FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
FACULDADE DE CIÊNCIAS BIOLÓGICAS E AMBIENTAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA GERAL/BIOPROSPECÇÃO

CARLA ROBERTA FERREIRA VOLOBUFF

AVALIAÇÃO BIOLÓGICA E ESTUDO FITOQUÍMICO DE QUATRO ESPÉCIES DE *Psychotria* (RUBIACEAE)

v. 1

Dourados

2016

CARLA ROBERTA FERREIRA VOLOBUFF

AVALIAÇÃO BIOLÓGICA E ESTUDO FITOQUÍMICO DE QUATRO ESPÉCIES DE *Psychotria* (RUBIACEAE)

Dissertação apresentada à Universidade Federal da Grande Dourados para obtenção do título de Mestre em Biologia Geral/Bioprospecção.

Área de concentração: Bioprospecção

Orientadora: Profª. Drª. Anelise Samara Nazari Formagio

Dourados

2016

AGRADECIMENTOS

À Deus, por sempre me guiar e por permitir tantas bênçãos em minha vida.

À minha mãe Valdirene, por ser meu porto seguro e me incentivar nos estudos. Nunca me deixou desanimar diante dos percalços durante minha jornada acadêmica e sempre fez aumentar a minha fé em Deus e que Ele tem um propósito para nossa vida. Basta acreditar que tudo é possível e Ele não desampara aquele que se esforça e se dedica. Muito obrigada mãe, sem a senhora eu não teria chegado até aqui.

Aos meus irmãos Roberta e Paulo pelo companheirismo e pelos risos constantes, sempre me acolheram e estiveram ao meu lado nos momentos mais difíceis.

Ao meu namorado e amigo Marcos Vinícius, por cuidar tão bem de mim e permanecer ao meu lado dando apoio e incentivo para não desistir. Obrigada por ser tão presente e por trazer mais felicidade para a minha vida.

Aos da minha família Ferreira, que torceram pelo meu crescimento e pelo auxílio que prestaram quando necessitei.

À minha orientadora Profa. Dra. Anelise Formagio pela paciência, por todas as oportunidades que me proporcionou e pelos valiosos ensinamentos. Um exemplo de pessoa e profissional a ser seguido, por total competência e eficiência no trabalho. Muito obrigada por estar contribuindo na minha formação pessoal e profissional.

Aos colegas do laboratório de Plantas Medicinais Yara, Diego, Raquel e Kamila por auxiliarem nos testes biológicos e aos demais que não estão mais presentes no nosso grupo de pesquisa. Agradeço também a técnica do laboratório Suzana Heim por sempre cuidar de nós e ser tão querida com todos. Agradeço a Joyce Alencar por dispor de seus dias de trabalho para nos acompanhar nas atividades realizadas na Faculdade de Ciências da Saúde.

Agradeço também Profa. Dra. Mary Ann Foglio, Profa. Dra. Candida Leite Kassuya, Profa. Dra. Maria Helena Sarragiotto pelo auxílio com as atividades biológicas e RMN.

À Profa. Dra. Virgínia Demarchi Kappel Trichez e Profa. Dra. Adriana Mestriner por participarem da banca de qualificação e pelas valiosas contribuições.

Ao Prof. Dr. Jonas da Silva Mota e Prof. Dr. Ubirajara Lanza Júnior por terem aceito o convite de participar da banca de defesa e pelas valiosas contribuições.

Ao PPG Biologia Geral/Bioprospecção pela oportunidade de participar do Programa e dar continuidade aos meus objetivos.

À Capes pela bolsa concedida.

À Fundect pelo auxílio financeiro para desenvolvimento do projeto de pesquisa.

Muito obrigada a todos que contribuíram direta ou indiretamente para a realização deste trabalho.

LISTA DE ABREVIATURAS E SIGLAS

786-0	Renal
ABTS	2,2’-azinobis(3-etilbenzotiazolina-6-ácido sulfônico)
AChE	Acetilcolinesterase
AcSCh	Iodeto de acetilcolina
AMF	Fração hidrometanólica
ANVISA	Agência Nacional de Vigilância Sanitária
BCG	*Mycobacterium bovis*
BChE	Butirilcolinesterase
BSA	Abumina sérica bovina
CC	Cortex cerebral
CD_3OD	Metanol deuterado
CF	Fração clorofórmio
Cg	Carragenina
$CHCl_3$	Clorofórmio
COX	Ciclo-oxigenase
DA	Doença de Alzheimer
DMSO	Dimetilsulfóxido
DNA	Ácido Desoxirribonucléico
DPPH	2,2 difenil-1-picrilhidrazil
DTNB	5,5’-dithiobis-(2-ácido nitrobenzóico)
EAF	Fração acetato de etila
EMPL	Extrato metanólico de *Psychotria leiocarpa*
ERNs	Espécies Reativas de Nitrogênio
EROs	Espécies Reativas de Oxigênio
EtOAc	Acetato de etila
GI_{50}	Atividade citostática
HaCaT	Queratinócitos
HF	Fração hexano
HP	Hipocampo
HPLC-DAD	Cromatografia Líquida de Alta Eficiência com arranjo diodo

HT-29	Cólon
K-562	Leucemia
MAO-A	Monoamina Oxidase A
MAO-B	Monoamina Oxidase B
MCF-7	Mama
NCI-ADR/RES	Mama Resistente a Múltiplas Drogas
NCI-H460	Pulmão
NMR	Ressonância Magnética Nuclear
OECD	Organization for Economic Cooperation and Development
OMS	Organização Mundial da Saúde
OVCAR-3	Ovário
PBS	Tampão fosfato salina
PC-3	Próstata
PL-1	Vincosamida
REMUME	Relação Municipal de Medicamentos Essenciais
RENAME	Relação Nacional de Medicamentos Essenciais
RESME	Relação Estadual de Medicamentos Essenciais
SNC	Sistema Nervoso Central
ST	Estriado
THA	Tetrahidroaminocridina
TLC	Cromatografia em camada fina
TMS	Tetrametilsilano
TNF- α	Fator de Necrose Tumoral α
U251	Glioma
UV	Ultravioleta

LISTA DE ESQUEMAS

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

RESUMO

VOLOBUFF, C. R. F. **Avaliação biológica e estudo fitoquímico de quatro espécies de *Psychotria* (RUBIACEAE).** 2016. Dissertação (Mestrado) – Faculdade de Ciências Biológicas e Ambientais, Universidade Federal da Grande Dourados, Dourados, 2016.

Espécies do gênero *Psychotria* (Rubiaceae) são utilizadas na medicina popular para tratamento de diversas enfermidades. Devido a ocorrência de espécies do gênero na região da Grande Dourados, este trabalho objetivou realizar a avaliação biológica e o estudo fitoquímico de *P. capillacea*, *P. deflexa*, *P. carthagenensis* e *P. leiocarpa*. **Manuscrito I:** O extrato metanólico das quatro espécies de *Psychotria* foi utilizado para quantificar o teor de flavonoides, flavonol, fenóis totais e taninos condensados, além da atividade antioxidante pelos métodos DPPH, ABTS e β-caroteno/ácido linoleico e análise por HPLC/DAD. *P. carthagenensis* e *P. capillacea* apresentaram alta atividade de sequestro de radicais livres com IC_{50} de 16,92 e 30,05 µg/mL, quando comparados ao padrão BHT. *P. carthagenensis* apresentou alta atividade (79,1%) com o método de β-caroteno quando comparado com quercetina. *P. capillacea* apresentou alta concentração de compostos fenólicos (148,42 mg/ácido gálico g/extrato). Alta concentração de flavonoides foi observado em *P. carthagenensis* (182,07 mg quercetina/g extrato). Elevada concentração de flavonol foi encontrado nos extrato de *P. deflexa* e *P. carthagenensis* (275,07 e 241,19). Altos níveis de taninos condensados foram encontrados em *P. carthagenensis* (632,39 mg/catequina/g extrato). Na análise por HPLC/DAD somente o ácido p-cumárico foi encontrado em *P. carthagenensis* e *P. capillacea*. **Manuscrito II:** Foi realizado o fracionamento, isolamento e identificação dos principais constituintes presentes no extrato metanólico das folhas de *P. leiocarpa* e avaliação da atividade antitumoral, anticolinesterásica, anti-inflamatória e toxicidade aguda de *P. leiocarpa*. O estudo fitoquímico realizado com o extrato metanólico das folhas de *P. leiocarpa* resultou no isolamento do alcaloide vincosamida. O extrato não apresentou toxicidade em nenhuma dose testada. Extrato e vincosamida foram avaliados frente a atividade anti-inflamatória nos modelos de edema de pata induzido por carragenina e pleurisia em camundongos e ensaio anticâncer testados em dez linhagens de células tumorais. O extrato não foi efetivo no modelo de edema de pata. Entretanto, extrato exibiu migração leucocitária de 86,71% e 88,85% nas doses de 100 e 300 mg/kg^{-1}. Vincosamida apresentou migração leucocitária de 75,41% e 88,35% nas doses

de 3 e 30 mg/kg^{-1}. Para a atividade antiproliferativa, extrato apresentou $GI_{50} \geq 56,89$ µg/mL. Vincosamida apresentou potente atividade com valores de Gi50 11,75 – 24,96 µM para todas as células testadas. A atividade anticolinesterásica foi realizada *in vitro* e *in vivo* em cérebro de ratos. No ensaio *in vitro*, o extrato apresentou inibição da acetilcolinesterase para as estruturas de córtex (64,00%), hipocampo (51,80%) e estriado (18,00%). No modelo *in vivo*, o extrato nas doses de 0,5 e 3 mg/kg exibiu uma inibição para córtex de 61% e 48%, não apresentando efeito significativo para hipocampo e estriado. Extrato e vincosamida apresentaram efeitos antiproliferativo, anticolinesterásico e anti-inflamatório frente aos modelos experimentais testados.

Palavras-chave: Alcaloides indolicos, anticolinesterásica, inflamação, toxicidade, antioxidante, antiproliferativo

ABSTRACT

VOLOBUFF, C. R. F. **Biological evaluation and study phytochemical species *Psychotria* sp. (Rubiaceae).** 2016. Master (MSc) – Faculty of Biological and Environmental Sciences, Federal University of Grande Dourados, Dourados, 2016.

Species of the genus *Psychotria* (Rubiaceae) are used in folk medicine to treat various diseases. Because the occurrence of species of the genus in the Grande Dourados, this study aimed to carry out biological evaluation and phytochemical study of *P. capillacea*, *P. deflexa*, *P. carthagenensis* and *P. leiocarpa*. **Manuscript I:** Methanol extract of the four species of *Psychotria* was used to quantify the flavonoid content, flavonol, total phenols and tannins in addition to the antioxidant activity by DPPH methods, ABTS and β-carotene / linoleic acid and analyzed by HPLC / DAD. *P. carthagenensis* and *P. capillacea* showed high activity of scavenging free radicals with IC_{50} 16.92 and 30.05 mg/mL, compared to the standard BHT. *P. carthagenensis* showed high activity (79.1%) with β-carotene method when compared to quercetin. *P. capillacea* showed high concentration of phenolic compounds (148.42 mg/gallic acid/g extract). High concentration of flavonoids was observed in *P. carthagenensis* (182.07 mg quercetin/g extract). High concentration of flavonols found in *P. deflexa* and *P. carthagenensis* extracts (275.07 and 241.19). High levels of condensed tannins were found in *P. carthagenensis* (632.39 mg/catechin/g extract). In the analysis by HPLC / DAD only the p-coumaric acid was found in *P. carthagenensis* and *P. capillacea*. **Manuscript II:** This was the fractionation, isolation and identification of the main constituents present in the methanol extract of the leaves of *P. leiocarpa* (EMPL) and evaluation of antitumor activity, acetylcholinesterase, anti-inflammatory and acute toxicity P. leiocarpa. The phytochemical study of the methanol extract of EMPL leaves resulted in the isolation of vincosamide alkaloid. The extract showed no toxicity at any dose tested. EMPL and vincosamide were evaluated against anti-inflammatory activity in the rat paw edema model induced by carrageenin pleurisy and anticancer test mice and tested in ten cancer cell lines. The EMPL was not effective in the rat paw edema model. However, leukocyte migration extract exhibited a 86.71% and 88.85% at doses of 100 and 300 mg/kg^{-1}. Vincosamide presented leukocyte migration 75.41% and 88.35% at doses of 3 and 30 mg/kg^{-1}. For the antiproliferative activity, extract showed $GI_{50} \geq 56.89$ mg / mL. Vincosamide showed potent activity with GI_{50} values from 11.75

to 24.96 µM for all tested cells. The anticholinesterase activity was carried out *in vitro* and *in vivo* in rat brain. *In vitro* assay the extract showed inhibition of acetylcholinesterase for cerebral cortex (64.00%), hippocampus (51.80%) and striatum (18.00%). In the *in vivo* model, the extract at doses of 0.5 and 3 mg/kg exhibited an inhibition to the cortex of 61% and 48%, but had no significant effect on the hippocampus and striatum. EMPL and vincosamide showed antiproliferative effects, anticholinesterase and anti-inflammatory against the tested experimental models.

Keywords: indole alkaloids, acetylcholinesterase, inflammation, toxicity, antioxidant, antiproliferative

1. INTRODUÇÃO

A utilização de plantas com fins medicinais, para tratamento, cura e prevenção de doenças, é uma das mais antigas formas de prática medicinal da humanidade. No início da década de 1990, a Organização Mundial de Saúde (OMS) divulgou que 65-80 % da população dos países em desenvolvimento dependiam das plantas medicinais como única forma de acesso aos cuidados básicos de saúde (PONTES, 2006; RUAS, 2013).

No Brasil, as plantas medicinais da flora nativa são utilizadas com pouca ou nenhuma comprovação de suas propriedades biológicas, propagadas por usuários ou comerciantes. Muitas vezes essas plantas são, inclusive, empregadas para fins medicinais diferentes daqueles utilizados pelos silvícolas. Resoluções da Agência Nacional de Vigilância Sanitária (ANVISA), de 16 de março de 2004 visam a normatização do registro de medicamentos fitoterápicos. A Resolução-RDC no. 48 determina que todos os testes referentes ao controle de qualidade de fitoterápicos deverão ser realizados em rede credenciada no sistema REBLAS (Rede Brasileira de Laboratórios em Saúde) ou por empresas que possuam certificado de BPFC (Boas Práticas de Fabricação e Controle).

A diferença entre planta medicinal e fitoterápico está na elaboração da planta para uma fórmula farmacêutica, o que caracteriza um fitoterápico. A OMS define planta medicinal como sendo "todo e qualquer vegetal que possui, em um ou mais órgãos, substâncias que podem ser utilizadas com fins terapêuticos ou que sejam precursores de fármacos semi-sintéticos" (WHO, 1998). Destaca-se também o fitofármaco, que por definição "é a substância ativa, isolada de matérias-primas vegetais ou mesmo, mistura de substâncias ativas de origem vegetal".

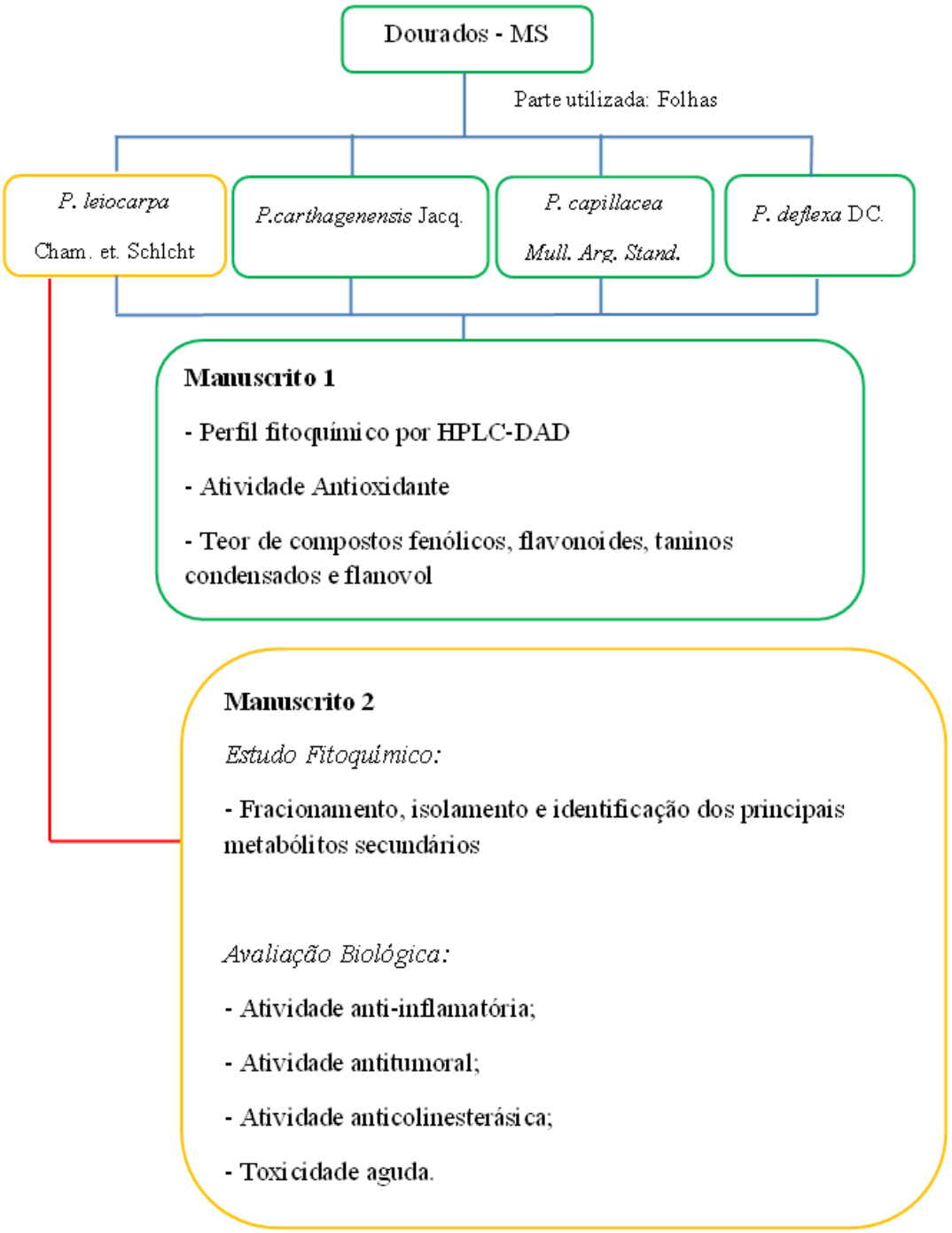
A realização da I Conferência Nacional de Assistência Farmacêutica, em 2003, sob o tema "Efetivando o acesso, a qualidade e a humanização na Assistência Farmacêutica, com controle social", reforçou a necessidade de atualização constante das listas de medicamentos essenciais de cunho nacional (Rename), estadual (Resme) e municipal (Remume). A Rename (Relação Nacional de Medicamentos Essenciais) sofreu quatro revisões mais recentes que foram a de 2002, 2006, 2008 e 2010, sendo a última publicada pela Portaria MS/GM n.º 1044, de 05 de maio de 2010, possuindo 343 fármacos, 08 correlatos e 33 imunoterápicos, em 574 apresentações farmacêuticas (PEPE, 2010). Dentre os fármacos destaca-se sulfato de vimblastina (utilizado para tratamento de câncer de mama, leucemia, linfoma, câncer de pulmão e testicular) e sulfato de vincristina (utilizada em casos de leucemia), ambos isolados de *Catharanthus roseus* (L.) G. Don. (MORAES et al., 2011). Alguns fitoterápicos utilizados são o xarope de guaco (*Mikania glomerata*), xarope de agrião (*Nasturtium officinale*) e

Calmatoss®, um xarope utilizado como antitussígeno, expectorante, antisséptico respiratório e broncodilatador, composto por tinturas de *Mikania glomerata* Spreg., *Grindelia robusta* Nutt., *Copaifera officinalis* (Jacq.) L., *Myroxylon toluifera* L. Harms., *Nasturtium officinal* R. Br., além de própolis e mel (SOARES et al., 2006).

Assim, as comprovações científicas farmacológicas e a investigação fitoquímica de plantas são de grande importância, pois o isolamento de princípios ativos e a modificação química destes podem resultar em descobertas de novos fármacos com aplicação terapêutica e menor efeito tóxico.

Em função dos aspectos discutidos acima, o nosso grupo de pesquisa vem desenvolvendo o estudo do potencial químico e biológico de plantas presentes no cerrado e mata atlântica na região de Dourados-MS. Das várias famílias presentes nesta região, destaca-se a Rubiaceae, por sua ampla distribuição e pelo número de gêneros e espécies ocorrentes, dentre eles o gênero *Psychotria* com oito espécies registradas segundo Pereira (2007) realizando um estudo sobre a florística, sistema reprodutivo, distribuição e relações alométricas de espécies da família Rubiaceae no Mato Grosso do Sul.

Neste contexto, o trabalho foi elaborado para avaliar a ação farmacológica e o estudo fitoquímico de quatro espécies de *Psychotria* coletadas em Dourados-MS (**Esquema 1**).



Esquema 1. Proposta de trabalho com espécies de *Psychotria* coletadas em Dourados-MS.

2. REVISÃO DE LITERATURA

2.1 Gênero *Psychotria*

O gênero *Psychotria* (Rubiaceae) compreende aproximadamente 2000 espécies, taxonomicamente complexo e largamente empregado na medicina popular para o tratamento de diversas enfermidades, como diarreia e parasitas intestinais (MCGAW et al., 2000), infecções

virais e bacterianas (LOCHER et al., 1995; KUO et al., 2001), disfunções cardiovasculares, distúrbios mentais e alimentares (CABALLERO-GEORGE et al., 2001). Do ponto quimiotaxonômico é caracterizado principalmente pela presença de alcaloides polindolicos derivados da condensação de várias unidades *N*-metiltriptamina, além da presença de iridoides e alcaloides indolicos monoterpênicos (LOPES, 1998) os quais são responsáveis por algumas atividades biológicas. Estes alcaloides são obtidos pela rota metabólica cujo aminoácido de origem é o triptofano e de um único precursor a estrictosidina produzida pela condensação de uma molécula de triptamina (**Figura 1)** com a secologanina, elaborada via geraniol, a partir das moléculas do ácido mevalônico (BRUNETON, 1991).

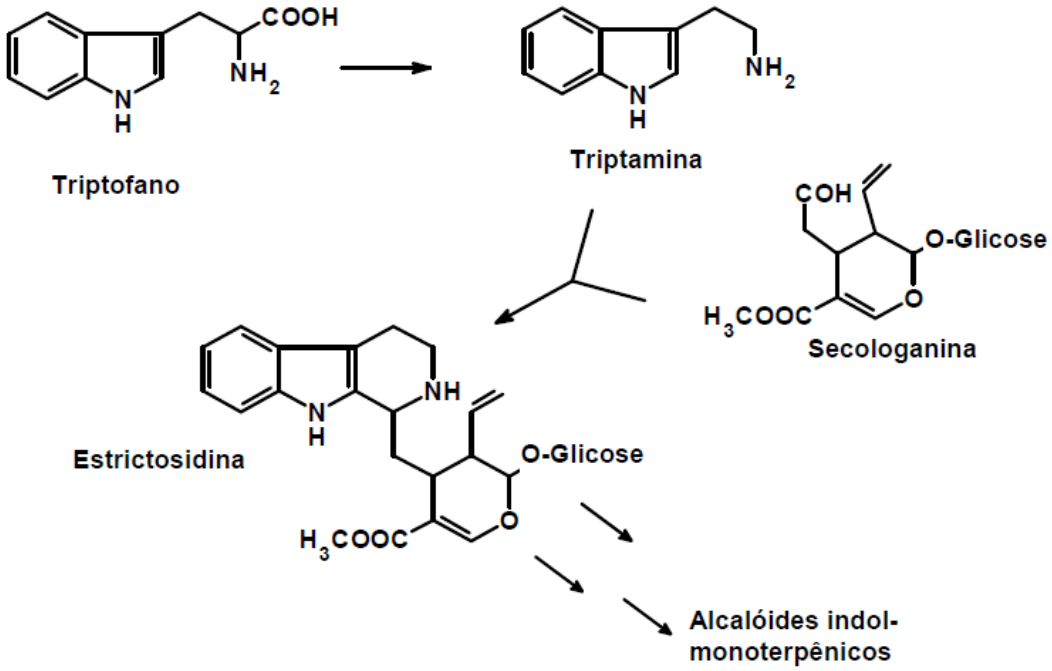


Figura 1. Rota biossintética para os alcaloides indol-monoterpênicos (DEWICK, 1997).

A pesquisa no gênero foi motivada principalmente pelo uso de duas espécies *Palicourea viridis* e *P. carthagenensis*, juntamente com decocto de *Banisteriopsis caapi*, produziram uma bebida alucinógena "ayahuasca", utilizada para fins religiosos e medicinais por povos da Amazônia (MCKENNA et al., 1984; LIWSZYC et al., 1992). Da espécie *P. viridis* foi isolado um alcaloide N,N-dimetiltriptamina **(I) (Figura 2)**, o qual possui semelhança estrutural com a serotonina **(II)**, que o torna uma alternativa no tratamento de distúrbios do sistema nervoso central (SÉRPICO e CAMURÇA, 2001).

De *P. umbelata* isolou-se a umbelatina ou psicolatina **(III)** que apresenta atividade ansiolítica e anticonvulsivante (BOTH, 2006). Dos estudos com *P. myriantha* Mull. Arg. foram isoladas as myrianthoisinas A **(IV)** e B **(V)** e o ácido estrictosidínico **(VI)**.

Da espécie *P. brachyceras* isolou brachycerina, e em *P. leiocarpa* foram encontrados o alcaloide N,β-D-glucopiranosil vincosamida **(VII)** e os iridoides asperulosideo **(VIII)** e deacetilasperulosídeo **(IX)**. Lialosídeo **(X)** e estrictosamida **(XI)** foram isolados de *P. suterella* (HENRIQUES, 2004; SANTOS, 2001; KERBER, 2001).

Das folhas da espécie *P. myriantha* Mull. Arg., foi isolado o ácido strictosidínico **(VI)**, um alcaloide indolico monoterpênico glicosilado apresentando efeito analgésico dose-dependente parcialmente revertido por naloxona em modelo de placa quente (BOTH et al., 2002). O ácido estrictosidínico (10 mg/kg) também foi ativo para o aumento do metabolismo de monoaminas na Doença de Alzheimer em estriado de ratos (FARIAS et al., 2010).

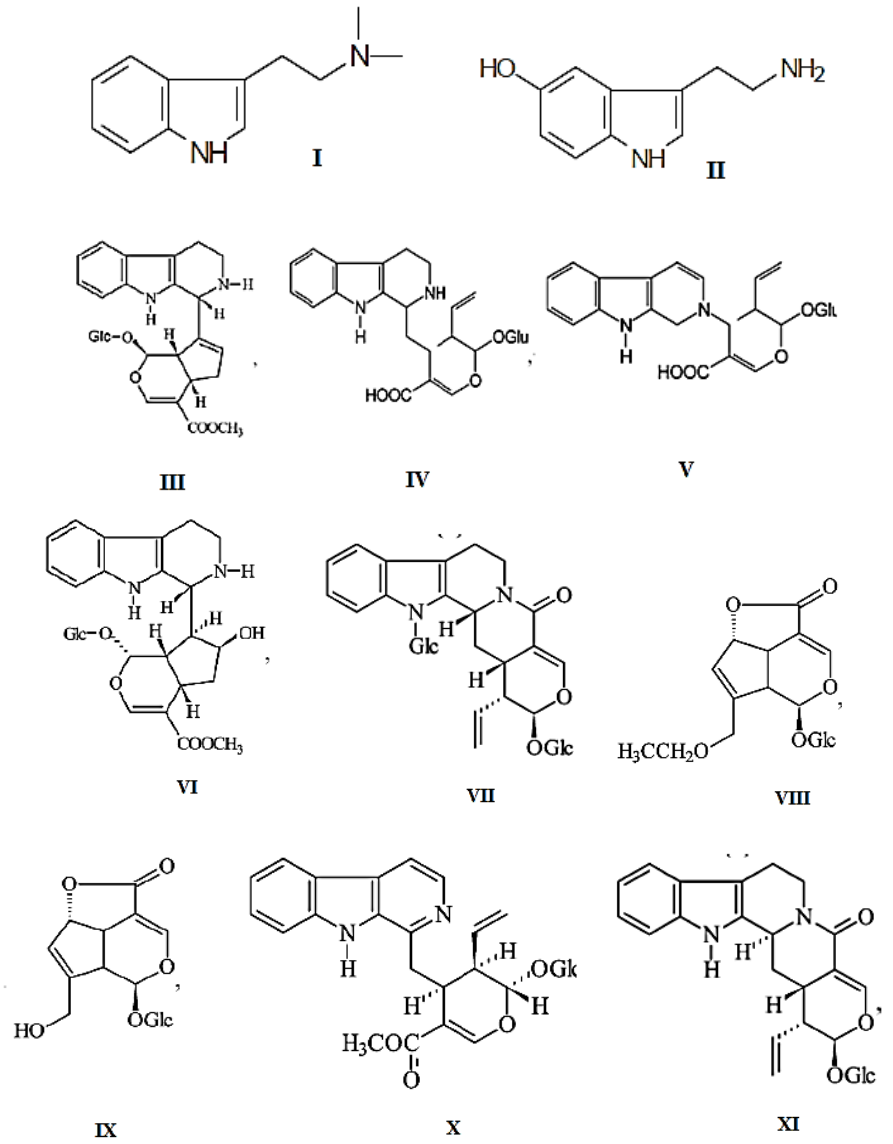


Figura 2. Estruturas de alcaloides e iridoides isolados de *Psychotria*.

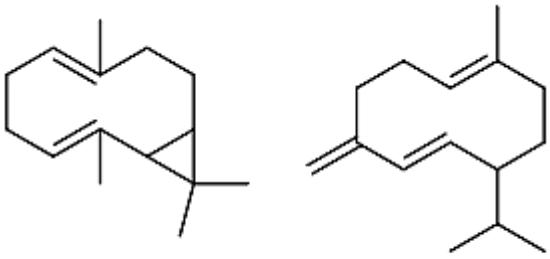
2.2 *Psychotria leiocarpa* Cham. e Schlcht.

Psychotria leiocarpa (**Figura 3**) popularmente conhecida como "grandiúva-de-anta" ou "cafeeiro-do-mato", é um arbusto que atinge 2 m de altura o qual se distribui da Bahia e Minas Gerais até o Rio Grande do Sul, bem como no Mato Grosso do Sul e Paraguai (ANDERSSON, 1992; DELPRETE et al., 2005) . Dillenburg e Porto (1985) relataram o isolamento do alcaloide indolico monoterpênico *N*-glicosilado (*N*, β-D-glucopiranosil vincosamida) **(VII)** e de iridoides asperulosídeo **(VIII)** e deacetilasperulosídeo **(IX) (Figura 2)** das folhas dessa espécie (HENRIQUES, 2004; SANTOS, 2001; KERBER, 2001).

Figura 3. Folhas e galhos de *Psychotria leiocarpa* (Fonte: Volobuff, 2014).

Estudo de Leal (1994) em extratos etanólicos de *P. leiocarpa*, *P. brachyceras*, *P. myriantha* e *P. suterella* avaliando a toxicidade e atividade analgésica em camundongos, indicou que os alcaloides relatados nas espécies foram os principais responsáveis pelas atividades testadas. Atividade analgésica inespecífica a partir do extrato etanólico das folhas de *P. leiocarpa* foi evidenciado por Elisabetsky et al. (1997).

O óleo essencial extraído das folhas apresentou 33 compostos caracterizados exclusivamente por sesquiterpenos, destacando o biciclogermacreno com 35,6% e germacreno D com 17,6% **(Figura 4)** (ANDRADE et al., 2010).



XII XIII

Figura 4. Principais constituintes biciclogermacreno **(XII)** e germacreno **(XIII)** encontrados no óleo essencial de *P. leiocarpa*.

2.3 *Psychotria carthagenensis* Jacq.

P. carthagenensis **(Figura 5)** popularmente conhecida como "cafeeiro-do-mato", "carne-de-vaca" ou "erva-de-rato-branca", ocorre nas regiões Norte, Sudeste e Centro-Oeste do Brasil (ANDERSSON, 1992). Esta planta é um dos componentes da bebida alucinógena ayahuasca, usada pelos povos da floresta Amazônica. Dimetiltriptamina foi identificada como o componente principal do extrato das folhas (RIVIER e LINDGREN, 1972).

Figura 5. Folhas e galhos de *P. carthagenensis* (Fonte: Volobuff, 2014).

2.4 *Psychotria capillacea* Müll. Arg. Stand.

P. capillacea **(Figura 6)**, popularmente conhecida como "café", ocorre no Brasil nos Estados do Amazonas, Mato Grosso do Sul e Paraná, bem como no Paraguai e Argentina (ANDERSSON, 1992). As propriedades biológicas e químicas não foram relatadas na literatura consultada para esta espécie.

Figura 6. Folhas e galhos de *P. capillacea* (Fonte: Volobuff, 2014).

2.5 *Psychotria deflexa* DC.

P. deflexa **(Figura 7)** é conhecida popularmente como "erva-de-rato" ou "café-selvagem", ocorre do México à Argentina. No Brasil ocorre em quase todos os estados brasileiros, incluindo o Mato Grosso do Sul (ANDERSSON, 1992). Investigação química anterior relata o alcalóide cromóforo indol.

Figura 7. Folhas e galhos de *P. deflexa* (Fonte: Volobuff, 2014).

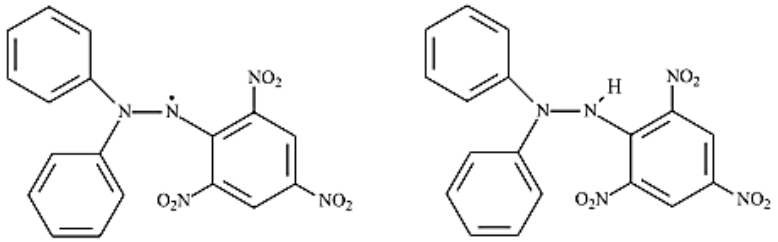
2.6 Atividades Biológicas

2.6.1 Estresse oxidativo

Espécies reativas de oxigênio (EROs) e espécies reativas de nitrogênio (ERNs) são geradas por processos metabólicos normais em todos os organismos aeróbicos (FINKEL e HOLBROOK, 2000). Entretanto, a produção excessiva destas espécies pode exceder as defesas antioxidantes celulares e levar a uma condição denominada estresse oxidativo, que tem sido relacionado à patogênese de várias doenças por meio de diferentes mecanismos, que podem envolver mutação no DNA, oxidação de proteínas e peroxidação lipídica (FINKEL e HOLBROOK, 2000).

Existem vários métodos para a determinação da atividade antioxidante *in vitro* de substâncias biologicamente ativas, envolvendo desde ensaios químicos com substratos lipídicos a ensaios mais complexos utilizando as mais diversas técnicas instrumentais, tais como: DPPH, ABTS e β-caroteno/ácido linoleico. Os testes antioxidantes empregados em alimentos e sistemas biológicos podem ser classificados em ensaios para avaliar a peroxidação lipídica, em que um lipídio ou um substrato lipoproteico sob condições padrão é utilizado e o grau de oxidação é mensurado, e os ensaios para medir o percentual de sequestro de radicais livres (SÁNCHEZ-MORENO, 2002).

Um dos métodos para determinação da atividade antioxidante é pelo sequestro do radical 2,2-difenil-1-picril-hidrazil (DPPH), que consiste no radical livre estável DPPH conferindo uma coloração violeta a esta molécula. Este modelo é baseado na medida da capacidade antioxidante de uma substância sequestrar o radical DPPH, reduzindo-o a hidrazina. Quando uma determinada substância que age como doador é adicionada a uma solução de DPPH, a hidrazina é obtida com mudança simultânea na coloração de violeta a amarelo pálido **(Figura 8)**.



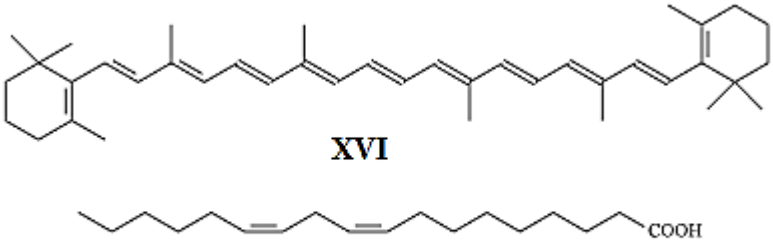
XIV **XV**

Figura 8. Formas radicalar **(XIV)** e não radicalar **(XV)** do DPPH.

O sistema de co-oxidação do β-caroteno/ácido linoleico **(XVI; XVII) (Figura 9)**, permite avaliar a capacidade de uma determinada substância de prevenir a oxidação do β-

caroteno, protegendo-o dos radicais livres gerados durante a peroxidação do ácido linoleico. A reação pode ser monitorada espectrofotometricamente pela perda da coloração do β-caroteno em 470 nm (BROINIZI et al., 2007).

Outro ensaio baseado na inibição de antioxidantes com a inibição de cátion é ABTS 2,2-azinobis-(3-etilbenzotiazolina-6-sulfonato), que tem por característica um espectro de absorção de longo comprimento de onda característico mostrando absorção mínima de 415 nm e absorção máxima de 660, 734 e 820 nm (SÁNCHEZ-MORENO, 2002).



XVI

XVII

Figura 9. Estrutura do β-caroteno **(XVI)** e ácido linoleico **(XVII)**.

O grande interesse científico por substâncias antioxidantes se deve principalmente ao envolvimento dos radicais livres na etiologia de várias doenças, incluindo inflamação, câncer, as doenças degenerativas do SNC como o mal de Alzheimer, o mal de Parkinson e também no processo de envelhecimento. As células e os tecidos vivos estão permanentemente expostos a processos oxidativos contra os quais podem ser protegidos pela ação de antioxidantes naturais (ZHAO et al., 2001; CUZZOCREA et al., 2001).

2.6.2 Doença de Alzheimer

A doença de Alzheimer (DA) é uma desordem neurodegenerativa, que afeta cerca de 1,5% da população em idade entre 65-69 anos, 21% entre 85-86 anos e 39% acima dos 90 anos. Do ponto de vista etiológico, DA pode ser definida como uma doença multifatorial. A maioria dos casos da doença é esporádica com uma ocorrência tardia na vida, mas a manifestação precoce da doença tem sido associada a fatores genéticos (FRANK e GUPTA, 2005; ROSSI et al., 2008). Alguns estudos sugerem que a falta de interações sociais e atividades que estimulem a memória, bem como um estilo de vida sedentário podem aumentar o risco de DA (FRATIGLIONI et al., 2004).

A DA causa muitas mudanças no cotidiano das famílias, e como consequência, abalos e desgastes emocionais a caracterizam como uma doença de acometimento familiar (FREITAS et

al., 2008). Os sintomas comportamentais e psicológicos são os resultados da exposição ao estresse sofrido pelos cuidadores de pacientes com a doença. Na maior parte dos casos, cuidadores são parentes próximos aos pacientes, e a qualidade de vida de ambas as partes costuma ser afetada de forma negativa devido à difícil tarefa de convivência (PAULA et al., 2008).

Além do comprometimento da memória recente, vários sintomas paralelos são associados à doença, incluindo o humor depressivo, a diminuição de prazer aos contatos sociais ou usuais, o isolamento ou retraimento social, alterações de apetite e do ciclo vigília-sono, alterações psicomotoras, irritabilidade, fadiga, sentimentos de inutilidade, desesperança ou culpa excessiva e inapropriada e pensamentos recorrentes de morte (VITAL et al., 2010).

Esta desordem é caracterizada por deterioração dos neurônios colinérgicos, o que resulta em notável déficit do neurotransmissor acetilcolina **(XVII) (Figura 10)** (WHITEHOUSE et al., 1982) e se manifesta em nível das regiões cerebrais associadas às funções mentais superiores, especialmente ao córtex frontal e hipocampo (VIEGAS JUNIOR et al., 2004).

Dentre as causas mais evidentes da gênese da doença estão a ocorrência de deposição extracelular de peptídeo β-amilóide (derivado do precursor amilóide de proteína) em plaquetas senis e a formação errática de neurofibrilas intracelulares (VIEGAS JUNIOR et al., 2004).

Uma das estratégias para o tratamento da DA é baseada na 'hipótese colinérgica', o que sugere que a causa da deterioração da memória em pacientes com DA é um déficit da função colinérgica no hipocampo e seções corticais no cérebro (BARTUS et al., 1982; BECKER et al., 1988; PERRY, 1986). Os inibidores da acetilcolinesterase podem restaurar o nível de acetilcolina em sinapses colinérgicas do córtex cerebral (GIACOBINI, 2000; TARIOT et al., 2004), que são responsáveis pela melhora da função cognitiva. Desta forma, os inibidores da acetilcolinesterase são usados como uma abordagem para minimizar os sintomas da doença.

Dentre os anticolinesterásicos de uso clínico está o tetrahidroaminocridina (THA), genericamente chamado de Tacrina **(XVIII) (Figura 10)**, o qual foi liberado para comercialização com o nome de Cognex®. Em contrapartida, este fármaco mostrou-se inconveniente por ter de ser administrado quatro vezes ao dia e estar associado a hepatotoxicidade e incidências de efeitos colaterais (FARLOW et al., 1992; KNAPP et al., 1994).

O mais potente inibidor o (R,S)-1-benzil-4-[(5,6-dimetoxi-1-indanona)2-il] metil piperidina, conhecido por Donepezil **(XIX) (Figura 10)** e comercializado como Aricept®, foi aprovado pelo *Food and Drug Admnistration* em 1996 (NIGHTINGALE, 1997). Esse fármaco resultou no estudo e planejamento racional de drogas a partir do conhecimento do sítio ativo da

enzima acetilcolinesterase e de suas interações químicas com os ligantes. Além disso, ele representa uma nova geração de inibidores anticolinesterásicos que oferece a possibilidade de ser administrado uma vez ao dia e com poucos efeitos colaterais (KRYGER et al., 1999).

Compostos isolados a partir de plantas medicinais também foram empregados para o tratamento da doença. A galantamina **(XX) (Figura 10)** ou (4aS, 6R, 8aS)-4a, 5, 9, 10, 11, 12-hexaidro-3-metoxi-11-metil-6H-benzofuro [3a, 3, 2, -ef] denominado comercialmente de Remynil® é um alcaloide isolado de flores e bulbo da *Galanthus woronowii*, que foi posteriormente sintetizado (SANCHEZ et al., 1984; CZOLLNER et al., 2001).

O Huperzine A **(XXI) (Figura 10)**, um alcaloide isolado de uma erva chinesa conhecida como *Huperzia serrata* é um potente e seletivo inibidor da enzima acetilcolinesterase. Embora não tenha sido aprovado para comercialização pelo *Food and Drug Admnistration*, é muito utilizado na medicina tradicional chinesa como fitoterápico.

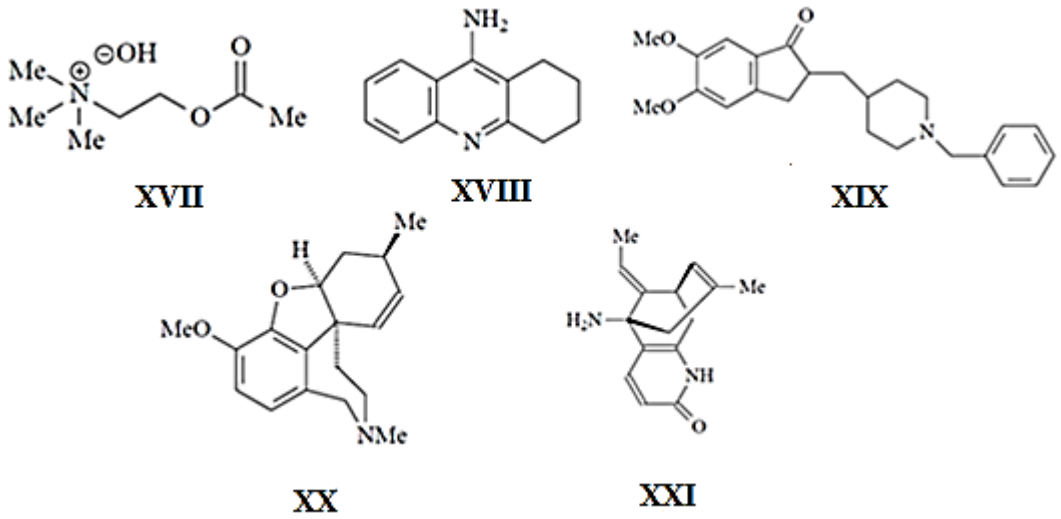


Figura 10. Estruturas químicas de acetilcolina **(XVII)**, tacrina **(XVIII)**, donepezil **(XIX)**, galantamina **(XX)** e Huperzine A **(XXI)**.

Devido a busca por novos inibidores da acetilcolinesterase, há o interesse na pesquisa com plantas com potencial medicinal, isolando e sintetizando compostos que sejam biologicamente ativos.

2.6.3 Inflamação

A inflamação é uma resposta biológica complexa de tecidos vasculares a estímulos nocivos, tais como patógenos, danos celulares ou irritantes (físicos ou químicos). É um

mecanismo de defesa destinado a remover os estímulos lesivos e iniciar o processo de cicatrização do tecido (MALDINI et al., 2009).

De acordo com Tedgui e Mallat (2001) os mecanismos envolvidos na resposta inflamatória formam uma reação em cascata, envolvendo células inflamatórias (neutrófilos, linfócitos, monócito/macrófago) e células vasculares (endoteliais e células da musculatura lisa). No entanto, a resposta inflamatória visa à proteção do organismo frente a um agente infeccioso, tendo como objetivo a destruição do invasor.

A inflamação ocorre em três eventos principais: aumento do suprimento sanguíneo para a área afetada; aumento da permeabilidade capilar ocasionado pela retração das células endoteliais, como consequente escape de moléculas maiores permitindo, então, que os mediadores solúveis da imunidade atinjam o local da infecção; migração dos leucócitos, dos capilares para os tecidos circundantes. Na fase final da inflamação, os neutrófilos são particularmente prevalentes, mas tardiamente no processo, os monócitos e linfócitos também migram para o local inflamado (ROITT et al., 2003).

A inflamação pode ser aguda ou crônica. A aguda é a de curta duração, ocorrendo nas primeiras horas, dias e caracteriza-se pela não especificidade e grande quantidade de exsudação do fluido e de proteínas do plasma para o interstício com o objetivo de eliminar os tecidos mortos, proteger contra infecção local e permitir o acesso do sistema imune à área danificada (STEVENS e LOWE, 1998). Persistindo o agente lesivo, inicia-se a fase crônica, que é de longa duração e está, na maioria das vezes, associada com a presença de células (linfócitos, macrófagos, dentre outras), angiogênese, fibrose e necrose de tecidos (DRAY, 1995; POBER e COTRAN, 1990).

Dentre os modelos experimentais utilizados em estudos com fármacos anti-inflamatórios estão: o estudo da quimiotaxia de leucócitos "*in vitro*"; teste do edema inflamatório na pata do animal induzido por substâncias químicas; angiogênese; modelos de inflamação por mecanismos autoimunes; modelos que utilizam agentes infecciosos, teste da permeabilidade capilar induzida pelos marcadores da inflamação pleurisia ou peritonite e edema inflamatório de orelha induzido por agentes irritantes (LAPA et al., 2001).

Anti-inflamatórios são direcionados a inibir enzimas específicas e/ou antagonizar receptores específicos e a resposta de translação de proteínas envolvidas na inflamação. Os inibidores de ciclo-oxigenase (COX), o fator de necrose tumoral α (TNF-α) e os corticosteroides são exemplos dessa abordagem utilizada com o objetivo de bloquear a produção e/ou ação de mediadores químicos pró-inflamatórios (SERHAN, 2008).

Compostos naturais com diferentes mecanismos de ação podem ser utilizados no tratamento de doenças inflamatórias, sobretudo a partir de plantas que estão sendo utilizadas com esse propósito. Dentre os metabólitos secundários com potencial atividade anti-inflamatória estão a curcumina, extraída da *Curcuma longa* L. (Zingiberaceae); a rutina, a quercetina, a apigenina, a morina e a narigenina, obtidas do *Ginkgo biloba*; a silimarina, extraída do *Silybum marianum* (L.) Gaertn. (Asteraceae); alguns flavonoides, como a baicaleína, obtida da *Scutellaria baicalensis* Georgi (Lamiaceae) e o cirsiliol, derivado da *Achillea fragantissima* (Forssk.), entre outros compostos (CALIXTO et al., 2003).

Desta forma, estudos a partir de plantas com potencial medicinal são necessários para contribuir para a descoberta de novos anti-inflamatórios que possam ser utilizados com eficácia e segurança para o tratamento da doença.

2.6.4 Câncer

Câncer, uma doença heterogênea multifatorial, é uma das principais causas de mortalidade no mundo (SIEGEL et al., 2013). Apesar dos avanços na descoberta de medicamentos anticâncer, em 2012, 14,1 milhões de novos casos de câncer foram diagnosticados em todo o mundo, com 8,2 milhões de mortes (FERLAY et al., 2012).

Vários mecanismos bioquímicos então envolvidos na gênese e desenvolvimento de cancro, incluindo o stress oxidativo, que é induzido por radicais livres e causa danos no DNA (AMES e GOLD, 1991). Tem sido sugerido que este dano oxidativo pode ser evitado ou limitado pela dieta de fitoquímicos (LIU, 2013). Estudo têm demonstrado que a dieta de fitoquímicos possui mecanismos de ação complementares e sobrepostos para a prevenção do câncer, incluindo a eliminação de radical livre e a redução do estresse oxidativo, a inibição da proliferação celular, indução de diferenciação celular, a inibição da expressão do oncogene, a indução do gene supressor de tumor, a regulação do ciclo celular, e a indução da apoptose (LIU e FINLEY, 2005; LIU, 2013).

A química de produtos naturais constitui uma das principais linhas de pesquisa na busca de novos agentes anticancerígenos. Alcaloides de origem vegetal utilizados para o tratamento de alguns tipos de câncer são a vincristina e a vimblastina, isoladas da *Catharantus roseus* (CARVALHAES et al., 2002).

3. OBJETIVOS

3.1 Objetivo Geral

Avaliação biológica e estudo fitoquímico de quatro espécies de *Psychotria* (Rubiaceae).

3.2 Objetivos Específicos

Manuscrito 1:

- Preparação do extrato metanólico das folhas de *P. leiocarpa*, *P. carthagenensis*, *P. capillacea* e *P. deflexa*;
- Teor de flavonoides, flavonol, taninos condensados e fenóis totais do extrato metanólico;
- Avaliação da atividade antioxidante pelos métodos DPPH, ABTS e β-caroteno/ácido linoleico;
- Análise por HPLC/DAD do extrato metanólico.

Manuscrito 2:

- Fracionamento, isolamento e identificação dos principais constituintes presentes do extrato metanólico das folhas de *P. leiocarpa*;
- Avaliação da atividade antitumoral, anticolinesterásica e anti-inflamatória do extrato metanólico e compostos isolados.

4. REFERÊNCIAS

AMES, B. N.; GOLD, L. S. Endogenous mutagens and the causes of aging and câncer. **Mutation Research**, v. 250, p. 3-16, 1991.

ANDERSSON, L.A. 1992. A provisional checklist of Neotropical Rubiaceae. Scripta Botanica Belgica 1: 1-199.

ANDRADE, J. M. M.; BIEGELMEYER, R.; XAVIER, C. A. G.; BORDIGNON, S. A. L.; MORENO, P. R. H.; ZUANAZZI, J. A. S.; HENRIQUES, A. T.; APEL, M. A. Essential oil constituents of *Psychotria leiocarpa*. **Chemistry of Natural Compounds**, v. 46, p. 649-650, 2010.

BARTUS, R. T.; DEAN, R. L.; BEER, B.; LIPPA, A. S. The cholinergic hypothesis of geriactric memory dysfunction. **Science**, v. 217, p. 408-417, 1982.

BECKER, R.; GIACOBINI, E.; ELBLE, R.; MCILHANY, M.; SHERMAN, K. Potential pharmacotherapy of Alzheimer's disease. A comparison of various forms of physostigmine administration. **Acta Neurologica Scandinavica**, v. 77, p. 19-32, 1988.

BOTH, F. L.; MENEGUINI, L.; KERBER, V. A.; HENRIQUES, A. T.; ELISABETSKY, E. Role of glutamate and dopamine receptors in the psychopharmacological profile of the índole alkaloid psychollatine. **Journal of Natural Products**, v.69, p. 342-345, 2006.

BOTH, F. L. **Avaliação do perfil farmacológico de psicolatina isolada de *Psychotria umbellata* (Rubiaceae).** 2005. Tese (Doutorado) – Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul.

BOTH, F. L.; FARIAS, F. M.; NICOLÁO, L. L.; MISTURINI, J.; HENRIQUES, A.; ELISABETSKY, E. Avaliação da atividade analgésica de extratos alcaloídicos de espécies de *Psychotria*. **Revista Brasileira de Plantas Medicinais**, v.5, p. 41-5, 2002.

BROINIZI, P. R. B.; ANDRADE-WARTHA, E. R. S. DE; SILVA, A. M. O.; NOVOA, A. J. V.; TORRES, R. P.; AZEREDO, H. M. C.; ALVES, R. E.; MANCINI, J. Avaliação da atividade antioxidante dos compostos fenólicos naturalmente presentes em subprodutos do pseudofruto de caju (*Anacardium occidentale* L.) **Ciência e Tecnologia de Alimentos,** v. 27, p. 902, 2007.

BRUNETON, J. **Elementos de fitoquímica y de farnaconosia.** 1 ed. Espanha: Editorial Acribia, S.A., p.467-517, 1991.

CABALLERO-GEORGE, C.; VANDERHEYDEN, P. M. L.; SOLIS, P. N.; PIETERS, L.; SHAHAT, A. A.; GUPTA, M. P.; VAUQUELIN, G.; VLIETNICK, A. J. Biological screening of selected medicinal Panamenian plants by radioligand-binding techniques. **Phytomedicine**, v. 8, p. 59-70, 2001.

CALIXTO, J. B.; OTUKI, M. F.; SANTOS, A. R. S. Antiinflammatory compounds of plant origin. Part I. Action on arachidonic acid pathway, nitric oxide and nuclear factor Kb (NFKb). **Planta Medica**, v. 69, p. 1-12, 2003.

CARVALHAES, S. F.; COSTA, D. L.; MAZZEI, J. L.; TADDEI, L. E. M.; D'AVILA, L. A. Alternative extraction of alkaloid anticarcinogens from Brazilian "vinca rosea" using ion exchange chromatography. **Revista Brasileira de Farmacognosia**, v. 12, p. 83-84, 2002.

CUZZOCREA, S.; RILEY, D. P.; CAPUTI, A. P.; SALVEMINI, D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. **Pharmacological Reviews**, v. 53, p. 135-159, 2001.

CZOLLNER, L.; TREU, M.; FROEHLIC, J.; KUEENBURG, B.; JORDIS, U. Synthesis of galanthamine. **Arkivoc**, p. 191-200, 2001.

DRAY, A. Inflammatory mediators of pain. **The British Journal of Anaesthesia**, v. 75, p. 125-131, 1995.

DELPRETE, P. G.; SMITH, L. B.; KLEIN, R. M. **Rubiaceae.** Flora Ilustrada Catarinense, 2005. I: p. 542-549.

DEWICK, P. M. **Medicinal natural products: a biosynthetic approach**. Chichester, Ed. John Wiley & Sons, v.2, p. 351-359, 1997.

DILLENBURG, C. R.; PORTO, M. L. Rubiaceae-tribo *Psychotriae*. **Boletim Instituto de Biosciências – UFRGS** 1985, 39, 1-76.

ELISABETSKY, E.; AMADOR, T. A.; LEAL, M. B.; NUNES, D. S.; CARVALHO, A. C. T.; VEROTTA, L. Merging ethnopharmacology with chemotaxonomy: an approach to unveil bioactive natural products. The case of *Psychotria* alkaloids as potential analgesics. **Ciência e Cultura**, v.49, p. 378-385, 1997.

FARIAS, F.M.; PASSOS, C.S.; ARBO, M. D.; ZUANAZZI, J. A.; STEFFEN, V. M.; HENRIQUES, A. T. Monoamine levels in rat striatum after acute intraperitoneal injection of

strictosidinic acid isolated from *Psychotria myriantha* Mull. Arg. (Rubiaceae). **Phytomedicine**, v. 17, p. 289–91, 2010.

FARLOW, M.; GRACON, S. I.; HERSEY, L. A.; LEWIS, K. W.; SADOWSKY, C. H. A controlled trial of tacrine in Alzheimer's disease. **The Journal of the American Medical Association**, v. 268, p. 2523, 1992.

FERLAY, J.; SOERJOMATARAM, I.; ERVIK, M.; DIKSHIT, R.; ESER, S.; MATHERS, C.; REBELO, M.; PARKIN, D. M.; FORMAN, D.; BRAY, F. GLOBOCAN 2012 v. 1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Disponível em: http://globocan.iarc.fr Acessado em 07/08/2015.

FINKEL, T.; HOLBROOK, N. J. Oxidants, oxidative stress and the biology of ageing. **Nature**, v. 408, p. 239-247, 2000.

FORMAGIO, A. S. N.; VOLOBUFF, C. R. F.; SANTIAGO, M.; CARDOSO, C. A. L.; VIEIRA, M. C.; PEREIRA, Z. V. Evaluation of antioxidant activity, total flavonoids, tannins and phenolic compounds in *Psychotria* leaf extracts. **Antioxidants**, v. 3, p. 745-757, 2014.

FRANK, B.; GUPTA, S. A review of antioxidants and Alzheimer's disease. **Annals of Clinical Psychiatry**, v. 17, p. 269-86, 2005.

FRATIGLIONI, L.; PAILLARD-BORG, S.; WINBLAD, B. An active and socially integrated lifestyle in late life might protect against dementia. **Lancet Neurology**, v. 3, p. 343-53, 2004.

FREITAS, I. C. C.; PAULA, K. C. C.; SOARES, J. L.; PARENTE, A. C. M. Convivendo com o Portador de Alzheimer: perspectivas do familiar cuidador. **Revista Brasileira de Enfermagem**, v. 61, p. 508-13, 2008.

GIACOBINI, E. Cholinesterase Inhibitors Stabilize Alzheimer Disease. **Neurochemical Research**, v. 25, p. 1185-1190, 2000.

HENRIQUES, A. T.; LOPES, S. O.; PARANHOS, T. J. T.; GREGIANINI, T. S.; POSER, G. L. V.; FETT-NETO, A. G; SCHRPSEMA J. N,β-D-glucopyranosyl vincosamide, a light regulated índole alkaloid from the shoots of *Psychotria leiocarpa*. **Phytochemistry**, v.65, p.449-454, 2004.

KERBER, V. A.; PASSOS, C. S.; VERLI, H.; FETT-NETO, A. G.; QUIRION, J. P.; HENRIQUES, A. T. Psychollatine, a glucosidic monoterpene índole alkaloid from *Psychotria umbellata*. **Journal of Natural Products**, v.71, n.4, 697-700, 2008.

KERBER, V.A.; GREGIANINI, T. S.; PARANHOS, J. T.; SCHWAMBACH, J.; FARIAS, F.; FETT, J. O.; FETT-NETO, A.; ZUANAZZI, J. A. S.; QUIRION, J. C.; ELISABETSKY, E.; HENRIQUES, A. T. Brachycerine, a novel monoterpene índole alkaloid from *Psychotria brachyceras*. **Journal of Natural Products**, v. 67, p.677-679, 2001.

KNAPP, M. J.; KNOPMAN, D. S.; SOLOMON, P. R.; PENDLEBURY, W. W.; DAVID, C. S.; GRACON, S. I. A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease. **The Journal of the American Medical Association**, v. 271, p. 985, 1994.

KRYGER, G.; SILMAN, I.; SUSSMAN, J. L. Structure of acetylcholinesterase complexed with E2020 (Aricept): implications for the design of new anti-Alzheimer drugs. **Structure**, v. 7, p. 297-307, 1999.

KUO, Y. C.; CHIEN, C. C.; TSAI, W. J.; HO, Y. H. Regulation of herpes simplex virus type 1 replication in Vero cells by *Psychotria serpens*: relationship to gene expression, DNA replication, and protein synthesis. **Antiviral Research**, v. 51, p. 95-109, 2001.

LAPA, A. J.; SOUCCAR, C.; LIMA-LANDMAN, M. T. R.; LIMA, T. C. M. **Métodos de avaliação da atividade farmacológica de plantas medicinais**. São Paulo: CYTED/CNPq, 2001.

LEAL, M. B. Estudo psicofarmacológico de espécies de *Psychotria* (Rubiaceae) do Estado do Rio Grande do Sul. **Dissertação de Mestrado.** 1994. Curso de Pós-Graduação em Ciências Farmacêuticas. Universidade Federal do Rio Grande do Sul.

LIU, R. H. Dietary bioactive compounds and their health implications. **Journal of Food Science**, v. 78, p. A18–A25, 2013.

LIU, R. H.; FINLEY, J. Potential cell culture models for antioxidant research. **Journal of Agricultural and Food Chemistry**, v, 53, p. 4311–4314, 2005.

LIWSZYC, G. E.; VUORI, E.; RASANEN,I.; LSSAKAINEN, J. Daime - a ritual herbal potion. **Journal of Ethnopharmacology**, 36, 91-92, 1992.

LOCHER, C. P.; BURCH, M. T.; MOWER, H. F.; BERESTECKY, J.; DAVIS, H.; VAN POEL, B.; LASURE, A.; VANDEN BERGHE, D. A.; VLIETINCK, A. J. Anti-microbial activity and anti-complement activity of extracts obtained from selects Hawaian plants. **Journal of Ethnopharmacology**, v. 49, p. 23-32, 1995.

LOPES, S. O. Análise química e culivo *in vitro* de *Psychotria leiocarpa* Cham. et Schltcl. e *Psychotria cathagenensis* Jacq. (Rubiaceae). **Dissertação de Mestrado**. 1998. Porto Alegre: Universidade Federal do Rio Grande do Sul, Curso de Pós-Graduação em Ciências Farmacêuticas.

MALDINI, M.; SOSA, S.; MONTORO, P.; GIANGASPERO, A.; BALICK, M. J.; PIZZA, C.; DELLA LOGGIA, R. Screening of the topical anti-inflammatory activity of the bark of *Acacia cornigera* Willdenow, *Byrsonima crassifolia* Kunth, *Sweetia panamensis* Yakovlev and the leaves of *Sphagneticola trilobata* Hitchcock. **Journal of Ethnopharmacology,** v. 122, p. 430-433, 2009.

MCGAW, L. J.; JÄGER, A. K.; VAN STADEN, J. Antibacterial, antihelmintic and antiamoebic activity in South African medicinal plants. **Journal of Ethnopharmacology,** v. 72, p. 247-63, 2000.

MCKENNA, D. J.; TOWERS, G. N. T.; ABBOT, F. Monoamine oxidase inhibitors in South American hallucinogenic plants: triptamine and B-carboline constituents of Ayahuasca. **Journal of Ethnopharmacology**, v. 10, p. 195-223, 1984.

MORAES, L. G.; ALONSO, A. M.; OLIVEIRA-FILHO, E. C. Plantas medicinais no tratamento do câncer: uma breve revisão da literatura. **Universitas: Ciências da Saúde**, v.9, n.1, p.77-99, 2011.

NIGHTINGALE, S. L. Donepezil approved for treatment of Alzheimer's disease. **The Journal of the American Medical Association**, v. 277, p. 10, 1997.

PAULA, J. A.; ROQUE, F. P.; ARAUJO, F. S. Qualidade de Vida em Cuidadores de Idosos Portadores de Demência de Alzheimer. **Jornal Brasileiro de Psiquiatria**, v. 57, p. 283-287, 2008.

PEPE, V. L. E. A Relação Nacional de Medicamentos Essenciais (Rename): a seleção de medicamentos no Brasil. Disponível em: http://www.ensp.fiocruz.br/portal-ensp/judicializacao/pdfs/514.pdf. Acesso em: 03 set. 2015.

PEREIRA, Z. V. Rubiaceae Juss. do Parque Estadual das Várzeas do Rio Ivinhema, Mato Grosso do Sul: florística, sistema reprodutivo, distribuição espacial e relações alométricas de espécies distílicas. (Tese de Doutorado). Instituto de Biologia. Universidade Estadual de Campinas. Campinas, 2007.

PERRY, E. K. The cholinergic hypothesis—tem years on. **British Medical Bulletin**, v. 42, p. 63-69, 1986.

POBER, J. S.; COTRAN, R. S. Overview: the role of endothelial cell in inflammation. **Transplantation**, v. 50, p. 537, 1990.

PONTES, R. M. F.; MONTEIRO, P. S.; RODRIGUES, M. C. S. O uso da fitoterapia no cuidado de crianças atendidas em um centro de saúde do Distrito Federal. Brasília, 2006.

RIVIER, L.; LINDGREN, J. E. "Ayahuasca", the South American hallucinogenic drink: An ethnobotanical and chemical investigation. **Society for Economic Botany**, v. 26, p. 101-129, 1972.

ROITT, I.; BROSTOFF, J.; MALE, D. **Imunologia** (tradução e revisão científica de Gubert IC, Malone eds), 6. ed., São Paulo, p. 10-11. 2003.

ROSSI, L.; MAZZITELLI, S.; ARCIELLO, M.; CAPO, C.; ROTILIO, G. Benefits from dietary polyphenols for brain aging and Alzheimer's disease. **Neurochemical Research**, v. 33, p. 2390-2400, 2008.

SANCHEZ, I. H.; SORIA, J. J.; LOPEZ, F. J.; LARRAZA, M. I.; FLORES, H. J. Total synthesis of racemic lycoramine. **The Journal of Organic Chemistry**, v. 49, p. 157-163, 1984.

SÁNCHEZ-MORENO, C. Review: Methods Used to Evaluate the Free Radical Scavenging Activity in Foods and Biological Systems. **Food Science & Technology**, v. 8, p. 121, 2002.

SANTOS, L. V.; FETT-NETO, A. G.; KERBER, V. A.; ELISABETISKY, E.; QUIRION, J. C.; HENRIQUES, A. T. Indole monoterpene alkaloids from leaves of Psychotria suterella Mull. Arg. (Rubiaceae). **Biochemical Systematics and Ecology**, v.29, p.1185-1187, 2001.

SERHAN, C. N. Systems approach with inflammatory exudates uncovers novel antiinflammatory and pro-resolving mediators. **Prostaglandins, leukotrienes, and essential fatty acids,** n.79, p. 157-163, 2008.

SÉRPICO, R. L.; CAMURÇA, D. M. Ayahuasca: Revisões teóricas e considerações botânicas sobre as espécies *Banisteriopsis caapi* Morton, 1931 e *Psychotria viridis* Ruíz e Pavon, 1779. Guarulhos, 2006. Monografia – Curso de Licenciatura Plena em Ciências Biológicas, Universidade de Guarulhos.

SIEGEL, R.; NAISHADHAM, D.; JEMAL, A. Cancer statistics, 2013. **CA: A Cancer Journal for Clinicians**, v. 63, p. 11-30, 2013

SOARES, A. K. A.; CARMO, G. C.; QUENTAL, D. P.; NASCIMENTO, D. F.; BEZERRA, F. A. F.; MORAES, M. O.; MORAES, M. E. A. Avaliação da segurança clínica de um fitoterápico contendo *Mikania glomerata*, *Grindelia robusta*, *Copaifera officinalis*, *Myroxylon toluifera*, *Nasturtium officinale*, própolis e mel em voluntários saudáveis. **Brazilian Journal of Pharmacognosy**, v. 16, p. 447-454, 2006.

STEVENS, A.; LOWE, J. Doenças do sistema urinário. In: **Patologia**. 2ed. São Paulo: Manole, p. 317-349, 1998.

TARIOT, P. N.; FARLOW, M. R.; GROSSBERG, G. T.; GRAHAM, S. M.; MCDONALD, S.; GERGEL, I. Memantine treatment in patients with moderate to severe Alzheimer disease already receiving donepezil: a randomized controlled trial. **The Journal of the American Medical Association,** v. 291, p. 317-324, 2004.

TEDGUI, A.; MALLAT, Z. Anti-inflammatory mechanisms in the vascular wall. **Circulation research**, v. 11, p. 877-887, 2001.

VIEGAS JUNIOR, C.; BOLZANI, V. S.; FURLAN, M. Produtos naturais como candidatos a fármacos úteis no tratamento do mal de Alzheimer**. Química Nova**, v. 27, p. 655-660, 2004.

VITAL, T. M.; HERNANDEZ, S. S. S.; GOBBI, S.; COSTA, J. L. R.; STELLA, F. Atividade Física Sistematizada e Sintomas de Depressão na Demência de Alzheimer: uma revisão sistemática. **Jornal Brasileiro de Psiquiatria**, v. 59, p. 58-64, 2010.

WHITEHOUSE, P. J.; PRICE, D. L.; STRUBLE, R. G. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. **Science**, v. 215, p. 1237-1239, 1982.

WHO. Bulletin of the World Health Organization. Regulatory situation of herbal medicines. A worldwide review, Geneva, 1998.

ZHAO, B., MOOCHHALA, S. M.; THAM, S. Y. Biologically active components of *Physostigma venenosum*. **Journal of Chromatography B-Analytical Technologies in tje Biomedical and Life Sciences**, v. 812, p. 135-159, 2001.

5. MANUSCRITO 1

Antioxidants **2014**, *3*, 745-757 manuscripts; doi:10.3390/antiox3040745

OPEN ACCESS

antioxidants

ISSN 2076-3921

www.mdpi.com/journal/antioxidants

Article

Evaluation of Antioxidant Activity, Total Flavonoids, Tannins and Phenolic Compounds in *Psychotria* Leaf Extracts

Anelise Samara Nazari Formagio [1,*], Carla Roberta Ferreira Volobuff [2], Matheus Santiago [2], Claudia Andrea Lima Cardoso [3], Maria do Carmo Vieira [1] and Zefa Valdevina Pereira [2]

[1] Faculty of Agricultural Sciences, University Federal of Grande Dourados (UFGD), 79804-970/533 Dourados-MS, Brazil; E-Mail: aneliseformagio@ufgd.edu.br (A.S.N.F.); mariavieira@ufgd.edu.br (M.C.V.)

[2] Biological and Environmental Sciences, University Federal of Grande Dourados (UFGD), 79804 970/533 Dourados-MS, Brazil; E-Mail: volobuff81@ gmail.com (C.R.F.V.); matheus_ss@hotmail.com (M.S.); zefapereira@ufgd.edu.br (Z.V.P.)

[3] Chemistry Program, State University of Mato Grosso do Sul (UEMS), Mato Grosso do Sul, 79804-970/351 Dourados-MS, Brazil; E-Mail: claudia@uems.br (C.A.L.C.)

***** Author to whom correspondence should be addressed; E-Mail: aneliseformagio@ufgd.edu.br;
Tel.: +5567-34102426.

External Editor: Luca Sebastiani

Received: 16 May 2014; in revised form: 21 May 2014 / Accepted: 8 September 2014 / Published:

Abstract: The antioxidant activity of *Psychotria carthagenensis*, *P. leiocarpa*, *P. capillacea* and *P. deflexa* (Rubiaceae) extracts were investigated, and the concentrations of total phenolics, flavonoids, condensed tannins and flavonols were determined. The chemical compositions of the extracts were investigated using the high performance liquid chromatography (HPLC/PAD) method. We used 1,1-diphenyl-1-picrylhydrazyl free radical (DPPH), β-Carotene bleaching and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cations to

determine antioxidant activity. The ability to scavenge radical was measured in these experiments by the discoloration of the solution. Concentrations of constituents were measured spectrophotometrically. *P. carthagenensis* and *P. capillacea* exhibited the highest antioxidant activity, in the DPPH test, β-carotene bleaching and ABTS system. The highest phenolic, flavonoid, condensed tannin and flavonol concentration was found in *P. carthagenensis* and *P. capillacea* extracts. HPLC-PDA analysis of *P. carthagenensis* and *P. capillacea* revealed hydroxycinnamic acid (*p*-coumaric acid). This is the first report on the antioxidant properties and constituent analysis of these *Psychotria* extracts.

Keywords: *Psychotria*; antioxidant activity; *p*-coumaric acid

1. Introduction

Antioxidants, molecules with a radical-scavenging capacity, are thought to exert a protective effect against free radical damage. These biomolecules may contribute to the prevention of many chronic diseases, such as cancer, cardiovascular disease, atherosclerosis, diabetes, asthma, hepatitis and arthritis [1,2]. The consumption of traditional diets prepared with spices and medicinal and aromatic herbs has attracted increasing interest among consumers and scientists because these spices and herbs exhibit antioxidant properties attributed to a variety of bioactive phytochemicals [3,4].

The species of the genus *Psychotria* are used by the population in the form of infusion and with external application. The internal uses are indicated for diseases of the treat disorders gastrointestinal, bronchial diseases and reproductive disorders. In external use, in applications, skin tumors, ulcers, ocular disorders, such as poultices, and baths for the treatment of fever, sore head and ear [5,6]. Reported phytochemical studies on the *Psychotria* genus showed alkaloids, mainly polypirrolidinoindole [7–13], quinolines [14–19], and monoterpene indole alkaloids [20–26]. Some of these alkaloids display pharmacological effects such as inhibition of human platelet aggregation [27], cytotoxicity [28], and analgesic activity [29], as well as antimalarial and antileishmanial effects [30].

Psychotria leiocarpa Cham. and Schlecht., popularly known as "*grandiúva-de-anta*" or "*cafeeiro-do-mato*", is an understorey woody shrub native to the forests of Southern Brazil [8]. From leaves, was isolated an N-glycosylated monoterpenoid indole alkaloid N, β-D-glucopyranosyl vincosamide, constitutes up to 2.5% of the dry weight in leaves [31], and iridoid glucosides asperuloside and deacetylasperuloside [32]. Study showed that this alkaloid may act indirectly in *P. leiocarpa* protection against oxidative stress generated upon wounding, UV exposure, and perhaps other environmental stresses [33]. *Psychotria carthagenensis* Jacq., popularly known as "*cafeeiro-do-mato*", "*carne-de-vaca*" or "*erva-de-rato-branca*", occurs in most parts of the southern Brazilian State, Rio Grande do Sul. This plant is one of the

components of the hallucinogenic beverage *ayahuasca*, used by the people from the Amazonian Forest [34]. Dimetyl-tryptamine was identified as the major component of a leaf extract [35]. *Psychotria deflexa* DC., popularly known as "*erva-de-rato*" or "*café selvagem*", occurs from Mexico to Argentina. Chemical investigation has previously reported the alkaloid indole chromophore. *Psychotria capillacea* Müll. Arg. Standl., popularly known as "coffee," occurs in the Brazil states Amazonas, Mato Grosso do Sul, and Parana, as well as in Paraguay and Argentina [24]. The pharmacological properties and chemical for this species was not reported on in the consulted literature.

However, the few biological studies for the species indicate the importance of the continuity of phytochemical studies and activities, with these species reported. Thus, this study represents the first antioxidant investigation of *Psychotria carthagenensis*, *P. leiocarpa*, *P. deflexa* and *P. capillacea* with a different analytical methodology. We also determined the concentrations of phenolic compounds, condensed tannins, flavonoids, and flavonol.

2. Results and Discussion

2.1. Antioxidant Activity

Due to the complexity of some plant extracts, the use of several different methods is recommended for the evaluation of antioxidant activity [36]. Currently used methods include the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay [37], which measures the ability of a substance to scavenge the DPPH radical, reducing it to hydrazine. When a substance that acts as a donor of hydrogen atoms is added to a solution of DPPH, hydrazine is obtained, with a change in color from violet to pale yellow. 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$) is a decolorization technique, in that the radical is generated directly in a stable form prior to reaction with putative antioxidants, which involves the production of the blue/green $ABTS^{+}$ chromophore through the reaction of ABTS with potassium persulfate. The β-carotene bleaching method [38] evaluates the ability of a substance to prevent the oxidation of β-carotene, protecting it from the free radicals generated during the peroxidation of linoleic acid.

The effects of methanolic extracts of *Psychotria* species in the DPPH, linoleic acid peroxidation and ABTS assays are shown in Table 1. *P. carthagenensis* and *P. capillacea* exhibited the highest scavenging activity with an IC_{50} of 16.92 ± 4.58 and 30.05 ± 6.22 µg/mL, respectively (Table 1), which was comparable to that of the standard antioxidant butylated hydroxytoluene (BHT) (IC_{50} = 16.72 ± 1.34 µg/mL) and ascorbic acid (IC_{50} = 22.28 ± 0.53 µg/mL). The comparison of the obtained free-radical scavenging data indicated potent activity for the *P. carthagenensis* and *P. capillacea* at 100 µg/mL, with %FRS values of 93.52% ± 8.41% and 91.78% ± 4.23%, respectively. These results show that the DPPH radical scavenging activity of these extracts was similar to BHT and ascorbic acid (Table 1). Gallic acid (6.56%, standard), *P. carthagenensis* (6.47%) and *P. capillacea* (7.32%) also resulted in a remarkable reduction of the DPPH remaining, compared with *P. leicocarpa* and *P. deflexa* (56.26% and 63.51%), respectively, when recorded after 60 min (Figure 1). It is clear that the

more DPPH that remains, the lower the radical-scavenging activity of the tested samples is. All these data clearly indicate that *P. carthagenensis* and *P. capillacea* extracts are effective electrons or hydrogen atoms donor to DPPH.

Figure 1. Kinetic behavior of methanol extracts (100 µg/mL) against DPPH: *P. carthagenensis*, *P. leiocarpa*, *P. capillacea*, *P. deflexa* and gallic acid standard.

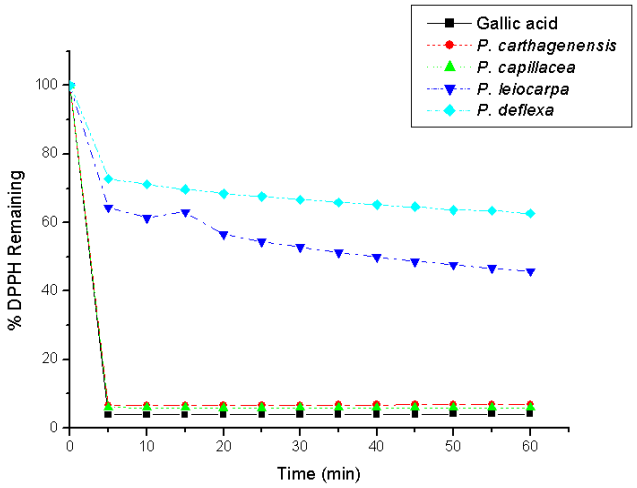


Table 1. Antioxidant activity of *Psychotria* leaf extracts by DPPH, β-carotene/linoleic acid and ABTS assays.

Extracts	Test			
	DPPH		β-Carotene/Linoleic Acid	$ABTS^+$
	IC_{50} (μg/mL) (Limit Confidence 95%)	%FRS *	(%AA)	(%)
P. carthagenensis	16.92 ± 4.58 (12.03–22.71)	93.52 ± 8.41	79.12 ± 3.70	92.5 ± 7.43
P. leiocarpa	127.00 ± 10.55 (129.50–155.94)	54.13 ± 11.10	22.30 ± 7.14	12.20 ± 4.44
P. capillacea	30.05 ± 6.22 (36.68–47.27)	91.78 ± 4.23	33.40 ± 15.22	87.34 ± 8.32
P. deflexa	146.40 ± 12.47 (93.81–140.42)	66.37 ± 8.12	26.05 ± 10.60	15.58 ± 5.22
Standards				
BHT	16.72 ± 1.34 (14.08–17.22)	92.19 ± 1.29	91.20 ± 4.54	96.4 ± 2.44
Ascorbic acid	22.28 ± 0.53 (22.20–23.43)	96.40 ± 0.27	4.13 ± 1.42	80.9 ± 5.56
Quercetin	n.d.	n.d.	80.65 ± 1.25	n.d.

Values are expressed as the mean ± SD (n = 3); n.d. = not determined; IC_{50} = concentration resulting in 50% inhibition of DPPH, derived from the graph of I% (inhibition percentage) *versus* concentration in μg/mL; %FRS = free-radical scavenging percentage (***** antioxidant activity evaluated by DPPH free-radical scavenging at a final concentration equivalent to 100 μg/mL of extract); %AA = antioxidant activity, evaluated by the β-carotene/linoleic acid method. (%) = ABTS radical scavenging activity.

Higher antioxidant activity was also found for *P. carthagenensis* (79.1% ± 3.7%) with the β-carotene bleaching method compared to quercetin and BHT (Table 1). This higher activity was not observed for ascorbic acid, which has relatively high polarity. We propose that this extract contains lipophilic compounds, which act by inhibiting or retarding oxidation of β-carotene.

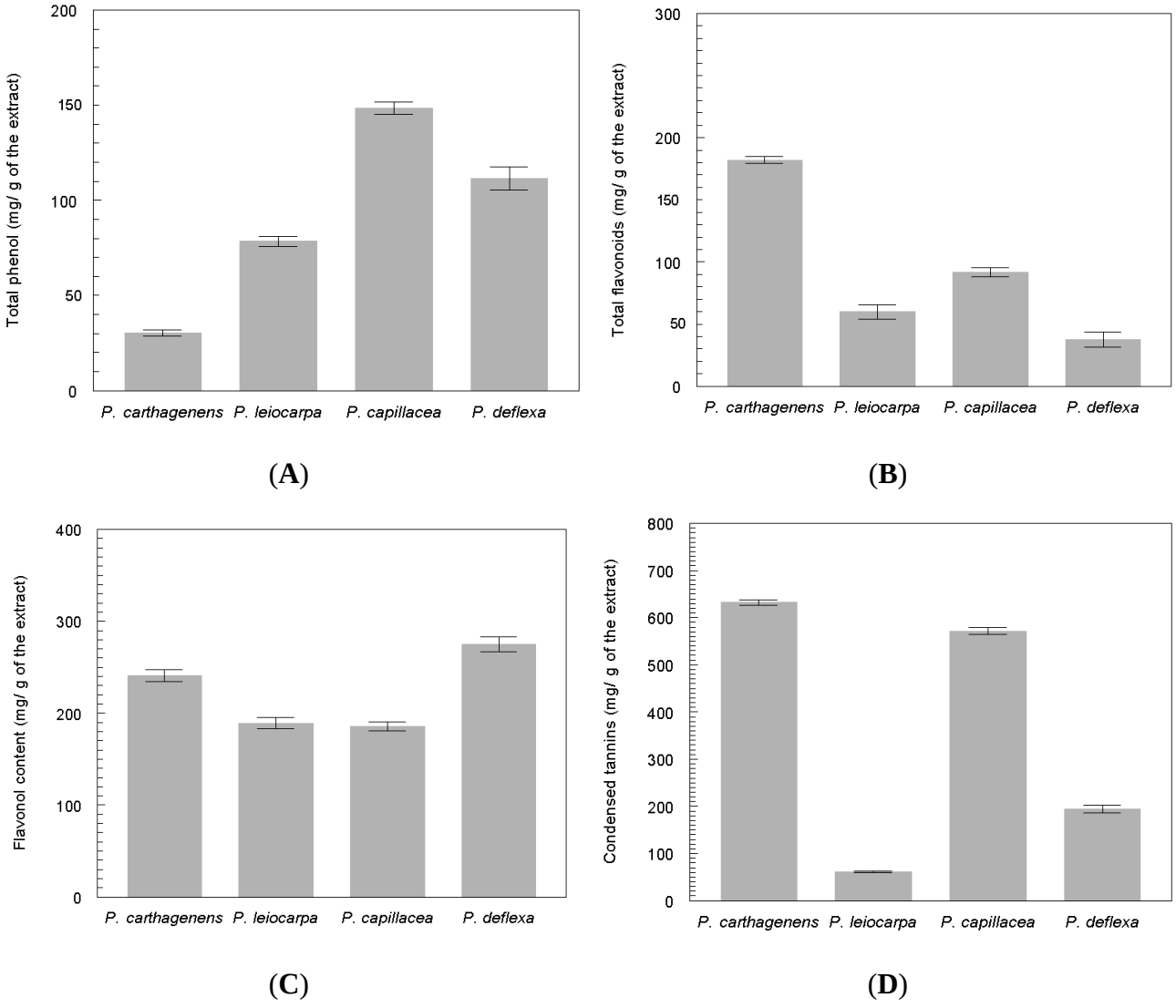
The scavenging effect of extracts from *Psycotria* and standard on $ABTS^+$ decreased in the order: BHT ~ *P. carthagenensis* > *P. capillacea* ~ ascorbic acid > *P. deflexa* ~ *P. leicocarpa* (96.4% ± 2.44%, 92.5% ± 7.43%, 87.34% ± 8.32%, 80.9% ± 5.56%, 15.58% ± 5.22% and 12.20% ± 4.44%, respectively) at the same concentration (100 μg/mL). *P. carthagenensis* and *P. capillacea* exhibited effective radical cation scavenging activity, when compared to other tested extracts.

2.2. Levels of Constituents

The total concentrations of phenolic compounds in the extracts are shown in Figure 2A. *P. capillacea* had the highest total concentration (148.42 ± 4.69 mg/acid galic g/extract), followed by *P. deflexa* and *P. leiocarpa* (11.42 ± 8.12 and 78.45 ± 5.20 mg/g GAE, respectively). *P.*

carthagenensis, with 182.07 ± 2.78 mg/g quercetin, exhibited the highest flavonoid concentration (Figure 2B). The flavonoid concentrations of *P. capillacea*, *P. leiocarpa*, and *P. deflexa* were 91.58 ± 3.74, 59.80 ± 6.45, and 37.64 ± 10.14 mg/g QE, respectively (Figure 2B). The highest flavonol concentrations were found in extracts *P. deflexa* and *P. carthagenensis* yielded 275.07 ± 8.40 and 241.19 ± 9.48. *P. leiocarpa* and *P. capillacea* also yielded 189.20 ± 6.44, and 185.54 ± 5.33 mg/g QE, respectively (Figure 2C). High levels of condensed tannins were also found in *P. carthagenensis* (632.39 ± 5.63 mg/catechin g/extract) and *P. capillacea* (571.95 ± 7.22 mg/g CE), while the lowest concentrations were observed in *P. deflexa* and *P. leiocarpa* (194.67 ± 9.02 and 60.97 ± 10.45 mg/g CE, respectively (Figure 2D).

Figure 2: Total phenols (**A**); Total flavonoids (**B**); Total flavonols (**C**); and condensed tannins (**D**) in *P. carthagenis*, *P. leiocarpa*, *P. capillacea* and *P. deflexa* extracts. The data represent the mean ± SD.



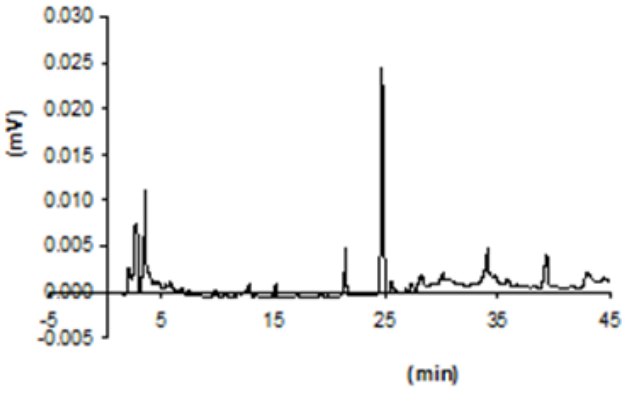
In plants, flavonoids occasionally occur as aglycones, although the most common forms are glycoside derivatives. These compounds account for 60% of total dietary phenolic compounds [39,40]. Flavonols are the most common flavonoids in the plant kingdom, and glycosides of quercetin are the most common naturally occurring flavonols [39].

Phenolic compounds are known as high-level antioxidants because of their ability to scavenge free radicals and active oxygen species, such as singlet oxygen, superoxide free radicals and hydroxyl radicals [41]. The radical-scavenging activity is attributed to replacement of hydroxyl groups in the aromatic ring systems of the phenolic compounds as a result of their hydrogen donating ability [38].

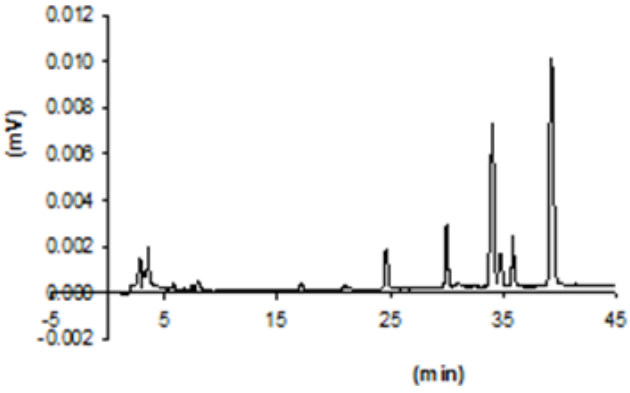
2.3. HPLC/PAD Analysis from Psychotria Extracts

After determination of the antioxidant potential and levels of constituents, we analyzed the methanolic extracts obtained from *Psychotria* in an analytical LC (Figure 3). The standards were easily identified based on their UV absorption spectra and retention times. The substances found in the extracts were unambiguously identified by performing co-injection experiments in which aliquots of extract and standard were mixed and diluted to a known volume, and analyzed by HPLC. Only *p*-coumaric acid (*tr* = 24.91 min) was found in *P. carthagenensis* and *P. capillacea* (Figure 3A,B).

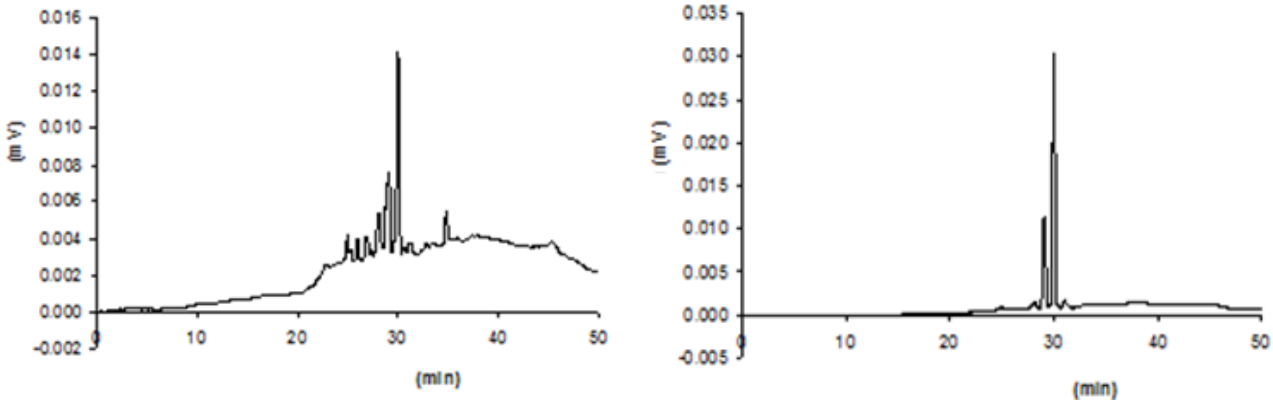
Figure 3. Chromatogram of *P. carthagenensis* (**A**); *P. capillacea* (**B**); *P. leiocarpa* and (**C**) *P. deflexa*; (**D**) extracts by HPLC/PDA analysis.



(**A**)



(**B**)



(**C**) (**D**)

The antioxidant activity of the pure *p*-coumaric acid at 40 µg/mL concentration was determined. DPPH radical scavenging activity of *p*-coumaric acid was found to be 55.6% ± 3.4%; the β-carotene bleaching with 78.6% ± 2%; and 87.14% ± 5.7% in ABTS radical scavenging activity. Comparison of the obtained of *P. carthagenensis* and *P. capillacea* data indicated the potent activity for the pure *p*-coumaric acid.

p-Coumaric acid (4-hydroxycinnamic acid), a phenolic acid, hydroxyl derivative of cinnamic acid, is widely used in the chemical, food, health, cosmetic, and pharmaceutical industries. Studies reported that antioxidants, such as *p*-coumaric acid and others hydroxycinnamic acids, function as chemoprotective agents by quenching carcinogenic nitrosating agents in several biological compartments, including salivary and gastric fluids [42]. However, minor attention has also been directed to the activity of simple phenolic acids, such as benzoic or cinnamic acids, and their derivatives.

3. Experimental Section

3.1. General Information

2,4-Dinitrophenylhydrazine (DNPH), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyltoluene (BHT), 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), quercetin, catechin, β-carotene and *p*-coumaric acid (98%) were purchased from Sigma Chemical Co., (St. Louis MO, USA). Potassium persulfate, tween 40, Folin-Ciocalteau, and sodium carbonate were purchased from Dinamina. Sulfuric acid, methanol, ethanol, hydrochloric acid, ascorbic acid, chloroform, linolenic acid, gallic acid, aluminum chloride, sodium acetate, and vanillin were obtained from Vetec (Duque de Caxias, RJ, Brazil). Spectroscopic grade acetonitrile was purchased from Merck (Darmstadt). Standards (caffeic acid (98%), *p*-coumaric acid (98%), luteolin (98%), quercetin (98%) and apigenin (95%)) were purchased from Sigma Aldrich (St. Louis MO, USA).

3.2. Plant Material

Leaves were collected in Dourados, Mato Grosso do Sul, Brazil. Botanical identification was performed by Dr. Zefa Valdevina Pereira (Faculty of Biological Sciences and Ambiental, Federal University of Grande Dourados (UFGD)). Specimens of *P. carthagenensis* (DDMS 5006), *P. deflexa* (DDMS 5005), *P. leiocarpa* (DDMS 5007) and *P. capillacea* (DDMS 5008) were deposited in the Herbarium of the Faculty of Biological Sciences and Ambiental, Federal University of Grande Dourados (UFGD), Mato Grosso do Sul, Brazil.

3.3. Preparation of Extracts

Dried leaves of *P. carthagenensis* (710 g), *P. deflexa* (620 g), *P. leiocarpa* (560 g) and *P. capillacea* (660 g) were exhaustively extracted by maceration with methanol (8 × 4 L) at room temperature. After filtration, evaporation of the solvent under vacuum furnished the extract. The extract yields (% dry weight) of the *P. carthagenensis*, *P. deflexa*, *P. leiocarpa*, and *P. capillacea* samples were 20.00%, 18.50%, 18.00% and 21.75% (w/w), respectively.

3.4. Measurement of Antioxidant Activity

3.4.1. β-Carotene/Linoleic Acid Method

The β-carotene solution was prepared by dissolving 2 mg β-carotene in 10 mL chloroform; 1 mL of this β-carotene-chloroform solution was mixed with 20 mg linoleic acid and 0.2 g Tween 40. Subsequently, the chloroform was removed by a rotary evaporator at 45 °C. Distilled water (50 mL) was slowly added with vigorous agitation to form an emulsion. Emulsion aliquots (5 mL) were transferred with 0.2 mL of the extracts different concentrations (10–200 µg/mL, sample stock 1.0 mg/mL). Control samples were prepared with 0.2 mL methanol devoid of extract [43–45]. As soon as the emulsion was added to each tube, absorbance was read at 470 nm against blank (zero time). Tubes were placed in a water bath at 50 °C, and oxidation was monitored by absorbance at 15 min intervals until the color of β-carotene in the control sample had disappeared (105 min). BHT was used as reference. The analyses were performed in triplicate. Antioxidant activity (AA) was calculated as percent inhibition relative to the control:

$$\%AA = [1 - (Ai - At)/(A'i - A't)] \times 100 \quad (1)$$

Ai = absorbance of sample at zero time, At = absorbance of sample after incubation (105 min) at 50 °C, A'i = absorbance of control at zero time, and A't = absorbance of control after incubation (105 min) at 50 °C.

3.4.2. Scavenging of 1,1-Diphenyl-2-Picrylhydrazyl (DPPH)

Sample stock solutions (1.0 mg/mL) were diluted to final concentrations of 200, 125, 50, 25, 10 and 5 µg/mL in methanol. Samples were added to 3 mL of methanolic DPPH (0.1 mM), prepared daily. The mixture was shaken and left to stand at room temperature in the dark. After 30 min, absorbance was measured at 517 nm against a blank containing all reagents except the

test samples [37]. BHT was used as the positive control. Assays were carried out in triplicate. The percentage of inhibition of DPPH (I%) was calculated using the following equation:

$$I\% = (A_0 - A/A_0) \times 100 \quad (2)$$

A_0 is the absorbance of the blank solution and A is the absorbance of the methanolic extract.

The IC_{50}, the concentration giving 50% inhibition of DPPH, was read off a graph of I% (percentage inhibition) *versus* extract concentration.

3.4.3. $ABTS^+$ Scavenging Activity

Total antioxidant activity was measured using an improved azinobis (ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging method [46] with minor modifications. Sample stock solutions (1.0 mg/mL) were diluted to final concentrations of 250, 125, 50, 25, 10 and 5 µg/mL in methanol. Briefly, 7.0 mM ABTS and 140 mM potassium persulphate were mixed and kept in the dark for 16 h at ambient temperature. Before usage, the $ABTS^+$ solution was diluted to get an absorbance of 0.700 ± 0.05 at 734 nm with ethanol (P.A.). Then, 3 mL of $ABTS^+$ solution was added to 30 µL of different sample concentrations (5–250 µg/mL). After 30 min, the absorbance was taken at 734 nm using spectrophotometer. The $ABTS^+$ scavenging activity was calculated using the following equation:

$$\text{ABTS radical scavenging activity (\%)} = (A_0 - A/A_0) \times 100 \quad (3)$$

A_0 is the absorbance of the blank solution and A is the absorbance of the methanolics extracts.

3.5. Concentrations of Constituents

3.5.1. Total Phenol Concentration

The total phenol concentration of the samples was determined using folin reagent [47]. Briefly, 100 µL of extract in methanol (1 g/L) were mixed with 1.0 mL of distilled water and 0.5 mL of folin-ciocaleu's reagent (1:10 v/v). After mixing, 1.5 mL of 2% aqueous sodium bicarbonate were added, and the mixture was allowed to stand for 30 min with intermittent shaking. The absorbance was measured at 765 nm using a spectrophotometer. Total phenolic concentration is expressed as gallic acid equivalent (GAE) in mg per gram of extract. The methanol solution was used as a blank. All assays were carried out in triplicate.

3.5.2. Total Flavonoid Concentration

The amount of total flavonoids in the extracts was measured spectrophotometrically as previously reported [48]. Briefly, 500 µL of each extract was mixed with 1.50 mL of 95% ethanol, 0.10 mL of 10% aluminium chloride ($AlCl_3.6H_2O$), 0.10 mL of sodium acetate ($NaC_2H_3O_2.3H_2O$) (1 M) and 2.80 mL of distilled water. After incubation for 40 min, absorbance was measured at 415 nm using a spectrophotometer. To calculate the concentration of flavonoids, we prepared a calibration curve using quercetin as standard. The flavonoid

concentration is expressed as quercetin equivalents (QE) in mg per gram of extract. All assays were carried out in triplicate.

3.5.3. Condensed Tannin Concentration (CT)

CT concentrations were determined by a modified version of a method reported previously [47,48]. Samples were mixed with 5 mL vanillin-HCl (8% conc. aq. HCl and 4% vanillin in methanol). Absorbance at 500 nm was read after 20 min. Catechin was used as the standard. The condensed tannin concentration is expressed as catechin equivalents (CE) in mg per gram of extract.

3.5.4. Flavonol Concentration

Total flavonols in the plant extracts were estimated using the method reported previously [36,47]. To 2 mL of sample, 2 mL $AlCl_3$ (2%)/ethanol and 3 mL (50 g/L) sodium acetate were added. The mixture was shaken and incubated for 2.5 h at 20 °C. Absorbance was read at 440 nm. Total flavonols are expressed as mg of quercetin equivalents per gram of dry weight (mg/g QE) using the calibration curve with quercetin.

3.6. HPLC/PDA Analysis

The *Psychotria* extracts and standards were analyzed using an analytical HPLC (Varian 210) system, with a ternary solvent delivery system and an auto-sampler. A photodiode array detector (PAD) was monitored at λ = 200–800 nm. The HPLC column was C-18 (25 cm × 4.6 mm; particle size, 5 μm; Luna, Phenomenex, Torrance, CA, USA), with a small pre-column (2.5 cm × 3 mm) containing the same packing to protect the analytical column. The flow rate and injected volume were 1.0 mL min^{-1} and 10 μL, respectively. All chromatographic analyses were performed at 22 °C. The elution of *P. leicocarpa* and *P. deflexa* extracts and standards was carried out using acetonitrile with formic acid (pH 2.64) (solvent A) and water (solvent B). The solvent gradient program was as follows: 0 min, 70% B; 30 min, 37% B; in 45 min returning to the initial condition. The *P. carthagenensis* and *P. capillacea* extracts and standards were eluted using acetonitrile with formic acid (pH 2.64) (solvent A) and water (solvent B). The solvent gradient program was as follows: 0 min, 84% B; 15 min, 59% B; 20 min, 79% B; 40 min, 0% B, in 50 min returning to the initial condition.

Stock solutions of standards (caffeic acid, *p*-coumaric acid, luteolin, quercetin and apigenin), created by dissolving individual solutions in acetonitrile at 10 μg/mL and were employed in the identification of compounds using a PDA detector (200–800 nm). This did not reveal coeluting substances.

4. Conclusions

According to data obtained from the present study, *P. carthagenensis* and *P. capillacea* were found to be effectives antioxidants in different *in vitro* assay including DPPH radical, ABTS

radical and β-carotene bleaching activities when they are compared to standard antioxidant compounds, such as BHT, gallic acid, quercetin and ascorbic acid. The results indicate that the antioxidant activity to these plants can be attributed the presence the *p*-coumaric acid. Further, more detailed, studies on the chemical composition of those extracts, as well as studies with other models, such *in vivo* assays, are essential to characterize them as biological antioxidants.

Acknowledgments

We are grateful to Fundect, Capes, and CNPq for fellowships.

Author Contributions

Anelise Samara Nazari Formagio, Carla Roberta Ferreira Volobuff and Matheus Santiago designed the study; performed extractions; determined the levels of total phenols, flavonoids, tannins and flavonols; assessed antioxidant activity; and helped in writing and editing. Claudia Andrea Lima Cardoso participated in the HPLC-DAD analysis. Maria do Carmo Vieira and Zefa Valdevina Pereira participated in the collection and identification of plant material. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that they have no competing interests.

References

1. Lindley, M. The impact of food processing on antioxidants in vegetable oils, fruits and vegetables. *Trends Food Sci. Technol.* **1998**, *9*, 336–340.
2. Middleton, E.; Kandaswamy, C.; Theoharides, T.C. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000**, *52*, 673–751.
3. Salah, N.; Miller, N.J.; Paganga, G.; Tijburg, L.; Rice-Evans, C.A. Polyphenolic flavonols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch. Biochem. Biophys.* **1995**, *322*, 339–346.
4. Whitehead, T.P.; Robinson, D.; Allaway, S.; Syms, J.; Hale, A. Effect of red wine ingestion on the antioxidant capacity of serum. *Clin. Chem.* **1995**, *41*, 32–35.
5. Perry, L.M.; Metzger, J. *Medicinal Plants of East and Southeast*; MIT Press: Cambridge, UK, 1980.
6. Lajis, N.H.; Mahmud, Z.; Toia, R.F. The alkaloids of *Psychotria rostrata*. *Planta Med.* **1993**, *59*, 383–384.
7. Hart, N.K.; Johns, S.R.; Lamberton, J.A.; Summons, R.E. Psychotridine, a $C_{55}H_{62}N_{10}$ alkaloid from *Psychotria beccarioides* (Rubiaceae). *Austrian J. Chem.* **1974**, *27*, 639–646.

8. Roth, A.; Kuballa, B.; Bounthanh, C.; Cabalion, P.; Sévenet, T.; Beck, J.P.; Anton, R. Cytotoxic activity of polyindoline alkaloids of *Psychotria fosteriana* (Rubiaceae). *Planta Med.* **1986**, *5*, 450–453.
9. Libot, F.; Miet, C.; Kunesch, N.; Poisson, J.E.; Pusset, J.; Sévenet, T. Rubiacées d'océanie: Alcaloïdes de *Psychotria oleoides* de Nouvelle-Calédonie et de *Calycodendron milnei* du Vanuatu (Nouvelle-Hébrides). *J. Nat. Prod.* **1987**, *50*, 468–473.
10. Adjibade, Y.; Weniger, B.; Quirion, J.C.; Kuballa, B.; Cabalion, P.; Anton, R. Dimeric alkaloids from *Psychotria fosteriana*. *Phytochemistry* **1992**, *31*, 317–319.
11. Verotta, L.; Pilati, T.; Tato, M.; Elisabetsky, E.; Amador, T.A.; Nunes, D.S. Pyrrolidinoindoline alkaloids from *Psychotria colorata*. *J. Nat. Prod.* **1998**, *61*, 392–396.
12. Jannic, V.; Gueritte, F.; Laprevote, O.; Serani, L.; Martin, M.T.; Sevenet, T.; Potier, P. Pyrrolidinoindoline alkaloids from *Psychotria oleoides* and *Psychotria lyciiflora*. *J. Nat. Prod.* **1999**, *62*, 838–843.
13. Takayama, M.; Hamano, H.; Ochi, Y.; Seaegusa, H.; Komatsu, K.; Murati, T.; Arakura, N.; Imai, Y.; Hasebe, O.; Kawa, S.; *et al*. Recurrent attacks of autoimune pancreatites results in pancreatic stone formation. *Am. J. Gastrienterol.* **2004**, *99*, 932–937.
14. Itoh, A.; Tanahashi, T.; Nagakura, N. Six tetrahydroisoquinoline-monoterpene glucosides from *Cephaelis ipecacuanha*. *Phytochemistry* **1991**, *30*, 3117–3123.
15. Itoh, A.; Tanahashi, T.; Nagakura, N.; Nayeshiro, H. Tetrahydroisoquinoline-monoterpene glucosides from *Alangium lamarckii* and *Cephaelis ipecacuanha*. *Phytochemistry* **1994**, *36*, 383–387.
16. Itoh, A.; Ikuta, Y.; Baba, Y.; Tanahashi, T.; Nagakura, N. Ipecac alkaloids from *Cephaelis acuminata*. *Phytochemistry* **1999**, *52*, 1169–1176.
17. Nagakura, N.; Itoh, A.; Tanahashi, T. Four tetrahydroisoquinoline-monoterpene glucosides from *Cephaelis ipecacuanha*. *Phytochemistry* **1993**, *32*, 761–765.
18. Solis, P.; Ravelo, A.G.; Palenzuela, J.A.; Gupta, M.P.; Gonzalez, A.; Phillipson, J.D. Quinoline alkaloids from *Psychotria glomerata*. *Phytochemistry* **1997**, *44*, 963–969.
19. Muhammad, I.; Dunbar, C.D.; Shabana, I.K.; Tekwani, B.L.; Bedir, E.; Takamatsu, S.; Ferreira, D.; Walker, L.A. Antiparasitic alkaloids from *Psychotria klugii*. *J. Nat. Prod.* **2003**, *66*, 962–967.
20. Achenbach, H.; Lottes, M.; Waibel, R.; Karias, G.A.; Correa, M.D.; Gupta, M.P. Alkaloids and other compounds from *Psychotria correae*. *Phytochemistry* **1995**, *38*, 1537–1545.
21. Kerber, V.A.; Gregianni, T.S.; Paranhos, J.S.; Schwanbach, J.; Farias, F.; Fett, J.P.; Fett-Neto, A.G.; Zuanazzi, J.A.; Quirion, J.C.; Elisabetsky, E.; *et al*. Brachycerine, a novel monoterpene indole alkaloid from *Psychotria brachyceras*. *J. Nat. Prod.* **2001**, *64*, 677–679.
22. Santos, L.V.; Fett-Neto, A.G.; Kerber, V.A.; Elisabetsky, E.; Quirion, J.C.; Henriques, A.T. Indole monoterpene alkaloids from leaves of *Psychotria suterella* Müll. Arg. (Rubiaceae). *Biochem. Syst. Ecol.* **2001**, *29*, 1185-1187.
23. Paul, J.H.A.; Maxwell, A.R.; Reynolds, W.F. Novel bis(monoterpenoid) indole alkaloids from *Psychotria bahiensis*. *J. Nat. Prod.* **2003**, *66*, 752–754.

24. Henriques, A.T.; Lopes, S.O.; Paranhos, J.T.; Gregianini, T.S.; von Poser, G.L.; Fett-Neto, A.G.; Schripsema, J. *N*,β-D-Glucopyranosyl vincosamide, a light regulated indole alkaloid from the shoots of *Psychotria leiocarpa*. *Phytochemistry* **2004**, *65*, 449–454.
25. Simoes-Pires, C.A.; Farias, F.N.; Marston, A.; Queiroz, E.F.; Chaves, C.G.; Henriques, A.T.; Hostettman, K. Indole monoterpenes with antichemotactic activity from *Psychotria myriantha*: Chemotaxonomic significance. *Nat. Prod. Commun.* **2006**, *1*, 1101–1106.
26. Nomura, T.; Quesada, A.L.; Kutchan, T.M. The New β-D-glucosidade in terpenoide isoquinoline alkaloid biosynthesis in *Psychotria ipecacuanha*. *J. Biol. Chem.* **2008**, *283*, 34650–34659.
27. Beretz, A.; Roth-Georger, A.; Corre, G.; Kuballa, B.; Anton. R.; Cazenale, J.P. Polyindolinic alkaloids from *Psychotria fosteriana*. Potent inhibitors of the aggregation of human platelets. *Planta Med.* **1985**, *4*, 300–303.
28. Leal, M.B.; Elisabetsky, E. Opioid-like activity of *Psychotria brachypoda*. *Int. J. Pharmacogn.* **1996**, *34*, 267–272.
29. Lopes, S.O. Análise Química e Cultivo *in Vitro* de *Psychotria leiocarpa* Cham. et Schlecht. e *Psychotria carthagenensis* Jacq. (Rubiaceae). Master's Thesis, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, **1998**, 177.
30. Lopes, S.; Poser, G.L.; Kerber, V.A.; Farias, F.M.; Konrath, E.L.; Moreno, P.; Sobral, M.E.; Zuanazzi, J.A.S.; Henriques, A.T. Taxonomic significance of alkaloids and iridoid glucosides in the tribe *Psychotrieae* (Rubiaceae). *Biochem. Syst. Ecol.* **2004**, *32*, 1187–1195.
31. Dillenburg, C.R.; Porto, M.L. Rubiaceae-tribo *Psychotriae. Boletim Instituto de Biociências—UFRGS* **1985**, *39*, 1–76.
32. Rivier, L.; Lindgren, J.E. "Ayahuasca", the South American hallucinogenic drink: An ethnobotanical and chemical investigation. *Econ. Bot.* **1972**, *26*, 101–129.
33. McKenna, D.J.; Towers, G.N.T.; Abbot, F. Monoamine oxidase inhibitors in South American hallucinogenic plants: Trypatamine and β-carboline constituents of Ayahusca. *J. Ethnopharmacol.* **1984**, *10*, 195–223.
34. Adedapo, A.A.; Jimoh, F.O.; Koduru, S.; Afolayan, A.J.; Masika, P.J. Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of *Calpurnia aurea*. *BMC Complement. Altern. Med.* **2008**, *53*, 1–8.
35. Blois, M.S. Antioxidant determinations by the use of a stable free radical. *Nature* **1958**, *181*, 1199–1200.
36. Brand-Williams, W.; Cuvelier, M.E.; Benset, C. Use of free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.* **1995**, *28*, 25–30.
37. Harborne, J.B.; Williams, C.A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481–504.
38. Naczk, M.; Shahidi, F. Extraction and analysis of phenolics in food. *J. Chromatogr.* **2004**, *1054*, 95–111.

39. Hall, C.A.; Cuppett, S.L. Structure-activities of natural antioxidants. In *Antioxidant Methodology: in Vivo and in Vitro Concepts*; Aruoma, O.I., Cuppett, S.L., Eds.; AOCS Press: Champaign, IL, USA, 1997; pp. 141–172.
40. Robbins, R.J. Phenolic acids in foods: An overview of analytical methodology. *J. Agric. Food Chem.* **2003**, *51*, 2866–2887.
41. Torres, J.L.; Rosazza, J.P.N. Microbial transformations of *p*-coumaric acid by *Bacillus megaterium* and *Curvularia lunata*. *J. Nat. Prod.* **2001**, *64*, 1408–1414.
42. Jayaprakasha, G.K.; Singh, R.P.; Sakariah, K.K. Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models *in vitro*. *Food Chem.* **2001**, *73*, 285–290.
43. Kaur, C.; Kapoor, H.C. Antioxidant activity and total phenolic content of some Asian vegetables. *Int. J. Food Sci. Technol.* **2002**, *37*, 153–161.
44. Shahidi, F.; Chavan, U.D.; Naczk, M.; Amarowicz, R. Nutrient distribution and phenolic antioxidants in air-classified fractions of beach pea (*Lathyrus maritimus* L.). *J. Agric. Food Chem.* **2001**, *49*, 926–933.
45. Rufino, M.S.M.; Alves, R.E.; Brito, E.S.; Morais, S.M.; Sampaio, C.G.; Pérez-Jiménez, J.; Saura-Calixto, F.D. Determinação da atividade antioxidante total em frutas pela captura do radical livre ABTS. *Embrapa Agroindústria Tropical/Comunicado Técnico* **2007**, *128*, 1-4.
46. Djeridane, A.; Yousfi, M.; Nadjemi, B.; Boutassouna, D.; Stocker, P.; Vidal, N. Antioxidant activity of some algerian medicinal plants extracts containing phenolic compounds. *Food Chem.* **2006**, *97*, 654–660.
47. Maxson, E.D.; Rooney, L.W. Evaluation of methods for tannin analysis in sorghum grain. *Cereal Chem.* **1972**, *49*, 719–729.
48. Lin, J.Y.; Tang, C.Y. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. *Food Chem.* **2007**, *101*, 140–147.

6. MANUSCRITO 2

Anti-inflammatory, antiproliferative and anticolinesterasic activity of the extract and vincosamide isolated from of *Psychotria leiocarpa*

Carla Roberta Ferreira Volobuff[a], Joyce Alencar Santos[b], Candida Aparecida Leite Kassuya[b], Mary Ann Foglio[c], João Ernesto de Carvalho[c], Ana Lúcia Tasca Gois Ruiz[c], Maria do Carmo Vieira[d], Zefa Valdevina Pereira[a] and Anelise Samara Nazari Formagio[a*]

[a]Faculty of Biological and Environmental Sciences, Federal University of Grande Dourados, 79804-970, Dourados – MS, Brazil.

[b]Faculty of Health Sciences, Federal University of Grande Dourados, 79804-970 Dourados – MS, Brazil.

[c]Campinas State University, 13083-970, Campinas – SP, Brazil.

[d]Faculty of Agricultural Sciences, Federal University of Grande Dourados, 79804-970, Dourados – MS, Brazil.

Corresponding author: E-mail address: aneliseformagio@ufgd.edu.br (A. Formagio).

Abbreviations: CC, cerebral cortex; HP, hippocampus; ST, striatum; AChE, acetylcholinesterase; AcSCh, acetylthiocholine iodide; ATC, acetyltiocolina; PBS, phosphate-buffered saline.

ABSTRACT

Objectives: The methanolic extract of *Psychotria leiocarpa* (leaves) (EMPL) collected in Dourados-MS, was investigated antiproliferative, anti-inflammatory and anticolinesterasic activity, as well as the acute toxicity and chemical composition.

Material and methods: Fractionation of the chloroform fraction through chromatographic method yielded vincosamide (PL-1) was found as the main chemical constituent of the EMPL. The EMPL and PL-1 was evaluated for anti-inflammatory activity using carrageenan-induced paw edema and pleurisy models in mice and tested in an anticancer assay against ten human cancer cell lines. The response parameter (GI_{50}) was calculated for the cell lines tested. The microplate assay was used to investigate the EMPL for acetylcholinesterase (AChE) inhibitors using Ellman's reagent. The three brain structure from the rats were selected for the experiment cerebral cortex (CC), hippocampus (HP), hypothalamus (HT) and striatum (ST). The protein concentration of homogenized samples was determined by method Coomasie blue, using

bovine serum albumin as standard. The acute toxicity *in vivo* in Swiss mice treated with the EMPL at doses of 175, 560, 1,792 and 2,000 mg/kg were investigate.

Results and discussion: The oral administration of EMPL as well as the vincosamide (PL-1) significantly inhibited the carrageenan (Cg) induced pleurisy, reducing the migration of total leukocytes in mice by 86.71 ± 4% (100 mg kg^{-1} of EMPL), 88.85 ± 6% (300 mg kg^{-1} of EMPL), 75.41 ± 6% (3 mg kg^{-1} of PL-1) and 88.35 ± 2% (30 mg kg^{-1} of PL-1). The EMPL showed antitumor activity with $GI_{50} \geq 56.89$ µg/ mL against all human tumor cell lines assayed and PL-1 exhibited broad-spectrum antitumor and potent activity at GI_{50} values ranging from 11.75 – 24.96 µM. The oral administration with EMPL (0.5 and 3 mg/kg) showed an inhibition of AChE activity in the cerebral cortex (CC) by 61% and 48%, respectively 1 h after oral administration. The acute administration of the EMPL of body weight did not cause signs of toxicity in the treated animals. Our results reveal for the first time the isolation and identification the monoterpene indole alkaloid known vincosamide (PL-1), as well marked anti-inflammatory, anticancer and anticholinesterasic effects of EMPL and PL-1 (vincosamide).

Introduction

Psychotria leiocarpa Cham. & Schlecht., "grandiúva-de-anta", is an small shrub (2 m in Height) native of Argentina, Paraguay, and Brazil (Smith and Downs, 1956). Chemical studies reported the isolation monoterpene indolic alkaloid, N, β-glucopyranosyl vincosamide, as main constituent from the leaves (Lopes, 1998; Matsuura, Fett-Neto, 2013), and iridoid glucosides, asperuloside and deacetylasperuloside (Lopes et al., 2004). Pharmacological effects have also been reported, such as the following: anti-inflammatory in NO production inhibitory (IC_{50} = 43.09 ± 1.75 %) in macrophages, antioxidant using a DPPH assay at concentrations of 10 µg/mL (70.89 ± 5.17 %) and 100 µg/mL (23.42 ± 2.11 %), antimycobacterial on the growth of

Mycobacterium bovis BCG (IC_{50} = 14.24 ± 1.08 %) (Moraes et al., 2011) and a non-specific analgesic activity (Elisabetsky et al., 1997). Study showed that N, β-glucopyranosyl vincosamide may act indirectly in *P. leiocarpa* protection against oxidative stress generated upon wounding, UV exposure, and perhaps other environmental stresses (McKenna et al., 1984). The data indicate the importance of continuity in the phytochemical studies and especially in investigation of major evidence of these studies, in addition other biological activities.

Recent investigations of our research group showed that *Psychotria* species were effective antioxidants in different *in vitro* assay including DPPH radical, ABTS radical and β-carotene bleaching activities (Formagio et al., 2014) and in the isolation of a new dimeric alkaloid tryptamine, brachybotryne and *N*-oxide derivative together with a known compound identified as bufotenine from the P. *brachybotrya* (Ribeiro et al., 2016).

Continuing our studies in search of species of *Psychotria* with pharmacological activity potential, this study aims to evaluate the antiproliferative, anti-inflammatory and anticolinesterasic activity, as well as the acute toxicity and chemical study of the methanolic extract of *P. leiocarpa* (leaves) collected in Dourados-MS.

Material and Methods

General methods

^{1}H and ^{13}C-NMR spectra were recorded on a Varian Mercury Plus spectrometer operating at 300 MHz and 75.5 MHz, respectively, using CD_3OD as solvent, and tetramethylsilane (TMS) as internal reference. Chromatography columns (CC) were performed on silica gel 60 (0.063-0.200 mm, Merck). TLC was performed on normal phase pre-coated

silica gel 60 G or 60 GF254 (Merck) plates. Visualization of the compounds on TLC was accomplished by UV irradiation at 254 and 366 nm, and/or by spraying with a dragendorf solutions.

Animals

The experiments were conducted using mice male and female *Swiss* (20-25g, n=6) and rats male Wistar (200-300 g, n=6) obtained from the University Federal da Grande Dourados (UFGD). The animals were maintained at a constant temperature (23 ± 1ºC) on a 12 hours light/dark cycle with free access to food and water. All experimental procedures were carried out in accordance with U.S. National Institute of Health, and were approved by the Animal Ethics Committee from UFGD (Nbr. 14/2015).

Collection and plant identification

Fresh leaves oh the *P. leiocarpa* were collected in Dourados (S 22º 08' 25", W 55º 08' 17"), Mato Grosso do Sul, Brazil. Botanical identification was performed by Profa. Dr. Zefa Valdevina Pereira, and specimen (DDMS-5007) was deposited in the Herbarium of the Faculty of Biological Sciences and Ambiental, Federal University of Grande Dourados -UFGD, Mato Grosso do Sul, Brazil.

Isolation and identification of alkaloid

The air-dried and powdered aerial parts of *P. leiocarpa* (560 g) were exhaustively extracted by maceration with methanol at room temperature. After filtration, evaporation of the solvent under vacuum furnished the extract methanolic (EMPL) (21 g).

A portion of the EMPL (13 g) was dissolved in MeOH/ H_2O (1:1) and partitioned with n-hexane, chloroform ($CHCl_3$) and ethyl acetate (EtOAc). Evaporation of the solvents resulted

in the *n*-hexane (HF; 1.04 g), chloroform (CF; 2.45 g), EtOAc (EAF; 1.68 g) and aqueous-methanol (AMF; 7.45 g) fractions. Part the CF (680 mg) was fractionated by CC on silica gel ((*n*-hexane/ EtOAc 10 to 80% and EtOAc/ MeOH 10 to 70%) to resulting in sub-fractions CF-1 to CF-13. The purification of sub-fraction CF-4 on preparative thin-layer chromatography eluted in EtOAc/ MeOH 30% yielded the PL-1.

Vincosamide (PL-1)

^{1}H NMR (δ_H CD_3OD, 300 MHz): 8.54 (NH), 4.95 (d, J = 11.5 Hz, H-3), 2.94 (ddd, J = 13.5; 11.8; 5.7 Hz, H-5a), 5.05 (ddd, J = 13.1; 3.0; 1.2 Hz, H-5b), 2.69 (ddd, J = 15.2; 11.8; 3.0 Hz, H-6a), 2.75 (ddd, J = 15.2; 5.7; 1.2 Hz, H-6b), 7.40 (d, J = 7.8 Hz, H-9), 7.02 (t, J = 7.8; 1.2 Hz, H-10), 7.12 (t, J = 7.8; 1,2 Hz, H-11), 7.31 (d, J = 7.8 Hz, H-12), 1.40 (dddd, J = 12.9; 12.9; 11.5; 1.5Hz, H-14a), 2.48 (dt, J = 12.9 Hz, H-14b), 3.23 (m, H-15), 7.44 (d, J = 2.4 Hz, H-17), 5.17 (dd, J = 10.2; 1.8 Hz, H-18a), 5.28 (dd, J = 17.1; 1.8 Hz, H-18b), 5.54 (ddd, J = 17.1; 10.2; 1.8 Hz, H-19), 2.71 (m, H-20), 5.50 (d, J = 1.8 Hz, H-21), 4.69 (d, J= 8.1 Hz, H-1'), 3.23 - 3.55 (4 H, m, H-2'-H-5'), 3.91 (1H, dd, J = 12.6; 1.8, H-6'a), 3.67 (1H, m, H-6'b). ^{13}C NMR (δ_C CD_3OD, 75.5 MHz): δ_C 134.5 (C-2), 54.79 (C-3), 41.21 (C -5), 22.06 (C -6), 109.03 (C -7), 127.93 (C -8), 118.84 (C-9), 120.0 (C-10), 122.53 (C-11), 111.98 (C-12), 138.27 (C -13), 32.64 (C-14), 27.32 (C -15), 109.01 (C-16), 149.01 (C -17), 120.5 (C-18), 133.92 (C-19) 44.52 (C -20), 97.36 (C -21), 166.05 (C-22), 99.57 (C-1'), 74.82 (C-2'), 77.96 (C-3'), 71.56 (C-4'), 78.35 (C-5'), 62.66 (C-6').

Acetylcholinesterase assay

In vitro AChE activity

After euthanasia of 3 animals, each brain structural (cerebral cortex, hippocampus, hypothalamus and striatum) were collected and homogenized separately in buffer Tris-HCl pH

7.2 with 10 mmol, 160 mmol sucrose (1:10, w/v), and EMPL (10 mg/mL). Find activity of homogenate was determined by the method of Ellman (1961). The test medium containing DTNB 1.04 mmol and potassium phosphate buffer pH 7.2, 24 mmol was incubated for 2 minutes at 30º C with 25 mL of the sample and the reaction initiated by addition of the acetylthiocholine iodide (ACSCh, 0.8 mM). The product of the reaction was determined at 412 nm for 2 min. The enzyme activity was expressed in mmol ACSCh/h/mg protein.

The protein concentration of homogenized samples was determined by method Coomasie blue (Bradford, 1976), using bovine serum albumin (BSA) as standard and adjusted for each structure: CC (0.7mg/ml), HP (0.8 mg/ml) and ST (0.4 mg/ml).

In vivo AChE activity

Groups of six male rats separated were treated with two doses of *P. leiocarpa* extract EMPL (0.5 and 3 mg/kg). The control group was treated with 0.9% saline and 20% MeOH. The animals were anesthetized and decapitated 60 minutes after of oral treatment EMPL. The three brain structure was selected for the experiment cerebral cortex (CC), hippocampus (HP) and striatum (ST), were maintained refrigerated in a solution of 10 mM Tris-HCl, pH 7.4, on ice. The tissues were homogenized in a glass potter in Tris-HCl solution at a proportion of 1:10 (w/v) and then centrifuged at 3500 rpm for 10 minutes to yield a supernatant that was used for the enzyme assay. The procedure was performed at 4 ºC and AChE activity was measured according to the sprectrophotometric method of Ellman (1961). In each homogenate was added a buffered solution of Ellman's reagent (10 mM DTNB) and acetylthiocholine iodide (ACSCh) at a concentration of 0.8 mM. The rate of hydrolysis was measured at 412 nm for 3 minutes with 3 second intervals, occurring formation of the thiolate dianion of DTNB. The enzyme activity was expressed in mmol ACSCh/h/mg protein.

The protein concentration of homogenized samples was determined by method Coomasie blue (Bradford, 1976), using BSA as standard.

Anticancer assay in vitro

The methanolic extract and the compound isolated vincosamide were tested against ten tumor cell lines, provide by the National Cancer Institute (Frederick, MD, USA), which were: U251 (glioma, CNS), MCF-7 (breast), NCI-ADR/RES (breast expressing the multiple drug resistance phenotype), 786-0 (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT-29 (colon), K-562 (leukemia) and HaCaT (keratinocytes). The cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content employing the sulforhodamina B assay (Monks et al., 1991). Doxorubicin (0.025-25 μg/mL) was used as a positive control. Three measurements were obtained: first at time point zero (T_0, at the beginning of incubation) and then 48 h post-incubation for both the compound-free (C) and tested (T) cells. Cell proliferation was determined using the equation $100 \times [(T - T_0)/C - T_0]$. A cytostatic effect was observed when $T \geq T0$, while a cytocidal effect occurred when $T < T_0$. The experiments were performed in triplicate. From the concentration–response curve for each cell line, the GI_{50} values (growth inhibitory activity or cytocidal effect) value was determined through nonlinear regression analysis using Origin 8.0® software (OriginLab Corp.). The experiments were performed in triplicate.

Carrageenan-induced paw oedema

Different groups of male mice were orally treated (1 hour before intrapleural injection) with vehicle and EMPL (100 or 300 mg/kg). Another group of mice was treated subcutaneously (0.5 hour before subcutaneously injection) with the anti-inflammatory drug dexamethasone (1 mg/kg). The animals received a 50 μL s.c. injection into the right hindpaw of

carrageenan (300 µg/paw) suspended in sterile 0.9% saline. The contralateral paw received only saline and was used as control. The thickness of the paw oedema was measured using a Plethysmometer Paw (PanLab s.l.u. LE7500) 1 hour before any treatment and at different time points (0.5, 1, 2, and 4) after the injection of carrageenan. Results were expressed in µm and the difference between basal and post-injection values quantified as oedema (Henriques et al., 1987).

Pleural cell migration

Different groups of female mice were orally treated (1 hour before intrapleural injection) with vehicle, MEPL (100 or 300 mg/kg), and PL-1 (3 or 30 mg/kg). Another group of mice was treated subcutaneously (0.5 hour before intrapleural injection) with the anti-inflammatory drug dexamethasone (1 mg/kg). Pleurisy was induced by the intrapleural injection of 100 µL of 1% carrageenan as previously described (Velo, et al, 1973). Briefly, an adapted needle was inserted into the right side of the thoracic cavity of the animals to enable intrapleural (i.p.) administration of carrageenan. Naive mice received an equal volume (100µL) of sterile, pyrogenfree saline. After 4 hour, the animals were killed and the pleural cavity was washed with 1mL of phosphate-buffered saline (PBS). The exudate volume was measured, and an aliquot of 20 µL was diluted in Turck solution (1:20) and used to determine the total number of leukocytes in a Neubauer chamber. Total cell count was performed under light microscopy and the results are reported as the number of cells per ml of pleural fluid.

Acute toxicity

The acute toxicity study was based on protocol 425 conducted according to the Organization for Economic Cooperation and Development (OECD, 2008) and the protocols established by the Brazilian Health Surveillance Agency (ANVISA, 2004). According to the

protocol established, 9 animals were used; each received a single oral administration by gavage of the MEPL. Initially, one of the animals received a dose of 175 mg/kg and was observed at 30 minutes, 1 h, 2 h, 4 h, 6 h, 12 h, 24 h and 48 h. After this period, a second animal received a dose of 560 mg/kg, and after 48 hours, the third animal received a dose of 1792 mg/kg. After an additional 48 hours, a fourth animal received a dose of 2000 mg/kg. After the last dose administered, no deaths were observed, and according to the protocol, 4 more animals received 2000 mg/kg. The control group received the vehicle used for diluting CF (drops of DMSO + distilled water). The animals were observed for signs of toxicity over 14 days. Behavioural observations (reflexes, tremors, convulsions, lacrimation, cyanosis, salivation, piloerection, muscle tone and motor coordination), mortality, the weight of the animals, and the amount of water and feed were analysed. After 14 days of treatment, the animals were weighed and subsequently euthanized by cervical dislocation. Organs such as the spleen, heart, liver, lungs and kidneys were assessed for the presence of macroscopic alterations in appearance, colour, size, weight and consistency.

Statistical analyses

Data are presented as mean ± standard error of the mean (SEM). Difference among groups was evaluated by analyses of variance (one-way ANOVA) followed by Tukey-Kramer or Newman-Keuls tests. Statistical differences were considered to be significant at $p < 0.05$. Graphs were performed using GraphPad Prism Software (San Diego, CA, U.S.A).

Results and discussion

The monoterpene indole alkaloid known vincosamide (PL-1) (Fig. 1), was found as the main chemical constituent of the extract methanolic from the leaves of *P. leiocarpa* (EMPL), isolated of the chloroform (CF) fraction through chromatographic fractionation and

characterized by NMR spectral data and by comparing their ^{1}H and ^{13}C NMR data with data reported in the literature (Erdelmeier et al., 1991) and subsequently their pharmacological activity was evaluated.

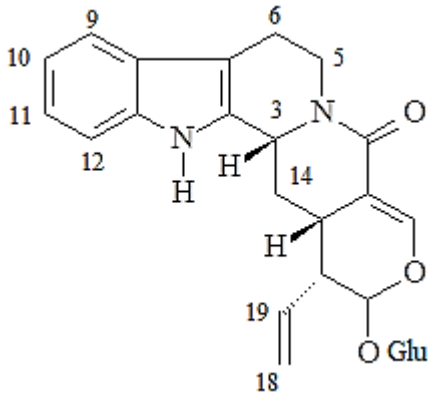


Fig. 1. Structure of vincosamide (PL-1) isolated from the extract methanolic of *P. leiocarpa* collected in Dourados-MS.

The ^{1}H and ^{13}C NMR data of compound PL-1 were characteristic by the signals for a indole ring at 7.40 (d, J = 7.8 Hz, H-9)/ δ_C 118.84, 7.31 (d, J = 7.8 Hz, H-12)/ δ_C 111.98 , 7.12 (ddd, J = 7.8; 7,5; 1,2 Hz, H-11) / δ_C 122.53, 7.02 (ddd , J = 7.8; 7.5; 1.2 Hz, H-10)/ δ_C 120.00 in the region of aromatics. The comparison in the literature of vincosamide data and its epimer strictosamide, maintains the relative configuration at position α of the H- 3 (Faria, 2009). The signals for H-19 at δ_H 5.54 (ddd, J = 17.1; 10.2; 1.8 Hz) and for 5.17 (dd, J = 10.2; 1.8 Hz, H-18a) and δ_H 5.28 (dd, J = 17.1; 1.8 Hz, H-18b) together with the methylene carbon at δ_C 120.5 (C-18) confirmed the terminal vinylidene unit. The carbonyl group was evidenced by the signal at δ_C 166.05 (C-22). The signal for the β-glucopyranosyl moiety was observed at δ_H 3.23-3.91/ δ_C 62.66 – 78.35 and δ_H 4.69 (d, J= 8.1 Hz, H-1')/ δ_C 99.57 in the ^{1}H and ^{13}C NMR spectra.

These data were consistent with of those described in literature for vincosamide (Erdelmeier et al., 1991) isolated from *Nauclea orientalis* (Rubiaceae). To our knowledge this is the first time reported in *P. leiocarpa*. The N-β-glucopyranosyl vincosamide was found in

the leaves of *P. leiocarpa* colleted in Porto Alegre/Brazil (Henriques et al., 2004; Lopes 1998). These variations may be related to the environmental conditions to which the plant is exposed such as mineral, water supply and sunlight.

In addition to these compounds reported for *P. leiocarpa*, studies with others native species this genus in Brazil showing indole chromophores led to the isolation of the to tryptamine-iridoid alkaloids, as for example the strictosamide isolated from the *P. prunifolia* (Faria et al., 2010), lialosideo of the *P. suturella*, psicollatine of the *P. umbellate*, braquicerine of the *P. brachyceras* and from *P. myriantha* the strictosidinic acid (Farias, et al., 2012), and some of them display a large range of effects on the central nervous system, such as anxiolytic, antidepressant, analgesic, and impairment of learning and memory acquisition (Farias et al., 2010; Simões et al., 2006; Both, et al., 2002, 2005; 2006; Farias 2012).

The effect of EMPL (10 mg/mL) was evaluated for AChE inhibition *in vitro* assay from four rat brain (Fig. 2), using methanol for sample preparation. In these assay, EMPL was able for AChE inhibition in the cerebral cortex (CC), hippocampus (HP) and striatum (ST) with 64.00 %, 51.80 % and 18.00 %, respectively, when compared with control. In addition, *in vivo* assay the EMPL at doses of 0.5 and 3 mg/kg showed an inhibition of AChE activity in the cerebral cortex (CC) by 61% and 48 % ($P < 0.01$) respectively, 1 h after oral administration of treatment when compared with control rats (Fig. 3A). Oral administration of EMPL had no significant effect on HP and ST in AChE activity compared to the control/saline ($P < 0.05$, Fig. 3B and 3C).

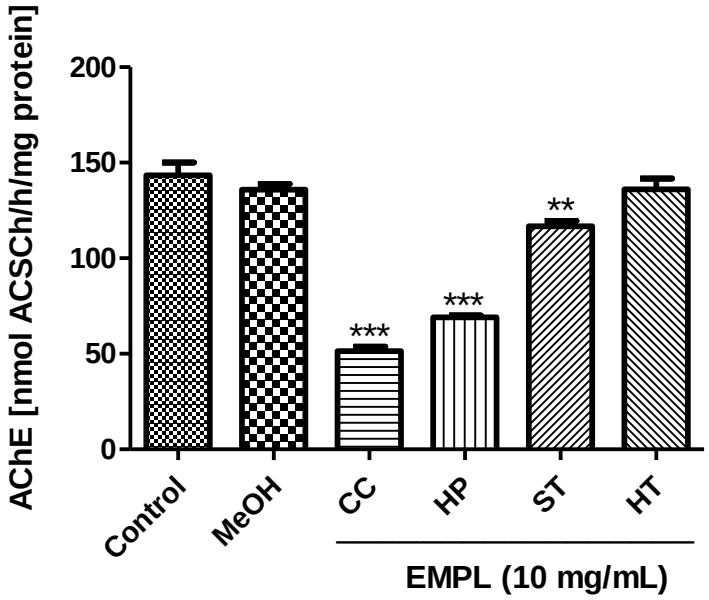
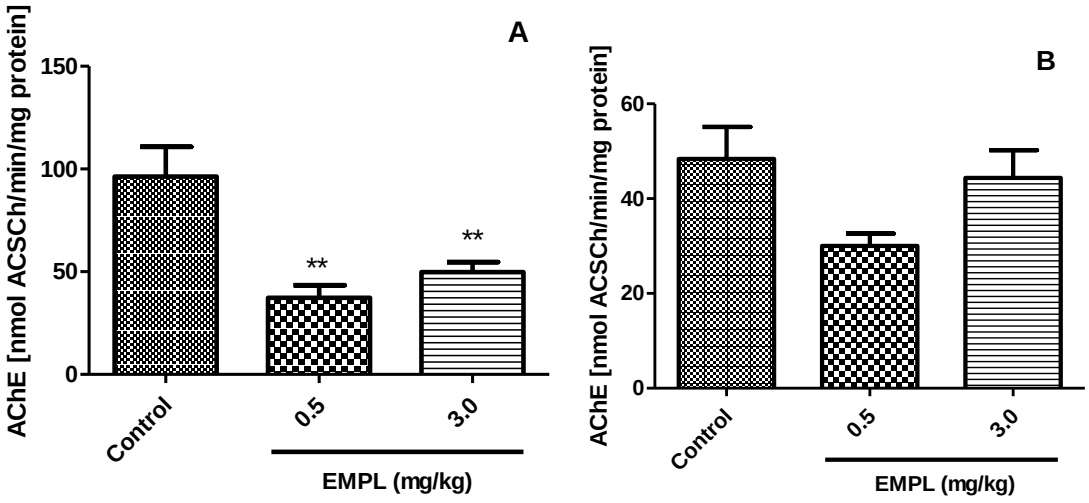


Fig. 2. Effect of extract methanolic from the leaves of *P. leiocarpa* (EMPL, 10 mg/mL) on AChE activity in the cerebral cortex (**CC**), hippocampus (**HP**), striatum (**ST**) and hypothalamus (**HT**). Values are expressed as mean ± S. E. M. n = 6 observations per group. ***, ** - Statisitical Difference vs. Control (H_2O + saline); $P < 0.001$ or $P < 0.05$



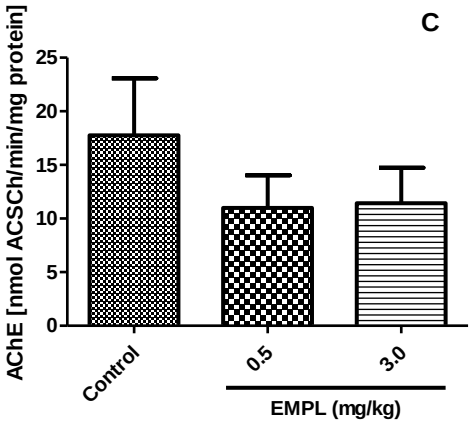


Fig. 3. Effect of 1 h of oral treatment with extract methanolic of *P. leiocarpa* at of 0.5 and 3.0 mg/kg on AChE activity in the cerebral cortex (**A**), hippocampus (**B**) and striatum (**C**). Values are expressed as mean ± SEM n = 6 observations per group. The ** symbol indicate significant differences among the groups (** $P < 0.05$).

Alzheimer's disease (AD) is a chronic neurological disorder characterized by memory impairment, cognitive dysfunction, behavioral disturbances, and deficits in activities of daily living. Acetylcholinesterase (AchE) inhibition is the major therapeutic strategy employed for the treatment of Alzheimer's disease (Orhan et al., 2004). A study by Passos et al. (2013) describes the interaction of the many compounds extracted from *Psychotria* in ability to AChE, butyrylcholinesterase (BChE), monoamine oxidases A and B (MAO-A and MAO-B) inhibitory potencies, highlighting two β-carboline alkaloids, prunifoleine and 14-oxoprunifoleine, and four monoterpene indole alkaloids, angustine, vallesiachotamine lactone, E-vallesiachotamine and Z-vallesiachotamine inhibited BChE and MAO-A.

Previous studies from extracts of *Psychotria* were screened at the US National Cancer Institute for their activities against human cancer (one-dose/60-cell line prescreen), and were selected as 'hot' genera (Cragg et al., 2006). The alkaloids related this genus as tryptamine and pyrrolidinoindole isolates of *P. henryi* showed inhibitory activity of less than 10 μM, against human osteosarcoma cell line (Liu et al., 2013).

In the search to find potential antitumor constituents from *P. leiocarpa*, the *in vitro* antiproliferative effect of the EMPL and PL-1 was evaluated against ten human cancer cell lines. The GI_{50} values (growth inhibitory activity or cytocidal effect) refer to the drug concentration that produces a 50% reduction of cellular growth when compared to untreated control cells. The EMPL showed antitumor activity with $GI_{50} \geq 56.89$ µg/ mL against all human tumor cell lines assayed (data not presented). Compound PL-1 exhibited broad spectrum antitumor and potent activity at GI_{50} values ranging from 11.75 – 24.96 µM , against all of the tested human tumor cell lines, showing effective growth inhibition against NCI-H460 (GI_{50}= 11.75 µM), PCO-3 (GI_{50}= 12.54 µM), OVCAR-3 (GI_{50}= 13.00 µM), HT-29 (GI_{50}= 12.17 µM), K-562 (GI_{50}= 12.17 µM) e HaCaT (GI_{50}= 13.00 µM) (Fig. 4), not showing cytostatic effect.

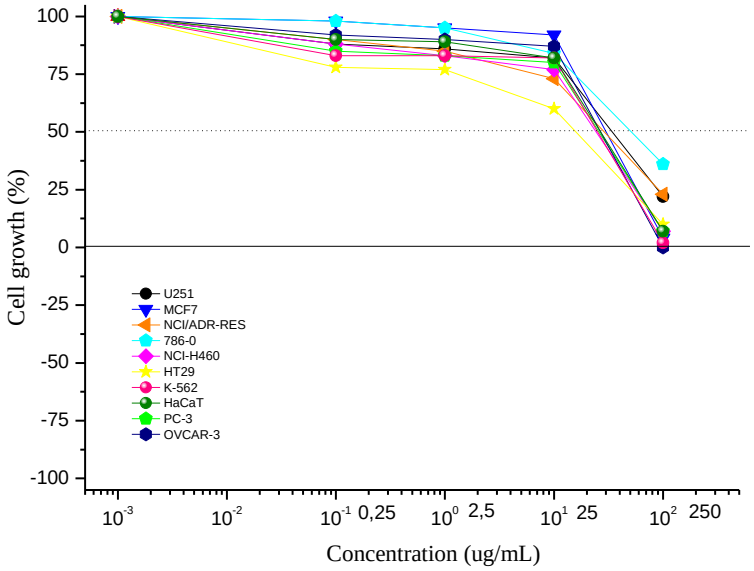


Fig. 4. Antiproliferative activity the vincosamide (PL-1) of *P. leiocarpa*.

The EMPL (100 and 300 mg kg^{-1}) also was available in inflammation model, not showed anti-inflammatory effect when evaluated by the method of carrageenan-induced paw edema at 4 hours. Additional, the EMPL and PL-1 was evaluated in a direct leukocyte migration pleurisy model of carrageenan induced inflammation. Fig. 5 shows that the oral

administration of EMPL at doses of 100 and 300 mg kg^{-1} inhibited leukocyte migration by 86.71 ± 4 % e 88.85 ± 6%, respectively, 4 hours after the carrageenan injection when compared to the control group. In relation to PL-1 administration, both doses of 3 and 30 mg kg^{-1} by oral route inhibited the leukocyte migration, with inhibitions o 75.41 ± 6 % and 88.35 ± 2 % respectively (Fig. 5). The dexamethasone (91.52 ± 2%) (Fig. 5) inhibited in inflammation, showing it effectiveness as anti-inflammatory agent.

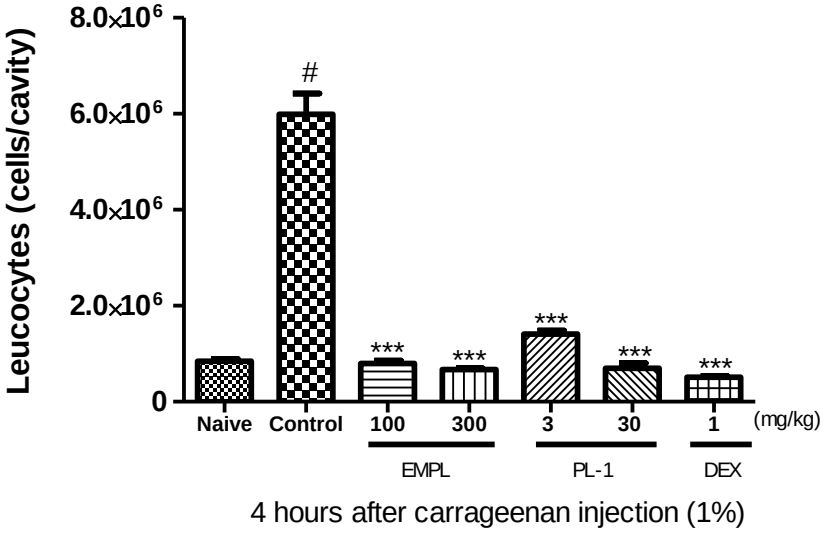


Fig. 5. Effects of EMPL and major compound isolated from EMPL on total leucocytes induced by carrageenan in the pleural cavity of mice. Animals received oral treatment with EMPL (100 and 300 mg/kg), PL-1 (3 and 30 mg/kg), vehicle (Control) or dexamethasone (DEX), and after 1 h, they received an intrapleural injection of carrageenan of a 1% solution/cavity. Control animals received only the vehicles. The data are represented the means ± SEM of animals. The # symbol indicate the statistical differences of naïve and control group ($p < 0.001$) while the * compared treated group in relation to control group: *** $p<0.001$, one-way ANOVA followed by Student-Newman-Keuls.

The assessment of acute toxicity was conducted for 14 days to determine the maximum tolerated dose (MTD). The animals used in this study were exposed to EMPL, and no clinical signs of toxicity were observed for any dose. No significant differences occurred regarding drinking water and food between the control group and any of the treated groups. No deaths

were reported. Furthermore, the relative and absolute weights of the organs (liver, kidneys, and lungs) showed no difference compared to the control group.

Conclusion

Compound PL-1 (vincosamide) is being described for the first time in *P. leiocarpa*; however, the presence of this compound was reported in species belonging to the *Psychotria* genus. The assays results demonstrated the antiproliferative, anticolinesterasic and anti-inflammatory effects of *P. leiocarpa* and vincosamide in the experimental models tested. Future studies of *P. leiocarpa* (EMPL) and vincosamide in chronic treatment on AChE and BuChE activities in rat brain, and also molecular docking will with the vincosamide to evaluate their interaction.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

The authors are grateful to FUNDECT (Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul), Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Unicamp (Universidade Estadual de Campinas - Centro Multidisciplinar de Química, Biológicas e Agrícolas/CPQBA) and UFGD (Universidade Federal da Grande Dourados) for financial support and fellowships. The authors are grateful to Dra. Zefa Valdevina Pereira (Faculdade de Ciências Biológicas e Ambiental –

UFGD, Dourados-MS) for botanical identification of plant material and Dra. Maria Helena Sarragiotto (Departamento de Química – UEM, Maringá-PR) for technical support.

References

ANVISA. Guia para a realização de estudos de toxicidade pré-clínica de fitoterápicos. 2004 [21 February 2015]. Available: http://www.diariodasleis.com.br/busca/exibelink.php?numlink=1-9-34-2004-03-16-90.

Both, F. L., Kerber, V. A., Henriques, A.T., Elisabetsky, E. 2002. Analgesic properties of umbellatine from *Psychotria umbellata*. Pharmaceutical Biology 40, 336–41.

Both, F. L., Meneghini, L., Kerber, V. A., Henriques, A. T., Elisabetsky, E. 2005. Psychopharmacological profile of the alkaloid psychollatine as a 5HT2A/C serotonin modulator. Journal of Natural Products 68, 374–80.

Both, F. L., Meneghini, L., Kerber, V. A, Henriques, A. T., Elisabetsky, E. 2006. Role of glutamate and dopamine receptors in the psychopharmacological profile of the indole alkaloid psychollatine. Journal of Natural Products 69, 342–5.

Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. 1976. Analytical Biochemistry 72, 248-254.

Cragg, G. M., Newman, D. J., Yang, S. S. 2006. Natural product extracts of plants and

marine origin having antileukemia potential The NCI experience. Journal of Natural Products 69, 488–498.

De Santos, L.V., Fett-Neto, A.G., Kerber, V.A., Elisabetsky, E., Quirion, J.C., Henriques, A.T. 2001. Indole monoterpene alkaloids from leaves of *Psychotria suterella* Müll. Arg. (Rubiaceae). Biochemical Systematic and Ecology 29, 1185–7.

Ellman, G. L. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7, 88-95.

Elisabetsky, E., Amador, T. A., Leal, M. B., Nunes, D. S., Carvalho, A. C. T., Verotta, L. 1997. Merging ethnopharmacology with chemotaxonomy: an approach to unveil bioactive natural products. The case of *Psychotria* alkaloids as potential analgesics. Ciência e Cultura 49, 378-385.

Erdelmeier, C. A. J., Wright, A. D., Orjala, J., Baumgartner, B., Rali, T., Sticher, O., 1991. New indole alkaloid glycosides from *Nauclea orientalis*. Planta Medica 57, 149–152.

Faria, E. O. Estudo fitoquímico das folhas de *Psychotria prunifolia* (Kunth) Steyerm (Rubiaceae). 2009. Dissertação (Mestrado) – Universidade Federal de Goiás, Instituto de Química. 126 f.

Faria, E. O., Kato, L., de Oliveira, C. M. A., Carvalho, B. G., Silva, C. C., Sales, L. S. 2010 Quaternary b-carboline alkaloids from *Psychotria prunifolia* (Kunth) Steyerm. Phytochemistry Letters 3, 133-116.

Farias, F. M., Passos, C. S., Arbo, M. D., Barros, D. M., Gottfried, Steffen, V. M., Henriques, A. T. 2012. Strictosidinic acid, isolated from *Psychotria myriantha* Mull. Arg. (Rubiaceae), decreases serotonin levels in rat hippocampus. Fitoterapia 86, 1138-1143.

Farias, F. M., Passos, C. S., Arbo, M. D., Zuanazzi, J. A., Steffen, V. M., Henriques, A. T. 2010. Monoamine levels in rat striatum after acute intraperitoneal injection of strictosidinic acid isolated from *Psychotria myriantha* Mull. Arg. (Rubiaceae). Phytomedicine 17, 289–91.

Formagio, A. S. N., Volobuff, C. R. F., Santiago, M., Cardoso, C. A. L., Vieira, M. C., Pereira, Z. V. 2014. Evaluation of antioxidante activity, total flavonoids, tannins and phenolic compounds in *Psychotria* leaf extracts. Antioxidants 3, 745-757.

Henriques, A.T., Lopes, S.O., Paranhos, J.T., Gregianini, T.S., Von Poser, G.L., Fett-Neto, A.G., Schripsema, J. 2004. N, β-D-glucopyranosyl vincosamide, a light regulated índole alkaloid from the shoots of *Psychotria leiocarpa*. Phytochemistry 65, 449-454.

Henriques, M.G., Silva, P.M., Martins, M.A, Flores, C.A, Cunha, F.Q, Assreuy-Filho, J., Cordeiro, R.S. 1987. Mouse paw edema. A new model for inflammation? Brazilian Journal of Medical and Biological Research 20, 243-9.

Liu, Y., Wang, J. S., Wang, X. B., Kong, L. Y. 2013. Two novel dimeric indole alkaloids from the leaves and twigs of *Psychotria henryi*. Fitoterapia 86, 178-182.

Lopes, S. O. 1998. Análise química e cultivo *in vitro* de *Psychotria leiocarpa* Cham. et Schlecht. e *Psychotria carthagenensis* Jacq. (Rubiaceae). Master Thesis, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Lopes, S., von Poser, G. L., Kerber, V. A., Farias, F. M., Konrath, E. L., Moreno, P., Sobral, M. E., Zuanazzi, J. A. S., Henriques, A. T. 2004. Taxonomic significance of alkaloids and iridoid glucosides in the tribe Psychotrieae (Rubiaceae). Biochemical Systematics and Ecology 32, 1187-1195.

Matsuura, H. N., Fett-Neto, A. G. 2013. The major indole alkaloid N, β-D-glucopyranosyl vincosamide from leaves of *Psychotria leiocarpa* Cham. & Schltdl. is not an antifeedant but shows broad antioxidant activity. Natural Product Research 27, 402-11.

McKenna, D.J., Towers, G.N.T., Abbot, F. 1984. Monoamine oxidase inhibitors in South American hallucinogenic plants: Trypatamine and β-carboline constituents of Ayahusca. Journal of Ethnopharmacology 10, 195–223.

Monks, A. D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D.; Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J., Boyd, M. J. 1991. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. Journal of National Cancer Institute 83, 757-766.

Moraes, T. M. S., de Araújo, M. H., Bernardes, N. R., de Oliveira, D. B., Lasunskaia, E. B., Muzitano, M. F., Cunha, M. 2011. Antimycobacterial activity and alkaloid prospection of

Psychotria species (Rubiaceae) from the Brazilian Atlantic Rainforest. Planta Medica 77, 964-970.

OECD. Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure. Guidelines for the Testing of Chemicals. 2008. doi: 10.1787/9789264071049-en.

Orhan, I., Sener, B., Choudhary, M.I., Khalid, A. 2004. Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Turkish medicinal plants. Journal of Ethnopharmacology 91, 57–60.

Passos, C. S., Simões-Pires, C. A., Nurisso, A., Soldi, T. C., Kato, L., de Oliveira, C. M. A., de Faria, E. O., Marcourt, L., Gottfried, C., Carrupt, P. A., Henriques, A. T. 2013. Indole alkaloids of *Psychotria* as multifunctional cholinesterases and monoamine oxidases inhibitors. Phytochemistry 86, 8-20.

Ribeiro, M. A. S., Gomes, C. M. B., Formagio, A. S. N., Pereira, Z. V., Melo, U. Z., Basso, E. A., da Costa, W. F., Baldoqui, D. C., Sarragiotto, M. H. 2016. Structural characterization of dimeric índole alkaloids from *Psychotria brachybotrya* by NMR spectroscopy and theoretical calculations. Tetrahedron Letters 57, 1331-1334.

Simões-Pires, C. A., Farias, F. M., Marston, A., Queiroz, E. F., Chaves, C. G., Henriques, A. T., Hostettmann, K. 2006. Indole monoterpenes with antichemotactic activity from *Psychotria myriantha*: chemotaxonomic significance. Nat Prod Commun 1, 1101–6

Smith, L. B., Downs, R. J. 1956. Resumo preliminar das rubiáceas de Santa Catarina; Sellowia 7, 13-86.

Velo, G. P., Dunn, C. J., Giroud, J. P., Timsit, J., Willoughby, D. A. 1973. Distribution of prostaglandins in inflammatory exudate. The Journal of Pathology 111, 149–158.

7 CONSIDERAÇÕES FINAIS

De acordo com dados obtidos no manuscrito 1, *P. carthagenensis* e *P. capillacea* foram encontrados para ser efetivos antioxidantes no ensaio diferente *in vitro* incluindo radical DPPH, ABTS radical e β-caroteno atividades de branqueamento, quando são comparados com compostos antioxidantes convencionais, tais como BHT, ácido gálico, quercetina e ácido ascórbico. Os resultados indicam que a atividade antioxidante para estas plantas podem ser atribuído à presença do ácido p-cumárico. Além disso, estudos mais detalhados, relativos à composição química destes extratos, bem como estudos com outros modelos, tais ensaios *in vivo*, são essenciais para caracterizá-los como antioxidantes biológicos.

No manuscrito 2, o composto PL-1 (vincosamida) é descrito pela primeira vez em *P. leiocarpa*, no entanto, a presença deste composto foi relatada em espécies que pertencem ao gênero *Psychotria*. Os resultados demonstraram efeitos nos ensaios antiproliferativos, anticolinesterasicos e anti-inflamatórios de *P. leiocarpa* e vincosamida nos modelos experimentais testados. Estudos futuros de *P. leiocarpa* (EMPL) e vincosamida serão realizados para tratamento crônico na atividade da acetilcolinesterase e BuChE no cérebro de ratos, e também de encaixe molecular, juntamente com a vincosamida para avaliar sua interação.

8 ANEXOS

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 22 de fevereiro de 2016.

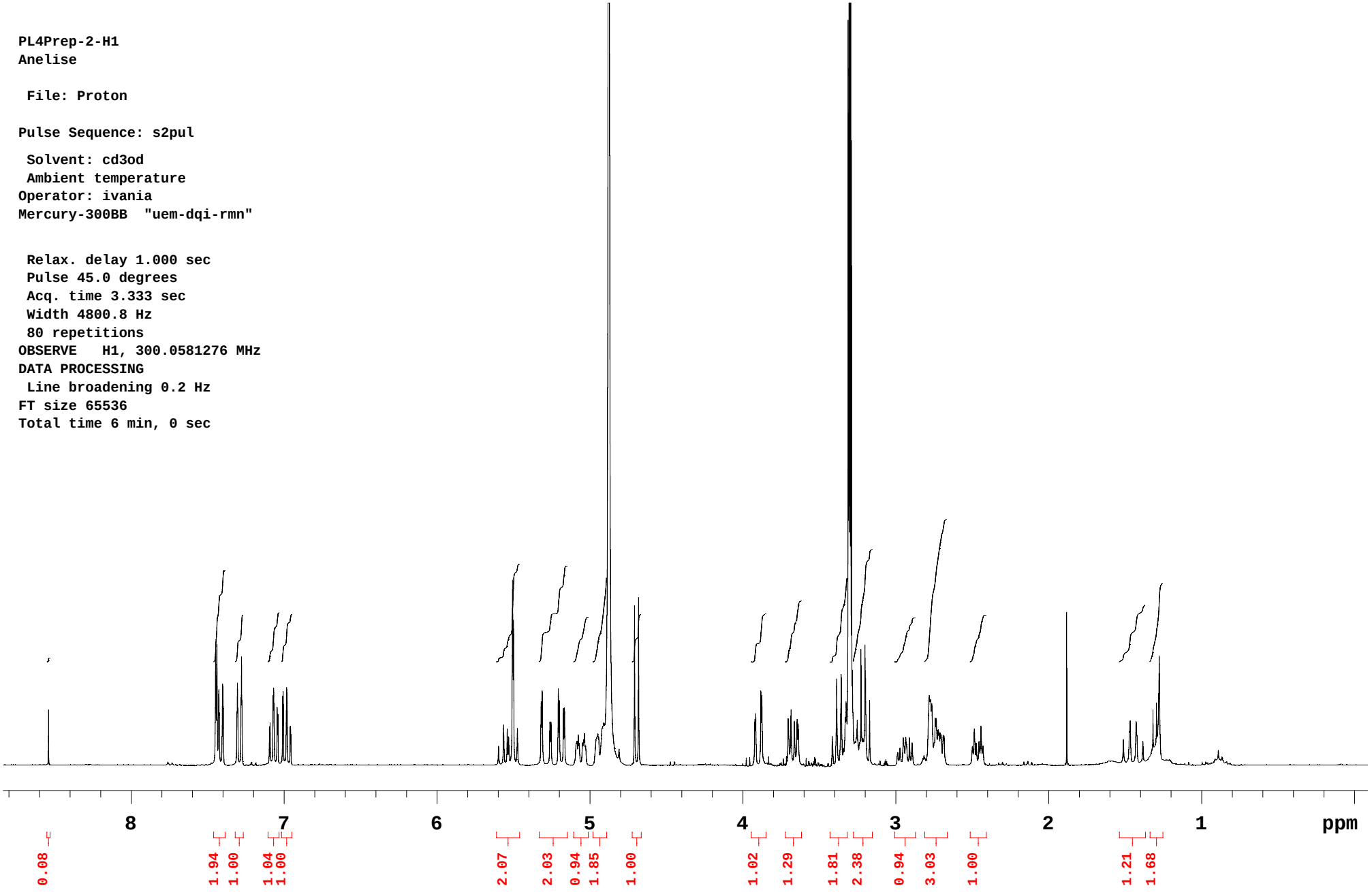
CERTIFICADO

Certificamos que o projeto intitulado **"Avaliação da atividade antitumoral, anticolinesterásica, anti-inflamatória e toxicidade de espécies de Psychotria (Rubiaceae)",** protocolo nº 14/2015, sob responsabilidade de Anelise Samara Nazari Formagio – 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 11 de dezembro de 2015.

Vigência do Projeto	*07/03/2016 – 15/04/2016*
Espécie/linhagem	*Rattus norvegicus/* Wistar e *Mus musculus/* Swiss
Nº de animais	195
Peso/idade	200-300 g/ 2-3 meses 25-30 g/ 45 dias
Sexo	87 Machos e 24 Fêmeas / 84 Machos
Origem	Biotério da Faculdade de Ciências da Saúde-FCS/UFGD

Melissa Negrão Sepulvida

Melissa Negrão Sepulvida
Coordenadora CEUA


PL4Prep-2-H1
Anelise
File: Proton
Pulse Sequence: s2pul
Solvent: cd3od
Ambient temperature
Operator: ivania
Mercury-300BB "uem-dqi-rmn"
Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 3.333 sec
Width 4800.8 Hz
80 repetitions
OBSERVE H1, 300.0581276 MHz
DATA PROCESSING
Line broadening 0.2 Hz
FT size 65536
Total time 6 min, 0 sec
8
7
6
5
4
3
2
1
ppm
0.08
1.94
1.00
1.04
1.00
2.07
2.03
0.94
1.85
1.00
1.02
1.29
1.81
2.38
0.94
3.03
1.00
1.21
1.68

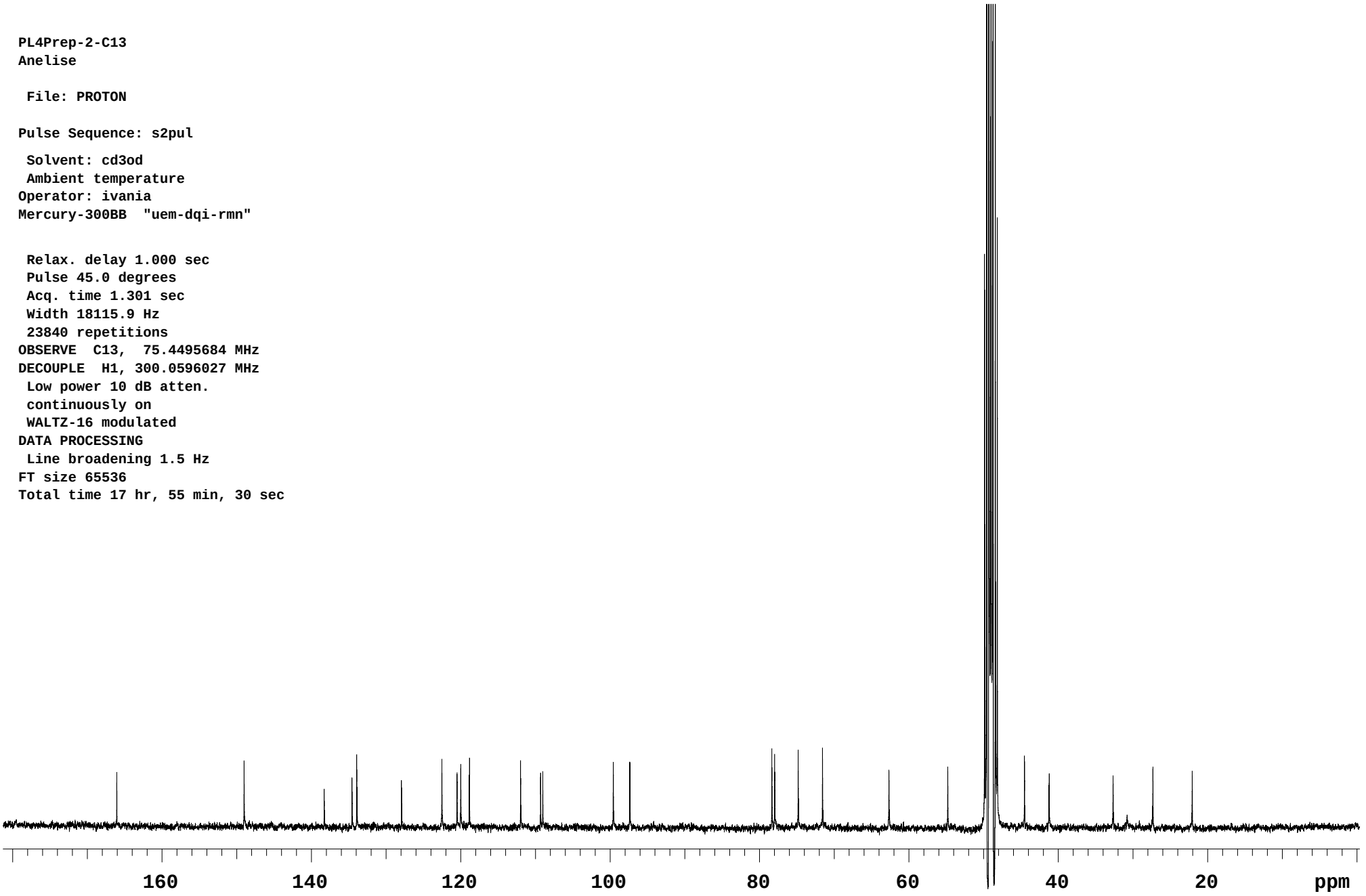
PL4Prep-2-C13
Anelise

File: PROTON

Pulse Sequence: s2pul

Solvent: cd3od
Ambient temperature
Operator: ivania
Mercury-300BB "uem-dqi-rmn"

Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 1.301 sec
Width 18115.9 Hz
23840 repetitions
OBSERVE C13, 75.4495684 MHz
DECOUPLE H1, 300.0596027 MHz
Low power 10 dB atten.
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.5 Hz
FT size 65536
Total time 17 hr, 55 min, 30 sec



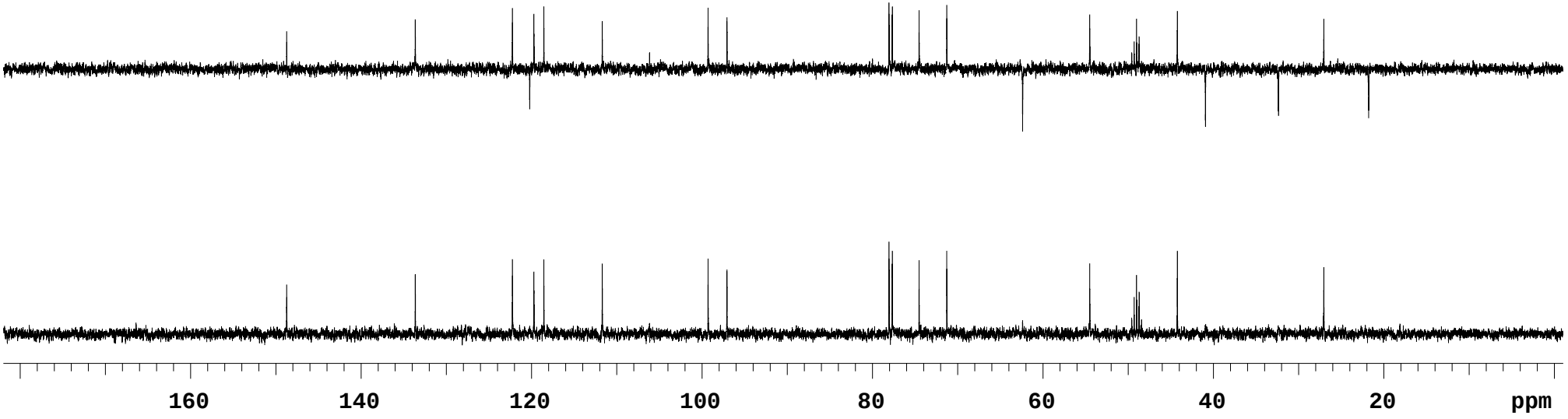
PL4Prep-2-DEPT
Anelise

File: PROTON

Pulse Sequence: DEPT

Solvent: cd3od
Ambient temperature
Operator: ivania
Mercury-300BB "uem-dqi-rmn"

Relax. delay 1.000 sec
Pulse 90.0 degrees
Acq. time 1.000 sec
Width 18115.9 Hz
5120 repetitions
OBSERVE C13, 75.4495899 MHz
DECOUPLE H1, 300.0596027 MHz
Low power 10 dB atten.
on during acquisition
off during delay
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 65536
Total time 6 hr, 14 min, 33 sec



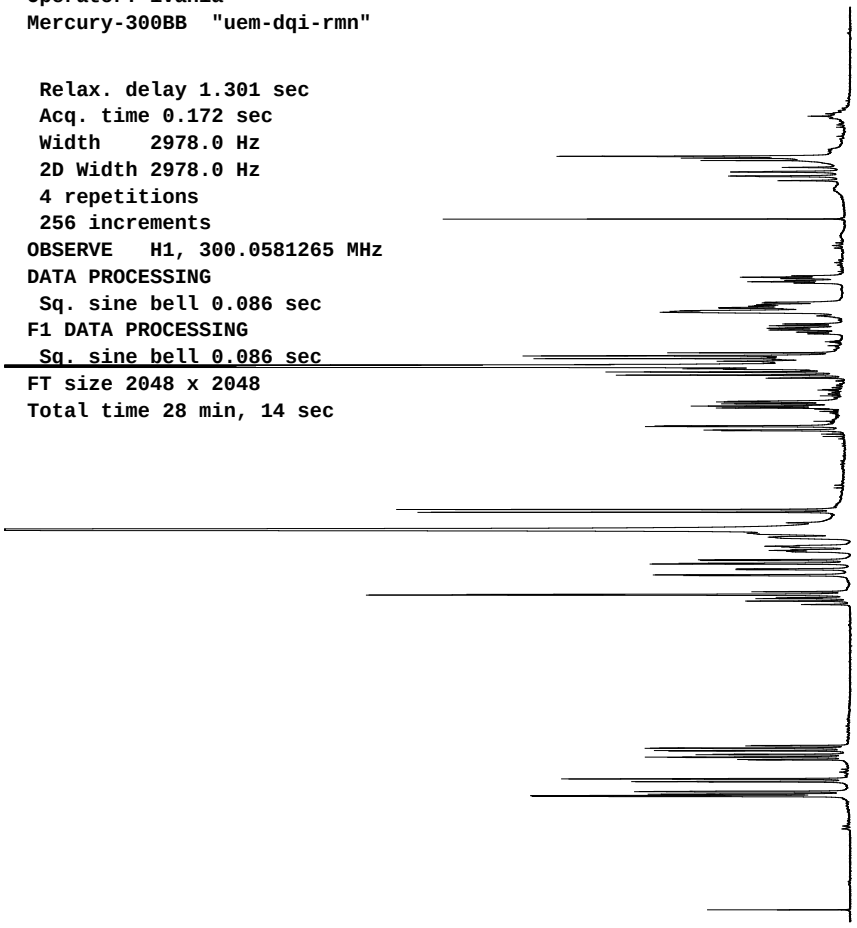
PL4Prep-2-gCOSY
Anelise

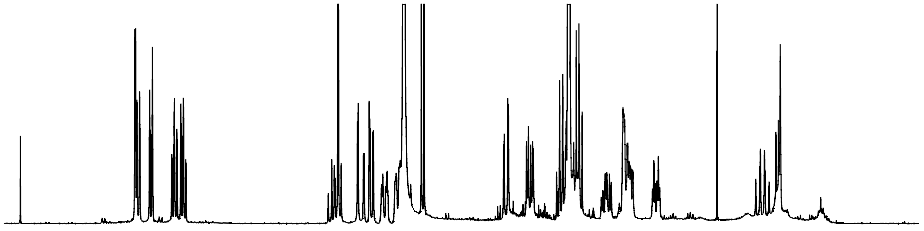
File: PROTON

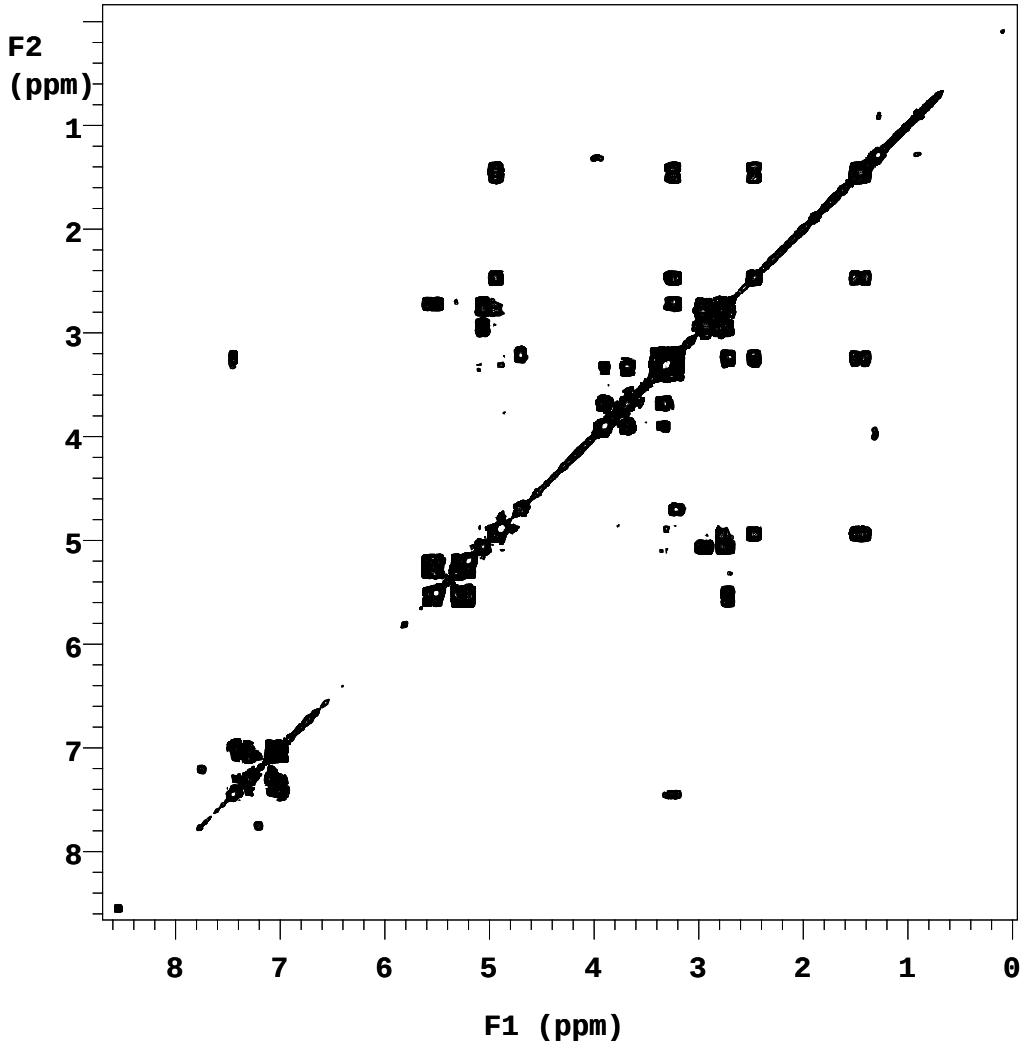
Pulse Sequence: gCOSY

Solvent: cd3od
Ambient temperature
Operator: ivania
Mercury-300BB "uem-dqi-rmn"

Relax. delay 1.301 sec
Acq. time 0.172 sec
Width 2978.0 Hz
2D Width 2978.0 Hz
4 repetitions
256 increments
OBSERVE H1, 300.0581265 MHz
DATA PROCESSING
Sq. sine bell 0.086 sec
F1 DATA PROCESSING
Sq. sine bell 0.086 sec
FT size 2048 x 2048
Total time 28 min, 14 sec







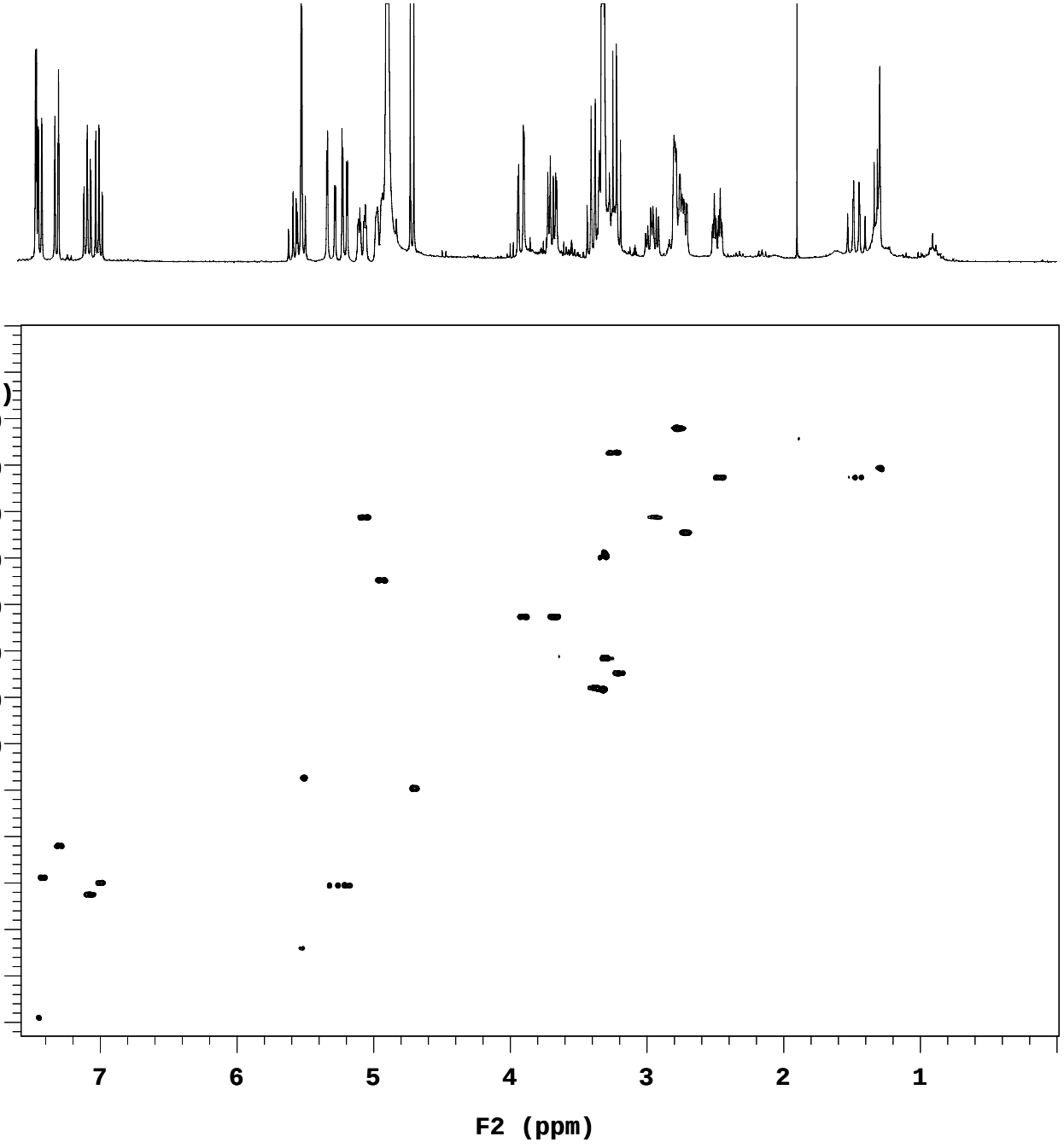
PL4Prep-2-gHSQC
Anelise

File: PROTON

Pulse Sequence: gHSQC

Solvent: cd3od
Ambient temperature
Operator: ivania
Mercury-300BB "uem-dqi-rmn"

Relax. delay 1.301 sec
Acq. time 0.199 sec
Width 3046.0 Hz
2D Width 12826.7 Hz
20 repetitions
2 x 128 increments
OBSERVE H1, 300.0581265 MHz
DECOUPLE C13, 75.4553346 MHz
Low power 10 dB atten.
on during acquisition
off during delay
GARP-1 modulated
DATA PROCESSING
Gauss apodization 0.092 sec
F1 DATA PROCESSING
Gauss apodization 0.009 sec
FT size 2048 x 2048
Total time 2 hr, 22 min, 16 sec



PL4Prep-2-gHMBC
Anelise

File: PL4Prep-2-gHMBC

Pulse Sequence: gHMBC

Solvent: cd3od
Ambient temperature
Operator: ivania
File: PL4Prep-2-gHMBC
Mercury-300BB "uem-dqi-rmn"

Relax. delay 1.500 sec
Mixing 0.080 sec
Acq. time 0.128 sec
Width 3046.0 Hz
2D Width 18107.7 Hz
80 repetitions
200 increments
OBSERVE H1, 300.0581265 MHz
DATA PROCESSING
Sine bell 0.064 sec
F1 DATA PROCESSING
Sine bell 0.011 sec
FT size 2048 x 2048
Total time 7 hr, 49 min, 18 sec