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Avaliação do potencial diurético, anti-hipertensivo e cardioprotetor da planta medicinal *Gomphrena celosioides* Mart. (Amaranthaceae) em ratos

PAULO CÉSAR DE PAULA VASCONCELOS

Dourados - MS
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Dedico este trabalho a meus filhos
Sem os quais ele teria sido realizado com muito menos alegria
E em muito menos tempo...

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EPÍGRAFE

Nossa vida é uma roda
Que vive sempre a girar
Entra a moda e sai a moda
E depois torna a voltar
Quem pensar que o tempo passa
A vida é que vai passar
O tempo fica parado
Sempre no mesmo lugar

(CANUTO ALVES VASCONCELOS)

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

1K1C	1 Kidney, 1 Clip
2K1C	2 Kidneys, 1 Clip
ACE	Angiotensin-Converting Enzyme
ACh	Acetylcholine
AII	Angiotensina II
ALDO	Aldosterona
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CEUA-UFGD	Comissão de Ética no Uso de Animais – Universidade Federal da Grande Dourados
CK	Creatine Kinase
CK-MB	Creatine Kinase MB
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
DBP	Diastolic Blood Pressure
DCV	Doença Cardiovascular
DM	Diabetes Melito
DRC	Doença Renal Crônica
ECA	Enzima Conversora da Angiotensina
EDTA	Ethylenediaminetetraacetic Acid
EEGC	Extrato Etanólico de *Gomphrena celosioides* / Ethanolic Extract of *Gomphrena celosioides*
EEGC100	Extrato Etanólico de *Gomphrena celosioides* 100 mg/kg / Ethanolic Extract of *Gomphrena celosioides* 100 mg/kg
EEGC30	Extrato Etanólico de *Gomphrena celosioides* 30 mg/kg / Ethanolic Extract of *Gomphrena celosioides* 30 mg/kg
EEGC300	Extrato Etanólico de *Gomphrena celosioides* 300 mg/kg / Ethanolic Extract of *Gomphrena celosioides* 300 mg/kg
ELISA	Enzyme Linked Immunosorbent Assay
eNOS	endothelial Nitric Oxide Synthase
ESI-MS	Electrospray Ionisation Mass Spectrometry

ESI-MS/MS	Electrospray Ionization Tandem Mass Spectrometry
FAEPEX	Fundo de Apoio ao Ensino, à Pesquisa e Extensão
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FFCLRP/USP	Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo
GGT	Gamma-Glutamyl Transferase
HA	Hipertensão Arterial
HAS	Hipertensão Arterial Sistêmica
HCTZ	Hydrochlorothiazide
L-NAME	Nω-Nitro-L-arginine Methyl Ester
LOA	Lesão em Órgão-Alvo
MAP	Mean Arterial Pressure
NO	Óxido Nítrico / Nitric Oxide
NPS	Sodium Nitroprusside
PA	Pressão Arterial
PAD	Pressão Arterial Diastólica
PAS	Pressão Arterial Sistólica
PG	Prostaglandins
PGE	Prostaglandina E
PGI2	Prostacyclin
Phe	Phenylephrine
PNA	Peptídeo Natriurético Atrial
PSS	Physiologic Saline Solution
PUC-RJ	Pontifícia Universidade Católica do Rio de Janeiro
RAAS	Sistema Renina Agiotensina Aldosterona (do inglês: Renin Angiotensin Aldosterone System)
RBC	Glóbulos Vermelhos (do inglês: Red Blood Cells)
SBP	Systolic Blood Pressure
SEM	Standard Error of the Mean
SNPS	Sistema Nervoso Parassimpático
SNS	Sistema Nervoso Simpático
SUS	Sistema Único de Saúde
TBARS	Thiobarbituric Acid Reactive Substances

UFGD	Universidade Federal da Grande Dourados
UNa	Urine Sodium
Unicamp	Universidade Estadual de Campinas
UV	Urine Volume

Avaliação do potencial diurético, anti-hipertensivo e cardioprotetor da planta medicinal *Gomphrena celosioides* Mart. (Amaranthaceae) em ratos

RESUMO

Introdução: *Gomphrena celosioides* Mart., Amaranthaceae, conhecida como "perpétua", é uma planta medicinal nativa em várias partes do mundo e tem muitos usos populares, incluindo o tratamento de cálculos renais e desordens do trato urinário. Embora *G. celosioides* seja usada como diurética, nenhum estudo havia sido conduzido para avaliar esta declaração etnofarmacológica. Um possível efeito diurético suscita questionamento sobre sua utilidade como anti-hipertensivo. **Objetivos do estudo:** Avaliar o potencial diurético, anti-hipertensivo e, em última instância, cardioprotetor do extrato etanólico de G. *celosioides* (EEGC) e elucidar seus mecanismos de ação. **Material e métodos:** EEGC (30, 100 e 300 mg/kg) foi administrado por via oral em ratos e o volume (UV) e o sódio (UNa) urinários foram medidos pelas 8 h seguintes com o auxílio de gaiolas metabólicas. Para avaliar a participação das vias do óxido nítrico (NO), prostaglandinas e bradicinina no seu efeito, foi avaliado se a menor dose efetiva (EEGC 100 mg/kg) mantinha atividade diurética na presença de inibidores de cada uma dessas vias. A atividade diurética também foi aferida diariamente em modelo de 7 dias, após o qual sangue foi coletado para análise de atividade da enzima conversora de angiotensina (ECA), aldosterona e vasopressina. Depois foi verificado o efeito hipotensor do EEGC em modelo agudo e prolongado com ratos hipertensos que sofreram cirurgia de Goldblatt. A pressão arterial média (MAP) dos animais 1K1C (1 kidney, 1 clip) foi aferida diretamente por canulação da artéria carótida antes e após a administração aguda intraduodenal de 30, 100 ou 300 mg/kg de EEGC. Para o ensaio de 4 semanas, os animais 2K1C (2 kidneys, 1 clip) receberam tratamentos orais diários com EEGC 100 mg/kg ou controles (veículo e enalapril 15 mg/kg) por 28 dias. Foram avaliadas semanalmente a MAP, através de manguito de calda e ao final por canulação da artéria carótida, e a diurese. Seus leitos mesentéricos foram isolados e canulados para avaliar sua variação de pressão após o contato com drogas vasoativas. Órgãos internos foram pesados e processados para histologia e mediu-se a espessura da parede do ventrículo cardíaco esquerdo. Amostras de sangue seguiram para quantificação de atividade da ECA, aldosterona, nitrito e substâncias reativas ao ácido tiobarbitúrico (TBARS). **Resultados:** EEGC aumentou significativamente o UV e o UNa após 8 h em relação ao grupo controle. Observou-se que este efeito é dependente das vias de NO, prostaglandinas e bradicinina pois seus inibidores reduziram os efeitos diuréticos do EEGC. Além disso, no modelo prolongado, essa atividade se manteve pelos 7 dias e houve diminuição na aldosterona sérica. EEGC foi capaz de reduzir de forma aguda a MAP dos animais hipertensos. No teste de 4 semanas, o EEGC também atuou como diurético e como anti-hipertensivo, tendo sua MAP, no final do experimento, próxima aos valores pré-cirúrgicos. Além disso, o EEGC reduziu o prejuízo à reatividade vascular induzido pela hipertensão renovascular, uma vez que os seus leitos mesentéricos, assim como o grupo de animais não operados, apresentaram menor contratilidade e maior dilatabilidade em relação ao controle hipertenso. Além disso, foi verificada inibição da atividade da ECA pelo extrato, o que levou também a menores níveis de aldosterona. No grupo EEGC, houve aumento do nitrito e a técnica TBARS mostrou diminuição de malondialdeído. Por fim, observou-se cardioproteção ao se constatar que o grupo EEGC apresentava menor espessura das paredes ventriculares. **Conclusão:** EEGC mostrou efeitos diurético, natriurético e anti-hipertensivo tanto agudos como prolongados. Esses efeitos foram suficientes para promover cardioproteção, sendo proposto como principal mecanismo de ação a inibição da ECA. Portanto, estes resultados podem ter relevância clínica e permitir o desenvolvimento de novos medicamentos naturais a partir de *G. celosioides*.

Palavras-chave: plantas medicinais; hipertensão; inibição da ECA; etnofarmacologia

Evaluation of the diuretic, antihypertensive and cardioprotective potential of the medicinal plant *Gomphrena celosioides* Mart. (Amaranthaceae) in rats

ABSTRACT

Introduction: *Gomphrena celosioides* Mart., Amaranthaceae, known as "perpétua", is a medicinal plant native to many parts of the world and has several popular uses, including the treatment of kidney stones and urinary tract disorders. Although *G. celosioides* is used as a diuretic agent, no study had been conducted to evaluate this ethnopharmacological statement. A possible diuretic effect raises questions about its usefulness as an antihypertensive. **Objectives of the study:** To evaluate the diuretic, antihypertensive and, ultimately, cardioprotective potential of the ethanolic extract of *G. celosioides* (EEGC) and to elucidate its mechanisms of action. **Material and methods:** EEGC (30, 100 and 300 mg/kg) was orally administered in rats and urinary volume (UV) and sodium (UNa) were measured for the following 8 h with the aid of metabolic cages. In order to evaluate the role of the nitric oxide (NO), prostaglandin and bradykinin pathway in this effect, it was evaluated whether the lowest effective dose (EEGC 100 mg/kg) maintained diuretic activity in the presence of inhibitors of each of these pathways. Diuretic activity was also measured daily in a 7-day model, after which blood was collected for analysis of angiotensin converting enzyme (ACE) activity, aldosterone and vasopressin. Then the hypotensive effect of EEGC in an acute and in a prolonged model was assessed using hypertensive rats that had undergone Goldblatt surgery. The mean arterial blood pressure (MAP) of the 1K1C (1 kidney, 1 clip) animals was directly measured, by cannulation of the carotid artery, before and after the acute intraduodenal administration of 30, 100 or 300 mg/kg EEGC. For the 4 week trial, 2K1C (2 kidneys, 1 clip) animals received daily oral treatments with EEGC 100 mg/kg or controls (vehicle and enalapril 15 mg/kg) for 28 days. MAP (by tail cuff and at the end by cannulation of the carotid artery) and diuresis were evaluated weekly. Their mesenteric beds were isolated and cannulated to evaluate their pressure variation after addition of vasoactive drugs. Internal organs were weighed and processed for histology and the thickness of the left cardiac ventricle wall was measured. Blood samples were taken for quantification of ACE activity, aldosterone, thiobarbituric acid reactive substances (TBARS) and nitrite. **Results:** EEGC significantly increased UV and UNa after 8 h comparing to the control group. It was observed that this effect is dependent on the NO, prostaglandin and bradykinin pathways since their inhibitors reduced the diuretic effects of EEGC. In addition, in the prolonged model, this activity was maintained for 7 days and there was a decrease in serum aldosterone. EEGC was able to acutely reduce the MAP of hypertensive animals. In the 4-week test, the EEGC also served as a diuretic and as an antihypertensive, with its MAP at the end of the experiment being close to pre-surgical values. In addition, EEGC reduced the impairment to vascular reactivity induced by renovascular hypertension, since its mesenteric beds, similarly to the group of non-operated animals, presented lower contractility and greater dilatability comparing to the hypertensive control. In addition, the extract promoted inhibition of ACE activity, which also led to lower levels of aldosterone. In the EEGC group, nitrite was increased and TBARS assay showed decreased malondialdehyde. Finally, cardioprotection was observed as the EEGC group showed thinner ventricular walls. **Conclusion:** EEGC showed acute and prolonged diuretic, natriuretic and antihypertensive effects. These effects were sufficient to promote cardioprotection, and ACE inhibition was proposed as the main mechanism of action. Therefore, these results may have clinical relevance and allow the development of new natural medicines from G. celosioides.

Keywords: herbal medicine; hypertension; ACE inhibition; ethnopharmacology

SUMÁRIO

1. INTRODUÇÃO

Desde tempos antigos, o ser humano busca na natureza cura para suas doenças. O uso de plantas como finalidade curativa sempre esteve presente na espécie e, como em outros animais, era instintivo (STOJANOSKI, 1999). A primeira evidência escrita de uso de plantas para a preparação de drogas data de cerca de 5000 anos atrás (KELLY, 2009). Até o século XIX, as plantas medicinais e seus extratos constituíam quase a totalidade dos medicamentos utilizados e somente no início século passado começaram a surgir os fármacos nos moldes atuais, com a descoberta dos primeiros anestésicos e antibióticos (SIMÕES et al., 1999).

O uso de plantas medicinais ainda é ubíquo, porém, muitas vezes são utilizadas plantas com fins medicinais com pouco ou nenhum conhecimento de suas propriedades farmacológicas (VEIGA JUNIOR et al., 2005). Mesmo se considerarmos o uso tradicional como evidência de eficácia, dados científicos sobre segurança e mecanismos de ação úteis à prática clínica são indispensáveis e, infelizmente, estão disponíveis para poucas plantas medicinais (CLAESON, 2014). Assim, pesquisas com produtos naturais guiadas pelo conhecimento popular (etnofarmacologia) são necessárias e têm contribuído bastante para a renovação dos fármacos por proporcionar o conhecimento de novas substâncias químicas e a caracterização de novos mecanismos de ação (RATES, 2001). É consenso que a pesquisa envolvendo plantas medicinais é complexa e se faz necessária uma seleção criteriosa das espécies a serem estudadas, com a participação de uma equipe multidisciplinar em todas as etapas da pesquisa, além de levar em consideração a indicação popular de uso medicinal (DI STASI, 1996; SOUZA BRITO; NUNES, 1997).

De acordo com Newman e Cragg (2016), entre os anos de 1981 e 2014, de todas as novas moléculas introduzidas no mercado, em torno de 33% eram substâncias isoladas de produtos naturais, semi-sintéticos derivados de produtos naturais ou então moléculas sintetizadas tomando como modelo estruturas de origem natural e essa média tem se mantido semelhante na década atual. Para eles, a descoberta de medicamentos envolvendo a geração de uma diversidade molecular verdadeiramente nova a partir de fontes de produtos naturais, juntamente com metodologias sintéticas totais e combinadas e incluindo a manipulação de caminhos biossintéticos, continuará a oferecer a melhor solução para a atual crise de produtividade que enfrenta a comunidade científica envolvida na descoberta e desenvolvimento de fármacos. Mesmo a química combinatória (revisada em MIERTUS et al., 2000), que

atualmente está presente em cerca de 80% das novas descobertas, é uma técnica que está sendo bastante utilizada tendo como ponto de partida compostos de origem natural, sendo esse conceito chamado de química biocombinatória (GANESAN, 2004).

Segundo a Organização Mundial da Saúde (WHO, 2013), muitas populações ainda fazem uso de plantas medicinais como principal recurso no atendimento básico à saúde. Além disso, é crescente o uso de medicamentos fitoterápicos, inclusive no Brasil (CACCIA-BAVA et al., 2017). Medicamentos fitoterápicos, como diz a legislação brasileira, são aqueles produzidos utilizando-se exclusivamente de matérias-primas ativas de origem vegetal que exibam constância em sua qualidade, com eficácia e segurança baseadas em evidências clínicas e dados publicados na literatura técnico-científica (BRASIL, 2014). De acordo com Caccia-Bava et al., 2017, o uso de medicamentos fitoterápicos no Sistema Único de Saúde brasileiro (SUS) vem crescendo a um ritmo maior que o uso da planta medicinal em preparação caseira, portanto, há uma necessidade de um fortalecimento do elo entre pesquisa científica e indústria farmacêutica nacional para entregar produtos que atendam essa demanda crescente.

Com o aumento da expectativa de vida no Brasil e no mundo aumenta-se também a necessidade de tratamentos contra doenças crônicas, como é o caso da hipertensão (GIACOMELLI et al., 2016). E de fato o uso de plantas medicinais e de outras terapias alternativas para o tratamento da hipertensão é crescente mundialmente falando, devido em grande parte à crença das pessoas no "conceito holístico" da medicina e da percepção de que as plantas são eficazes, apresentam menos efeitos colaterais (o que nem sempre é verdade) e são mais baratas que as terapias convencionais. Além disso, o controle sobre o próprio tratamento, a maior facilidade, além de costumes e tradições religiosas pesam a favor do uso das plantas (BISHOP et al., 2010).

Um bom exemplo de sucesso de planta medicinal no combate à hipertensão é o alho, cujo consumo por hipertensos é inclusive recomendado pela Sociedade Brasileira de Cardiologia (MALACHIAS et al., 2016). Já foi demonstrado que ele é rico em componentes bioativos que regulam a produção de óxido nítrico (NO) e sulfeto de hidrogênio (H_2S), sendo o principal a alicina, capaz de promover inibição da enzima conversora de angiotensina (ECA), sendo todos esses efeitos responsáveis por diminuição na pressão (RIED; FAKLER, 2014).

A carência de pesquisas sobre eficácia e principalmente segurança das plantas medicinais e a falta maior ainda de ensaios clínicos constitui uma limitação significativa em seu uso contra hipertensão no presente. A esse respeito, vale destacar que pode haver interações farmacológicas dos metabólitos secundários plantas com anti-hipertensivos existentes, além de

outras drogas, que podem levar a efeitos adversos graves e, portanto, sua administração deve ser monitorada criteriosamente (EKOR, 2014).

Portanto, há uma grande relevância farmacológica na pesquisa com produtos naturais para o combate à doença hipertensiva, que vem a guiar uso das plantas com base em dados confiáveis sobre efeitos adversos e interações medicamentosas, além de comprovação de eficácia e estabelecimento de doses efetivas e seguras e de mecanismos de ação. Isso, somado à elevada demanda social pela disponibilização de medicamentos anti-hipertensivos de origem vegetal constitui importante motivação para o desenvolvimento deste trabalho.

2. REVISÃO DE LITERATURA

2.1. *Gomphrena celosioides*

Gomphrena celosioides Mart., pertencente à família Amaranthaceae e conhecida popularmente no Brasil como perpétua e em outros países como "bachelor's button" ou "prostrate globe-amaranth", é uma espécie de erva daninha anual ou perene de vida curta, descoberta pela primeira vez em Queensland em 1930 (MYERS et al., 2000). Possui caule ereto ascendendo a 10 a 20 cm de comprimento, pouco ramificada e recoberta com pelos brancos sedosos. As folhas são oblongo-lanceoladas a oblongo-obovadas, com 1,5 a 4,5 cm de comprimento e 0,5 a 1,3 cm de largura. As flores são terminais e axilares com pontas cilíndricas, medindo 1 a 2 cm de comprimento e 1 cm de diâmetro (WAGNER et al., 1999). Ela cresce ao longo das estradas, margens de rios, ferrovia e em pousio, ocasionalmente invadindo pastagens. É bem distribuída na América do Sul, Ásia, África Oriental e Ocidental (TAKIM et al., 2013). No Brasil, ela é descrita como uma erva daninha nociva que é muito comum em culturas de terras secas e plantações (SUZANE et al., 2010). É raramente comida por animais. Desconhece-se seu consumo por cabras e é tóxico para os cavalos quando consumida em excesso por longos períodos (FANK-DE-CARVALHO; GRACIANO-RIBEIRO, 2005).

G. celosioides é usada no tratamento de várias doenças da pele e como abortiva na América do Sul (BURKILL, 1984). A decocção de toda a planta e da espécie relacionada *G. globosa* Linn é aplicada a feridas gangrenosas (ARENAS; AZOREARO, 1977). *G. martiana* e *G. boliviana*, que têm uso medicinal popular, tiveram pronunciada atividade antimicrobiana comprovada (Pomilio et al., 1992). Esta atividade foi atribuída a flavonas isoladas dessas plantas. Espécies brasileiras de *Gomphrena* são empregadas no tratamento de afecções brônquicas, diarreia, febre, como analgésico, tônico, carminativo ou diurético (VIEIRA et al., 1994). Na Índia, o suco de *G. celosioides* (planta inteira) com *Piper nigrum* e suco de limão é

tomado duas vezes ao dia durante 10 dias para prevenir cálculos renais e expulsá-los (PRACHI et al., 2009).

Um estudo inicial farmacoquímico com *Gomphrena celosioides* revelou a presença de saponinas, esteroides, aminoácidos e açúcares não redutores em todas as partes da planta; fenois e flavonoides nas folhas, caule e inflorescências. Betacianinas ocorreram apenas na haste; açúcares redutores nas inflorescências, enquanto cetoses foram relatados na raiz e caule (BOTHA; GERRITMA-VAN DER VIJVER, 1986).

O fracionamento dos extratos das partes aéreas feito por Moura et al., (2004) levou ao isolamento do ácido 4-hidroxi-benzóico e ácido 4-hidroxi-3-metoxibenzóico, ou ácido vanílico, além de estigmasterol, sitosterol e campesterol. Extratos das raízes renderam ecdisterona metil palmitato, stigmast-6-en-3-Ob-(Dglicopiranosídeo) e estigmasterol.

Uma triagem antimicrobiana *in vitro* de extratos e compostos puros de *G. celosioides* indicou atividade positiva contra *Staphylococcus aureus* e *Salmonella typhi,* mas nenhum efeito foi observado para *Pseudomonas aeruginosa*, *Proteus mirabilis* e *Escherichia coli* (Moura et al., 2004).

Onocha et al. (2005) relataram efeito antibacteriano, antifúngico e antiparasitário em extratos de *G. celosioides*. Em seu trabalho, o crescimento de *S. aureus* foi inibido pelo extrato metanólico em baixa concentração. O extrato acetato de etila inibiu o crescimento de *Salmonella typhimurium, P. aeruginosa* e *Bacillus subtillis* sendo o efeito de alta concentração comparável ao da ampicilina. *S. typhi, P. aeruginosa* e *S. aureus* foram apenas sensíveis ao extrato metanólico em baixa concentração. No mesmo estudo, observou-se que a atividade antifúngica do extrato metanólico em três fungos (*Candida albicans*, *Aspergillus niger* e *Dermatophyte specie*) era elevada, mesmo em baixa concentração, e foi comparável ao efeito do tioconazol. Quanto à atividade anti-helmíntica, os extratos acetato de etila e metanólico exibiram efeito pronunciado contra os helmintos *Fasciola gigantica*, *Taenia solium* e *Pheretima pasthuma*. Os extratos, além de causar paralisia dos vermes, também causaram morte dos mesmos. *G. celosioides* também apresentou atividade antimalárica em uma triagem feita por Gessler et al. (1994).

Botha e Gerritma-Van der Vijver (1986) haviam relatado que as raízes apresentaram uma fraca atividade antibacteriana contra três bactérias Gram-positivas (*Bacillus cereus Micrococcus luteus* e *Bacillus licheniformis*). Além disso, relataram em estudo toxicológico que ação leve sobre o sistema nervoso autônomo em ratos foi demonstrada por todas as partes da planta, quando administradas em doses elevadas (> 2000 mg/kg) sendo observados sintomas simpáticos e parassimpáticos, como diarreia e redução da taxa de respiração.

A atividade diurética de *G. celosioides* foi apenas mencionada por Dhawan et al. (1977). Seu efeito contra urolitíase também foi demonstrado em um estudo científico em ratos (GOSWAMI; SRIVASTAVA, 2015), e sua eficácia foi atribuída à redução das concentrações urinárias de constituintes de pedra, bem como aumento da diurese, embora este parâmetro não tenha sido avaliado.

Com base nos achados farmacológicos envolvendo *G. celosioides* e considerando seus usos populares, a boa ocorrência de compostos fenólicos em sua constituição e sua facilidade de obtenção, esta planta constitui um bom alvo de investigação por efeito diurético e anti-hipertensivo, dada a carência de dados a respeito.

2.2. Fisiologia da Pressão Arterial

De forma simplificada, a pressão arterial (PA) depende do produto entre o débito cardíaco e a resistência vascular periférica. Uma grande quantidade de fatores pode influenciar esses dois determinantes, como observado na Figura 1. O débito cardíaco depende principalmente da frequência cardíaca e do volume sistólico. A frequência cardíaca é regida por receptores β-1 e colinérgicos sob controle de estimulação simpática e parassimpática, respectivamente. O volume sistólico é determinado pela força de contração ventricular (também sob controle autonômico) e pela pressão de enchimento, que por sua vez é determinada pelo estado do volume de fluido intravascular e pela capacitância venosa. A resistência vascular sistêmica é influenciada por mecanismos vasoativos múltiplos sob o controle de fatores neurais, humorais e renais locais, regionais e sistêmicos (GUYTON, 1989; HALL et al., 1989). Todos esses determinantes fisiológicos de fluxo e resistência são interdependentes e governados mecanismos de ação rápidos, intermediários e tardios, que mantêm a pressão arterial dentro do intervalo normal, apesar de variações significativas nos parâmetros individuais (SOBEL; BAKRIS, 1999).

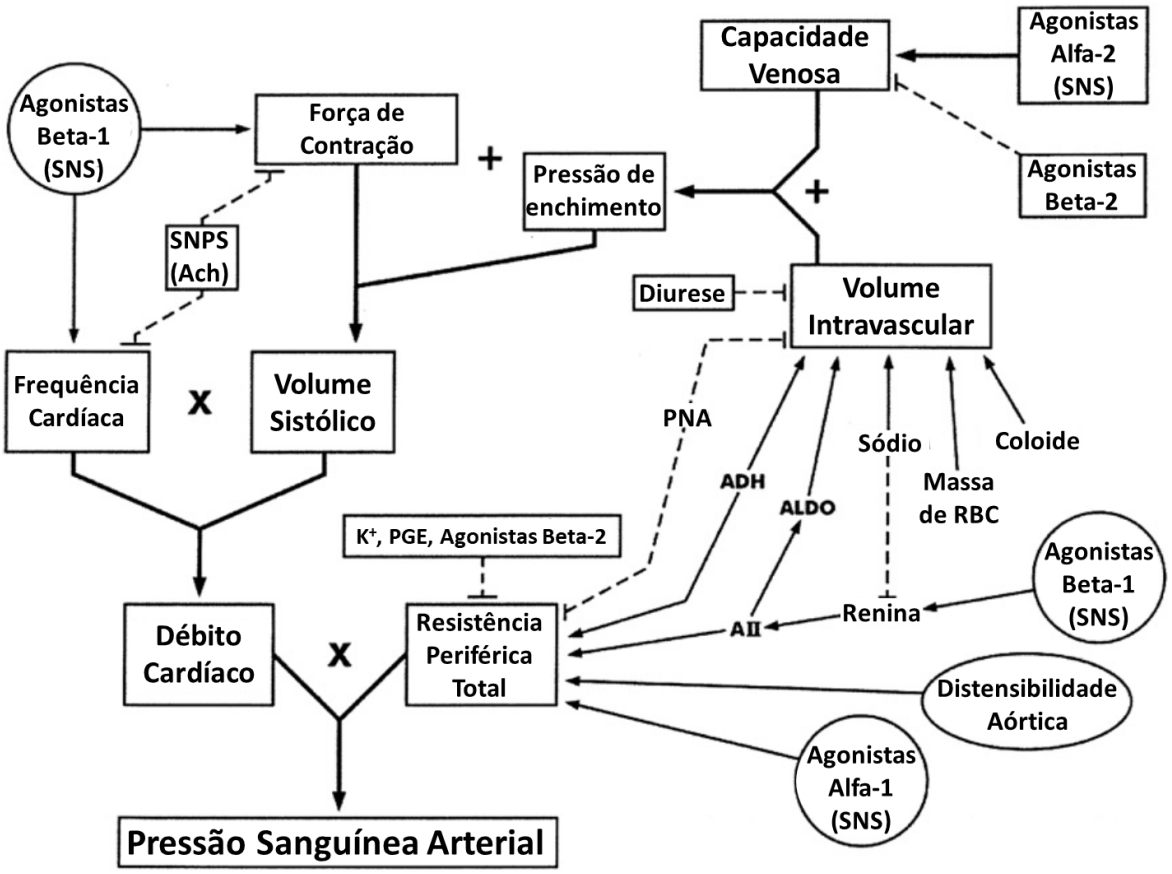

Figura 1: Fatores que determinam a pressão arterial. Ach, acetilcolina; ADH, hormônio antidiurético; ALDO, aldosterona; AII, angiotensina II; PNA, peptídeo natriurético atrial; K^+, potássio; PGE, prostaglandina E; SNPS, sistema nervoso parassimpático; RBC, glóbulos vermelhos; SNS, sistema nervoso simpático; ┤, inibitório (influência negativa); →, Influência estimulante (positiva). (Traduzido de SOBEL; BAKRIS, 1999).

2.2.1. O sistema renina-angiotensina-aldosterona

O sistema renina-angiotensina-aldosterona (RAAS) é um dos mais importantes sistemas de regulação do volume e da pressão sanguínea. A renina é uma enzima proteolítica sintetizada no rim em resposta a queda na perfusão renal. Ela é responsável pela clivagem do decapeptídeo angiotensina I a partir do angiotensinogênio, um substrato proteico circulante que é produzido no fígado. A angiotensina I, apesar de não possuir efeitos vasoativos por si só, é biotransformada com o auxílio da ECA, presente na superfície endotelial, em angiotensina II (ERDOS, 1990).

A angiotensina II é um importante mediador vasoativo com ação resultante hipertensiva. Ela atua por meio dos receptores AT_1 e AT_2, sendo o primeiro com efeito dominante. O receptor AT_1 é um receptor acoplado à proteína G e está presente em vários locais, sendo de grande relevância no músculo liso vascular, onde sua ativação leva a vasoconstrição e estimulação da

remodelação hipertrófica da musculatura lisa (DZAU, 2001). Além do efeito hipertensor direto, a ativação dos receptores AT_1 leva a uma série de efeitos que culminam com o aumento da pressão sanguínea sistêmica, como aumento da força de contração cardíaca, do tônus do sistema nervoso simpático (SNS), da sede, da liberação de vasopressina e, finalmente, da produção de aldosterona (DZAU, 2001). A aldosterona, por sua vez, atua nos túbulos e ductos coletores renais aumentando a expressão das proteínas constituintes dos canais de sódio, resultando em um aumento na reabsorção deste eletrólito e, consequentemente, de água, aumentando o volume sanguíneo circulante (FERRARIO, 2010; GUYTON, 1991).

Os receptores AT_2 quando ativados, têm ação hipotensora pelo aumento da liberação de NO e bradicinina, embora esses efeitos sejam sobrepujados pela ação hipertensora perpetrada pelos receptores AT_1 (KASCHINA; UNGER, 2003).

A ativação aumentada e persistente do RAAS está intimamente envolvida com o desenvolvimento de hipertensão arterial (HA), discutida a seguir, induzindo alterações sistêmicas que elevam a pressão basal do indivíduo, incluindo aumento da resistência vascular renal, o que desencadeia ativação ainda maior do RAAS, e facilitação do processo aterosclerótico pelo aumento da produção de espécies reativas de oxigênio na vasculatura (Touys, 2003).

2.3. Hipertensão arterial

O indivíduo é considerado hipertenso quando sua PA, medida em mais de uma ocasião por profissional treinado e equipamento calibrado, for maior que 140 mm Hg para a pressão arterial sistólica (PAS) e/ou 90 mm Hg para pressão arterial diastólica (PAD). Com frequência, a HA está associada a distúrbios metabólicos e alterações funcionais e/ou estruturais de órgãos-alvo, como coração, encéfalo, rins e vasos sanguíneos (MALACHIAS et al., 2016).

Atualmente considera-se como pré-hipertensão valores de PAS entre 121 e 139 e/ou PAD entre 81 e 89 mm Hg (CHOBANIAN et al., 2003). Essa classificação é relevante pelo fato de os portadores de pré-hipertensão serem mais predispostos ao desenvolvimento de HA (ARIMA et al., 2012) e de complicações cardiovasculares (SANTOS et al., 2016). A prevalência mundial de pré-hipertensão fica em média entre 21 e 37,7% (EGAN; STEVENS-FABRY, 2015).

A doença hipertensiva costuma ser agravada pela presença de fatores de risco, como dislipidemia, obesidade abdominal, intolerância à glicose e diabetes melito (MALACHIAS et al., 2016) (Tabela 1). Os principais fatores que contribuem para o desenvolvimento de HA dependem de idade (incidência aumenta com a idade), sexo (mais comum em mulheres após a

menopausa), etnia (predominante em negros), excesso de peso e obesidade (relação direta), ingestão de sódio (um dos principais fatores de risco), ingestão de álcool (o excesso pode elevar a pressão), sedentarismo (o exercício é fator protetor), fatores socioeconômicos (maior incidência em baixa escolaridade e renda) e genética (fator de difícil mensuração) (MALACHIAS et al., 2016).

Tabela 1: Estratificação de risco no paciente hipertenso de acordo com fatores de risco adicionais, presença de lesão em órgão alvo e de doença cardiovascular ou renal

	PAS 130-139 ou PAD 85-89	HAS Estágio1 PAS 140-159 ou PAD 90-99	HAS Estágio 2 PAS160-179 ou PAD 100-109	HAS estágio 3 PAS ≥ 180 ou PAD ≥ 110
Sem fator de risco	Sem risco Adicional	Risco Baixo	Risco Moderado	Risco Alto
1-2 fatores de risco	Risco Baixo	Risco Moderado	Risco Alto	Risco Alto
≥ 3 fatores de risco	Risco Moderado	Risco Alto	Risco Alto	Risco Alto
Presença de LOA, DCV, DRC ou DM	Risco Alto	Risco Alto	Risco Alto	Risco Alto

PAS: pressão arterial sistólica; PAD: pressão arterial diastólica; HAS: hipertensão arterial sistêmica; DCV: doença cardiovascular; DRC: doença renal crônica; DM: diabetes melito: LOA: lesão em órgão-alvo. (Malachias et al., 2016)

No Brasil a HA atinge praticamente 30% da população, sendo que na população idosa esse número ultrapassa 60%, além de ser o principal fator responsável pelas mortes por doenças cardiovasculares (Scala et al., 2015). Isso porque a hipertensão é considerada um fator predisponente para infarto, acidente vascular encefálico, doença coronariana, desordens arteriais periféricas, insuficiência cardíaca e insuficiência renal, estando essas doenças entre as que mais matam no mundo (WILLIAMS et al., 2004; GODFRAIND, 2006).

A patogenia da HA é complexa, sendo que somente em 5% dos casos a causa é clara, nos quais ela é secundária. A HA secundária é decorrente principalmente de doenças renais, como insuficiência renal crônica ou estenose da artéria renal. Outras causas incluem distúrbios endócrinos, especialmente envolvendo as adrenais, além de defeitos genéticos raros. Nos outros cerca de 95% dos casos a HA é idiopática e referida com essencial (KUMAR et al., 2013).

A hipertensão essencial deve ser encarada como um conjunto de fatores ambientais e genéticos que culminam com a elevação da pressão basal do indivíduo. Segundo a teoria da via unificada para o desenvolvimento da hipertensão, a lesão nos rins por vasoconstrição renal repetitiva e intermitente ocasionada por uma variedade de fatores é um ponto crucial

(JOHNSON et al., 2005a). Esses fatores incluem hiperativação do SNS induzida pelo estresse ou por um mecanismo genético, alterações no RAAS (polimorfismos genéticos, contraceptivos orais ou secundários à ativação do SNS renal, isquemia renal, hipocalemia, etc.) resultando em aumento da angiotensina II circulante, ou hiperuricemia induzida pela dieta, genética ou ativação do SNS (JULIUS, 1996; JEUNEMAITRE et al., 1999; JOHNSON et al., 2005b).

As estratégias clínicas comuns para levar à queda na pressão sanguínea incluem terapias farmacológicas e não farmacológicas. A abordagem não farmacológica envolve mudanças no estilo de vida, como controle do peso, prática regular de exercícios físicos, alimentação rica em fibras e pobre em gorduras saturadas, redução do consumo de sódio e de álcool e cessação do tabagismo (MALACHIAS et al., 2016).

As terapias farmacológicas, que não dispensam as mudanças no estilo de vida, são feitas com o uso de agentes que funcionam reduzindo a resistência arterial e/ou diminuindo o débito cardíaco. Isso é alcançado principalmente com o uso de diuréticos, inibidores da ECA, antagonistas da angiotensina II, beta-bloqueadores e bloqueadores de canais de cálcio. É comum que haja necessidade de associação entre mais de uma classe medicamentosa para que se alcance o efeito desejado (revisado em LAURENT, 2017). A seguir são descritas as duas das principais classes de fármacos comumente prescritos para a hipertensão cujos mecanismos de ação mais se relacionam com os objetivos deste trabalho.

2.3.1. Diuréticos

Estritamente falando, diuréticos são substâncias que agem no rim promovendo a eliminação de líquido do corpo pela urina (BRATER, 2000), o que leva à redução no volume de sangue circulante, reduzindo, portanto, a pressão sanguínea (GALLAGHER et al., 2006; WILLIAMS et al., 2004). Para serem clinicamente eficazes, no entanto, esses compostos devem induzir a perda de sódio (LALHOU et al., 2006). Isto é conseguido através de compostos que interferem com a reabsorção de íons, e consequentemente de água, através das paredes dos túbulos renais culminando com a sua eliminação do organismo (BRATER, 2000; MATERSON, 1983; PUSCHETT, 1994;).

Atualmente os diuréticos estão entre os medicamentos mais utilizados no mundo e, além de também serem úteis contra edemas e urolitíase, a sua principal aplicação é no controle de desordens cardiovasculares relacionadas à hipertensão (WRIGHT et al., 2007).

Os principais tipos de diuréticos podem ser divididos de acordo com seus locais de ação, sendo eles o túbulo contorcido proximal, a porção ascendente da alça de Henle, o túbulo contorcido distal e o ducto coletor. Com ação no túbulo proximal temos os diuréticos osmóticos,

que aumentam a osmolaridade da urina por serem filtrados no glomérulo e não reabsorvidos, e os inibidores da anidrase carbônica, já obsoletos. Na alça de Henle, temos os saluréticos potentes: os diuréticos de alça, como a furosemida. Eles inibem o cotransportador $Na^+/K^+/2Cl^-$, responsável pela reabsorção de sódio, potássio e cloreto, levando a uma grande concentração desses eletrólitos na urina, o que obriga a saída de água. No túbulo distal, os diuréticos tiazídicos (saluréticos moderados) inibem o cotranspotador Na^+/Cl^-, responsável pela reabsorção de sódio e cloreto, concentrando esses íons na urina e indiretamente estimulando a excreção de potássio. Por fim, no ducto coletor, os antagonistas do receptor de aldosterona inibem os canais reabsorvedores de sódio sensíveis à aldosterona. São pouco potentes, mas têm a vantagem de serem poupadores de potássio (MATERSON, 1983).

Apesar da boa eficácia dos diuréticos convencionais no controle da PA, eles não são isentos de efeitos colaterais. Os diuréticos denotados como perdedores de potássio, como os tiazídicos e os de alça, têm como principais efeitos colaterais a hipocalemia, que pode ocasionar desordens musculares e cardíacas, e a médio e longo prazo diabetes do tipo 2 (ZILLICH et al., 2006). Pouco se sabe sobre o mecanismo pelo qual o uso desses diuréticos está associado ao aparecimento da diabetes, mas sabe-se que quanto menor o valor do potássio sérico, menor o valor da insulinemia e, portanto, maior o valor da glicemia, o que, com o tempo, leva a alteração na tolerância à glicose (HOUSTON, 1988; MEISINGER et al., 2013).

Desta forma, a busca por novos agentes diuréticos é de grande demanda para a saúde pública, particularmente se tratando de plantas medicinais, pelas quais há um crescente interesse por seus benefícios. De fato, há um crescente número de artigos publicados sobre plantas ou princípios ativos derivados de plantas com uso popular como agentes diuréticos, sendo que a maior parte dessas pesquisas comprovou eficácia clínica dos agentes testados (WRIGHT et al., 2007).

2.3.2. Antagonistas do RAAS

Os principais representantes desse grupo, já estabelecidos amplamente na prática clínica, são os inibidores da ECA e os antagonistas de receptores AT_1. Ambas as classes têm efeitos finais semelhantes, reduzindo a influência do RAAS sobre a PA. Os efeitos hipotensores desses medicamentos são moderados em indivíduos normotensos, porém são pronunciados em hipertensos, principalmente os que apresentam níveis elevados de renina plasmática (RANG et al., 2012).

O antagonismo do RAAS é protetor contra a doença hipertensiva, tanto por manter a PA dos indivíduos em níveis próximos aos fisiológicos, prevenindo os danos diretos da hipertensão,

quanto por inibir a remodelação vascular e cardíaca. É sabido que a angiotensina II e a aldosterona são capazes de induzir diretamente a hipertrofia e hiperplasia da musculatura lisa vascular e a hipertrofia e fibrose miocárdica, condições que elevam a resistência vascular e a PA e facilitam o desenvolvimento de insuficiência cardíaca (ZHANG et al., 2008).

Os inibidores da ECA, cujos exemplos incluem o captopril e o enalapril, agem por prevenir a transformação da angiotensina I em angiotensina II pela ECA. A menor produção de angiotensina II promovida por esse processo também diminui a estimulação da produção de aldosterona (RANG et al., 2012). Estes medicamentos são bem tolerados e entre seus efeitos colaterais está a tosse seca e angioedema (mais raro) devido ao acúmulo de bradicinina (a kinase II, que é semelhante à ECA e é responsável pela degradação de cininas, também é inibida). Sugere-se que o aumento da disponibilidade de bradicinina, além de poder causar efeitos indesejados, seja um mecanismo de ação hipotensor adicional dessa classe de medicamentos por conta de a bradicinina ser um mediador vasodilatador (GAVRAS et al., 2002). Outros efeitos adversos incluem, insuficiência renal em pacientes com estenose das artérias renais e hipercalemia (KOSTIS et al., 2004; SCHOOLWERTH et al., 2001).

Os inibidores da ECA não são capazes de prevenir a produção de angiotensina II que é feita em menor escala por vias alternativas à ECA, como a quimase e outras proteases teciduais (PETRIE et al., 2001). Portanto, a inibição direta dos receptores AT_1 surge como solução a este problema. Como exemplos de antagonistas de receptores AT_1 temos as sartanas (losartana, valsartana, entre outras). Elas bloqueiam a atividade da angiotensina II diretamente nos seus locais de ação, por antagonizar seus receptores responsáveis por mediar, entre outros efeitos, a vasoconstrição e a estimulação da liberação de aldosterona (RANG et al., 2012). Estes fármacos, como dito anteriormente, se comportam de forma semelhante aos inibidores de ECA, porém sem acarretar o acúmulo de bradicinina, não desencadeando, portanto, os efeitos adversos tosse e angioedema. São raros seus efeitos colaterais, porém, como os inibidores da ECA, podem causar insuficiência renal em pacientes com estenose das artérias renais (SCHOOLWERTH et al., 2001).

No âmbito dos produtos naturais, vem sendo demonstrado que algumas plantas, principalmente as ricas em flavonoides, apresentam atividade anti-hipertensiva por atuar antagonizando o RAAS (XIE; ZHANG, 2012). Alguns flavonoides, como a isoquercitrina, já tiveram demonstrada atividade inibitória da ECA (GASPAROTTO JUNIOR et al., 2011). Portanto, esse alvo farmacológico demonstra grande valor na pesquisa com plantas medicinais.

3. OBJETIVOS

GERAL

Avaliar o potencial diurético, anti-hipertensivo e, em última instância, cardioprotetor do extrato da planta medicinal *Gomphrena celosioides* e elucidar seus possíveis mecanismos de ação.

ESPECÍFICOS

Verificar o efeito da administração aguda do extrato etanólico de *Gomphrena celosioides* (EEGC) em diferentes doses sobre a diurese de ratos normotensos.

Avaliar se o possível efeito diurético do EEGC se mantém duradouro.

Investigar a participação das vias do NO, da bradicinina e das prostaglandinas como mecanismos de ação do efeito diurético do EEGC.

Mensurar o potencial hipotensor da administração aguda do EEGC em diferentes doses em ratos com hipertensão renovascular.

Aferir o efeito diurético e hipotensor da administração prolongada do EEGC em animais com hipertensão renovascular.

Avaliar o efeito da administração prolongada do EEGC sobre a reatividade dos vasos de resistência do leito mesentérico a drogas vasoativas.

Investigar o potencial de inibição da ECA, de redução da concentração de aldosterona, da manutenção de NO e redução dos níveis de MDA como parâmetro de estresse oxidativo.

Verificar se o EEGC possui efeito cardioprotetor sobre animais hipertensos em administração prolongada.

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5. APÊNDICES

5.1. Artigo 1: Mechanisms Underlying the Diuretic Effect of *Gomphrena celosioides* Mart. (Amaranthaceae)

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Abstract

Ethnopharmacological relevance: *Gomphrena celosioides* (Amaranthaceae) is a native medicinal plant found in Mato Grosso do Sul State that is used for treating urinary tract and kidney stones. This study aimed to evaluate the diuretic effects of ethanolic extract from *G. celosioides* (EEGC) on acute and extended diuresis to provide a pharmacological basis for its use in traditional medicine.
Aim of the study: To evaluate the diuretic and natriuretic activity of EEGC and its mechanism of action in an animal model.
Materials and methods: EEGC (30, 100 and 300 mg/kg) was orally administered in male Wistar rats, and urinary excretion was measured at intervals of up to 8 h after administration. To evaluate participation of the nitric oxide (NO), prostaglandin and bradykinin pathways in its effect, respective inhibitors were also administered together with effectives doses of EEGC and compared with control groups. A 7-day model with daily administration and urine measurement was also carried out.
Results: Oral administration of doses of 100 and 300 significantly increased urine output after 8 h compared to the control group. It was observed this effect is dependent on the NO, prostaglandin and bradykinin pathways because their inhibitors reduced the diuretic effects of EEGC. Moreover, after 7 days of treatment, the effect was sustained and a decrease in serum aldosterone was observed in the extract group.
Conclusion: According to the results, *G. celosioides* extract showed diuretic and natriuretic effects associated with more than one mechanism of action. Considering that all diuretic drugs are currently available for the treatment of volume and electrolyte disturbances, especially hypertensive status, the present results may have clinical relevance and open new possibilities for the development of new natural diuretics from *G. celosioides*.

Keywords: *Gomphrena celosioides*; diuretic plant; natriuresis; aldosterone; hypertension.

1. INTRODUCTION

Hypertension is a predisposing factor for stroke, coronary heart disease, peripheral arterial disorders, heart failure and renal failure (Williams et al., 2004; Godfraind, 2006). Common clinical strategies to lower blood pressure include the use of agents that function by

reducing arterial resistance and/or decreasing cardiac output. Diuretics are among the drugs most used to promote increased urinary sodium and urine volume output, which leads to a reduction in the volume of circulating blood, thus reducing blood pressure (Williams et al., 2004; Gallagher et al., 2006)

Gomphrena celosioides Mart. belongs to the Amaranthaceae family and is a weed up to 20 cm tall and popularly known as "perpétua", "bachelor's button" or "prostrate globe-amaranth" (Myers et al., 2000). It is a native medicinal plant that is found and used in Mato Grosso do Sul State (Cunha and Bortolotto, 2011), where it was collected for the present study. It is also well distributed throughout South America, Asia, and East and West Africa (Takim et al., 2013). It is used in the treatment of various skin diseases and as an abortifacient in South America (Burkill, 1984). In Brazil, it has been employed to treat infectious and renal diseases as well as respiratory and gastrointestinal disorders. Gastroprotective effects were even scientifically shown (Oluwabunmi and Abiola, 2015). A decoction of the whole plant and of its related species *G. globosa* Linn. is applied to gangrenous wounds (Arenas and Azorearo, 1977). *Gomphrena* species are also employed in the treatment of bronchial disorders, diarrhoea, and fever and as an analgesic, tonic, diuretic and carminative (Vieira et al., 1994). The diuretic activity of *G. celosioides* was mentioned by Dhawan et al. (1977) and Chauhan et al. (2009). Whole *G. celosioides* juice with *Piper nigrum* and lemon juice is taken twice a day for 10 days to prevent kidney stones and expel them (Chauhan et al., 2009). *G. celosioides* activity in urolithiasis was also demonstrated in a scientific study on rats (Goswami and Srivastava, 2015), and its effectiveness was attributed to increased diuresis and lowering of urinary concentrations of stone constituents.

An initial phytochemical study with *G. celosioides* revealed the presence of saponins, steroids, amino acids, reducing and non-reducing sugars, phenols, flavonoids, betacyanins and ketoses (Botha and Gerritsma-van der Vijver, 1986). Fractionation of the extract of the aerial parts conducted by Moura et al. (2004) led to the isolation of 4-hydroxy-benzoic acid and 4-hydroxy-3-methoxybenzoic acid (or vanillic acid), stigmasterol, sitosterol, campesterol, methyl palmitate and stigmast-6-en-3-Ob-(D-glycopyranoside).

This study aimed to experimentally evaluate the diuretic and natriuretic potential of acute and extended treatment with the ethanolic extract of *G. celosioides* (EEGC) in rats, as well as to elucidate possible mechanisms of action.

2. MATERIAL AND METHODS

2.1. Plant material, extraction and ESI-MS/MS analysis

Aerial parts of *G. celosioides* were collected at Paranaíba, Mato Grosso do Sul, Brazil [*lat:* -19.666667 *long:* -51.183333 WGS84], in April of 1994 and 2014 and identified by Prof. Dr. Josafá Carlos de Siqueira, an expert in the family Amaranthaceae from Pontifical Catholic University of Rio de Janeiro (PUC-RJ), Brazil. A voucher specimen (SPFR-2962) was deposited in the herbarium of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo (FFCLRP/USP).

Air-dried powdered aerial parts (16.0 Kg) were exhaustively extracted (maceration at room temperature) with ethanol in the proportion of plant powder mass /solvent 1:2 (w/v). The spent biomass was filtered and the solvent was removed in a rotary evaporator under reduced pressure and temperature below 40ºC, yielding 901 g of ethanolic crude extract (EEGC).

EEGC (1 mg/mL) was diluted in a solution containing 50% (v/v) chromatographic grade methanol, 50% (v/v) deionized water and 0.5% ammonium hydroxide (Merck, Darmstadt, Germany). Fingerprinting ESI-MS analyses were performed according to Salvador et al. (2011) using an UPLC-MS instrument, model ACQUITY TQD (Waters Corporation, Milford, MA, USA), and the general conditions were: source temperature of 100 °C, capillary voltage of 3.0 kV and cone voltage of 30 V. ESI-MS was performed by direct infusion using a syringe pump with a flow rate of 10 μL. min/mL. Structural analysis of single ions in the mass spectra from the extract was performed by ESI-MS/MS. Ions with *m/z* of interest were selected and submitted to 15–45 eV collisions with argon in the collision quadrupole. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation. The compounds were identified by comparison of their ESI-MS/MS fragmentation spectra with fragmentation spectra of authentic standard samples and with literature data (Dosumu et al., 2014; Salvador et al., 2011; Moura et al., 2004).

The extract sodium and potassium content was determined by flame spectrometer and the results 0,47 mmol/g of sodium and 1,32 mmol/g of potassium were considered not interferent to the assessment of its diuretic effect.

2.2. Animals

Male *Wistar* rats (200 to 250 g) were used from the animal facilities of Universidade Federal da Grande Dourados (UFGD) an were acclimated under a temperature of 23 ± 2 °C, humidity of 60-80%, and 12-hour light/dark cycle; and they received food and water *ad*

libitum. The animals were handled according to internationally accepted standard guidelines for animal use, and all experiments obeyed experimental protocols previously approved by the Ethics Commission on the Use of Animals from UFGD (CEUA-UFGD) under process number 01/2015.

2.3. Single-dose model of diuretic assessment

Diuretic activity was determined according to the method of Kau et al. (1984) with some modifications. Rats, after fasting overnight with water *ad libitum*, were randomly divided into five groups (n = 5). Before treatment, all animals received an oral load of isotonic saline (0.9% NaCl, 50 ml/kg) to establish a uniform water and salt balance. Then, EEGC was administered by the oral route (p.o.) to animals at doses of 30, 100 and 300 mg/kg (EEGC30, EEGC100 and EEGC300). A negative control group received the same amount of vehicle, and a positive control group received 25 mg/kg of hydrochlorothiazide (HCTZ). Immediately afterwards, the animals were placed in metabolic cages. Urine was collected and measured after 1, 2, 4, 6 and 8 h. Cumulative urinary excretion was calculated relative to body weight and was expressed as ml/100 g.

2.4. Assessment of the involvement of the prostaglandin, bradykinin and NO pathways in the single-dose model

The dose of EEGC that was considered to be the best dose according to the previous assay was selected to evaluate the role of the prostaglandin, bradykinin and NO pathways on EEGC. A model similar to that above was constructed with twelve groups of animals divided into three treatment groups (vehicle, EEGC and HCTZ) for each of four pretreatments administered before the water and salt load: 1- indomethacin (prostaglandin synthesis inhibitor) 5 mg/kg, p.o.; 2- L-NAME (NO synthesis inhibitor) 60 mg/kg, p.o.; 3- HOE-140 (bradykinin antagonist) 1.5 mg/kg, i.p.; or 4- no pretreatment. Urine was collected at the same intervals as described above, and cumulative excretion was calculated.

2.5. Extended model with daily administration

The dose that was considered to be the best according to the previous assay was selected for an extended seven-day model with daily dosage. For this purpose, the experimental group vehicle, EEGC and HCTZ were placed individually in metabolic cages and received treatment orally once a day for seven days. The 24-hour urine of each animal was collected, its volume was measured daily during the experiment and it was stored for biochemical analysis.

2.6. Urine and serum analysis

Urine samples were collected from each animal using metabolic cages. Blood samples were obtained at the end of the experiments by caudal puncture and immediately transferred to paediatric tubes with separating gel. Serum was obtained by centrifugation for 10 minutes at 4000 rpm. Urine pH was measured with a standard pH metre, and urinary density was estimated by weighing samples using a digital analytical balance. Urine and/or serum Na^+, K^+, Cl^-, calcium, urea, creatinine, glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and total proteins were quantified by a Roche® cobas Integra 400 plus automated biochemistry analyser.

2.6.1. Angiotensin converting enzyme (ACE) assay and determination of aldosterone and vasopressin

Samples were collected from rats after 7 days of treatment with EEGC (100 mg/kg) or vehicle (control). Blood was collected into glass tubes after decapitation and serum was obtained by centrifugation (800 g, 10 min, 4 ºC). The aldosterone and vasopressin levels were measured by Enzyme Linked Immunosorbent Assay (ELISA, Immuno-Biological Laboratories, Inc.) and radioimmunoassay, respectively.

ACE activity was determined as previously described (Santos et al., 1985). Serum (10 µL) was incubated for 15 min at 37 °C with 490 µL of assay solution (composition: NaCl 0.9 M and Hip-His-Leu at 5 mM in 0.4 M sodium borate buffer, pH 8.3). The reaction was stopped by the addition of 1.2 ml of NaOH (0.34 M). The production of His-Leu was fluorometrically measured (365 nm excitation and 495 emission, Aminco Model J4-7461 fluoromonitor, American Instrument Co., Silver Springs, MD) after the addition of 100 µL of o-phthaldialdehyde (20 mg/ml in methanol) and 200 µL of HCl (3 M), followed by centrifugation (800 g, 5 min) at room temperature. To correct for intrinsic plasma fluorescence, zero-time blank samples were prepared by adding plasma after NaOH treatment. All measurements were performed in triplicate.

2.7. Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). Differences between means were tested by Student's *t* test or one-way analysis of variance (ANOVA) followed by Bonferroni's post-test. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Electrospray ionization mass spectrometry fingerprinting

ESI-MS fingerprints of the EEGC in negative mode showed a characteristic distribution of organic compounds with acidic sites, such as phenolic organic acids (Salvador et al., 2011; Moura et al., 2004). These analyses showed that the constituents detected in the analysed sample coincided with the mass of phenolic acids and flavonoids (Table 1, Figure 1).

Table 1: Compounds identified in ethanol extract of *Gomphrena celosioides* aerial parts (EEGC), using negative ion mode ESI-MS/MS.

Compound	Ethanol extract of *Gomphrena celosioides* (EEGC)	Deprotonated ions $[M-H]^-$, *m/z*	MS/MS ions *m/z*
Malic acid	+	133	25 eV: 133→115
Caffeic acid	+	179	25 eV: 179→135
Ferulic acid	+	195	25 eV: 193→178, 149,134
Catechin	+	290	25 eV: 290
Vanillic acid	+	165	25 eV: 165
Irisone B	+	297	25 eV: 297
Dimethoxy-flavone	+	329	25 eV: 329
Caffeoyl- glucose	+	339	25 eV: 339

+: detected compound.

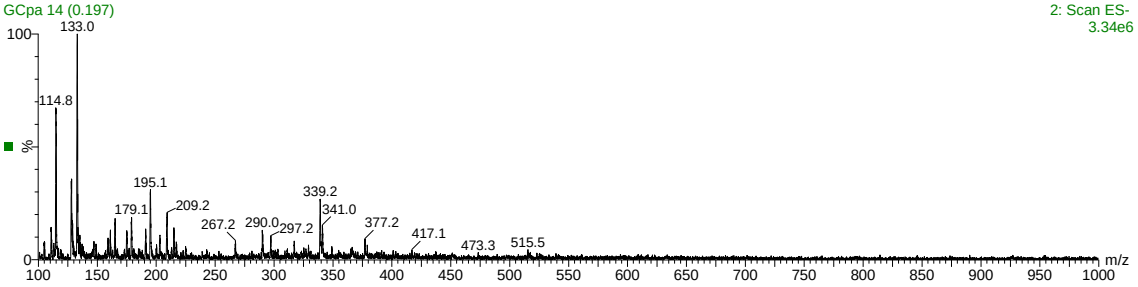

Figure 1: ESI(−)-MS fingerprints of ethanol extract of *Gomphrena celosioides* aerial parts (EEGC).

3.2. EEGC promotes diuresis and natriuresis in the single-dose model of diuretic assessment

After 8 h of diuresis monitoring, we observed that the urine volume, standardized by body weight, was significantly higher in the EEGC30 and EEGC300 groups compared to the control, by an amount similar to HCTZ (Figure 2). When we calculated the diuretic index (DI), we observed that EEGC100 and EEGC300 promoted a 1.74 ± 0.25 and 1.86 ± 0.28-fold increase in urine volume output, respectively, compared to control, while HCTZ's index was 1.75 ± 0.08. Time course curves showed that the extract started to promote effects from 6 h on, whereas HCTZ was effective after the second hour (Figure 2). Urine sodium (UNa) was also significantly higher in the EEGC100 (1.97 ± 0.25-fold) and EEGC300 (2.25 ± 0.38-fold) groups compared to the control and was even higher in the HCTZ (2.75 ± 0.20-fold) group (Figure 3). The urine K^+ and Cl^- values were only significantly modified by HCTZ (2.42 ± 0.13 and 2.54 ± 0.18 fold, respectively), while urine calcium, pH and density and were similar in all groups (data not shown). Serum electrolytes, urea and creatinine were not different among the groups either (data not shown). After this assay, we selected EEGC100 as the best dose for further analysis because it showed significant diuretic and natriuretic effects that were very close to the threefold higher dose EEGC300. Hence, EEGC100 is hereafter referred to as EEGC.

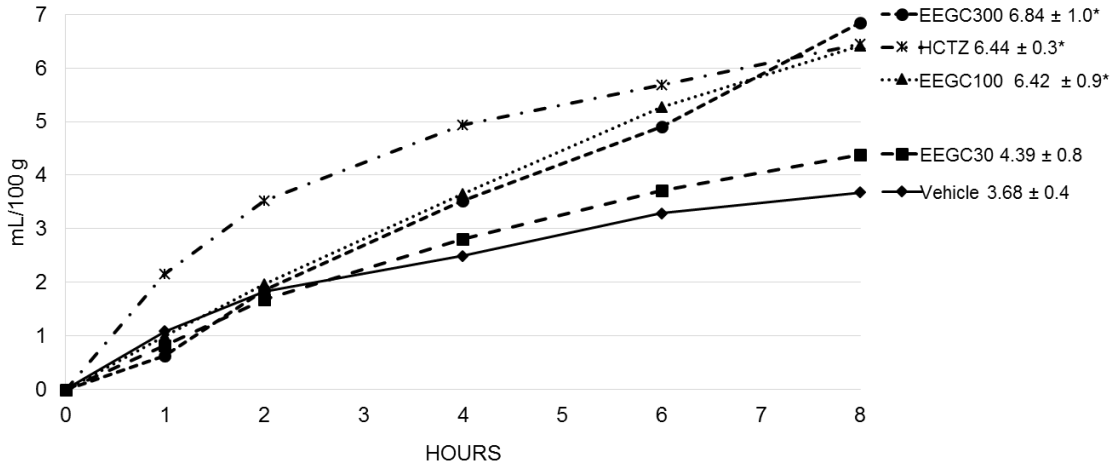

Figure 2: Cumulative urine volume from single-dose model. Results are mean ± S.E.M., n = 7. One-way ANOVA followed by Bonferroni's post-test; * means $p<0.05$ comparing to vehicle group. EEGC30, 100 and 300 = Ethanolic Extract of *Gomphrena celosioides* 30, 100 and 300 mg/kg respectively. HCTZ = Hydrochlorothiazide.

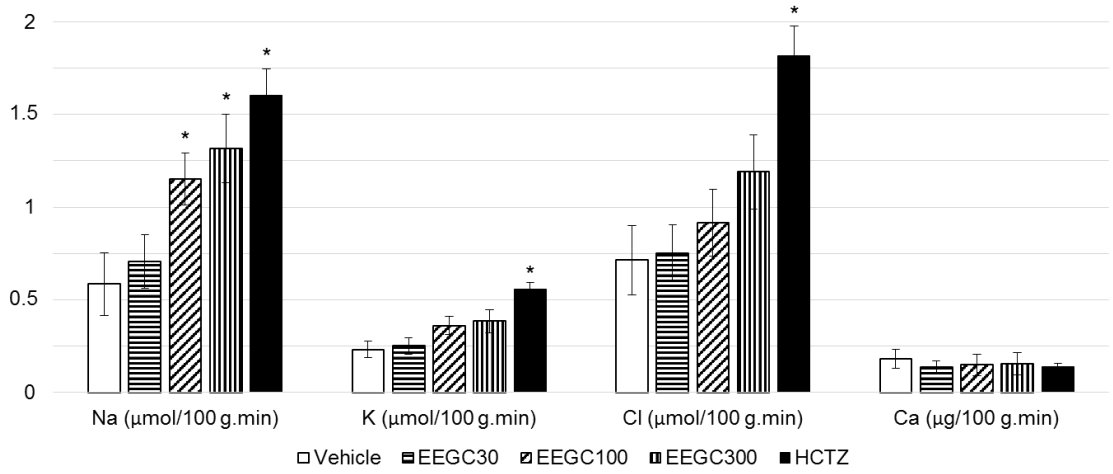

Figure 3: Urine electrolytes from single-dose model. Results are mean ± S.E.M., n = 7. One-way ANOVA followed by Bonferroni's post-test; * means $p<0.05$ comparing to vehicle group. EEGC30, 100 and 300 = Ethanolic Extract of *Gomphrena celosioides* 30, 100 and 300 mg/kg respectively. HCTZ = Hydrochlorothiazide.

3.3. L-NAME, indomethacin and HOE-140 can diminish the diuretic and natriuretic effects of EEGC

After pretreatment with L-NAME, indomethacin or HOE-140, EECG failed to significantly promote diuresis or natriuresis (Figure 4). After pretreatment with indomethacin, the EEGC group additionally showed significantly lower diuresis and natriuresis compared to the non-pretreated EEGC group. This was not found in the L-NAME – EEGC or HOE-140 – EEGC groups, that were not significantly different from the respective EEGC groups without pretreatment. Only the HCTZ groups were able to increase diuresis, urine Na^+, (Figure 4) K^+, and Cl^- (data not shown) regardless of pretreatment. Other urine parameters and all serum parameters (data not shown) remained unaltered, except that the indomethacin and HOE-140 pretreated groups showed lower urinary calcium compared to the non-pretreated groups, which is expected as an effect of low renal prostaglandins (Gomaa et al., 1990).

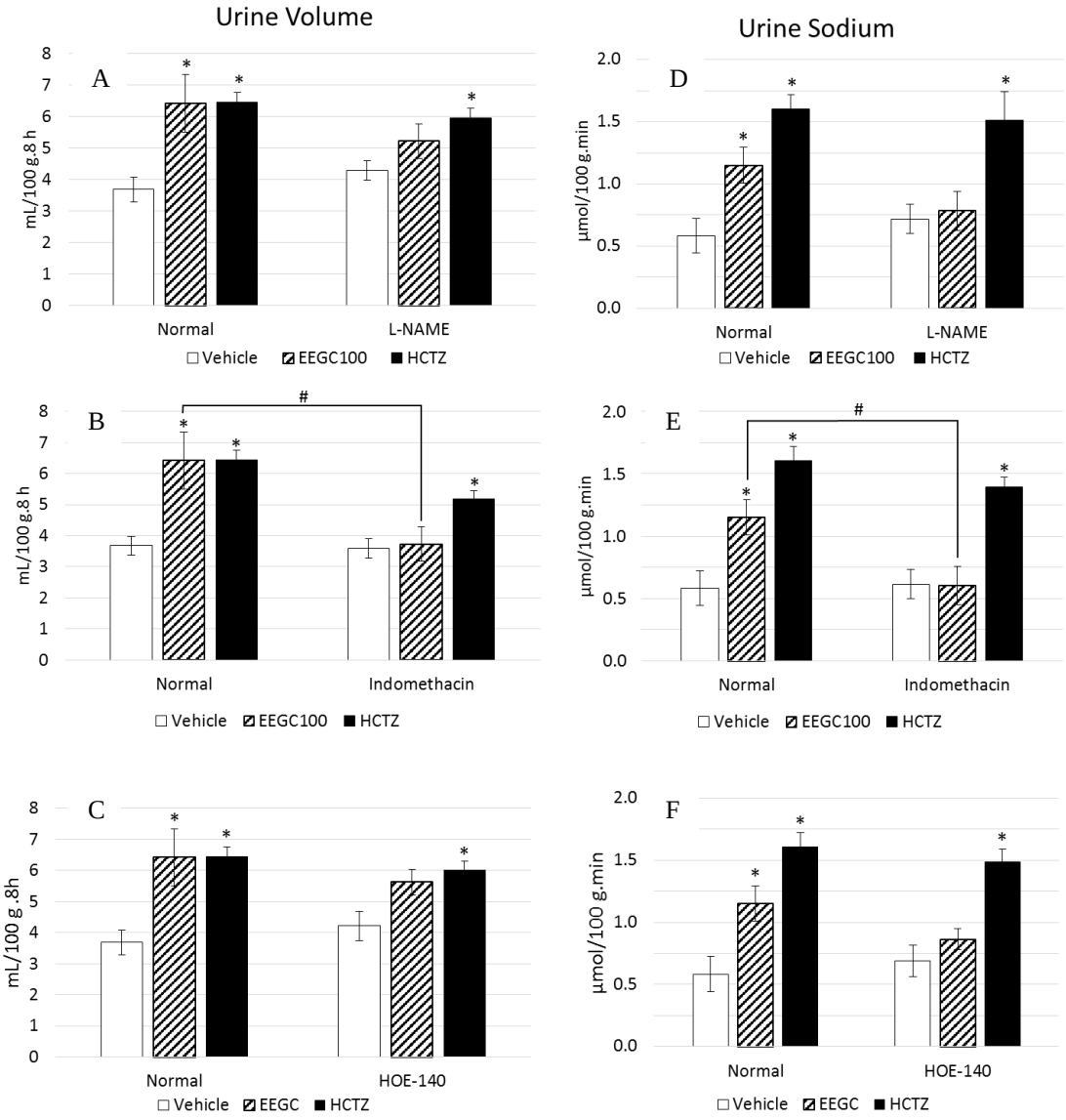

Figure 4: Urine Volume (A, B, C) and Sodium (D, E, F) from single-dose model for assessment of NO (A, D), prostaglandins (B, E) and bradykinin (C, F) pathways involvement, without pretreatment (Normal) or after pretreatment with L-NAME, Indomethacin or HOE-140. Results are mean ± S.E.M., Normal groups: n = 7, others: n = 6. One-way ANOVA followed by Bonferroni's post-test; * means $p<0.05$ comparing to same pretreatment vehicle group. # means $p<0.05$ comparing to correspondent non-pretreated group (Normal). EEGC = Ethanolic Extract of *Gomphrena celosioides* 100 mg/kg. HCTZ = Hydrochlorothiazide.

3.4. Diuretic and natriuretic effects of EEGC are sustained during 7-day treatment in the extended model with lower aldosterone levels

During the 7 days of treatment with vehicle, EEGC or HCTZ, we observed sustained increases of UV and UNa in the groups treated with EEGC and HCTZ compared to vehicle

(Figure 5). The mean diuretic index was 1.29 ± 0.08 for EEGC and 1.40 ± 0.09 for HCTZ. Water intake was uniform among the groups (Figure 5).

After 7 days of treatment in the extended model, analysis of blood samples showed significantly lower aldosterone levels in the EEGC group compared to vehicle. ACE activity was numerically lower in the EEGC group compared to vehicle, but not significantly different (p = 0.06) (Figure 6). The vasopressin levels were similar in all three groups (Figure 6), as were all of the serum biochemical parameters analysed (data not shown).

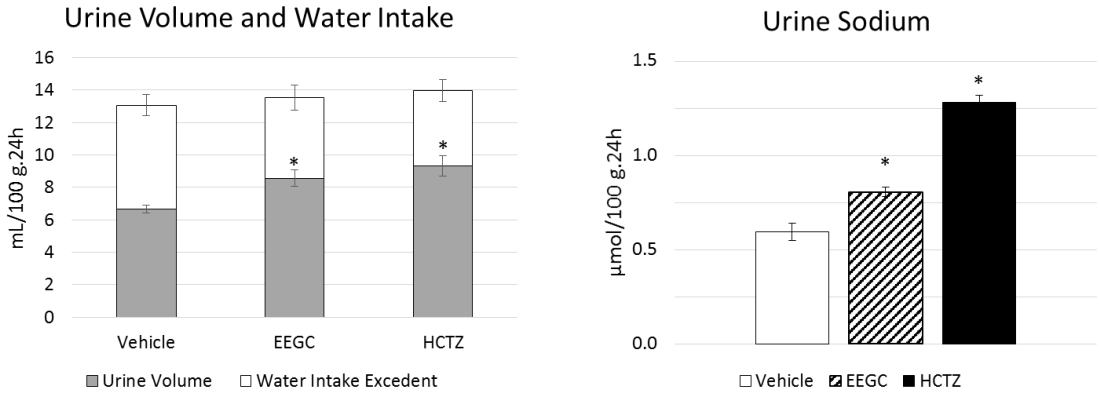

Figure 5: Urine Volume (gray), water intake (white + gray) (Panel A) and urine Sodium (Panel B) from extended model with daily doses. Results are mean ± S.E.M., n = 6. One-way ANOVA followed by Bonferroni's post-test; * means p<0.05 comparing to vehicle group. EEGC = Ethanolic Extract of *Gomphrena celosioides* 100 mg/kg. HCTZ = Hydrochlorothiazide.

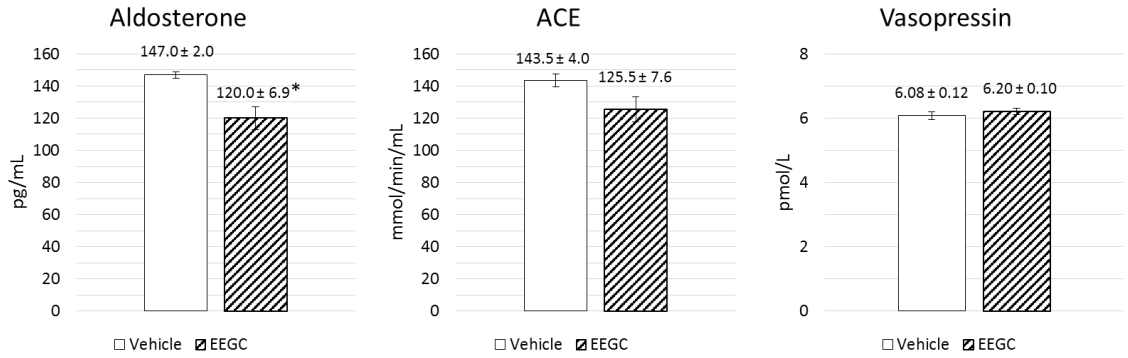

Figure 6: Serum Aldosterone, ACE and Vasopressin from extended model with daily doses. Results are mean ± S.E.M., n = 6. Student's t test; * means p<0.05 comparing to vehicle group. EEGC = Ethanolic Extract of *Gomphrena celosioides* 100 mg/kg. HCTZ = Hydrochlorothiazide. ACE = Angiotensin Converting Enzyme Activity.

4. DISCUSSION

To our knowledge, this is the first study to show the diuretic activity of the *Gomphrena celosioides* plant in detail and to seek to understand some of the mechanisms of its action.

With acute administration of its ethanolic extract, we observed relatively rapid diuretic action with statistical significance after 8 h. Although it takes longer to act than the standard drug used as a reference, hydrochlorothiazide, the magnitude of the effect of the extract after 8 h at doses of 100 and 300 mg/kg closely resembles the drug, which is an interesting finding from the point of view of clinical use. A significant effect was also observed for natriuresis, but with a slight loss potassium, chloride or calcium in the urine, which can be an advantage similar to that of commercial potassium-sparing diuretics.

With the observations that the administration of 100 mg/kg EEGC presented significant effects compared to the vehicle group and that this effect was similar to the three-fold higher dose, it was selected for use in subsequent experiments. Intending to evaluate the participation of the prostaglandin, NO and bradykinin pathways, we checked whether EEGC sustained its effects in the presence of inhibitors of these pathways. In fact, the three inhibitors reduced or eliminated the diuretic and natriuretic effects of the extract, showing that these pathways are important to its mechanism of action.

Bradykinin interferes with the homeostasis of renal blood flow, increasing the local release of NO and prostaglandins and causing vasodilation and increased glomerular filtration. The degradation is carried out by angiotensin converting enzyme (ACE), a fact that explains why increased activity of bradykinin is implicated as one of the antihypertensive action mechanisms of ACE inhibitors (for review see Manolis et al., 2010). It has been postulated that some plants rich in polyphenols have diuretic and antihypertensive effects that act in this system, for example, inhibiting ACE, thus increasing the local availability of bradykinin (Gasparotto Jr. et al., 2012; de Souza et al., 2013; Prando et al., 2016). This increased availability of bradykinin probably contributes to the effects of EEGC as well as the increase of prostaglandins because the inhibition of prostaglandin production by indomethacin also reduced the diuretic effect of the extract.

Nitric oxide also plays a key role in the regulation of the vascular tone, promoting vasodilatation of the afferent renal arterioles and the resulting increase in glomerular filtration. The production of NO is increased by eNOS, which is one of the mechanisms of action of the renal activity of bradykinin (Chappell, 2012). We observed that its presence is also important for the role of EEGC. A possible increase in the availability of bradykinin has the ability to

raise the levels of NO to contribute to diuresis, which would explain the usefulness of this mediator in the plant's mechanism of action. Moreover, NO appears at lower levels in the presence of oxidative stress. It is likely that as this plant is rich in polyphenols, it has important antioxidant effects, contributing to the maintenance of the levels of NO, thus aiding in the diuretic effect.

Another important finding from this study was that the diuretic and natriuretic effects of EEGC remain present for several days during prolonged treatment without upregulation of water intake, as was seen in the seven-day model. This means that, at least within seven days, there is no compensatory mechanism of the body that is able to counteract its effect. In addition, renal toxicity was not observed within the dose tested since the levels of urea and creatinine remained unaltered.

After evaluating the serum of animals treated for seven days, we found reduced levels of aldosterone in the EEGC group compared to the vehicle group. Although the ACE activity was not significantly different between the two groups, it is also possible that with a statistical p-value = 0.06, the extract exerted some ACE inhibitory activity, which would explain the reduced concentration of aldosterone and the consequent increase in natriuresis and also corroborate the notion that the extract causes increased availability of bradykinin. This activity would be of great clinical utility, resembling one of the most used classes of commercial drugs: ACE inhibitors.

Previous chemical studies with species of this genus were related to the isolation of steroids, terpenoids, ecdysteroids, flavonoids, aurantiamide and protoalkaloids Salvador et al., 2012. Even though some phytochemical and biological activity studies have been performed in certain species, overall, the genus *Gomphrena* still remains poorly studied. Its aerial parts are rich in phenolic acids and flavonoids, chemical compounds that may act synergistically and contribute to the diuretic profile of *G. celosioides*. According to ESI-MS fingerprinting, it was possible to identify the presence of phenolic acids and flavonoids, which could explain, in part, the observed effects.

Based on this information, we can state that the ethanol extract of EEGC has pronounced and prolonged diuretic and natriuretic activities and that this effect is probably due to downregulation of aldosterone, involving local maintenance of bradykinin and prostaglandins as well as local maintenance of NO. Future studies are needed to investigate the usefulness of this diuretic effect in the treatment of hypertension.

Acknowledgements

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5.2. Artigo 2: Diuretic herb *Gomphrena celosioides* Mart. (Amaranthaceae) promotes sustained arterial pressure reduction and cardioprotection on rats with renovascular hypertension

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Abstract

Ethnopharmacological relevance: *Gomphrena celosioides* Mart., belonging to the Amaranthaceae family, is a weed known as "perpétua," and its ethnopharmacological use is to treat of urinary tract disorders and kidney stones. Urinary tract disorders and kidney stones could include several pathological conditions such hypertension, diuretic and lithiasic problems. In the present work a model of renovascular hypertension was developed *in vivo* to investigate its usefulness as an antihypertensive drug.

Aim of the study: Evaluate the effect of acute and 28 day oral administration of *G. celosioides* extract on systemic arterial pressure and diuresis of renovascular-hypertensive rats, as well as its effect on cardiac remodeling and vascular reactivity.

Materials and Methods: Ethanolic extract of *G. celosioides* (EEGC) was used. To induce renovascular hypertension, adult male Wistar rats were submitted to Goldblatt 1K1C or 2K1C surgery. The mean arterial pressure (MAP) of 1K1C animals was directly assessed by cannulation of the carotid artery before and after intraduodenal acute administration of 30, 100 or 300 mg/kg of EEGC. For the 4-week assay, 2K1C animals received daily treatments with water (control group), 100 mg/kg EEGC or 15 mg/kg enalapril for 28 days. Diuresis and caudal blood pressure were assessed weekly, and at the 28th day of treatment, the MAP was directly quantified shortly before euthanasia. Internal organs were removed, weighed and routinely processed for histology and the left ventricle wall was measured. Blood was collected for biochemical analysis and mechanism investigation by quantification of angiotensin converting enzyme (ACE) activity and aldosterone, nitrite and thiobarbituric acid reactive substances (TBARS) concentration. The rats' mesenteric beds were isolated and cannulated to have their pressure variation assessed after crescent doses of phenylephrine (Phe), acetylcholine (ACh) and sodium nitroprusside (SNP).

Results: EEGC acutely reduced MAP the dose of 100 mg/kg. In the 4-week assay, EEGC acted as diuretic after acute administration after 1, 2, 3 and 4 weeks of treatment. EEGC also acted as an antihypertensive and it showed significant difference already after 1 week (and after 3 and 4 weeks) compared to control, with its MAP close to pre-surgery values at the end of the experiment. It promoted ACE inhibition, which led to lower aldosterone levels. The lower TBARS and higher nitrite concentration found in the EEGC group suggest antioxidant activity and NO maintenance. Moreover, EEGC counteracted the impairment of vascular reactivity induced by renovascular hypertension. Cardioprotection in the extract group was confirmed by a thinner left ventricle wall compared to the control.

Conclusions: The *G. celosioides* diuretic effect is maintained on renovascular hypertensive rats and can reduce the blood pressure after the first week of treatment by inhibiting ACE and these

effects are longstanding and strong enough to promote cardioprotection. Therefore, it shows potential as an antihypertensive drug.

Keywords: herbal medicine; diuretic; antihypertensive; ace inhibitor; 2k1c

1. INTRODUCTION

Gomphrena celosioides Mart. (Amaranthaceae) is a weed up to 20 cm tall and popularly known as “perpétua”, “bachelor’s button” or “prostrate globe-amaranth” (Myers et al., 2000). It is a native medicinal plant that is found and used in Mato Grosso do Sul State (Cunha and Bortolotto, 2011). It is rich in phenolic acids and flavonoids (Vasconcelos et al., 2017), and it has several popular uses worldwide (Burkill, 1984; Takim et al., 2013; Vieira et al., 1994). Prachi et al. (2009) and Sharma and Vijayvergia (2011) reported the wide and effective use of the extract of *G. celosioides* in folk medicine in the treatment of urinary tract disorders and kidney stones. Renal and urinary tract disorders include several pathological conditions such as hypertension, diuretic and lithiasic problems.

Diuretic activity from this plant had already been mentioned (Prachi et al. 2009; Dhawan et al. 1977) and recently was investigated in detail by our research group. It was determined that the ethanolic extract of *G. celosioides* oral administration can promote sustained increases in diuresis and natriuresis and decrease the aldosterone levels and that this effect is dependent on the prostaglandin (PG), bradykinin and nitric oxide (NO) pathways (Vasconcelos et al., 2017).

Hypertension is a major public health problem and predisposes the occurrence of stroke, coronary heart disease, peripheral arterial disorders, heart failure and renal failure (Williams et al., 2004; Godfraind, 2006). Experimental models of hypertension are commonly used to investigate new drugs and the effects of elevated blood pressure on target organs. Renovascular hypertension is a useful model that satisfactorily mimics the development of human primary hypertension and allows the evaluation of drugs cardioprotective effect (Sakironda et al., 2009).

One of the most widely used class of drugs in the world against cardiovascular disorders related to hypertension is the diuretics class (Wright et al., 2007). Diuretics promote increased urinary sodium and urine volume output, which leads to a reduction in the volume of circulating blood, thus reducing blood pressure (Williams et al., 2004; Gallagher et al., 2006).

Angiotensin-converting enzyme (ACE) inhibitors, as well as angiotensin II receptor antagonists, are also used in the treatment of hypertension by lowering the activity of the renin-angiotensin system (RAS) (Williams et al., 2004). Increased RAS activity is associated not only with the pathophysiology of chronic hypertension but also with the process of myocardial

hypertrophy and remodeling, by the action of both angiotensin II and aldosterone (Barauna et al., 2008; Iwai et al., 1995, Zhang et al., 2008).

Therefore, the finding that *G. celosioides* acts as diuretic and decreases the aldosterone levels raises the following questions. Are these effects able to lower blood pressure? More so, are these effects able to treat hypertensive individuals leading to significant cardioprotection?

With these questions in mind, the aim of this study is to evaluate the effect of the ethanolic extract of *Gomphrena celosioides* (EEGC) on the diuresis and blood pressure of renovascular-hypertensive rats by acute administration and 4-week treatment, as well as to evaluate its possible mechanisms of action and cardioprotective potential.

2. MATERIAL AND METHODS

2.1. Plant material and extraction

Aerial parts of G. celosioides were collected at Paranaíba, Mato Grosso do Sul, Brazil [lat: -19.666667 long: -51.183333 WGS84] in April 1994 (in 2014, the same plant material with similar chemical profile and bioactivity was also collected in April for extract preparation) and identified by Prof. Dr. Josafá Carlos de Siqueira from the Pontifical Catholic University of Rio de Janeiro (PUC-RJ), Brazil. A voucher specimen (SCAB 4051) is deposited in the herbarium of Pontifical Catholic University, Rio de Janeiro.

Air-dried powdered aerial parts (16.0 kg) were exhaustively extracted (maceration at room temperature) with ethanol in a proportion of plant powder mass /solvent 1:2 (w/v). The spent biomass was filtered, and the solvent was removed in a rotary evaporator under reduced pressure and temperature below 40°C, yielding 901 g of ethanolic crude extract (EEGC).

The extract ESI-MS/MS fingerprint analysis and sodium and potassium content were previously described (Vasconcelos et al., 2017).

The oral doses of the extract (30, 100 and 300 mg/kg) were selected to cover a logarithmic range within which crude extracts tend to have pharmacologic action without significant adverse effects. Moreover, it was based on our previous studies with this extract (Vasconcelos et al., 2017).

2.2. Animals

All the experiments used a total of 51 male *Wistar* rats (200 to 250 g), from the animal facilities of Universidade Federal da Grande Dourados (UFGD). They were acclimated under a temperature of 23 ± 2 °C, humidity of 60-80%, and 12 h light/dark cycle. Food and water

were given *ad libitum*. All experiments obeyed experimental protocols previously approved by the Ethics Commission on the Use of Animals from UFGD (CEUA-UFGD) under process number 01/2015 and the animals were handled according to internationally accepted standard guidelines for animal use

2.3. Renovascular hypertension induction

The method first described by Goldblatt et al. (1934) was used with some modifications. The 1K1C (1 Kidney 1 Clip) method was used for the acute model and 2K1C (2 Kidneys 1 Clip) was used for the 4-week treatment model due to better animal survivability. For the 2K1C surgery, the rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) (i.p.) and suffered an incision in the left side of the abdomen for renal artery exposure. A silver clip of 0.2 mm internal diameter was placed over the left renal artery near the aorta leading to a constriction greater than 50% and the incision was sutured. For the 1K1C model, in addition to left renal clipping, the right kidney was completely excised. Operated animals rested for 28 days to establish hypertension.

2.4. Acute model of direct blood pressure measurement on 1K1C rats

The method described by Souza et al. (2011) was used. For this measurement, 1K1C hypertensive rats (as described above) were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) (i.m.). Then, 30 IU of heparin (s.c.) was administered. The animals were kept under spontaneous breathing. The left carotid artery was cannulated with a 0.7 x 19 mm catheter and connected to a pressure transducer linked to a monitoring system associated with its software, allowing the recording of the mean arterial pressure (MAP). For the stabilization of blood pressure after the surgical procedure, a 15-min interval was adopted prior to the injection of any drug. After this, the animals received EEGC (30, 100 or 300 mg/kg diluted in distilled water; final volume: 300 µl) by intraduodenal injection as an alternative enteral route, since the animals were anesthetized, therefore unable to receive oral administration. The MAP was then monitored for the following 30 min. At the end of the experiments, the animals were euthanized with a halothane overdose. Since MAP was measured before and after treatment, each rat served as its own reference control.

2.5. 4-week model of daily administrations on hypertensive rats

2.5.1. Group design

This model was used to mimic chronic hypertension treatment, as its duration is sufficient for the renovascular hypertension to induce cardiac remodeling (de Simone et al., 1992). The 2K1C hypertensive rats were distributed in 15 pairs of animals, all of which had a similar combined baseline urinary excretion volume (UV). These 15 pairs were then randomly divided into 3 groups with similar mean baseline MAPs. The first group received distilled water (control), the second one received enalapril 15 mg/kg (100 µl/kg in distilled water) and the third received the best dose of EEGC selected in our previous studies and confirmed in the acute model (100 mg/kg, 100 µl/kg in distilled water). The treatments were given orally once a day for 4 weeks. Diuresis and blood pressure were assessed weekly, as described below.

2.5.2. Diuretic assessment

Diuretic activity was determined as described by Kau et al. (1984) with some modifications. Before the beginning of the daily treatments, the 2K1C rats, after fasting overnight with water *ad libitum*, received an oral load of isotonic saline (0.9% NaCl, 50 mL/kg) to establish a uniform water and salt balance. Then, the animals were placed in metabolic cages for the collection of urine for 8 h to determine the baseline UV, which was calculated relative to the body weight and was expressed as mL/100 g. The rat with the highest urine volume was paired with the one that had the lowest urine volume; the second highest one with the second lowest one, etc. in order to obtain similar UVs and reduce deviation. After the beginning of the treatments, diuresis was assessed acutely and then once a week using the same method, now placing the pairs of animals in the metabolic cages (see topic 2.5.1).

2.5.3. Blood pressure assessment

The 2K1C rats had their baseline MAP, systolic (SBP) and diastolic (DBP) blood pressure assessed with tail cuff equipment (Letica Scientific Instruments), and the animals with SBPs lower than 130 mm Hg were excluded from the experiment. Then, after the beginning of the treatments, they had their MAP, SBP and DBP measured again after 1 and 3 weeks of treatment. At the end of the 4-week experiment, their direct MAP was measured by carotid cannulation similar to the acute model.

2.5.4. Urine and serum analysis

Urine samples were collected from each animal using metabolic cages. Blood samples were obtained at the end of the experiment from the cannulated carotid artery and immediately

transferred to analytic tubes with separating gel. Serum was obtained by centrifugation for 10 min at 4000 rpm. Urine Na^+, K^+, Cl^- and calcium, and serum Na^+, K^+, Cl^-, calcium, urea, creatinine, glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), creatine kinase (CK) and creatine kinase MB (CK-MB) were quantified by a Roche® cobas Integra 400 plus automated biochemistry analyzer.

2.5.5. Mechanisms investigation: ACE activity, aldosterone, nitrite and TBARS

Serum ACE activity was determined by indirect fluorimetry according to the methods described by Santos et al. (1985). Aldosterone levels were measured by an Enzyme Linked Immunosorbent Assay (ELISA, Immuno-Biological Laboratories, Inc). Thiobarbituric acid reactive substance (TBARS) levels were measured using TBARS assay kits (Cayman Chemical, Ann Arbor, Michigan, USA) according to the manufacturer's instructions. Finally, the plasma nitrite concentration was determined by enzymatically reducing nitrate according to the technique described by Schmidt et al. (1989).

2.5.6. Isolation of the mesenteric bed and assessment of vascular reactivity to phenylephrine, acetylcholine and sodium nitroprusside

Four weeks after initiation of the protocols, the animals were anesthetized with a combination of ketamine (100 mg / kg) and xylazine (20 mg / kg) and the mesenteric beds were rapidly isolated and prepared for infusion as previously described (Kawasaki et al. 1988; Kawasaki et al., 1991). The superior mesenteric artery was cannulated and washed gently with a solution of PSS (physiological saline) (pH 7.4, composition in mM: 119 NaCl, 4.7 KCl, 2.4 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, 0.03 EDTA and 11.1 dextrose) to remove the blood from the inside. After removal of the entire intestine and associated vascular bed, the mesenteric bed was gently separated. The isolated mesenteric beds were packed in glass vats, kept at 37 ° C, aerated with carbogen solution (95% O_2 and 5% CO_2) and perfused at a constant flow of 4 mL/min with a PSS solution and the aid of a peristaltic pump. A force transducer connected to a signal amplifier and coupled to a computer with specific software determined changes in the perfusion pressure.

After a period of stabilization of the infusion pressure (~ 28 mm Hg; 15 min) a dose-response curve of phenylephrine (1, 3, 10 and 30 nmol) was obtained in the preparations from the animals of each experimental group (control, enalapril and EEGC). A fourth group of three untreated non-operated normotensive rats was included for reference. Then, the preparations were subjected to an increase in perfusion pressure (approximately 90 mm Hg) by infusion of a PSS solution containing phenylephrine (10 μM). After the stabilization period, two dose-

response curves were performed, one with acetylcholine (ACh) and one with sodium nitroprusside (SNP) (both 3, 10, 30, 100, 300 and 1000 nmol) to produce vascular endothelium-dependent and independent relaxation, respectively, consequently dropping perfusion pressure.

2.5.7. Organ weighing and histopathology

At the end of the 4-week experiment, the heart, liver and kidneys were isolated for weighing and were routinely processed to histological slides that were stained in hematoxylin and eosin. The heart was cross-sectioned to display the left ventricle wall and to enable the measurement of its thickness using standard image analyzing software. The resulting weight of the organs was calculated relative to the body weight. Histopathological analysis was performed by two pathologists that were unaware of the experimental groups.

2.6. Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). Differences between the means were tested by Student's *t* test for the acute model. For the extended model, one-way analysis of variance (ANOVA) was followed by Dunnett's post-test. When there were data representing the different time points of the same group, two-way ANOVA was used followed by Dunnett's or Tukey's post-test for multiple comparisons and the data was treated as paired. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. 1K1C and 2K1C surgery successfully promoted renovascular hypertension

Before the renal clipping surgeries, the naive rats' mean MAP (mm Hg) was 92.74 ± 2.09. 1K1C operated animals 28 days after surgery presented a strongly augmented MAP of 167.90 ± 4.4, while 2K1C animals showed a mean MAP value of 111.83 ± 2.12.

3.2. EEGC lowers MAP of hypertensive rats after acute treatment

The direct MAP measurement before and after the intraduodenal administration of 30, 100 or 300 mg/kg of EEGC on 1K1C rats showed that the extract was capable of significantly lowering MAP at the doses of 100 and 300 mg/kg, presenting a classical dose/response curve (Figure 1). At 100 mg/kg EEGC, MAP was lowered (expressed as mm Hg) from 165.1 ± 7.7 to 128.4 ± 8.8, while the 300 mg/kg dose brought a baseline MAP of 173.5 ± 8.3 to 135.3 ± 7.8. This meant a MAP reduction of 36.7 and 38.2 mm Hg, respectively (Figure 1). After this assay,

we selected 100 mg/kg as the best dose of the extract for further analysis because it showed significant hypotensive effect that was very close to the threefold higher dose, in addition to being the same dose used in our previous studies with this extract.

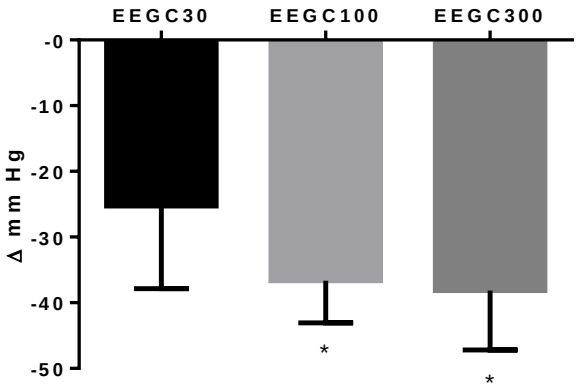

Figure 1: Mean arterial pressure reduction after acute administration of 30, 100, and 300 mg/kg of EEGC on 1K1C rats. Student's *t* test; * means $p < 0.05$ comparing the MAP before and after treatment; n = 5, 6 and 7 for, 30, 100, and 300 mg/kg. EEGC30, 100 and 300 = ethanolic extract of *Gomphrena celosioides* 30, 100 and 300 mg/kg.

3.3. EEGC promotes sustained augmented diuresis and natriuresis on 2K1C-operated rats

G. celosioides extract acted as a diuretic after acute treatment and after 1, 2, 3 and 4 weeks of treatment, leading to a mean 8 h-diuresis (mL/100 g) of 6.42 ± 0.16 (baseline 4.99 ± 0.04), against 4.84 ± 0.13 from the control group (baseline 4.81 ± 0.13) (Figure 2). Interestingly, at this model, enalapril reduced the renal function with mean values of 8 h- diuresis of 3.35 ± 0.18 (baseline 5.03 ± 0.06). EEGC also significantly increased the urine sodium excretion (UNa) compared to the control group. EEGC mean UNa (µmol/100 g.min) was 1.45 ± 0.05 (baseline 1.04 ± 0.04), while the control group was 0.99 ± 0.04 (baseline 0.94 ± 0.07) (Figure 2). Urine potassium and chloride were not significantly different from the control (data not shown), and none of the serum parameters tested were different among the groups (data not shown), except that the urea and creatinine levels were significantly higher in the enalapril group compared to the control (creatinine: enalapril = 0.47 ± 0.04 mg/dL vs control = 0.34 ± 0.03 mg/dL; urea: enalapril = 71.0 ± 7.0 mg/dL vs control = 50.7 ± 7.0 mg/dL).

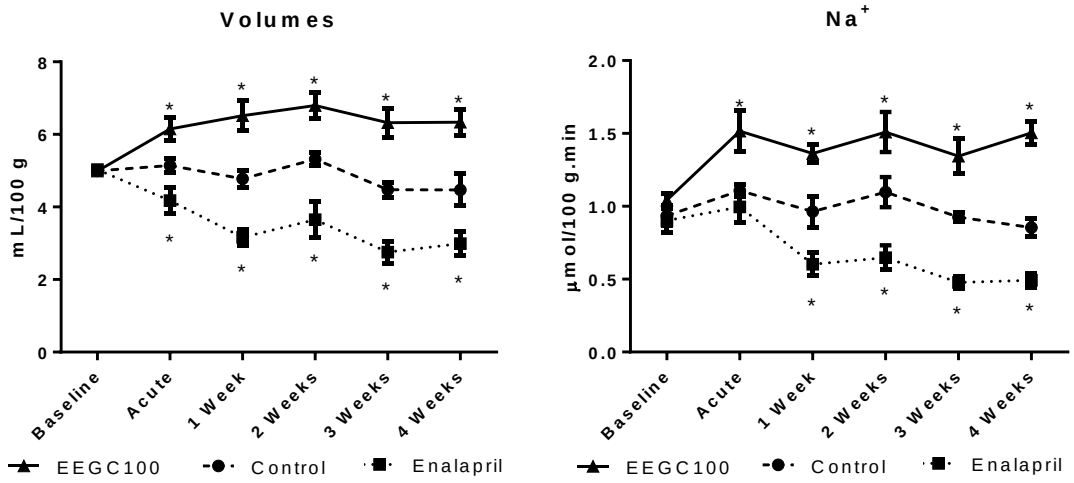

Figure 2: Urine volume and sodium from the 2K1C rats of the 4-week model. The results are the mean ± S.E.M. Two-way ANOVA followed by Dunnett's post-test for multiple comparisons; * means $p < 0.05$ comparing the control group at the same time point; n = 5 (pairs of rats); EEGC100 = ethanolic extract of *Gomphrena celosioides* 100 mg/kg.

3.4. EEGC extended treatment maintains the MAP of 2K1C-operated rats' at lower levels comparing to the control

EEGC acted as an antihypertensive and already showed significant difference after 1 week of treatment (and also after 3 and 4 weeks) compared to the control, with its MAP values (mm Hg) ranging from baseline 111.7 ± 2.7 to 102.1 ± 3.0 at the first week and 92.2 ± 3.8 at the end of the experiment, which was not different from the MAP values before surgery (see topic 3.1.), although the pressure reduction was significantly lower than the enalapril group. The control group ranged from baseline 113.5 ± 2.7 to 117.2 ± 6.7 at the end, while enalapril brought a baseline of 110.3 ± 2.3 to 72.4 ± 6.9 after 4 weeks (Figure 3).

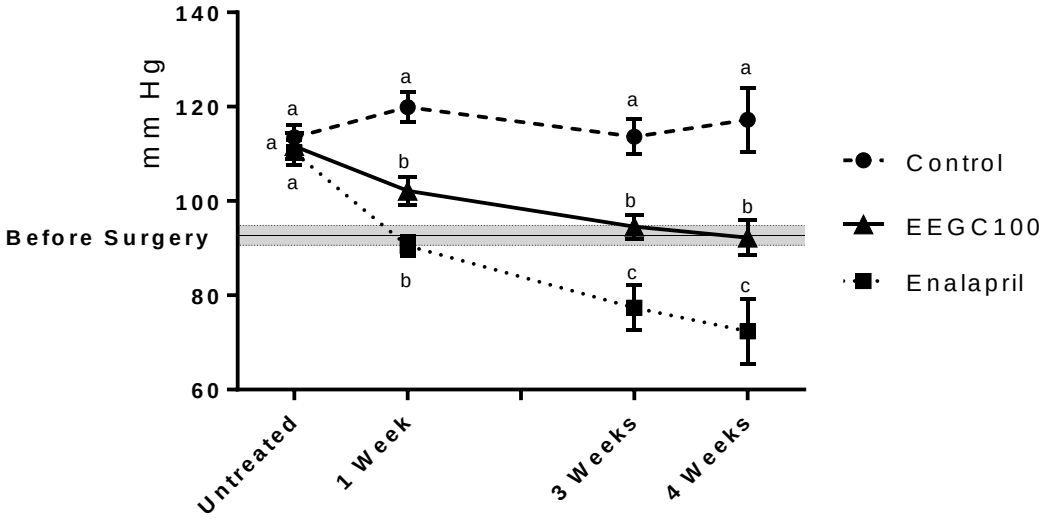

Figure 3: The mean arterial pressure of 2K1C animals in the 4-week model. Before surgery, untreated, 1 week and 3 week measures made by tail cuff. Four week measures made by direct carotid cannulation. The results are the mean ± S.E.M. Two-way ANOVA followed by Tukey's post-test for multiple comparisons; Different letters (a, b, c) mean statistic difference ($p < 0.05$) compared within the same time point; n = 10; EEGC100 = ethanolic extract of *Gomphrena celosioides* 100 mg/kg.

3.5. EEGC promoted ACE activity inhibition, lower aldosterone and TBARS levels and higher values of nitrite.

EEGC, as well as enalapril, acted as an ACE inhibitor, since the serum ACE activity (mmol/mL.min) of the EEGC group was 98.62 ± 11.36, while the control group it was 159.43 ± 25.26. The enalapril result was even lower at 75.75 ± 9.13. Consequently, the serum aldosterone concentration (pg/mL) on the EEGG and enalapril groups was also lower: 87.62 ± 11.04 and 63.88 ± 8.32, respectively, against 144.29 ± 23.17 in the control group (Figure 4).

Serum nitrite (µM), an indirect assessment of the NO levels, was found to be augmented on the EEGC group: 134.75 ± 15.63 against 53.86 ± 9.67 in the control group. In addition, the oxidative stress biomarker TBARS (mmol/L) was lower in the EEGC and enalapril groups compared to the control (control = 8.73 ± 1.41, enalapril = 5.42 ± 0.69, EEGC = 3.48 ± 0.47) (Figure 4).

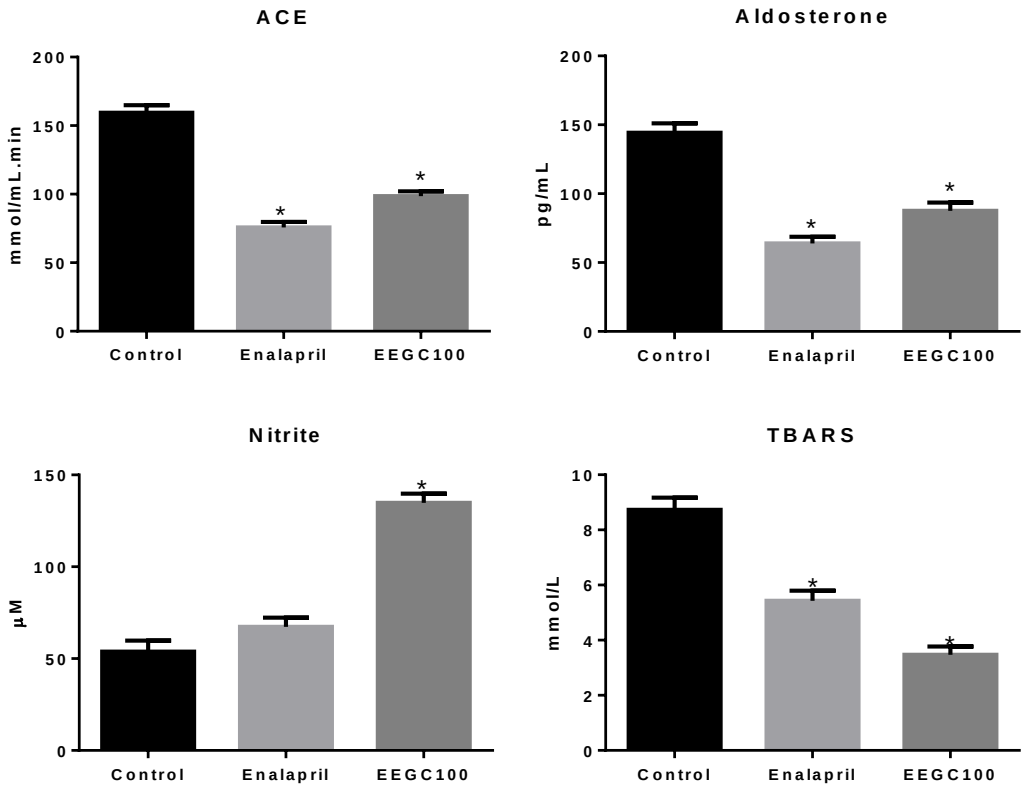

Figure 4: Serum ACE activity and aldosterone, nitrite and TBARS concentrations on the 2K1C rats submitted to the 4-week model. The results are the mean ± S.E.M. One-way ANOVA followed by Dunnett's post-test; * means $p < 0.05$ compared to the control group; n = 10. ACE = angiotensin converting enzyme; TBARS = thiobarbituric acid reactive substances; EEGC100 = ethanolic extract of *Gomphrena celosioides* 100 mg/kg.

3.6. EEGC counteracted the impairment of vascular reactivity induced by renovascular hypertension

The isolated mesenteric beds from the EEGC group, similar to the ones from the enalapril and normotensive groups, exhibited lower contractility following Phe administration compared to the hypertensive control (Figure 5). After 30 nmol of phenylephrine, the hypertensive control vascular bed pressure (mm Hg) increased by 58.52 ± 15.90, while EEGC increased by 31.41 ± 8.70 and enalapril increased by 18.36 ± 4.50. The EEGC group also showed augmented relaxation following ACh and SNP administration compared to the hypertensive control. The relaxation of PSS+Phe pre-contracted vascular beds was measured as the area under curve (mm Hg.min), since the duration of the effect of the dilator drugs were observed to be a determinant parameter in addition to its magnitude. This way, the areas under the curve after the administration of ACh (1 nmol) were -42.73 ± 6.95 for the control group, -74.64 ± 18.24 for enalapril and -75.09 ± 21.30 for EEGC. Similar results were found after

administration of SNP (1 nmol): -43.15 ± 6.81 for the control group, -58.04 ± 12.86 for enalapril and -75.44 ± 15.56 for EEGC (Figure 5).

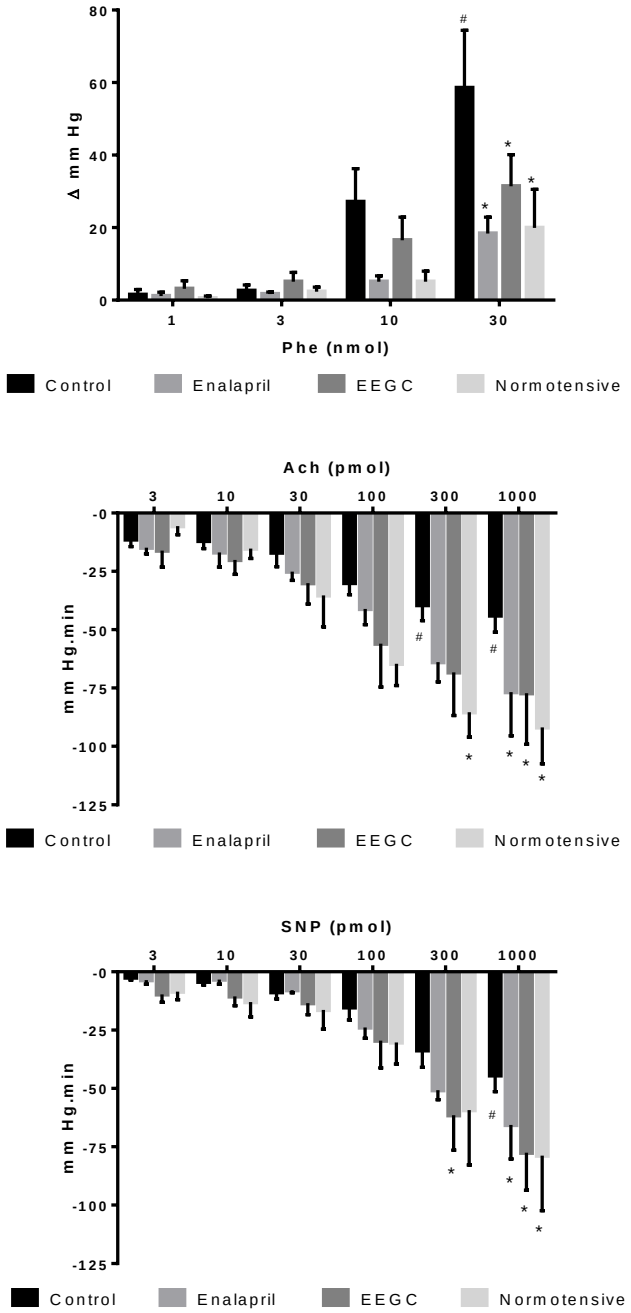

Figure 5: Isolated mesenteric bed pressure curves of Phe, ACh and SNP from 2K1C rats after 4 weeks of treatments and from normotensive reference rats. The results are the mean ± S.E.M. Two-way ANOVA followed by Dunnett's post-test for multiple comparisons; * means $p < 0.05$ compared to the control group; # means $p < 0.05$ compared to the normotensive group; n = 7, 5, 5 and 3 for the control, enalapril, EEGC and normotensive groups, respectively; Phe = phenylephrine, ACh = acetylcholine, SNP = sodium nitroprusside, EEGC = ethanolic extract of *Gomphrena celosioides* 100 mg/kg.

3.7. Histopathology - EEGC prevented left ventricle hypertrophy

2K1C surgery led control rats to have, at the end of the experiment, a thicker left ventricle wall (2.59 ± 0.07 mm), as seen in the comparison to the normotensive rats (2.19 ± 0.10 mm) (Figure 6). The control group heart weight was 0.90 ± 0.02.

After four weeks, enalapril-treated rats had thinner left ventricle myocardium (2.20 ± 0.06 mm) and lighter hearts (0.78 ± 0.03 g) compared to the control group. Similarly, to enalapril, the EEGC group left ventricle wall was also thinner (2.33 ± 0.08 mm) than the control (Figure 6), although no difference was found in the heart weight (0.87 ± 0.03 g).

All the other isolated organs (liver and kidneys) showed no weight difference among the groups, except that the enalapril group's left kidney was lighter than the control (data not shown). In addition, the left kidneys from the enalapril group showed noticeably more severe alterations than the ones from the other groups, including multifocal to coalescent areas of interstitial fibrosis associated with lymphoplasmacytic infiltrate with tubular degeneration and renal surface depressions (Figure 7).

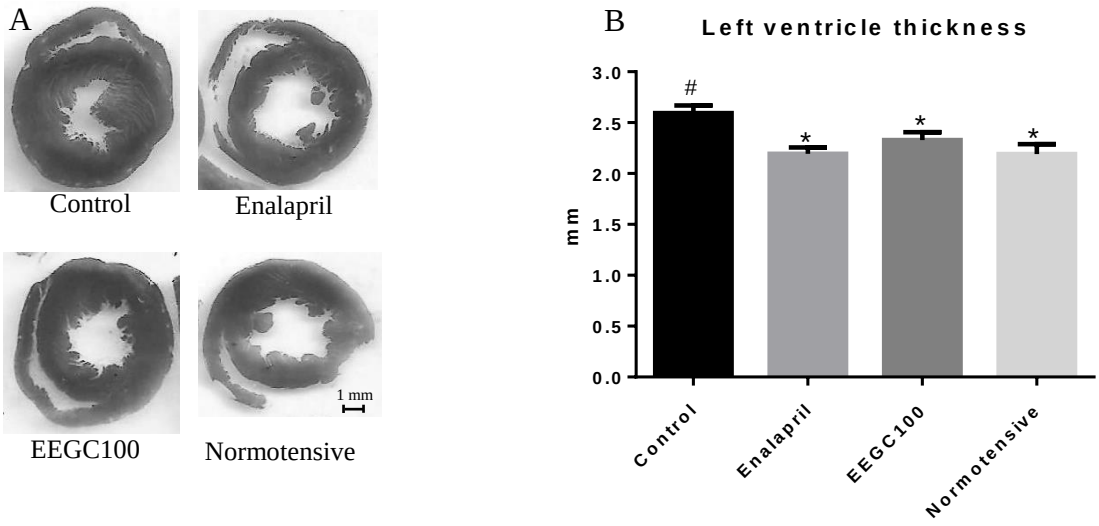

Figure 6: Cross-section of representative left ventricles (A) and measurements of their wall thickness (B) from the hearts of the 2K1C rats after 4 weeks of treatment and from the normotensive reference rats. A: routine hematoxylin and eosin histology. B: Results are the mean ± S.E.M. One-way ANOVA followed by Dunnett's post-test; * means $p < 0.05$ compared to the control group; $^{\#}$ means $p < 0.05$ compared to the normotensive group; n = 9, 10, 10 and 3 for the control, enalapril, EEGC and normotensive groups, respectively; EEGC100 = ethanolic extract of *Gomphrena celosioides* 100 mg/kg

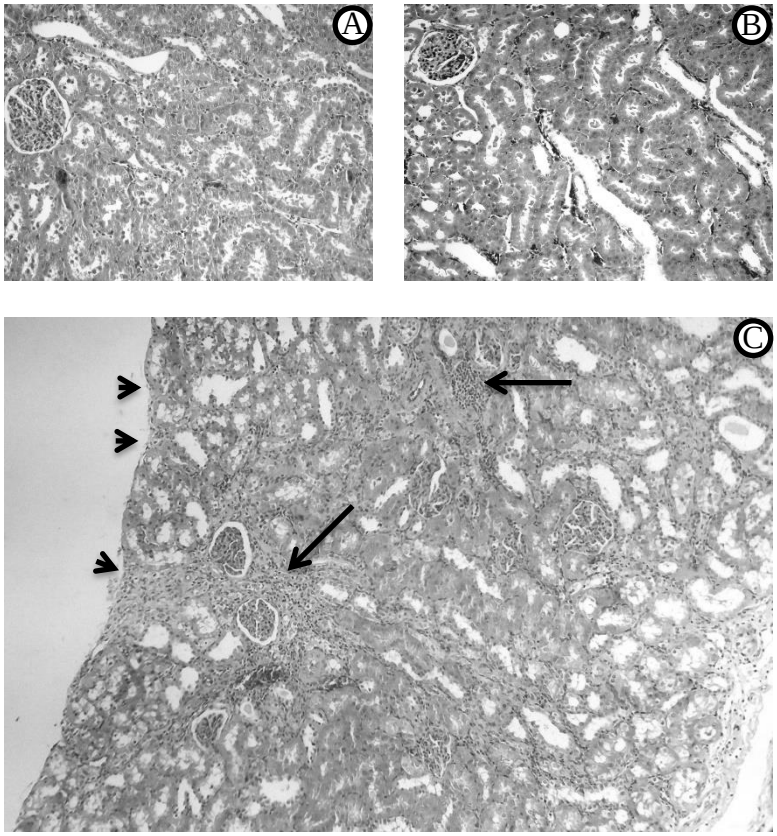

Figure 7: Histomicrography of representative left kidneys from the control (A), EEGC (B) and enalapril (C) groups. Notice in the enalapril group: renal fibrosis with multifocal to coalescent areas of interstitial fibrosis (large arrows) associated with lymphoplasmacytic infiltrate with tubular degeneration and renal surface depressions (small arrows). Other groups are included as reference. Hematoxylin and eosin, 10X.

4. DISCUSSION

This is the first time that the plant *G. celosioides* has had its antihypertensive action proven in an *in vivo* model. Previously, our group had already shown its diuretic potential in normotensive animals and found that treated rats exhibited lower levels of aldosterone and that the diuretic effect was inhibited when blocking bradykinin receptors, NO or PG production (Vasconcelos et al., 2017). The obvious course of action would be to test whether it would be effective as an antihypertensive drug, and this study showed great potential in this regard.

The extract of *G. celosioides* was able to reduce the arterial pressure of the hypertensive animals after 20 min of acute administration, which led us to believe that, besides the diuretic action, it also has direct hypotensive effect.

During the 4-week treatment, the diuretic and natriuretic effect of the extract was reproduced in hypertensive animals. Blood pressure was reduced from the first week until the last, showing that, at least in the 4 weeks assessed, there is no compensatory effect from the

organism that decreases its activity. Although the EEGC did not promote hypotensive effect as pronounced as enalapril, it was sufficient to bring MAP to the same values as before the 2K1C surgery.

The renovascular hypertension induction model by placement of a renal artery flow reducer clip aims to mimic the advanced phase of primary hypertension in humans, in which the histopathological finding that narrowing the lumen of the afferent arteriole is common (Moritz & Oldt, 1937). It is believed that there is an initial reflex contraction of the afferent arteriole in response to elevations of the systemic pressure to normalize the intrarenal pressure, thus protecting the sensitive glomeruli against possible damages. With the excessive activation of this mechanism, there is a possibility of injury, atherosclerosis, and hypertrophy of this arteriole, leading to a loss of dilatability and consequent chronic reduction of renal blood flow, leading to increased renin release and, finally, aldosteronism, which is a key event for the installation of primary hypertension (Johnson & Schreiner, 1997; Johnson et al., 2005).

This model responds to treatments with ACE inhibition similarly to human hypertension, but, interestingly, in this study, the ACE inhibitor enalapril at the dose used, despite reducing the pressure to normal levels, also reduced the renal function and so the animals urinated less and eliminated less sodium. This reduction was corroborated by the fact that this group had significantly higher serum urea and creatinine levels than the others and their left kidneys were lighter and presented severe interstitial damage. This kind of damage has been already observed in the clipped kidney after prolonged ACE inhibition (Jackson et al., 1990; Wenzel et al., 1992), which most likely explains the decrease in renal function promoted by enalapril. It is important to highlight that EEGC acted as an antihypertensive without worsening the renal function.

It has already been shown that some flavonoids can promote vasodilation, for example, by inducing the synthesis and/or release of PGI2 (Khaza'ai and Wahle, 1996; Schramm and German, 1998; Schramm et al., 2001; Gasparotto Junior et al., 2012). The finding that EEGC, which is flavonoid-rich, inhibits ECA activity and consequently strongly lowers the serum aldosterone suggests that this is its main antihypertensive mechanism of action. This mechanism was also found to be present in other plants rich in polyphenols, which show diuretic and antihypertensive effects by inhibiting ACE and increasing the local availability of bradykinin (de Souza et al., 2013; Gasparotto Jr. et al., 2012; Prando et al. al., 2016).

The presence of polyphenols has also been associated with an antihypertensive effect due to its antioxidant action, which contributes to the maintenance of vascular levels of NO (Sha et al., 2016), since NO appears at lower levels in the presence of oxidative stress (Kojda

and Harrison, 1999). It is very likely that this pathway contributes to the EEGC antihypertensive effect, since, as demonstrated by at least one oxidative stress biomarker (TBARS), the extract may act as antioxidant. Moreover, the higher serum nitrite levels found in the EEGC group suggest that the extract increases the NO bioavailability, presumably for its antioxidant action, which further contributes to vasodilation.

The 2K1C Goldblatt surgery is known to lead to impairment of the vascular reactivity of resistance arteries from the animal's mesenteric bed (Arruda et al., 2005). Our results show that EEGC, as well as enalapril, counteracted the vascular hyper-reactivity to constriction caused by renovascular hypertension. Moreover, the extract can restore both endothelium-dependent and independent relaxation of these vessels, leading to a similar dose response curve as the normotensive non-operated rats. We propose that the ACE activity inhibition by EEGC, along with NO maintenance, is responsible for this effect.

Finally, it is possible to say that EEGC exerts cardioprotection since it prevented hypertension-associated cardiac remodeling. Augmented RAS activity is directly linked to cardiac hypertrophy (Zhang et al., 2008), and the 2K1C model is capable of inducing this alteration by promoting a high plasma renin activity (de Simone et al., 1992). Therefore, it is expected that ACE inhibition promoted by the extract was involved in this protective effect.

With these findings, we can conclude that the ethanolic extract of *Gomphrena celosioides* promotes augmented diuresis and natriuresis not only on normotensive animals but also on renovascular-hypertensive subjects and that this effect persists for at least four weeks. Moreover, this diuretic effect, together with a direct hypotensive effect, can counteract renovascular hypertension, promoting cardioprotection and ACE inhibition, which is proposed to be its main mechanism of action.

Acknowledgements

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5.3. Considerações finais

As etapas deste estudo objetivaram responder perguntas sobre as atividades biológicas de *G. celosioides* em uma ordem lógica, contando uma história. A partir da constatação do uso popular bastante diverso e da boa presença de substâncias potencialmente ativas biologicamente buscou-se a comprovação de um dos usos medicinais dessa planta: como diurética.

A constatação de que ela possui sim uma importante atividade diurética motivou avaliações mais aprofundadas, como as vias envolvidas nos mecanismos de ação. Considerando que o principal uso dos medicamentos diuréticos é no combate à hipertensão, o próximo passo óbvio foi verificar se a planta teria potencial para ser usada no tratamento desta doença. Os resultados mostraram-se bastante expressivos no sentido de redução aguda da pressão, até gerando algum ceticismo por parte dos pesquisadores. Porém, a atividade anti-hipertensiva foi confirmada cronicamente e a observação de que o extrato promove significativa inibição da ECA reforçou a percepção do seu potencial.

Acreditamos que seu efeito não derive apenas de uma substância, mas que mais provavelmente provenha da combinação da ação de diversas moléculas. É possível que a continuação dos estudos com essa planta através da obtenção de frações enriquecidas a partir do extrato utilizado venha a dar pistas sobre qual ou quais grupos de substâncias contribuem mais para o efeito farmacológico.

A sua associação com medicamentos comerciais contra hipertensão também constitui objeto interessante de estudos futuros. A avaliação da possibilidade de sinergismo entre planta e fármacos já existentes e, principalmente, da segurança do uso concomitante pode abrir portas para o desenvolvimento de um novo fitoterápico.

G. celosioides é, portanto, uma fonte promissora para novos tratamentos anti-hipertensivos.

6. ANEXOS

6.1. Aprovação da Comissão de Ética (CEUA-UFGD)

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 9 de julho de 2015.

CERTIFICADO

Certificamos que o projeto intitulado **"Avaliaçao dos Efeitos Cardioprotetores de *Gomphrena celosioides* Mart. (Amaranthaceae) em Ratos",** protocolo nº 01/2015, sob responsabilidade de Cândida Aparecida Leite Kassuya – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto n° 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 02 de junho de 2015.

Vigência do Projeto	01/08/2015 – 31/10/2017
Espécie/linhagem	*Rattus norvegicus*/ Wistar
Nº de animais	228
Peso/idade	200-300 g/ 2-3 meses
Sexo	Machos
Origem	Biotério Central da Universidade Federal de Mato Grosso do Sul /UFMS – Campus Campo Grande

Melissa Negrão Sepulvida

Melissa Negrão Sepulvida
Coordenadora CEUA

6.2. Artigo 1: Páginas da Publicação

Journal of Ethnopharmacology 202 (2017) 85–91

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Mechanisms underlying the diuretic effect of *Gomphrena celosioides* Mart. (Amaranthaceae)

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ABSTRACT

Ethnopharmacological relevance: *Gomphrena celosioides* (Amaranthaceae) is a native medicinal plant found in Mato Grosso do Sul State that is used for treating urinary tract and kidney stones. This study aimed to evaluate the diuretic effects of ethanolic extract from *G. celosioides* (EEGC) on acute and extended diuresis to provide a pharmacological basis for its use in traditional medicine.

Aim of the study: To evaluate the diuretic and natriuretic activity of EEGC and its mechanism of action in an animal model.

Materials and methods: EEGC (30, 100 and 300 mg/kg) was orally administered in male *Wistar* rats, and urinary excretion was measured at intervals of up to 8 h after administration. To evaluate participation of the nitric oxide (NO), prostaglandin and bradykinin pathways in its effect, respective inhibitors were also administered together with effectives doses of EEGC and compared with control groups. A 7-day model with daily administration and urine measurement was also carried out.

Results: Oral administration of doses of 100 and 300 significantly increased urine output after 8 h compared to the control group. It was observed this effect is dependent on the NO, prostaglandin and bradykinin pathways because their inhibitors reduced the diuretic effects of EEGC. Moreover, after 7 days of treatment, the effect was sustained and a decrease in serum aldosterone was observed in the extract group.

Conclusion: According to the results, *G. celosioides* extract showed diuretic and natriuretic effects associated with more than one mechanism of action. Considering that all diuretic drugs are currently available for the treatment of volume and electrolyte disturbances, especially hypertensive status, the present results may have clinical relevance and open new possibilities for the development of new natural diuretics from *G. celosioides*.

1. Introduction

Hypertension is a predisposing factor for stroke, coronary heart disease, peripheral arterial disorders, heart failure and renal failure (Williams et al., 2004; Godfraind, 2006). Common clinical strategies to lower blood pressure include the use of agents that function by reducing arterial resistance and/or decreasing cardiac output. Diuretics are among the drugs most used to promote increased urinary sodium and urine volume output, which leads to a reduction in the volume of circulating blood, thus reducing blood pressure (Williams et al., 2004; Gallagher et al., 2006).

Gomphrena celosioides Mart. belongs to the Amaranthaceae family and is a weed up to 20 cm tall and popularly known as “perpétua”, “bachelor's button” or “prostrate globe-amaranth” (Myers et al., 2000). It is a native medicinal plant that is found and used in Mato Grosso do Sul State (Cunha and Bortolotto, 2011), where it was collected for the present study. It is also well distributed throughout South America, Asia, and East and West Africa (Takim et al., 2013). It is used in the treatment of various skin diseases and as an abortifacient in South America (Burkill, 1984). In Brazil, it has been employed to treat infectious and renal diseases as well as respiratory and gastrointestinal disorders. Gastroprotective effects were even scientifically shown (Oluwabunmi and Abiola, 2015). A decoction of the whole plant and of its related species *G. globosa* Linn. is applied to gangrenous wounds (Arenas and Azorearo, 1977). *Gomphrena* species are also employed in the treatment of bronchial disorders, diarrhoea, and fever and as an analgesic, tonic, diuretic and carminative (Vieira et al., 1994). The diuretic activity of *G. celosioides* was mentioned by Dhawan et al.

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(1977) and Chauhan et al. (2009). Whole *G. celosioides* juice with *Piper nigrum* and lemon juice is taken twice a day for 10 days to prevent kidney stones and expel them (Chauhan et al., 2009). *G. celosioides* activity in urolithiasis was also demonstrated in a scientific study on rats (Goswami and Srivastava, 2015), and its effectiveness was attributed to increased diuresis and lowering of urinary concentrations of stone constituents.

An initial phytochemical study with *G. celosioides* revealed the presence of saponins, steroids, amino acids, reducing and non-reducing sugars, phenols, flavonoids, betacyanins and ketoses (Botha and Gerritsma-van der Vijver, 1986). Fractionation of the extract of the aerial parts conducted by Moura et al. (2004) led to the isolation of 4-hydroxy-benzoic acid and 4-hydroxy-3-methoxybenzoic acid (or vanillic acid), stigmasterol, sitosterol, campesterol, methyl palmitate and stigmast-6-en-3-Ob-(D-glycopyranoside).

This study aimed to experimentally evaluate the diuretic and natriuretic potential of acute and extended treatment with the ethanolic extract of *G. celosioides* (EEGC) in rats, as well as to elucidate possible mechanisms of action.

2. Material and methods

2.1. Plant material, extraction and ESI-MS/MS analysis

Aerial parts of *G. celosioides* were collected at Paranaíba, Mato Grosso do Sul, Brazil [*lat:* −19.666667 *long:* −51.183333 WGS84], in April of 1994 and 2014 and identified by Prof. Dr. Josafá Carlos de Siqueira, an expert in the family Amaranthaceae from Pontifical Catholic University of Rio de Janeiro (PUC-RJ), Brazil. A voucher specimen (SPFR-2962) was deposited in the herbarium of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo (FFCLRP/USP).

Air-dried powdered aerial parts (16.0 kg) were exhaustively extracted (maceration at room temperature) with ethanol in the proportion of plant powder mass /solvent 1:2 (w/v). The spent biomass was filtered and the solvent was removed in a rotary evaporator under reduced pressure and temperature below 40 °C, yielding 901 g of ethanolic crude extract (EEGC).

EEGC (1 mg/ml) was diluted in a solution containing 50% (v/v) chromatographic grade methanol, 50% (v/v) deionized water and 0.5% ammonium hydroxide (Merck, Darmstadt, Germany). Fingerprinting ESI-MS analyses were performed according to Salvador et al. (2011) using an UPLC-MS instrument, model ACQUITY TQD (Waters Corporation, Milford, MA, USA), and the general conditions were: source temperature of 100 °C, capillary voltage of 3.0 kV and cone voltage of 30 V. ESI-MS was performed by direct infusion using a syringe pump with a flow rate of 10 μL. min/ml. Structural analysis of single ions in the mass spectra from the extract was performed by ESI-MS/MS. Ions with *m/z* of interest were selected and submitted to 15–45 eV collisions with argon in the collision quadrupole. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation. The compounds were identified by comparison of their ESI-MS/MS fragmentation spectra with fragmentation spectra of authentic standard samples and with literature data (Dosumu et al., 2014; Salvador et al., 2011; Moura et al., 2004).

In addition, the Sodium and Potassium content of the extract was determined by flame spectrometer and were verified 0.47 mmol/g of Sodium and 1.32 mmol/g of Potassium, concentrations that not interfere in the diuretic effect of this extract.

2.2. Animals

Male *Wistar* rats (200–250 g) were used from the animal facilities of Universidade Federal da Grande Dourados (UFGD) an were acclimated under a temperature of 23 ± 2 °C, humidity of 60–80%, and 12-h light/dark cycle; and they received food and water *ad libitum*. The animals were handled according to internationally accepted standard guidelines for animal use, and all experiments obeyed experimental protocols previously approved by the Ethics Commission on the Use of Animals from UFGD (CEUA-UFGD) under process number 01/2015.

2.3. Single-dose model of diuretic assessment

Diuretic activity was determined according to the method of Kau et al. (1984) with some modifications. Rats, after fasting overnight with water *ad libitum*, were randomly divided into five groups (n =5). Before treatment, all animals received an oral load of isotonic saline (0.9% NaCl, 50 ml/kg) to establish a uniform water and salt balance. Then, EEGC was administered by the oral route (p.o.) to animals at doses of 30, 100 and 300 mg/kg (EEGC30, EEGC100 and EEGC300). A negative control group received the same amount of vehicle, and a positive control group received 25 mg/kg of hydrochlorothiazide (HCTZ). Immediately afterwards, the animals were placed in metabolic cages. Urine was collected and measured after 1, 2, 4, 6 and 8 h. Cumulative urinary excretion was calculated relative to body weight and was expressed as ml/100 g.

2.4. Assessment of the involvement of the prostaglandin, bradykinin and NO pathways in the single-dose model

The dose of EEGC that was considered to be the best dose according to the previous assay was selected to evaluate the role of the prostaglandin, bradykinin and NO pathways on EEGC. A model similar to that above was constructed with twelve groups of animals divided into three treatment groups (vehicle, EEGC and HCTZ) for each of four pretreatments administered before the water and salt load: 1- indomethacin (prostaglandin synthesis inhibitor) 5 mg/kg, p.o.; 2- L-NAME (NO synthesis inhibitor) 60 mg/kg, p.o.; 3- HOE-140 (bradykinin antagonist) 1.5 mg/kg, i.p.; or 4- no pretreatment. Urine was collected at the same intervals as described above, and cumulative excretion was calculated.

2.5. Extended model with daily administration

The dose that was considered to be the best according to the previous assay was selected for an extended seven-day model with daily dosage. For this purpose, the experimental group vehicle, EEGC and HCTZ were placed individually in metabolic cages and received treatment orally once a day for seven days. The 24-h urine of each animal was collected, its volume was measured daily during the experiment and it was stored for biochemical analysis.

2.6. Urine and serum analysis

Urine samples were collected from each animal using metabolic cages. Blood samples were obtained at the end of the experiments by caudal puncture and immediately transferred to paediatric tubes with separating gel. Serum was obtained by centrifugation for 10 min at 4000 rpm. Urine pH was measured with a standard pH metre, and urinary density was estimated by weighing samples using a digital analytical balance. Urine and/or serum Na^+, K^+, Cl^-, calcium, urea, creatinine, glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and total proteins were quantified by a Roche® cobas Integra 400 plus automated biochemistry analyser.

2.6.1. Angiotensin converting enzyme (ACE) assay and determination of aldosterone and vasopressin

Samples were collected from rats after 7 days of treatment with EEGC (100 mg/kg) or vehicle (control). Blood was collected into glass tubes after decapitation and serum was obtained by centrifugation

(800*g*, 10 min, 4 °C). The aldosterone and vasopressin levels were measured by Enzyme Linked Immunosorbent Assay (ELISA, Immuno-Biological Laboratories, Inc.) and radioimmunoassay, respectively.

ACE activity was determined as previously described (Santos et al., 1985). Serum (10 µL) was incubated for 15 min at 37 °C with 490 µL of assay solution (composition: NaCl 0.9 M and Hip-His-Leu at 5 mM in 0.4 M sodium borate buffer, pH 8.3). The reaction was stopped by the addition of 1.2 ml of NaOH (0.34 M). The production of His-Leu was fluorometrically measured (365 nm excitation and 495 emission, Aminco Model J4-7461 fluoromonitor, American Instrument Co., Silver Springs, MD) after the addition of 100 µL of o-phthaldialdehyde (20 mg/ml in methanol) and 200 µL of HCl (3 M), followed by centrifugation (800*g*, 5 min) at room temperature. To correct for intrinsic plasma fluorescence, zero-time blank samples were prepared by adding plasma after NaOH treatment. All measurements were performed in triplicate.

2.7. Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). Differences between means were tested by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's post-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Electrospray ionization mass spectrometry fingerprinting

ESI-MS fingerprints of the EEGC in negative mode showed a characteristic distribution of organic compounds with acidic sites, such as phenolic organic acids (Salvador et al., 2011; Moura et al., 2004). These analyses showed that the constituents detected in the analysed sample coincided with the mass of phenolic acids and flavonoids (Table 1, Fig. 1).

3.2. EEGC promotes diuresis and natriuresis in the single-dose model of diuretic assessment

After 8 h of diuresis monitoring, we observed that the urine volume, standardized by body weight, was significantly higher in the EEGC30 and EEGC300 groups compared to the control, by an amount similar to HCTZ (Fig. 2). When we calculated the diuretic index (DI), we observed that EEGC100 and EEGC300 promoted a 1.74 ± 0.25 and 1.86 ± 0.28-fold increase in urine volume output, respectively, compared to control, while HCTZ's index was 1.75 ± 0.08. Time course curves showed that the extract started to promote effects from 6 h on, whereas HCTZ was effective after the second hour (Fig. 2). Urine sodium (UNa) was also significantly higher in the EEGC100 (1.97 ± 0.25-fold) and EEGC300 (2.25 ± 0.38-fold) groups compared to the control and was even higher in the HCTZ (2.75 ± 0.20-fold) group (Fig. 3). The urine K^+ and Cl^- values were only significantly modified by HCTZ (2.42 ± 0.13 and 2.54 ± 0.18 fold, respectively), while urine calcium, pH and density and were similar in all groups (data not shown). Serum electrolytes, urea and creatinine were not different among the groups either (data not shown). After this assay, we selected EEGC100 as the best dose for further analysis because it showed significant diuretic and natriuretic effects that were very close to the threefold higher dose EEGC300. Hence, EEGC100 is hereafter referred to as EEGC.

Table 1
Compounds identified in ethanol extract of *Gomphrena celosioides* aerial parts (EEGC), using negative ion mode ESI-MS/MS.

Compound	Ethanol extract of *Gomphrena celosioides* (EEGC)	Deprotonated ions $[M-H]^-$, *m/z*	MS/MS ions *m/z*
Malic acid	+	133	25 eV: 133→115
Caffeic acid	+	179	25 eV: 179→135
Ferulic acid	+	195	25 eV: 193→178, 149,134
Catechin	+	290	25 eV: 290
Vanillic acid	+	165	25 eV: 165
Irisone B	+	297	25 eV: 297
Dimethoxy-flavone	+	329	25 eV: 329
Caffeoyl-glucose	+	339	25 eV: 339

+: detected compound.

3.3. L-NAME, indomethacin and HOE-140 can diminish the diuretic and natriuretic effects of EEGC

After pretreatment with L-NAME, indomethacin or HOE-140, EECG failed to significantly promote diuresis or natriuresis (Fig. 4). After pretreatment with indomethacin, the EEGC group additionally showed significantly lower diuresis and natriuresis compared to the non-pretreated EEGC group. This was not found in the L-NAME – EEGC or HOE-140 – EEGC groups, that were not significantly different from the respective EEGC groups without pretreatment. Only the HCTZ groups were able to increase diuresis, urine Na^+, (Fig. 4) K^+, and Cl^- (data not shown) regardless of pretreatment. Other urine parameters and all serum parameters (data not shown) remained unaltered, except that the indomethacin and HOE-140 pretreated groups showed lower urinary calcium compared to the non-pretreated groups, which is expected as an effect of low renal prostaglandins (Gomaa et al., 1990).

3.4. Diuretic and natriuretic effects of EEGC are sustained during 7-day treatment in the extended model with lower aldosterone levels

During the 7 days of treatment with vehicle, EEGC or HCTZ, we observed sustained increases of UV and UNa in the groups treated with EEGC and HCTZ compared to vehicle (Fig. 5). The mean diuretic index was 1.29 ± 0.08 for EEGC and 1.40 ± 0.09 for HCTZ. Water intake was uniform among the groups (Fig. 5).

After 7 days of treatment in the extended model, analysis of blood samples showed significantly lower aldosterone levels in the EEGC group compared to vehicle. ACE activity was numerically lower in the EEGC group compared to vehicle, but not significantly different ($p=0.06$) (Fig. 6). The vasopressin levels were similar in all three groups (Fig. 6), as were all of the serum biochemical parameters analysed (data not shown).

4. Discussion

To our knowledge, this is the first study to show the diuretic activity of the *Gomphrena celosioides* plant in detail and to seek to understand some of the mechanisms of its action.

With acute administration of its ethanolic extract, we observed relatively rapid diuretic action with statistical significance after 8 h. Although it takes longer to act than the standard drug used as a reference, hydrochlorothiazide, the magnitude of the effect of the extract after 8 h at doses of 100 and 300 mg/kg closely resembles the drug, which is an interesting finding from the point of view of clinical use. A significant effect was also observed for natriuresis, but with a slight loss potassium, chloride or calcium in the urine, which can be an advantage similar to that of commercial potassium-sparing diuretics.

With the observations that the administration of 100 mg/kg EEGC presented significant effects compared to the vehicle group and that this effect was similar to the three-fold higher dose, it was selected for use in subsequent experiments. Intending to evaluate the participation

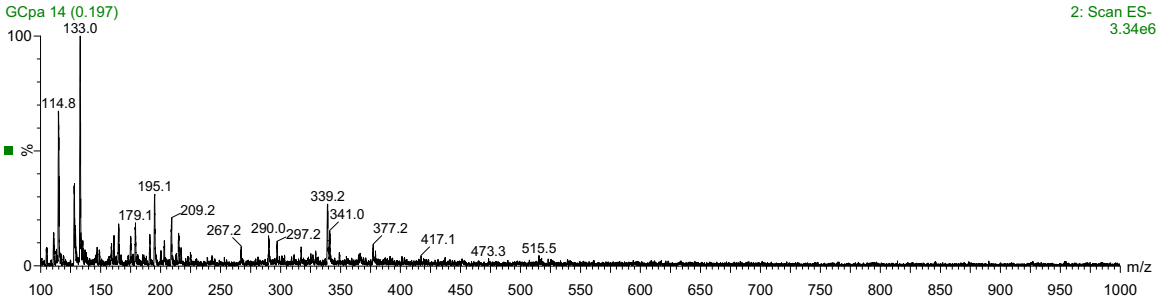

Fig. 1. ESI(−)-MS fingerprints of ethanol extract of *Gomphrena celosioides* aerial parts (EEGC).

of the prostaglandin, NO and bradykinin pathways, we checked whether EEGC sustained its effects in the presence of inhibitors of these pathways. In fact, the three inhibitors reduced or eliminated the diuretic and natriuretic effects of the extract, showing that these pathways are important to its mechanism of action.

Bradykinin interferes with the homeostasis of renal blood flow, increasing the local release of NO and prostaglandins and causing vasodilation and increased glomerular filtration. The degradation is carried out by angiotensin converting enzyme (ACE), a fact that explains why increased activity of bradykinin is implicated as one of the antihypertensive action mechanisms of ACE inhibitors (for review see Manolis et al., 2010). It has been postulated that some plants rich in polyphenols have diuretic and antihypertensive effects that act in this system, for example, inhibiting ACE, thus increasing the local availability of bradykinin (Gasparotto Jr et al., 2012; de Souza et al., 2013; Prando et al., 2016). This increased availability of bradykinin probably contributes to the effects of EEGC as well as the increase of prostaglandins because the inhibition of prostaglandin production by indomethacin also reduced the diuretic effect of the extract.

Nitric oxide also plays a key role in the regulation of the vascular tone, promoting vasodilatation of the afferent renal arterioles and the resulting increase in glomerular filtration. The production of NO is increased by eNOS, which is one of the mechanisms of action of the renal activity of bradykinin (Chappell, 2012). We observed that its presence is also important for the role of EEGC. A possible increase in the availability of bradykinin has the ability to raise the levels of NO to contribute to diuresis, which would explain the usefulness of this mediator in the plant's mechanism of action. Moreover, NO appears at lower levels in the presence of oxidative stress. It is likely that as this plant is rich in polyphenols, it has important antioxidant effects, contributing to the maintenance of the levels of NO, thus aiding in the diuretic effect.

Another important finding from this study was that the diuretic and natriuretic effects of EEGC remain present for several days during prolonged treatment without upregulation of water intake, as was seen in the seven-day model. This means that, at least within seven days, there is no compensatory mechanism of the body that is able to counteract its effect. In addition, renal toxicity was not observed within the dose tested since the levels of urea and creatinine remained unaltered.

After evaluating the serum of animals treated for seven days, we found reduced levels of aldosterone in the EEGC group compared to the vehicle group. Although the ACE activity was not significantly different between the two groups, it is also possible that with a statistical p-value =0.06, the extract exerted some ACE inhibitory activity, which would explain the reduced concentration of aldosterone and the consequent increase in natriuresis and also corroborate the notion that the extract causes increased availability of bradykinin. This activity would be of great clinical utility, resembling one of the most used classes of commercial drugs: ACE inhibitors.

Previous chemical studies with species of this genus were related to the isolation of steroids, terpenoids, ecdysteroids, flavonoids, aurantiamide and protoalkaloids (Salvador et al., 2012). Even though some phytochemical and biological activity studies have been performed in certain species, overall, the genus *Gomphrena* still remains poorly studied. Its aerial parts are rich in phenolic acids and flavonoids, chemical compounds that may act synergistically and contribute to the diuretic profile of *G. celosioides*. According to ESI-MS fingerprinting, it was possible to identify the presence of phenolic acids and flavonoids, which could explain, in part, the observed effects.

Based on this information, we can state that the ethanol extract of EEGC has pronounced and prolonged diuretic and natriuretic activities and that this effect is probably due to downregulation of aldosterone, involving local maintenance of bradykinin and prostaglandins as well as local maintenance of NO. Future studies are needed to investigate the usefulness of this diuretic effect in the treatment of hypertension.

Authors' contributions

All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; PCPV, AGJ and DRS conducted the experiments; MJS and JVM were involved in the

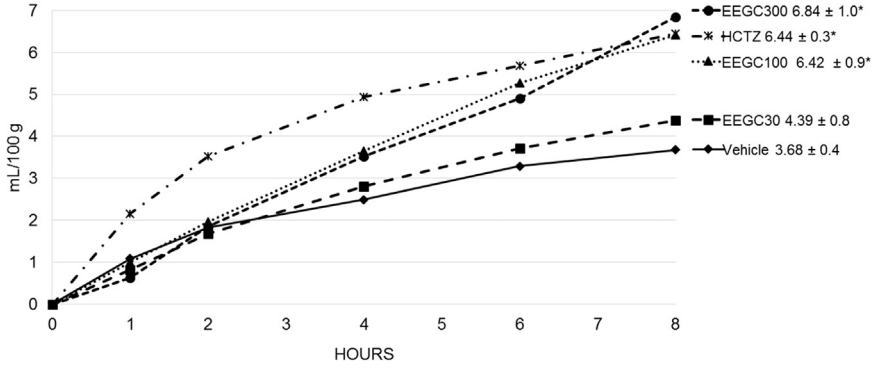

Fig. 2. Cumulative urine volume from single-dose model. Results are mean ± S.E.M., n =7. One-way ANOVA followed by Bonferroni's post-test; * means $p < 0.05$ comparing to vehicle group. EEGC30, 100 and 300= Ethanolic Extract of *Gomphrena celosioides* 30, 100 and 300 mg/kg respectively. HCTZ = Hydrochlorothiazide.

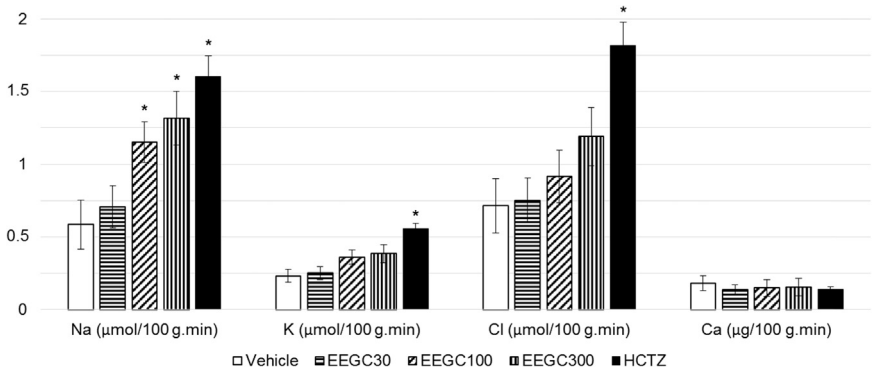

Fig. 3. Urine electrolytes from single-dose model. Results are mean ± S.E.M., n =7. One-way ANOVA followed by Bonferroni's post-test; * means $p < 0.05$ comparing to vehicle group. EEGC30, 100 and 300= Ethanolic Extract of *Gomphrena celosioides* 30, 100 and 300 mg/kg respectively. HCTZ = Hydrochlorothiazide.

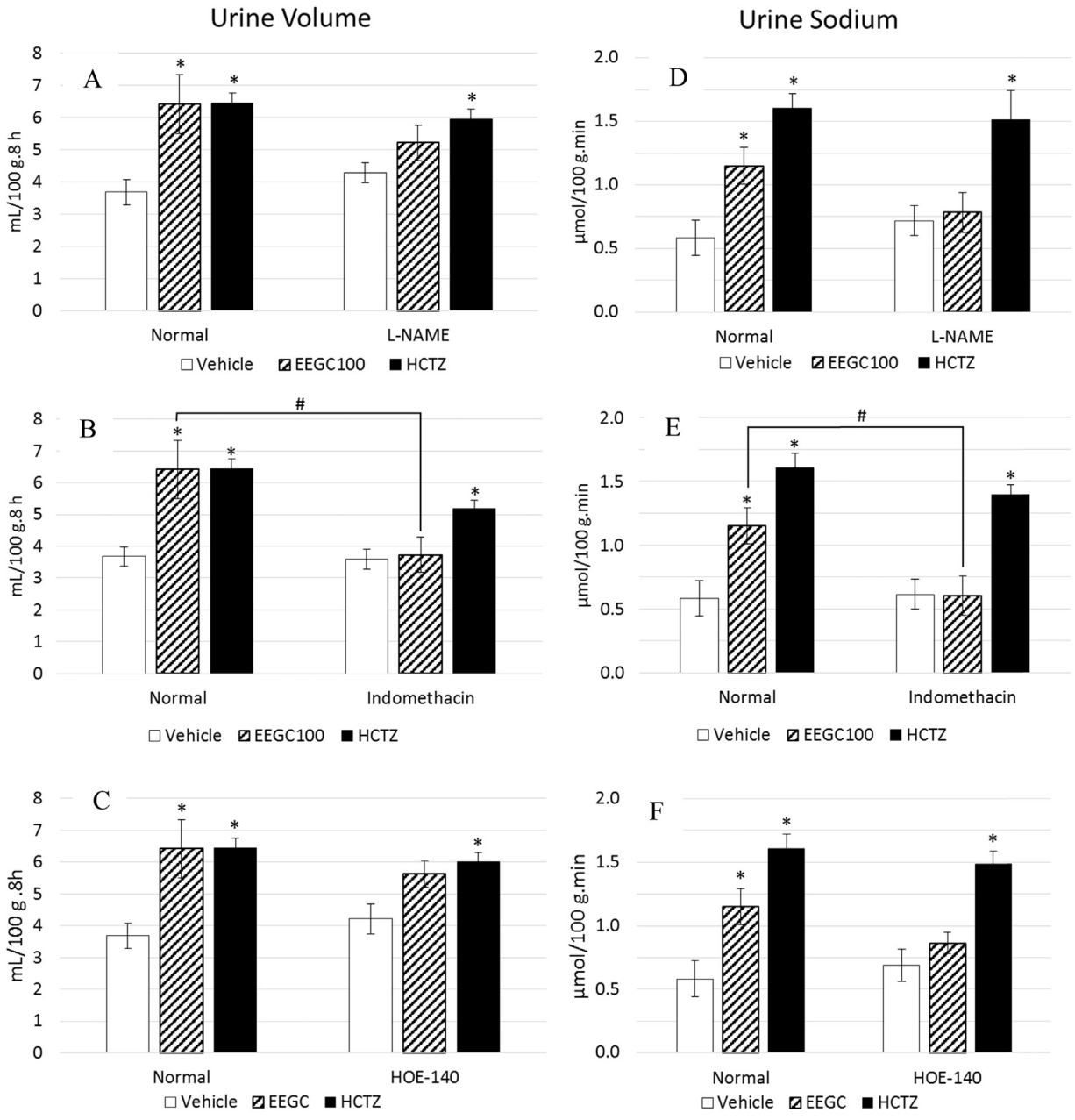

Fig. 4. Urine Volume (A, B, C) and Sodium (D, E, F) from single-dose model for assessment of NO (A, D), prostaglandins (B, E) and bradykinin (C, F) pathways involvement, without pretreatment (Normal) or after pretreatment with L-NAME, Indomethacin or HOE-140. Results are mean ± S.E.M., Normal groups: n =7, others: n =6. One-way ANOVA followed by Bonferroni's post-test; * means $p < 0.05$ comparing to same pretreatment vehicle group. # means $p < 0.05$ comparing to correspondent non-pretreated group (Normal). EEGC = Ethanolic Extract of *Gomphrena celosioides* 100 mg/kg. HCTZ = Hydrochlorothiazide.

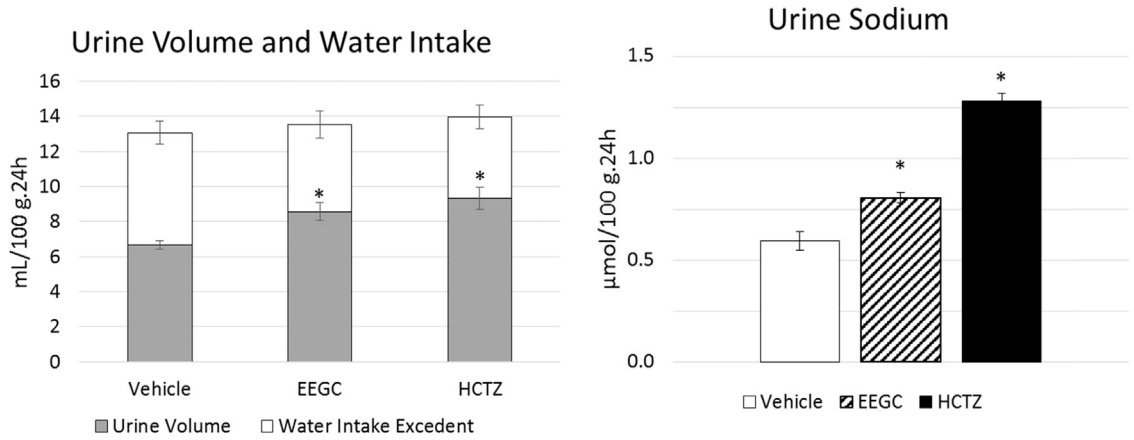

Fig. 5. Urine Volume (gray), water intake (white + gray) (Panel A) and urine Sodium (Panel B) from extended model with daily doses. Results are mean ± S.E.M., n =6. One-way ANOVA followed by Bonferroni's post-test; * means $p < 0.05$ comparing to vehicle group. EEGC = Ethanolic Extract of *Gomphrena celosioides* 100 mg/kg. HCTZ = Hydrochlorothiazide.

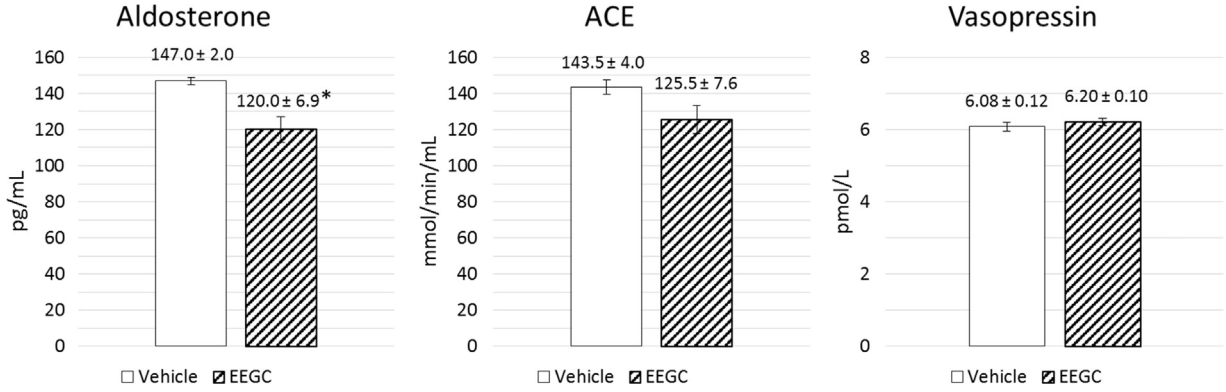

Fig. 6. Serum Aldosterone, ACE and Vasopressin from extended model with daily doses. Results are mean ± S.E.M., n =6. Student's *t*-test; * means $p < 0.05$ comparing to vehicle group. EEGC = Ethanolic Extract of *Gomphrena celosioides* 100 mg/kg. HCTZ = Hydrochlorothiazide. ACE = Angiotensin Converting Enzyme Activity.

preparation and chemical analysis of extract; CALK and PCPV performed data analyses and wrote the manuscript. All authors read and approved the final manuscript.

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