.

SUMÁRIO

1. INTRODUÇÃO

Cerca de dois terços das mortes no mundo estão relacionados a doenças crônicas não transmissíveis (DCNTs) (OMS, 2015). Dentre elas está a hipertensão arterial sistêmica (HAS), um assassino silencioso que afeta 1,13 bilhões de pessoas ao redor do mundo e é um importante fator de risco para as doenças cardiovasculares (DC), incluindo o infarto agudo do miocárdio (IAM), o acidente vascular encefálico (AVE) e as doenças renais crônicas (DRC) (BROWN et al., 2003; OMS, 2013a).

Um em cada três adultos possuem HAS, a partir dos 50 anos ou mais esta proporção aumenta para uma em cada duas pessoas, em ambos os sexos (OMS, 2013a). Isto se mostra mais evidente dentre a população feminina, uma vez que durante o período reprodutivo a pressão arterial (PA) assim como o risco de desenvolver HAS é menor em comparação aos homens da mesma faixa etária. Contudo, no período pós-menopausa a porcentagem de HAS é maior em mulheres do que em homens, em decorrência da queda dos níveis de estrogênio, um importante cardioprotetor (MOZAFFARIAN et al., 2015; COLAFELLA et al., 2018).

Normalizar os níveis de PA é importante para reduzir a ocorrência prematura de infartos, AVE, DRC e para prevenir, a longo prazo, milhares de mortes ao redor do mundo (BIBBINS-DOMINGO et al., 2010; OMS, 2013a). Embora o tratamento da hipertensão seja teoricamente fácil, uma vez que os medicamentos são baratos e o diagnóstico simples (MALACHIAS et al., 2016), apenas cerca de 13% dos pacientes têm o controle adequado da doença (CHOW et al., 2013). Esta baixa taxa de adesão é devido ao curso assintomático da doença, falta de conhecimento sobre a patologia, relacionamento inadequado com o médico, longo período de tratamento, fatores demográficos como educação e idade, e ainda, a dificuldade no acesso ao sistema de saúde básico (OMS, 2003). Para superar estas dificuldades, a população faz uso de plantas medicinais ou fitoterápicos no tratamento da HAS, uma vez que esses produtos naturais são considerados “seguros”, acessíveis, e eventualmente de menor custo (PRANDO et al., 2015; WET et al., 2016).

Apesar das plantas medicinais serem amplamente utilizadas para o tratamento de DC, seus efeitos cardioprotetores prolongados ainda são pouco conhecidos e estudados. Uma planta medicinal extensivamente utilizada pela população no tratamento da HAS, dislipidemias e doenças circulatórias é a *Cuphea carthagenensis* J. F. Macbr. (Lythraceae), popularmente no Brasil conhecida como ‘sete-sangrias’. Essa espécie é encontrada no Brasil, Havaí e em outras ilhas do sul do pacífico (GRAHAM et al., 2006; VENDRUSCULO et al., 2006; BOLSON et al., 2015). Ensaios *in vitro* confirmaram a atividade antioxidante

(SCHULDT et al., 2004; BERGMEIER et al., 2014) e a inibição da enzima conversora de angiotensina (BRAGA et al., 2000). Além disso, estudos pré-clínicos relatam efeitos antiateroscleróticos e hipocolesterolêmicos (BIAVATTI et al., 2004; BARBOZA et al., 2016).

Mesmo com a ampla utilização desta espécie pela população e as evidências farmacológicas sobre seus efeitos cardiovasculares, a atividade reno e cardioprotetora da *C. carthagenensis* frente a um modelo de HAS associado a privação de estrogênio não foi cientificamente avaliada. Deste modo, investigamos os efeitos reno e cardioprotetores da *C. carthagenensis* em ratas ovariectomizadas e com hipertensão renovascular, a fim de simular grande parte da população de mulheres acima de 50 anos afetadas pela HAS.

2. REVISÃO DE LITERATURA

As DCNTs, tais como as DC, doenças respiratórias crônicas, cânceres e diabetes, são alguns dos principais problemas de saúde do século XXI. Cerca de 38 milhões de pessoas no mundo morrem a cada ano de DCNTs, e no Brasil, estima-se que estas sejam responsáveis por 74% do total de mortes (OMS, 2014a). Além disso, os gastos gerados decorrentes destas doenças se aproximam a U$ 7 trilhões em países de baixa e média renda (OMS, 2014b).

2.1 Hipertensão Arterial Sistêmica

Apesar da população em geral não a considerar uma ‘doença’ por ser assintomática, a HAS é um problema mundial que causa indiretamente 9,4 milhões de mortes por ano e afeta 32,5% dos indivíduos adultos e mais de 60% dos idosos no Brasil. Os fatores de risco que estão associados ao desenvolvimento da HAS são idade, sexo, etnia, obesidade, ingestão de sal e álcool, sedentarismo, fatores socioeconômicos e genéticos (OMS, 2013a; MALACHIAS et al., 2016).

Existem dois tipos de HAS, a primária ou essencial que corresponde aos casos em que não existe uma única causa identificável para o desenvolvimento da doença, e a secundária, que corresponde 5-10% dos pacientes hipertensos e possui uma causa identificável que pode ser categorizadas em vascular, renal, endócrina ou neural (BHAGANI et al., 2018).

A HAS é caracterizada pela PA elevada (≥ 140/90 mm Hg), ou seja, é uma condição na qual os vasos sanguíneos encontram-se sob pressão persistentemente elevada. Se esta condição não for controlada pode levar a uma série de eventos deletérios, começando pela alteração da reatividade vascular e, posteriormente, conduzindo ao aumento persistente da

resistência vascular periférica, com profundas alterações estruturais, mecânicas e funcionais das arteríolas (OPARIL et al., 2003). Desta forma, com o decorrer do tempo, o remodelamento vascular causa danos a órgãos alvos, acarretando diversas DC como o AVE, o IAM, a insuficiência cardíaca (IC), a doença arterial periférica (DAP) e a DRC (OMS, 2013a).

2.1.1 Hipertensão arterial renovascular

Dentre os pacientes com pressão arterial elevada, 5% destes correspondem à hipertensão arterial renovascular (HARV). Normalmente essa condição é ocasionada pela estenose total ou parcial, uni ou bilateral da artéria renal (EAR). Em 90% dos casos é acarretada pela aterosclerose, seguido pela displasia fibromuscular e pela arterite de Takayasu (MALACHIAS et al., 2016).

O desenvolvimento de HARV está ligada inicialmente à ativação do sistema renina-angiotensina-aldosterona (Figura.1). Devido à oclusão da artéria renal a pressão sanguínea nos rins passa a ser menor, ocasionando um aumento sustentado na liberação de renina pelas células justaglomerulares localizadas nos rins. A renina age enzimaticamente sobre o angiotensinogênio liberando angiotensina I que é convertida posteriormente em sua forma ativa, a angiotensina II (Ang II), pela enzima conversora de angiotensina (ECA). A Ang II exerce diversos mecanismos vasoconstritores como a estimulação na síntese de aldosterona, diminuição da excreção de água e sal, estimulação das vias adrenérgicas simpáticas, remodelação vascular, estimulação da produção de oxidantes e modificação da vasodilatação dependente das prostaglandinas, ocasionando assim o aumento da PA (OPARIL, 2003; GUYTON, 2006; STEPHEN, 2017). Existem dois modelos bem consolidados desenvolvidos por Goldblatt et al. (1934) que mimetizam a HARV. No modelo 2-rins-1-clipe (2K1C) a artéria renal é ocluída pela inserção de um clipe de prata, enquanto o rim contralateral é mantido, ao contrário do modelo 1-rim-1-clipe (1K1C) onde o rim contralateral é removido cirurgicamente.

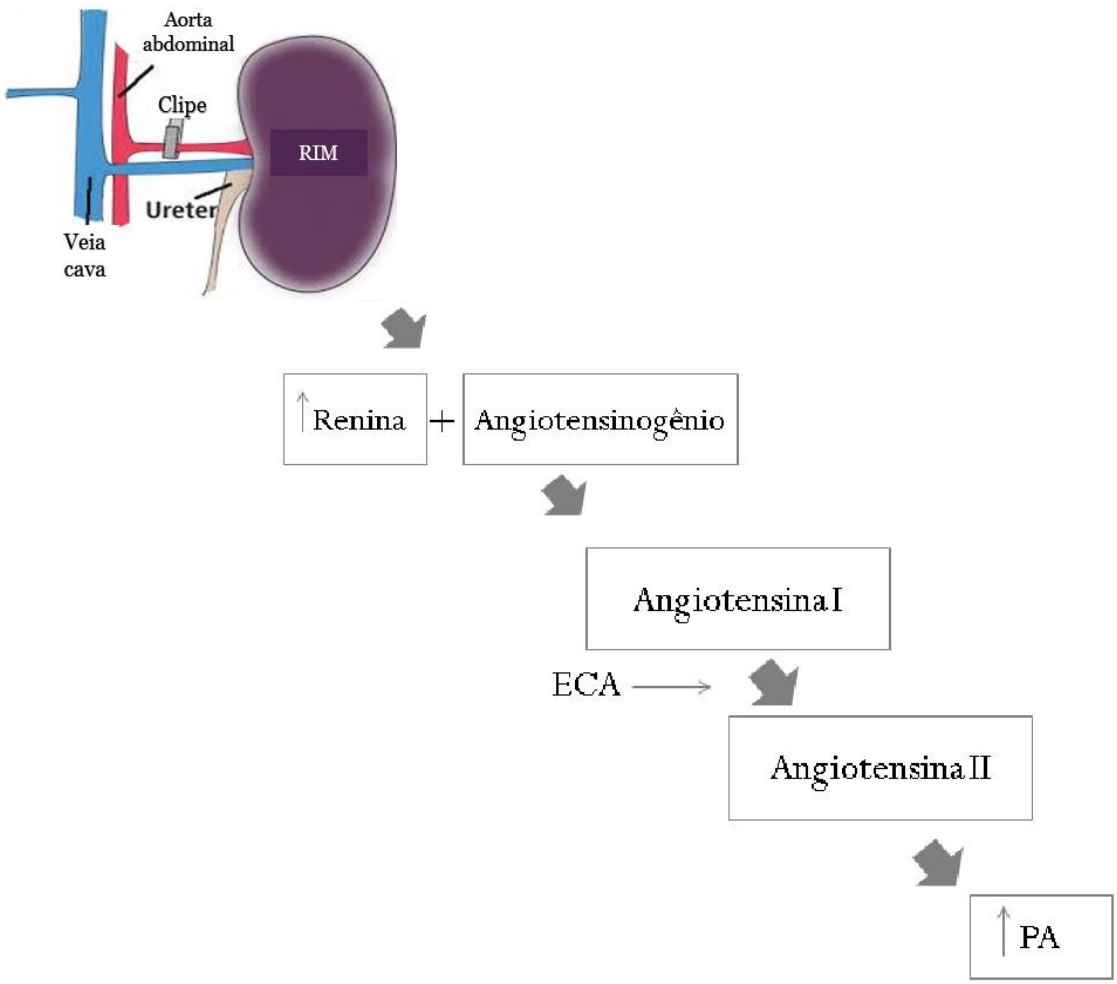

Figura 1. Sistema Renina-Angiotensina-Aldosterona (Fonte: Adaptado de GUYTON, 2006, p. 223).

2.1.2 Estresse oxidativo e HAS

Quando um átomo ou molécula possuem elétrons desemparelhados em sua última camada eletrônica tornam-se altamente reativos e são chamados de radicais livres. Estes quando derivados do metabolismo do oxigênio são chamados de espécies reativas de oxigênio (EROs) e desempenham importante papel biológico, incluindo a defesa contra agentes infecciosos, resposta mitogênica e manutenção das funções das células endoteliais e da musculatura lisa vascular (VALKO et al., 2007; CHEN et al., 2018).

Contudo, o estresse oxidativo causado pelo desequilíbrio entre os níveis de pró-oxidantes e antioxidantes é responsável pelo desenvolvimento e manutenção de diversas patologias, dentre elas a HAS (CHEN et al., 2018). A associação entre o desenvolvimento da HAS e o estresse oxidativo ainda não está totalmente elucidada, entretanto existem diversas fontes de EROs no sistema circulatório (Figura. 2). Dentre elas destacam-se a

xantina oxidase, a óxido nítrico sintase endotelial (eNOS) e principalmente a enzima NADPH oxidase (NOX) (WARD et al., 2006).

O aumento da produção de Ang II ocasionado pelo modelo de Goldblatt estimula a atividade da NOX, responsável pela produção do radical superóxido ($O_2^{-\bullet}$). Este reage com o óxido nítrico (NO) formando o peroxinitrito (ONOO−), um composto altamente reativo. O ONOO− oxida a tetrahidrobiopterina (BH_4), um importante cofator da eNOS, desta forma acarretando o desacoplamento desta enzima, que deixa de produzir NO para sintetizar mais $O_2^{-\bullet}$. Assim, ocorre a diminuição da biodisponibilidade do NO, um importante vasodilatador, além do aumento sustentado de EROs, o que por sua vez também ativa o fator de transcrição NF-kB, desencadeando uma resposta inflamatória local. De fato, isso conduz a uma importante disfunção endotelial seguida pelo aumento sustentado da resistência vascular periférica, e consequentemente, o aumento da PA (GONZÁLEZ et al., 2014; GARCÍA-REDONDO et al., 2016).

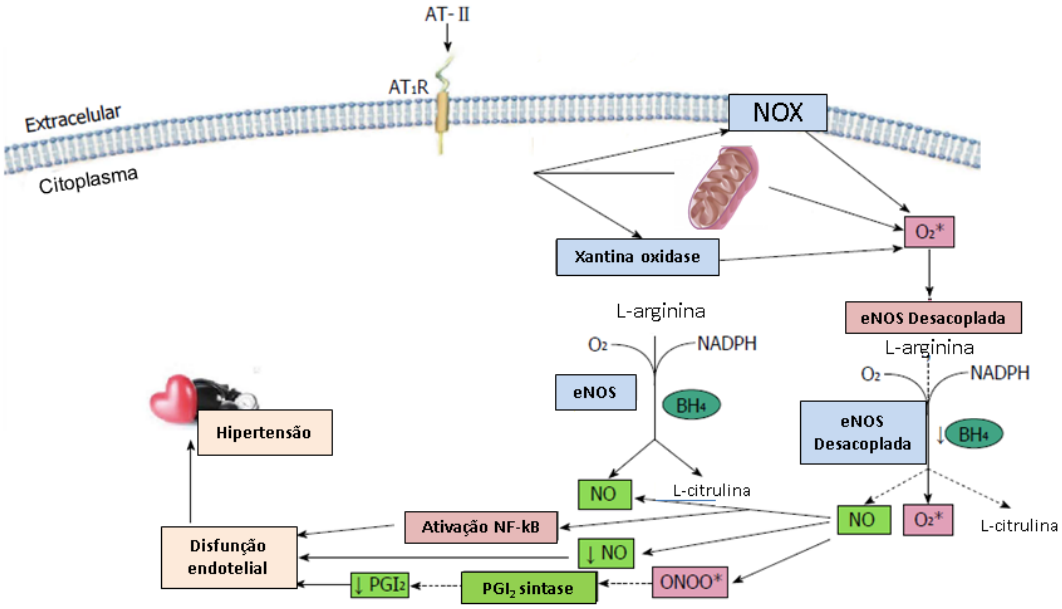

Figura 2. Resumo esquemático do papel do estresse oxidativo na patogênese da hipertensão. NOX: NADPH oxidase; NO: Óxido nítrico; BH_4: Tetrahidrobiopterina; $O_2^{-\bullet}$: Superóxido; ONOO−: Peroxinitrito; eNOS: Óxido nítrico sintase endotelial; PGI_2: Prostaglandinas; NF-κB: Fator nuclear kappa B (Fonte: Adaptado de GONZÁLEZ J. et al. 2014, p.357).

2.1.3 Mulheres e HAS

Durante o período reprodutivo, as mulheres possuem uma PA menor do que os homens, assim como uma menor prevalência de HAS. De fato neste período a proporção é de

36% em homens e 33% em mulheres. Contudo, a capacidade cardioprotetora é perdida no período pós-menopausa, onde a prevalência de HAS passa a ser de 73% e 81% em homens e mulheres, respectivamente (Figura 3). A causa das diferenças entre os gêneros no desenvolvimento de HAS é multifatorial, mas é atribuída principalmente ao estrogênio que sofre uma queda abrupta em sua produção no período pós-menopausa (COLAFELLA et al. 2018).

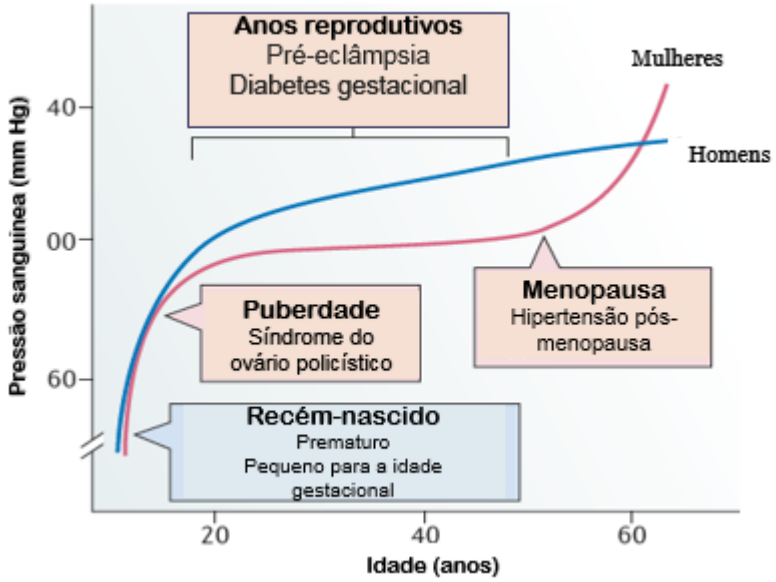

Figura 3. Mudanças na pressão sanguínea em ambos os sexos ao longo do tempo de vida (Fonte: Adaptado de Colafella et al., 2018).

Os estrogênios são produzidos principalmente nos ovários, corpos lúteos e placenta em mulheres na pré-menopausa. Contudo estes hormônios podem ser sintetizados em menores quantidades em outros órgãos como o fígado, coração, músculos, ossos e cérebro, tornando-se as principais formadores de estrogênio no período pós-menopausa. Existem três principais formas de estrogênio nas mulheres, sendo a estrona (E1), o estradiol (E2 ou 17β-estradiol) e o estriol (E3). Dentre estas, a forma mais ativa e produzida é a E2 (CUI; SHEN; LI, 2013).

Os receptores de estrogênio REα e REβ presentes em diversos tipos de células, desempenham um importante papel na proteção do sistema cardiovascular em mulheres. A sinalização dos REβ promove a vasodilatação por meio da regulação da biodisponibilidade do NO, alteração da permeabilidade iônica das células lisas vasculares e regulação do controle adrenérgico das artérias. Desta forma, ocorre um efeito protetor quanto às patologias

desencadeadas pela vasoconstrição excessiva. Além disso, o REβ tem propriedades angiogênicas e anti-inflamatórias, o que confere proteção extra contra os danos vasculares (MUKA et al. 2016).

2.2 Tratamentos disponíveis para a HAS

A HAS é um importante fator de risco para o desenvolvimento das DC, e por isso o tratamento adequado é imprescindível, e pode ser feito através do uso de medicamentos e/ou através de mudanças do estilo de vida. Dentre as formas de tratamento não medicamentoso incluem-se uma alimentação saudável, com baixo teor de sódio e gorduras saturadas, a prática de atividades físicas regulares, cessação do tabagismo, diminuição do consumo de álcool e o controle do peso corporal (OPARIL et al., 2015; MALACHIAS et al., 2016).

No Brasil, existem diversos fármacos disponíveis para o tratamento da HAS. Dentre os principais, incluem-se os diuréticos, os agentes de ação central, alfabloqueadores, betabloqueadores, vasodilatadores diretos, bloqueadores de canais de cálcio, bloqueadores dos receptores AT_1 da Ang II, inibidores da ECA e inibidores diretos de renina. Apesar de sua indiscutível eficácia, esses agentes podem acarretar diferentes efeitos adversos, incluindo taquicardia, fraqueza, disfunção erétil, tosse seca, broncoespasmo e distúrbios da condução atrioventricular (MALACHIAS et al., 2016).

Apesar da vasta gama de opções para o tratamento da HAS, de 24% a 34,5% dos pacientes não aderem às medicações indicadas. Isso ocorre, pelo menos em parte, devido ao longo curso assintomático da doença, alterações necessárias no estilo de vida, uso de diversas classes de medicamentos para controle adequado da PA, e o aparecimento de efeitos adversos (MIRANDA et al., 2002; OPARIL et al., 2015). Desta forma, a busca por novas alternativas para o tratamento de pacientes hipertensos é de suma importância, sobretudo visando ao aumento da adesão, da qualidade de vida e a diminuição dos desfechos desfavoráveis da HAS.

2.3 Polifenóis e HAS

Um importante passo para o controle adequado da HAS é o consumo de alimentos saudáveis, uma vez que são fontes de substâncias bioativas importantes, tais como os polifenóis, encontrados em muitas frutas, vegetais e plantas medicinais (GÓMEZ-GUZMÁN et al., 2018).

Dentre estes compostos, os flavonoides são os mais estudados quanto ao seu papel na proteção do sistema cardiovascular, pois possuem atividade antioxidante devido à eliminação

de EROs, através da doação de hidrogênio, ligação á íons de metal, dentre outras vias. Além disso, atuam na sinalização de moléculas por meio da ligação aos principais receptores celulares ou proteínas que estão envolvidas em cascatas de sinalização, resultando em respostas fisiológicas ou expressão gênica (HUGEL et al., 2016).

Dentre os flavonoides a quercetina merece destaque, pois diversos estudos pré-clínicos e clínicos apontam que a suplementação com este composto resulta em uma diminuição significativa da PA. Isto ocorre por múltiplas vias, como a inibição da ECA, melhora da função endotelial, ativação direta sobre o músculo liso vascular, efeito antioxidante e a modulação da sinalização celular e expressão gênica (PATEL et al. 2018).

A quercetina possui também importante papel na renoproteção, uma vez que regula a expressão de canais de Na^+ em células epiteliais renais, além disso, estimula o cotransportador 1 Na^+- K^+-$2Cl^-$ regulando assim a reabsorção renal de Na^+ e volume do líquido extracelular (VARGAS et al. 2018). Dessa forma, é importante a pesquisa com plantas que possua grandes quantidades destes compostos secundários para sua utilização no tratamento de pessoas com HAS.

2.4 Plantas medicinais

Achados arqueológicos evidenciam que o uso das plantas medicinais para o tratamento de diversas doenças ocorre desde a era pré-histórica, e a sua utilização perdura até os dias de hoje. Atualmente, estima-se que 30% dos medicamentos disponíveis são derivados de recursos naturais. Além disso, o mercado global de fitoterápicos alcança 20 bilhões de dólares anualmente (HALBERSTEIN, 2005; DUTRA et al., 2016).

Segundo a Organização Mundial da Saúde (OMS), 80% da população de países em desenvolvimento utilizam de práticas tradicionais na atenção primária, sendo que 85% destes fazem uso de plantas medicinais. Desta forma, foi fundado pela OMS em 1970 o Programa de Medicina Tradicional, que tem como objetivo estimular políticas de integração da medicina complementar e tradicional nos sistemas de atenção a saúde dos estados-membros (OMS, 2002; MINISTÉRIO DA SAÚDE, 2006).

Nesse contexto, o Brasil implementou a Política e Programa Nacional de Plantas Medicinais e Fitoterápicos, que tem como objetivo garantir à população brasileira o acesso seguro e o uso racional de plantas medicinais e fitoterápicos, promovendo a utilização sustentável da biodiversidade e o desenvolvimento da cadeia produtiva e da indústria nacional (MINISTÉRIO DA SAÚDE, 2006).

O Brasil merece destaque nas pesquisas de plantas medicinais, pois detêm de 15% a 20% da biodiversidade mundial. Além disso, o país conta também com grande diversidade cultural e étnica, que dispõe do conhecimento tradicional passado de geração em geração. Contudo, apenas 8% do total de espécies vegetais catalogadas (60 mil) foram estudadas para compostos bioativos e cerca de 1.100 foram avaliadas em suas propriedades medicinais, isto mostra a importância do estudo de novas plantas utilizadas popularmente para que o mercado de fitoterápicos se desenvolva cada vez mais no Brasil (GUERRA et al., 2001; MINISTÉRIO DA SAÚDE, 2006).

2.5 Cuphea carthagenensis (Jacq.) J. F. Macbr.

No contexto da importância do estudo de espécies medicinais brasileiras, uma planta herbácea merece destaque. Amplamente utilizada pela população brasileira, a *Cuphea carthagenensis* (Figura 4) pertencente à família Lythraceae, pode ser encontrada principalmente no leste e oeste da América do Sul, em áreas costeiras dos Estados Unidos e nas Ilhas do Havaí e do Pacífico Sul (GRAHAM et al., 2006) (Figura 5). Esta planta conhecida popularmente no Brasil como ‘sete-sangrias’ é utilizada para o tratamento de várias doenças do sistema gastrointestinal, cardiovascular e como diurético (VENDRUSCOLO et al., 2006; PRANDO et al., 2015).

Figura 4. *Cuphea carthagenensis* (Fonte: Horto de Plantas medicinais da Universidade Paranaense - UNIPAR).

Figura 5. Distribuição geográfica da *Cuphea carthagenensis* (Fonte: Gbif.org.br)

Estudos fitoquímicos demonstram que o extrato aquoso de *C. carthagenensis* possui altas concentrações de compostos fenólicos tais como, flavonoides, taninos e proantocianidinas. De fato, os compostos isolados mais abundantes são a quercetina-3-sulfato, quercetina-5-O-β-glicopiranosídeo e quercetina-3-O-β-arabinofuranoside (KREPSKY et al., 2012).

Diversos estudos pré-clínicos foram realizados utilizando a *C. carthagenensis* (Tabela 1). Ensaios *in vitro* realizados por Braga et al. (2000), Schuldt et al. (2004), Bergmeier et al. (2014), Andrighetti-Fröhner et al. (2005) e Campana et al. (2015) mostraram que esta espécie pode inibir a ECA, o TNF-α e possuir atividade antioxidante e antiviral. Schuldt et al. (2000) mostraram que a *C. carthagenensis* é capaz de proporcionar a vasodilatação em anéis de aorta em ratos por meio de dois mecanismos, dependente e independente de endotélio. Barboza et al. (2016) constataram importante atividade antiaterosclerótica da *C. carthagenensis* em coelhos Nova Zelândia submetidos a uma dieta rica em colesterol. Além disso, Biavatti et al. (2004) mostraram uma significante atividade hipocolesterolêmica em ratos normotensos, além de não haver quaisquer indícios de toxicidade após o tratamento oral por 90 dias.

Tabela 1. Pesquisas pré-clínicas realizadas com *C. carthagenensis*

Autores	**Parte da Planta**	**Teste**	**Tipo de extrato**	**Conclusão**

Braga et al. 2000	Folhas	*in vitro*	Etanólico	Inibição da enzima conversora de angiotensina I
Schuldt et al. 2000	Parte aérea	*in vitro*	Hidroalcoólico, butanólico e acetato de etila	Vasodilatação em anéis de aorta
Schuldt et al. 2004	Parte aérea	*in vitro*	Hidroalcoólico, butanólico e acetato de etila	Antioxidante
Biavatti et al. 2004	Folhas	*in vivo*	Aquoso	Hipocolesterolêmico
Andrighetti-Fröhner et al. 2005	Parte aérea	*in vitro*	Etanólico	Antiviral
Krepsky et al. 2012	Parte aérea	*in vitro*	Aquoso, etanólico e butanólico	Vasodilatação em anéis de aorta
Campana et al. 2015	Parte aérea	*in vitro*	Butanólico	Atividade anti TNF-α
Barboza et al. 2016	Parte aérea	*in vivo*	Aquoso	Ateroprotetor
Bergmeier et al. 2014	Parte aérea	*in vitro*	Etanólico e acetônico	Antioxidante

Apesar do uso muito difundido desta espécie pela população, e as evidências farmacológicas sobre seus efeitos cardiovasculares, a atividade reno e cardioprotetora da *C. carthagenensis* frente a um modelo de HAS associado a privação de estrogênio não foi cientificamente avaliada. Deste modo, avaliamos os efeitos reno e cardioprotetores da *C. carthagenensis* em ratas ovariectomizadas e com hipertensão renovascular para simular grande parte da população de mulheres acima de 50 anos afetadas pela HAS.

3. OBJETIVOS

GERAL

Investigar os efeitos reno e cardioprotetores do sobrenadante etanólico obtido de *Cuphea carthagenensis* em ratas ovariectomizadas e com hipertensão renovascular.

ESPECÍFICOS

- Produzir o extrato aquoso purificado da *C. carthagenensis* e determinar o perfil fitoquímico da espécie;
- Realizar a ovariectomia e induzir a hipertensão renovascular (2K1C) em ratas da linhagem Wistar;
- Avaliar a função renal de ratas ovariectomizadas com hipertensão renovascular tratadas por via oral com o veículo (água destilada), enalapril (15 mg/kg) e com o extrato aquoso purificado de *C. carthagenensis* nas doses de 30, 100 e 300 mg/kg por 28 dias;
- Determinar a atividade anti-hipertensiva do extrato aquoso purificado de *C. carthagenensis* após o tratamento prolongado;
- Investigar o sistema de defesa antioxidante tecidual e marcadores de estresse oxidativo sérico;
- Averiguar a reatividade vascular do leito mesentérico isolado de ratas ovariectomizadas e com hipertensão renovascular após o tratamento prolongado com o extrato aquoso purificado de *C. carthagenensis;*.
- Avaliar os efeitos vasodilatadores induzidos pelo extrato aquoso purificado de *C. carthagenensis* em leito mesentérico isolado e perfundido;
- Investigar os mecanismos moleculares envolvidos nos efeitos vasodilatadores do extrato aquoso purificado de *C. carthagenensis.*

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5. APÊNDICES

5.1 Artigo: Redox regulation and NO/cGMP plus K+ channels activation contributes to cardiorenal protection induced by *Cuphea carthagenensis* (Jacq.) J.F. Macbr. in ovariectomized hypertensive rats. (Qualis A2)

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Phytomedicine 51 (2018) 7–19

Contents lists available at ScienceDirect

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journal homepage: www.elsevier.com/locate/phymed

Original Article

Redox regulation and NO/cGMP plus K^+ channel activation contributes to cardiorenal protection induced by *Cuphea carthagenensis* (Jacq.) J.F. Macbr. in ovariectomized hypertensive rats

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ARTICLE INFO

Keywords:
Antihypertensive
Antioxidant
Cardioprotective
Diuretic
Renoprotective

ABSTRACT

Background: One of the medicinal plants widely used by the population in the treatment of hypertension, atherosclerosis and circulatory disorders is *Cuphea carthagenensis* (Jacq.) J.F. Macbr. (Lythraceae), popularly known as 'sete sangrias', being found in Brazil, Hawaii and in South Pacific Islands. Despite the widespread use of this species by the population, its long-term antihypertensive and cardioprotective activities have not yet been scientifically evaluated.

Purpose: To evaluate the possible cardioprotective effects of an ethanol-soluble fraction obtained from *C. carthagenensis* (ESCC) using ovariectomized hypertensive rats to simulate a broad part of the female population over 50 years of age affected by hypertension. In addition, the molecular mechanism that may be responsible for its cardiorenal protective effects was also explored.

Methods: Female Wistar rats were submitted to surgical procedures of bilateral ovariectomy and induction of renovascular hypertension (two-kidneys, one-clip model). The sham-operated group was used as negative control. ESCC was obtained and a detailed phytochemical investigation about its main secondary metabolites was performed. ESCC was orally administered at doses of 30, 100 and 300 mg/kg, daily, for 28 days, 5 weeks after surgery. Enalapril (15 mg/kg) was used as standard antihypertensive drug. Renal function was evaluated on days 1, 7, 14, 21 and 28. At the end of the experimental period, systolic, diastolic, mean arterial pressure and heart rate were recorded. The activity of the tissue enzymatic antioxidant system, thiobarbituric acid reactive substances, nitrotyrosine, nitrite, aldosterone and vasopressin levels, in addition to the activity of the angiotensin-converting enzyme were also evaluated. Additionally, vascular reactivity to acetylcholine, sodium nitroprusside, and phenylephrine, and the role of nitric oxide, prostaglandins, and K^+ channels in the vasodilator response of ESCC on the mesenteric vascular bed were also investigated.

Results: ESCC-treatment induced an important cardiorenal protective response, preserving renal function and

Abbreviations: 2K1C, two kidney, one clip; 4-AP, 4-aminopyridine; ACE, angiotensin converting enzyme; ACh, acetylcholine; ANOVA, analysis of variance; AP, arterial pressure; BW, body weight; Ca^{++}, calcium; CAT, catalase; cGMP, cyclic guanosine monophosphate; Cl^-, chloride; DBP, diastolic blood pressure; e-NOS, endothelial nitric oxide sintase; EDTA, ethylenediaminetetraacetic acid; EI, excretion load; ELISA, enzyme-linked immunosorbent assay; ENAL, enalapril; ESCC, ethanol soluble fraction from *Cuphea carthagenensis*; EtOH, ethanol; GLB, glibenclamide; GSH, reduced glutathione; GST, glutathione S-transferase; HR, heart rate; HR-MS, high-resolution mass spectrometry; i-NOS, inducible nitric oxide sintase; K^+, potassium; L-NAME, N(G)-nitro-L-arginine methyl ester; LC–MS, liquid chromatography–mass spectrometry; LPO, lipid peroxidation; MAP, mean arterial pressure; MeOH-H_2O, methanol-water; MVB, mesenteric vascular bed; Na^+, sodium; NO, nitric oxide; NT, nitrotyrosine; O_2^-, superoxide anion; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; OH^-, hydroxyl anion; $ONOO^-$, peroxynitrite; Phe, phenylephrine; PP, perfusion pressure; PSS, physiological saline solution; ROS, reactive oxygen species; SBP, systolic blood pressure; S.E.M, standard error of the mean; Sham, placebo surgery; SNP, sodium nitroprusside; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive species; TEA, tetraethylammonium; UHPLC, ultra-high performance liquid chromatography

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https://doi.org/10.1016/j.phymed.2018.05.011
Received 5 December 2017; Received in revised form 30 April 2018; Accepted 18 May 2018

preventing elevation of blood pressure and heart rate in ovariectomized hypertensive rats. In addition, prolonged treatment with ESCC recovered mesenteric vascular reactivity at all doses used. This effect was associated with an important modulation of the antioxidant defense system with a possible increase in NO bioavailability. Additionally, NO/cGMP activation and K^+ channel opening-dependent vasodilator effect was observed on the mesenteric vascular bed, indicating a potential mechanism for the cardiovascular effects of ESCC.
Conclusion: A 28-days ESCC treatment reduces the progression of the cardiorenal disease in ovariectomized hypertensive rats. These effects seem to be involved with an attenuation of oxidative and nitrosative stress, affecting endothelial nitric oxide production and K^+ channel opening in smooth muscle cells.

Introduction

Around two third of deaths in the world are consequence of non-communicable diseases (WHO, 2014). Hypertension is among them, a silent killer that affects 1.13 billion people worldwide and is an important risk factor for cardiovascular diseases like atherosclerosis, heart attack and stroke, as well as chronic kidney disease (Brown et al., 2003; WHO, 2013). One in three adults has hypertension, increasing to one in two individuals aged 50 years or more (Mozaffarian et al., 2015). If on the one hand, hypertension affects both genders, women are more affected after a pronounced drop in estrogen levels, a fact that usually occurs after menopause. The causes for the high-prevalence of hypertension in older postmenopausal women are not fully understood, although some data point to an increase in the activity of the renin-angiotensin system, especially an increase in plasma renin activity, a fact similar to that occurring in renovascular hypertension (Lima et al., 2012).

Although hypertension is theoretically easy to treat, since medications are inexpensive and diagnosis is simple, only about 13% of patients have adequate control of the disease (Chow et al., 2013). This low rate is due to asymptomatic course and lack of knowledge about the disease, relationship with physicians, prolonged duration of treatment, demography factors like education and age, and difficult access to the basic health system (WHO, 2003). To overcome these difficulties, many individuals use medicinal plants for the treatment of hypertension, since they consider them safe, accessible and inexpensive (Wet et al., 2016).

One of the medicinal plants widely used by the population in the treatment of hypertension, atherosclerosis and circulatory disorders is *Cuphea carthagenensis* (Jacq.) J.F. Macbr. (Lythraceae), popularly known as 'sete sangrias', being found in Brazil, Hawaii and in South Pacific Islands (Bolson et al., 2015; Vendruscolo et al., 2006). Among the main compounds, quercetin-3-phosphate, quercetin-5-O-β-glucopyranoside, quercetin-3-O-α-arabinofuranoside (Krepsky et al., 2010), triterpenes (González et al., 1994), tannins and proanthocyanidins stand out (Krepsky et al., 2012). *In vitro* assays confirmed antioxidant activities (Bergmeier et al., 2014; Prando et al., 2015; Schuldt et al., 2004) and angiotensin-converting enzyme inhibition (Braga et al., 2000). Furthermore, preclinical studies have reported vasodilatory, antiatherosclerotic and hypocholesterolemic effects (Barboza et al., 2016; Biavatti et al., 2004; Krepsky et al., 2012).

Despite the widespread use of this species by the population, its long term antihypertensive and cardioprotective activities have not yet been scientifically evaluated. Thus, the prolonged cardiorenal activities of *C. carthagenensis* in ovariectomized hypertensive rats was investigated to simulate a broad part of the female population over 50 years of age affected by hypertension. In addition, the molecular mechanism that may be responsible for its cardiorenal protective effects was also explored.

Material and methods

Drugs

The following drugs, salts and solutions were used: xylazine and ketamine hydrochloride (Syntec, São Paulo, SP, Brazil) and heparin (Hipolabor, Belo Horizonte, MG, Brazil). Acetylcholine chloride, phenylephrine, indomethacin, enalapril, Nω-Nitro-L-arginine methyl ester, tetraethylammonium, ODQ, 4-aminopyridine, glibenclamide, sodium nitroprusside, NaCl, KCl, $NaHCO_3$, $MgSO_4$, $CaCl_2$, KH_2PO_4, dextrose, ethylenediaminetetraacetic acid, 2′,7′-dichlorofluorescein-diacetate, 5,5′-dithiobis, bovine serum albumin, ethylenediaminetetraacetic acid, reduced glutathione, Tris–HCl, and xylenol orange were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were obtained in analytical grade.

Plant material and extract preparation

Cuphea carthagenensis leaves were collected from the botanical garden of the Paranaense University (UNIPAR, Umuarama, Brazil) at 430 m above sea level (S23°47′55–W53°18′48), during the summer of 2016. The plant was identified and voucher specimens were deposited at UNIPAR Herbarium under number 2401.

Extracts were obtained by infusion in a similar manner to that popularly used in Brazil (Bolson et al., 2015) and prepared according to Prando et al. (2015). For this, the plant material was air-dried in an oven at 37 °C for 5 days and then cut and pulverized. One liter of boiling water was poured for every 60 g of dried ground leaves; the container was sealed and extraction occurred until room temperature was reached (around 6 h). The infusion was treated with 3 volumes of ethanol, originating a precipitate and an ethanol-soluble fraction (ESCC) that yielded 10.34%. ESCC was lyophilized, maintained at −20 °C, and resuspended in filtered water to preclinical assays.

Phytochemical analysis – liquid chromatography–mass spectrometry

The liquid chromatography–mass spectrometry (LC–MS) analysis was carried out in an ultra-high performance liquid chromatograph (UHPLC, Acquity – Waters) coupled to a high-resolution mass spectrometer (HR-MS). The chromatographic separation was developed on a C18 HSS T3 column (Waters) with 100 × 2.1 mm and 1.7 m of particle, at 60 °C. The solvent was composed of 0.1% formic acid (v/v) in water (A) and acetonitrile (B), with a gradient increasing solvent B: 0–30% in 7 min, 30–80% in 12 min, then returning to initial condition in 15 min, with flow rate of 400 µl/min. The sample was prepared at 2 mg/ml in MeOH−H_2O (1:1, v/v) and the injection volume was 5 µl.

The mass spectrometry analysis was performed in electrospray ionization in LTQ-Orbitrap XL (Thermo Scientific), operating in the positive and negative ionization modes. The sample was introduced from LC eluate and dried by nitrogen flows in the sheath gas and auxiliary gas (at 50 and 15 arbitrary units, respectively), aided by a source temperature of 350 °C. Ionization energies were: for positive ions, spray at 4.5 kV, tube lens at 110 V and capillary at 40 V; and for negative ionization, spray at 3.2 kV, tube lens at −130 V and capillary at −25 V. The mass resolution was set at 15000 FWHM and the mass calibration (100–2000 *m/z*) was performed prior to analysis with Pierce™ LTQ ESI Positive Ion (Caffeine, MRFA, Ultramark-1621) and Negative Ion (SDS, sodium taurocholate, Ultramark-1621) calibration solutions (Thermo-Fischer). MS data was acquired in total ion current (TIC) and a data-dependent event was used to fragment the most

abundant ion from each peak using a normalized collision induced dissociation (CID) at 10.

Pharmacological studies

In vivo and ex vivo studies

Animals: Twelve-week-old female Wistar rats weighing 200–300 g were randomized and housed in plastic cages, with environmental enrichment, at 22 ± 2 °C under 12/12 h light dark cycle, 55 ± 10% humidity conditions, and *ad libitum* access to food and water. All procedures were previously approved by Institutional Ethics Committee of the Federal University of Grande Dourados (Brazil; protocol number 46/2016) and conducted in accordance with Guidelines for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Health Institute.

Ovariectomy and hypertension induction (Goldblatt model; two kidneys, one clip; 2K1C)

Initially, female rats were anesthetized by intraperitoneal route (ketamine 100 mg/kg plus xylazine 20 mg/kg). Then, animals were submitted to laparotomy and the two ovaries were gently removed. In the same procedure, the left renal artery was isolated and a silver clip with 1.5 mm lumen was placed to partially limit blood flow (Umar et al., 2010). In some animals (Sham-operated), the ovaries were not removed and the artery was not clipped. After the procedure, animals were hydrated with saline solution (2 ml/animal, subcutaneously, single administration), and received antiinflammatory (indomethacin, 2 mg/kg, orally, every 12 h, during 3 days) and antibiotic (enrofloxacin, 10 mg/kg, subcutaneously, single dose). Systolic blood pressure (SBP) was weekly measured (for 28 days) by the tail-cuff method. Only animals with SBP above 140 mmHg were used in experiments.

Experimental design

Four weeks after surgery, animals were randomized and divided into 6 groups (n = 8–10). Rats were treated once a day (by gavage), during 28 days, with vehicle (filtered water; positive control), enalapril (ENAL, 15 mg/kg, standard antihypertensive drug) or ESCC (30, 100 or 300 mg/kg). The Sham-operated group (negative control) was treated with vehicle only.

Diuretic activity

The diuretic activity was evaluated according to methods previously described by Gasparotto Junior et al. (2009). Weekly, immediately after treatments, rats were placed in metabolic cages and urine was collected for 8 h. Urinary volume was measured and expressed as ml/100 g of body weight. Urinary sodium (Na^+), potassium (K^+), chloride (Cl^-) and calcium (Ca^{+2}) levels were quantified in an ion selective meter (COBAS INTEGRA 400 plus; Roche®). pH was determined on fresh urine samples using digital pH meter (Q400MT; Quimis Instruments, Brazil). Density was estimated by handheld refractometer (NO107; Nova Instruments, Brazil). Excretion load (El) of Na^+, K^+, Cl^- and Ca^{+2} was obtained by multiplying the concentration of electrolytes (mEq/l) by the urinary flow (ml/min). Results are expressed as µEq/min/100 g.

Arterial pressure and heart rate evaluation

On the last day of treatments, rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) intramuscularly administered. Immediately, a *bolus* injection of heparin (15 IU) was subcutaneously applied. Then, the left carotid artery was isolated, cannulated and connected to a pressure transducer coupled to a PowerLab® recording system, and an application program (Chart, v 4 0.1; all from ADI Instruments; Castle Hill, Australia) recorded the systolic (SBP) and diastolic (DBP) blood pressure, mean arterial pressure (MAP) and heart rate (HR). After 15 min of stabilization, changes in arterial pressure (AP) and HR were recorded for 5 min.

Vascular reactivity

After AP and HR evaluation, and before euthanasia, mesenteric vascular beds (MVBs) were isolated and prepared for perfusion-according methods described by McGregor (1965). MVBs were placed in a water-jacketed organ bath and perfused (at 4 ml/min) with PSS (composition in mM: NaCl 119; KCl 4.7; $CaCl_2$ 2.4; $MgSO_4$ 1.2; $NaHCO_3$ 25.0; KH_2PO_4 1.2; dextrose 11.1; and EDTA 0.03) at 37 °C and gassed with 95% O_2/5% CO_2. Changes in the perfusion pressure (PP; mmHg) were detected by a pressure transducer coupled to a PowerLab® recording system, and an application program (Chart, v 4 0.1; all from ADI Instruments; Castle Hill, Australia). After equilibrium (30 min), its integrity was checked by a *bolus* injection of KCl (120 mmol). Then, a phenylephrine dose-response administration (Phe 3, 10, and 30 nmol; 50–100 µl) was performed. After a new equilibration period (30 min), MVBs were continuously perfused with PSS plus Phe (3 µM) to induce a prolonged increase in PP. Under these conditions, vascular reactivity to sodium nitroprusside (SNP; 3, 10 and 30 pmol; 50–100 µl) and acetylcholine (ACh 3, 10 and 30 pmol; 50–100 µl) was evaluated. An equilibration period (15 min) was allowed between each drug administration.

Serum biochemical parameters

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, Na^+ and K^+ levels were measured using automated biochemical analyzer (Roche® Cobas Integra 400 plus). Nitrotyrosine (NT), vasopressin, and aldosterone levels were measured by enzyme-linked immunosorbent assay (ELISA; BD Biosciences, CA, USA). Plasma nitrite concentrations were enzymatically determined by reducing nitrate according to methods described by Schmidt et al. (1989). 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 TBARS assay kit (Cayman Chemical, Ann Arbor, Michigan, USA).

Tecidual antioxidant system

After euthanasia (by suprapharmacological isoflurane dose), kidney, aorta, and heart samples were removed and homogenized in K^+ phosphate buffer (0.1 M, pH 6.5), in a 1:10 dilution. Part of the homogenate was used to determine reduced glutathione (GSH) levels and another part was centrifuged at 9000 *g* for 20 min at 4 °C. The enzyme superoxide dismutase (SOD) was analyzed by the method of pyrogallol oxidation (Gao et al., 1998). Lipid peroxidation (LPO) rate was measured by the FOX method according to protocol described by Jiang et al. (1992). The GSH concentration was measured based on a previously described technique (Sedlak and Lindsay, 1968), with few modifications. The results were expressed by the amount of protein in the homogenates, determined by a method described elsewhere (Bradford, 1976).

In vitro studies

Molecular mechanisms involved in the vascular effects of ESCC

MVBs and aortic rings from normotensive female rats (n = 5), without any previous treatment, were used in this study. In the first step, PSS containing Phe (3 µM) were continuously perfused in MVBS according to previous descriptions. After equilibrium (30 min), different preparations received *bolus* injections containing 0.0003, 0.001,

analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. The significance level was set at 95% ($p < 0.05$) and results were expressed as mean ± standard error of the mean (S.E.M.).

Results

Phytochemical characterization

In a previous work, we have identified several flavonol glycosides from *Cuphea carthagenensis*, which main compound was identified as quercetin glucuronide, then, monosaccharides were assessed by thin layer chromatography (Barboza et al., 2016; Prando et al., 2015). Similarly, the current extract showed the same compounds, mainly flavonol glycosides, composed of kaempferol, quercetin and myricetin as aglycones, identified from their negative regular/radical product-ions at *m/z* 285/284˙, 301/300˙ and 317/316˙, respectively. Monosaccharides were the same previously found and quercetin-3-sulfate was observed *m/z* 380.99245 $[M\text{-}H]^-$.

In our previous analysis, however, many of the low-abundant peaks were not identified. Now, these compounds were identified as hydrolysable tannins. Different from condensed tannins, hydrolysable tannins are usually composed of central core of a β-glucopyranosyl group attached by galloyl groups (gallotannins) or their oxidative linked products, ellagitannins. In *C. carthagenensis*, several hydrolysable tannins, including some isomers, have been found.

Compounds **1** and **2** appeared at *m/z* 633.072, with low-abundant fragments at *m/z* 481.06 and 463.05, consistent with loss of gallic acid residue, and the main product at *m/z* 300.99, consistent with corilagin-type ellagitannin (galloyl-hexahydroxydiphenoylglucoside) (Chen et al., 2015). Initially, compound **3** was not well defined, since it produced ions at *m/z* 681.056, 505.046, 329.034 and 153.022 with a constant difference among ions of 176.01 atomic mass units. The ion at *m/z* 153.022 is consistent with butyl-sulfate $[M\text{-}H]^-$ and the other ions are equivalent to sodium butyl-sulfate clusters (neutral mass of 176.012 Da), yielding ions at *m/z* 329.034 $[M_2 + Na–H]^-$, *m/z* 505.046 $[M_3 + Na_2–H_2]^-$ and *m/z* 681.056 $[M_4 + Na_3–H_3]^-$. The isotopic mass distribution corroborates this statement, since the M + 2 isotope has mass and intensity consistent with the presence of sulfur atom [*e.g.* *m/z* 153.0223 ($C_4H_9O_4S^-$ monoisotopic mass, 100%), *m/z* 154.0264 (M + 1, ~4.5%), *m/z* 154.0177 ($C_4H_9O_4{}^{34}S^-$, M + 2, ~4.4%)].

Compound **4**, at *m/z* 785.083 was tentative identified as digalloyl-hexahydroxydiphenoylglucoside, producing fragments at *m/z* 633.07 and *m/z* 615.06 due to the loss of gallic acid residue. A product-ion at *m/z* 483.07 was interpreted as from a digalloyl-glucose fragment and that at *m/z* 300.99 as the ellagic acid residue. Compound **5** appeared at *m/z* 483.077 and fragments at *m/z* 331.06 and 313.05 were tentative identified as digalloy-glucose. Compound **6** was similar to compound **4**, tentative identified as a digalloyl-hexahydroxydiphenoylglucoside isomer. Compound **7** was observed at *m/z* 635.087, with fragments at *m/z* 483.07, 465.06, 313.05, consistent with trigalloyl-glucoside.

Compound **8** gave rise to ion at *m/z* 387.165 with fragments at *m/z* 369.15, 341.10, 249.06, 225.11, 207.10 and 163.11. Similar fragmentation profile was observed for tuberonic (12-OH jasmonic) acid-glucoside (Quirantes-Pine et al., 2010). Compound **9** was observed at *m/z* 385.186 (lower ion), 421.163 and 431.191 (main ion), being consistent with $[M\text{-}H]^-$, $[M + Cl]^-$ and $[M + HCOO]^-$, respectively. This compound did not produce fragments enough to be an identified tentative. Compound **10**, at *m/z* 479.082 and fragment at *m/z* 316.02 was identified as myricetin-glucoside, as previously described (Barboza et al., 2016).

The main compounds observed in the chromatogram were tentative identified as quercetin-5-*O*-β-glucopyranoside (**11**), quercetin-glucoronide (**14**), quercetin-3-*O*-β-glucopyranoside (**15**), quercetin-3-O-arabinofuranoside (**18**), kaempferol-rutinoside (**19**), kaempferol-glucoside (**20**) and quercetin (**22**) based on their mass spectra profile (Fig. 1), and previous reports (Krepski et al., 2012; Souza et al., 2008, 2009). Compound **23** gave ion at *m/z* 533.129 with main fragment at *m/z* 300.027, consistent with the radical ion from quercetin. Compounds with similar *m/z* value were obtained from *Oenothera cheiranthifolia* (Nakanishi et al., 2007) and *Gaultheria procumbens* (Michel et al., 2014), being identified as quercetin-glucuronide *n*-butyl ester. The overall phytochemical analysis is summarized in Table 1.

Table 1
Phytochemical analysis of ESCC obtained from *Cuphea carthagenensis* (LC-MS).

Peak	Rt	$MS^1(-)$	$MS^2(-)$	Tentative identification
1	1.96	633.0724	481.06 463.05, 300.99	Galloyl-hexahydroxydiphenoylglucoside
2	2.48	633.0722	481.06 463.05, 300.99	Galloyl-hexahydroxydiphenoylglucoside
3	3.30		681.0560, 505.0461, 329.034, 153 0.022	Butyl sulfate
4	4.12	785.0830	633.07, 615.06, 483.07, 300.99	Digalloyl-hexahydroxydiphenoylglucoside
5	4.30	483.0770	331.06, 313.05	Digalloyl-glucose
6	5.04	785.0822	633.07, 615.06, 483.07, 300.99	Digalloyl-hexahydroxydiphenoylglucoside
7	5.51	635.0877	483.07, 465.06, 313.05	Trigalloyl-glucose
8	5.60	387.1654	369.15, 341.10, 207.10, 163.12	Tuberonic acid glycoside
9	5.91		431.192, 421.163, 385.186	n.i.
10	6.52	479.0825	316.022,	Myricetin-glucoside
11	7.04	463.0882	301.035	Quercetin-5-O-β-glucopyranoside
12	7.15	615.0981	313.056, 301.035/300.027	Quercetin-galloylhexoside
13	7.37	609.1457	301.035/300.027	Rutin
14	7.46	477.0677	301.035	Quercetin-glucoronide
15	7.54	463.0882	301.035/300.027	Quercetin-3-O-β-glucopyranoside
16	7.73	380.9920	301.035	Quercetin-3-sulfate
17	7.88	551.2704	–	n.i.
18	8.15	433.0782	300.027	Quecertin-3-O-arabinofuranoside
19	8.22	593.1509	284.032	Kaempferol-rutinoside
20	8.41	447.0931	284.032	Kaempferol-glucoside
21	9.15	519.1865	357.133	n.i.
22	9.63	301.0353	273.03, 179.00, 151.00, 107.01	Quercetin
23	9.94	533.1297	301.035, 300.027	Quercetin 3-O-n-butyl-glucuronide
24	10.39	327.2177	–	n.i.
25	10.64	329.2333	–	n.i.

n.i. – not identified.

ESCC induces an important renoprotective effect in ovariectomized-hypertensive rats

On the 1st day of treatment, all hypertensive and ovariectomized animals, regardless of treatment, presented a significant reduction in urinary volume when compared to the Sham-operated group (Table 2). Similarly, animals in the positive control group also had a significant reduction in renal excretion of Cl^-. On the other hand, while animals treated with ENAL showed a significant reduction in urinary excretion of Na^+, Cl^- and Ca^{+2}, female rats that received ESCC, especially at the highest dose (300 mg/kg), showed a significant increase in renal elimination of Na^+, Cl^- and Ca^{+2}, with values similar to those obtained for the Sham-operated group (Table 2).

The effects of oral administration of ESCC on urinary volume and electrolyte excretion, pH and density on the 7th day of treatment are presented in Table 3. At this time, animals in the positive control, ESCC or ENAL groups had a significant reduction in urinary volume when compared to the sham-operated group. In addition, positive control animals also showed a significant reduction in renal elimination of Na^+ and Cl^-. Similarly, animals treated with ENAL showed an important reduction in the renal elimination of Na^+, Cl^- and K^+. On the other hand, animals treated with ESCC at doses of 30 and 300 mg/kg showed a significant increase in renal Na^+ elimination, and only at the dose of 300 mg/kg, in the Cl^-excretion, with values similar to the Sham-operated group.

On the 14th day of treatment, positive control animals showed a significant reduction in urinary volume and renal excretion of Na^+ and Cl^-. Interestingly, all animals treated with ESCC (30, 100 or 300 mg/kg) or ENAL did not present significant differences in the urinary volume or renal excretion of Na^+, Cl^- and K^+ when compared to the Sham-operated group. On the other hand, animals that received ENAL showed a significant reduction in the Ca^{2+} elimination when compared to the positive control or Sham-operated group (Table 4).

The effects of oral administration of ESCC on urinary volume and electrolyte excretion, pH and density on the 21st day of treatment are presented in Table 5. Rats in the positive control group continued to present a significant reduction of urinary volume and renal excretion of Na^+ and Cl^-. Similarly, animals receiving ENAL showed a significant reduction in renal Na^+ excretion and a significant increase in urinary density. On the other hand, ovariectomized hypertensive rats treated with ESCC (300 mg/kg) presented a significant increase in renal Na^+, Cl^- and K^+ elimination, whereas animals treated with doses of 30 and 100 mg/kg had their renal function preserved, with effects similar to those obtained with Sham-operated animals.

On the 28th day of treatment, all ovariectomized hypertensive rats that received no treatment had a significant reduction in urinary volume and Na^+, Cl^- and Ca^{2+} excretion when compared to Sham-operated rats (Table 6). Similar to what was observed in previous weeks, ENAL-treated rats also showed a significant reduction in urinary volume and renal elimination of Na^+, Cl^-, K^+ and Ca^{2+}. On the other hand, all animals treated with ESCC had urinary volume similar to the Sham-operated control group. In addition, urinary Na^+ and K^+ excretion remained significantly increased in animals treated with ESCC (300 mg/kg) when compared to animals in the positive control group (Table 6). Additionally, none of the experimental groups had a significant change in serum Na^+, K + , or creatinine levels. However, only animals treated with ENAL showed an expressive increase in serum urea levels when compared to Sham-operated or positive control rats (Table 7).

ESCC prevent SBP, DBP, MAP, and HR rise in 2K1C-ovariectomized rats

The values obtained for blood pressures levels and heart rate for Sham-operated and 2K1C-ovariectomized rats treated with vehicle, ENAL or ESCC (30, 100, and 300 mg/kg) are shown in Fig. 2A–D. SBP, DBP, MAP, and HR of Sham-operated animals were 108 ± 7.8, 70 ± 6.8, 83 ± 6.5 mmHg, and 197 ± 21 bpm, respectively. All animals in the positive control group presented a significant increase in blood pressure and heart rate, with values of 145 ± 4.1, 92 ± 4.2, 110 ± 5.8 mmHg, and 299 ± 19 bpm for SBP, DBP, MAP, and HR, respectively. Prolonged oral treatments with ESCC (at all doses) were able to prevent the significant increase in blood pressure and heart rate observed in animals of the positive control group. In addition, ESCC maintained hemodynamic values close to those obtained with Sham-operated rats. Only animals treated with ENAL had an additional reduction in blood pressure levels even when compared to the Sham-operated group.

Prolonged treatment with ESCC does not affect plasma ACE activity or serum aldosterone and vasopressin levels

None of the ovariectomized hypertensive rats treated for 28 days with ESCC showed any change in plasma ACE activity or serum aldosterone and vasopressin levels. As expected, animals treated with ENAL showed a significant reduction in ACE activity and in serum aldosterone levels, without affecting serum vasopressin levels (Supplementary material).

ESCC treatment reduces lipid peroxidation and nitrosative stress and increases nitrate levels in 2K1C-ovariectomized rats

Animals in the positive control group had an expressive increase in serum NT and TBARS concentration (~80%) when compared with Sham-operated rats (Fig. 3B and C). In addition, a significant reduction (~40%) in nitrite concentration, an indirect marker of the nitric oxide bioavailability, was also observed (Fig. 3A). ESCC treatment was able to increase nitrite levels to values close to those observed in the Sham-operated group (Sham-operated: 98 ± 8.6 µM; ESCC 30: 84 ± 8.7 µM; ESCC 100: 92 ± 8.6 µM; and ESCC 300: 108 ± 7.3 µM) (Fig. 3A). Similarly, serum TBARS and NT levels were also significantly reduced

Table 2
Effects of oral administration of ESCC obtained from *Cuphea carthagenensis* on urinary volume and electrolyte excretion, pH and density on 1st day of treatment.

Group	Urinary Volume (ml/100 g/8 h)	El_{Na^+} (µEq/min/100 g)	El_{k^+} (µEq/min/100 g)	$El_{Ca^{++}}$ (µEq/min/100 g)	El_{Cl^-} (µEq/min/100 g)	pH	Density
Sham	9.50 ± 0.47	1.034 ± 0.082	0.345 ± 0.037	0.028 ± 0.003	1.221 ± 0.104	6.66 ± 0.15	1009 ± 0.84
C^+	6.22 ± 0.51[b]	1.034 ± 0.049	0.323 ± 0.019	0.017 ± 0.001	0.870 ± 0.039[b]	6.82 ± 0.09	1010 ± 1.05
ESCC (30 mg/kg)	6.02 ± 0.39[b]	1.079 ± 0.029	0.344 ± 0.027	0.028 ± 0.001	1.050 ± 0.033	6.69 ± 0.12	1011 ± 0.66
ESCC (100 mg/kg)	6.24 ± 0.75[b]	1.019 ± 0.049	0.316 ± 0.021	0.029 ± 0.003	1.054 ± 0.038	6.74 ± 0.14	1011 ± 1.76
ESCC (300 mg/kg)	6.65 ± 0.26[b]	1.167 ± 0.071[a]	0.396 ± 0.029	0.035 ± 0.007[a]	1.167 ± 0.061[a]	6.92 ± 0.12	1011 ± 0.33
ENAL	5.40 ± 0.79[b]	0.519 ± 0.081[ab]	0.239 ± 0.028	0.009 ± 0.002[b]	0.539 ± 0.069[ab]	6.64 ± 0.18	1010 ± 0.92

Values are expressed as mean ± S. E. M. of 8–10 rats in each group in comparison with the positive control (C^+; [a]$p < 0.05$) or Sham-operated group ([b]$p < 0.05$) using one-way ANOVA followed by Dunnett's test. El: Excreted load; ENAL: enalapril.

Table 3
Effects of oral administration of ESCC obtained from *Cuphea carthagenensis* on urinary volume and electrolyte excretion, pH and density on 7th day of treatment.

Group	Urinary Volume (ml/100 g/8 h)	El_{Na^+} (μEq/min/100 g)	El_{k^+} (μEq/min/100 g)	$El_{Ca^{++}}$ (μEq/min/100 g)	El_{Cl^-} (μEq/min/100 g)	pH	Density
Sham	8.13 ± 0.34	1.23 ± 0.04	0.32 ± 0.03	0.02 ± 0.002	1.34 ± 0.06	6.60 ± 0.16	1009 ± 0.52
C^+	6.19 ± 0.40[b]	0.91 ± 0.06[b]	0.35 ± 0.05	0.02 ± 0.006	1.06 ± 0.09[b]	6.63 ± 0.13	1010 ± 0.70
ESCC (30 mg/kg)	5.71 ± 0.45[b]	1.23 ± 0.07[a]	0.28 ± 0.01	0.02 ± 0.002	1.17 ± 0.07	6.58 ± 0.12	1011 ± 0.72
ESCC (100 mg/kg)	5.33 ± 0.42[b]	1.06 ± 0.03	0.25 ± 0.00	0.02 ± 0.003	1.13 ± 0.03	6.43 ± 0.18	1011 ± 0.52
ESCC (300 mg/kg)	6.23 ± 0.32[b]	1.43 ± 0.07[a]	0.40 ± 0.02	0.03 ± 0.003	1.48 ± 0.08[a]	6.25 ± 0.19	1011 ± 0.47
ENAL	5.42 ± 0.49[b]	0.62 ± 0.05[ab]	0.21 ± 0.02[a]	0.01 ± 0.004	0.65 ± 0.06[ab]	6.54 ± 0.13	1010 ± 0.70

Values are expressed as mean ± S. E. M. of 8–10 rats in each group in comparison with the positive control (C^+; [a]$p < 0.05$) or Sham-operated group ([b]$p < 0.05$) using one-way ANOVA followed by Dunnett's test. El: Excreted load; ENAL: enalapril.

(Fig. 3B and C). In fact, prolonged treatment with the highest ESCC dose (300 mg/kg) was able to reduce TBARS levels to values statistically lower than those observed in Sham-operated animals (Sham operated: 7.1 ± 0.9 mmol/l; ESCC 300: 3.8 ± 0.5 mmol/l).

ESCC modulates the tissue antioxidant defense system in ovariectomized hypertensive rats

The results obtained from tissue antioxidant defense system evaluation are presented in Table 8. Ovariectomy plus hypertension increased lipid peroxidation levels by ~65%, 22% and 23% in the kidney, heart and aorta samples, respectively, when compared to Sham-operated animals. In addition, GSH levels and SOD activity were also reduced in positive control group. ESCC-treatment (100 and 300 mg/kg) significantly raised SOD activity and GSH levels, besides prevented lipid peroxidation in all tissues evaluated. Unlike ESCC, ENAL was only able to reverse the changes found in kidney and aortic GSH levels, and renal lipid peroxidation.

Prolonged treatment with ESCC restores vascular reactivity in MVBs from ovariectomized hypertensive rats

In MVBs from ovariectomized hypertensive rats, the administration of ACh or SNP was able to induce a vasodilatory effect ~45% lower that in Sham-operated rats (Fig. 4A and B). Similarly, the vasoconstrictive response to Phe was significantly higher in animals from positive control group when compared to Sham-operated rats (Fig. 4C). On the other hand, in animals treated with ESCC or ENAL (15 mg/kg), the effects of ACh, SNP or Phe were not different from those observed in Sham-animals.

ESCC-administration induces vasodilation and increases intracellular cGMP levels

The continuous perfusion of MVB with Phe induced a sustained increase in the vascular perfusion pressure, which was dose-dependently reduced by ESCC (0.003, 0.01, and 0.03 mg) into the perfusion apparatus (Supplementary material). In addition, incubation of ESCC (0.01 and 0.03 mg/ml) with the aortic rings of rats increased the cGMP levels by ~62% and 103%, respectively, when compared with basal levels, whereas its co-incubation with ODQ (100 μm) completely abolished this effect. The NO-donor SNP increased the cGMP levels by ~156%, whereas co-incubation with ODQ completely vanished SNP-mediated increases in cGMP (Fig. 5C).

The vascular effects of ESCC appear to be involved with endothelial nitric oxide production and K^+ channel opening in smooth muscle cells

In MVBs, the effects of 0.003, 0.01, and 0.03 mg ESCC was decreased by around 50% in MVB perfused with L-NAME (Fig. 5B). On the other hand, the vasodilatory effect of ESCC remained unchanged in preparations perfused with indomethacin (Fig. 5A). The perfusion of MVB with PSS added of 40 mM KCl vanished the effects of ESCC (Fig. 6A). Moreover, the perfusion decrease generated by the administration of three ESCC doses was reduced by around 50–70% in MVB perfused with TEA, GLB, or 4-AP (Fig. 6B–D). Interestingly, the co-administration of TEA plus GLB and 4-AP (Fig. 6E), or L-NAME plus GLB and 4-AP (Fig. 6F) abolished the vascular effects of ESCC.

Discussion

The main aim of this study was to show that ESCC obtained from *Cuphea carthagenensis*, a natural product widely used in Brazil, has cardiorenal protective effects on ovariectomized hypertensive rats. Notably, this was the first study to show that ESCC modulates oxidative stress and reduces the tone of resistance arteries, which are mainly responsible for regulating systemic blood pressure and renal function. Our findings reinforce the cardiovascular benefits previously associated with the popular use of *C. carthagenensis*.

Although *C. carthagenensis* is widely used in Brazilian traditional

Table 4
Effects of oral administration of ESCC obtained from *Cuphea carthagenensis* on urinary volume and electrolyte excretion, pH and density on 14th day of treatment.

Group	Urinary Volume (ml/100 g/8 h)	El_{Na^+} (μEq/min/100 g)	El_{k^+} (μEq/min/100 g)	$El_{Ca^{++}}$ (μEq/min/100 g)	El_{Cl^-} (μEq/min/100 g)	pH	Density
Sham	6.22 ± 0.51	0.66 ± 0.04	0.20 ± 0.02	0.02 ± 0.003	0.71 ± 0.05	6.96 ± 0.05	1010 ± 0.88
C^+	4.22 ± 0.34[b]	0.35 ± 0.05[b]	0.25 ± 0.01	0.02 ± 0.004	0.42 ± 0.05[b]	7.40 ± 0.10	1010 ± 0.64
ESCC (30 mg/kg)	5.32 ± 0.38	0.76 ± 0.04	0.22 ± 0.01	0.02 ± 0.003	0.77 ± 0.03	7.05 ± 0.12	1010 ± 0.52
ESCC (100 mg/kg)	5.21 ± 0.32	0.70 ± 0.03	0.20 ± 0.01	0.02 ± 0.001	0.68 ± 0.01	6.80 ± 0.22	1010 ± 0.44
ESCC (300 mg/kg)	5.98 ± 0.45	0.77 ± 0.10	0.26 ± 0.04	0.02 ± 0.004	0.85 ± 0.12	7.41 ± 0.22	1011 ± 0.35
ENAL	5.99 ± 0.59	0.74 ± 0.04	0.20 ± 0.01	0.01 ± 0.002[a]	0.79 ± 0.04	6.92 ± 0.10	1012 ± 1.07

Values are expressed as mean ± S. E. M. of 8–10 rats in each group in comparison with the positive control (C^+; [a]$p < 0.05$) or Sham-operated group ([b]$p < 0.05$) using one-way ANOVA followed by Dunnett's test. El: Excreted load; ENAL: enalapril.

Table 5
Effects of oral administration of ESCC obtained from *Cuphea carthagenensis* on urinary volume and electrolyte excretion, pH and density on 21st day of treatment.

Group	Urinary Volume (ml/100 g/8 h)	El_{Na^+} (μEq/min/100 g)	El_{k^+} (μEq/min/100 g)	$El_{Ca^{++}}$ (μEq/min/100 g)	El_{Cl^-} (μEq/min/100 g)	pH	Density
Sham	5.97 ± 0.49	0.79 ± 0.06	0.19 ± 0.01	0.02 ± 0.001	0.84 ± 0.06	7.56 ± 0.10	1010 ± 0.53
C^+	4.10 ± 0.23[b]	0.45 ± 0.09[b]	0.17 ± 0.02	0.03 ± 0.006	0.52 ± 0.09[b]	7.53 ± 0.16	1010 ± 0.52
ESCC (30 mg/kg)	5.17 ± 0.28	0.71 ± 0.03[a]	0.18 ± 0.01	0.03 ± 0.004	0.75 ± 0.03	7.33 ± 0.18	1010 ± 0.61
ESCC (100 mg/kg)	5.29 ± 0.27	0.60 ± 0.01[a]	0.16 ± 0.02	0.03 ± 0.002	0.63 ± 0.01	7.30 ± 0.13	1011 ± 0.67
ESCC (300 mg/kg)	5.87 ± 0.22[a]	1.32 ± 0.06[ab]	0.40 ± 0.02[ab]	0.02 ± 0.003	1.49 ± 0.07[ab]	7.24 ± 0.17	1010 ± 0.44
ENAL	5.11 ± 0.37	0.57 ± 0.04[b]	0.24 ± 0.02	0.03 ± 0.004	0.66 ± 0.05	7.62 ± 0.12	1012 ± 0.70[a]

Values are expressed as mean ± S. E. M. of 8–10 rats in each group in comparison with the positive control (C^+; [a]$p < 0.05$) or Sham-operated group ([b]$p < 0.05$) using one-way ANOVA followed by Dunnett's test. El: Excreted load; ENAL: enalapril.

medicine (Bolson et al., 2015; Vendruscolo et al., 2006), studies aimed at hypertension conditions associated with low estrogen levels have never been conducted. Thus, we chose to use a renovascular hypertension model associated with ovariectomy, which mimicked clinical conditions very close to those of hypertensive women over 50 years of age (Lima et al., 2012). In this study, all ovariectomized hypertensive rats showed a significant decline in renal function throughout the experimental period, characterized by persistent reduction of urinary volume and renal excretion of electrolytes. In addition, a significant increase in the function of plasma ECA and serum aldosterone levels, associated with increased oxidative and nitrosative stress, may have directly contributed to the persistent increase in AP and HR and for the changes in vascular responsiveness. In fact, a potent stimulator of NADPH oxidase in vascular smooth muscle cells, and consequently $O_2^{\bullet-}$-production, is angiotensin II (Dusting et al., 2004). Moreover, some therapeutic actions of antihypertensive drugs (angiotensin II receptor blockers and angiotensin converting enzyme inhibitors) are attributed to the inhibition of NADPH oxidase, with a consequent reduction in the ROS production (Touyz, 2004).

An interesting data of this study was obtained in animals treated with ENAL. Despite the evident and predictable cardioprotective effect, including the reduction of SBP, DBP, MAP, and HR, in addition to its vascular protective and antioxidant properties, an important reduction of renal function was observed in all ENAL-treated rats. Literature data have shown that ACE inhibitors often cause unrecognized significant worsening renal failure in patients with reduced renal function, sometimes irreversible, and that more caution is required regarding their use, especially in older hypertensive patients, with likely ischemic hypertensive nephropathy (Onuigbo et al., 2011). Therefore, as the hypertension model used in this study is involved with renovascular hypertension by partial ischemia of the renal artery, it is probable that ENAL significantly worsened renal function in detriment of its cardioprotective effects, opening the discussion about the safety of this class of drugs in this specific clinical condition.

In recent years, many evidences have brought light on the possible cardiovascular benefits of ESCC. Recent studies have shown that ESCC, in addition to expressive *in vitro* antioxidant activity, was also capable of modulating the enzymatic antioxidant defense systems and reducing atherosclerotic lesions in male rabbits (Barbosa et al., 2016; Prando et al., 2015). In addition, a preliminary vasodilatory effect induced by *C. carthagenensis* preparations in the aortic rings of rats has also been reported (Krepsky et al., 2012). Although data are quite suggestive, the effects of ESCC on long-term renal and cardiovascular function in hypertension model associated with estrogen deprivation remained unclear. So, the first relevant data obtained with our study came with the evaluation of the renal function. In fact, during the first two weeks with ESCC treatment, animals began to show signs of decreased urinary excretion. Nevertheless, in a different way than with enalapril, ESCC not only completely prevented renal dysfunction after two weeks of treatment, but also showed a significant saluretic activity, especially at dose of 300 mg/kg. In addition, at the end of 28 days, ESCC prevented changes in vascular responsiveness and the establishment of renovascular hypertension, with values very close to those obtained with the Sham-operated group.

An emerging question that arose after the analysis of the abovementioned data was how much the cardiorenal protection induced by ESCC is a consequence of the systemic reduction of oxidative and nitrosative stress. In fact, this possibility was considered after we verified that ESCC had no effect on the renin-angiotensin system or on the levels of the antidiuretic hormone. Several recent studies have shown a direct relation of the cardiorenal effects of different natural products, including diuretic, vasodilator and antihypertensive responses, with their antioxidant properties and consequent increase in the NO bioavailability (Lívero et al., 2017; Bai et al., 2015). It is now well established that NO resulting from e-NOS plays a crucial role in protecting blood vessels. This action is associated with maintenance of vascular tone, AP regulation, prevention of platelet aggregation, inhibition of monocyte and neutrophil adhesion to vascular endothelium, antiproliferative and antioxidative effect. NO produced by e-NOS induces the SOD expression in the muscular layer of the vessel and in the extracellular side,

Table 6
Effects of oral administration of ESCC obtained from *Cuphea carthagenensis* on urinary volume and electrolyte excretion, pH and density on 28th day of treatment.

Group	Urinary Volume (ml/100 g/8 h)	El_{Na^+} (μEq/min/100 g)	El_{k^+} (μEq/min/100 g)	$El_{Ca^{++}}$ (μEq/min/100 g)	El_{Cl^-} (μEq/min/100 g)	pH	Density
Sham	7.29 ± 0.63	1.45 ± 0.07	0.27 ± 0.03	0.05 ± 0.004	1.52 ± 0.09	6.97 ± 0.12	1011 ± 0.64
C^+	5.11 ± 0.12[b]	0.97 ± 0.07[b]	0.21 ± 0.01	0.03 ± 0.002[b]	1.15 ± 0.07[b]	7.24 ± 0.09	1010 ± 0.25
ESCC (30 mg/kg)	5.92 ± 0.49	1.03 ± 0.12	0.23 ± 0.02	0.04 ± 0.006	1.35 ± 0.13	7.25 ± 0.18	1013 ± 1.05
ESCC (100 mg/kg)	5.83 ± 0.80	0.97 ± 0.08	0.23 ± 0.02	0.04 ± 0.002	1.40 ± 0.05	7.01 ± 0.26	1012 ± 0.42
ESCC (300 mg/kg)	6.28 ± 0.88	1.30 ± 0.08[a]	0.34 ± 0.02[a]	0.04 ± 0.003	1.42 ± 0.07	7.17 ± 0.12	1012 ± 0.55
ENAL	5.39 ± 0.29[b]	0.61 ± 0.05[ab]	0.16 ± 0.01[b]	0.03 ± 0.003[b]	0.69 ± 0.05[ab]	7.04 ± 0.24	1010 ± 1.00

Values are expressed as mean ± S. E. M. of 8–10 rats in each group in comparison with the positive control (C^+; [a]$p < 0.05$) or Sham-operated group ([b]$p < 0.05$) using one-way ANOVA followed by Dunnett's test. El: Excreted load; ENAL: enalapril.

Table 7
Effects of oral administration of ESCC obtained from *Cuphea carthagenensis* on serum Na^+, K^+, urea, and creatinine on 28th day of treatment.

Group	Na^+ (mmol/l)	K^+ (mmol/l)	Urea (mg/dl)	Creatinine (mg/dl)
Sham	129.5 ± 2.13	5.63 ± 0.32	57.32 ± 0.93	0.32 ± 0.02
C^+	134.5 ± 1.97	6.36 ± 0.33	54.80 ± 2.75	0.39 ± 0.03
ESCC (30 mg/kg)	132.9 ± 1.14	6.46 ± 0.16	56.08 ± 1.36	0.39 ± 0.02
ESCC (100 mg/kg)	133.0 ± 1.13	6.03 ± 0.50	52.43 ± 1.57	0.43 ± 0.02
ESCC (300 mg/kg)	133.0 ± 1.03	5.87 ± 0.24	51.46 ± 1.29	0.38 ± 0.03
ENAL	131.3 ± 1.43	6.43 ± 0.26	64.85 ± 4.37[ab]	0.44 ± 0.05

Values are expressed as mean ± S. E. M. of 8–10 rats in each group in comparison with the positive control (C^+; $^a p < 0.05$) or Sham-operated group ($^b p < 0.05$) using one-way ANOVA followed by Dunnett's test.

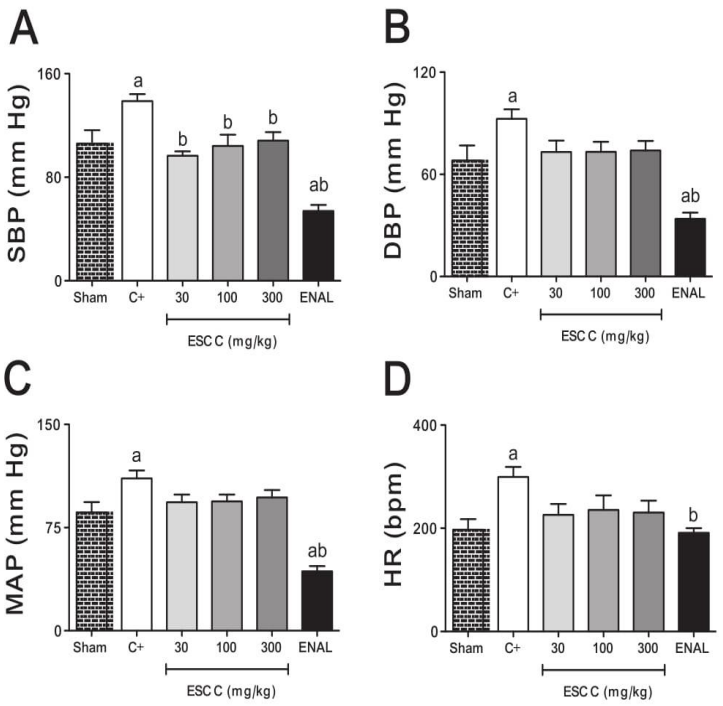

Fig. 2. Prolonged administration of ESCC obtained from *Cuphea carthagenensis* prevents SBP (A), DBP (B), MAP (C), and HR (D) rise in 2K1C-ovariectomized rats. Vehicle, ESCC (30, 100, and 300 mg/kg), or ENAL (15 mg/kg) was given orally for 28 days. The letter "C^+" indicates the effect measured after administration of vehicle only. The results show the mean ± S.E.M. (n = 8–10). Statistical analyses were performed by means of two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. $^a p < 0.05$ when compared to Sham-operated group. $^b p < 0.05$ when compared to positive control group (C^+). ENAL: enalapril; DBP: diastolic blood pressure; HR: heart rate; MAP: mean arterial pressure; SBP: systolic blood pressure; Sham: placebo surgery.

decreasing the available superoxide anion (O_2^-) and, consequently, the production of peroxynitrite ($ONOO^-$). NO also induces the synthesis of ferritin, which binds to free iron ions and prevents the generation of O_2^-. On the other hand, in the presence of some important cardiovascular disorders, including advanced atherosclerotic disease, activated macrophages produce O_2^-, express i-NOS and produce NO. In this way, $ONOO^-$ and OH^- are produced, further compromising tissue integrity, favoring the activation of coagulation and contributing to

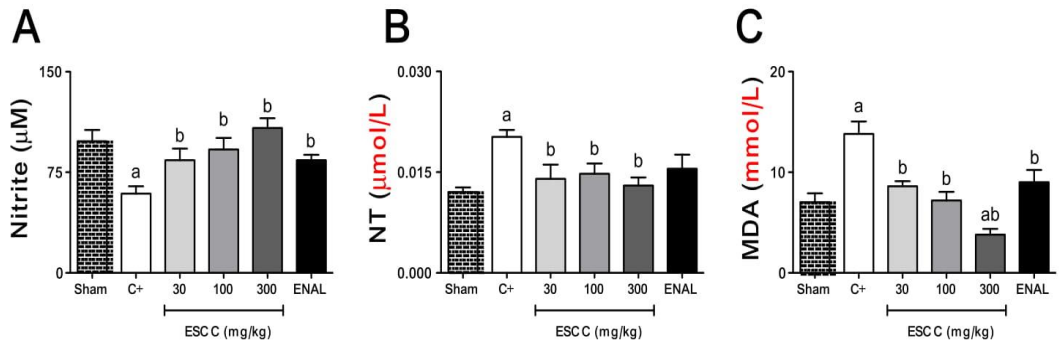

Fig. 3. ESCC- treatment increases nitrite concentration (A), and reduces NT (B) and TBARS (C) levels. The serum samples were obtained after 28-days of treatment with vehicle, ESCC (30, 100, and 300 mg/kg), or ENAL (15 mg/kg). The results show the mean ± S.E.M. (n = 8–10). Statistical analyses were performed by means of two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. $^a p < 0.05$ when compared to Sham-operated group. $^b p < 0.05$ when compared to positive control group (C^+). ACE: angiotensin converting enzyme; ENAL: enalapril; NT: nitrotyrosine; MDA: malondialdehyde; Sham: placebo surgery.

Table 8
Effects of oral administration of ESCC obtained from *Cuphea carthagenensis* on antioxidant tissue defense system.

Parameter	Sham	C^+	ESCC (30 mg/kg)	ESCC (100 mg/kg)	ESCC (300 mg/kg)	Enalapril
Kidney						
SOD	79.40 ± 4.41	52.17 ± 3.73[b]	63.65 ± 2.68[b]	70.36 ± 3.65[a]	77.21 ± 2.21[a]	61.68 ± 1.80[b]
GSH	406.10 ± 11.82	252.80 ± 5.77[b]	302.40 ± 3.53[ab]	353.10 ± 4.73[ab]	412.80 ± 9.21[a]	303.00 ± 4.24[ab]
LPO	11.90 ± 1.07	19.73 ± 1.30[b]	14.30 ± 0.88[a]	12.63 ± 0.68[a]	11.02 ± 0.51[a]	11.40 ± 0.48[a]
Heart						
SOD	40.57 ± 1.61	30.54 ± 0.61[b]	34.89 ± 1.49[b]	38.03 ± 1.23[a]	40.46 ± 1.40[a]	34.84 ± 1.84[b]
GSH	588.10 ± 9.33	352.6 ± 9.15[b]	441.60 ± 6.68[b]	528.90 ± 8.61[a]	610.00 ± 9.21[a]	400.50 ± 75.35[b]
LPO	16.48 ± 0.97	20.17 ± 0.35[b]	18.10 ± 0.65	17.99 ± 0.73	17.42 ± 0.59[a]	17.93 ± 0.37
Aorta						
SOD	71.73 ± 5.00	62.37 ± 2.55	67.48 ± 1.36	68.61 ± 1.37	69.64 ± 1.73	62.83 ± 1.53
GSH	259.90 ± 5.79	136.20 ± 4.58[b]	204.90 ± 5.79[ab]	241.00 ± 4.38[ab]	265.60 ± 4.25[a]	182.00 ± 4.31[ab]
LPO	6.90 ± 0.53	8.54 ± 0.38[b]	7.61 ± 0.41	7.29 ± 0.41	6.91 ± 0.47[a]	7.22 ± 0.33

Values are expressed as mean ± S. E. M. of 8–10 rats in each group in comparison with the positive control (C^+; [a]$p < 0.05$) or Sham-operated group ([b]$p < 0.05$) using one-way ANOVA followed by Dunnett's test. SOD: Superoxide dismutase (Unit of SOD/mg of protein); GSH: reduced glutathione (µg GSH/g of tissue); LPO: lipid peroxidation (nmol hydroperoxides/mg of protein).

vascular lumen obstruction (Wolin, 2000).

Thus, in an attempt to clarify these questions, we start from two different approaches. First, we aimed to verify if ESCC would be capable of affecting the endogenous antioxidant defense system and, consequently, the NO/GMPC pathway activity. Then, we also investigated the vascular effects of ESCC on resistance vessels using MVBs. Surprisingly, prolonged treatment with ESCC positively modulated the cardiorenal enzymatic antioxidant defense systems, directly reflecting in the reduction of LPO, TBARS and NT levels. In addition, an expressive increase in tecidual GSH and serum nitrite levels was observed. Some data has showed that the production of nitric oxide by e-NOS is not the only route available for the formation of NO. NO can also be produced from nitrite under hypoxic situations (Planchet and Kaiser, 2006). Furthermore, nitrite showed antihypertensive effects on 2K1C hypertensive rats, which may be due to its antioxidant properties resulting from vascular NADPH oxidase activity inhibition (Montenegro et al., 2011). Corroborant with the previous results, a considerable increase in intracellular cGMP levels in the aortic smooth muscle cells were also observed, suggesting the activation of the NO/cGMP pathway by ESCC. In fact, the administration of ESCC in the perfusion apparatus was also able to induce a significant dose-dependent vasodilator response in MVBs, showing a possible effect of ESCC on the control of vascular tone, possibly influenced by NO.

Homeostatic control of vascular tone is directly dependent on the synthesis and release of vasoconstricting and vasodilatory substances in response to different endogenous or exogenous stimuli, including chemical or physical agents, such as shear stress and pulsatile stretch (Vanhoutte, 2003). Two endothelial mediators, NO and prostacyclin, play an important role in the control of the vascular tone (Shimokawa et al., 1996). In our study, using MVBs, it was shown that pre-treatment with L-NAME, a NO synthase inhibitor, partially reduced the vasodilatory effects of ESCC. However, as the effects have not been completely suppressed, it is reasonable to state that ESCC is capable of inducing vasodilator effect on resistance vessels by both endothelium-independent and endothelium-dependent mechanisms, since the inhibition of cyclooxygenase by indomethacin did not influence the vascular effects of ESCC.

To explore the mechanisms involved in the endothelium-independent activity of ESCC, we perfused some MVBs preparations with 40 mM KCl to evaluate the putative blockade of the K^+ current across cellular membranes (Brayden, 1996). Since the blockade of the K^+ current completely abolished the vasodilatory effects of ESCC, we understand that modulation of the potassium efflux may be directly involved in these effects. This hypothesis was addressed by using classic K^+ channel blockers (TEA, GLB, and 4-AP), which resulted in a partial reduction in the vascular effects of ESCC when used alone, but completely blocked when administered in combination. In fact, even replacing TEA by L-NAME, the vasodilatory effects were completely blocked, showing that part of the vasodilatory response depends on the activation of the NO/cGMP pathway. These data indicate that the activation of Kir6.1 ATP-sensitive K^+ channels (blocked by GLB), KV K^+ channels (blocked by 4-AP), and Kca K^+ channels (blocked by TEA) are a crucial step for the vasodilatory effects of ESCC. If we take into account that the vasodilatory effects of NO include the opening of K^+ channels (Archer et al., 1994), and the blockade of the vasodilator response after L-NAME plus GLB and 4-AP association, it is reasonable to

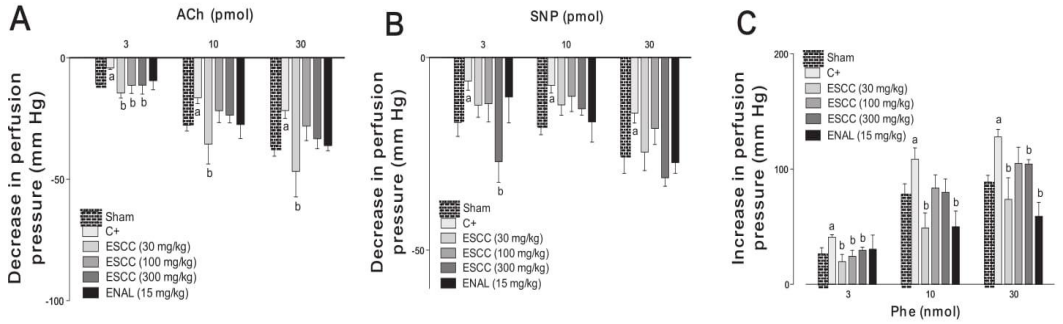

Fig. 4. Prolonged treatment with ESCC restores vascular reactivity in MVBs from ovariectomized hypertensive rats. Effects of ACh (3, 10, and 30 pmol; A), SNP (3, 10, and 30 pmol; B), or Phe (3, 10, and 30 nmol; C) on the perfusion pressure of the MVBs from normotensive (Sham) or ovariectomized hypertensive rats (C^+), in the presence or absence of prolonged treatment with ESCC (30, 100, 300 mg/kg) or ENAL (15 mg/kg). Values in panel are expressed as mean ± S.E.M. of 8–10 experiments. [a]indicates $p < 0.05$ compared with the perfusion pressure in Sham-operated rats. [b]indicates $p < 0.05$ compared with the perfusion pressure in ovariectomized hypertensive rats (C^+). ACh: acetylcholine; ENAL: enalapril; SNP: sodium nitroprusside; Phe: phenylephrine.

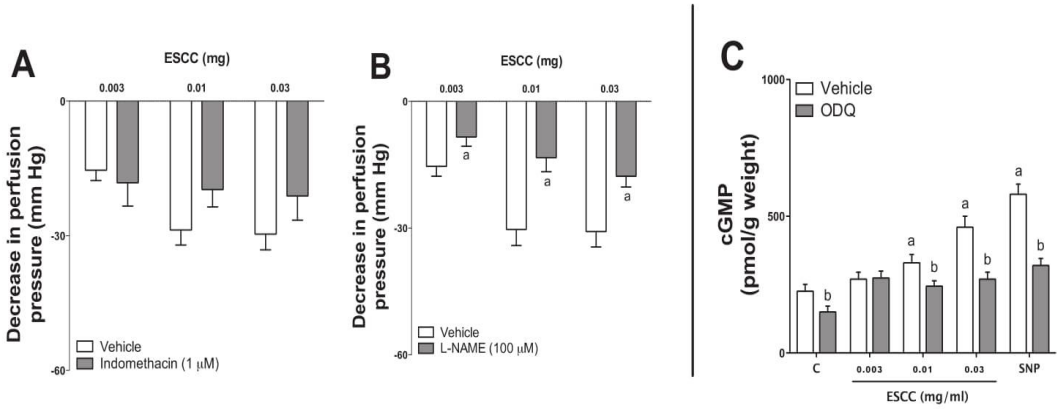

Fig. 5. Role of NO/cGMP pathway on the vascular effects of ESCC. Effects of ESCC (0.003, 0.01, and 0.03 mg) on endothelium-intact mesenteric vascular beds continuously perfused with L-NAME (A) or indomethacin (B) are presented. In addition, intracellular cGMP levels from rat aortic rings incubated with ESCC (0.003, 0.01, and 0.03 mg/ml) or sodium nitroprusside (SNP), in the absence and in the presence of ODQ (100 μm) are showed (C). The results show the mean ± S.E.M. of 5 preparations per group. For A and B [a]indicate $p < 0.05$ compared with the effects of ESCC on the respective vehicle group. For cGMP levels $p < 0.05$ *vs.* [a]control (C) or after incubation with [b]ODQ.

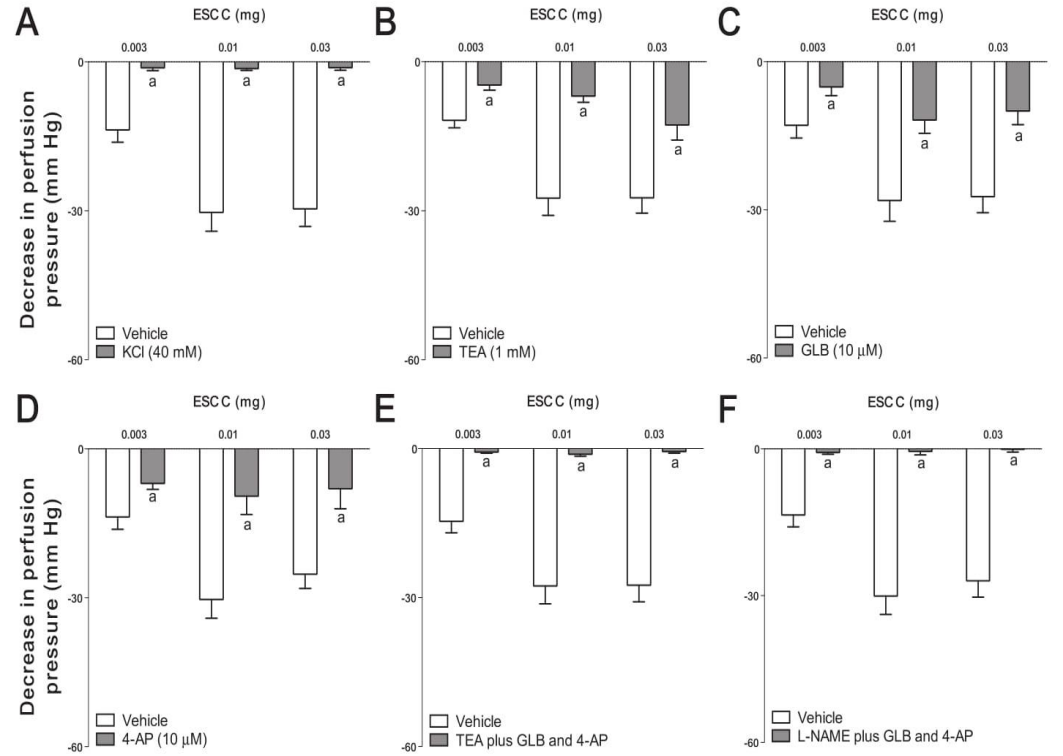

Fig. 6. Vascular effects of ESCC are related to the NO/cGMP pathway activation and opening of different potassium channels in the MVBs. The effects of ESCC were evaluated in preparations perfused with PSS containing 3 μM Phe added of 40 mM KCl (A), tetraethylammonium (TEA, B), glibenclamide (GLB, C), 4-aminopyridine (4-AP, D), TEA plus GLB and 4-AP (E), and L-NAME plus GLB and 4-AP (F). The results show the mean ± S.E.M. of 5 preparations. [a]indicate $p < 0.05$ compared with the effects of ESCC on the respective vehicle group. All experiments were performed in endothelium-intact preparations. MVBs: mesenteric vascular beds; Phe: phenylephrine.

suggest that potassium channels participate in both endothelium-dependent and independent effects of ESCC. In fact, if endothelium-independent effects may involve both Kir6.1 ATP-sensitive and KV K^+ channels, the endothelium-dependent effects appear to be dependent on secondary NO-mediated opening of Kca K^+ channels.

A limitation of our study was that it did not to show the exact mechanism by which NO production is triggered by ESCC; but the expressive modulation of the tissue antioxidant and antinitrosant system, may suggest that the reduction of oxidative and nitrosative stress can directly participate in this process. Despite the evidence, the secondary metabolites present in ESCC are also capable of directly influencing the release of endothelial NO and the opening of different potassium channels, regardless of its antioxidant effects. In fact, flavonoids such as quercetin (Marunaka, 2017) and kaempferol (Devi et al., 2015) are classic antioxidants that activate the NO/cGMP pathway and may influence the opening of several ion channels. Future studies can clarify the true role of each of the compounds present in the ESCC and show whether the effect evidenced in this work is due to only one compound, or to the set of them acting in a coordinated way.

Conclusions

The data showed that a 28-day ESCC treatment reduces the progression of the cardiorenal disease in ovariectomized hypertensive rats. These effects seem to be involved with an attenuation of the oxidative and nitrosative stress, affecting endothelial NO production and K^+ channel opening in smooth muscle cells.

Author's contribution

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

Acknowledgments

This work was supported by grants from the Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT, Brazil, 59/300.046/2015), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil, 449464/2014-8). The authors are grateful to the University Hospital of the Federal University of Grande Dourados for some biochemical analyzes.

Conflict of interest

The authors declare that there are no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.05.011.

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6. CONCLUSÕES

Este trabalho mostrou que o tratamento prolongado com o sobrenadante etanólico, obtido a partir da *C. carthagenensis*, promove a cardio e renoproteção frente á um modelo de HARV em ratas ovariectomizadas, através da redução do estresse oxidativo e nitrosativo, causados por este modelo. Além disso, promove tanto a produção de NO endotelial quanto a ativação de canais de K^+ em células musculares lisas.

7. ANEXO

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, 4 de dezembro de 2016.

CERTIFICADO

Certificamos que a proposta intitulada **"Investigação dos efeitos cardioprotetores *de* *Cuphea carthagenensis*** (Jacq.) J. F. Macbr. **em ratas ovariectomizadas com hipertensão renovascular",** registrada sob o protocolo de nº 46/2016, sob a responsabilidade de *Arquimedes Gasparotto Junior* – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata, subfilo Vertebrata* (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto n° 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 30/09/2016.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*05/12/2016 a 01/11/2018*
Espécie/linhagem/raça	*Rattus norvegicus/* Wistar
Nº de animais	60
Peso/idade	60 dias
Sexo	Fêmeas
Origem	Biotério Central da UFGD

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Coordenadora CEUA