UNIVERSIDADE FEEVALE

MESTRADO EM TOXICOLOGIA E ANÁLISES TOXICOLÓGICAS

ANA VALÉRIA DE OLIVEIRA GONÇALVES PRIETSCH

PESQUISA FITOQUÍMICA PRELIMINAR, INVESTIGAÇÃO DA ATIVIDADE ANTIOXIDANTE *IN VITRO* E AVALIAÇÃO TOXICOLÓGICA PRÉ-CLÍNICA DO EXTRATO ENRIQUECIDO DE COMPOSTOS FENÓLICOS DAS PARTES AÉREAS DE *Acacia mearnsii* De Wild

Novo Hamburgo

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Dissertação apresentada ao Curso de Mestrado em Toxicologia e Análises Toxicológicas como requisito para obtenção do título de Mestre em Toxicologia e Análises Toxicológicas.

Linha de Pesquisa: Toxicologia Experimental

Orientadora: Prof.ª Drª. Edna Sayuri Suyenaga

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ANA VALÉRIA DE OLIVEIRA GONÇALVES PRIETSCH

Dissertação intitulada: “PESQUISA FITOQUÍMICA PRELIMINAR, INVESTIGAÇÃO DA ATIVIDADE ANTIOXIDANTE *IN VITRO* E AVALIAÇÃO TOXICOLÓGICA PRÉ-CLÍNICA DO EXTRATO ENRIQUECIDO DE COMPOSTOS FENÓLICOS DAS PARTES AÉREAS DE *Acacia mearnsii* De Wild”, apresentada ao Programa de Pós-Graduação em Toxicologia e Análises Toxicológicas, da Universidade Feevale, como requisito necessário para obtenção do grau de Mestre.

Aprovado por:

_____

Orientadora: Profª Drª Edna Sayuri Suyenaga
Universidade Feevale

_____

Profª Drª Ana Rita Breier
Banca Examinadora – Universidade Vale do Rio dos Sinos

_____

Prof Dr Olyr Celestino Kreutz
Banca Examinadora – Universidade Feevale

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O Senhor é a minha luz e a minha salvação; de quem terei temor? O Senhor é o meu forte refúgio, de quem terei medo?

Salmos 27:1 Bíblia Sagrada

RESUMO

A acácia-negra (*Acacia mearnsii* De Wild) é uma espécie vegetal de grande importância econômica no Rio Grande do Sul, usada como matéria-prima para a obtenção de carvão e fonte de taninos para as indústrias de curtimento de couro. Suas partes aéreas são geralmente descartadas em pastagens e, no intuito de redução de geração de resíduos sólidos, torna-se interessante o seu reaproveitamento, como possível fonte de compostos bioativos para a indústria farmacêutica. Desta forma, o presente trabalho teve como objetivo analisar o perfil fitoquímico do das folhas de *A. mearnsii*, bem como investigar sua ação antioxidante *in vitro* e avaliar seu potencial toxicológico, através de ensaios pré-clínicos *in vitro* e *in vivo* do extrato enriquecido de compostos fenólicos (EECF). Como resultados, a análise fitoquímica preliminar das folhas indicou a presença de compostos fenólicos, principalmente. A obtenção do extrato hidroalcoólico foi feito através da maceração de 50g das partes aéreas a frio, em solução constituída ´por etanol: água (4:1-V:V), com agitação a cada 24 horas em vapor rotatório, sob pressão reduzida (temperatura não superior à 40ºC). A partir do extrato hidroalcoólico, realizou-se o fracionamento por meio da cromatografia *flash,* empregando-se solventes de diferentes polaridades*.* Após, realizou-se doseamento de compostos fenólicos totais pelo método de Folin-Ciocalteu, sendo obtidos aproximadamente 65 mg equivalente grama de ácido gálico. Deste extrato, por cromatografia preparativa, foram isolados 2 compostos, os quais foram identificados por cromatografia líquida de alta eficiência acoplada a espectrometria de massas (CLAE-EM), os resultados mostraram o ácido cinâmico e cumarina. Quanto a pesquisa da ação antioxidante, revelou potencial efeito indicado por 621,748 µmol equivalentes de trolox/g de EECF pela técnica de 2,2’ azinobis-3 etibenziltiazolina-6 ácido sulfônico (ABTS), 314,085 µmol equivalentes de trolox/g do EECF pelo 1,1-difenil-2-picrilhidrazil (DPPH), 299,758 µmol equivalentes de sulfato ferroso/g de EECF pelo poder antioxidante da redução do ferro (FRAP), 65,046 mg equivalentes de ácido gálico/g de EECF e 19,632 mg equivalentes de catequina/g de EECF. Em relação a uma possível ação citotóxica do EECF sobre as células HaCat, observou-se que àquelas expostas às concentrações de 10, 100 e 500 µg/mL houve redução da viabilidade celular, através da redução da viabilidade lisossomal. Quanto à citotoxicidade frente ao brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difenil-tetrazolio (MTT) as células expostas à concentração de 2500

µg/mL sofreram redução da viabilidade celular, devido à redução da viabilidade mitocondrial. Entretanto, pelo método de laranja de acridina as concentrações de 1000 e 2500 µg/mL do EECF da *A. mearnsii* promoveram redução da viabilidade celular por apoptose, indicando morte celular. Através do ensaio de genotoxicidade *in vitro* utilizando os bulbos de *Allium cepa*, não foram observadas alterações cromossômicas nas concentrações de 10 e 20% (p:V). Quanto aos testes *in vivo* foi pesquisada a ação do EECF sobre a atividade locomotora (campo aberto) e não foi verificado tal efeito em ratos tratados com as doses de 100 e 200 mg/kg. Porém, pelo ensaio de labirinto em cruz, os animais tratados com a dose de 200 mg/kg, apresentaram leve efeito ansiogênico, quando comparados ao grupo controle. Tem-se como perspectiva promissora nos estudos realizados até o presente momento, principalmente na investigação de compostos anticâncer devido à ação citotóxica e antioxidante observadas, sem alterações genotóxicas e efeito nocivo sobre o sistema nervoso pelos ensaios pré-clínicos.

Palavras-Chave: *Acacia mearnsii*, fitoquímica, toxicologia experimental

ABSTRACT

The black acacia (*Acacia mearnsii* De Wild) is a plant species of great economic importance in Rio Grande do Sul, used as raw matter for the production of coal and a source of tannins for tannery industries. Their aerial parts are usually disposed in pastures, and in order to reduce the generation of solid waste, their reuse as source of bioactive compounds for the pharmaceutical industry becomes interesting. Therefore, the aim of this study was to analyze the phytochemical profile of phenolic compounds-enriched extract (PCEE) of *A. mearnsii* leaves, in order to investigate their antioxidant action *in vitro*, and to evaluate their toxicological potential through *in vitro* and *in vivo* assays. As a result, preliminary phytochemical analysis of the leaves indicated the presence of phenolic compounds, mainly. The hydroalcoholic extract was obtained by cold maceration of 50g of the aerial parts, in solution consisting of ethanol: water (4:1 – V: V), with stirring every 24 hours in rotary steam under reduced pressure (temperature not exceeding 40°C). From the hydroalcoholic extract, a fractionation by flash chromatography was accomplished, using solvents of different polarities. After, the dosage of total phenolic compounds was accomplished by Folin-Ciocaltey method, obtaining 65 mg of gallic acid equivalent/g. From this extract, by preparative chromatography, 2 compounds were isolated, which were identified by high performance liquid chromatography coupled to mass spectrometry (CG-MS), the results demonstrated the cinnamic acid and coumarin. The research of antioxidant action revealed the antioxidant potential of the material tested, and obtained 621.748 µmol equivalent trolox/g PCEE by 2,2'azinobis-(3-ethylbenzthiazoline-6-sulfonic acid radical method (ABTS), 314.085 µmol equivalent trolox/g PCEE by 1,1-difenil-2-picrilhidrazil radical method (DPPH), 299.758 µmol ferrous sulphate equivalent/g PCEE by ferric reducing antioxidant power method (FRAP), 65.046 mg gallic acid equivalent/g PCEE, and 19.632 mg catechin equivalent/g PCEE. Regarding a possible cytotoxic action of EECF on HaCat cells, it was observed that those exposed at 10, 100 and 500 µg/mL concentrations had a reduction in cell viability, through the reduction of lysosomal viability. As for cytotoxicity towards 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyl-tetrazolium bromide (MTT), cells exposed at concentration of 2500 µg/mL reduced their viability due to the reduction of mitochondrial viability. However, by the acridine orange method concentrations of 1000 and 2500 µg/mL of EECF of *A. mearnsii* promoted reduction of cell viability by

apoptosis, indicating cell death. Though the *in vitro* genotoxicity assay, using the bulbs of *Allium cepa*, no chromosomic alterations were observed at the concentrations of 10% and 20%. In relation to the *in vivo* assay on the action of the PCEE on the locomotor activity (open field), no action on the rats treated with doses of 100 and 200 mg/kg was verified. However, by the maze assay (plus maze) with the dose of 200 mg/kg, the animals presented anxiogenic effect when compared to the control group due to the higher entrance in the closed arms. It is a promising prospect in the studies conducted up to the present moment, mainly in the investigation of anticancer compounds due to the observed cytotoxic and antioxidant action, without genotoxic alterations and harmful effect on the nervous system by preclinical tests.

Keywords: *Acacia mearnsii*, phytochemistry, experimental toxicology

LISTA DE ABREVIATURAS E SIGLAS

ABTS: ácido 2,2’azinobis-(3-etilbenziltiazolina-6- sulfônico)

CG/EM: Cromatografia gasosa acoplada à espectrometria de massa

CLAE/EM: Cromatografia líquida de alta eficiência acoplada à espectrometria de massa

DL_{50}: Dose letal em 50%

DMSO: Dimetil sulfóxido

DPPH: 2,2-difenil-1-picrilhidrazil

EECF: extrato enriquecido de compostos fenólicos

FRAP: *Ferric reducing antioxidant power*

LA: Laranja de acridina

mL: Mililitro

MTT: 3-[4,5- dimetiazol-2-il] 2,5- difenil brometo de tetrazolium

PBS: Soro fetal bovino

VN: Vermelho neutro

µl: Microlitro

CCD: Cromatografia de Camada Delgada

CEUA: Comitê de Ética no Uso de Animais

EM: Espectrometria de massa

PPM: Partes por milhão

RPM: rotações por minuto

PLUS MAZE: Labirinto em cruz elevado

OPEN FIELD: Campo aberto

Hacat: linhagem de células de pulmão

CN: controle negativo

CP: Controle positivo

MN: Micronúcleo

EBA: Entrada em braços abertos

EBF: Entrada em braços fechados

IC50: Índice inibitóriode 50%

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1 APRESENTAÇÃO GERAL

A dissertação apresenta inicialmente uma INTRODUÇÃO GERAL sobre a planta *Acacia mearnsii* De Wild. Nesta parte são abordados os seguintes tópicos:

- Histórico
- Características botânicas
- Usos populares do gênero *Acacia*
- Composição química
- Estudos farmacológicos
- Estudos toxicológicos

Os resultados das análises fitoquímica, antioxidante e toxicológica experimental estão relatados em um artigo a ser submetido para a revista *Journal Ethnopharmacology* (suas normas encontram-se no Anexo IV).

Abaixo, seguem os trabalhos apresentados durante o desenvolvimento desta dissertação:

1. OLIVEIRA, T. M. S.; DALZOCHIO, T; HAUBERT, R. ; PRIETSCH, A. V. G.; GEHLEN, G.; SUYENAGA, E. S.. Investigação da citogenotoxicidade in vitro do extrato aquoso das folhas de *Acacia mearnsii* De Wild em modelo de *Allium cepa* L.. In: XII Congresso Mundial de Farmacêuticos de Língua Portuguesa, 2016, Gramado. XII Congresso Mundial de Farmacêuticos de Língua Portuguesa: Área 2- Plantas Medicinais e Fitoterapia, 2016.

2. WEIMER, P.; NUNES, C. ; PRIETSCH, A. V. G.; ROSSI, R. C.; SUYENAGA, E. S. Avaliação da atividade antioxidante *in vitro* e quantificação de compostos fenólicos e flavonóides totais das partes aéreas de acácia-negra *Acacia mearnsii* De Wild).. In: XII Congresso Mundial de Farmacêuticos de Língua Portuguesa,

2016, Gramado. XII Congresso Mundial de Farmacêuticos de Língua Portuguesa: Área 2- Plantas Medicinais e Fitoterapia, 2016.

3. HAUBERT, R.; SPIES, L. M.; PRIETSCH, A. V. G.; SUYENAGA, E. S.. Avaliação do potencial efeito antiedematogênico do extrato hidroalcoólico das folhas de *Acacia mearnsii* EM RATOS WISTAR. In: Feira de Iniciação CientÍfica (FIC) - Inovamundi 2017, 2017, Novo Hamburgo. Feira de Iniciação CientÍfica (FIC) - Anais A.9, V.9, Outubro de 2017. Novo Hamburgo: Editora Feevale, 2017. v. 9. p. 217.

2. **INTRODUÇÃO GERAL**

2.1 Histórico

Acacia mearnsii De Wild é uma leguminosa que apareceu no cenário econômico mundial por volta de 1868, quando foi transportada de seu *habitat* natural, a Austrália, para a África do Sul, como planta de crescimento rápido e produtora de lenha. Vinte anos mais tarde, sua casca começava a ser empregada como substância tanante, para curtimento de couros, e também utilizada em fabricação de adesivos e produtos farmacêuticos (MARINHO, 2017). O primeiro plantio de *A. mearnsii* no Brasil foi datada em 1918, na região do vale do Rio Taquari/RS (SEGURA, 2010).

Os plantios comerciais tiveram início em 1928, por Júlio Lohmann, no Município de Estrela/RS, tendo como objetivo a utilização da casca em curtumes. Em 1941, iniciou-se a utilização comercial desta espécie como fonte de taninos, através da criação da SETA - Sociedade Extrativa de Tanino de Acácia Ltda. Em 1957, existiam 81 milhões de árvores de *A*. *mearnsii* plantadas, e em 2005 estimou-se 156.377 hectares com plantios desta espécie vegetal, atualmente sua área plantada está em 160.872 hectares em todo o País (IBÁ, 2015).

2.2 Características botânicas

A. *mearnsii* é nativa da Austrália e da Tasmânia. Pertence à família Fabaceae, subfamília Mimosoideae. Trata-se de uma árvore de porte médio, arbusto grande, copa arredondada e casca castanho-escuro (CALEGARI et al., 2016). Caracteriza-se por ser uma árvore de 10 a 30 m de altura, que cresce bem em qualquer tipo de solo, porém em seu ambiente natural é um arbusto ou pequena árvore, comumente com 6 a 10 m de altura, podendo alcançar até 15m (ELOY et al., 2015). O caule é reto e dominante, quando em conjunto com outras árvores. A casca de árvores adultas é de coloração preto-amarronzada, dura e fissurada. As folhas são compostas, bipenadas e de coloração verde-escuro (por este motivo o nome acácia-negra), o comprimento total das folhas compostas varia entre 8 e 12 cm. Já a folhagem das mudas apresenta de 4 a 8 pares opostos de pinas, de coloração

verde-escuro e cada pina é formada por 20 a 25 pares de folíolos oblongos (Figura 1). As inflorescências são em panículas terminais ou axilares, mais ou menos do tamanho da folha, com 20 a 30 flores, as quais são hermafroditas de coloração amarelo-creme claro. Os frutos são legumes mais ou menos retos, finamente peludos, comprimidos entre as sementes. Essa espécie se distribui no sudeste da Austrália, Tansmânia, África do Sul, América do Norte, América do Sul, Ásia, Pacífico e Europa (IBF, 2018).

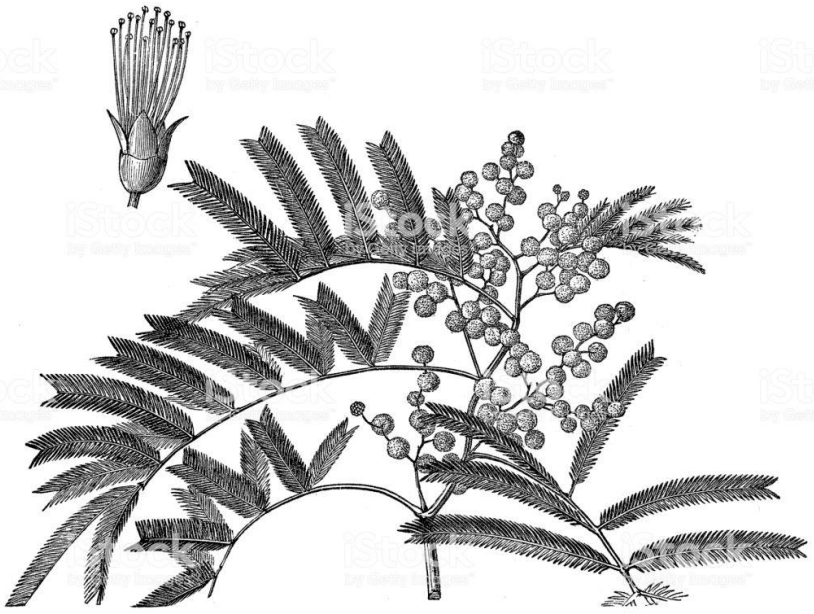


Figura 1: Partes aéreas de *Acacia mearnsii*
Fonte*:* INSTITUTO BRASILEIRO DE FLORESTAS - IBF (2018)

2.3 Usos populares do gênero *Acacia*

A espécie vegetal *A. mearnsii* trata-se de uma planta de exploração econômica, portanto não é empregada na medicina popular. Porém, há relatos de diferentes espécies do gênero *Acacia* serem empregadas para o tratamento de várias doenças.

Na Índia, a espécie *A. catechu* é utilizada para o tratamento da diarréia, leucorréia, hemorroidas e erisipela. O suco da casca fresca tem sido utilizado em tratamento de gonorréia. É relatado que *A. catechu* tem atividade hipoglicemiante, antipirético, hepatoprotetor e propriedades digestivas (RAY et al., 2006).

A casca da *A. columbrina* é empregada na medicina popular em muitas regiões do Brasil. É considerada amarga, adstringente, depurativa, hemostática, sendo utilizada contra leucorréia e gonorréia. O decocto e o xarope da casca do caule são empregados contra tosse, bronquite e coqueluche. O ferimento de sua casca libera uma goma-resina usada no fabrico de goma-de-mascar e no tratamento de problemas respiratórios (ALBUQUERQUE UP, 2007).

Quanto *A. adstringens* é indicada contra leucorréia, hemorragias, diarréia, hemorroidas, para limpeza de ferimentos e na forma de gotas contra conjuntivite. O decocto de sua casca é empregado para hemorragias uterinas, corrimento vaginal, feridas ulcerosas e para pele excessivamente oleosa (LORENZI & MATTOS, 2008).

As folhas da *A. senegal* são utilizadas para o tratamento de doenças como gonorreia, diarreia, tosse, desordem gástrica e lepra. O extrato da casca do caule é comumente usado para infecções do trato respiratório (MUDI & SALISU, 2009).

No Paquistão, o extrato de *A. leucophloea* é usado na medicina tradicional como antitérmico, hemostático, prevenção de infecção, anti-helmíntico e antídoto para veneno de cobra, mas o estudo concluiu que o extrato possui atividade espasmolítica, broncodilatadora e contra distúrbios gastrintestinais (IMRAN, 2011).

2.4 Composição química

As cascas de *A. mearnsii* apresentam como seus principais metabólitos secundários os compostos fenólicos, em especial taninos condensados (catequinas, galocatequinas). Além destes, destacam-se também como metabólitos secundários os terpenos (monoterpenos e diterpenos), protocianinas, saponinas, flavonóides (molisacacidina, mircetina, quercetina, robinetinidol), estes compostos são encontrados na folhas e flores da *A. meransii* segundo SILVA, 2018.

2.5 Estudos farmacológicos

Um extrato seco das cascas *A. mearnsii* em acetona que apresentou atividade antibacteriana nas concentrações de 20 a 10000 ng/mL, as quais todas foram efetivas frente a *Proteus vulgaris, Staphylococcus aureus*, *Escherichia coli* e *Serratia marcescens* (OLAJUYIGBE & AFOLAYAN, 2012).

Verificou-se efeito anti-obesidade e antidiabético em camundongos tratados com o extrato seco enriquecido de compostos polifenólicos, na sua dieta durante uma semana (2,5% e 5% p:p). Tais efeitos foram atribuídos pela capacidade do extrato em aumentar a expressão de RNAm da adiponecina e diminuição da expressão de TNF-alfa dos adipócitos, sugerindo reduzir os efeitos da síndrome de metabólicos (IKARASHI et al., 2011a).

Foi relatada atividade anti-inflamatória do óleo volátil obtido das cascas de *A. mearnsii* em modelo de inflamação induzida pela albumina em ratos, sendo conferido tal efeito ao monoterpeno majoritário cis-verbenol (AVOSEH et al., 2015).

Observou-se diminuição de sintomas de dermatite atópica em camundongos tratados com extrato aquoso enriquecido de compostos fenólicos a 3% de *A. mearnsii* (p:p, v.o.), apresentou efeito inibitório na expressão de ceramidase (IKARASHI et al., 2011b).

O polifenol robinetinidiol (4 beta-8) - epigalocatequina 3-*O*- galato isolado das cascas de *A. mearnsii*, mostrou efeito neuroprotetor frente ao ensaio de citotoxicidade induzida pela acroleína em células de neuroblastoma SH-SY5Y humana, sugerindo ser indicado para o tratamento da doença de Alzheimer (LU et al., 2010).

2.6 Estudos toxicológicos

Em um experimento com camundongos *Swiss* sobre a toxicidade aguda do extrato pirolenhoso de *A. mearnsii* nas concentrações de 1:100; 1:50; 1:25; 1:12,5; 1:5; 1:4; 1:2 e 1:1, diluídos em água (V:V). Nas concentrações 1:100; 1:50; 1:25; e 1:12,5, não foram relatados óbitos, porém nas concentrações 1:1 e 1:2 todos os camundongos morreram em 24 horas (PELISOLI FORMAGIO et al., 2012).

Olajuyigbe e Afolayan (2012) avaliaram a citotoxicidade *in vivo* do extrato em acetona da casca seca da *A. mearnsii*, utilizando camarões da espécie *Artemia nauplii*. A DL_{50} (dose letal em 50 %) determinada neste estudo foi de 112,36 µg/mL.

3 OBJETIVOS

3.1 Objetivo Geral

Investigar o perfil fitoquímico do extrato enriquecido de compostos fenólicos (EECF) das partes aéreas de *Acacia mearnsii,* bem como avaliar a potencial atividade antioxidante *in vitro* e possível toxicidade *in vitro* e *in vivo.*

3.2 Objetivos Específicos

- Obter o extrato enriquecido de compostos fenólicos (EECF) a partir da maceração hidroalcoólica das partes aéreas de *A. mearnsii;*

- Utilizar cromatografia analítica e preparativa para o isolamento dos compostos majoritários do extrato hidroalcoólico enriquecido de compostos fenólicos de *A. mearnsii*;

- Identificar os compostos fenólicos isolados em cromatografia gasosa acoplada em espectrometria de massas (CG/EM);

- Avaliar a atividade antioxidante *in vitro* pelo método de captura de radicais livres DPPH (radical 1,1-difenil-2-picriiidrazil) e ABTS (radical 2,2’azinobis- (3-etillbenz-tiazolina- ácido 6- sulfônico) do EECF;

- Verificar a citotoxicidade *in vitro* em queratinócitos humanos (linhagem HaCaT), no ensaio MTT (viabilidade mitocondrial), frente vermelho neutro (viabilidade lisossomal), laranja de acridina (endocitose /apoptose) do EECF;

- Avaliar o possível efeito do EECF sobre o sistema nervoso de ratos, através dos ensaios de *plus maze* e *open field.*

4 ARTIGO

Artigo: Pesquisa Fitoquímica Preliminar da Atividade Antioxidante e Avaliação Toxicológica do Extrato Enriquecido de Compostos Fenólicos das Partes Aéreas de *Acacia Mearnsii* De Wild

Artigo a ser submetido ao periódico *Journal of Ethnopharmacology* no formato Original Research Article (FI: 2.466; QUALIS CB II: B1).

Preliminary phytochemical research, in vitro antioxidant activity investigation and pre-clinical toxicological evaluation of the enriched extract of phenolic compounds from the aerial parts of *Acacia mearnsii* De Wild

Ana Valéria de Oliveira Gonçalves Prietsch[a]; Edna Sayuri Suyenaga[b*]

[a]Master's degree student at Toxicology and Toxicological Analysis Post-Graduation Program, Feevale University, Novo Hamburgo, RS, Brazil.
[b] Professor at Toxicology and Toxicological Analysis Post-Graduation Program, Feevale University, Novo Hamburgo, RS, Brazil.
Health Science Institute, Feevale University
Campus II - RS 239, nº 2755, Zip Code: 93352-000 Novo Hamburgo, RS, Brazil
[*]Corresponding author: Edna Sayuri Suyenaga, Av. Dr. Maurício Cardoso, 510 - Hamburgo Velho, Novo Hamburgo-RS,93510-250,Brazil; Telephone:555199185965; E-mail: suyenaga@feevale.br

Abstract

Acacia mearnsii De Wild (black acacia) is an economic important plant in Rio Grande do Sul State, Brazil. It is used as a source of charcoal and tannins for leather tanning. Their aerial parts usually discarted in fields, and in order to reduce the generation of solid waste, their reuse as source of bioactive compounds for the pharmaceutical industry becomes interesting. Therefore, the aim of this study was to analyze the preliminary phytochemical profile of *A. mearnsii* leaves, as well as to investigate their antioxidant action *in vitro*, and to evaluate their toxicological potential through *in vitro* and *in vivo* assays. Our preliminary results by phytochemical screening of the leaves indicated mainly phenolic compounds. Leaves were macerated at room tempertature, for seven days in hydroalcoholic solution 80% (v: v). From this extrat which was obtained some phenolic compounds enriched extract (PCEE) by fractionation carried out with the use of solvents of different degrees of polarity by flash chromatography, with 25% yield (w: w). Total phenolic compounds were quantified by the Folin-Ciocalteu method and were obtained 65 mg approximately, represented as gram gallic acid equivalent. Two compounds were isolated from preparative chromatography, which were identified as cinnamic acid and coumarin by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS). PCEE revealed potential antioxidant action, it was obtained 621.748 µmol equivalent trolox/g PCEE by 2,2'azinobis-(3-ethylbenzthiazoline-6-sulfonic acid radical method (ABTS), 314.085 µmol equivalent trolox/g PCEE by 1,1-difenil-2-picrilhidrazil radical method (DPPH), 299.758 µmol ferrous sulphate equivalent/g PCEE by ferric reducing

antioxidant power method (FRAP), 65.046 mg gallic acid equivalent/g PCEE, and 19.632 mg catechin equivalent/g PCEE. Regarding a possible cytotoxic action of EECF on HaCat cells, it was observed that those exposed at 10, 100 and 500 µg/mL concentrations had a reduction in cell viability, through the reduction of lysosomal viability. As for cytotoxicity towards 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyl-tetrazolium bromide (MTT), cells exposed at concentration of 2500 µg/mL were reduced their viability due to reduction of mitochondrial viability. Already by the acridine orange method concentrations of 1000 and 2500 µg/mL of EECF of *A. mearnsii* promoted reduction of cell viability by apoptosis, indicating cell death. Though the *in vitro* genotoxicity assay, which were used bulbs of *Allium cepa*, no chromosomic alterations were observed at the concentrations of 10% and 20% (w: V). In relation to *in vivo* assay on the central nervous system of Wistar rats of the PCEE, was did not verify any action on locomotor activity (open field) in animals treated with doses of 100 and 200 mg/kg (oral route). However, by plus maze test rats treated with 200 mg/kg dose they had presented tenuous anxiogenic effect when compared with to control group. It is a promising prospect in the studies conducted up to the present moment, mainly in the investigation of anticancer compounds due to cytotoxic and antioxidant action observed, without genotoxic alterations and injurious effect on the nervous system by preclinical tests.

Keywords: *Acacia mearnsii*, phytochemistry, experimental toxicology

1. Introduction

Acacia mearnsii De Wild is popularly known in Brazil as black acacia or mimosa because of its dark green leaves as an adult (Silva, 2018). It is an exotic species in Brazil, which belongs to family Fabaceae (Calegari et al., 2016). It has social-economic importance in the South region of Brazil, for supplying tannins for the tanning of leather and charcoal. Regarding the popular use, *A. mearnsii* has some medicinal applications, which can be utilized as hemostatic or astringent (Suyenaga et al., 2015). Researches for new sources of bioactive compounds are very important, and requires previous studies in relation to phytochemicals, pharmacological and toxicological aspects. Thus, the present work aimed at the preliminary phytochemical research of extracts obtained from aerial parts of *A.*

mearnsii, as well as to investigate its toxicological potential through *in vitro* and *in vivo* pre-clinical assays.

2 Methodology

2.1 Plant material

The *A. mearnsii* leaves were collected in Novo Hamburgo-RS, Brazil, in September 2016. The identification of the material was accomplished with the preparation of exsicata, under number HEFE 445. Leaves were dried at room temperature, free of humidity and under light protection. After the drying, the plant material was previously milled with pistil and gral, whose granulometry was according Brazilian Pharmacopoeia recommendations (Farmacopéia Brasileira, 2010).

2.2 Animals and ethics

Male *Wistar* rats weighting 180 to 200 g were utilized (n= 10 animals per group), kept under controlled room temperature (23° - 25°C) with 12h light/dark cycle. Food and water were freely available. Doses of 100 mg/kg and 200 mg/kg were used (oral route – gavage method).

The present study was approved by Ethics Committee for Animal Research of Feevale University, under the CEUA number of 01.14.037. The euthanasia of the animals obeyed the Resolution n° 714, dated June 20th, 2002 of the Federal Council of Veterinary Medicine.

2.3 HaCaT cell culture

Human keratinocytes from the HaCaT cell line were derived from the collection of the Cytotoxicity Laboratory of the Cell Bank in Rio de Janeiro. The cells were cultivated in DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum, kept under humid atmosphere of 5% CO_2 and at 37°C. Negative control (NC) cells were kept in standard conditions, and cultivations exposed to 1% hydrogen peroxide for 30 minutes constituted the positive control (PC).

2.4 Obtaining hydroalcoholic extract

Hydroalcoholic extract was obtained through maceration of 50 g of cold aerial parts, in solution constituted by ethanol: water (80 % – v: v), agitated every 24 hours for 7 days, at room temperature. The macerated was concentrated in rotary

evaporator, under reduced pressure, with heating in water bath at a temperature not higher than 40°C, to remove the solvent.

2.4.1 Obtaining the Phenolic Compounds-Enriched Extract (PCEE)

A fraction of the concentrated hydroalcoholic extract was performed, through flash chromatography (Andrade, 2010) utilizing silica gel as stationary phase and different eluent systems. The solutions of the eluent system were prepared utilizing different proportions of petroleum ether, ethyl acetate and methanol which were prepared by increasing the polarity. These fractions were concentrated in rotatory evaporator with heating in water bath not higher than 40°C, under reduced pressure, and later dried with nitrogen gas.

2.5 Quantification of phenolic compounds and total flavonoids

For the quantification of total phenolic compounds, 0.5 mg/mL of PCEE solution of *A. mearnsii* solubilized in ultra-pure water was used, and to assure the stability of all reagents, all procedures were performed under light protection.

The phenolic compounds were analyzed by the Folin-Ciocalteu method, at 760 nm and with application of reaction time of 2 h in aqueous medium (Singleton et al., 1999; Meda et al., 2005; Pires, 2016). For the quantification a standard curve of gallic acid was prepared in the concentration range of 0.001 to 0.005 mg/mL and the final result was expressed as gallic acid equivalent/g dried extracts.

2.6 Chromatography analysis

The fractions were analyzed in thin layer chromatography (TLC), using chromate sheets of silica gel GF 254 and the eluent system composed by hexane: acetone, in proportions 2:1 (v:v). The majority bands were then isolated by preparative chromatography, using the same eluent system. The isolated substances were analyzed in Focus GC chromatography coupled to ISQ single quadrupole mass detector (*Thermo Scientific*) with Auto sampler (*TriPlus*) applicator, and using helium as drag gas, whose profile was compared to database of identification files. (STOBBE, 2011).

2.7 Evaluation of in vitro antioxidant activity of PCEE

The antioxidant capacity of dried extract of *A. mearnsii* was measured through the stabilization of $ABTS^{+\bullet}$ (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radicals, $DPPH^{\bullet}$ (2,2-difenil-1-picrilhidrazil), and by reduction of metallic ions (FRAP). A 0.5 mg/mL of stock solution of PCEE was prepared and the analysis were performed under light protection at concentrations of 2.50 to 40.00 µmol/L. All analytical techniques were performed in triplicate and the results were expressed as mean (± standard deviation).

2.7.1 $ABTS^{\bullet+}$ (2,2'azinobis-3-ethylbenzthiazoline-6-sulfonic acid) radical method

For the determination of the antioxidant activity by the $ABTS^{\bullet+}$ radical method, the methodology described by Torres et al. (2017) was used. Initially, the $ABTS^{\bullet+}$ radical was formed from the reaction of 7 mM ABTS with 2.45 mM potassium persulfate, which were then incubated at room temperature and under light protection for 16 hours. After this period, the solution was diluted in ethanol until the obtention of a solution with absorbance of 0.70 (± 0.01). To perform the analyzes, 40 µL of the diluted sample was added to 1960 µL of the solution containing the radical and the absorbance was determined in a spectrophotometer (Coleman 33 D) at 734 nm after 30 minutes of reaction. As standard solution, the synthetic antioxidant Trolox at concentrations of 0.00 to 15.00 µmol/L in ethanol was used. The final results were expressed as µmol/L equivalent trolox/g of dried extracts, after 30 minutes contact of the samples with the radical.

2.7.2 $DPPH^{\bullet}$ (2,2-difenil-1-picrilhidrazil) method

For the analysis of the samples, 1.5 mL of methanolic solution of $DPPH^{\bullet}$ ($6x10^{-5}$ M) was added to an aliquot of 0.5 mL of the samples containing different concentrations of the extract. The readings were performed in spectrophotometer (Coleman 33D) at 517 nm, after 2; 5; 10 and 20 minutes from the beginning of the reaction. All the determinations were performed in triplicate and followed by a control (without antioxidant), (Brand-Williams et al., 1995; Pires et al., 2016). The decrease in the absorbance reading of the samples was correlated with the control, establishing the percentage of discoloration of the $DPPH^{\bullet}$ radical according to the formula below:

$$\% \text{ protection} = [Abs_{control} - Abs_{blank}) / Abs_{control}] \times 100$$

For the calculation of the values of EC_{50} (concentration of the extract necessary to reduce 50% of DPPH radical) of different extracts, the antioxidant activity in different concentrations was calculated, in order to draw a linear curve between the antioxidant capacity of the respective extract and its concentration. These data were submitted to a linear regression and an equation of the straight line was obtained for the calculation of the IC_{50} (50% inhibitory concentration) (Brand-Williams et al., 1995; Pires et al., 2016).

2.7.3 FRAP (Ferric Reducing Antioxidant Power) method

The FRAP analysis (Ferric Reducing Antioxidant Power) was measured in the presence of the reagent TPTZ (2,4,6-tri (2-piridil)-1,3,5-triazine), using the technique described by Urrea-Victoria et al. (2016). To evaluate the reduction of metallic ions a standard curve of ferrous sulfate was prepared in acidified aqueous medium, at 593 nm, 37°C and with reaction time of 4 minutes, the final result was determined in µmol/L ferrous sulphate equivalent/g dried extracts.

2.8 In vitro cytotoxicity assessment

The cytotoxic profile of PCEE was analyzed through classic colorimetric assays, which the lysosomal viability was evaluated by the incorporation of Neutral Red (NR) described by Borenfreund and Puerner, (1985). The mitochondrial functionality was performed through the reduction of MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) according to the technique described by Fotakis and Timbrell (2006) and the endocytosis/apoptosis by the acridine orange assay described by Shin et al. (2012).

All sub confluent cultures were exposed, in triplicate, for 24 hours to PCEE, in six concentrations (1 to 2500 µg/ mL), along with NC and PC.

2.9 Evaluation of in vitro citogenotoxicity of PCEE in Allium cepa model

For the evaluation of citogenotoxicity, a bioassay was performed in *A. cepa* based on the method described by Fiskejo (1985). The PCEE was analyzed in the concentrations of 10% and 20% (w:v) in water. The bulbs of *A. cepa* were germinated at 22°C in Petri dishes with distilled water for five days. Then, the roots were exposed to the samples for 24 hours, the negative control was exposed to

distilled water, and positive control to paracetamol solution (800 mg/L). After the exposure, the roots were collected, fixed in Carnoy's solution for 6 hours and stored in 70% ethanol. For the preparation of the slides, the roots were washed, subjected to acid hydrolysis with HCl and stained with 1% acetic orcein. The analysis was performed in optical microscope, in which the mitotic index, frequency of micronuclei and chromosomal aberrations were evaluated.

2.10 Evaluation of the PCEE action on the central nervous system in Wistar rats

To evaluate the effect of PCEE on the central nervous system (CNS) in Wistar rats, the open field tests and the pluz maze test were applied. The animals were treated with the infusion of the plant in doses of 100 and 200 mg/kg, and the controls received water, by oral route through gavage 01 hour before the experiment, o EECF foi preparado segundo Netto et al., 1986).

For the open field test, a varnished wooden box (50 x 60 x 40 cm) was used, with front face of glass. The floor was covered with linoleum and divided into 12 rectangles (15.0 x 13.3 cm), with dark lines. The test section lasted 5 minutes. The animals were placed in the left rear corner of the box and left free to explore the environment. The latency time in the first rectangle, the orientation responses, the number of crossings of a rectangle to another, and the number of fecal *bolus* were evaluated (Izquierdo, 1979; Izquierdo et al., 1984; Netto et al.,1986).

Regarding the plus maze test, an apparatus was used consisting of an elevated box, approximately 50 cm from the ground, in the shape of a cross with two open arms (50 x 10 cm) arranged perpendicular to two closed arms (50 x 10 x 40 cm) by sidewalls devoid of ceiling, forming an angle of 90°. The animals were placed in the center of the box and the animals' entrance in the open and closed arms, as well as their length of stay were measured (Pellow, 1985; Fontanive, et al., 2010).

2.11 Statistical Analysis

The results of the *in vitro* and *in vivo* assays were analyzed by ANOVA (One-way) and followed by Tukey post-hoc test and MSD (minimum significant difference). The results with $p < 0.05$ were considered statistically different.

3 Results

3.1 Phytochemical screening

Through phytochemical screening, the presence of phenolic compounds, flavonoids, tannins, coumarins and saponins were verified, as it can be observed in Table 1. The presence of anthraquinones, anthocyanin, alkaloids and cardiac glycosides were not verified (Table 1).

Table 1: Phytochemical screening results

Secondary metabolite	Result
Phenolic compounds	+
Flavonoids	+
Tannins	+++
Anthraquinones	-
Alkaloids	-
Cardiac glycosides	-
Saponins	+
Coumarins	+++
Anthocyanins	-

(+) moderate positive, (+++) intense positive, (-) absent

3.2 Chromatography analysis in the hydroalcoholic extract of A. mearnsii leaves

Among the fractions obtained from the concentrated hydroalcoholic extract, the fraction of 100% ethyl acetate showed higher yield (25%), being nominated as PCEE, due to the characterization of phenolic compounds compared to the 3% ferric chloride solution, which was storage in amber bottle under refrigeration, until the identification of their majority compounds.

An analytical chromatography of PCEE was performed, followed by preparative chromatography. For this, GF_{254} silica gel and eluent system consisting of hexane: acetone mixture in the proportions of 2:1 (v:v) were used. Two fluorescent bands were observed, blue and red color, visualized by ultraviolet light.

These majority substances were nominated as P1 and P2 and demonstrated Rf (retention factor) values equal to 0.47 and 0.43, respectively. These were analyzed by CG/MS (chromatography coupled to mass spectrometry) for their identification.

3.3 Quantification of phenolic compounds of PCEE

A standard curve of gallic acid was obtained for the determination of total phenolic compounds by Folin Ciocalteu method, represented as equation of a straight-line y= 115.07x – 0.0125, with r value equal to 0.9997, demonstrating good linearity (Figure 1).

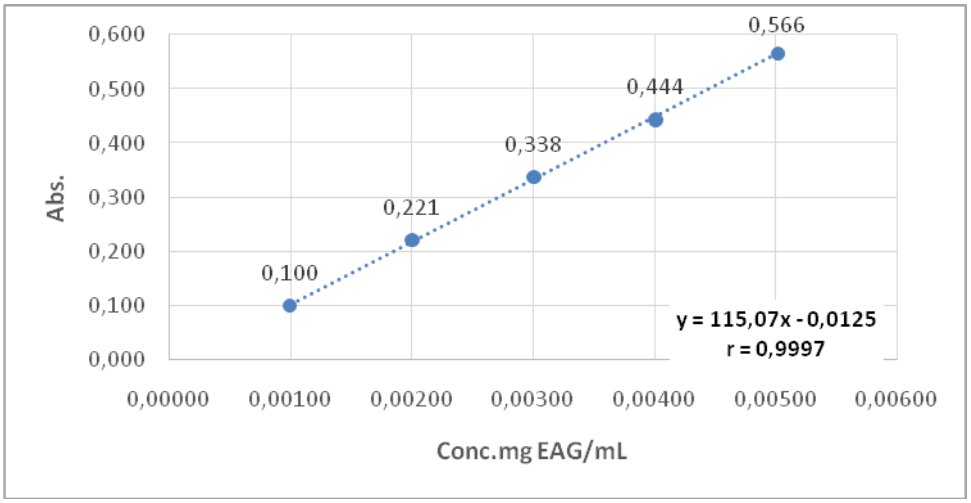


Figure 1: Standard curve of the gallic acid assay

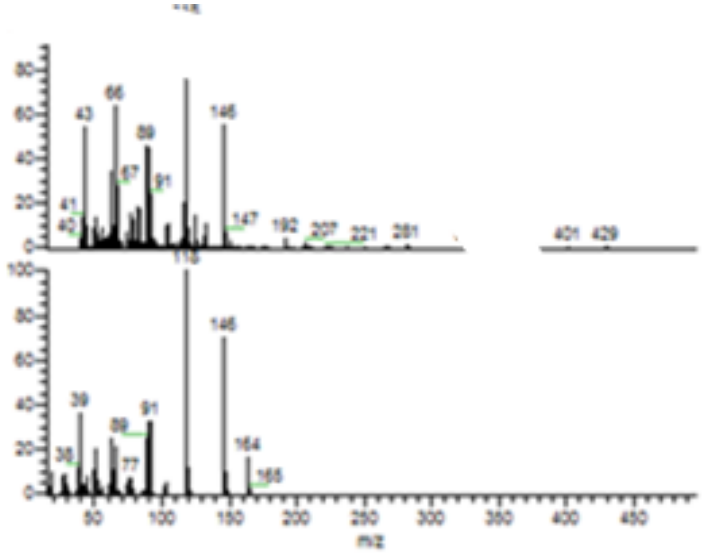
The content of total phenolic compounds presented in the PCEE was 65.046 mg ± 0.4792, represented as gram gallic acid equivalent (GAE/g).

3.4 Analysis in gas chromatography/ mass spectrometry (GC/MS)

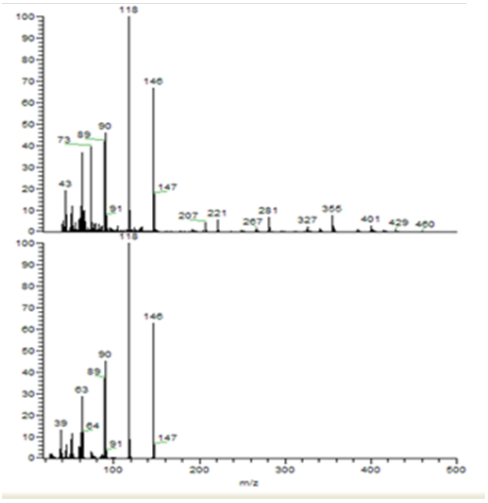
Based on the analysis of GC/MS (gas chromatography coupled to mass spectrometry), the substances P1 and P2 presented molecular weight of 148.16 g/mol and 146 g/mol, respectively. In both compounds were observed fragments that characterize phenylpropyl group by 118.18 m/z peak and phenol group, characterized by 89 m/z peak; the 43 m/z fragment characterizes the carboxyl group. These data were compared to the database, coupled to GC/MS from the Toxicology Laboratory. The high similarity of the fragmentations led to the suggestions that the compounds P1 and P2 are respectively phenolic compounds: cinnamic acid and coumarin. For confirmation, analyzes in the TLC (thin layer chromatography) were performed compared to the corresponding reference substances (Sigma) in three different eluent systems, confirming their identity (data not presented).

The coumarins are structurally lactones of o-hydroxy cinnamic acid (2H-1-benzopyran-2-ones) being the simplest representative of coumarin. The coumarins are derivative from the cinnamic acid by cyclization of the side chain of o-coumaric acid (Simões et al., 2017). Thus, it corroborates the possible chemical elucidation of

the isolated compounds of the aerial parts of *A. mearnsii* being the coumarin and the cinnamic acid, unpublished substances for this plant species. À baixo seguem os respectivos cromatogramas:



Substância P1: ácido cinâmico



Substância P2: cumarina

3.5 Evaluation of in vitro antioxidant activity

Between the activities that these secondary metabolites can perform in the organism, there is the intrinsic antioxidant activity due to the presence of free hydroxyls, mainly from the phenolic compounds (Ncube and Van Staden, 2015). Thus, as presented in Table 2, it was possible to verify this potential antioxidant *in vitro* of the dried extract of *A. mearnsii*, which is exercined both by free radical stabilization and by metal ion reduction.

The PCEE presented IC_{50} equal to 621.748 µmol equivalent trolox/g, 314.085 µmol equivalent trolox/g and 299.758 µmol ferrous sulphate equivalent/g, respectively to $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP (Table 2).

Table 2: Results of the $ABTS^{\bullet+}$ radical, $DPPH^{\bullet}$ and FRAP of the PCEE of *A. mearnsii*

Method	Equation of a Straight Line	r	IC_{50} (mg/mL)
$ABTS^{\bullet+}$	y = 3.7971x	r = 0.9988	621.748 ± 28.023 µmol eq trolox/g
$DPPH^{\bullet}$	y = 2316.9x	r = 0.9998	314.085 ± 19.717 µmol eq trolox/g
FRAP	y = 0.0234x - 0.0009	r = 0.9999	299.758 ± 13.061 µmol eq ferrous sulphate/g

IC_{50}: (50% inhibitory concentration)

The antioxidant potential of the plant extracts is associated against various inflammatory processes, through the stabilization of the free radicals generated, preventing the oxidation of lipid, protein and cellular membrane structures and protecting the organism of possible damages of these processes. The antioxidant activity observed is also in agreement with the use of leaves of this plant in popular medicine, due to its antioxidant, anti-inflammatory, antiviral and healing properties (Silva, 2018). Phenolic compounds presented a range of biological activities and are capable of stabilizing free radicals and chelating metals, which could provoke the production of other reactive species. From a structural point of view, the presence of OH (hydroxyl) groups in the phenolic compounds is essential for the performance of their antioxidant capacity (Adebayo et al., 2015).

3.6 In vitro cytotoxicity assessment

3.6.1 In vitro assessment of the neutral red test

The method of incorporation with neutral dye is carried out with the incorporation of the dye into the cell membrane, concentrating on the lysosomes, by electrostatic bonding with anionic group. However, when the damage in the cell membrane occurs, they consequently release the dye within the cells. Thus, it is

possible to verify the alive, damaged and dead cells, by the intensity or absence of staining of the cellular culture presented at the end of the test (Borenfreund and Puerner, 1985; Mattana et al., 2014).

Table 3 express the results of exposed HaCaT cells to PCEE solutions regarding the neutral red (NR).

Table 3: Cellular viability of exposed HaCaT to PCEE of *A. mearnsii* regarding the NR

PCEE (µg/mL)	**Cellular viability % in HaCaT cells (Mean ± CV)**
1	164.17 ± 30.95
10	127.8573 ± 11.55[a]
100	97.74 ± 59.39[a]
500	126.38 ± 38.65[a]
NC	228.84 ± 171.34
PC	18.01 ± 15.89

One-way ANOVA, followed by Tukey post hoc test ([a]: $p < 0.05$) NC: negative control; PC: positive control; CV: coefficient of variance.

The cells exposed at concentrations of 10, 100 and 500 µg/mL of PCEE presented reduction on the cellular viability, being an indicative of the cytotoxic effect by lysossomal reduction. Regarding those exposed to the concentration of 1 µg/mL, they did not suffer effects on the cellular viability, although it presented a mean percentage below the negative control, but not significant.

3.6.2 In vitro cytotoxicity assessment through MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide)

This assessment is based on the damage induced by the extract on the cellular metabolism in relation to the activity of mitochondrial enzymes, such as dehydrogenases. The evaluation of mitochondrial viability consequently allows to verify the cellular viability, which is quantified by the reduction of MTT (a yellow salt and soluble in water) to formazan (a purple salt and insoluble in water) by the action of these enzymes. Thus, the reduction of MTT to formazan is directly proportional to the mitochondrial activity and to cellular viability according to Fotakis and Timbrell (2006).

The results of this assessment can be visualized in Table 4, which evaluated the cellular viability of HaCaT cells, exposed in the concentrations of 1; 25; 100; 250; 1000 and 2500 µg/mL of the PCEE extract.

Table 4: Evaluation of PCEE of *A. mearnsii* regarding the MTT Assay

PCEE (µg/mL)	**Cellular viability % in HaCat cells (Mean ± CV)**
1	141.89 ± 5.80
25	122.84 ± 11.36
100	127.29 ± 12.88
250	130.141 ± 12.42
1000	110.404 ± 3.12
2500	70.956 ± 5.23 [a]
NC	87.487 ± 60.13
PC	0.00035 ± 0.00024

One-way ANOVA followed by Tukey post hoc test ([a]: $p < 0.05$ compared to PC; [b]: $p < 0.05$ in relation to NC; [*]: in relation to the highest concentration). NC: negative control; PC: positive control; CV: coefficient of variance

No significant difference was observed between the concentrations of PCEE analyzed, presenting cellular viability superior to 100%, except for the cells exposed to the concentration of 2500 µg/mL, which presented a cellular viability of around 71%, which can mean and expressive increase in the mitochondrial functionality and can be related to pre-apoptotic event (cell death without extravasation of content) (Fotakis and Timbrell, 2006).

3.6.3 Result of in vitro evaluation of acridine orange test

The acridine orange fluorescent marker is a weak base. At acid pH, weak bases accept a proton and are converted into positively charged substances which are no longer able to freely cross the cell membranes. Because of this property, the acridine orange accumulates and is retained in acid compartments. This allows its use as a marker of mature autophagosomes. In cells marked with acridine orange, the cytoplasm and the nucleolus fluoresce green, while acid compartments fluoresce red. The intensity of the red fluorescence is proportional to the degree of acidity, thus allowing both the number of autophagic cells and the intensity of autophagy, inferred

in the evaluation of the volume of acid compartments, to be quantified (Shin et al., 2012).

Table 5 demonstrates the results of cells exposed to PCEE regarding the acridine orange (AO) test.

Table 5: Evaluation of PCEE of *A. mearnsii* regarding the AO test

PCEE (µg/mL)	**Cellular viability % in HaCat cells (Mean ± CV)**
1	168.816 ± 17.038
25	146.142 ± 48.951[b]
100	125.139 ± 5.903[b]
250	137.025 ± 15.952[b]
1000	95.762 ± 15.189[a]
2500	72.125 ± 2.917[a]
NC	155.124 ± 93.820
PC	74.894 ± 47.835 [b]

One-way ANOVA followed by Tukey post hoc test ([a]: $p < 0.05$ compared to PC; [b]: $p < 0.05$ compared to NC). NC: negative control; PC: positive control; CV: coefficient of variance

According to the statistical data mentioned above, the acridine orange test presented a reduction of cellular viability by apoptosis at concentrations of 1000 and 2500 µg/mL, indicating its capacity to induce death of the HaCaT cells. Regarding the other concentrations (1, 25, 100 and 250 µg/mL), they maintained the cellular viability, however their mean values were lower than the negative control (100% viable cells), except for the concentration of 1 µg/mL, which was slightly higher but not significant

3.7 *In vitro cytogenotoxicity investigation in Allium cepa model*

The parameters for the identification of cytotoxicity, mutagenicity and genotoxicity were determined through the observation of mitotic index (MI) and frequency of micronuclei (MN), in 1000 cells of *Allium cepa*. Regarding the chromosomic aberrations (CA), 200 cells were analyzed, whose bulbs were exposed to the concentrations of 10 and 20% of PCEE solubilized in water (Table 6).

Table 6: Mitotic index (MI), frequency of micronuclei (MN), and chromosomic aberrations (CA) in *A. cepa* roots exposed to *A. mearnsii* samples at 10% and 20% concentrations

Group	Mitotic Index (%)	MN/ 1000 cells	CA (%)
NC	11.7 ± 1.4 [a]	2.0 ± 0.7 [a]	0.0 ± 0.0 [a]
PC	0.69 ± 0.3	0.86 ± 0.5	0.31 ± 0.2
10%	12.3 ± 1.7[a]	2.0 ± 0.7 [a]	0.2 ± 0.4
20%	12.6 ± 1.4[a]	2.2 ± 0.8[a]	0.1 ± 0.3 [a]

Negative Control (NC): distilled water; Positive Control (PC): paracetamol 800 mg/mL. Values are expressed as mean ± standard deviation of different parameters analyzed. For the statistical analysis, the Kruskal-Wallis test was used, and the differences were considered significative when $p < 0.05$.

A difference between the groups were verified regarding the mitotic index, and the positive control presented a low mitotic index. The frequency of micronuclei was low in 1000 cells in interphase, in both concentrations of the extract and in the negative control. The frequency of chromosomic aberrations in 200 cells in anaphase-telophase was also low, with no statistic difference between the treatments.

3.8 Evaluation of PCEE action on locomotor activity (open field) in Wistar rats

Regarding the evaluation of animals in the open field (Table 7), the animals treated with doses of 100 and 200 mg/kg presented no significative difference between the doses tested, nor the number of orientations and defecation ($p > 0.05$) compared to the control group. In fact, no reports were found in the literature on central actions related to the substances isolated in this study that could compromise the animals' motor capacity.

Table 7: Evaluation of PCEE action on open field in Wistar rats

Criteria	100 mg/kg	200 mg/kg	Control
Number of crossing	94.8 ± 33.37	96.5 ± 15.82	117.20 ± 22.6

Number of orientation responses	32.9 ± 14.07	39.8 ± 11.711	39.10 ± 16.67
Number of fecal *bolus*	2.7 ± 1.90	1.55 ± 2.15	2.00 ± 0.93

The results are expressed as mean ± standard deviation. *p < 0.05 compared to control; ANOVA/ Duncan. n= 10 animals

Regarding the plus maze (Table 8), a significant difference was observed between the control group and the animals treated with a dose of 200 mg/kg (p < 0.05) in relation to the closed arms. No significative effect was observed regarding the time of permanence in the open arms. Through this analysis, an anxiogenic effect is suggested.

Table 8: Evaluation of PCEE of *A. mearnsii* on the CNS in plus maze

Criteria	100 mg/kg	200 mg/kg	Control
EOA	7.2 ± 3.65	8.5 ± 4.92	9.50 ± 1.96
ECA	7.3 ± 3.90	6.9 ± 2.84*	10.7 ± 3.53

EOA: number of entrances in open arms. ECA: number of entrances in closed arms. The results are expressed as mean ± standard deviation. *p < 0.05 compared to control; ANOVA/Duncan tests. n=10 animals.

4. Discussion

Although only a small number of species of the genus *Acacia* has been evaluated regarding their chemical composition, the presence of saponins, coumarins, flavonoids, condensed and hydrolysable tannins were reported (Calegari et al., 2016; Silva, 2018). Barks of *A. mearnsii* have as their main secondary metabolite the presence of condensed tannins (Catechins and gallocatechins). In addition to these, terpenes (monoterpenes and diterpenes), protocyanins, saponins, flavonoids (molisacacidine, mycetin, quercetin, robinetinidol), in the *A. mearnsii* leaves are also highlighted (Silva, 2018).

Based on the preliminary phytochemical analysis, the presence of flavonoids, saponins, coumarins, as well as tannins and the absence of alkaloids, anthraquinones, and cardiotonic heterosides were observed (Table 1).

Corroborating with the study of Silva (2018), who stated that *Acacia* inflorescences, in general, were very perfumed. Coumarin, which may also be

presented in the leaves, was identified through the CG/MS analysis in present study. Coumarin are derived from the cinnamic acid, originated from the cyclization of the side chain of *o*-coumaric (Simões, 2017). Thus, corroborates the chemical elucidation of these two compounds isolated in the aerial pasts of *A. mearnsii* being the coumarin and cinnamic acid.

In the present study, it was verified that *A. mearnsii* leaves presented considerable phenolic compounds, through phytochemical screening. The enriched extract, the PCEE presented around 65 mg of total phenolic compounds in gram gallic acid equivalent.

Regarding the antioxidant potential, data obtained suggested an important observed activity of the PCEE obtained from the aerial parts of *A. mearnsii*. Possibly, such an effect has been observed due to the presence of phenolic compounds, such as flavonoids, tannins, and coumarins. The phenolic compounds presented a range of biological activities and are capable of stabilizing free radicals and chelating metals, which can provoke the production of other reactive species. From the structural point of view hydroxyls (-OH) presence in phenolic compounds are essential for their antioxidant capacity (Adebayo et al., 2015). Over the last decade, several secondary metabolites from many different classes have been isolated and tested regarding their antioxidant properties. These substances have demonstrated significant results and constitute a new possibility to reduce the cellular damage caused by the oxidative stress. As an example, the tannins, the coumarins, and a wide range of references emphasizing the use of flavonoids (Adebayo et al., 2015). A study concerning the antioxidant effect with the *Acacia catechu* stated that the gallic acid presented a potent antioxidant action, being able to exhibit anticancer effect (Sahoo et al., 2016).

The results of cytotoxicity screening, thus, could help to decide which materials are to be subjected to a fractionation/ purification process. In that way, the phytochemical investigation of medicinal plants used for cancer treatment has undeniably resulted in the development of many important anticancer substances (Nath et al., 2013).

Thus, when analyzing the *in vitro* cytotoxic profile of PCEE, it was observed through the incorporation of the Neutral Red assay a decrease in the cellular viability in cells exposed to 10, 100 and 500 µg/mL. Regarding the MTT assay in HaCaT cells, only the concentration of 2500 µg/mL promoted the reduction of cellular viability. This data corroborates with the results obtained by Mendonça (2017), after

the exposure of the human bladder cancer T24 to the extract of *A. mearnsii* for 24 hours, the MTT assay presented a reduction of cellular viability when exposed to the crude extract of *A. mearnsii* (100 and 200 µg/mL).

Lipophilic compounds are able to cross the biological membranes through passive diffusion process. The acridine orange is a lipophilic and fluorescent substance capable of easily cross cellular membranes, which allows its use as a mature autophagosome marker (Traganos and Darzynkiewicz, 1994). The PCEE of *A. mearnsii* was capable of inducing the death of HaCaT cells at the concentrations of 1000 and 2500 µg/mL, indicating apoptosis. According to Konan et al. (2012), the compounds that promote apoptosis as a cell death induction pathway are promising drugs candidates with antitumor action. It is known that antitumor activity of various flavonoids are attributed to its efficiency in inhibiting the topoisomerases I and II enzymes, reducing cellular proliferation through the inhibition of protein kinases, and/or inducing apoptosis and the release of cytochrome c (Lu et al., 2010).

Several studies have shown that most antitumor drugs promote the death of cancer cells through the mechanism of apoptosis, a type of programmed cell death. Mendonça et al. (2017) showed reduction of bladder cancer by apoptose promoted by coumarinic derivates isolated from *Acacia mearnisii.* Drugs that promote apoptosis as a cell death induction pathway are strong candidates for antitumor drugs (Pereira, 2015).

Regarding the cytogenotoxic results of *A. mearnsii*, it was not observed at the concentrations tested (10 and 20%), due to the low frequency of micronuclei, low number of chromosomic aberrations, as well as in the number of cells in anaphase-telophase, which was also low.

Studies of cytotoxicity and genotoxicity were conducted with species of *Acacia aroma* in *Allium cepa* model. The dose-dependence effect was observed, at concentrations of 1000 and 10000 ppm and macroscopic and microscopic anomalies were observed, which can be related to the tissue regeneration and healing processes of the plant, as well as their potential antitumor activity (Arias et al., 2004).

According to the results presented by the open field test, no statistically significant difference was observed between the doses tested and the control group, suggesting no interference in the rat's locomotor activity. However, the animals treated with the dose of 200 mg/kg demonstrated an anxiogenic effect when compared to the controls in relation to the number of entrances in the closed arms in

the Plus Maze test. To date, no reports have been found in the literature on central actions regarding the *A. mearnsii* action on the CNS.

5 Conclusions

The results obtained in the present study suggested the pharmacological potential of the *Acacia mearnsii De Wild* leaves. Through phytochemical screening, the presence of phenolic compounds, flavonoids, tannins, coumarins and saponins were characterized.

Quantification of phenolic compounds in PCEE was performed in approximately 65 of gallic acid equivalent/g. Its 2 major compounds, cinnamic acid and coumarin, were isolated and identified.

As regards to the evaluation of the potential antioxidant activity *in vitro*, the antioxidant effect was observed against the ABTS, DPPH and FRAP methods, with IC 50 values of 621.74 µmol eq trolox/g, 314.085 µmol eq trolox/g and 299.758 µmol eq ferrous sulphate/g, respectively. This fact can be justified due to the presence of phenolic compounds.

Regarding the research of the *in vitro* cytotoxic effect on keratinocytes of the PCEE HaCat strain, cytotoxic activity was verified by the MTT method, neutral red and acridine orange. However, it did not show cytogenotoxic changes in *Allium cepa* cells at the concentrations tested.

The PCEE did not affect the locomotor action of Wistar rats at the doses evaluated but promoted an anxiogenic effect at a dose of 200 mg / kg.

The results of this study may contribute to further research regarding the obtainment of substances as drug candidates obtained from *A. mearnsii*.

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4 CONSIDERAÇÕES FINAIS

Os resultados obtidos no presente estudo sugerem o potencial farmacológico das folhas de *Acacia mearnsii* De Wild. Através da triagem fitoquímica, foram caracterizadas a presença de compostos fenólicos, flavonóides, taninos, cumarinas e saponinas.

Realizou-se a quantificação de compostos fenólicos no EECF em aproximadamente 65 equivalente-grama de ácido gálico. Foram isolados e identificados seus 2 compostos majoritários, o ácido cinâmico e cumarina.

Quanto a avaliação da potencial atividade antioxidante *in vitro*, foi observado efeito antioxidante frente aos métodos ABTS, DPPH e FRAP, com valores de IC_{50} de 621.74 µmol eq trolox/g, 314.085 µmol eq trolox/g e 299.758 µmol eq sulfato ferroso/g, respectivamente. Tal fato pode ser justificado devido à presença de compostos fenólicos.

Em relação à pesquisa do efeito citotóxico *in vitro* em queratinócitos da linhagem HaCat do EECF, foi verificado atividade citotóxica pelo método MTT, vermelho neutro e laranja de acridina. Porém, não apresentou alterações citogenotóxicas em células de *Allium cepa* nas concentrações testadas.

O EECF não afetou a ação locomotora de ratos Wistar nas doses avaliadas, porém, promoveu efeito ansiogênico na dose de 200 mg/kg.

Os resultados deste estudo podem contribuir para pesquisas posteriores quanto à obtenção de substâncias a candidatas a fármacos obtidos a partir de *A*. *mearnsii.*

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ANEXO I

Protocolo do MTT:

Cultura e Manutenção das Células A linhagem celular humana de queratinócitos do pulmão. linhagem HaCaT. foi cultivada em meio RPMI e suplementado com 10% de soro fetal bovino (SFB) e mantida em temperatura de 37°C. com umidade relativa do ar de 95% e atmosfera de 5% CO2. Para a realização dos experimentos as células foram tripsinizadas e plaqueadas conforme a necessidade. As culturas foram expostas aos tratamentos com EECF da *A. mearnsii* (1. 25. 100. 250. 1000 e 2500µg/mL). A viabilidade celular foi determinada pela técnica de óxido-redução e formação de cristais de Formazan. As células foram plaqueadas em poços de 96 poços e após a confluência foram tratadas com as diferentes concentrações do EECF. Após 24h de tratamento as células foram lavadas com PBS e na sequência. foi adicionado uma solução de MTT 0.5%. Após 3 horas de incubação. o meio foi removido e a placa foi mantida em temperatura ambiente por 24h. para secagem. Os cristais de formazan foram eluídos em DMSO e a intensidade de coloração foi determinada por espectrofotômetro a 570nm. Os resultados foram determinados em percentual da absorbância dos tratados em relação ao controle.

ANEXO II

Protocolo do Vermelho Neutro:

A citotoxidade é avaliada pelo ensaio de viabilidade celular por meio do corante vermelho neutro que se baseia na capacidade de captura e acúmulo do corante nos lisossomos das células viáveis não injuriadas (BORENFREUND; BABICH; MARTIN-ALGUACIL, 1988). As células da linhagem L929 foram semeadas em placas de 96 poços a uma densidade de 1 x 105 células/poço e incubadas em estufa a 37oC com 5% de CO2 e. 12 horas depois. foi realizado o tratamento com várias diluições do extrato em meio não suplementado com SBF (meio incompleto). Vinte e quatro horas após o tratamento com o extrato. foi adicionado 200µL de vermelho neutro (50 µg/mL) em cada poço e a placa foi incubada na estufa por 3 horas. Após este período. o vermelho neutro foi removido e as células foram lavadas com uma solução de formaldeído 1% (v/v) e CaCl2 1% (p/v); e. em seguida. foram adicionados 200µL de etanol com ácido acético 1% (v/v). A leitura da absorbância foi realizada após 30 minutos à temperatura ambiente em leitor de microplacas (µQuant) em 540nm (BORENFREUND & PUERNER, 1985). A viabilidade celular foi expressa em porcentagem de células viáveis em relação ao grupo controle sem tratamento. A citotoxidade é avaliada pelo ensaio de viabilidade celular por meio do corante vermelho neutro que se baseia na capacidade de captura e acúmulo do corante nos lisossomos das células viáveis não injuriadas (BORENFREUND & PUERNER, 1985). As células da linhagem HaCaT foram semeadas em placas de 96 poços a uma densidade de 1 x 105 células/poço e incubadas em estufa a 37oC com 5% de CO2 e. 12 horas depois. foi realizado o tratamento com várias diluições do extrato em meio não suplementado com SBF (meio incompleto). Vinte e quatro horas após o tratamento com o extrato. foi adicionado 200 µL de vermelho neutro (50µg/mL) em cada poço e a placa foi incubada na estufa por 3 horas. Após este período. o vermelho neutro foi removido e as células foram lavadas com uma solução de formaldeído 0.5% (v/v) e CaCl2 1% (p/v); e. em seguida. foram adicionados 0.2 ml de etanol (50%) com ácido acético 1% (v/v). A leitura da absorbância foi realizada após 30 minutos à temperatura ambiente em leitor de microplacas (µQuant) em 540nm (BORENFREUND & PUERNER, 1985). A escolha deste método para avaliar a citotoxidade do extrato de *A. mearnsii* foi devido à menor interferência na leitura da absorbância em 540 nm. A viabilidade celular foi

expressa em porcentagem de células viáveis em relação ao grupo controle sem tratamento.

ANEXO III

Protocolo do ensaio Laranja de acridina:

Os estágios de apoptose tardia e inicial foram contados por coloração com laranja de acridina (LA) e examinados por microscopia de fluorescência. Para isto. as células HaCaT foram semeadas ($2x10^4$ células/poço) em placas contendo 96 poços com meio de cultura RPMI-1640. suplementado com SBF 10% e s/p 1%. Para isto. 20 µL desta suspensão de células foram solubilizados em 180 µL de azul de tripano e adicionado à Câmara de Neubauer para a contagem das células. Após 24 horas. o meio de cultura suplementado foi retirado. os poços foram lavados com PBS e foram adicionados meio de cultura com as frações do EECF da *A. mearnsii* nas concentrações de tratamento. Assim. as frações foram aplicadas nas concentrações: 1. 25 µg/mL. 100 µg/mL. 250. 500. 1000e 2500µg/mL (1% de DMSO). respectivamente. Após 24 horas de tratamento. o meio contendo os extratos foi aspirado. as células foram lavadas com PBS e tripsinizadas com 40 µL de tripsina por poço. Em seguida. as células foram centrifugadas a 2000 rpm por 5 minutos. ressuspendidas em 50 µL e PBS e coradas com 10 µL de Laranja de Acridina (LA 10 µg/mL). Também foi aplicado peróxido de hidrogênio (10 µg/mL) como controle positivo. As células coradas foram aplicadas em lâminas de vidro e a morfologia das células foi avaliada utilizando microscópio de fluorescência usando o filtro FITC e aumento de 43 400X. Foram obtidas 20 imagens em campos aleatórios e as células presentes foram contadas. As células foram classificadas em viáveis. em apoptose. Para o cálculo de porcentagem em cada uma das etapas. foi realizada regra de três simples considerando o número total de células encontradas como 100%. O Laranja de Acridina marca as células em verde. sendo que as células viáveis aparecem com núcleos esféricos. brilhantes e bem delimitados. e por fim. as células em apoptose inicial apresentam fragmentos e vacúolos (BEHZAD, et al., 2016).

ANEXO IV

Normas para publicação no Journal Ethnopharmacology

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
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A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

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Cancer Research UK, 1975. Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ (accessed 13 March 2003).
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ANEXO V

Comprovantes de trabalhos realizados durante o mestrado

XII Congresso Mundial de Farmacêuticos de Língua Portuguesa

V Simpósio de Plantas Medicinais e Fitoterápicos no Sistema Público de Saúde
Congresso Internacional de Fitoterapia
I Congresso Brasileiro de Farmácia Estética
I Simpósio Farmacêutico de Nutracêuticos

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Certificado

Certificamos que foi apresentado o trabalho científico intitulado "AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE IN VITRO E QUANTIFICAÇÃO DE COMPOSTOS FENÓLICOS E FLAVONOIDES TOTAIS DAS PARTES AÉREAS DE ACÁCIA-NEGRA (*Acacia mearnsii* De Wild)" de autoria de PATRÍCIA WEIMER; CRISTIANE NUNES; RONETE HAUBERT; ANA VALÉRIA DE GONÇALVES PRIETSCH; ROCHELE CASSANTA ROSSI; EDNA SAYURI SUYENAGA, no XII Congresso Mundial de Farmacêuticos de Língua Portuguesa; V Simpósio de Plantas Medicinais e Fitoterápicos no Sistema Público de Saúde; Congresso Internacional de Fitoterapia; I Congresso Brasileiro de Farmácia Estética e I Simpósio Farmacêutico de Nutracêuticos, com carga horária de 30 horas, realizados pelos Conselho Federal de Farmácia, Associação de Farmacêuticos dos Países de Língua Portuguesa e Fundação Brasileira de Ciências Farmacêuticas, com parceria institucional do Conselho Regional de Farmácia do Rio Grande do Sul, nos dias 8, 9 e 10 de novembro de 2016, em Gramado, Rio Grande do Sul.

Gramado - RS, 10 de novembro de 2016.

Walter da Silva Jorge João
Presidente do Conselho Federal de Farmácia

Valmir de Santi
Presidente do XII Congresso Mundial de Farmacêuticos de Língua Portuguesa

Carlos André Oeiras Sena
Presidente da Fundação Brasileira de Ciências Farmacêuticas

INVESTIGAÇÃO DA ATIVIDADE ANTI-INFLAMATÓRIA DO EXTRATO HIDROALCOÓLICO OBTIDO DAS RAÍZES DE BRUNFELSIA UNIFLORA

Lenise Maria Spies[1]; Ronete Elisabete Haubert[1]; Ana Valéria de Oliveira Gonçalves Prietsch[1]; Edna Sayuri Suyenaga[2];

Brunfelsia uniflora (Solanaceae) é uma espécie nativa da região sul e sudeste do Brasil, popularmente conhecida como primavera, manacá-de-cheiro. Suas folhas são empregadas na medicina popular, para o tratamento de artrite, reumatismo, sífilis, febre amarela e antitérmica. O objetivo do presente trabalho foi avaliar o efeito antiedematogênico do extrato hidroalcoólico das raízes. O material vegetal foi coletado em Cachoeira do Sul – RS e identificado botanicamente. O extrato hidroalcoólico foi obtido pela maceração da raiz (10 %, p:V) em álcool 80%, durante 7 dias e o solvente eliminado em evaporador rotatório a vácuo. O modelo de avaliação do efeito antiedematogênio seguiu-se a técnica descrita por WINTER e colaboradores (1962). Utilizou-se ratos Wistar (machos, 3 meses de idade, n= 6 animais/grupo). Os animais controles receberam água e os tratados, as doses de 100 mg/kg e 200 mg/kg do extrato hidroalcoólico ressolubilizado em água, por via oral (gavage). Após uma hora do tratamento, o edema foi induzido na região aponevrose subplantar da pata traseira esquerda dos ratos, através da injeção de 0,1 mL de suspensão de carragenina em solução salina, e as patas direitas serviram de controle (solução salina). O volume das patas foi medido em triplicata, nos tempos 1, 2 e 4 horas após a injeção do agente flogístico, com auxílio do pletismômetro Ugo Basile, modelo 7140. Os dados obtidos foram analisados estatisticamente pelo teste t de Student. Através dos resultados obtidos, todos os animais tratados com o extrato hidroalcoólico de B. uniflora apresentaram redução do edema formado em comparação ao grupo controle ($p < 0,01$), em especial na dose de 200 mg/kg, com inibição de 89,1% após 1 hora da indução do edema. Pelos dados observados, sugere-se potencial efeito antiedematogênico do extrato obtido das raízes. Estudos fitoquímicos estão em andamento para a caracterização das substâncias bioativas, bem como estudos farmacológicos e toxicológicos.

Palavras-chave: Brunfelsia uniflora. Carragenina. Edema. Raízes. Rato.

[1]Autor(es) [2]Orientador(es)

E-mails: lenise_spies@hotmail.com e suyenaga@feevale.br

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Gramado - RS, 10 de novembro de 2016.

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Presidente do Fundação Brasileira de Ciências Farmacêuticas