UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
FACULDADE DE CIÊNCIAS DA SAÚDE
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Caracterização química e potencial farmacológico de espécies de *Psychotria* e modelagem molecular de substâncias majoritárias

CARLA ROBERTA FERREIRA VOLOBUFF

Dourados – MS
2019

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o meu refúgio, a minha fortaleza, e
nele confiarei".

(Salmo 91:2)

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LISTA DE ABREVIATURAS

786-0	Rim
AcetilCoA	Acetil coenzima A
ACh	Acetilcolina
AChE	Acetilcolinesterase
AIEs	Anti-inflamatórios esteroidais
AINEs	Anti-inflamatórios não-esteroidais
BHT	Butil-hidroxitolueno
BuChE	Butirilcolinesterase
CC	Coluna cromatográfica
CG-EM	Cromatografia gasosa acoplada a espectrometria de massa
ChAT	Colina acetiltransferase
COX-1	Ciclooxigenase 1
COX-2	Ciclooxigenase 2
DA	Doença de Alzheimer
DPPH	2,2-difenil-1-picrilhidrazilo
DTNB	Ácido 5,5-ditiobis-2-nitrobenzoico
EOPP	Óleo essencial de *Psychotria poeppigiana*
EROs	Espécies Reativas de Oxigênio
GI_{50}	Concentração para 50% da inibição máxima da proliferação celular
HaCaT	Queratinócitos
HPLC	High performance liquid chromatography / Cromatografia líquida de alta eficiência
HT29	Cólon
IAChE	Inibidor da acetilcolinesterase
IL	Interleucina
IR	Índice de retenção
K-562	Leucemia
mAChE	Acetilcolinesterase de *Mus musculus*
MAO-A	Monoamina oxidase A
MAO-B	Monoamina oxidase B

MCF-7	Mama
MDA	Malondialdeído
MEPB	*Psychotria brachybotrya*
MEPC	*Psychotria capillacea*
MEPCA	*Psychotria carthagenensis*
MEPCR	*Palicourea crocea*
MEPD	*Psychotria deflexa*
MEPL / ME-PL	*Psychotria leiocarpa*
MEPP	*Psychotria poeppigiana*
MIC	Concentração inibitória mínima
NCI-ADR/RES	Ovário multirresistente
NCI-H460	Pulmão
OVCAR-3	Ovário
PC-3	Próstata
PL-1	Vincosamida
TBA	Ácido tiobarbitúrico
TLRs	Receptores Toll-like
U251	Glioma
UHPLC-HRMS/MS	*Ultra-high pressure liquid chromatography-high resolution mass spectrometry* / Cromatografia líquida de alta pressão acoplada a espectrometria de massa de alta resolução

Caracterização química e potencial farmacológico de espécies de *Psychotria* e modelagem molecular de substâncias majoritárias

RESUMO

Espécies de *Psychotria* (Rubiaceae) são utilizadas na medicina popular para tratamento de doenças inflamatórias e distúrbios no sistema nervoso central, caracterizados quimicamente pela presença de uma variedade de metabólitos secundários. Além disso, várias espécies deste gênero tem ocorrência em Mato Grosso do Sul. Neste sentido, o objetivo deste trabalho foi realizar a caracterização química e potencial farmacológico de espécies de *Psychotria* e modelagem molecular de substâncias majoritárias. **Artigo 1**: Avaliar o efeito antiproliferativo e anticolinesterásico do extrato metanólico de *Psychotria deflexa, P. carthagenensis, P. leiocarpa, P. capillacea, P. poeppigiana, P. brachybotrya* e *Palicourea crocea* e quantificação de alcaloides. Os alcaloides foram quantificados espectrofotometricamente e apresentaram valores entre 47.6 a 21.9 µg/g. O extrato metanólico de *P. leiocarpa* apresentou inibição do crescimento celular frente as células de ovário (GI_{50}: 3.28 µg/mL), leucemia (GI_{50}: 5.26 µg/mL), queratinócitos (não-tumoral) (GI_{50}: 27.20 µg/mL), próstata (GI_{50}: 34.92 µg/mL), mama (GI_{50}: 35.80 µg/mL). *P. capillacea* apresentou frente a ovário (GI_{50}: 2.33 µg/mL) e glioma (GI_{50}: 16.66 µg/mL). A inibição da acetilcolinesterase foi significativa no hipocampo para os extratos de *P. deflexa* (57%), *P. brachybotrya* (50%) e *P. leiocarpa* (40%), seguidos de *P. poeppigiana* e *P. capillacea* (ambas com 21%), quando comparados ao controle. **Artigo 2:** Avaliar o efeito anti-inflamatório e inibição da acetilcolinesterase do extrato metanólico e vincosamida de *Psychotria leiocarpa*. Do extrato metanólico das folhas de *P. leiocarpa* isolou-se o alcaloide vincosamida. O extrato e alcaloide foram submetidos à avaliação da atividade anti-inflamatória, anti-acetilcolinesterase e docagem molecular. O extrato metanólico foi submetido à toxicidade aguda, não evidenciou sinais de toxicidade aos animais tratados. O extrato metanólico (30, 100 e 300 mg/kg) e vincosamida (3, 30 e 100 mg/kg) demonstraram inibição significativa quando avaliada nos modelos de edema de pata, pleurisia e hiperalgesia térmica e mecânica. A atividade anti-acetilcolinesterase *in vitro* do extrato metanólico (30 e 100 mg/kg) e vincosamida (30 mg/kg) foi significativa no córtex frontal. O acoplamento molecular de vincosamida demonstrou interações significativas com o sítio catalítico e periférico da enzima acetilcolinesterase, corroborando com a atividade no ensaio de inibição. **Artigo 3:** Realizar a composição química, avaliar a atividade antioxidante, inibição da

acetilcolinesterase e realizar a docagem molecular dos sesquiterpenos majoritários do óleo essencial de *Psychotria poeppigiana*. Foram identificadas 19 substâncias, com predominância de sesquiterpenos, sendo germacreno D (29,38%) e biciclogermacreno (25,21%) os majoritários. O óleo essencial apresentou inibição na lipoperoxidação (IC_{50} = 12,78 ± 1,36 µg/mL), quando comparado ao controle BHT (IC_{50} = 38,71 ± 3,22 µg/mL). A inibição da acetilcolinesterase realizada em quatro estruturas cerebrais de ratos *Wistar*, foi significativa no hipocampo (81,50%), córtex cerebral (70,0%) e hipotálamo (55,88%). A modelagem molecular enzima-ligante, evidenciou que os principais constituintes do óleo podem interagir nos sítios ativos catalítico e periférico da enzima. **Artigo 4:** Avaliar as atividades antioxidante, acetilcolinesterase, antinociceptiva e quantificar os alcaloides do extrato metanólico das folhas de *P. poeppigiana*. O cromatograma de íons totais do extrato metanólico foi analisado por CL-EM/EM e evidenciou dois alcaloides (calycanthine e hodgkinsine), uma cumarina (escopoletina), um iridoide (asperulosídeo) e dois terpenos (vomifoliol e loliolide). Elevados teores de fenóis, flavonoides, flavonóis e taninos condensados foram encontrados no extrato. Foi evidenciado significativa atividade antioxidante no teste de DPPH, β-caroteno e MDA. O extrato metanólico (30 e 100 mg/kg) inibiu a acetilcolinesterase *in vivo* no hipocampo, córtex cerebral, hipotálamo e estriado quando comparado ao controle. Ressalta-se que na literatura existem poucos relatos quanto aos estudos químicos e biológicos para as espécies em questão. Neste sentido, este estudo corrobora com os dados da literatura e uso popular descritos para a família e gênero com relação aos constituintes químicos e atividades biológicas relatados.

Palavras-chave: Inflamação, anti-acetilcolinesterásicos, alcaloides, sesquiterpenos, docagem molecular

Chemical characterization and pharmacological potential of *Psychotria* species and molecular modeling of major substances

ABSTRACT

Psychotria (Rubiaceae) species are used in folk medicine to treat inflammatory diseases and disorders of the central nervous system, chemically characterized by the presence of a variety of secondary metabolites. In addition, several species of this genus occur in Mato Grosso do Sul. In this sense, the objective of this work was to perform the chemical characterization and pharmacological potential of *Psychotria* species and molecular modeling of major substances. **Article 1:** To evaluate the antiproliferative and anticholinesterase effect of the methanolic extract of *Psychotria deflexa, P. carthagenensis, P. leiocarpa, P. capillacea, P. poeppigiana, P. brachybotrya* and *Palicourea crocea* and quantification of alkaloids. Alkaloids were quantified spectrophotometrically and showed values between 47.6 to 21.9 µg/g. *P. leiocarpa* methanolic extract showed inhibition of cell growth against ovarian cells (GI_{50}: 3.28 µg/mL), leukemia (GI_{50}: 5.26 µg/mL), non-tumor keratinocytes (GI_{50}: 27.20 µg/mL), prostate (GI_{50}: 34.92 µg/mL), breast (GI_{50}: 35.80 µg/mL). *P. capillacea* presented ovary (GI_{50}: 2.33 µg/mL) and glioma (GI_{50}: 16.66 µg/mL). Acetylcholinesterase inhibition was significant in the hippocampus for *P. deflexa* (57%), *P. brachybotrya* (50%) and *P. leiocarpa* (40%) extracts, followed by *P. poeppigiana* and *P. capillacea* (both 21%) when compared to the control. **Article 2:** To evaluate the inflammatory effect and acetylcholinesterase inhibition of methanolic extract and vincosamide from *Psychotria leiocarpa*. The methanolic extract of *P. leiocarpa* leaves resulted in the isolation of the alkaloid vincosamide, which were evaluated of anti-inflammatory, anti-acetylcholinesterase activity and molecular docking. Oral administration of the methanolic extract showed no signs of toxicity to the treated animals. The anti-inflammatory activity of methanolic extract (30, 100 and 300 mg/kg) and vincosamide (3, 30 and 100 mg/kg) showed significant inhibition when evaluated in paw edema, pleurisy and termal and mechanical hyperalgesic models. The anti-acetylcholinesterase activity of methanolic extract (30 and 100 mg/kg) and vincosamide (30 mg/kg) *in vitro* was significant in the frontal cortex. The molecular coupling of vincosamide demonstrated significant interactions with the catalytic and peripheral acetylcholinesterase enzyme site, corroborating the activity in the inhibition assay. **Article 3:** Carry out chemical composition, evaluate antioxidant activity, acetylcholinesterase inhibition and molecularly screen the major sesquiterpenes of *Psychotria*

poeppigiana essential oil. Nineteen substances were identified, with predominance of sesquiterpenes, being germacrene D (29.38%) and bicyclogermacrene (25.21%) the majority. The essential oil showed a reduction in malondialdehyde generation (IC_{50} = 12.78 ± 1.36 µg/mL) when compared to the BHT control (IC_{50} = 38.71 ± 3.22 µg/mL). Acetylcholinesterase inhibition performed in four brain structures of *Wistar* rats was pronounced in the hippocampus (81.50%), cerebral cortex (70.0%) and hypothalamus (55.88%). Enzyme-binder molecular modeling showed that the main constituents of the oil can interact in the catalytic and peripheral active sites of the enzyme. **Article 4:** To evaluate the inhibition of antioxidant, acetylcholinesterase, antinociceptive activities and quantify the alkaloids of the methanolic extract of *P. poeppigiana* leaves. The total ion chromatogram of the methanolic extract was analyzed by UHPLC-HRMS/MS and showed two alkaloids (calycanthine and hodgkinsine), one coumarin (scopoletin), one iridoid (asperuloside) and two terpenes (vomifoliol and loliolide). High levels of phenols, flavonoids, flavonols and condensed tannins were found in the extract. Significant antioxidant activity was evidenced in the DPPH, β-carotene and MDA test. Methanolic extract (30 and 100 mg/kg) inhibited acetylcholinesterase *in vivo* in the hippocampus, cerebral cortex, hypothalamus and striatum when compared to the control. It is noteworthy that there are few reports in the literature regarding chemical and biological studies for the species in question. In this sense, this study corroborates the literature and popular use data described for the family and gender regarding the chemical constituents and biological activities reported.

Keywords: Inflammation, anti-acetylcholinesterase, alkaloids, sesquiterpenes, molecular docking

SUMÁRIO

1 INTRODUÇÃO

Produtos naturais exercem uma influência benéfica sobre a saúde humana e assim são utilizados como adjuvantes terapêuticos, desde a antiguidade, incentivando cada vez mais, a pesquisa de produtos naturais. O interesse por produtos obtidos de plantas também ocorre por outras razões como o uso abusivo ou incorreto de medicamentos sintéticos e falta de acesso de uma grande porcentagem da população mundial ao tratamento farmacológico convencional (MAHESH; SATISH, 2008). Neste sentido, a busca por opções terapêuticas para diferentes doenças faz da pesquisa de produtos naturais um campo fértil em opções de moléculas com diferentes atividades biológicas. Estudos mostram que 64% dos medicamentos disponíveis são oriundos de produtos naturais (VALLI *et al*., 2013), tais como a aspirina® (obtido a partir da salicilina, a qual foi isolada de *Salix alba*) e morfina (isolado de *Papaver somniferum),* ambos apresentando ação analgésica (VEIGA JUNIOR; PINTO; MACIEL, 2005). Apesar do grande número de trabalhos publicados em relação a estudos fitoquímicos e farmacológicos, ainda é pouco explorada a utilização do potencial de plantas superiores, em relação ao número de espécies relatadas no Brasil, como uma fonte de novos fármacos (MAHESH; SATISH, 2008; NEWMAN; CRAGG, 2016).

Nesse sentido, nosso grupo de pesquisa tem desenvolvido o estudo do potencial químico e biológico de plantas superiores presentes no Cerrado e Mata Atlântica na região de Dourados-MS. Das várias famílias presentes nesta região, destaca-se Rubiaceae, por sua ampla distribuição e número de gêneros e espécies ocorrentes. Pereira e Kinoshita (2013), realizaram um estudo sobre florística, sistema reprodutivo, distribuição e relações alométricas de espécies desta família em Mato Grosso do Sul, sendo o gênero *Psychotria* um dos mais representativos com dez espécies registradas. *Psychotria* é quimicamente caracterizado como fonte de alcaloides indólicos monoterpênicos (VAN DE SANTOS *et al.,* 2001; FARIAS *et al*., 2010; FRAGOSO *et al*., 2008; HENRIQUES *et al*., 2004; KERBER *et al*., 2014; MAGEDANS *et al*., 2017) e estão correlacionados principalmente às atividades antioxidante, anti-inflamatória e atuantes no sistema nervoso central (FARIAS *et al*., 2010; FRAGOSO *et al*., 2008; KLEIN-JÚNIOR *et al*., 2016)

Neste contexto, o objetivo deste trabalho foi realizar a avaliação farmacológica e o estudo fitoquímico de espécies de *Psychotria* relatadas em Dourados-MS, com estudos multidisciplinares envolvendo a etnobotânica, a química e a farmacologia para viabilizar o conhecimento do uso terapêutico dessas plantas (**Figura 1)**.

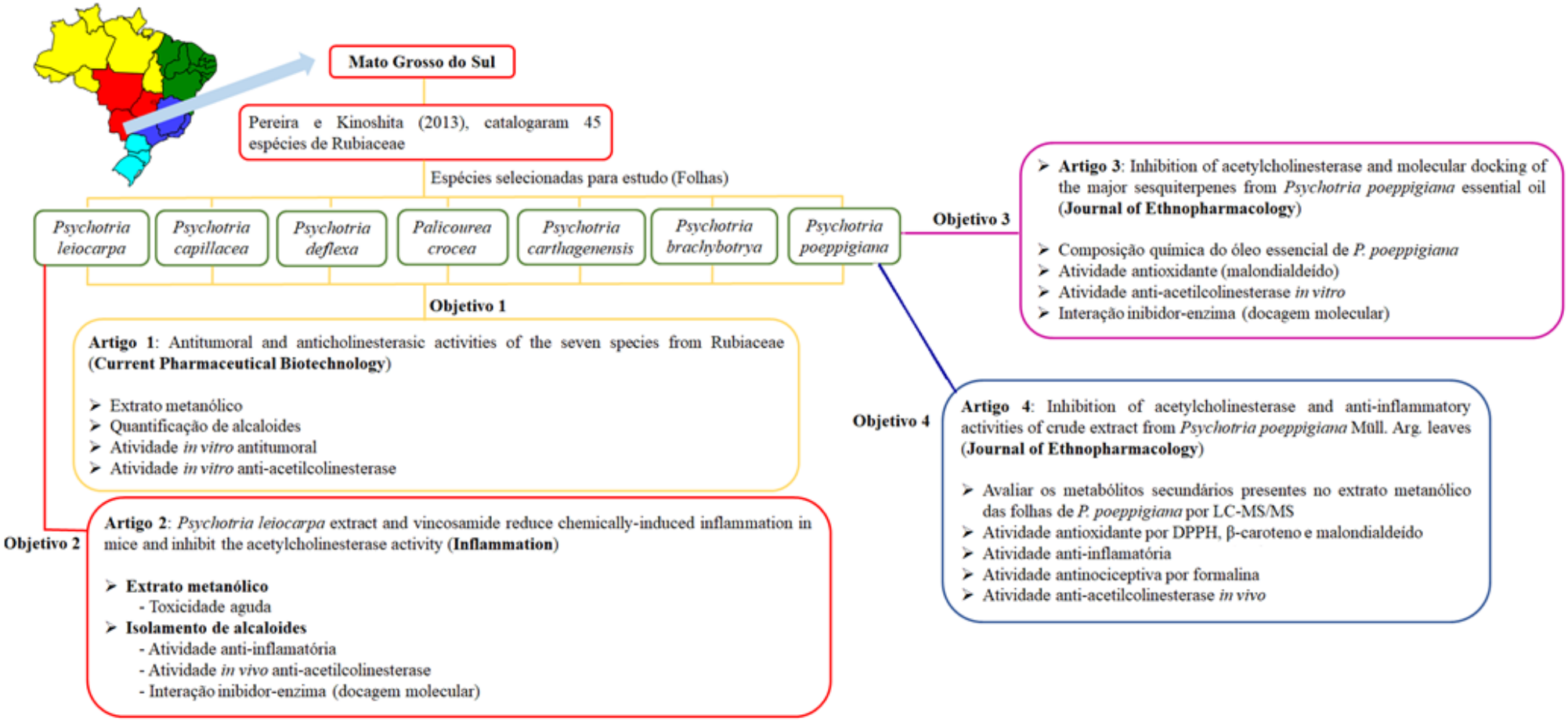


Figura 1. Proposta de trabalho com espécies de Rubiaceae coletadas em Mato Grosso do Sul.

2 REVISÃO DE LITERATURA

2.1 Rubiaceae – aspectos gerais

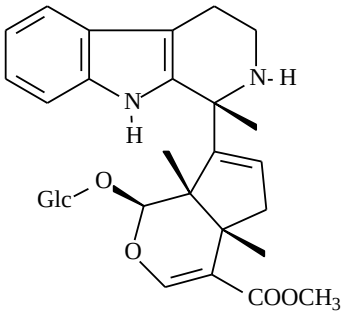
A família Rubiaceae é constituída por cerca de 650 gêneros e aproximadamente 13000 espécies (ROCHA SILVA; CHOZE, 2013), subdividida em três subfamílias: Cinchonoideae, Ixoroideae e Rubioideae, compreendendo cerca de 30 tribos (BREMER; ANDREASEN; OLSSON, 1995; ROVA *et al.*, 2002). No Brasil, encontram-se cerca de 125 gêneros e 1392 espécies (TORRES-LEITE; HOLUNDER; GARBIN; CARRIJO, 2018). Espécies desta família são amplamente utilizadas na medicina popular e na fabricação de fitofármacos e fitoterápicos, como por exemplo, *Uncaria tomentosa* (Willd.) DC. conhecida popularmente como unha de gato (comercializada como Unha de Gato®), utilizada para tratamento de processos inflamatórios (MONTSERRAT-DE LA PAZ *et al*., 2015). Diversas atividades biológicas são relatadas para esta família, como antinociceptiva, anti-inflamatória, antidiabética e efeitos no sistema nervoso central (AGUILAR *et al.*, 2002; DÉCIGA-CAMPOS *et al*., 2006; FARIAS *et al*., 2012; GUERRERO-ANALCO *et al*., 2007; TAÏWE *et al*., 2014; ZHU *et al*., 2012). Estudos fitoquímicos demonstram a presença de iridoides (MOURA, 2006), alcaloides (HENRIQUES *et al*., 2004), antraquinonas (LING *et al*., 2002), lignanas (SILVA *et al*., 2006), flavonoides, derivados fenólicos, triterpenos, diterpenos e cumarinas (LUCIANO *et al*., 2004).

2.2 Gênero *Psychotria* L.

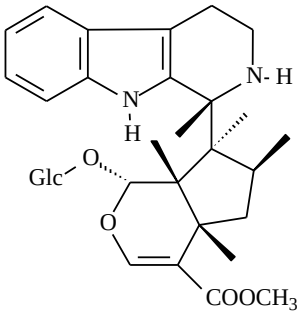
Psychotria pertencente à subfamília Rubioideae e tribo Psychotrieae, engloba cerca de 1650 espécies amplamente distribuídos em matas tropicais (NEPOKROEFF *et al*., 1999; LOPES *et al*., 2004). Taxonomicamente complexo, este gênero apresenta grandes similaridades morfológicas com *Palicourea* e *Cephaelis*, por este motivo, sua classificação tem sido motivo de controvérsias. Com isso, o conhecimento quimiotaxonômico, com base nos seus constituintes químicos isolados, torna-se uma ferramenta importante para sua diferenciação (FARIAS, 2006). De acordo com estudos anteriores, os alcaloides são a principal classe de metabólitos secundários isolados de *Psychotria*, destacando os alcaloides indólicos monoterpênicos em maior abundância, cujo aminoácido de origem é o triptofano e um único precursor a estrictosidina produzida pela condensação de uma molécula de triptamina com a secologanina, resultante da via do geraniol, a partir de moléculas do ácido mevalônico (BRUNETON, 1991).

Investigações fitoquímicas realizadas em *Psychotria*, levaram à identificação de vários alcaloides, com diversas atividades farmacológicas. Psicolatina ou umbelatina (**1**) (**Figura 2**) isolada de *Psychotria umbellata*, exibiu efeitos analgésicos moderados em resposta a estímulos algogênicos (BOTH *et al*., 2002a), efeito ansiolítico, antidepressivos e amnésicos em modelos de camundongos (BOTH *et al*., 2005; BOTH *et al*., 2006). O extrato alcaloidal de *P. myriantha* apresentou efeito analgésico dose-dependente parcialmente revertido por naloxona no modelo de placa quente (BOTH *et al*., 2002b). Das folhas, também foi isolado o ácido estrictosidínico (**2**) capaz de inibir a quimiotaxia de leucócitos polimorfonucleares *in vitro* e atividade analgésica e antipirética após a administração oral, além do isolamento de myriantosinas A (**3**) e B (**4**). Frações de alcaloides obtidos das folhas *P. suterella e P. laciniata*, foram capazes de inibir a monoamina oxidase A do cérebro de ratos, e a análise química revelou a presença dos alcaloides E/Z-vallesiachotamina (**5, 6**) (PASSOS *et al*., 2013). Outro alcaloide isolado foi a emetina (**7**) de *P. ipecacuanha*, demonstrando ser indutor de apoptose em células de leucemia (MÖLLER *et al*., 2007).

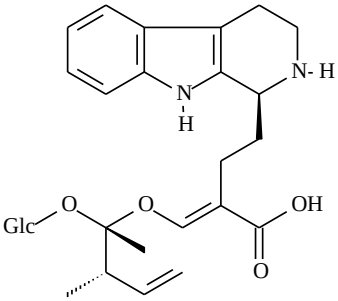
Estudo realizado a partir das folhas e flores de *P. colorata*, identificaram a presença de alcaloides pirrolindinoindolínicos como hodkinsina (**8**), quimonantina (**9**), quadrigemina C (**10**) e psicotridina (**11**) e alcaloides quinolínicos calicantina (**12**) e isocalicantina (**13**). Hodgkinsina demonstrou atividade analgésica com resultados semelhantes aos da morfina em estudos *in vivo* e *in vitro*, atuando por meio de receptores opioides e do antagonismo de receptores glutamatérgicos N-Metil-D-Aspartato (NMDA) (AMADOR *et al*., 2000; KODANKO *et al*., 2007), enquanto que a quimonantina apresentou atividade analgésica semelhante à hodkinsina (VEROTTA *et al*., 2002). Psicotridina demonstrou atividade analgésica dose dependente em testes de retirada de cauda em camundongos (AMADOR *et al*., 2001). De *P. viridis* foi isolado *N*,*N*-dimetiltriptamina (**14**) e estudos relatam potencial atuação no sistema nervoso central (ALMEIDA; SILVA; ASSIS, 2018), além disso é conhecida por sua ação alucinógena serotoninérgica, sendo um dos componentes das plantas utilizadas na preparação de bebidas ritualísticas, como “ayahuasca” e “vinho de Jurema”, usadas em cerimônias de seitas religiosas como Santo Daime, União do Vegetal, dentre outras (SIMÕES *et al*., 2017).



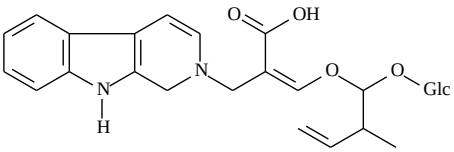
(**1**)



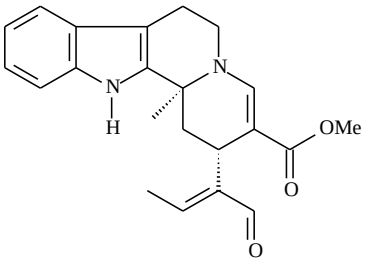
(**2**)



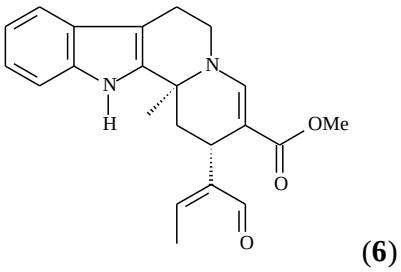
(**3**)



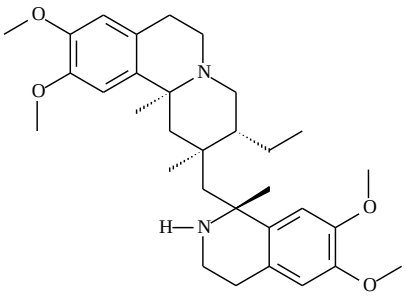
(**4**)



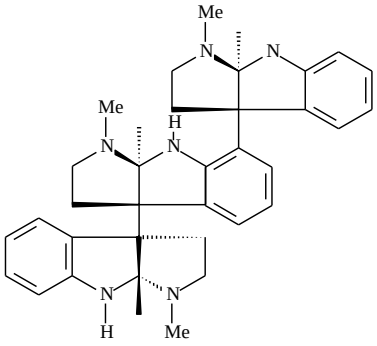
(**5**)



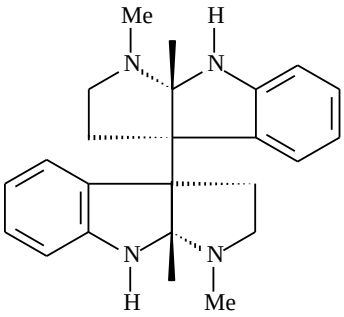
(**6**)



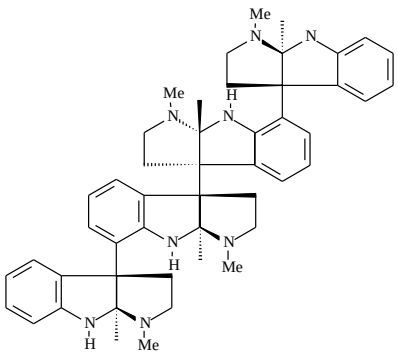
(**7**)



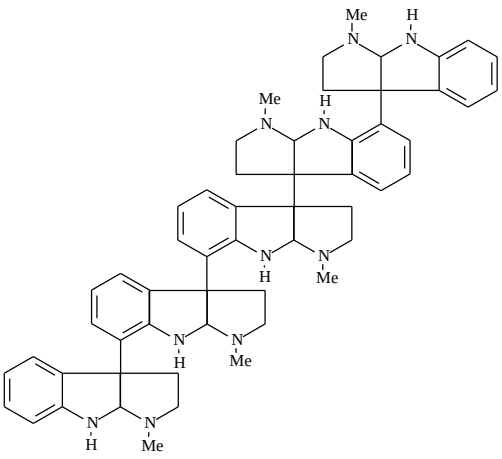
(**8**)



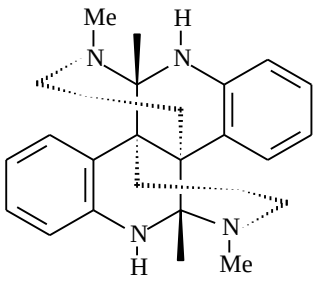
(**9**)



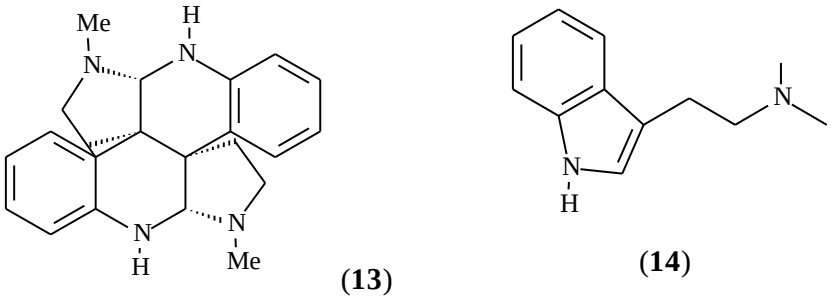
(**10**)



(**11**)



(**12**)



(**13**) (**14**)

Figura 2. Substâncias encontradas em espécies de *Psychotria*.

2.2.1 *Palicourea crocea* (Sw.) Schult.

Popularmente conhecida como "palicourea vermelha e/ou amarela", “douradinha” (**Figura 3**) é um arbusto que pode ser encontrado na América Central e América do Sul. No Brasil, tem ocorrência em quase todo o território nacional (ANDERSSON, 1992) e devido a ausência de monofluocerato de sódio, não é considerada tóxica (ANDRADE; MATOS, 1968; PEIXOTO *et al.*, 1987; PEREIRA *et al.*, 2003). Estudos químicos realizados com o extrato metanólico das folhas, relatam a presença de alcaloides como croceaina A (**15**) e B (**16**), psicolatina (**17**), braquicerina (**18**) e palicroceína (**19**) (**Figura 4**) (BERGER *et al.*, 2015; DUSMAN *et al.*, 2004; NARINE; MAXWELL, 2009). O extrato metanólico das folhas e croceaina A apresentaram efeito antiedematogênico, redução da hiperalgesia e inibiu a migração leucocitária. Além disso, no extrato metanólico foi evidenciado altas concentrações de compostos fenólicos e a presença de ácido cafeico, ácido ferúlico, rutina e quercetina (FORMAGIO *et al.*, 2019).

Figura 3. Folhas e inflorescência de *Palicourea crocea* (FONTE: NMNH - Botany Dept. 2018).

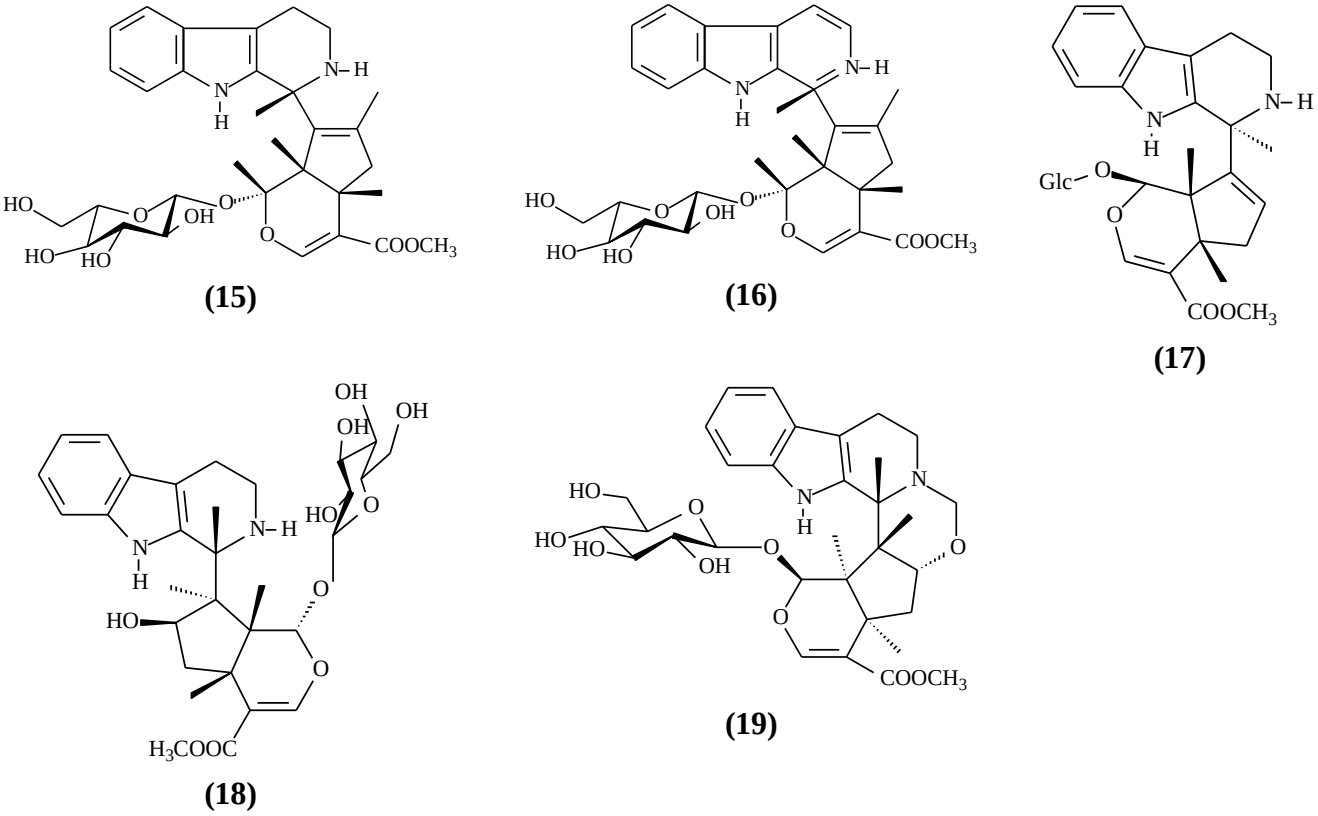


Figura 4. Alcaloides indólicos monoterpênicos isolados de *P. crocea*.

2.2.2 *Psychotria brachybotrya* Müll. Arg.

Psychotria brachybotrya (**Figura 5**) é um arbusto de ocorrência em Minas Gerais e Mato Grosso do Sul (LOMBARDI; GONÇALVES, 2000). A partir do extrato metanólico das partes aéreas desta espécie, foram isolados os alcaloides bufotenina (**20**), brachybotryne (**21**) e seu derivado *N*-óxido (**22**) (**Figura 6**) (RIBEIRO *et al.*, 2016). O extrato etanólico das folhas foi avaliado contra *Mycobacterium tuberculosis*, com concentração inibitória mínima (MIC) > 250 μg/mL (ARAÚJO *et al.*, 2014).

Figura 5. Folhas de *P. brachybotrya* (sinonímia: *P. gracilenta*) (Fonte: ZAPPI).

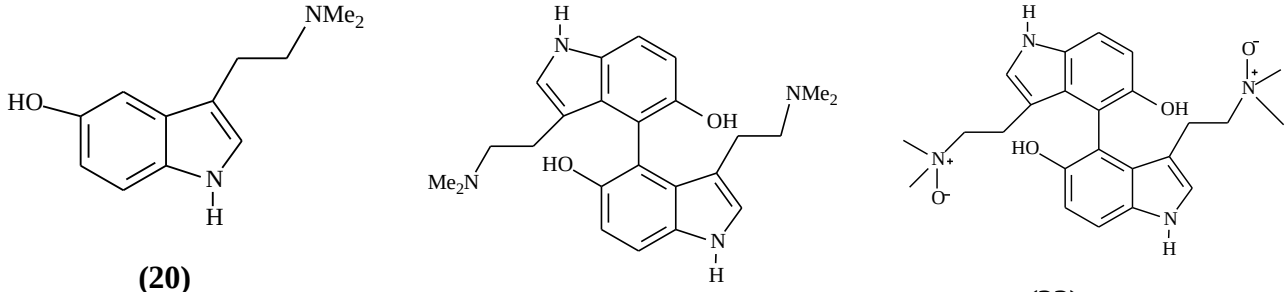


Figura 6. Alcaloides indólicos isolados de *P. brachybotrya*.

2.2.3 *Psychotria capillacea* Müll. Arg. Standl.

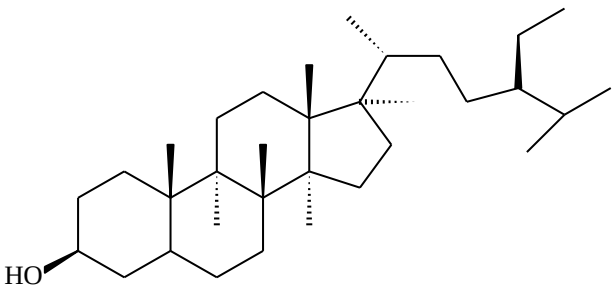
Popularmente conhecida como "café", *P. capillacea* (**Figura 7)** tem ocorrência nos estados do Amazonas, Mato Grosso do Sul, São Paulo e Paraná, em formações de Floresta de Galeria e Florestas Ombrófilas (BARBOSA *et al.*, 2015). Foi evidenciado baixo teor de fenóis totais, flavonoides, flavonol e taninos condensados e moderada capacidade antioxidantes frente ao DPPH, ABTS e β-caroteno (FORMAGIO *et al.*, 2014). Além desta triagem fitoquímica, não foram encontrados relatos na literatura quanto ao estudo fitoquímico e farmacológico para esta espécie.

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

2.2.4 *Psychotria carthagenensis* Jacq.

Conhecida popularmente como “carne-de-vaca”, “cafeeiro-do-mato” ou “maria-mole”, *P. carthagenensis* (**Figura 8**) é um arbusto que atinge de 2 a 3 metros de altura, encontrada em florestas sazonais e ombrófilas. Tem ocorrência em quase todos os estados brasileiros e é um dos componentes da bebida alucinógena da ayahuasca, utilizada originalmente pelos povos da Floresta Amazônica (RIVIER; LINDGREN, 1972; SMITH; DOWNS, 1956). Estudos químicos relatam a presença de dois triterpenos principais β-sitosterol (**23**) (**Figura 9**) e ácido ursólico (**24**) no extrato de acetato de etila de *P. carthagenensis* por cromatografia gasosa acoplada a espectrometria de massas (CG/EM) (LOPES; MORENO; HENRIQUES, 2000) e no extrato metanólico obtido das folhas, ácido p-cumárico (**25**) (FORMAGIO *et al.*, 2014). Além disso, o extrato metanólico apresentou pronunciada atividade antioxidante frente aos ensaios de DPPH, ABTS, β-caroteno e teor de flavonoides, flavonol, fenóis totais e taninos condensados (FORMAGIO *et al.*, 2014). De acordo com Rivier; Lidgren (1972), nas folhas de *P. carthagenensis* há a presença do alcaloide *N*,*N*-dimetiltriptamina (**26**) e *N*-monometiltriptamina e 2-metil-1,2,3,4-tetrahidro-β-carbolina.

Figura 8. Folhas e galhos de *P. carthagenensis* (Fonte: Volobuff, 2014).



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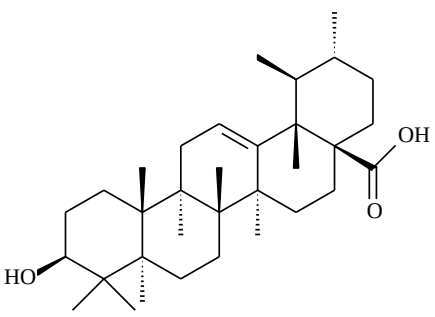

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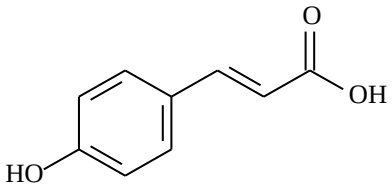

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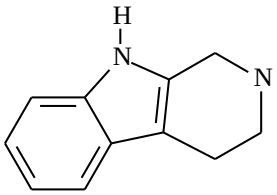


(26)

Figura 9. Substâncias presentes em *P. carthagenensis.*

2.2.5 *Psychotria deflexa* DC.

Psychotria deflexa (sinonímia: *Palicourea deflexa*) (**Figura 10**) é conhecida como "erva-de-rato" ou "café-selvagem", com ocorrência do México à Argentina. Estudo realizado com folhas de *Palicourea deflexa*, relata o isolamento do ácido harman-3-carboxílico da fração alcaloidal total, juntamente ao potencial inibitório da acetilcolinesterase em cérebros de peixe-zebra (BERTELLI *et al.*, 2017). O extrato metanólico foi avaliado quanto ao teor de constituintes fenóis totais, flavonoides, flavonol e taninos condensados, além da atividade antioxidante pelos modelos de DPPH, ABTS e β-caroteno (FORMAGIO *et al.*, 2014).

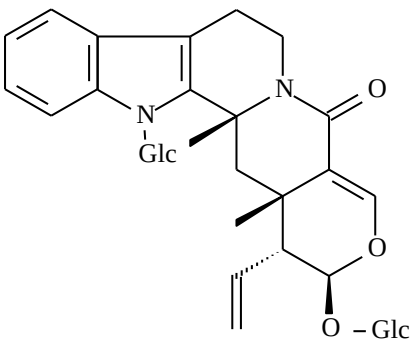
Figura 10. Folhas e galhos de *P. deflexa* (Fonte: Volobuff, 2014).

2.2.6 *Psychotria leiocarpa* Cham. & Schlecht.

Psychotria leiocarpa (“grandiúva-de-anta”) (**Figura 11**) é um arbusto que pode atingir até 2 m de altura, tem ampla ocorrência na Argentina, Paraguai e Brasil (ANDERSSON, 1992). Estudos químicos reportam o isolamento do alcaloide indolico monoterpênico *N*-β-D-

glucopiranosil vincosamida (**27**) (**Figura 12**), como constituinte principal das folhas juntamente com iridoides asperulosídeo (**28**) e deacetilasperulosídeo (**29**) (HENRIQUES *et al.*, 2004; LOPES *et al.*, 2004) e moderado teor de fenóis totais, flavonoides flavonol e taninos condensados (FORMAGIO *et al.*, 2014). No que se refere a atividades biológicas, o extrato etanólico das folhas demonstrou potencial anti-inflamatório na inibição de óxido nítrico em macrófagos (ELISABETSKY *et al.*, 1997); antioxidante frente ao reagente DPPH, ABTS e β-caroteno (FORMAGIO *et al.*, 2014), antimicobacteriano no crescimento de *Mycobacterium bovis* BCG (MORAES *et al.*, 2011) e atividade analgésica no teste de retirada de cauda (ELISABETSKY *et al.*, 1997). No óleo essencial extraído das folhas, foi evidenciado a presença de 33 compostos caracterizados exclusivamente por sesquiterpenos, destacando o biciclogermacreno (**30**) com 35,6% e germacreno D (**31**) com 17,6% (ANDRADE *et al.*, 2010).

Figura 11. Folhas e galhos de *P. leiocarpa* (Fonte: Volobuff, 2014).



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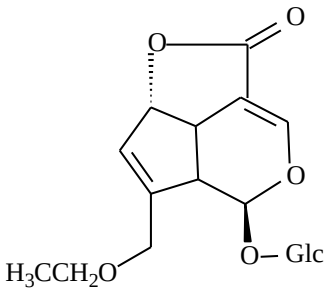

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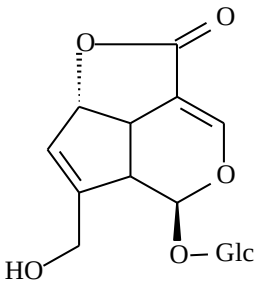

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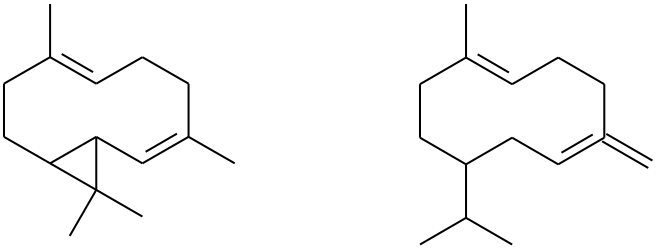


(30) **(31)**

Figura 12. Alcaloides, sesquiterpenos e iridoides isolados de *P. leiocarpa*.

2.2.7 *Psychotria poeppigiana* Müll. Arg.

Conhecida como "beijo de negro" e "chapéu do diabo" (**Figura 13**), tem vasta ocorrência na América Latina, sendo também encontrada no estado de Mato Grosso do Sul. O banho de vapor de suas folhas é utilizado no tratamento de febre alta, diarreia e astenia (VALADEAU *et al.*, 2009). Na Colômbia, a decocção a quente da raiz é administrada via oral e massageada no peito, sendo considerada eficaz contra doenças pulmonares (SCHULTES, 1985). No leste da Nicaragua, as folhas são utilizadas para tratamento de candidíase bucal (COE, 2008). A triagem fitoquímica a partir do extrato etanólico das folhas evidenciou a presença de cumarinas, fenois, flavonoides, esteroides, triterpenoides e alcaloides (PINO-BENÍTEZ, 2006). Quanto ao estudo biológico, o extrato etanólico das flores não apresentou efeito citotóxico e não inibiu o crescimento tumoral, mas exibiu efeito inibitório das metástases pulmonares em 50% (VILLASMIL *et al.*, 2006).

Figura 13. Flor e folhas de *P. poeppigiana* (Fonte: Dos Santos, 2018).

2.3 Atividades biológicas

2.3.1 Radicais livres e antioxidantes

Os radicais livres são moléculas que possuem um ou mais elétrons não-emparelhados no orbital externo, o que os torna mais reativos a qualquer outro tipo de molécula, incluindo lipídeos, proteínas e ácidos nucleicos, podendo ser classificados como espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN) (VASCONCELOS *et al.*, 2014). Em condições normais, há o equilíbrio entre produção e inativação normal dos radicais livres, impedindo a ocorrência de danos às biomoléculas. Quando há o rompimento deste equilíbrio, seja por aumento na produção de radicais livres ou redução nos mecanismos antioxidantes, é iniciado o processo de estresse oxidativo, o qual pode ocasionar a peroxidação de lipídeos, agressão às proteínas dos tecidos e membranas, enzimas, carboidratos e DNA promovendo o desencadeamento de diversas doenças crônicas, degenerativas, cardiovasculares, alterações do sistema imunológico e distúrbios no sistema nervoso (BARREIROS; DAVID; DAVID, 2006; SINHA; DABLA, 2015; TEIXEIRA, 2013).

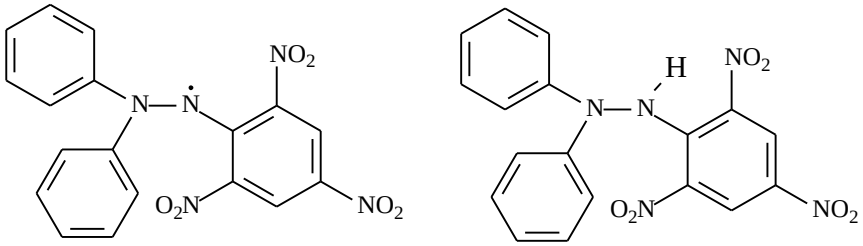
A geração de radicais livres é normalmente regulada pela ação de antioxidantes endógenos e exógenos. Os endógenos, produzidos pelo próprio organismo, são classificados como enzimáticos (superóxido dismutase, catalase e sistema antioxidante dependente de glutationa, formado pela glutationa-peroxidase e glutationa-redutase) e não enzimáticos (ácido lipoico, albumina, ácido úrico e glutationa). Os exógenos ou dietéticos são adquiridos por meio da ingestão alimentar, como vitaminas C e E, ácido ascórbico, tocoferol, carotenoides e compostos fenólicos (QUISPE *et al.*, 2019). O interesse em antioxidantes de fontes naturais tem sido cada vez mais acentuado, devido à proteção que exercem em nosso organismo contra os efeitos nocivos dos radicais livres, impedindo a progressão de muitas doenças (GÜLÇIN, 2012).

Na busca por antioxidantes exógenos, as plantas constituem uma fonte importante de substâncias que diferem amplamente em termos de estrutura e propriedades biológicas, como a curcumina obtida da *Curcuma longa* L., utilizada como corante de alimentos e antioxidante natural atuando no sequestro de radicais livres e inibição da peroxidação lipídica, além de potencial bacteriostático contra *Mycobacterium smegmatis*, usada para rastreamento da atividade antitubercular (MISHRA *et al.*, 2018).

2.3.1.1 Modelos experimentais *in vitro*

Existem vários modelos *in vitro* para a determinação da atividade antioxidante. Um dos métodos utilizados é o 2,2-difenil-1-picril-hidrazil (DPPH) de coloração violeta, o qual consiste na medida da capacidade antioxidante de uma substância sequestrar o radical DPPH, reduzindo-

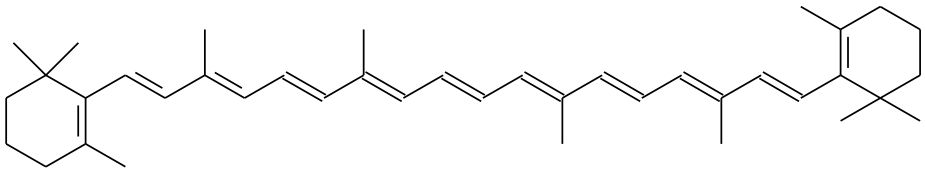
o a hidrazina (**Figura 14**). Quando uma determinada susbtância que age como doador é adicionada a uma solução de DPPH, a hidrazina é obtida com mudança simultânea na coloração de violeta a amarelo pálido, mensurado espectrofotometricamente a 515 nm (ALVES *et al.*, 2010).



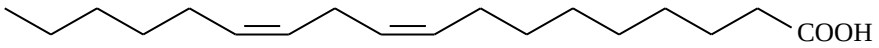
(32) **(33)**

Figura 14. Formas radicalar (**32**) e não radicalar (**33)** do DPPH.

O sistema de co-oxidação do β-caroteno/ácido linoleico, permite avaliar a capacidade de uma determinada substância em prevenir a oxidação do β-caroteno (**34**), protegendo-o dos radicais livres gerados durante a peroxidação do ácido linoleico (**35**) (**Figura 15**). A reação pode ser monitorada espectrofotometricamente pela perda da coloração do β-caroteno em 470 nm (ALVES *et al.*, 2010).



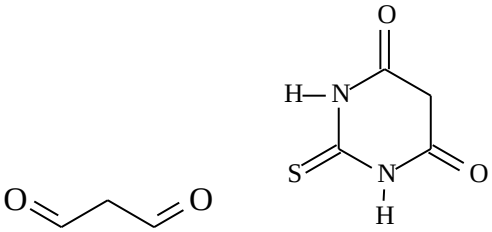
(34)



(35)

Figura 15. Estrutura química do β-caroteno e ácido linoleico.

Outro teste antioxidante é o método de malondialdeído (**36**). Neste ensaio, uma molécula de malondialdeído reage com duas moléculas de ácido tiobarbitúrico (**37**) (**Figura 16**) formando como produto um cromógeno de cor rosa fluorescente capaz de ser detectado espectrofotometricamente na faixa de 532-535 nm. A reação ocorre em meio ácido (pH 1-2) e a alta temperatura (100 ºC), no sentido de aumentar a sua velocidade e sensibilidade (FRANÇA *et al.*, 2013).



(36) **(37)**

Figura 16. Estrutura química do malondialdeído e ácido tiobarbitúrico.

2.3.2 Inflamação - aspectos gerais

Inflamação é derivada do "estado de se estar inflamado" (TROWBRIDGE; EMLING, 1997). A resposta inflamatória é um mecanismo benéfico, complexo e fisiológico pelo qual o organismo se defende contra infecções e tenta reparar danos teciduais, promovendo a regulação da homeostase (LAWRENCE *et al.*, 2002; RIOS *et al.*, 2009). O processo inflamatório pode ser dividido em duas fases: inflamação aguda e crônica. A inflamação aguda considerada de curta duração (horas ou dias) é caracterizada pela presença de vasodilatação (ocasionada por mediadores como óxido nítrico e prostaglandinas), exsudação de líquido (plasma), infiltração de células polimorfonucleares (neutrófilos), seguido por monócitos que se diferenciam em macrófagos e finalmente os fibroblastos que se proliferam podendo restabelecer a estrutura do tecido danificado (SHERWOOD; TOLIVER-KINSKY, 2004). A inflamação crônica é caracterizada por ter maior duração (semanas a meses) e, está correlacionada à presença de linfócitos e macrófagos, proliferação de vasos sanguíneos e as tentativas de reparo tecidual com consequente destruição do tecido e a formação de fibrose e necrose tecidual (FUJIWARA; KOBAYASHI, 2005).

Os componentes básicos de um processo inflamatório envolvem aspectos vasculares e celulares, mediadores derivados de células e da ativação plasmática, que produzem os sinais clínicos clássicos da inflamação (GILROY *et al.*, 2004). Os eventos vasculares ocorrem em resposta à ação dos mediadores inflamatórios. Iniciam-se imediatamente e desenvolvem-se durante as primeiras horas após o estímulo inflamatório. Eles induzem a produção de mediadores, como as aminas vasoativas, os peptídeos vasoativos, os fragmentos de componentes do complemento, os mediadores lipídicos, as citocinas, as quimiocinas e as enzimas proteolíticas (SERHAN; CHIANG; VAN DYKE, 2008). Todos os mediadores são conhecidos por promover dor pela ativação e/ou sensibilização das fibras aferentes primárias especializadas, ou seja, os nociceptores, e recrutam outros mediadores para exacerbar o sinal de dor (RITTNER; BRACK; STEIN, 2008). Os eventos celulares são marcados pela saída das

células circulantes da luz do vaso e a migração de leucócitos para o sítio inflamatório. Os leucócitos rolam na superfície do endotélio, depois são ativados e aderem a ele, ocasionando a transmigração por meio do endotélio. Então, atravessam a membrana basal e migram seguindo um gradiente quimiotático que se origina no local da lesão.

Dentre os mediadores envolvidos no processo inflamatório, estão as citocinas, eicosanoides e as cininas. Entre as citocinas mais conhecidas, TNF e IL-1 (IL-1α, IL-1β, IL-1Ra e IL-18) são vistas como de suma importância, devido aos seus papéis na patofisiologia de muitas respostas inflamatórias, além de terem um papel regulador sobre o início, a manutenção e o término das reações inflamatórias (EL ALWANI; OBEID; HANNUN, 2006; SAADANE *et al.*, 2011).

As citocinas IL-1β e TNF são as primeiras a serem formadas após o dano tecidual ou infecção, afetando diretamente os receptores específicos dos neurônios sensoriais (DE OLIVEIRA *et al.*, 2011). Em torno de 01 hora após o início da cascata inflamatória, estas citocinas são secretadas em grandes quantidades principalmente pelos macrófagos e aparecem na circulação e apresentam efeitos locais e sistêmicos (EL ALWANI; OBEID; HANNUN, 2006).

Os eicosanoides são mediadores lipídicos que compreendem os prostanoides, leucotrienos (LTs) e lipoxinas, todos derivados do metabolismo do ácido araquidônico (AA), que é um ácido graxo poli-insaturado esterificado presente nos fosfolipídios de membrana celular. Os eicosanoides estão envolvidos em vários processos fisiológicos e estão entre os mais importantes mediadores e moduladores da reação inflamatória (SERHAN, 2010).

Os prostanoides são produzidos por duas isoformas enzimáticas, as ciclooxigenases 1 e 2 (COX-1 e COX-2, respectivamente). A isoforma COX-1 é uma enzima constitutiva encontrada em vários tecidos, com função de promover homeostasia. Em contrapartida, a COX-2 é uma enzima induzida na inflamação, influenciando os eventos vasculares. Estas duas enzimas catalisam a conversão do ácido araquidônico em prostaglandina G_2 (PGG_2) e depois em prostaglandina H_2 (PGH_2) que é subsequentemente convertida nos prostanoides biologicamente ativos (HAWORTH; BUCKLEY, 2007).

O tratamento de distúrbios inflamatórios é realizado com a administração de anti-inflamatórios não-esteroidais (AINEs) e, em casos específicos, anti-inflamatórios esteroidais/glicocorticoides (AIEs). Os AINES atuam inibindo a síntese de prostaglandinas e tromboxanos através da inativação das enzimas COX-1 e COX-2, exibem ação analgésica, anti-inflamatória e antitrombótica, e por este motivo, são utilizados para o tratamento de diversas enfermidades como artrite reumatoide e esclerose sistêmica progressiva (SILVA *et al.*, 2019).

Os efeitos adversos gerais da inibição dos bloqueadores da ciclooxigenase, ocorrem, na maioria dos casos, por causa da inibição da isoforma constitutiva (COX-1). Tais efeitos, que são mais comuns em idosos, incluem: náuseas, vômitos, efeitos gastrintestinais, insuficiência renal reversível, efeitos cardiovasculares adversos, nefropatia associada a analgésicos, distúrbios hepáticos e depressão da medula óssea (SILVA *et al.*, 2019). Nesta classe, estão inclusos diversos grupos, como os salicilatos (aspirina), derivados indolacéticos (indometacina e diclofenaco) e os derivados do ácido propiônico (ibuprofeno e naxoprofeno) (GUERRA, 2011).

Os AIEs possuem ação analgésica e anti-inflamatória com redução do edema, além da prevenção de hiperalgesia através da inibição da fosfolipase A_2 e da COX. Além disso, atuam praticamente em todos os tecidos e órgãos, e por este motivo apresentam inúmeros efeitos adversos (FLAMMER; ROGATSKY, 2011). De maneira geral, agem na redução da síntese de prostaglandinas e citocinas pró-inflamatórias (IL-6, IL-2 e TNF-α), compreendendo efeitos imunossupressores e antiinflamatórios, sendo capazes de inibir manifestações iniciais e tardias do processo inflamatório. Dentre os fármacos desta classe, encontram-se hidrocortisona e cortisona (curta duração), prednisolona e predsona (média duração) e medicamentos de ação prolongada como a dexametasona e betametasona (ANTI; GIORGI; CHAHADE, 2008; BAVARESCO; BERNARDI; BATTASTINI, 2005). A dexametasona é um fármaco amplamente utilizado em modelos anti-inflamatórios, pois apresenta atividade farmacológica de dez a vinte vezes maior que o cortisol e a corticosterona. Além disso, atua como um potente anti-inflamatório e imunossupressor que atua inibindo a transcrição de genes que após a tradução originam receptores, proteínas que suprimem citocinas e, também, proteínas que controlam a ativação, migração, adesão e recrutamento celular. Como consequência, ocorre a inibição periférica da proliferação de linfócitos T, acompanhada por inibição da migração celular para sítios inflamatórios e controle da recirculação de leucócitos (BAVARESCO; BERNARDI; BATTASTINI, 2005).

No entanto, os medicamentos disponíveis apresentam efeitos adversos, como gastrointestinais, hipertensão arterial e insuficiência renal. Neste sentido, vários estudos buscam novas fontes oriundas de plantas para tratamento de processos inflamatórios com menores efeitos. Estudos evidenciam que além do potencial antioxidante, esta molécula inibe a atividade de enzimas pró-inflamatórias (MARMITT; REMPEL; GOETTERT; SILVA, 2015).

2.3.2.1 Modelos experimentais *in vivo*

Dentre os modelos utilizados para indução de inflamação, destacam-se pleurisia e edema de pata induzidos por carragenina. A carragenina é um polissacarídeo altamente

sulfatado obtido de algas vermelhas (Rodophyceae), com capacidade de ativar a resposta imune inata se ligando a TLR4/6 / TLR2/6 (receptor Toll-like) ou através da geração de espécies reativas de oxigênio (EROs). Após se ligar ao TLR, a carragenina ativa uma cascata de sinalização mediada por diversas proteínas adaptadoras e quinases. A via canônica do NF-κB é ativada por estas proteínas, além da via das proteínas quinases ativadas por mitógenos (MAPKs). Também tem a capacidade de reduzir as concentrações de proteína de choque térmico 27 (Hsp27) através da formação de EROs. A diminuição na concentração da Hsp27 está envolvida na ativação de quinases que por sua vez também tem relação com a ativação do NF-κB e via das MAPKs (BHATTACHARYYA; DUDEJA; TOBACMAN, 2008; DONG *et al.*, 2006).

O ensaio de pleurisia induzida por carragenina em camundongos é um modelo de inflamação neutrofílica, o qual permite a quantificação de diferentes mediadores pró-inflamatórios liberados na cavidade pleural. Neste modelo ocorre uma resposta inflamatória do tipo bifásica, semelhante a asma. Na primeira fase (4 horas após a administração da carragenina a 1%) desta resposta aguda, há formação de proteínas do exsudato, aumento da infiltração de leucócitos, principalmente neutrófilos, seguido de lesão pulmonar por espécies reativas de oxigênio e nitrogênio liberados por tais neutrófilos ativados no sítio inflamatório. Os radicais como o peróxido de hidrogênio, ânion superóxido, radical hidroxila e peroxinitrito são os principais mediadores liberados em grande quantidade na cavidade pleural dos camundongos. Na fase tardia (48 horas após a administração da carragenina a 1%), ocorre o início do remodelamento tecidual com a inversão no perfil leucocitário, ou seja, aumento de células mononucleares, além da presença do exsudato (SALEH; CALIXTO; MEDEIROS, 1996).

O modelo de edema de pata induzido por carragenina ocorre em duas fases distintas. A primeira fase (1-2 h) é caracterizada pela liberação de histamina, serotonina e bradicinina, enquanto que a segunda fase (2-4 h) é correlacionada à elevada produção de prostaglandinas. A infiltração local de neutrófilos também contribui para a resposta inflamatória neste modelo (DEL-VECHIO-VIEIRA *et al.*, 2016).

O teste de formalina consiste na aplicação de um estímulo algésico químico, utilizado como ferramenta para mimetizar uma condição de dor aguda e tônica. A administração da formalina promove a expressão de respostas nocifensivas em duas fases (fase I e II), as quais são separadas por um período quiescente (interfase) (MOGIL, 2009). Estudo realizado por MCCall; Tanner; Levine (1996), demonstraram haver ativação de fibras aferentes do tipo C durante todo o teste de formalina, de forma bifásica, com padrão análogo aos comportamentos induzidos pela formalina durante as fases I e II. A fase I do teste da formalina tem duração de

5 minutos e, parece ser resultante da ativação direta das fibras do tipo C e Aδ pela formalina (PUIG; SORKIN, 1996). Posteriormente, observa-se uma fase de quiescência, sem manifestação comportamental, a qual é independente da concentração de formalina utilizada. Esse período interfase é pensado por ser resultado de uma ativa inibição da entrada nociceptiva (HENRY *et al.*, 1999). A fase II do teste é caracterizada por reações inflamatórias e sensibilização central no corno dorsal da medula espinal (TJOLSEN *et al.*, 1992). A contribuição da estimulação de neurônios nociceptivos no desenvolvimento e manutenção dos comportamentos nociceptivos observados na fase II é sustentado pela observação de que o estímulo da formalina provoca liberação de neuromoduladores inflamatórios na medula espinal, como aminoácidos excitatórios (glutamato, aspartato) e substância P, que contribuem para sensibilização central (MALMBERG; YAKSH, 1992a; 1992b).

2.3.3 Câncer

O câncer é uma doença caracterizada por um desvio dos mecanismos de controle que regulam a sobrevida, proliferação e diferenciação das células. Engloba mais de 100 doenças, desencadeados por amplos fatores de risco como sexo, idade, raça, predisposição genética e exposição a carcinógenos ambientais (ALMEIDA *et al.*, 2005; STRATTON; CAMPBELL; FUTREAL, 2009).

O câncer compreende três etapas principais: transformação, proliferação e metástase. Durante a transformação, ocorre a mudança no fenótipo de uma célula com controle normal de seu crescimento descontrolado. Desta forma, as mutações podem ativar genes que promovem crescimento, inativar genes que inibem o crescimento, alterar genes que regulam a apoptose, conferir imortalização celular e inativar genes de reparo do DNA. A proliferação celular ocorre em fases distintas. Durante a fase *S*, ocorre a síntese de DNA e na fase *M* há a divisão da célula-mãe em duas células-filha durante a mitose. A fase entre a divisão celular e a síntese de DNA é denominada *lacuna 1* (*G1*) e a fase entre a síntese de DNA e a mitose é denominada *G2*. Para que ocorra a metástase, as células tumorais sofrem mutações que permitam sua invasão em tecidos e vasos, implantação em cavidades, disseminação por vasos linfáticos ou sanguíneos e crescimento em novo ambiente. Geralmente, tumores primários agressivos e de rápido crescimento têm mais tendência a metástases que os mais indolentes e de crescimento lento (ALMEIDA *et al.*, 2005) (**Figura 17**).

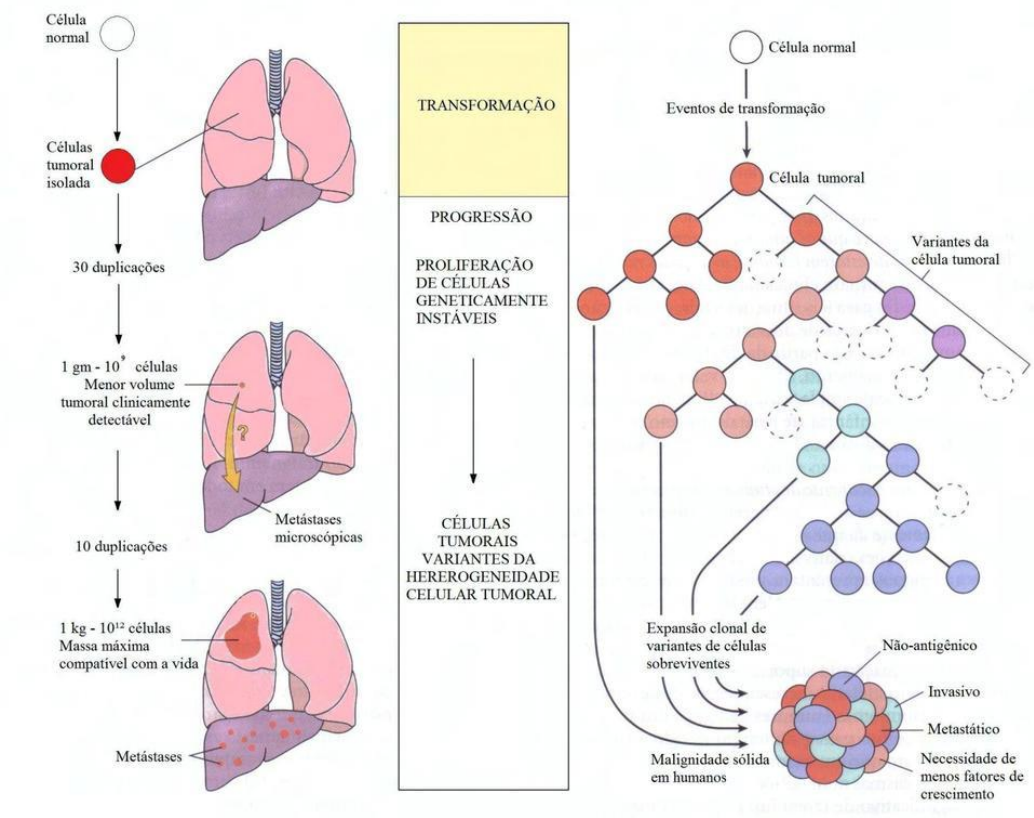


Figura 17. Biologia do crescimento tumoral. No lado esquerdo é demonstrado estimativas mínimas das duplicações das células tumorais que precedem a formação de uma massa tumoral clinicamente detectável. À direita está ilustrado a evolução clonal e a geração da heterogeneidade das células tumorais. Novos subclones surgem a partir dos descendentes da célula transformada original, e com o crescimento progressivo da massa do tumor são enriquecidos por variantes mais aptos a escapar das defesas do hospedeiro e provavelmente são os mais agressivos (FONTE: KUMAR; ABBAS; FAUSTO, 2005).

Vários fatores podem acarretar o desenvolvimento do câncer, como o processo inflamatório crônico e o estresse oxidativo. Como exemplo, encontra-se a pancreatite crônica, a qual está associada aos elevados índices de câncer no pâncreas e, doenças inflamatórias que afetam o intestino como a colite ulcerativa e doença de Crohn se associam ao desenvolvimento de adenocarcinoma de cólon. Os radicais livres são liberados e se depositam no local inflamado, devido ao recrutamento de células no tecido. O tecido com exposição prolongada à inflamação e oxidação, deterioram as células presentes e esse ciclo vicioso ocasiona carcinogênese (REUTER *et al.*, 2010). Além disso, há relatos na literatura que descrevem a interação do estresse oxidativo em todos os estágios do câncer, produzindo danos ao DNA por meio de

mutações; contribuição na expressão gênica anormal e alteração de DNA às células (SOSA *et al.*, 2013).

No Brasil, é estimado a ocorrência de 600 mil novos casos de câncer entre os anos de 2018-2019, sendo os cânceres de pulmão, próstata, mama feminina, reto e cólon os de maiores incidências, porém também há elevadas taxas de cânceres de estômago, esôfago e colo de útero. Os cânceres de próstata e mama serão os mais frequentes entre homens e mulheres, respectivamente. Na região Centro-Oeste, da qual faz parte o estado do Mato Grosso do Sul, os cânceres de estômago e colo do útero estão entre os de maior incidência (INCA, 2017; SANTOS, 2018).

Atualmente, grande parte dos agentes quimioterápicos tradicionais interfere na proliferação celular e é baseado em ciclo celular rápido e/ou promoção de apoptose para sua seletividade relativa contra as células cancerosas. A quimioterapia é um dos tratamentos farmacológicos mais utilizados para o câncer. Os tumores são mais sensíveis à quimioterapia quando apresentam crescimento rápido, basicamente por estarem progredindo pelo ciclo celular (ALMEIDA *et al.*, 2005). Visto que a quimioterapia pode acarretar danos no organismo, há a necessidade de novas fontes de tratamento, como o uso de plantas medicinais, pois apresentam menores impactos toxicológicos.

As plantas medicinais são fontes promissoras de princípios ativos com atividades anticâncer. A pesquisa de medicamentos anticancerígenos advindos de fontes naturais, iniciou-se em 1950, quando foram descobertos os alcaloides de vinca como a vimblastina, isolados de *Catharanthus roseus*, sendo a segunda classe de medicamentos mais usada na terapia contra o câncer (MOUDI; GO; YIEN; NAZRE, 2013). A partir do taxol, alcaloide derivado das espécies *Taxus brevifolia* e *T. baccata*, foi desenvolvido o medicamento Paclitaxel®, que possui ação anticancerígena semelhante aos alcaloides de vinca (CORRÊA, 1995).

2.3.3.1 Cultura de células *in vitro*

Visando a busca de moléculas bioativas para terapia do câncer, vários modelos experimentais tem sido utilizados como *screening*. Neste sentido, a cultura de células *in vitro* permite estudar o crescimento, diferenciação e morte celular, além de possibilitar mutações genéticas necessárias ao perfeito conhecimento da estrutura e funções dos genes (LOPES, 2015). Na pesquisa de antineoplásicos, este modelo é considerado uma ferramenta importante para descoberta de novas drogas, pois permite o estudo do comportamento das células em resposta a um tratamento e possibilita a compreensão dos efeitos e mecanismos envolvidos com

a ação de diferentes agentes (NIU; WANG, 2015). Por estes motivos, torna-se uma técnica alternativa aos modelos *in vivo* (LOPES, 2015).

O modelo de proliferação celular de metodologia de triagem *in vitro* para drogas anticâncer foi desenvolvida pelo National Cancer Institute NCI/NIH (Frederick, WA, EUA) (MONKS *et al.*, 1991). Após o período de ambientação das células, as amostras são aplicadas nas diferentes linhagens, e também uma avaliação da viabilidade celular na placa T0 (controle) que inferirá indiretamente a quantidade de células viáveis no início do tratamento das células. No final do experimento, a viabilidade celular das linhagens tratadas será comparada com a das linhagens não tratadas (T1), permitindo assim avaliar a ação antiproliferativa das amostras (MONKS *et al.*, 1991). A atividade antiproliferativa é avaliada através do ensaio colorimétrico com o reagente de sulforrodamina B. Este corante proteico liga-se aos aminoácidos básicos presentes nas proteínas das células viáveis fixadas. Portanto, quanto maior a quantidade de corante ligada ao compartimento, menor a proliferação celular (MONKS *et al.*, 1991; OKUBO, 2016).

2.3.4 Hipótese colinérgica

A doença de Alzheimer (DA) é uma doença neurodegenerativa, associada à neuroinflamação envolvendo a ativação de macrófagos, micróglia e linfócitos, estresse oxidativo e liberação de quimiocinas e citocinas pró-inflamatórias (FLOYD, 1999; PERRY, 2004), as quais apresentam papel fundamental no estágio inicial de neuroinflamação, devido a secreção de pró-inflamatórios intermediários que aumentam a permeabilidade da barreira hematoencefálica. Este aumento resulta em um potencial influxo de monócitos, linfócitos e macrófagos para o cérebro (TANSEY; MCCOY; FRANK-CANNON, 2007; TAUPIN, 2008).

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; GIACOBINI, 1988; PERRY, 1986). A acetilcolina (ACh) desempenha um papel importante na neurostransmissão periférica, e é sintetizada em uma única etapa a partir da colina e acetil coenzima A (acetilCoA) pela enzima colina acetiltransferase (ChAT), como segue:

$$\text{Acetil coenzima A + colina} \xrightarrow{\text{ChAT}} \text{Acetilcolina + coenzima A + } H_2O$$

Quando sintetizada no citoplasma, a ACh é transportada até o interior de vesículas sinápticas para armazenamento. A energia necessária para este processo é fornecida por uma ATPase que bombeia prótons para o interior da vesícula. O transporte de prótons para fora dela

está acoplado à captação de ACh para dentro da vesícula por meio de um canal antiportador de ACh-H^+. Este representa um alvo para alguns fármacos anticolinérgicos, como o vesamicol, e sua inibição resulta em déficit de armazenamento e liberação subsequente de ACh. A liberação da ACh na fenda sináptica ocorre por meio da fusão da vesícula sináptica com a membrana plasmática. O processo depende da despolarização da terminação axônica e da abertura dos canais de cálcio dependentes de voltagem. As vesículas que contem ACh fundem-se com a membrana plasmática quando os níveis intracelulares de cálcio aumentam em resposta a um potencial de ação pós-sináptico liberando o neurotransmissor na fenda sináptica. A ACh difunde-se na fenda sináptica e se liga a receptores pós e pré-sinápticos. Os receptores de ACh são divididos em muscarínicos e nicotínicos. Estes são canais iônicos regulados por ligantes, permeáveis a cátions, enquanto que os muscarínicos são acoplados à proteína G e alteram as vias de sinalização da célula, incluindo a ativação da fosfolipase C, inibição da adenilciclase e a abertura dos canais de K^+. A ACh ao atravessar a fenda sináptica é degradada pela acetilcolinesterase (AChE) a qual está ligada à membrana, resultando em colina e acetato (GOLAN *et al.*, 2014) (**Figura 18**).

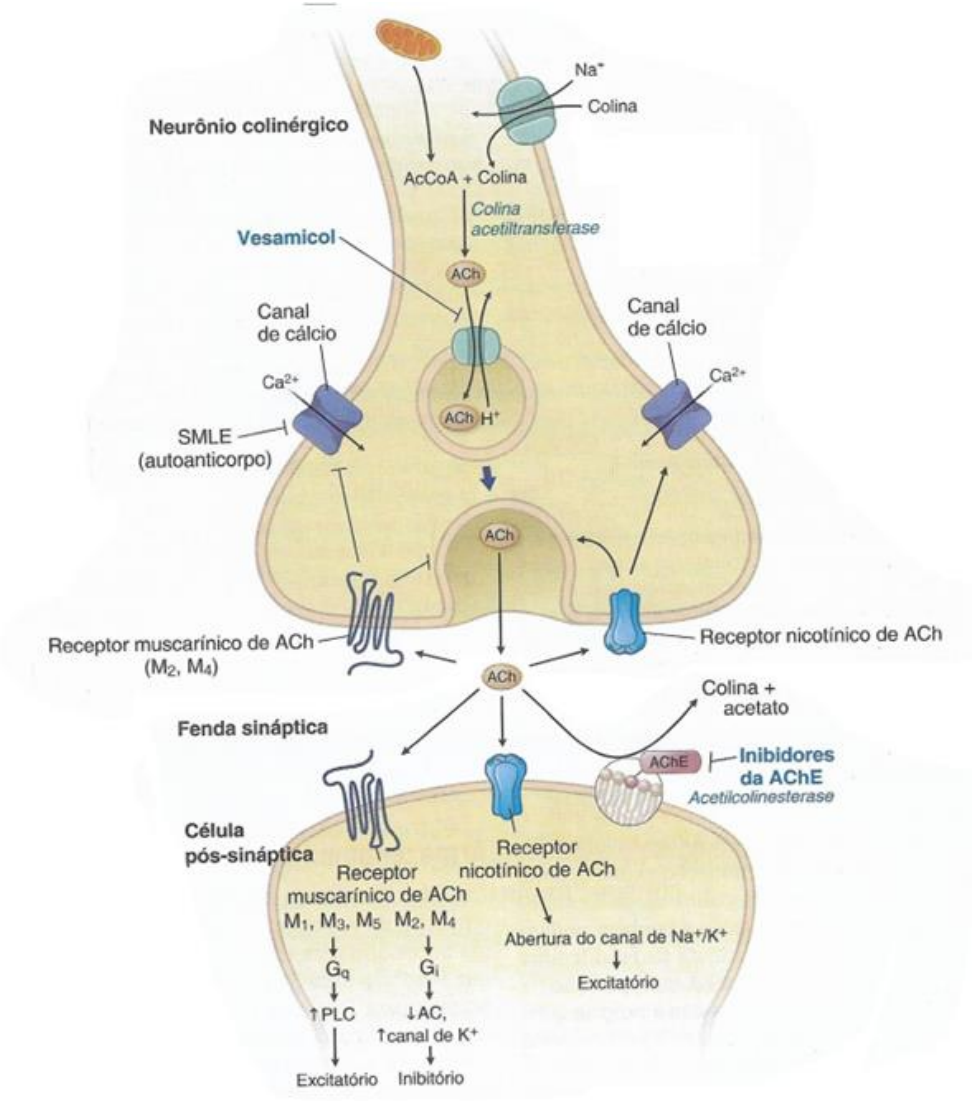


Figura 18. Vias de síntese, armazenamento, liberação e degradação da acetilcolina e agentes farmacológicos que atuam nestas vias (FONTE: Adaptado de GOLAN *et al*., 2014).

As enzimas conhecidas como colinesterases são responsáveis pela degradação da acetilcolina. A AChE é conhecida como colinesterase I ou específica, e se encontra principalmente em sinapses do sistema nervoso central, nos músculos esqueléticos e na membrana dos eritrócitos, sendo indispensável para a degradação de acetilcolina. A AChE está concentrada na membrana pós-sináptica, e a colina liberada sob sua ação é eficientemente transportada de volta à terminação pré-sináptica (ARAÚJO; SANTOS; GONSALVES, 2016). A butirilcolinesterase, também conhecida como colinesterase II ou inespecífica tem papel secundário na degradação de ACh, e é encontrada principalmente no plasma e fígado. Dentre as principais diferenças entre AChE e a BuChE, estão a presença de inibidores específicos,

diferentes níveis de sensibilidade ao substrato e parâmetros cinéticos próprios e possuem diferentes locais de ação nos organismos (ARAÚJO; SANTOS; GONSALVES, 2016).

O tratamento farmacológico mais utilizado para diminuir os efeitos do declínio colinérgico consiste em elevar os níveis do neurotransmissor acetilcolina no cérebro utilizando substâncias inibidoras da acetilcolinesterase. Ao inibir esta enzima, será disponibilizado uma quantidade maior deste neurotransmissor para as sinapses colinérgicas (BARBOSA FILHO *et al*., 2006; BERTÉ, 2009).

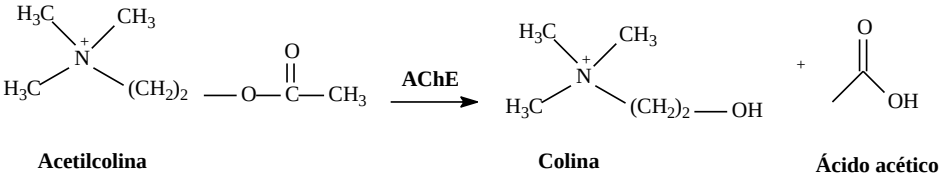
Dentre os anticolinesterásicos de uso clínico está o tetrahidroaminocridina, ou Tacrina, o qual foi liberado para comercialização com o nome de Cognex®. Em contrapartida, este fármaco mostrou-se inconveniente por ter de ser administrado quatro vezes ao dia e estar associado a incidência de efeitos colaterais como a hepatotoxicidade (FORLENZA, 2005). Compostos isolados a partir de plantas medicinais também foram empregados para o tratamento dos sintomas da doença. A galantamina denominado comercialmente de Remynil® é um alcaloide isolado de flores e bulbo da *Galanthus woronowii*, que foi posteriormente sintetizado (CZOLLNER *et al*., 2001).

Neste sentido, a busca por substâncias inibidoras da acetilcolinesterase oriundas de plantas, tem sido amplamente descritos em diferentes espécies vegetais e suas respectivas substâncias isoladas (AJAYI; ADEROGBA; OBUOTOR; MAJINDA, 2019; KONRATH *et al*., 2012; NEAGU; RADU; ALBU; PAUN, 2018), como tentativa de encontrar novos inibidores anticolinesterásicos com maior efeito e baixa toxicidade.

2.3.4.1 Método de Ellman para análise da atividade anticolinesterásica

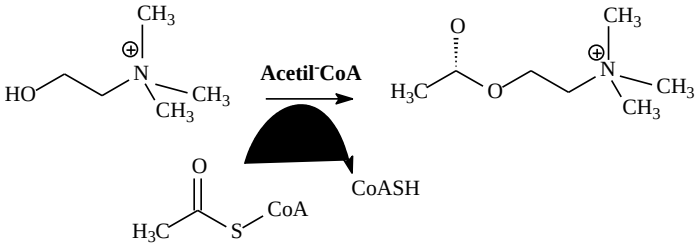
O método fotocolorimétrico de Ellman e colaboradores (1961) é bastante difundido e amplamente utilizado em diversas análises *in vitro* (onde a amostra a ser analisada é colocada em microplaca), *in vivo* (grupos de animais recebem por via oral as amostras) e em modelos de indução (grupos de animais recebem por via oral as amostras e, posteriormente, escopolamina via subcutânia mimetizando os efeitos de demência).

O princípio deste método consiste em determinar a atividade anticolinesterásica através da mensuração da taxa de produção da tiocolina à medida que a acetilcolina é hidrolisada pela acetilcolinesterase (**Figura 19**) (ELLMAN *et al*., 1961).

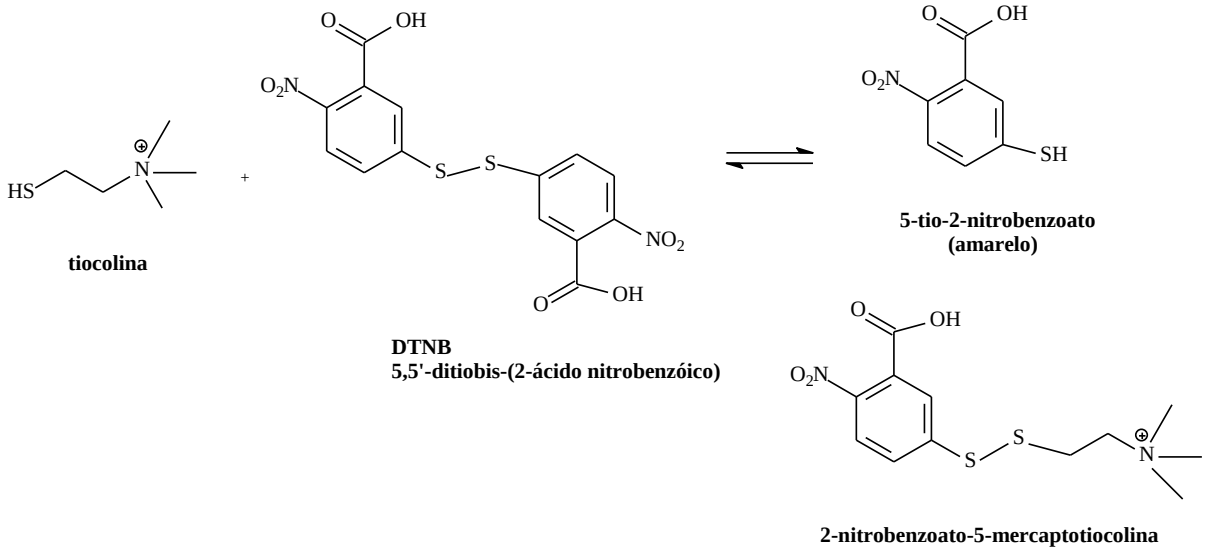


Acetilcolina **Colina** **Ácido acético**

Reação de hidrólise da acetilcolina



Reação de biossíntese de acetilcolina (ACh)



tiocolina

DTNB
5,5'-ditiobis-(2-ácido nitrobenzóico)

5-tio-2-nitrobenzoato
(amarelo)

2-nitrobenzoato-5-mercaptotiocolina

Figura 21. Reações ocorridas no método de Ellman.

O produto formado, tiocolina e o reagente de Ellman (ácido 5,5-ditiobis-2-nitrobenzoico – DTNB), reagem produzindo o ânion amarelo, permitindo a mensuração em espectrofotômetro a 412 nm. Este método é bastante sensível e pode ser aplicado a pequenas quantidades de tecidos e em baixas concentrações de enzima. O substrato utilizado é a acetiltiocolina, análogo do substrato natural (ELLMAN *et al.*, 1961).

2.3.5 Modelagem molecular – interação sítio ativo - AChE

A modelagem molecular consiste na predição do posicionamento (orientação e conformação) de um ligante (fármaco ou candidato a fármaco) dentro de um sítio de interação

alvo (KITCHEN *et al*., 2004). Nesta abordagem, a estrutura do alvo molecular permanece fixa e diversas posições de encaixe possíveis para o ligante são identificadas computacionalmente e por este motivo, são necessárias informações químicas do ligante e do receptor o mais detalhado possível (SILVA, 2013). Para avaliar os resultados da docagem utilizam-se os valores da energia de interação do complexo ligante-proteína, da energia intramolecular do ligante ou constante de inibição (MAGALHÃES; BARBOSA; DARDENNE, 2004). Para realização da modelagem molecular são utilizados vários softwares, como GOLD, AutoDock e Molegro Virtual Docker, capazes de fornecer dados de energia de ligação entre ligantes e macromoléculas, utilizando as estruturas tridimensionais da proteína e do ligante.

Dentre modelos de ancoragem molecular, destaca-se a interação sítio ativo-enzima com a AChE. A AChE é constituída por dois sítios de ligação, o esterásico e o periférico. O sítio ativo de ligação é localizado na parte inferior de uma cavidade estreita e profunda, denominada "cavidade aromática", alinhada com resíduos hidrofóbicos, a qual é composta principalmente por anéis aromáticos de resíduos de aminoácidos. Os aminoácidos Ser200, His440 e Glu327 constituintes do sítio esterásico são essenciais para a atividade catalítica da enzima, denominados de tríade catalítica. O sítio aniônico e periférico é localizado na parte superior da cavidade da aromática, apesar de não participar diretamente da atividade catalítica da enzima, este está envolvido na ligação de inibidores à enzima (SILVA, 2016) (**Figura 20**). Uma das estratégias para o tratamento de doenças colinérgicas, como a DA é baseada na inibição das enzimas colinesterases, elevando os níveis do neurotransmissor acetilcolina. Neste sentido, estudos de ancoragem molecular são de grande interesse, pois permitem evidenciar moléculas bioativas que podem ser utilizadas como possível alternativa para tratamento destas doenças.

Figura 20. Sítios ativos da enzima AChE (Fonte: Adaptado de Soreq e Seidman, 2001).

3 OBJETIVOS

GERAL

Realizar a avaliação farmacológica de espécies de Rubiaceae e posterior isolamento de alcaloides e modelagem molecular.

ESPECÍFICOS

- Estudos fitoquímicos:

Quantificar o teor de fenóis totais, flavonoides, flavonol e taninos condensados do extrato metanólico das folhas de *P. poeppigiana*;

Quantificar os alcaloides no extrato metanólico de *P. leiocarpa*, *P. deflexa*, *P. carthagenensis*, *P. capillacea*, *P. poeppigiana*, *P. brachybotrya* e *P. crocea*;

Isolar e identificar os alcaloides no extrato metanólico de *P. leiocarpa*;

Identificar a composição química do óleo essencial das folhas de *P. poeppigiana* por CG/EM;

Identificar as substâncias presentes no extrato metanólico das folhas de *P.poeppigiana* por técnicas acopladas CL-EM/EM.

- Atividade farmacológica:

Realizar a toxicidade aguda do extrato metanólico de *P. leiocarpa*;

Avaliar a atividade antiproliferativa frente a nove linhagens de células tumorais e uma linhagem não-tumoral do extrato metanólico de *P. leiocarpa*, *P. deflexa*, *P. carthagenensis*, *P. capillacea*, *P. poeppigiana*, *P. brachybotrya* e *P. crocea*;

Avaliar a atividade antioxidante do extrato metanólico e do óleo essencial das folhas de *P. poeppigiana*;

Avaliar a atividade anti-inflamatória do extrato metanólico de *P. poeppigiana*, *P. leiocarpa* e alcaloide isolado;

Avaliar a atividade hiperalgésica do extrato metanólico de *P. leiocarpa* e alcaloide isolado;

Avaliar a atividade antinociceptiva do extrato metanólico de *P. poeppigiana*;

Avaliar a inibição da acetilcolinesterase *in vitro* em quatro estruturas cerebrais obtidas de ratos *Wistar* do extrato metanólico de *P. leiocarpa*, *P. deflexa*, *P. carthagenensis*, *P. capillacea*, *P. poeppigiana*, *P. brachybotrya* e *P. crocea;* alcaloide isolado de *P. leiocarpa* e óleo essencial das folhas de *P. poeppigiana*;

Avaliar a inibição da acetilcolinesterase *in vivo* do extrato metanólico de *P. poeppigiana*;

Realizar a interação inibidor-enzima (docagem molecular) de substâncias majoritárias presentes no óleo essencial das folhas de *P. poeppigiana* e de alcaloide isolado de *P. leiocarpa*.

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5 APÊNDICES

5.1 Artigo I: Antitumoral and anticholinesterasic activities of the seven species from Rubiaceae

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RESEARCH ARTICLE

Antitumoral and Anticholinesterasic Activities of the Seven Species from Rubiaceae

Carla R.F. Volobuff[1], Pedro C.O. Junior[2], Sidney M. dos Santos[2], Zefa V. Pereira[2], Diego C. Ferreira[3], Claudia A.L. Cardoso[3], Ana L.T.G. Ruiz[4], Mary A. Foglio[4], João E. de Carvalho[4] and Anelise S.N. Formagio[1,2*]

[1]*Faculty of Health Sciences, Federal University of Grande Dourados, Dourados-MS, Brazil;* [2]*Faculty of Biological and Environmental Sciences, Federal University of Grande Dourados, Dourados-MS, Brazil;* [3]*Department of Chemistry, State University of Mato Grosso do Sul, Dourados-MS, Brazil;* [4]*Faculty of Pharmaceutical Science, University of Campinas Unicamp, SP, São Paulo, Brazil*

Abstract: ***Background***: The genus *Psychotria* and *Palicourea* are reported as a source of alkaloids and iridoids, which exhibit biological activities. This study aimed to evaluate antiproliferative and anticholinesterase activities and quantification of the alkaloids of seven species among the genus found in Mato Grosso do Sul region in Brazil.

Methods: Concentrations of alkaloids were measured spectrophotometrically. The extracts were submitted to antiproliferative activity against ten cell lines. The anticholinesterase activity of the extracts was developed using brain structures of male *Wistar* rats: cerebral cortex, hippocampus, hypothalamus and striatum by the Ellman method.

Results: Alkaloids from *Psychotria* and *Palicourea* species were quantified which showed values of 47.6 to 21.9 µg/g. Regarding the antiproliferative potential, *Palicourea crocea* demonstrated selectivity against the 786-0 cell line (GI_{50}: 22.87 µg/mL). *Psychotria leiocarpa* inhibited cell growth against OVCAR-3 (GI_{50}: 3.28 µg/mL), K-562 (GI_{50}: 5.26 µg/mL), HaCaT (GI_{50}: 27.20 µg/mL), PC-3 (GI_{50}: 34.92 µg/mL), MCF-7 (GI_{50}: 35.80 µg/mL) and *P. capillacea* showed activity against OVCAR-3 (GI_{50}: 2.33 µg/ml) and U251 (GI_{50}: 16.66 µg/ml). The effect of acetylcholinesterase inhibition was more effective in the hippocampus, demonstrating inhibition for *Paliourea crocea, Psychotria deflexa*, *P. brachybotrya* and *P. leiocarpa* of 70%, 57%, 50% and 40%, respectively, followed by *P. poeppigiana* and *P. capillacea*, inhibiting 21%, compared to the control.

Conclusion: Herein, the present work showed for the first time, anticholinesterasic and antiproliferative activities of extracts of *Palicourea* and *Psychotria* seem to be mainly associated with the levels of alkaloids in the leaves of these species.

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1. INTRODUCTION

The use of medicinal plants is a widespread practice, based mainly on folk medicine, which uses the therapeutic resource, and the efficacy attained by research corroborates the use. An example is the traditional use for religious practice in Amazon region of *Psychotria carthagenensis, P. viridis* and *Banisteriopsis caapi* with effects on the central nervous system, which is attributed to the hallucinogenic effects of the "ayahuasca" beverage [1-3]. Furthermore, species from *Psychotria* (Rubiaceae) were screened at the US National Cancer Institute for their activities against human cancer one-dose/60-cell-line [4]. Several leads of secondary metabolites from plant species have been reported. Tryptamine-iridoid alkaloids and polyindoline type are considered to be the main chemical constituents from *Psychotria* [5].

The State of Mato Grosso do Sul (MS), Brazil, encompasses biodiversity of three great Brazilian biomes, Atlantic Florest, Cerrado and Pantanal. Taxonomic studies with Rubiaceae in MS [6, 7], in the Parque Estadual das Várzeas do

*Address correspondence to this author at the Faculty of Health Sciences, Federal University of Grande Dourados (UFGD), Dourados, Brazil; Tel: +55-67 3410-2001; E-mail: aneliseformagio@ufgd.edu.br

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Rio Ivinhema, located in the Paraná river basin, demonstrated *Psychotria* (10 species) and *Palicourea* (4 species), are the most representative, with *Psychotria brachybotrya* being described for the first time on MS state. According to Barbosa (2018) the list was updated, presenting 14 from *Psychotria* and 9 from *Palicourea* [8].

The extensive distribution of *Psychotria* and *Palicourea* species in MS region and the medical use of some species without scientific evidence prompted the *in vitro* antiproliferative and anticholinesterase activities of six extracts from *Psychotria* and one *Palicourea* with quantification of alkaloids analysis. Chemistry and biological data of the species selected for this study are reported in Table **1** [9-22].

2. EXPERIMENTAL

2.1. Animals

The experiments were conducted using three male *Wistar* rats (200-300 g) from the Federal University of Grande Dourados – UFGD, Mato Grosso do Sul, Brazil. The animals were maintained at a constant temperature (23 ± 1ºC) on a 12-hour light/dark cycle with free access to food and water. The experimental procedures were carried out in accordance with the U.S. National Institutes of Health and approved by the Animal Ethics Committee from UFGD (Nbr. 14/2015 and Nbr. 17/2017).

2.2. Collection and Plant Identification

Leaves from *Psychotria* and *Palicourea* were collected in Dourados and identified by Profa. Dra. Zefa Valdevina Pereira. The specimens were registered in the herbarium of the Federal University of Grande Dourados, *P. deflexa* (DDMS 5005), *P. carthagenensis* (DDMS 5006), *P. leiocarpa* (DDMS 5007), *P. capillacea* (DDMS 5008), *P. poeppigiana* (DDMS 0006), *P. brachybotrya* (DDMS 984) and *Palicourea crocea* (DDMS 4487).

2.3. Preparation of the Methanol Extracts

Leaves from *Palicourea crocea*, *Psychotria brachybotrya*, *P. capillacea, P. carthagenensis, P. deflexa, P. leiocarpa* and *P. poeppigiana* were air-dried and exhaustively extracted by maceration using methanol at room temperature for 20 days. The solvent was evaporated under vacuum at 50ºC and freeze-dried to obtain the methanol extract of each sample, *P. crocea* (MEPCR; 67 g), *P. brachybotrya* (MEPB; 69 g), *P. capillacea* (MEPC; 25 g), *P. carthagenensis* (MEPCA; 19 g), *P. deflexa* (MEPD; 51 g), *P. leiocarpa* (MEPL; 66 g) and *P. poeppigiana* (MEPP; 23 g).

2.4. Quantification of the Alkaloids

The quantification of the total alkaloids of the samples was determined according to the procedure developed by [23]. 50 mg of berberine was weighed and transferred quantitatively to 50 mL volumetric flask and the volume was filled with distilled water, and then 5 mL of each solution was transferred) and acidified to pH 2-2.5 with 1N HCl; 5 mL of the acidified solution was transferred to each centrifuge tube and to each tube 2 mL of the Dragendorff reagent was added and centrifuged at 2400 rpm/30 minutes; the supernatant was discarded and the residue was treated with 1 mL of absolute ethyl alcohol; 2 mL of 1% sodium sulfite was added and centrifuged at 2400 rpm/30 minutes, then the supernatant was discarded and the residue was treated with 2 mL of concentrated nitric acid; the resulting contents were transferred to 50 mL volumetric flask, the volume was filled with distilled water; 1 mL of this solution was taken and 5 mL of 3% (w/v) thiourea was added; the mixture of nitric acid and thiourea was used as white; for the sample was read at 435 nm. Linearity was obtained between 40.0 and 200.0 µg/mL. In the analysis, 40 mL of extract at 6.66 µg/mL concentration was used and acidified to pH 2-2.5 with 1N HCl and 4 mL of Dragendorff reagent and centrifuged at 2400 rpm 30 minutes.

2.5. *In vitro* Antiproliferative Assay

The cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content employing cell inhibition growth with sulfohodamina B dye exposure [24]. Doxorubicin (0.025-25 µg/mL) was used as a positive control. The MEPCR, MEPB, MEPC, MEPCA, MEPD, MEPL and MEPP were tested against ten cell lines, U251 (glioma, CNS), MCF-7 (breast), NCI-ADR/RES (ovarian expressing the phenotype of multiple drug resistance phenotype), 786-0 (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT29 (colon), K-562 (leukaemia) and HaCaT (human keratinocytes, immortalized non-tumoural cell) provided by the National Cancer Institute (Frederick, MD, USA). The experiments were performed in triplicate. The GI_{50} (growth inhibitory activity or cytostatic effect) values were determined through nonlinear regression analysis using Origin 8.0® software (OriginLab Corp.).

2.6. Acetylcholinesterase (AChE) Activity Assay in rat Brain

Male rat brain was separated into the cerebral cortex, hippocampus, hypothalamus and striatum and placed in a 10 mM Tris-HCl solution, pH 7.4. The tissues were homogenized with Tris-HCl solution at a 1:10 proportion (w/v) and then centrifuged at 3500 rpm for 10 min to yield a supernatant that was used for the enzyme assay. The procedure was performed at 4ºC, and the AChE activity was measured [25]. The protein concentration of the homogenized samples was determined by the Coomassie blue [26], using bovine serum albumin (BSA) as a standard and adjusted for each structure: cerebral cortex (0.6 mg/mL), hippocampus (0.8 mg/mL), hypothalamus (0.6 mg/mL) and striatum (0.4 mg/mL). The test containing DTNB (1.04 mmol) and potassium phosphate buffer (pH 7.2, 24 mmol) was incubated for 2 minutes at 30°C with 25 mL of the seven extracts (1; 0.75; 0.5 and 0.25 mg/mL), and the reaction was initiated by the addition of acetylthiocholine iodide (ACSCh, 0.8 mM). The reaction product was determined at 412 nm and the percentage inhibition was calculated. All the reactions were performed in triplicate.

2.7. Statistical Analyses

Data are presented as the mean ± standard error of the mean (SEM). The difference among the groups was determined by analyses of variance (one-way ANOVA) followed by the Newman-Keuls test. $P<0.05$ was considered to represent a significant difference.

Table 1. Data reported in the literature of the selected species from *Psychotria* and *Palicourea*.

Species	Chemistry Study	Biological Study
Palicourea crocea (Sw.) Schult.	Alkaloids (croceaine A and B, [9]), psychollatine, 3, 4-dihydro-1-(1-β-glucopyranosyloxy-1,4a, 5, 7a-tetrahydro-4-methoxycarbonylcyclopenta[c]pyran-7-yl)-β-carboline-N^2-oxide [10], brachycerine and palicroceaine [11] and flavonoid (Kaempferol 3-O-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside) [12]	-
Psychotria brachybotrya Müll. Arg. (syn. *P. gracilenta* Müll. Arg.)	Alkaloids (brachybotryne, N-oxide-brachybotrine and bufotenine) [13]	Effect against *Mycobacterium tuberculosis* [14]
Psychotria capillacea (Müll. Arg.) Standl.	-	-
Psychotria carthagenensis Jacq.	β-sitosterol, ursolic acid [15] and p-coumaric acid [16]	
Psychotria deflexa DC., (syn. *Palicourea deflexa* (DC.) Borhidi)	Alkaloid (harman-3-carboxylic acid) [17]	Acetylcholinesterase inhibitory in zebrafish in brains [17]
Psychotria leiocarpa Cham. & Schltdl.	Alkaloid (N-β-glucopyranosyl vincosamide) [18], iridoid glucosides (asperuloside and deacetylasperuloside) [5]	Anti-inflammatory, antioxidant, antimycobacterial on *Mycobacterium bovis* BCG growth [19] and analgesic activity [2, 20]
Psychotria poeppigiana Müll. Arg. (syn. *Cephaelis barcellana* (Müll. Arg.) Standl.	-	Anti-inflammatory activity, pulmonary metastases by 50% [21] and anti-hallucinogenic effect [22]

3. RESULTS AND DISCUSSION

3.1. Quantification of the Alkaloids

The alkaloids from *Psychotria* and *Palicourea* species were quantified in the methanol extract. The MEPCR showed values of 47.6 μg/g followed by MEPD (42.2 μg/g), MEPB (39.8 μg/g), MEPL (35.1 μg/g), MEPC (25.4 μg/g), MEPP (25.3 μg/g) and MEPCA (21.9 μg/g). Alkaloids have been characterized in some these species (Table **1**), corroborating with the observed data, highlighting *Palicourea crocea*, *Psychotria deflexa*, *P. brachybotrya* and *P. leiocarpa*.

Tryptamine-iridoid alkaloids have previously been reported in other *Psychotria* species, such as strictosamide isolated from *P. prunifolia* [27], *P. laciniata* [28], *P. nuda* [29], lialosideo from *P. suturella*, psicollatine from *P. umbellate*, brachycerine from *P. brachyceras* and *Palicourea crocea* [30, 11], as also croceaine A and B from *Palicourea crocea* [9], strictosidinic acid from *P. myriantha* [31], *Palicourea padifolia* [11], *Palicourea coriacea* [32], *P. barbiflora* [33], *P. acuminate* [34], strictosidine from *Palicourea padifolia* [11], *P. acuminate* [34], bufotenine from *P. brachybotrya* [3], calycanthine from *P. collina* [35] with some of them displaying a large range of effects on the central nervous system, such as anxiolytic, antidepressant and analgesic effects as well as to treat impairment of learning and memory acquisition [26, 31, 36-41].

In Rubiaceae, this compounds class can be originated by the condensation of a monoterpene secologonin with amino acid or the corresponding amine: tyramine producing alkaloids isoquinolinicos, tryptophan forming indol-carboxilic and tryptamine forming alkaloids indol-monoterpenic. The main characteristics of all monoterpene indole alkaloids is the biosynthetic route which presents estrictosidine as a precursor and tryptophan precursor key originated from the indol system [41], that can be proposed by the biosynthetic route *via* anthranilic acid (Fig. **1**).

3.2. Biological Activity

3.2.1. Antiproliferative Assay

The antiproliferative properties of 7 extracts from Rubiaceae were assessed against 10 human cell lines, using doxorubicin as the chemotherapeutic drug (positive control), which showed GI_{50} (growth inhibitory activity) values ranging from 0.01-0.20 μg/mL against all the tested human tumor cell lines (Table **2**). In relation to extracts obtained from the aerial parts (leaves and branches), *Palicourea crocea* (MEPCR) (GI_{50}: 22.87 μg/mL) was particularly effective against 786-0 cell line (Table **2**). Regarding the extracts obtained from the leaves, *Psychotria capillacea* (MEPC) and *P. leiocarpa* (MEPL) demonstrated high activities against specific cell lines. MEPL showed effective growth inhibition against specific cell lines, including OVCAR-3 (GI_{50}: 3.28 μg/mL), K-562 (GI_{50}: 5.26 μg/mL), HaCaT (GI_{50}: 27.20 μg/mL), PC-3 (GI_{50}: 34.92 μg/mL), MCF-7 (GI_{50}: 35.80 μg/mL) (Table **2**). MEPC showed activity against the OVCAR-3 (GI_{50}: 2.33 μg/mL) and U251 (GI_{50}: 16.66 μg/mL) cell lines (Table **2**). Extracts from MEPB, MEPCA, MEPD, MEPP, showed GI_{50} values greater than 40 μg/mL against certain tested human tumor cell lines (Table **2**). For the first time, data from this study support and corroborate the folk use of *Psychotria* species showing cytocidal effect on various human tumor cell lines. Alkaloids from this genus such as tryptamine and pyrrolidinoindole isolates of *P. henryi* showed inhibitory activity values less than 10 μM, against human osteosarcoma cell line [42]. *Psychotria forsteriana*

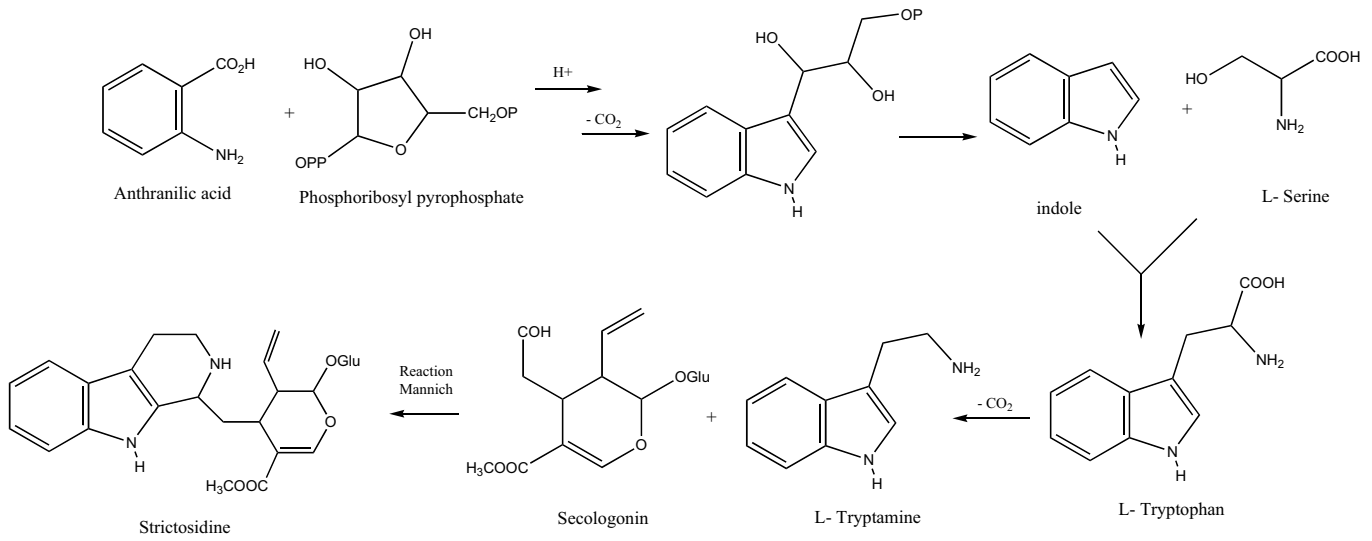

Fig. (1). Proposed biosynthetic indole monoterpene alkaloid route as strictosidine and tryptophan precursor.

Table 2. Cell growth inhibition (GI_{50} in µg/mL) for methanolic extracts from *Psychotria* and *Palicourea*.

Samples	Cell lines									
	U251	**MCF-7**	**NCI-ADR/RES**	**786-0**	**NCI-H460**	**PC-3**	**OVCAR-3**	**HT29**	**K-562**	**HaCaT**
Dox	0.03	0.02	0.44	0.02	0.01	0.08	0.16	0.20	0.07	0.03
MEPCR	70.79	>100	-	22.87	-	-	>100	-	80.26	>100
MEPB	78.35	85.20	-	-	>100	65.49	>100	-	81.71	>100
MEPC	16.66	40.11	62.81	-	-	-	2.33	83.69	86.23	-
MEPCA	51.87	50.65	60.60	73.81	-	>100	41.08	62.06	54.41	-
MEPD	93.19	>100	78.36	>100	49.45	51.56	46.03	>100	69.11	74.26
MEPL		35.80				34.92	3.28		5.26	27.20
MEPP	-	-	-	>100	-	57.77	-	-	>100	-

The response parameter (GI_{50}, growth inhibitory activity) was calculated for each tested extract cell line, and refer to the drug concentrations that resulted in a 50% reduction in the cellular growth relative to the untreated control cells.

leaf extracts showed significant cytotoxic activity in rat hepatoma cell lines and human leukemia cells [43]. Polyindole alkaloids isolated from *Psychotria oleoides, P. beccarioides* and *P. forsteriana*, with six, seven and eight tryptamine units tested on rat hepatoma cells, showed cytotoxic effect proportional to the increase in molecular weight [44]. Three novel cycles (Psyle A, C and E) of *Psychotria leptothyrsa* were monitored for cytotoxic activity, where cyclotides demonstrated potent cytotoxicity IC_{50} ¼ 0.64: 10 µM, and coexposure to cyclotides significantly elevating doxorubicin-induced toxicity, IC_{50} ¼ 0.39: 0.76 µM [45].

3.2.2. AChE Assay

Ellman method is rapid and simple, employing acetylthiocholine iodide which is hydrolyzed by AChE to liberate thiocholine, giving a reaction with Ellman's reagent, DTNB to form yellow coloured 5-thio-2-nitrobenzoate that can be quantified at 405 nm [25]. In this study, we evaluated *in vitro* effects of *Psychotria* and *Palicourea* extracts on AChE activity on rat's brain. Results demonstrated that *Psychotria* extracts altered AChE activity, but these alterations were not homogeneous in all evaluated brain structures. The bioactive extracts were tested at concentrations of 1.0 mg/mL. Significant AChE inhibition in the hippocampus, was observed by four extract, *Palicourea crocea* (MEPCR), *Psychotria deflexa* (MEPD), *P. brachybotrya* (MEPB) and *P. leiocarpa* (MEPL) with 70%, 57%, 50% and 40% ($P < 0.001$), respectively (Fig. **2A**). The data seem to be associated with the level of alkaloids present in the extracts these species.

Psychotria poeppgiana (MEPP) and *P. capillacea* (MEPC) inhibiting 21% ($P < 0.01$), compared to the control (Fig. **2A**). In this cerebral structure only, the extract of *P. carthagenensis* (MEPCA) increased AChE activity (13%) (Fig. **2A**). In striatum and hypothalamus, five extract significantly affected AChE inhibition, highlighting MEPCR (67%

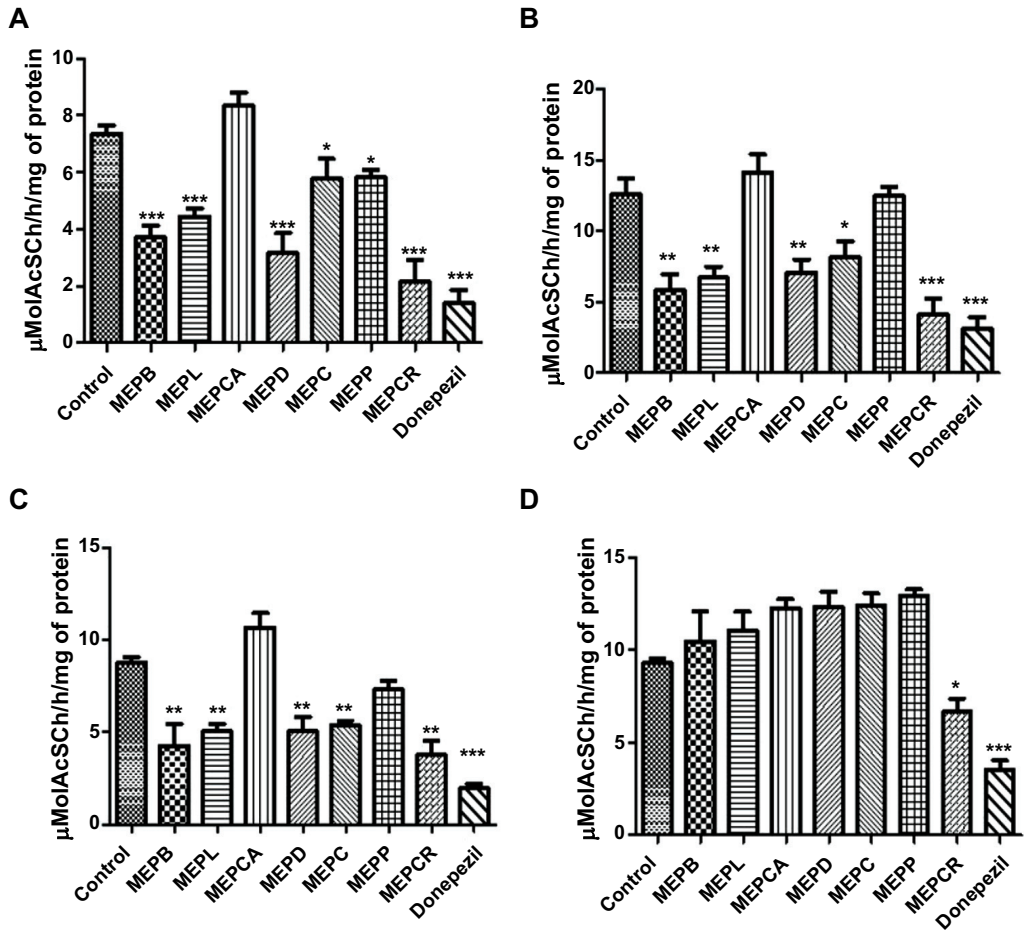

Fig. (2). *In vitro* effects from *Psychotria* and *Palicourea* on the AChE activity in the hippocampus (**A**), striatum (**B**), hypothalamus (**C**) and cerebral cortex (**D**) of rats. Each bar represents mean ± SEM. AChE activity is expressed as µmol of acetylthiocholine (AcSCh)/h/mg of protein. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with the control group. One-way ANOVA followed by the Newman-Keuls test.

and 57%), followed by MEPB (53% and 51%), MEPL (46% and 43%), MEPD (42% and 41%) and MEPC (38% and 35%) ($P < 0.01$), respectively, when compared to the control (Fig. **2B, C**), and the increase of the AChE by MEPCA (12% and 21%), respectively (Fig. **2B, C**). Differently, the enzyme activity was higher (≤ 38%) in all groups that received *Psychotria* extracts, when compared to the cerebral cortex control, and only MEPCR significantly inhibited AChE activity (28%) in the dose studied (Fig. **2D**). Donepezil inhibited AChE activity in all brain structures studied ($P < 0.001$) (Fig. **2**).

Palicourea crocea extract (MEPCR) presented ≤ 70% inhibition in all cerebral structures, and compounds that are able to activate this enzyme are a scientific tool to counteract organophosphate poisoning, highlighting the *P. carthagenensis* extract (MEPCA), ≤ 32% [46].

Whereas, in relationship to AChE activity in brain regions can be explained to the fact that AChE exists in a variety of molecular forms that differ in solubility and type of membrane attachment rather than in catalytic activity. The drugs that present AChE inhibition mechanisms are called anticholinesterases or indirect cholinergics. AChE when the blockade is unable to hydrolyze ACh, thus, this neurotransmitter tends to remain active for a longer period in the synaptic cleft, a fact that increases the cholinergic transmission, being indicated in the treatment of dementia associated with Alzheimer's (AD) and Parkinson's diseases [47, 48].

The inhibition of acetylcholinesterase (AChE) is the most effective therapeutic approach to optimize the cholinergic system in patients with AD. Several plant-derived alkaloids (rivastigmine, galanthamine and Huperzine A) that inhibit AChE can be used to treat early stages of AD, since these compounds increase the endogenous levels of acetylcholine to boost cholinergic neurotransmission [49, 50]. A review carried out in 2006 [51] identified 139 alkaloids active for AChE inhibition. The alkaloids with the indole nucleus synthetic and naturally have stimulated the search for this class of compounds in cancer therapy, which can act through multiple mechanisms, such as intercalating into DNA and inhibiting Topoisomerase I and II [52, 53]. Thus, a search for the alkaloids present in the studied species demonstrated by the preliminary quantification, corroborating with the biological data, arouses the interest in the continuation of the bioguided research, in the search to demonstrate the active molecules. Studies are now in progress to isolate and characterize the constituents present in the plant that could account for the reported pharmacological effects and also to further characterize their sites of action.

CONCLUSION

In conclusion, we reported here that the extracts from the *Psychotria* and *Palicourea* presented alkaloids in the preliminary analysis. The first evaluation of the biological activities of these species, showed that they possess potential anticholinesterase activity, with a greater effect on the cerebral structure of the hippocampus, and antiproliferative effect on various human tumor cell lines.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experimental procedures were carried out in accordance with the U.S. National Institutes of Health and approved by the Animal Ethics Committee from UFGD (Nbr. 14/2015 and Nbr. 17/2017). The experiments were conducted using three male *Wistar* rats (200-300 g) from the Federal University of Grande Dourados - UFGD, Mato Grosso do Sul, Brazil. The animals were maintained at a constant temperature (23 ± 1ºC) on a 12-hour light/dark cycle with free access to food and water.

HUMAN AND ANIMAL RIGHTS

No humans were involved in this study, the reported experiments on animals were in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/Guide-for-thecare-and-use-of-laboratory-animals.pdf) published by the National Academy of Sciences.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Zefa V. Pereira collected and identified plant; Carla R.F. Volobuff, Pedro C.O. Junior, Diego C. Ferreira, Claudia A.L. Cardoso and Anelise S.N. Formagio designed the chemistry and anticolinesterasic study, Ana L.T.G. Ruiz, Mary A. Foglio and João E. de Carvalho performed the antitumoral assays. All authors participated in the design, interpretation, and analysis of the data and approved the final manuscript.

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5.2 Artigo II: *Psychotria leiocarpa* extract and vincosamide reduce chemically-induced inflammation in mice and inhibit the acetylcholinesterase activity

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ORIGINAL ARTICLE

Psychotria leiocarpa Extract and Vincosamide Reduce Chemically-Induced Inflammation in Mice and Inhibit the Acetylcholinesterase Activity

Anelise Samara Nazari Formagio,[1,2,6] Carla Roberta Ferreira Volobuff,[2] Candida Aparecida Leite Kassuya,[2] Claudia Andréa Lima Cardoso,[3] Maria do Carmo Vieira,[4] Zefa Valdevina Pereira,[1] Mariane Cristovão Bagatin,[5] and Gisele de Freitas Gauze[5]

Abstract—Species from *Psychotria* are used in folk medicine against inflammatory diseases, respiratory disturbances, and anti-hallucinogenic. In the present study, the compound vincosamide (PL-1) was identified for the first time in methanolic extract of the *Psychotria leiocarpa* (ME-PL) leaves, as well as the anti-inflammatory and anticholinesteric effects in rodents and molecular docking simulations. The fractionation of the chloroform fraction (*CF*-PL) through chromatographic methods afforded the known compound PL-1. The anti-inflammatory activity of the ME-PL (30, 100, and 300 mg/kg) and PL-1 (3, 30, and 100 mg/kg) was analyzed using experimental models: paw edema, pleurisy, and mechanical and thermal hyperalgesia induced by carrageenan. The anticholinesterase activity of the ME-PL (30 and 100 mg/kg) and PL-1 (30 mg/kg) was showed by acetylcholinesterase (AChE) inhibitory in brain structures. The molecular docking simulations were performed using Molegro Virtual Docker v6.0. Overall, the results indicated that ME-PL and PL-1 demonstrated an anti-edematogenic effect in Cg-induced paw edema, leukocyte migration in the pleurisy model, and significantly reduced mechanical hyperalgesia, cold response to acetone in mice. The samples exhibited maximal inhibition of enzyme acetylcholinesterase (AChE) in the frontal cortex. The molecular coupling of PL-1 with the AChE showed significant

[1] Faculty of Biological and Environmental Sciences, Federal University of Grande Dourados UFGD, MS, Brazil, Dourados, Brazil
[2] Faculty of Health Sciences, Federal University of Grande Dourados UFGD, do Sul State, Mato Grosso, Brazil
[3] Course of Chemistry, University of Mato Grosso do Sul UEMS, do Sul State, Mato Grosso, Brazil
[4] Faculty of Agricultural Science, Federal University of Grande Dourados UFGD, do Sul State, Mato Grosso, Brazil
[5] Department of Chemistry, State University of Maringá UEM, Paraná, Brazil
[6] To whom correspondence should be addressed at Faculty of Biological and Environmental Sciences, Federal University of Grande Dourados UFGD, MS, Brazil, Dourados, Brazil. E-mail: aneliseformagio@ufgd.edu.br; aneliseformagio@gmail.com

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interactions with the catalytic and peripheral site, corroborating the activity presented in the inhibition assay. The acute administration of ME-PL did not cause signs of toxicity in the treated animals. The results showed that *P. leiocarpa* inhibited AChE and anti-inflammatory activity, and alkaloid vincosamide could be responsible, at least in part, for the observed effects, supporting the popular use of this genus.

KEY WORDS: Grandiúva-de-anta; alkaloid; acetylcholinesterase; inflammation; carrageenan; molecular docking.

INTRODUCTION

Grandiúva-de-anta as is known *Psychotria leiocarpa* Cham. & Schlecht. is a small shrub (2 m in height) native of Argentina, Paraguay, and Brazil [1]. Plants in this genus are used in folk medicine against inflammatory diseases, respiratory disturbances, and anti-hallucinogenic [2–5]. Monoterpene indolic alkaloid (*N*-β-glucopyranosyl vincosamide) is the main constituent of the extract from *P. leiocarpa* leaves collected in Morro Santana in Porto Alegre, Brazil [6, 7], along with iridoid glucosides, asperuloside, and deacetylasperuloside [8]. The essential oil of the leaves was characterized exclusively by sesquiterpenes, highlighting bicyclogermacrene, and germacrene D [9]. Pharmacological effects for extract have been reported, such as antioxidant, antimycobacterial on *Mycobacterium bovis* BCG growth [10], and analgesic [11, 12]. Also, anti-inflammatory effects were exhibited presenting dose analgesic activity non-dependent and non-reversible by naloxone, configuring non-specific action in experimental models in rodents [11].

In previous studies with species of *Psychotria*, our group reports the antioxidants activity in different *in vitro* assays, including DPPH, ABTS radicals, and β-carotene bleaching activities of four species of *Psychotria*, among them *P. leiocarpa* [13] and the isolation of a new dimeric tryptamine-related alkaloid, brachybotryne, and the corresponding N-oxide derivative, brachybotryne N-oxide, and bufotenine from *P. brachybotrya* [14].

In this sense, considering the folk use of *Psychotria* genus against process regulated by inflammatory mediators and mental disorders, but without scientific evidence of this potential therapeutic application, prompted research of *P. leiocarpa*. Thus, this study is aimed at evaluating the anti-inflammatory and anticholinesterasic effect in mice of the methanolic extract and alkaloid (vincosamide) obtained from *P. leiocarpa* leaves and molecular docking simulations.

Considering that molecular docking plays an important role in rational drug design, we also conducted a theoretical molecular docking study to evaluate how the alkaloid binds to acetylcholinesterase (AChE). Alzheimer's disease (AD) is a chronic neurodegenerative disease, pathologically associated with a highly atypical inflammatory response, which processes by the activation of the macrophage populations in the brain, characterized by memory impairment, cognitive dysfunction, behavioral disturbances, and deficits in the activities of daily living [15].

MATERIAL AND METHODS

Collection and Plant Identification

Psychotria leiocarpa fresh leaves were collected in Dourados (S 22° 17′ 38.4″, W 54° 95′ 94.2), Mato Grosso do Sul, Brazil. Botanical identification was performed by Profa. Dra. Zefa Valdevina Pereira, and a specimen (DDMS-5007) was deposited in the Herbarium of the Faculty of Biological and Environmental Sciences, Federal University of Grande Dourados - UFGD, Mato Grosso do Sul, Brazil. A scheme of experimental procedure conducted in this work from *P. leiocarpa* leaves is showed Fig. 1.

Isolation and Identification of Alkaloid

The air-dried and powdered leaves of *P. leiocarpa* (560 g) were extracted by maceration with methanol P.A. (2 L) at room temperature for 10 days. After filtration, evaporation of the solvent under vacuum provided the methanolic extract (ME-PL) (41 g). A portion of this extract (25 g) was dissolved in MeOH/H_2O (1:1) and partitioned with *n*-hexane, chloroform ($CHCl_3$), and ethyl acetate (EtOAc) with further evaporation of the solvents resulting in the *n*-hexane (HF-PL; 4.04 g), chloroform (*CF*-PL; 6.45 g), ethyl acetate (EAF-PL; 4.68 g), and aqueous-methanol (AMF-PL; 9.24 g) fractions. Part of the *CF*-PL was fractionated by CC on silica gel (*n*-hexane/EtOAc 10 to 80% and EtOAc/MeOH 10 to 70%), resulting in the subfractions *CF*-PL-1 to *CF*-PL-13. The purification of subfraction *CF*-PL-4 on preparative thin-layer chromatography eluted in EtOAc/MeOH 30% yielded PL-1–labeled sample. **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

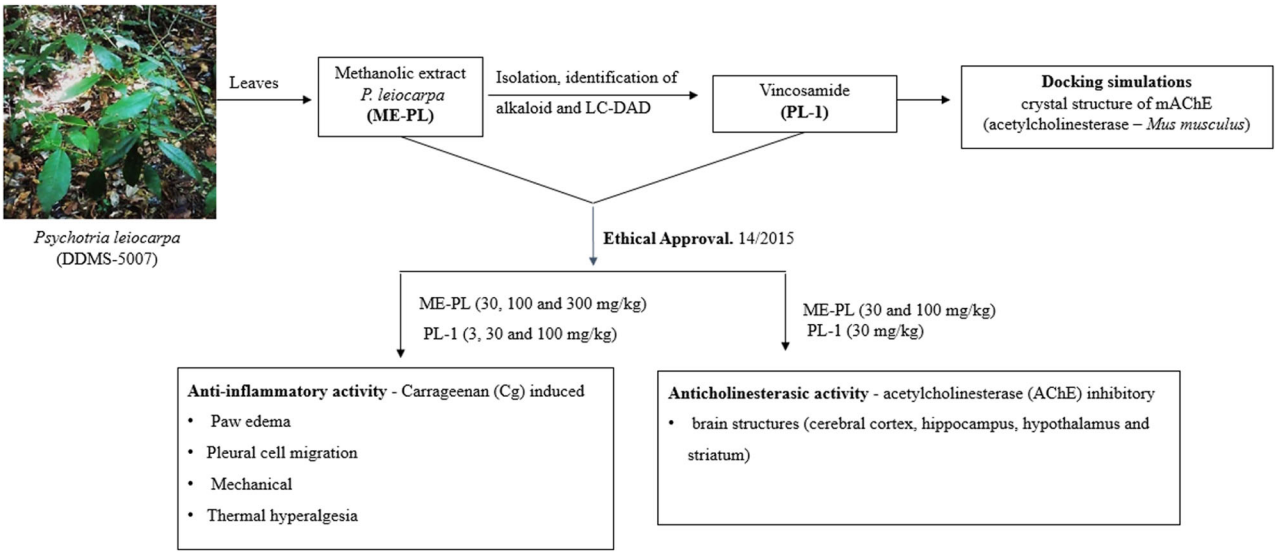

Fig. 1. Experimental procedure from *P. leiocarpa* leaves: Chemistry and biological assay.

(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; 1.5 Hz, 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), 54.4 (C-3), 41.2 (C-5), 22.1 (C-6), 109.03 (C-7), 127.9 (C-8), 118.8 (C-9), 120.0 (C-10), 122.5 (C-11), 111.9 (C-12), 138.3 (C-13), 32.6 (C-14), 27.3 (C-15), 109.0 (C-16), 149.0 (C-17), 120.5 (C-18), 133.9 (C-19) 44.5 (C-20), 97.3 (C-21), 166.0 (C-22), 99.6 (C-1′), 74.8 (C-2′), 77.9 (C-3′), 71.5 (C-4′), 78.3 (C-5′), 62.6 (C-6′).

LC Analysis

The ME-PL (10 μg/mL) was sonicated for 15 min and centrifuged at 10.000*g* for 15 min, further filtrated in 0.45 μm, and analyzed by LC. An analytical LC (LC-6AD, Shimadzu, Kyoto, Japan) system with a diode array detector (DAD) monitored at $\lambda = 200$–800 nm. The LC column was a C-18 (25 cm × 4.6 mm; particle size, 5 μm; Luna, Phenomenex, Torrance, CA, USA). In each analysis, the flow rate and the injected volume were set as 0.5 mL min^{-1} and 20 μL, respectively. All chromatographic analyses were performed at 22 °C. Elution was carried out using a binary mobile phase of eluent A: water with 0.05% trifluoroacetic acid and eluent B: acetonitrile; mobile phase gradient was as follows: 15–45% B in 35 min, 45–90% B in 17 min, 90% B for 3 min, and return of initial condition in the 5 min. Vincosamide standard was isolated in the laboratory diluted to 10 μg/mL initial concentration. Sample PL-1 was quantified by external calibration after appropriate dilutions in 0.01–10 μg/mL range.

Animals

The anti-inflammatory and anticholinesterasic assay were conducted using male and female *Swiss* mice (50 days old, 20–30 g, $n = 6$) obtained from the University Federal da Grande Dourados (UFGD). The animals were maintained at a constant temperature (23 ± 1 °C) on a 12-h light/dark cycle with free access to food and water.

Carrageenan (Cg)-Induced Paw Edema

The inhibitory effects on paw edema were evaluated as previously described [16]. For paw edema assay, male mice ($n = 6$) were orally treated with ME-PL (30, 100, and 300 mg/kg) and PL-1 (3, 30, and 100 mg/kg), control (saline solution), or positive control (DEX; 1 mg/kg), and after 1 h, paw edema was induced by injecting of Cg (300 μg/paw, 50 μL insterile 0.9% saline). Edema was

measured after 2, and 4 h with a paw plethysmometer (PANLAB Harvard).

Cg-Induced Pleurisy

The inhibitory effects on pleural cell migration were evaluated as previously described [17]. Female mice (n = 6) were treated with ME-PL (30, 100, and 300 mg/kg, v.o.), PL-1 (3, 30, and 100 mg/kg, v.o.), control (saline solution), DEX (1 mg/kg, v.o), and 1 h before, Cg (300 μg, 0.1 mL in PBS, pH = 7.4) was applied to the pleural cavity [18]. To determine the total number of leukocytes, an aliquot of 20 μL (exudates) was diluted in Turk solution (1:20) in a Neubauer chamber.

Mechanical Hyperalgesia

The mechanical sensitivity of the hind paw was measured by determination of withdraw thresholds. Nociceptive thresholds (g) were estimated using an electronic version of the Von Frey test (Insight ®, EFF 301, Digital analgesymeter). Separate groups of male mice (n = 6) were orally treated with ME-PL (30, 100, and 300 mg/kg) and PL-1 (3, 30, and 100 mg/kg), control or DEX. After 1 h, from respective treatment, an intraplantar injection of the carrageenan (300 μg/paw), in the right paw, was made while in the left paw an injection of saline. Constant pressure was applied to the plantar surface of the right hind paw with the analgesiometer until the mice vocalized or removed the paw, indicating the level of mechanical sensitivity induced by sensitization, 3 and 4 h after carrageenan administration [19].

Cold Thermal Stimulation

The sensitivity to cold was evaluated by the acetone test [20]. The animals were housed in suspended platform, and acetone (20 μL) was distributed in the skin of plantar surface of the right hind paw. The reaction, as indicated by paw licking, shaking, or rubbing the paw, was observed and recorded. The duration of the testing was 30 s.

AChE Assay in Brain Structures

Four groups of six male mice were separately treated by gavage 7 days with ME-PL (30 and 100 mg/kg), PL-1 (30 mg/kg), and control (0.9% saline). On the last day of the experiments, 1 h after samples' last dose, the animals were killed by decapitation and the mice brains were collected and separated into the cerebral cortex, hippocampus, hypothalamus, and striatum and placed in a 10 mM Tris–HCl solution, pH 7.4, on ice. The tissues were homogenized in a glass potter in the Tris–HCl solution at a 1:10 proportion (w/v) and then centrifuged at 3500 rpm for 10 min to yield a supernatant that was used for the enzyme assay. The procedure was performed at 4 °C, and the AChE activity was measured according to the spectrophotometric method previously described [21]. The test medium containing DTNB (1.04 mmol) and potassium phosphate buffer (pH 7.2, 24 mmol) was incubated for 2 min at 30 °C with 25 mL of the sample, and the reaction was initiated by the addition of acetylthiocholine iodide (ACSCh, 0.8 mM). The reaction product was determined at 412 nm for 2 min. The enzyme activity was expressed in μmol ACSCh/h/mg protein. The protein concentration of the homogenized samples was determined by the Coomassie blue method [22] using bovine serum albumin (BSA) as a standard, and protein concentrations were adjusted for each structure: cerebral cortex (0.7 mg/mL), hippocampus (0.8 mg/mL), hypothalamus (0.6 mg/mL), and striatum (0.4 mg/mL).

Docking Simulations

The crystal structure of mAChE (acetylcholinesterase—*Mus musculus*) complexed with choline (code ID: 2HA3) was obtained from the Protein Data Bank, and the molecular docking studies were performed using the Molegro Virtual Docker v6.0 [23]. This program restricts enzyme torsion angles but allows the flexibility of each tested ligand. All water molecules and ions were removed from the structures. The protocol for Molegro v6.0 uses the Moldock Score as a scoring function and the Moldock Optimizer as a search algorithm, with search sphere radiusset to 11 Å around the catalytic site. These protocols restricted the torsion angles of the enzyme, but allowed flexibility for the tested ligand. The results were ranked using the Rerank scores of the ligands, and all other options were set to the default value. Redocking simulations were repeated five times with each program, and the results were reproducible. The structure of vincosamide used in docking calculations was obtained from ChemSpider (http://www.chemspider.com) code id: 8339362.

Acute Toxicity

The acute toxicity study was based on protocol 425 [24, 25]. According to the protocol established, nine animals were used; each received a single oral administration by gavage of the ME-PL. Initially, one of the animals received a dose of 175 mg/kg and was observed at 30 min and 1, 2, 4, 6, 12, 24, and 48 h. After this period,

a second animal received a dose of 560 mg/kg, and after 48 h, the third animal received a dose of 1792 mg/kg. After an additional 48 h, a fourth animal received a dose of 2000 mg/kg. After the last dose had been administered, no deaths were observed, and according to the protocol, four more animals received 2000 mg/kg. The control group received the vehicle used for diluting ME-PA (drops of DMSO + distilled water). The animals were observed for signs of toxicity over 14 days. Behavioral observations (reflexes, tremors, convulsions, lacrimation, cyanosis, salivation, piloerection, muscle tone, and motor coordination) and mortality were analyzed. After 14 days of treatment, the animals were weighed and subsequently euthanized.

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). The difference among the groups was determined by analyses of variance (one-way ANOVA) followed by the Newman–Keuls test. $P < 0.05$ was considered to represent a significant difference.

RESULTS

Phytochemical Study

Compound PL-1 was quantified by HPLC resulting in 138.9 ± 0.3 mg/g yield (tr = 24.10 min) (Fig. 2).

The ^{1}H and ^{13}C NMR data of compound PL-1 were characterized by the signals for an indole ring at δ_H 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, and 7.02 (ddd, J = 7.8; 7.5; 1.2 Hz, H-10)/δ_C 120.00 in the region of aromatics. The signals for H-19 at δ_H 5.54 (ddd, J = 17.1; 10.2; 1.8 Hz) and at δ_H 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.

Biological Activity

Paw Edema

The ME-PL (300 mg/kg) ($P < 0.05$) and compound PL-1 (100 mg/kg) ($P < 0.01$) presented a decrease in the formation of edema in 1 h, with maximal inhibition of 52.38 ± 2% and 61.30 ± 3%, respectively. In the course of the experiment, ME-PL (100 and 300 mg/kg) ($P < 0.05$) and PL-1 (100 mg/kg) ($P < 0.001$) showed a significant decrease in edema compared to the group control with inhibitions of 40.47 ± 4%, 46.42 ± 3%, and 73.21 ± 2%, after 2 h, respectively, and 35.89 ± 1%, 39.10 ± 2%, and 67.94 ± 2%, after 4 h (Fig. 3). The positive control DEX (1 mg/kg) ($P < 0.001$) significantly reduced edema in 1 h (73.21 ± 2%), 2 h (76.19 ± 4%), and 4 h (77.56 ± 4%) (Fig. 3).

Pleural Cell Migration

It was observed that ME-PL at doses of 30, 100, and 300 mg/kg ($P < 0.001$) inhibited leukocyte migration by 84.76 ± 2%, 86.71 ± 1%, and 88.85 ± 1%, respectively, 4 h after carrageenan injection, when compared to the control group. In relation to PL-1, all doses of 3, 30, and 100 mg/kg demonstrated inhibition of 76.41 ± 1%, 88.35 ± 2%, and 88.51 ± 1%, respectively, (Fig. 4). DEX (91.52 ± 2%) inhibited inflammation, showing effectiveness as anti-inflammatory positive control (Fig. 4).

Anti-Hyperalgesic Effects

The treatment with ME-PL (100 and 300 mg/kg) ($P < 0.05$) and PL-1 (100 mg/kg) ($P < 0.01$) showed significantly anti-hyperalgesic effects, with reduction of 42.50 ± 6%, 55.00 ± 3%, and 70.00 ± 2% in 3 h (Fig. 5a), respectively. At 4 h after carrageenan-treated with maximal inhibition for ME-PL at dose 300 mg/kg ($P < 0.001$) (85.10 ± 3%) and for the PL-1 at dose 100 mg/kg ($P < 0.001$) (90.86 ± 4%), compared with control (Fig. 5b). Treatment with DEX (1 mg/kg) ($P < 0.001$) was able to reduce the mechanical hyperalgesia induced by carrageenan by 97.5 ± 1% after 3 h (Fig. 5a) and by 100% after 4 h (Fig. 5b).

Cold Sensitivity

The ME-PL demonstrated a potential reduction in sensitivity to acetone cold stimulus with a reduction of 63.15 ± 2% evaluated in 3 h (Fig. 6a) and 67.26 ± 2% in 4 h (Fig. 6b) for the doses 300 mg/kg ($P < 0.01$), respectively. PL-1 also presented a potential reduction in the dose evaluated (100 mg/kg) ($P < 0.001$) with 63.81 ± 2% in the time of 3 h (Fig. 6a) and 64.28 ± 2% in 4 h (Fig. 6b). The DEX (1 mg/kg) ($P < 0.001$) control showed high inhibition of 76.97 ± 1% and 85.11 ± 1% when evaluated in 3 h and 4 h, respectively (Fig. 6).

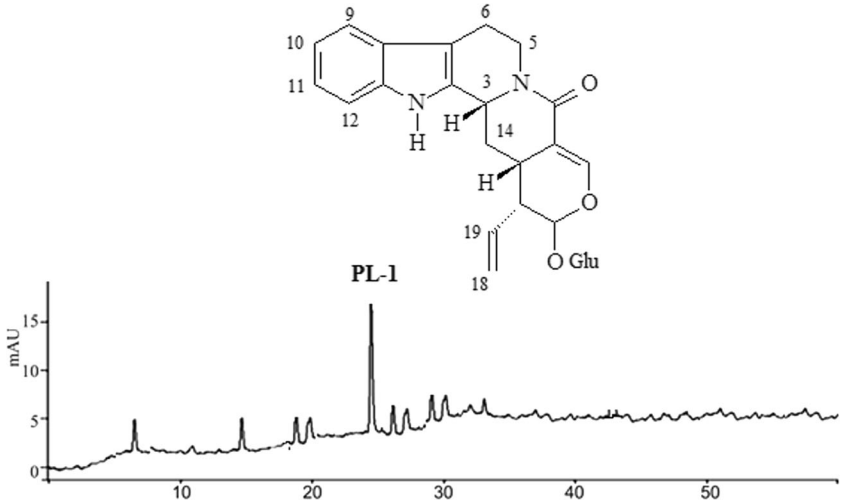

Fig. 2. Chromatogram representative of ME-PL with PL-1 isolated from methanolic extract of *P. leiocarpa* (ME-PL) collected in Dourados-MS.

Anticholinesterase Activity

Results demonstrated that in the oral administration with ME-PL and PL-1, the AChE activity was altered in different brain structures (Fig. 7). Figure 7a shows that in the groups treated with ME-PL (30 and 100 mg/kg) ($P < 0.001$) and PL-1 (30 mg/kg) ($P < 0.001$) acetylcholinesterase inhibitory activity was significantly decreased in the cerebral cortex, 47 ± 3%, 44 ± 1%, and 36 ± 1%, respectively, compared to the control group. In the hippocampus (Fig. 7b), the level of inhibition was also observed in the animals treated with ME-PL at 100 mg/kg ($P < 0.01$) (32 ± 4%), 30 mg/kg (31 ± 3%), and 30 mg/kg (28 ± 3%) doses of PL-1. Moreover, in the hypothalamus, a significant inhibition of 13 ± 1%, 12 ± 1%, and 14 ± 2% at doses of 100 and 30 mg/kg (ME-PL) ($P < 0.05$) and 30 mg/kg (PL-1) ($P < 0.05$), respectively, compared to the control group (Fig. 7c) was observed.

Enzyme-Inhibitor Interactions

Figure 8 shows that PL-1 interacts with both the anionic catalytic (active centre) and peripheral (PAS) sites, and according to the literature [26, 27], the following residues represent these sites in mAChE: His447, Trp86, Glu334, and Ser203; Tyr124, Trp286, and Tyr341.

Figure 9 supplements this information and highlights the hydrogen bonds with His447, Tyr124, and Arg296 that are very important for anchoring the ligand in the active site. In conclusion, vincosamide, a component from the methanolic extract of *P. leiocarpa* (ME-PL) leaves collected in Dourados-MS, is involved with acetylcholinesterase inhibitory activity.

Toxicity

The assessment of acute toxicity was conducted for 14 days to determine the lethal dose (LD_{50}). The animals used in this study were exposed to ME-PL, and no clinical signs of toxicity were observed at any dose. No deaths were reported.

DISCUSSION

Vincosamide (Fig. 2) was isolated from ME-PL collected in Dourados-MS and characterized by NMR spectral data and further evaluated for pharmacological activities. A comparison with the literature spectral data for vincosamide and the epimer strictosamide maintained the relative configuration at α position of H-3 [28]. Structural characterization for PL-1 was consistent with that described in the literature for vincosamide [29] isolated from *Nauclea orientalis* (Rubiaceae). To the best of our knowledge, this is the first time the compound was reported in *P. leiocarpa*. The N-β-glucopyranosyl vincosamide was found in the leaves of *P. leiocarpa* collected in Porto Alegre/Brazil, and vincosamide can be obtained with a small hydrolysis yield [6, 30]. These variations may have relationship to the environmental conditions to which the plant is exposed.

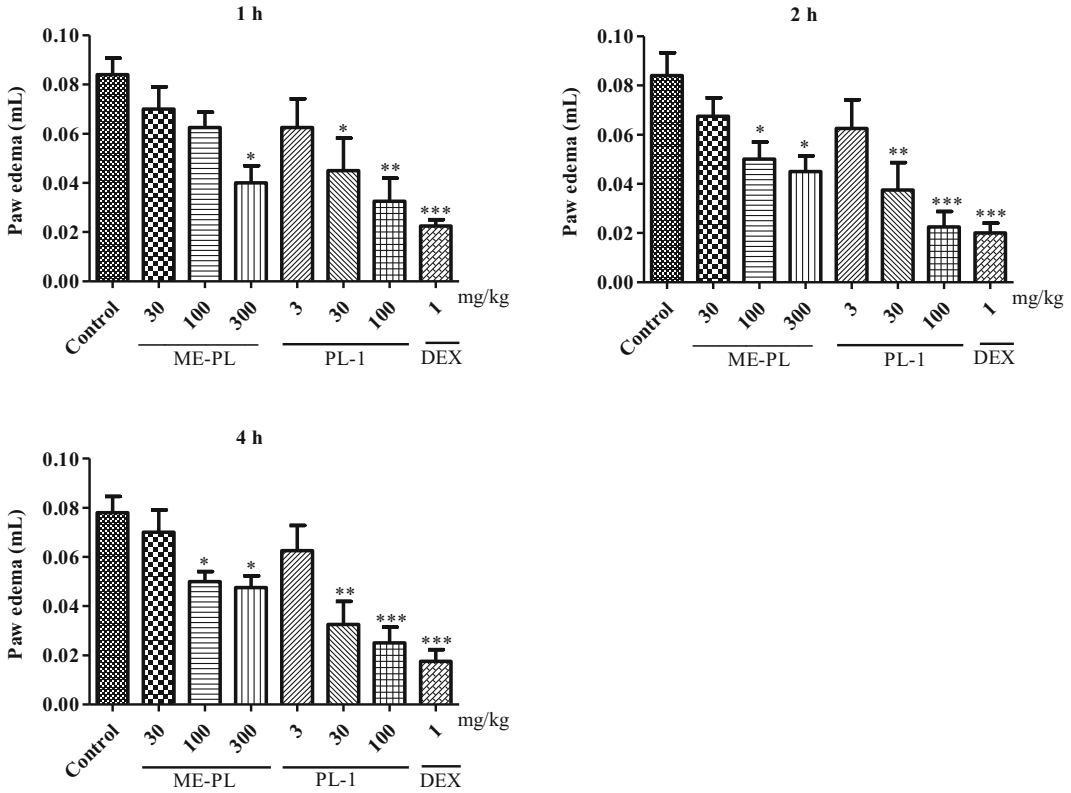

Fig. 3. Effects of ME-PL (30, 100, and 300 mg/kg) and PL-1 (3, 30, and 100 mg/kg), control (0.9% saline), or DEX (1 mg/kg), on paw edema evaluated in 1 h, 2 h, and 4 h after carrageenan induction. The data are represented as the means ± SEM of animals ($n = 6$). The * symbol compared the treated group in relation to the control group: *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, #$p < 0.001$, one-way ANOVA followed by Student–Newman–Keuls.

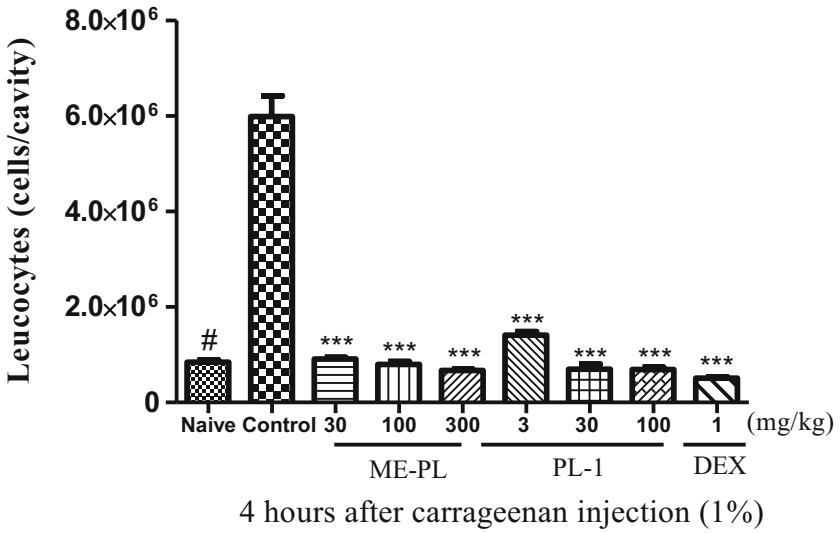

Fig. 4. Effects of ME-PL (30, 100, and 300 mg/kg) and PL-1 (3, 30, and 100 mg/kg), control (0.9% saline), or DEX (1 mg/kg), on total leucocytes induced by carrageenan in the pleural cavity of mice. The data are represented as the means ± SEM of animals ($n = 6$). The # symbol indicates the statistical differences of naïve and control group ($p < 0.001$) while the * compared treated group in relation to control group: *** $p < 0.001$. Differences between groups were analyzed by one-way ANOVA followed by the Newman–Keuls test.

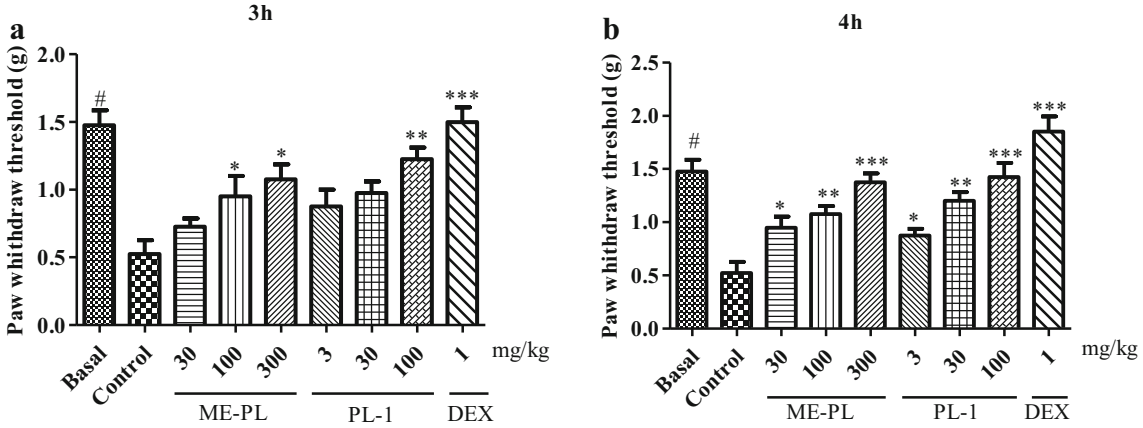

Fig. 5. Effect of oral administration of ME-PL (30, 100, or 300 mg/kg, p.o.), PL-1 (3, 30, or 100 mg/kg, p.o.), on mechanical hyperalgesia in mice. The animals received control (0.9 saline) or DEX (1 mg/kg). In **a,** the mechanical hyperalgesia was measured with a digital analgesy meter for 3, and in **b,** 4 h after carrageenan administration. Each bar represents the mean ± SEM of six animals. Differences between groups were analyzed by one-way ANOVA followed by the Newman–Keuls test, being $^{*}p < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $\#P < 0.001$ when compared with the control group.

In reviewing the literature, other species of this genus show indole chromophore (tryptamine-iridoid) alkaloids and some of them found in *Psychotria* display a large range of effects on the central nervous system, such as anxiolytic, antidepressant, and analgesic effects, as well as the impairment of learning and memory acquisition [18, 31–40].

The present study represents the first research into the anti-inflammatory and anticolinesterasic effects of the ME-PL and isolated compounds PL-1 (vincosamide) of leaves from *P. leiocarpa*. The focus of this work in inflammatory process was to widely assay the *P. leiocarpa* extract and isolated compound vincosamide in time response analysis (in paw inflammatory model), dose response aspects, and some inflammatory and nociceptive parameters in two models of *in vivo* inflammation. Anti-inflammatory activity of the ME-PL and PL-1 in acute inflammation was assessed by an induction model with Cg-induced paw edema and pleurisy (Figs. 3 and 4) because in the literature [10] only showed a potential *in vitro* activity of *P. leicocarpa* in nitric oxide activity. ME-PL and PL-1

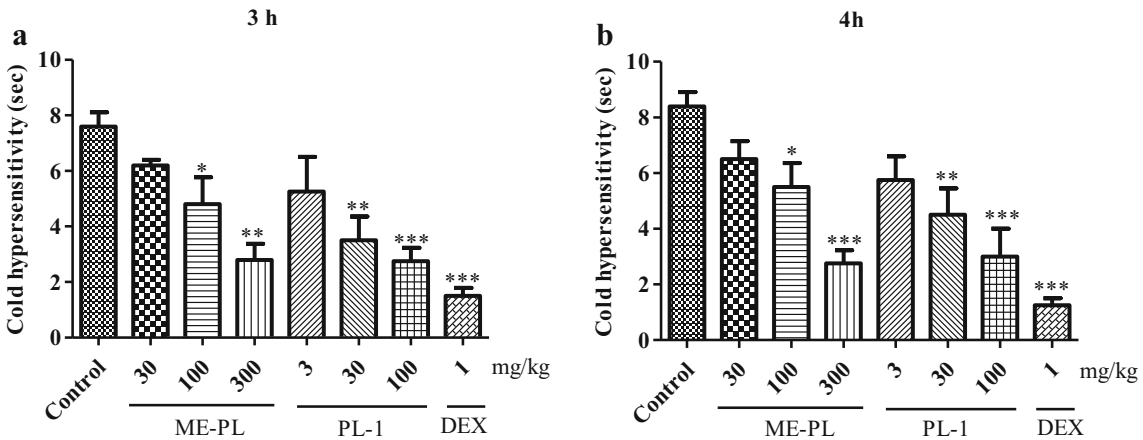

Fig. 6. Effect of oral administration of ME-PL (30, 100, or 300 mg/kg, p.o.), PL-1 (3, 30, or 100 mg/kg, p.o.), on the cold sensitivity induced by acetone in mice. The animal's control (0.9 saline) or DEX (1 mg/kg). The cold sensitivity was measured 3 and 4 h after carrageenan administration. Each bar represents the mean ± SEM of six animals. Differences between groups were analyzed by one-way ANOVA followed by the Newman–Keuls test, being $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $\#P < 0.001$ when compared with the control group.

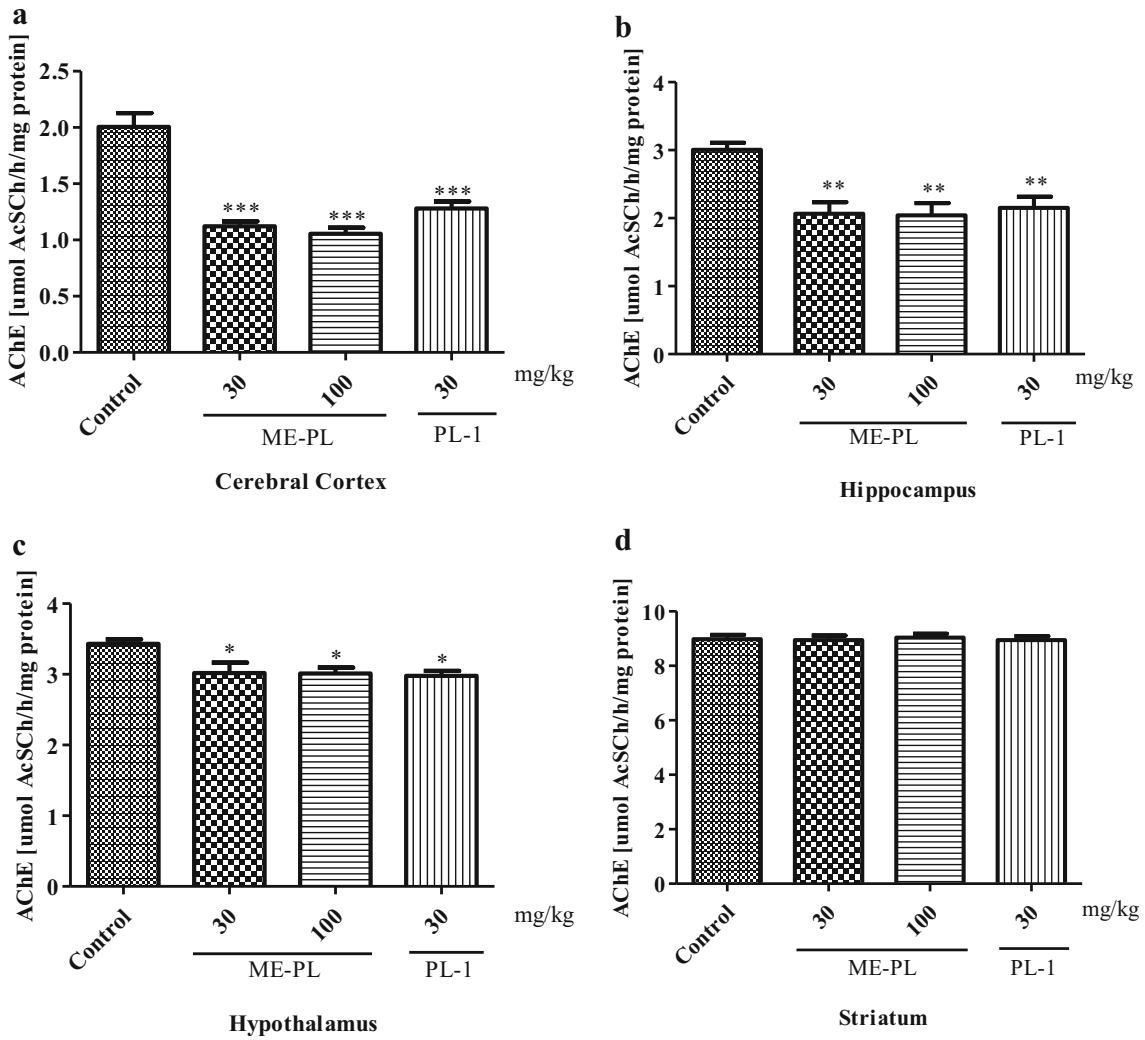

Fig. 7. Effect of the oral treatment with ME-PL and PL-1 on AChE activity in the cerebral cortex (a), hippocampus (b), hypothalamus (c), and striatum (d). Values are expressed as mean ± S. E. M. $n = 6$ observations per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared with the control group. One-way ANOVA followed by the Newman–Keuls test.

inhibited significantly (Fig. 3) the carrageenan inflammatory paw in a dose- and time-dependent way showing that ME-PL1 and PL-1 have a specific mechanism of action. In carrageenan paw inflammation, three parameters analyzed such as edema, mechanical hyperalgesia, and cold allodynia was inhibited by ME-PL and PL-1 (Figs. 5 and 6). Acute inflammation is characterized by cardinal signs of inflammation (edema, fever, redness, and pain), being widely evaluated by the described trials and used to analyze the anti-inflammatory agents involved during the inflammatory process [41]. The ME-PL and PL-1 showed significant inhibitory effect for edema formation in 2 h and 4 h, stage correlated with an increased production of prostaglandins, cyclooxygenase-2 (COX-2), and nitric oxide (NO) release in the inflammatory response [42], which confirmed the anti-edematogenic potential of *P. leiocarpa*. Li et al. [43] showed that compounds (including vincosamide) are able to inhibit some mediators (nitric oxide, Il-1β, and TNF) and transcriptional factors of inflammation without inducing cellular death. It is possible to suggest that *P. leiocarpa* acts like the main compound vincosamide. Another point is that ME-PL and PL-1 showed to be so effective as dexamethasone but not so potent but both inhibited all inflammatory parameters analyzed.

The ME-PL and PL-1 also showed inhibition of leukocyte migration above 80% for all doses evaluated and, in

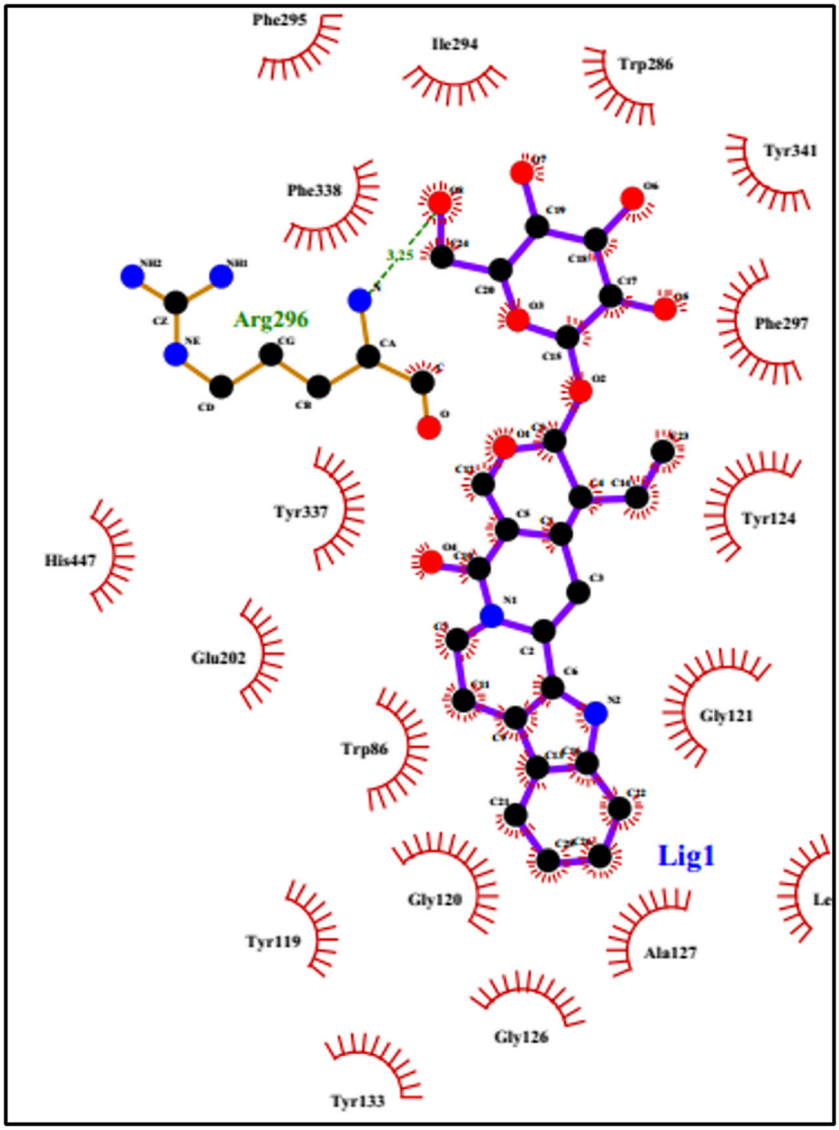

Fig. 8. Docking interactions between the active residues site of the protein with the vincosamide (PL-1) ligand.

the nociception caused by acetone, attenuated the duration of cold sensitivity, which demonstrated its anti-hyperalgesic and antinociceptive potentials. The increase in the pain sensitivity is a common characteristic of the inflammatory response that involves a reduction in the type C nerve fiber activation that is induced by mechanical stimuli [44].

Acetylcholine (ACh) is one of the factors involved in abating the inflammatory response and allowing the recovery of hemostasis and acts by attenuating the secretion of pro-inflammatory cytokines; however, the circulating AChE controls the levels of ACh, suggesting promotion of the inflammatory process under AChE excess [45, 46]. AChE plays an important role in the central and peripheral nervous systems, degrades the neurotransmitter ACh, and subsequently reduces the ACh level in the brain; thus, AChE inhibitor can increase ACh level in cholinergic synapses which have shown to alleviation the disease [47]. Study [48] describes the interaction of many compounds extracted from *Psychotria* with the ability to inhibit AChE, butyrylcholinesterase (BuChE), and monoamine oxidases A and B (MAO-A and MAO-B), highlighting two β-carboline alkaloids, prunifoleine and 14-oxoprunifoleine, and four monoterpene indole alkaloids,

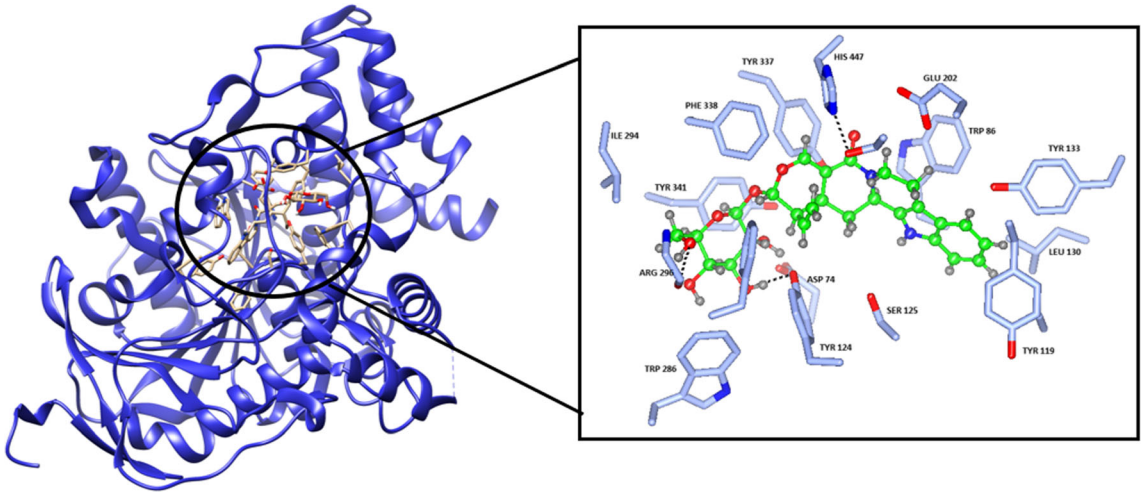

Fig. 9. Overall view of the mAChE subunit complexed with vincosamide (PL-1).

angustine, vallesiachotamine lactone, and E- and Z-vallesiachotamine, which inhibited BuChE and MAO-A. Recent research has increasingly suggested a central role of free radical–induced tissue damage in the pathogenesis of AD [49, 50].

The redocking protocol was evaluated based on the ability of the program to reproduce the choline binding modes observed in the corresponding crystallographic structure; after the validation of the methodology, vincosamide structure (PL-1) was docked in the active site of mAChE, and the protein-ligand interactions were evaluated. The docking study was preferable performed on mAChE, since inhibitory activity data used in the present study were measured on this enzyme [51].

The active AChE center, which consists of the triad Ser203, Glu334, and His447 in mammals [52], is nearly centrosymmetric to the subunit located at the bottom of a narrow gorge that is 20 Å in length. Inhibitors may bind the active site or at a distant allosteric site, the peripheral anionic site (PAS), that is located at the gorge rim [53].

The Trp286 residue plays a very important role in ligand binding with PAS [54]. The ligand interacts with Tyr 337 residue replacing Phe330 residue present in TAChE [53], and this residue belongs to a hydrophobic domain (Phe295, Phe297, Tyr337, Phe338, and Tyr341) that helps anchor the compound. This domain is crucial for enzymatic activity, constituting a difference between AChE and BuChE [55]. In addition, these residues Gly121, Gly126, Ala127, and Tyr119 help in stabilization and hydrophobic interactions. The losses of cholinergic neurons occur primarily in the cortex, the hippocampus, and the brain structures that play important roles in memory and cognitive function [56, 57]. Study has demonstrated that AChE activity is altered in CNS and lymphocytes in many pathological and experimental conditions [58–61]. Thus, synthetic and naturally compounds that may interfere with the activity of this enzyme may be important research targets regarding the treatment of inflammatory, cognitive, and neurochemical dysfunctions. In the present study, we demonstrated that ME-PL and PL-1 exerts an effect in the cholinergic system by altering the AChE activity in the mice' frontal cortex.

In conclusion, we have shown in this study that oral administration of 30 and 100 mg/kg ME-PL and 30 mg/ kg PL-1 inhibited AChE activity in the frontal cortex. Thus, the extract and compound of *P. leiocarpa* showed promising anti-inflammatory activity, in dose-response aspects in inflammatory paw and pleurisy, time curve, and nociceptive parameters. The presence of the alkaloid vincosamide could be responsible, at least in part, for the observed effects. To the best of our knowledge, these experimental trials using this species to evaluate the anti-inflammatory and AChE activity are the first reported in the literature and can contribute to validation of the popular use of this genus. Additional studies must be performed to define the mechanism of action.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no competing interests.

Ethical Approval. All experimental procedures were carried out in accordance with the U.S. National Institutes of Health and were approved by the Animal Ethics Committee from UFGD (Nbr. 14/2015).

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5.3 Artigo III: Inhibition of acetylcholinesterase and molecular docking of the major sesquiterpenes from *Psychotria poeppigiana* essential oil

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Inhibition of acetylcholinesterase and molecular docking of the major sesquiterpenes from *Psychotria poeppigiana* essential oil

Carla Roberta Ferreira Volobuff[a], Sidney Mariano dos Santos[b], Janaine Alberto Marangoni[b], Claudia Andrea Lima Cardoso[c], Zefa Valdevina Pereira[b], Diego Alberto dos Santos Yamazaki[d], Gisele de Freitas Gauze[d], Anelise Samara Nazari Formagio[a,b*]

[a]*Faculty of Health Sciences, Federal University of Grande Dourados, Dourados – MS, Brazil*

[b]*Faculty of Biological and Environmental Sciences, Federal University of Grande Dourados – UFGD, Dourados - MS, Brazil*

[c]*Department of Chemistry, University of Mato Grosso do Sul, Dourados –MS, Brazil*

[d]*Department of Chemistry, State University of Maringá, Maringá, PR, Brazil*

*Corresponding author at: Faculty of Health Sciences and Faculty of Environmental Sciences, Federal University of Grande Dourados – UFGD, Dourados – MS, Brazil.

E-mail address: aneliseformagio@ufgd.edu.br (A.S.N. Formagio).

Abbreviations: AChE, acetylcholinesterase; BHT, butylated hydroxytoluene; EOPP, essential oil of *Psychotria poeppigiana*; GC-MS, gas chromatography-mass spectrometry; mAChE, acetylcholinesterase – *Mus musculus*; MDA, malondialdehyde; ROS, reactive oxygen species; TBA, thiobarbituric acid

ABSTRACT

Ethnopharmacological relevance: Psychotria poeppigiana (Rubiaceae) has popularly been used in the form of decoction for mental disorder- related conditions.

Aim of the study: Considering the lack of scientific studies focused on chemical constituents and pharmacological activity of *P. poeppigiana* essential oil, this work aimed to chemical composition, antioxidant activity and acetylcholinesterase inhibition of essential oil from *P. poeppigiana* (EOPP), as well as molecular docking simulations of the main constituents.

Material and methods: The essential oil was extracted from the leaves of *P. poeppigiana* by hydrodistillation and analyzed by gas chromatography-mass spectrometry (GC–MS). The antioxidant activity was determined by malondialdehyde (MDA) assay and acetylcholinesterase (AChE) inhibition was evaluated in brain structures (cerebral cortex, hippocampus, hypothalamus and striatum) of the rodents *via* spectrophotometric quantification. Molecular docking simulations was carried using Autodock v.4.3.2, Molegro Virtual Docker v6.0 and Gold.

Results: The GC-MS identified 19 compounds, results confirmed the presence of germacrene D and bicyclogermacrene as major volatile compounds. The EOPP exhibited high antioxidant capacity (IC_{50} = 12.78 ± 1.36 μg/mL) when compared to BHT (38.71 ± 3.22 μg/mL). The AChE inhibition has been demonstrated in hippocampus (81.50%), cerebral cortex (70.0%) and hypothalamus (55.88%) in rats. The enzyme-ligand molecular modeling showed that the major constituents of EOPP can interact in both catalytic active site and peripheral active site.

Conclusions: To the best of our knowledge, this is first report on the chemical constituents and biological activity of essential oil of *P. poeppigiana* from Brazil. Overall, the results herein presented sustain and strengthen the use traditionally ascribed to *P. poeppigiana*.

Keywords: Beijo de negro; Rubiaceae; essential oil; germacrene; bicyclogermacrene; AChE.

Introduction

Essential oils are complex mixtures of odoriferous substances that usually present multiple pharmacology properties and, each of these constituents contributes to the biological effects, of this oil (Bakkali et al., 2008), highlighting the protection of biological systems due to their antioxidant activity (Pascual et al., 1994). The overproduction of reactive oxygen species in the body and the deficit of endogenous defense system can lead to various pathologies such as Alzheimer's disease (Liguori et al., 2018). According to the World Health Organization (Zhao et al., 2014), by 2030, the number of people with Alzheimer in the world will be 71.2 million, and it will be 106.8 million in 2050. The cholinergic hypothesis has been related to the onset of Alzheimer's disease due to decreased acetylcholine levels in specific brain regions such as hippocampus and cerebral cortex. The enzyme acetylcholinesterase has the ability to degrade acetylcholine released into the synaptic cleft during nerve impulses. In this sense, acetylcholinesterase inhibiting substances provide symptomatic relief and consequently prevent the progression of disease symptoms (Lalut et al., 2019). Currently, research aims to strengthen the endogenous defenses by natural substances derived from plants.

Psychotria poeppigiana Müll. Arg. (Synonym: *Cephaelis elata*) popularly known as "beijo de negro" and "chapéu do diabo", is a shrub and wide distribution being found in Central America, Bolívia, Mexico and Brazil (Burger & Taylor, 1993). Different parts of this plant in decoction form are popularly used for mental disorders-related conditions (Caballero-George et al., 2001). The authors are aware of two published data on the compositions of essential oils from other *Psychotria* plants have been studied. The results

showed the presence of the mono and sesquiterpenes in essential oil of the leaves *P. leiocarpa* (Andrade et al., 2010) and *P. eurycarpa* (Setzer, Noletto, Haber, 2006). However, studies concerning to the biological activity is relatively unexplored for essential oil for *Psychotria* species. Furthermore, extracts of *P. poeppigiana* reported phytochemical compound as alkaloids, phenols and flavonoids (Coe and Anderson, 1996, Guerrero et al., 2010; Volobuff et al., 2019) and studies concerning biological activity have demonstrated vasoactive (Guerrero et al., 2010), antimicrobial (candidiase) (Coe, 2008) and binding to serotonin receptors effects (Michel et al., 2007).

To the best of our knowledge, no studies have evaluated the biological activity and chemical of the essential oil from leaves for this specie. Thus, the aim of this study was to assess the chemical composition and acetylcholinesterase inhibitory the essential oil from leaves of *P. poeppigiana* (EOPP), and its effect on the enzyme activity and molecular docking simulations are also reported. Further, attention has been focused to explain the ethnobotanical uses, due to the lack of information available in the literature this specie.

Materials and methods

Chemicals

Phosphate buffer, trichloroacetic acid, thiobarbituric acid, butylated hydroxytoluene and coomasie blue were purchased from Sigma Chemical Co. (MO, USA). Donepezil is a product of Pfizer (Groton, CT, USA).

Plant material and Essential oil Extraction

Fresh leaves (600 g) of *Psychotria poeppigiana* (DDMS 0006 and SisGen A51F665) were collected early in the morning in August of 2017 in the Settlements Teijin, located in Nova Andradina, Mato Grosso do Sul, Brazil (22° 14' 0.05" S, 53° 20' 0.35" W) and identified by Prof. Dra. Zefa Valdevina Pereira.The fresh leaves were subjected to hydrodistillation in a Clevenger-type apparatus for approximately 4h to obtain the essential oil according to the procedure described in the Brazilian Pharmacopeia (Brasil, 2010). The obtained essential oil (EOPP) presented as a colour less liquid and had a distinct aroma.

Analysis of volatile compounds - Gas chromatography-mass spectrometry (GC–MS)

The analysis was performed using one gas chromatograph equipped with a mass spectrometer detector (GCMS-QP2010 Ultra, Shimadzu, Kyoto, Japan). DB-5 column (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness) was used, with helium (99.999% purity) as the carrier gas, at a flow rate of 1.0 mL min^{-1} and an injection volume of 1µL (in split mode, 1:10). The initial oven temperature was 50 ºC, with heating to 280 ºC at 3 ºC min^{-1}. The injector temperature was 220 ºC and the temperatures of the transfer line and the quadrupole detector were 280 ºC. The MS scan parameters included electron impact ionization voltage at 70 V, a mass range from 50 to 600 Daltons and a scan interval of 0.3 s.

The retention index was calculated using a mixture of linear alkanes (C_8-C_{40}) as an external reference. Compound identification was achieved by comparing the mass spectra of the samples with the spectra available in the NIST21 and WILEY229 libraries, as well as with data reported in the literature (Adams, 2007).

Animals

The antioxidant and anti-acetylcholinesterase activity were conducted using male *Wistar* rats (200-300 g) provided by the Federal University of Grande Dourados (UFGD), Mato Grosso do Sul, Brazil. The animals were maintained at a constant temperature (23 ± 1ºC) on a 12-hour light/dark cycle with free access to food (Nuvilab Cr-1) and drinking water. The experimental procedures were carried out in accordance with the U.S. National Institutes of Health and approved by the Animal Ethics Committee from UFGD (Nbr. 12/2017).

Antioxidant activity - Malondialdehyde (MDA) level

The EOPP was analyzed for its antioxidant capacity, according to the methodology descript by Stocks (1974). Spontaneous autoxidation of rat brain homogenates was evaluated by the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA). For the assay, three male *Wistar* rats were deprived of food overnight and then anesthetized via inhalation and were killed by decapitation and the mice brains were collected in saline, withdrawn and maintained in ice bath. The brain homogenate was obtained, which underwent spontaneous peroxidation. Aliquots (50 µL) of EOPP (25–100 µg/mL in methanol), were added with the objective of verifying the inhibition of peroxidation, or phosphate buffer (control) were added to 3 mL of homogenate and 1.2 mL of trichloroacetic acid, then the samples were centrifuged to collect the precipitated proteins. The supernatant (1 mL) was heated with 1 mL of an aqueous solution of 0.67% thiobarbituric acid for 15 min at 100 °C. The absorbance was measured at 532 nm. Butylated hydroxytoluene (BHT) was utilized as a positive control.

Acetylcholinesterase inhibitory activity

Three male *Wistar* rats were deprived of food overnight and then anesthetized via inhalation and were killed by decapitation and the mice brains were collected and separated into the cerebral cortex, hippocampus, hypothalamus, and striatum and placed in a 10 mM Tris–HCl solution, pH 7.4, on ice. The tissues were homogenized in a glass potter in the Tris–HCl solution at a 1:10 proportion (w/v) and then centrifuged at 3500 rpm for 10 min to yield a supernatant that was used for the enzyme assay, evaluated according to Ellman et al. (1961) measured at 412 nm. The protein concentration of the homogenized samples was determined by the Coomassie blue at 595 nm and adjusted for each structure: hypothalamus (0.6 mg/mL), cerebral cortex (0.6 mg/mL), hippocampus (0.8 mg/mL) and striatum (0.4 mg/mL) (Bradford, 1976). 25 mL of the EOPP (10 µg/mL) was incubated for 2 min at 30 °C with DTNB (1.04 mmol) and potassium phosphate buffer (pH 7.2, 24 mmol), and the reaction was initiated by the addition of acetylthiocholine iodide (ACSCh, 0.8 mM).

EOPP was evaluated at concentration of 10 µg/mL diluted in methanol, control used was 10% of MeOH in solution with Tris/HCL, total AChE activity = 100% and the positive control used was donepezil of 0.1 mM. The assay was performed in triplicate. The enzyme activity was expressed in µmol ACSCh/h/mg protein.

Docking simulations

The crystal structure of mAChE (acetylcholinesterase – *Mus musculus*) complexed with choline (code ID: 2HA3) was obtained from the Protein Data Bank. The virtual screening studies were performed using tree molecular docking programs: Autodock v.4.3.2 (Morris et al., 2009), Molegro Virtual Docker v6.0 (Thomsen; Christensen 2006) and Gold (Jones et al., 1997). The structure of all compound was obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/). All water molecules and

ions were removed from the structures of mAChE. The docking protocol using AutoDock v.4.2.3 was built using one box with dimensions of 50 x 50 x 50 Å, centered at the coordinates [27.0, 20.0, 14.8] with a grid spacing of 0.375 Å. The Lamarckian genetic algorithm was used and all options were set to the default value. For Molegro Virtual Docker v6.0, the docking protocol was carried out using the Moldock Score as a scoring function and the Moldock Optimizer as a search algorithm, with search sphere radiusset to 11 Å around the catalytic site and all other options were set to the default value (Formagio et al., 2019). The docking protocol for the GOLD program was performed using CHEMPLP as scoring function with search sphere radius set to 10 Å around the catalytic site and all other options were set to the default value. Redocking simulations were repeated five times and the results were reproducible. The docked compounds were ranked according to their best-scored conformation. The enzyme-ligand interactions were analyzed by CCP4mg molecular-graphics software (McNicholas et al., 2011).

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). The difference among the groups was determined by analyses of variance (one-way ANOVA) followed by the Newman–Keuls test.

Results and discussion

Chromatography

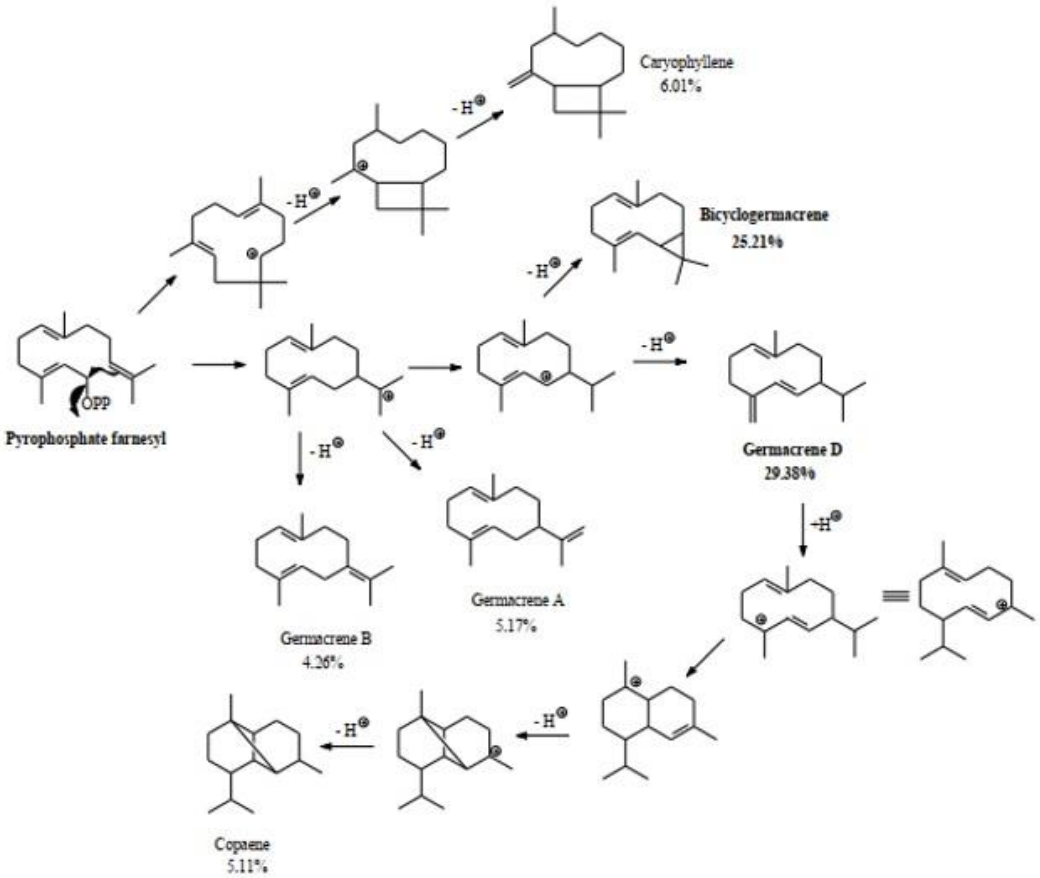
The hydrodistillation with leaves of *P. poeppigiana* resulted in 0.4% v/w (on the basis of the fresh leaves weight) essential oil (EOPP). The analysis by GC–MS identified 19 compounds, highlighting a lower proportion of monoterpenes compared to sesquiterpene (**Table 1**). Germacrene D (29.38%) and bicyclogermacrene (25.21%) were identified as

204 dominants (**Table 1**). These sesquiterpenes can be biosynthesized by formation of farnesyl
205 cation and the cyclization between C10 and C1 leads to the germacrylic cation formation
206 **(Fig. 1)** (Bülow and König, 2000). The caryophyllene, β-copaene, germacrenes A and B
207 which were also found in the *P. poeppigiana* essential oil with concentrations ≥ 4.26%, can
208 be originated by means of rearrangement and cyclization (**Table 1**, **Fig. 1**). Farnesyl
209 diphosphate is the common precursor of all sesquiterpenes (Bülow and König, 2000). The
210 chiral hydrocarbon germacrene D is a commonly found plant constituent and is considered
211 to be a key intermediate in the biosynthesis of many sesquiterpenes (Bülow and König,
212 2000). Considering previous studies on essential oil from *Psychotria* species, the essential
213 oil obtained from the *P. leiocarpa* leaves was also characterized exclusively by
214 sesquiterpenes been bicyclogermacrene (35.6%) and germacrene D (17.6%) the major
215 compounds (Andrade et al., 2010, Calixto et al., 2016). This is the first report of the chemical
216 composition of EOPP.
217
218 **Table 1.** Compounds identified in the essential oil *Psychotria poeppigiana* (EOPP).

Compounds	**RI[a]**	**RI[b]**	**(%)**
β-Pinene	975	974	1.45
Limonene	1024	1024	1.15
Terpinolene	1086	1086	1.10
α-Terpineol	1186	1186	1.01
Longifolene	1407	1407	2.33
α-Gurjunene	1409	1409	1.39
Caryophyllene	1417	1417	6.01
β-Copaene	1430	1430	5.11
β-Gurjunene	1431	1431	1.27
Aromadendrene	1439	1439	1.13
Germacrene D	1484	1484	29.38
Bicyclogermacrene	1500	1500	25.21
Germacrene A	1508	1508	5.17

Elemicin	1556	1555	4.07
Germacrene B	1559	1559	4.26
Spathulenol	1577	1577	3.37
Guaiol	1600	1600	1.20
α-Acorenol	1632	1632	1.51
α-muurolol	1640	1640	1.48
Total identified			**97.53**
Monoterpene			**3.70**
Oxygenated monoterpene			**1.01**
Sesquiterpene			**81.26**
Oxygenated sesquiterpene			**7.56**
Others			**4.07**

219 [a] Retention Index; [b] Retention index (Adams, 2007).



220

221 **Fig. 1.** Proposed biosynthetic pathway for formation of the germacrene e two derivatives (≥

222 4.26%) dominant in essential oil from *P. poeppigiana*.

223

Lipoperoxidation

The measurement of antioxidant activity EOPP was carried out to evaluate the inhibition of spontaneous lipoperoxidation of rat brain homogenate. In this model, EOPP contributed to decrease the generation of malondialdehyde (MDA) (IC_{50} = 12.78 ± 1.36 µg/mL) demonstrating high antioxidant activity in comparison to BHT (IC_{50} = 38.71 ± 3.22 µg/mL). The MDA is one of the oxidation products of polyunsaturated fatty acids been a biomarker of lipid peroxidation. For oxidative evaluation, the MDA reacts with thiobarbituric acid forming pink product with maximum absorption at 535 nm. Reactive oxygen species, such as superoxide anion (O^{2-}), may react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a highly oxidant compound that reacts with biological molecules, causing lipoperoxidation and oxidation of proteins and DNA. Determination of MDA, led to the conclusion that the natural antioxidants brought by the EOPP contributed to limit lipoperoxidation. The overproduction of ROS in the body and the deficit of endogenous defense system can lead to various pathologies such as AD. Currently, research aims to strengthen these endogenous defenses by natural substances derived from plants, which are endowed with antioxidants properties.

Acetylcholinesterase inhibition and docking simulations

Whereas the current medications used to treat of Alzheimer's disease have high cost and time to be acquired, in most cases, prolonged use causes adverse effects harmful to the body. In this context, the continuous search for better and safer drugs is helped by the great chemical variability of natural products (Newman and Cragg, 2016). Furthermore, considering the range of biological activities described for the genus *Psychotria* specially the effects in the central nervous system, *i.e.* association of

248 *Psychotria viridis* and *Banisteriopsis caapi* "ayahuasca" (Andrade et al., 2018), the
249 evaluation of *Psychotria* extracts for treatment of neurological pathologies.

250 Neurochemical and neuroanatomical studies suggest that cholinergic neurons pro-
251 jecting to the neocortex and hippocampus are those predominantly affected in Alzheimer.
252 Inhibition of AChE is considered one strategy for the treatment of neurological disorders.
253 Many natural products have been evaluated as potential AChE inhibitors (Mukherjee et
254 al. 2007). In this context, reversible inhibitors of this enzyme have been used as cognitive
255 enhancers in treatment of patients with Alzheimer's and other neurodegenerative
256 disorders (Lane et al. 2006).

257 First, considering the concentration required to inhibit 50% of spontaneous
258 lipoperoxidation, the acetylcholinesterase (AChE) inhibition by EOPP was evaluated at
259 10 µg/mL for four cerebral structures. Significant AChE inhibition has been demonstrated
260 in the hippocampus (81.50 ± 3%), cerebral cortex (70.0 ± 4%) and hypothalamus (55.88
261 ± 2%) (**Fig. 2**).

262

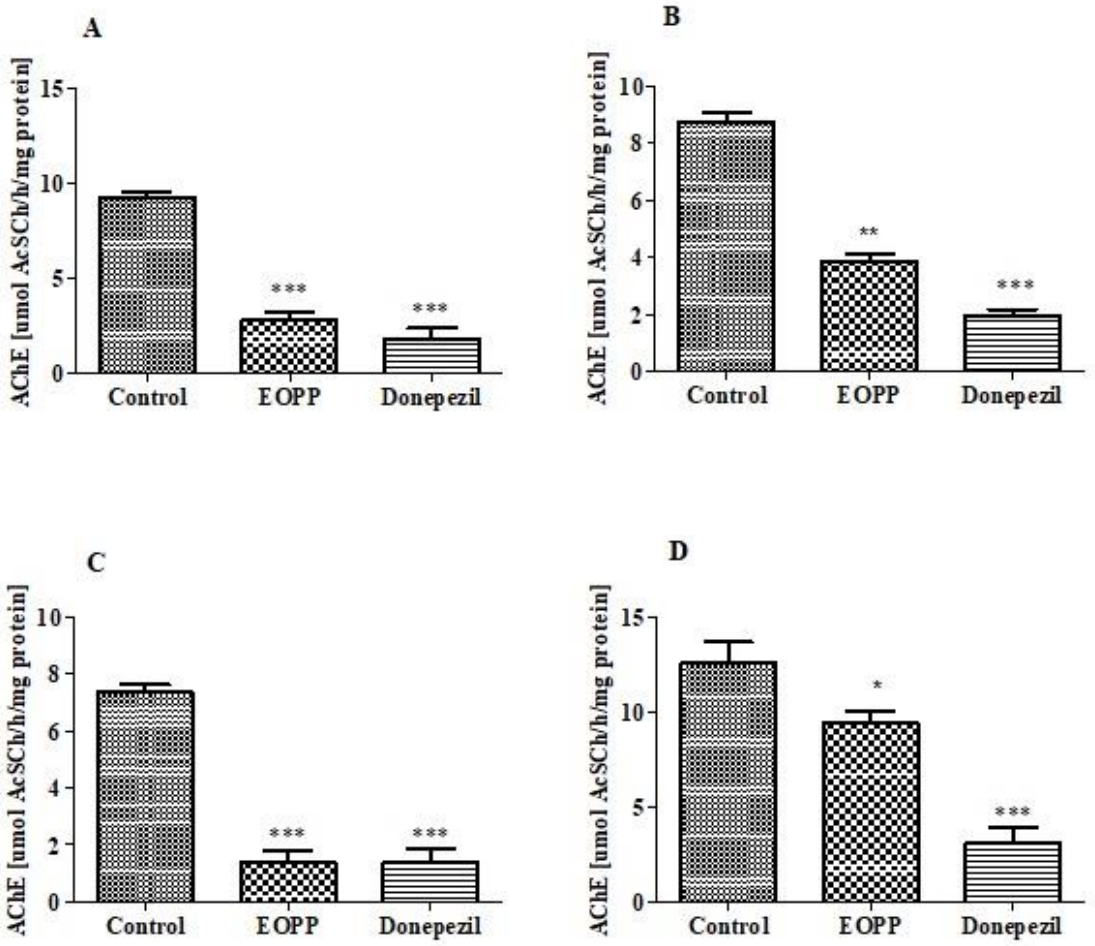


Fig. 2. *In vitro* effects of EOPP on the AChE activity in the cerebral cortex (A), hypothalamus (B), hippocampus (C) and striatum (D) of rats. Each bar represents mean ± SEM. AChE activity is expressed as µmol of acetylthiocholine (AcSCh)/h/mg of protein. Values are expressed as mean ± S. E. M. n = 6 observations per group. * $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, when compared with the control group (saline). One-way ANOVA followed by the Newman-Keuls test

Molecular modeling studies were conducted to clarify the possible mechanism of action of the EOPP. Firstly, we performed virtual screening simulations of the monoterpenes studied by Abdelgaleil et al. (2019) that exhibit high inhibitory effect on acetylcholinesterase. The simulations were carried out using three programs (Autodock

v.4.3.2, Molegro Virtual Docker v6.0 and Gold,) with different search and ranking algorithms. To evaluate which program best describe the experimental results. The program that best ranked the compounds, presenting binding energies according to the described IC_{50} values was Autodock v.4.3.2. Once the program has been choosing the EOPP compounds, monoterpenes studied by Abdelgaleil et al. (2019), donepezil and tacrine was subjected to three new screenings with the same program in order to verify the reproducibility of the scores (**Table 2**).

Table 2. Binding energy of EOPP compounds within the active sites of mAChE.

Ligand	Binding Energy (Kcal / mol)	Ligand	Binding Energy (Kcal / mol)
Donepezil [a]	-10.56	Germacrene D	-8.19
Guaiol	-8.74	α-Acorenol	-8.11
Spathulenol	-8.61	Bicyclogermacrene	-7.97
Longifolene	-8.6	**Pulegone** [b]	-6.52
β-Copaene	-8.57	α-Terpineol	-6.37
Germacrene A	-8.53	**Cineole** [b]	-6.27
Caryophyllene	-8.5	β-Pinene	-6.24
Germacrene B	-8.47	**α-Pipene** [b]	-6.2
α-Muurolol	-8.38	Terpinolene	-6.17
Aromadendrene	-8.34	**Terpineol** [b]	-6.02
Tacrine [a]	-8.32	Limonene	-5.95
β-Gurjunene	-8.28	**Citronellal** [b]	-5.23
α-Gurjunene	-8.21		

[a] reference drugs [b] monoterpenes studied by Abdelgaleil et al. (2019)

The docking results indicated that the most of EOPP compounds are better ranked than six monoterpenes previously studied by Abdelgaleil et al. (2019), with 14 compounds having higher binding energy than pulegone (IC_{50} = 8.79 mg/mL), the more

289 active monoterpene Moreover, 9 compounds of EOPP present higher binding energy than
290 tacrine (standard drug). This result suggested that many compounds identified in OEPP
291 may contribute to the observed AChE inhibitory activity.

292 Molecular docking simulations were performed to investigate the binding mode
293 of the major OEPP components germacrene D and bicyclogermacrene together with the
294 evaluation of how these sesquiterpenes interacs with mAChE active site (**Fig. 3**).

295 Germacrene D was highly hydrophobic and depth on the active site of the enzyme
296 (**Fig. 3A** and **3B**) binding near to the key residues constituting of the catalytic triad
297 (Ser203, and His447) and choline-binding site (Trp84). Germacrene D exhibited
298 favorable hydrophobic interactions with aromatic rings of five residues His447, Trp84,
299 Tyr334, Tyr124 and Phe338. More, the molecular orientation of bicyclogermacrene (**Fig.**
300 **3C** and **3D**) allowed binding near of the key residues of the peripheral site (Asp74, Tyr72,
301 Phe338, Tyr124 and Trp286). Further, these bindings were stabilized by hydrophobic
302 interactions with aromatic rings of amino acids Phe338, Phe297, Tyr124 and Trp286.
303 These results indicate that both major constituents of EOPP can interact in both catalytic
304 and peripheral active sites.

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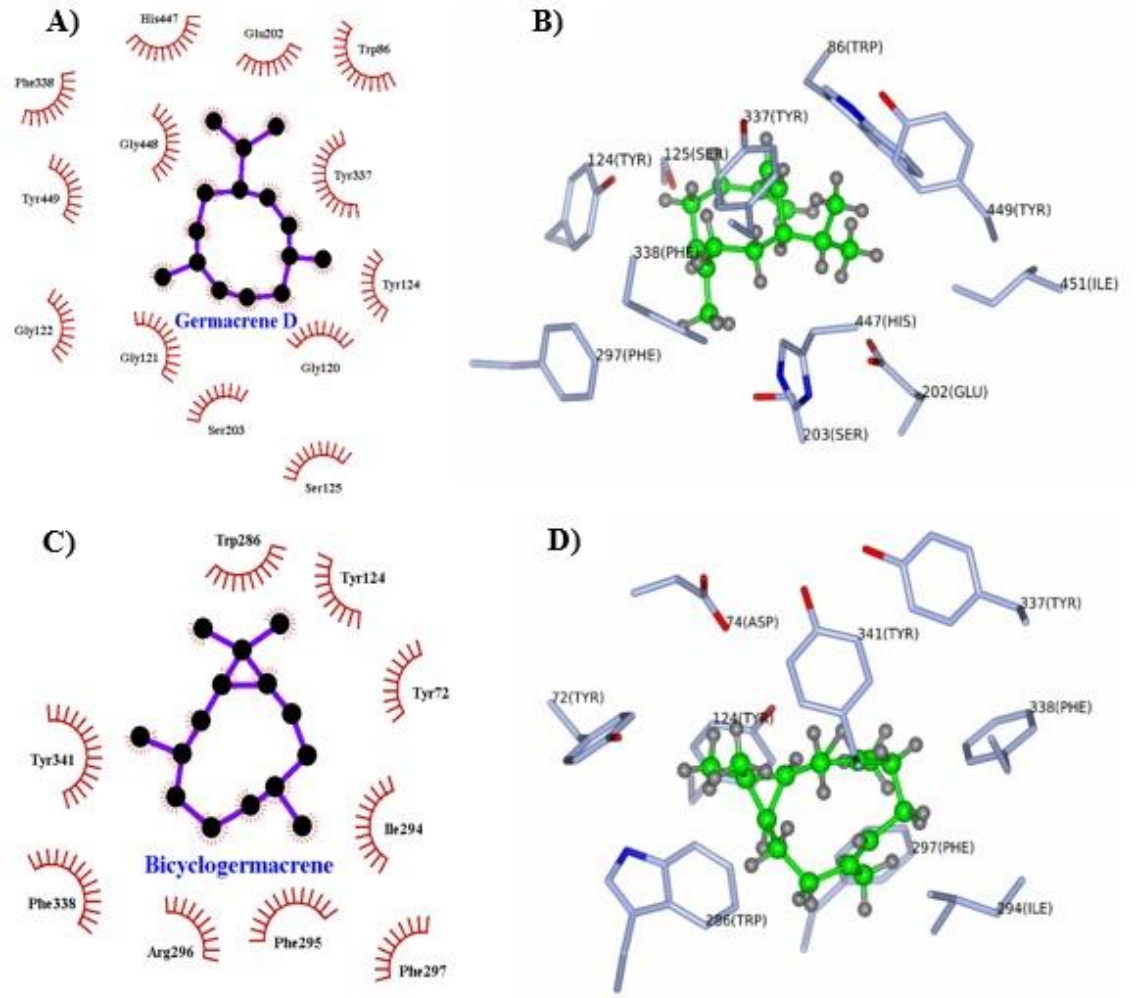

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307 **Fig. 3**. Molecular docking of germacrene D (A and B) and bicyclogermacrene (C and D)
308 with binding sites of mAChE; left 2D interaction diagram an right the complex structure
309 in stereo view (3D).

310

311 **Conclusion**

312 In this study, it was evidenced the presence of 19 compounds in the essential oil
313 of *P. poeppigiana* leaves (EOPP) with predominance of sesquiterpenes. This EOPP was
314 able to reduce lipid spontaneous oxidation and to inhibit AChE activity in brain
315 homogenates, potentially useful for the pharmacological management of conditions such
316 as Alzheimer's disease. The present study supports the ethnopharmacological use of *P.*

317 *poeppigiana* in the central nervous system. Finally, these results stimulate further
318 investigations of the EOPP.
319
320 **Author contribution**
321 ZVP collected and identified plant; CRFV, SMS, JAM, CALC and ASNF
322 designed the chemistry, antioxidant and anticholinesterase study; DASY and GF was
323 carried the molecular docking. All authors participated in the design, interpretation, and
324 analysis of the data and approved the final manuscript.
325
326 **Conflicts of interest**
327 The authors declare that the study has no competing financial interest.
328
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5.4 Artigo IV: Analgesic, anti-inflammatory and anticholinesterasic properties of crude extract from *Psychotria poeppigiana* leaves

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Analgesic, anti-inflammatory and anticholinesterasic properties of crude extract from *Psychotria poeppigiana* leaves

Carla Roberta Ferreira Volobuff[a], Pedro Cruz de Oliveira Junior[b], Zefa Valdevina Pereira[b], Marcia Regina Cabral[c], Maria Helena Sarragiotto[c], Candida Aparecida Leite Kassuya[a], Anelise Samara Nazari Formagio[a,b*]

[a]*Faculty of Health Sciences, Federal University of Grande Dourados, Dourados – MS, Brazil*

[b]*Faculty of Biological and Environmental Sciences, Federal University of Grande Dourados – UFGD, Dourados - MS, Brazil*

[c]*Department of Chemistry, State University of Maringá, Maringá, PR, Brazil*

[*]Corresponding author at: *Faculty of Biological and Environmental Sciences, Faculty of Health Sciences, UFGD, MS, Brazil*

E-mail address: aneliseformagio@ufgd.edu.br; aneliseformagio@yahoo.com.br (A.S.N.Formagio)

ABSTRACT

Ethnopharmacological relevance: The *P. poeppigiana* leaves are used for popular treatment of strong back pain and also against inflammatory reaction for snakebites. Scientific study did not explore the analgesic, anti-inflammatory, and anticholinesterasic properties of *P. poeppigiana* Leaves.

Aim of the study: This study was undertaken to investigate the potential *in vitro* antioxidant, *in vivo* acetylcholinesterase, anti-nociceptive, and anti-inflammatory properties, as well to quantifying the alkaloids of the methanolic extract of *P. poeppigiana* leaves (MEPP).

Material and methods: Alkaloid content was verified by UHPLC-HRMS/MS. The content of phenols, flavonoids, flavonol and condensed tannins and the antioxidant activity by DPPH, β-carotene and malondialdehyde methods were evaluated. MEPP anti-inflammatory activity was assayed by the carrageenan-induced models of paw edema and pleurisy in mice. MEPP anti-nociceptive potential was evaluated by the formalin method in mice. MEPP anticholinesterasic properties *in vivo* was evaluated in four brain structures of rats.

Results: The total ion chromatogram of MEPP was analyzed by UHPLC-HRMS/MS and evidenced two alkaloids, one coumarin, one iridoid and two terpenes derivatives. Highest phenols, flavonoids, flavonols and condensed tannins, concentration was found in extract. MEPP was presented significantly inhibition by the DPPH, β-carotene and MDA models. MEPP at doses 30 and 100 mg/kg, presented inhibition in the hippocampus, cerebral cortex, hypothalamus and striatum when compared to donepezil. In addition, MEPP presented anti-inflammatory and antinociceptive behavior in the evaluated models.

Conclusion: This is the first chemical and biological study performed with *P. poeppigiana* methanolic extract. The results indicate the anti-inflammatory, analgesic, and anticholinesterase potential, corroborating the popular use previously described for the species and genus.

Keywords: Acetylcholinesterse, methanolic extract, alkaloid, antioxidant, formalin.

Abreviattions: ACh, acetylcholine; AChE, acetylcholinesterase; BHT, butylated hydroxytoluene; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MeOH, methanol; MEPP, methanolic extract of *Psychotria poeppigiana*; MDA, malondialdehyde; TBA, thiobarbituric acid; UHPLC-HRMS/MS, ultra-high pressure liquid chromatography-high resolution mass spectrometry

1. Introduction

Psychotria poeppigiana Müll. Arg., Rubiaceae, folk name "beijo de negro" and "chapéu do diabo", is used for the treatment gastrointestinal disorders, stomachaches and fever (Gupta et al., 2005). Valadeau et al. (2009) showed that steam bath with *P. poeppigiana* leaves are used for popular treatment of strong back pain while Pino-Benítez (2006) showed that *P. poeppigiana* could be used as natural folk anti-inflammatory agent against inflammatory reaction for snakebites.

In Brazil, this plant is practically localized in all national territory (Andersson, 1992). Chemical study of dichloromethane and methanol extracts from the aerial parts of this specie identified bioactive compounds such as phenolic, aromatic acid, steroids and coumarin (Moreno et al., 2014). Recently, in our cytotoxic drug-screening program, we demonstrated the antitumoral and anticholinesterasic activities of the methanolic extract of several species of *Psychotria*, among them *P. poeppigiana*, which presented GI_{50} values 57.7 μg/mL against the prostate (PC-3) and acetylcholinesterase (AChE) inhibition in the hippocampus (Volobuff et al., 2019).

The inflammatory process plays a role in Alzheimer diseases (McLarnon, 2019, Tejera et al., 2019) because of this the use of Non steroidal anti-inflammatory (NSAIDS) decreases risk of Alzheimer's disease mortality (Benito-León et al., 2019) while some works indicate the

NSAIDS to be include in (Ali et al., 2019, Hershey; Lipton, 2019). The biological mechanism of this protection is not completely understood and may involve the anti-inflammatory properties of NSAIDs or inhibition of COX activity, inhibition of beta-amyloid(1-42) (Abeta42) production and aggregation, inhibition of beta-secretase activity, activation of PPAR-gamma or stimulation of neurotrophin synthesis (Imbimbo et al., 2010).

As strategy to novel Alzheimer drugs, the multi-target-directed ligand substances with anti-inflammatory and anticholinesterase activity are developed (AlFadly et al., 2019). Moreover, due to the limitation of pharmacological studies, this study aimed to evaluate inhibition of acetylcholinesterase, anti-nociceptive, and anti-inflammatory activities of the methanolic extract of *P. poeppigiana*, a genus with important bioactive compounds that is still unexplored. In addition, we further evaluate its antioxidant activity, phenols, flavonoids, flavonol content and alkaloids were identified by ultra-high pressure liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS/MS) from extract.

2. Materials and methods

2.1. Chemicals

Quercetin, catechin, gallic acid, Tris-HCl solution, β-carotene, phosphate buffer, 2,2-diphenyl-1-picrylhydrazyl (DPPH), carrageenan, dexamethasone, morphine, formalin, trichloroacetic acid, thiobarbituric acid, butylated hydroxytoluene and coomasie blue were purchased from Sigma Chemical Co. (MO, USA). Methanol, acetone and chloroform were obtained from Vetec (Rio de Janeiro, Brazil). Donepezil is a product of Pfizer (Groton, CT, USA).

2.2. Plant material and preparation of the methanolic extract

The leaves of *P. poeppigiana* were collected at Nova Andradina, Mato Grosso do Sul, Brazil (22° 14' 0.05" S, 53° 20' 0.35" W) in August 2018. A specimen (0006) was identified by one of the authors (Z.V. Pereira/UFGD) and deposited in the herbarium of this institution (DDMS 0006 and SisGen A51F665). Air-dried leaves of *P. poeppigiana* (188g) was extracted by maceration with methanol (6 x 2 L) at room temperature. After removal of MeOH under reduced pressure at 65 °C, and subsequent lyophilized in Alpha 1 - 2LD Plus, Christ equipment and vacuum parameters of 0.045 mbr and temperature of -42 °C, resulting in the extract methanolic (MEPP).

2.2.1. Content of phenol, flavonoids, flavonol and condensed tannins

A solution of the MEPP was dissolved in methanol at a concentration of 10µg/mL and was evaluated flavonoid content as described by Djeridane et al. (2006), using methanol (blank) and gallic acid as standard. The absorbance was measured at 760 nm, and expressed as mg of gallic acid equivalents (GAE)/g of extract. Flavonoids was determined using quercetin as standard, and absorbance was measured at 415 nm, as described by Lin and Tang (2007), expressed as mg of quercetin equivalents (QE)/g of extract. The concentration of flavonols was realized using the method reported previously (Lin; Tang, 2007; Maxson; Rooney, 1972) at 440 nm, and expressed as mg of quercetin equivalents (QE)/g of extract. The condensed tannins as according Maxson and Rooney (1972), and absorbance was performed at 500 nm. The results were expressed as mg of catechin equivalents (CAE)/ g of extract.

2.2.2. Analysis of the methanolic extract by UHPLC-HRMS/MS

The sample (MEPP) was prepared in MeOH (1.0 mg mL-1) and chromatographic separations were performed using UHPLC on a Symmetry C18 column (75 × 2.0 mm i.d.; 1.6 µm Shim-pack XR-ODS III), maintained at a temperature of 40 ºC. The mobile phase consisted

of H_2O (solvent A) and 0.1% formic acid in CH_3CN (solvent B). The gradient program was as follows: initial 0-1 min, using elution A-B (95:5, v/v), 1-3 min (30:70 v/v), 3-12 min (5:95 v/v) and kept at 95% B for 16 min at a flow rate of 0.2 mL min^{-1}. Injection volume was 3 µL. High resolution mass spectrometry analysis were carried out in a Q-TOF mass spectrometer via an electrospray ionization interface. The capillary voltage was operated in positive ionization mode, set at 4500 V, using sodium formate (10 µM) as calibrant. The dry gas parameters were set to 8 L min^{-1} at 200 ºC with a nebulization gas pressure of 4 bar. Collision-induced dissociation (CID) fragmentation was performed using argon (Ar) collision gas and collision energy from 0-30 eV. Spectra data of the investigated compounds were collected from m/z 50-1300 with a resolution of 50.000, and with an acquisition rate of 5 spectrums per second. The ions of interest were selected by auto MS/MS scan fragmentation. The data processing software was Data analysis 4.3 (Bruker). Moreover, the mass error value was calculated. Only molecular formulas ≤ 5 ppm of error were considered in this study (Harrison, 1999).

2.3. Animals

Male and female *Swiss* mice (25 - 35 g) for the anti-inflammatory assays, male *Wistar* rat (200 – 250 g) for anticholinesterasic and antioxidant were provided by the Federal University of Grande Dourados (UFGD), Mato Grosso do Sul, Brazil, under a 12 h light-dark cycle (lights on at 6:00 am), humidity 60-80% and free access to food (Nuvilab Cr-1) and water. The studies were carried out in accordance with demands of the U.S. National Institutes of Health and approved by the Animal Ethics Committee from UFGD (Nbr. 12/2017 CEUA).

2.3.1. Antioxidant activity

2.3.2. DPPH (2,2-diphenyl-1-picrylhydrazyl)

In DPPH assay, different concentrations of the MEPP (10 – 250 mg/mL) were added to 2 mL of methanol DPPH solution (0.1 mM), prepared daily. The mixture remained incubated for 30 min at room temperature in dark and the absorbance was measured at 515 nm. The BHT (butylated hydroxytoluene) was used to control. Reading will be done in triplicate (Brand-Williams; Cuvelier; Benset, 1995).

2.3.3. β-carotene/linoleic acid

To prepare the solution of β-carotene (Jayaprakasha, Singh, Sakariah, 2001; Kaur, Kapoor, 2002) were dissolved 2 mg β-carotene in 10 mL chloroform, with 1mL of this solution of β-carotene-chloroform was mixed with 20 mg linoleic acid and 0.2 g Tween 40. The chloroform was then removed by rotavaporation at 45°C. The solution was vigorously stirred for emulsion formation, after 50 ml of distilled water. 0.2mL of the different concentrations prepared were added to 5mL of the emulsion. The absorbance was read at 470nm at time zero. The solutions were placed in a 50°C water bath and the absorbance reading was performed every 15 minutes to monitor the oxidation until the β -carotene coloration disappeared (105 min). The assay was realized in triplicate.

2.3.4. Malondialdehyde (MDA) level

The lipid peroxidation assay was performed using malondialdehyde (MDA) (Stocks, 1974). The brains of the 4 rats *Wistar* were triturated in phosphate buffer pH 7.4, centrifuged to obtain the supernatant and diluted to three times their volume in phosphate buffer pH 7.4. Aliquots (50 μL) of MEPP (25-100 mg/mL in methanol) were added to 3 mL of diluted supernatant. They were incubated at 37 °C for 1 h, after which time 1.2 mL of trichloroacetic acid (280 mg/mL) was added and centrifuged. The supernatant obtained after centrifugation was withdrawn, 1mL of thiobarbituric acid (10 mg/ml) was added and incubated for 15 min at

100 °C. Absorbance reading was performed at 532 nm and BHT was used as a positive control. All data are the averages of triplicate analyses.

2.3.5. Carrageenan-induced paw edema

Groups of male *Swiss* mice (n = 6 animals/group) were treated orally with MEPP (100 and 300 mg/kg) and vehicle (control group). Dexamethasone (1 mg/kg) was administered subcutaneously and used as a positive control. All groups received intraplantar injection with 50 µL carrageenan (300 µg/paw) suspended in 0.9% sterile saline. The contralateral paw received 50 µL of saline and was used as a control. Paw edema thickness was measured at 1, 2 and 4 h after carrageenan injection (Kassuya et al., 2009).

2.3.6. Pleurisy

MEPP (30, 100 e 300 mg/kg) and vehicle (0.9% saline), were administered orally to diferents groups of female *Swiss* mice (n = 6 animals/group). Dexamethasone (1 mg/kg, subcutaneously) was used as a positive control. After 1 h of treatment, pleurisy was induced by intrapleural injection of 100 µl carrageenan (1%). The naive group received 100 µL sterile saline by intrapleural injection. After 4 h, the pleura was washed with 1 ml phosphate buffered saline. A 20 µl aliquot of exudate was collected and diluted with Turck solution (1:20) for leukocyte counting in a Neubauer chamber (Kassuya et al., 2009).

2.3.7. Formalin-induced nociception

Groups of male Swiss mice received oral MEPP (30, 100 and 300 mg/kg) and vehicle (0.9% saline, control). Morphine (4 mg/kg subcutaneously) was used as a positive control. After 1 h of treatment, the animals received 20 µL of saline containing 2.5% formalin in the right

hind paw. The nociceptive response from 0 to 5 min (phase 1 - neurogenic pain) and from 15 to 30 min (phase 2 - inflammatory response) were evaluated (Hunskaar and Hole, 1987).

2.3.8. In vivo acetylcholinesterase inhibitory activity

Male *Wistar* rats (n =7 animals/group) were treated for 7 days orally with MEPP (30 and 100 mg/kg), donepezil (1.3 mg/kg) as a positive control and vehicle (control, saline 0.9%). After the last day of treatment, the animals were anesthetized and decapted for removal of the brain structure and separated into the cerebral cortex, hippocampus, hypothalamus and striatum and placed in a 10 mM Tris–HCl solution, pH 7.4. The procedure was performed as previously described by Ellman et al. (1961). The protein concentration of the homogenized samples was determined by the Coomassie blue (Bradford, 1976) using bovine serum albumin as standard. All the reactions were performed in triplicate. The reaction product was determined at 412 nm. The enzyme activity was expressed in µmol AcSCh/h/mg protein.

2.11. Statistical analyses

Data are presented as the mean ± standard error of the mean (SEM). The difference among the groups was determined by analyses of variance (one-way ANOVA) followed by the Newman–Keuls test.

3. Results and Discussion

3.1. Phytochemical

Many species of the *Psychotria* are known as a rich source of indolics alkaloids (Berger et al., 2012; 2015), which are well studied due to their bioactivities and pharmacological effects, and comparatively few studies are applied in relation to other constituents, among them phenolic compounds. The analysis of MEPP from *P. poeppigiana* leaves demonstrated the

presence of phenolic (264.49 ± 3.45 mg GAE/g extract), polyphenolic (flavonoids 201.13 ± 2.67 mg QE/ g extract; flavonol 170.17 ± 1.54 mg QE/ g extract and condensed tannins content representing highest concentrations 834.30 mg CAE/g extract). Studies of the literature report glycosylated flavonoids derived from quercetin and kaempferol, phenolic acids and phenylpropane in several species from *Psychotria* (Harborne, 1977; Berger et al., 2016; Benevides, Young, Bolzani, 2005; Lu, Wang, Kong, 2014; Formagio et al., 2014), corroborating the data reported in our work. Simples phenolics, flavonoids and tannins exhibit strong pharmacological activities and nutraceuticals (Tapas, Sakarkar, Kakde, 2008).

The total ion chromatogram of MEPP was analyzed by UHPLC-HRMS/MS (**Fig. 1A**), to quickly provide structural information, leading to compounds identification. The extract was run under both positive condition and it showed several major and minor ionic species. Elucidating the chemical structure was performed through MS/MS fragmentations, and the identification of the metabolite was confirmed by comparing it to the published reports or databases such as ACD Lab, Inc. (Toronto, Canada), and by analyzing the ion fragmentation patterns. Based on the HRMS and tandem mass spectrometry (MS/MS) data, as well as comparison with reference standards and literature, six metabolites (two alkaloids, one coumarin, one iridoid and two terpenes derivatives) (**Fig. 1A, Fig. 2, Table 1**).

Studies with species of *Psychotria* have resulted in the isolation of several indole monoterpene alkaloids (Matsuura, Fett-Neto, 2013; Pimenta et al., 2011) which corroborates our results by presence of the calycanthine (**1**) (**Fig. 1B**) and hodgkinsine (**2**) (**Fig. 1C**). The presence of the m / z 173 ion in both spectra was identified as the noreleagnine alkaloid, indole monoterpenic alkaloid forming intermediate, whose MS spectrum analysis suggests the formula [$C_{11}H_{12}N_2 + H^+$] for the m / z ion 173.1068 (**Fig. 1B, C**).

Table 1 - The main compounds observed by LC-MS/MS in the positive mode of the methanolic extract from *P. poeppigiana* leaves (MEPP).

Compound N°	**Exact mass**	**Molecular formula**	**t_r min**	**Compound**	**MS/MS**
1	347.2225	$C_{22}H_{26}N_4$	0.74	calycanthine	173, 130
2	519.3222	$C_{33}H_{38}N_6$	0.92	hodgkinsine	173, 130
3	193.0491	$C_{10}H_8O_4$	3.76	scopoletin	147, 119, 91
4	437.2225	$C_{18}H_{22}O_{11}$	3.93	asperuloside	275, 247, 187, 147, 109
5	225.1485	$C_{13}H_{20}O_3$	4.00	vomifoliol	149, 123, 107, 95
6	197.1172	$C_{11}H_{16}O_3$	4.41	loliolide	179, 133, 107, 91

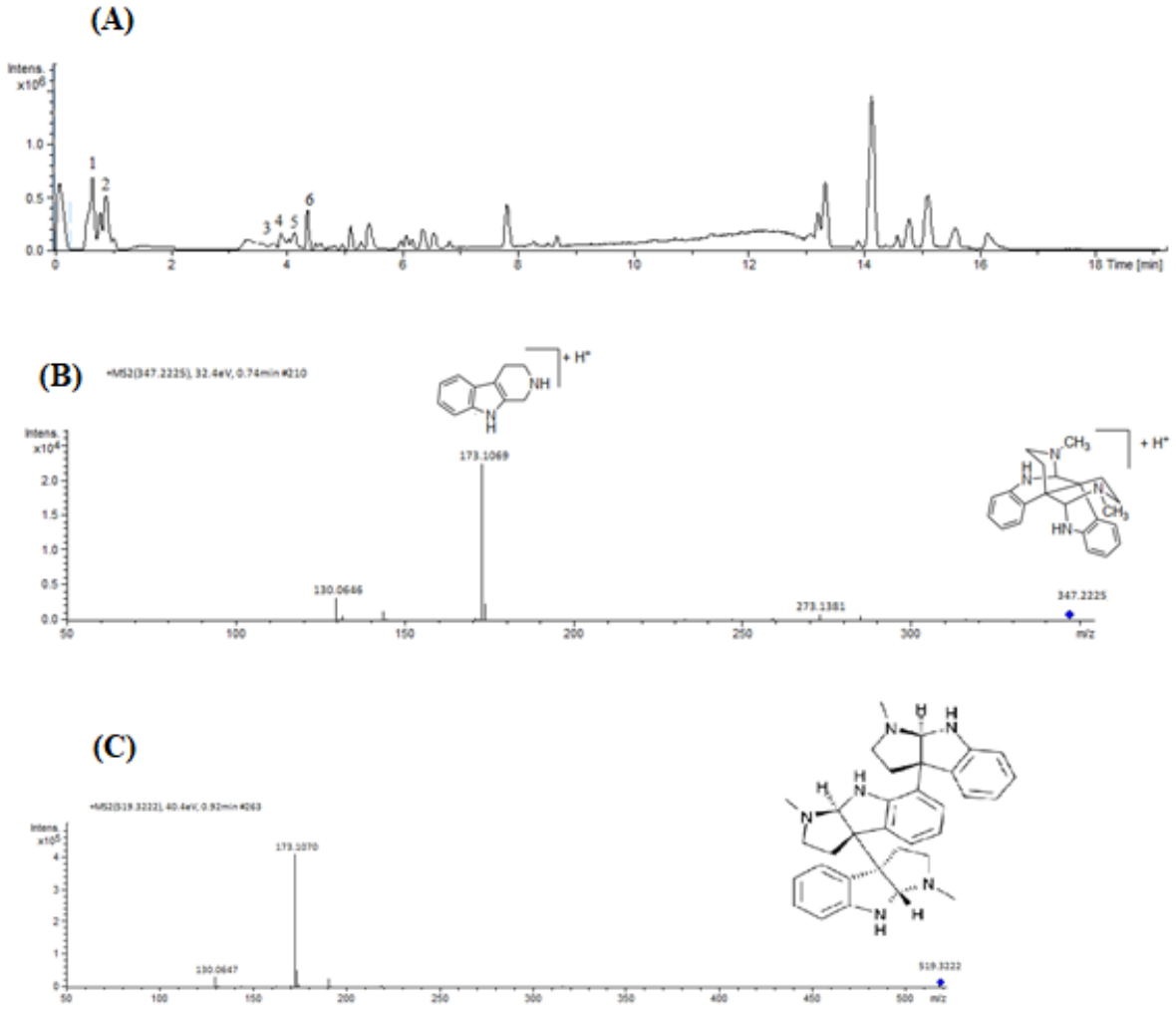

Fig. 1. (A) Total ion chromatogram of MEPP in positive ion mode, **(B)** chromatogram of calycanthine and **(C)** hodgkinsine.

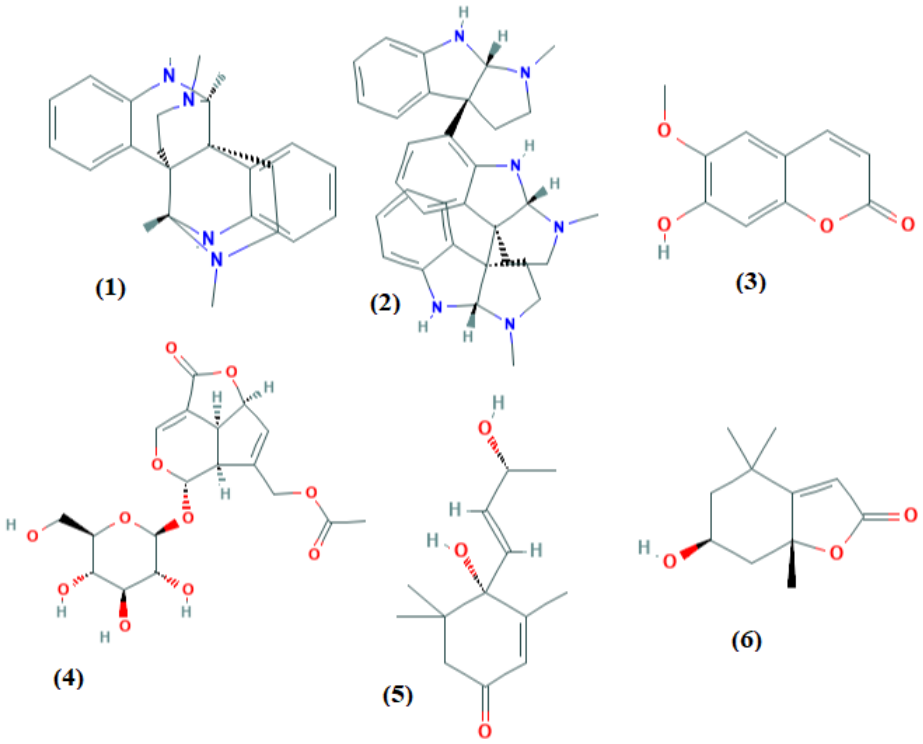

Fig. 2. Chemical structure of the calycanthine **(1)**, hodgkinsine **(2)**, escopoletin **(3)**, asperuloside **(4)**, vomifoliol **(5)** and loliolide **(6)** found in the MEPP.

Calycanthine shown antifungic, anticonvulsivant effect and inhibition of GABA neurotransmitter as a result of interactions with L-type Ca^{2+} channels and inhibiting GABA-mediated chloride currents at $GABA_A$ receptors (Chebib et al., 2003; Xu, Cheng, 2015). Hodgkinsine has demonstrated antinociceptive (Amador et al., 2000), antiviral, antimicrobial (Saad, Sharkawy, Shie, 1995) and analgesic potential (Amador et al., 2001; Kodanko et al., 2007). In this sense, the lack of correlated biological data for these substances reinforces our study regarding the inhibition of acetylcholinesterase, anti-inflammatory and antioxidant for this species.

3.2. Biological activity

Regarding antioxidant activity, MEPP was evaluated by the DPPH, β-carotene and MDA models, demonstrated values of the IC_{50} of 16.30 ± 1.22 (positive control BHT 16.72 ±

1.34 μg/mL); 47.30 ± 5.89 (BHT 91.20 ± 4.54 μg/mL) and 32.73 ± 0.89 μg/mL (BHT 38.71 ± 3.22 μg/mL), respectively.

In the anti-inflammatory paw edema model, MEPP (30, 100 and 300 mg/kg) significantly decreased edema formation by 1 h (88%; 88%; 68%), 2 h (72%; 725% and 70.28%) and 4 h (66%; 80% and 83%), when compared to the dexamethasone (94% at 1h, 84% at 2h and 85% at 4h) (**Fig. 3**).

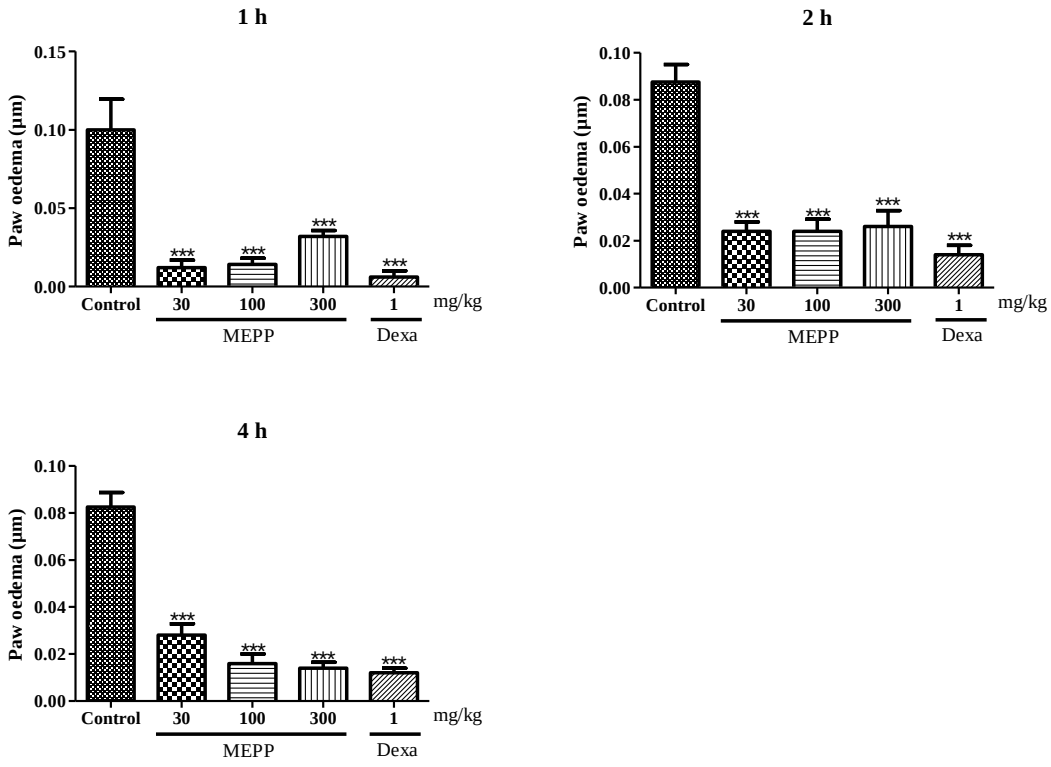

Fig. 3. Effects of MEPP (30, 100 and 300 mg/kg) in the carrageenan induced paw edema at 1, 2 and 4 h. The bars express the mean ± SEM of 6 animals, compared with vehicle vs treated group. ****P*<0.001, one-way ANOVA followed by Student-Newman-Keuls.

Oral administration with MEPP (30, 100 and 300 mg/kg) demonstrated significant inhibition of leukocyte migration of 79%, 93% and 89.60 %, respectively. The positive control dexamethasone inhibited 99.99% (**Fig. 4**).

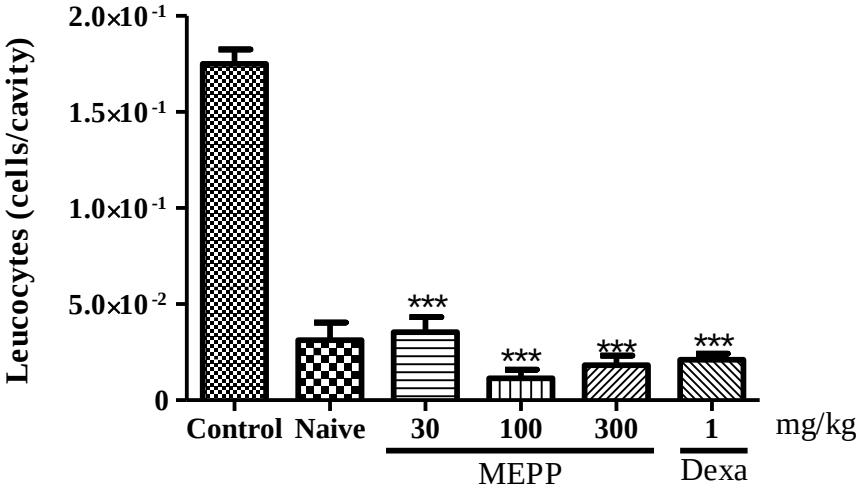

Fig. 4. Effects of MEPP on total leukocytes induced by carrageenan in the pleural cavity of mice. Animal received the oral treatment with MEPP (30, 100 and 300 mg/kg), or vehicle, and after 1 h they received an intrapleural injection of Cg (100 ul of a 1% solution/cavity). Control animals received only the vehicles. The bars express the mean ± SEM of 6 animals, compared with vehicle vs treated group. ****P*<0.001, one-way ANOVA followed by Student-Newman-Keuls.

MEPP was evaluated for antinociceptive action. In the first phase, at a dose of 30 mg/kg and 300 mg/kg, they had a maximum inhibition of 29.27% and 51% respectively, while dexamethasone inhibited 67.48%. The 100 mg/kg dose showed no significant inhibition. In the second phase, the doses of 30, 100 and 300 mg/kg presented inhibition of 11.74%, 14.89%, 47.16% and dexamethasone 95.74% (**Fig. 5**).

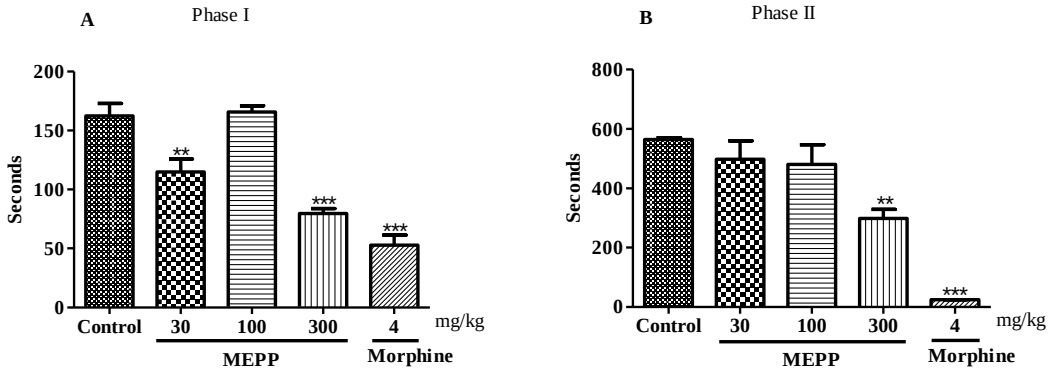

Fig. 5. Effect of the MEPP (30, 100 and 300 mg/kg), vehicle and morphine (4 mg/kg) on pain related behaviors in the formalin-induced nociception model. (**A**) Administration of the MEPP decreased pain in phase I (neurogenic origin pain). (**B**) Administration of the MEPP significantly decreased pain in phase II (inflammatory response). The results are presented as the mean ± SEM. **P<0.01, ***P<0.001 compared with the control group (vehicle). Differences between groups were analyzed by analysis of variance (one-way ANOVA) followed by the Newman–Keuls test.

Rubiaceae reports a range of medicinal uses such as treatment of malaria, diarrhea, digestive problems, skin diseases, fever, bleeding, urinary and respiratory infections, headache, eye and gum inflammation (Conserva; Ferrer, 2012). Previous studies report reversible analgesic evaluation of naloxone opioids by the formalin, writhing and tail movement methods of alkaloids in *P. colorata* leaves and flowers (Elisabetsky et al., 1995). In the ethanol extract of *P. vellosiana* was reported the potential antimycobacterial and anti-inflammatory potential activities (Moraes et al., 2011).

The MEPP was evaluated at 30 and 100 mg/kg for four cerebral structures and presented inhibition in the hippocampus (81.53 ± 0.03% and 83.31 ± 0.02%), cerebral cortex (86.83 ± 0.04% and 87.43 ± 0.05%), hypothalamus (85.81 ± 0.05% and 88.31 ± 0.07%) and striatum (87.03 ± 0.06% and 88.72 ± 0.06%), respectively (**Fig. 6**). Donepezil (1.30 mg/kg) was used to

control and showed inhibition to hypothalamus (88.68 ± 0.05%), cerebral cortex (88.30 ± 0.04%), striatum (82.68 ± 0.05%) and hippocampus (79.15 ± 0.04%) (**Fig. 6**).

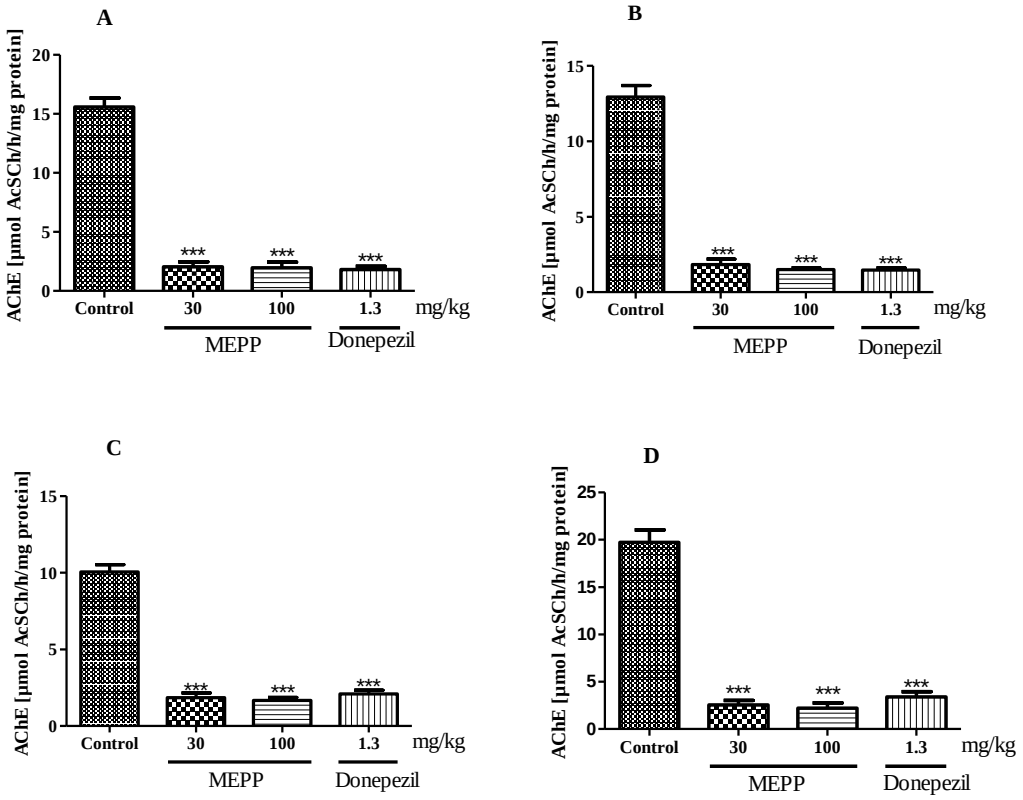

Fig. 6. *In vivo* effects of MEPP on the AChE activity in the cerebral cortex (A), hypothalamus (B), hippocampus (C) and striatum (D) of rats. Each bar represents mean ± SEM. AChE activity is expressed as µmol of acetylthiocholine (AcSCh)/h/mg of protein. Values are expressed as mean ± S. E. M. n = 6 observations per group. ***P < 0.001, when compared with the control group (saline). One-way ANOVA followed by the Newman-Keuls test.

This is the first chemical and biological study performed with *P. poeppigiana* methanolic extract. The results indicate the anti-inflammatory and anticholinesterase potential, corroborating the popular use previously described for the species and genus.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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6 CONCLUSÃO

De acordo com os dados obtidos neste estudo, foi evidenciado significativa concentração de alcaloides nos extratos metanólicos de espécies de *Psychotria* e inibição do crescimento celular e da atividade da acetilcolinesterase em modelos *in vitro*. A partir do extrato metanólico das folhas de *P. leiocarpa*, foi isolado o alcaloide vincosamida e, ambos apresentaram significativa atividade anti-inflamatória e inibição da atividade da acetilcolinesterase no córtex cerebral em modelo *in vitro*. O acoplamento molecular de vincosamida demonstrou interações significativas com o sítio catalítico e periférico da acetilcolinesterase. A composição química do óleo essencial de *P. poeppigiana*, resultou na identificação de 19 compostos, inibição da lipoperoxidação e significativa inibição da atividade da acetilcolinesterase no hipocampo, córtex cerebral e hipotálamo. Além disso, a modelagem molecular realizada com os sesquiterpenos majoritários biciclogermacreno e germacreno D, apresentaram interação com os sítios catalítico e periférico da enzima acetilcolinesterase. Com relação ao estudo químico do extrato metanólico de *P. poeppigiana*, foi evidenciado a presença de dois alcaloides, uma cumarina, um iridoide e dois terpenos. Além disso, o extrato metanólico apresentou elevado teor de constituintes e inibição significativa nos modelos antioxidantes avaliados. No que se refere a inibição da acetilcolinesterase no modelo *in vivo*, o extrato metanólico apresentou inibição em quatro estruturas cerebrais avaliadas.

Ressalta-se que na literatura existem poucos relatos quanto aos estudos químicos e biológicos para as espécies em questão. Neste sentido, este estudo corrobora com os dados da literatura e uso popular descritos para a família e gênero com relação aos constituintes químicos e atividades biológicas relatados.

7 ANEXOS

7.1 Aprovação do Comitê de Ética (CEUA) – 14/2015

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

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 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 nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi 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

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

7.2. Aprovação do Comitê de Ética (CEUA) – 12/2017

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

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 6 de setembro de 2017.

CERTIFICADO

Certificamos que a proposta intitulada "***Alcalóides indólicos de duas espécies de Rubiaceae: isolamento, avaliação farmacológica e modelagem molecular***", registrada sob o protocolo de nº 12/2017, sob a responsabilidade de *Anelise Samara Nazari Formagio e Carla Roberta Ferreira Volobuff* – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata, subfilo Vertebrata* (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 12/05/2017.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*02/10/2017 a 20/02/2020*
Espécie/linhagem/raça	*Rattus norvegicus*/Wistar e *Mus musculus*/Swiss
Nº de animais	458/ 164 Wistar e 294 Swiss
Peso/idade	60 dias e 45 dias
Sexo	149 machos e 15 fêmeas Wistar 204 machos e 90 fêmeas Swiss
Origem	Biotério Central da UFGD

Melissa Negrão Sepulvida

Melissa Negrão Sepulvida
Coordenadora CEUA

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

7.3 OUTRAS CONTRIBUIÇÕES

Journal of Ethnopharmacology 231 (2019) 66–72

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Anti-inflammatory action of an alkaloid, fraction and extract from *Alchornea glandulosa* in mice

Frederico Formagio-Neto[a], Carla Roberta Ferreira Volobuff[a], Milena Menezes Corrêa Pederiva[b], Zefa Valdevina Pereira[b], Maria Helena Sarragiotto[c], Claudia Andrea Lima Cardoso[d], Candida Aparecida Leite Kassuya[a], Anelise Samara Nazari Formagio[a,b,*]

[a] Federal University of Grande Dourados, Faculty of Health Science, Dourados, MS, Brazil
[b] Federal University of Grande Dourados, Faculty of Biological and Environmental Sciences, Dourados, MS, Brazil
[c] Maringá State University, Chemistry Department, Maringá, PR, Brazil
[d] Department of Chemistry, University of Mato Grosso do Sul, Dourados, MS, Brazil

ARTICLE INFO

Keywords:
Alchornea glandulosa
Euphorbiaceae
Guanidine alkaloid
Flavonoids, phenolic acids, inflammation

ABSTRACT

Ethnopharmacological relevance: *Alchornea glandulosa* (Euphorbiaceae) has traditionally been used in medicine for treating immune-mediated inflammatory diseases.

Aim of study: This work aimed to evaluate the anti-inflammatory effects of a methanolic extract of leaves from *A. glandulosa* (MEAG), as well as the ethyl acetate fraction (EAFAG) and isolated compound guanidine alkaloid *N*-1, *N*-2, *N*-3-triisopentenylguanidine (AG-1), in experimental *in vivo* models of inflammation in mice. We also investigated this extract's phenols, flavonoids and flavonol compounds.

Materials and methods: MEAG (extracted by maceration with methanol), EAFAG (fraction resulting from the partition of the methanolic extract with ethyl acetate) and AG-1 (alkaloid isolated by chromatographic methods) were analysed. MEAG and EAFAG were analysed by HPLC/DAD. The effects of MEAG (30, 100 and 300 mg/kg), EAFAG (30, 100 and 300 mg/kg) and AG-1 (5 and 30 mg/kg) were studied in the following experimental mouse models: paw oedema and myeloperoxidase (MPO) activity, croton-oil-induced ear oedema, leukocyte migration in a pleurisy model induced by carrageenan and zymosan induction of joint inflammation.

Results: MEAG and EAFAG were analysed by LC/DAD, and phenolic acids (gallic acid and caffeic acid) and flavonoids (myricetin-3-O-α-rhamnopyranoside and quercetin) were detected. MEAG, EAFAG and AG-1 were used in the carrageenan-induced paw oedema model and showed maximum inhibitions of 60.10% (MEAG, 2 h, 300 mg/kg) and 66.21% (EAFAG, 2 h, 300 mg/kg). AG-1 at 5 mg/kg showed significant inhibition, ranging from 60.92% to 63.13%, at all evaluated times, and the 30 mg/kg dose showed inhibition of 42.12% (1 h) and 40.36% (2 h). MEAG (37%, 46.1% and 68.11%) and EAFAG (31%, 42.21% and 48.93%), at doses of 30, 100 and 300 mg/kg, respectively, significantly reduced the increase in MPO activity, and AG-1 (5 and 30 mg/kg) showed inhibition of 64.62% and 65.12%, respectively. In the pleurisy model, MEAG (300 mg/kg), EAFAG (300 mg/kg) and AG-1 (30 mg/kg) significantly reduced the migration of total leukocytes with maximal inhibition of 80.90%, 83.17% and 89.39%, respectively. In the croton oil model, pretreatment with MEAG (0.1, 0.3 and 1 mg/ear) increased the diameter of the right ear (30.32%, 48.87% and 53.09%, respectively). Finally, MEAG (100 and 300 mg/kg; 33.11% and 56.03%) and EAFAG (100 and 300 mg/kg; 36.89% and 50.53%) reduced zymosan-induced oedema formation.

Conclusions: To the best of our knowledge, these results are the first to demonstrate that *A. glandulosa* exhibits oral and topical anti-inflammatory activity. This study detected alkaloid and phenol/polyphenolic compounds in *A. glandulosa*, which may help to explain the ethnobotanical use of this plant in traditional medicine in Brazil to treat immune-mediated inflammatory diseases.

Abbreviations: AG-1, *N-1, N-2, N-3*-triisopentenylguanidine; EAFAG, ethyl acetate fraction; DEX, dexamethasone; LPS, lipopolysaccharide; MEAG, methanolic extract of *Alchornea glandulosa*; MPO, myeloperoxidase; NO, nitric oxide; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; TNF-α, tumour necrosis factor-α

* Correspondence to: Faculty of Health Sciences, UFGD, MS, Brazil.
E-mail addresses: fredformagio@hotmail.com (F. Formagio-Neto), volobuff81@gmail.com (C.R.F. Volobuff), mimenezesbiomed@gmail.com (M.M.C. Pederiva), zefapereira@ufgd.edu.br (Z.V. Pereira), mhsarragiotto@uem.br (M.H. Sarragiotto), claudia@uems.br (C.A.L. Cardoso), candidakassuya@ufgd.edu.br (C.A.L. Kassuya), aneliseformagio@ufgd.edu.br (A.S.N. Formagio).

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Chemical constituents of *Cochlospermum regium* (Schrank) Pilg. root and its antioxidant, antidiabetic, antiglycation, and anticholinesterase effects in Wistar rats

Thiago Félix de Miranda Pedroso[a,1], Thaise Regine Bonamigo[a], Jenifer da Silva[a], Paulo Vasconcelos[a], Jessica Maria Félix[a], Claudia Andrea Lima Cardoso[b], Roosevelt Isaias Carvalho Souza[a], Ariany Carvalho dos Santos[a], Carla Roberta Ferreira Volobuff[a], Anelise Samara Nazari Formagio[a], Virginia Demarchi Kappel Trichez[a,1,*]

[a] Faculty of Health Sciences, Federal University of Grande Dourados, Dourados, MS, Brazil
[b] Course of Chemistry, State University of Mato Grosso do Sul, Dourados, MS, Brazil

ARTICLE INFO

Keywords:
Cochlospermum regium
Diabetes
Phenolic compounds
AGEs
Alloxan
Acetylcholinesterase

ABSTRACT

The main physiological consequence of diabetes mellitus is chronic hyperglycemia. This condition is related to the formation of free radicals including advanced glycation end products (AGES) and to an increase in inflammatory processes. *Cochlospermum regium* (Schrank) Pilg., part of the Bixaceae family, is a cerrado plant known for its anti-inflammatory effects. The objectives of this study were to analyze the constituent compounds of *C. regium* roots and to evaluate the antioxidant, antiglycation, antidiabetic, and anticholinergic effects of its hydromethanolic extract through *in vitro* and *in vivo* experimental models. The presence of phenols, flavonoids, condensed tannins, and flavonols was analyzed by liquid chromatography - photodiode array (LC/PDA) analysis. Whereas antioxidant activity was investigated *via* DPPH, ABTS, β-carotene/linoleic acid, and malondialdehyde colorimetric assays. Inhibition of AGEs was examined *via* a bovine serum albumin system whose glycosylating agent was glucose. Antidiabetic potential was examined in normoglycemic Wistar rats that received glucose overload, in alloxan-induced diabetic rats, and in rats that received a hyperglycemic diet. Disaccharidase inhibition was assessed using *in vitro* and *in vivo* methods, as was acetylcholinesterase (AChE) inhibition in brain structures. The hydromethanolic extract (CRHE) possessed a high concentration of phenolic compounds and showed antioxidant effects. The LC-DAD results revealed that CRHE contained a high concentration of phenolic acids and the majority was gallic acid. Treatment with CRHE caused significant inhibition of AGE formation. The oral glucose tolerance test (OGTT) in normoglycemic animals showed a reduction in blood glucose levels after treatment with 100 mg/kg CHRE, accompanied by an increase in hepatic glycogen content. There was also a significant reduction in the fasting glucose levels of alloxan-induced diabetic animals after 7 days of treatment with daily doses of 100 mg/kg. After 14 weeks of hyperglycemic diet, the last four or which were combined with 100 mg/kg CRHE treatment, there was a decrease in blood triglyceride levels. There was also a statistically significant decrease in the enzymatic activity of maltase, lactase and sucrase. The results demonstrate that oral administration of 30 and 100 mg/kg CRHE inhibited AChE activity in different brain structures. Thus, the extract of *C. regium* showed promising antioxidant, antiglycation, and antidiabetic effects that may be related to its high phenolic content.

1. Introduction

Diabetes mellitus (DM) is a multifactorial disease, caused by a combination of genetic, behavioral, social, and environmental factors. Normal glucose homeostasis is achieved by the precise regulation of insulin produced and secreted by the islets of Langerhans in the pancreas combined with an appropriate response from body tissues and organs including the liver, muscles, and adipose tissue. A absolute

* Corresponding author at: Federal University of Grande Dourados, Dourados Highway - Itahum, Km 12 - University City, Zip Code 364 - CEP 79804-970, Dourados, Mato Grosso do Sul, Brazil.
E-mail address: virginiakappel@ufgd.edu.br (V.D.K. Trichez).
[1] These authors equally contributed to this study.

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ORIGINAL ARTICLE

Anti-inflammatory Activity of Methanolic Extract and an Alkaloid from *Palicourea crocea* (Sw.) Roem and Schult

Anelise Samara Nazari Formagio,[1,2,5] Pedro Cruz de Oliveira Junior,[1] Carla Roberta Ferreira Volobuff,[2] Candida Aparecida Leite Kassuya,[2] Diego Cegobia Ferreira,[3] Claudia Andrea Lima Cardoso,[3] Maria Helena Sarragiotto,[4] and Zefa Valdevina Pereira[1]

Abstract— *Palicourea crocea* (Sw.) Roem. and Schult., "douradinha," are used by treat inflammation (edema). Croceaine A (PC-1) was isolated from *P. crocea* (MEPC) leaves and studied for its antioxidant and anti-inflammatory activity, as well as concentrations of constituents and acute toxicity. The phenols and polyphenolics compounds and HPLC/DAD were determined. The antioxidant activity were evaluated for DPPH, ABTS, and MDA. MEPC (300, 100, and 300 mg/kg) and PC-1 (10 and 30 mg/kg) were tested for anti-inflammatory effects in paw edema, pleurisy, cold sensitivity, and mechanical hyperalgesia. Acute toxicity is also described. MEPC contained high concentrations of phenolic and flavonoid compounds (≤ 800.35 mg/g), as well as caffeic acid, ferulic acid, rutin, and quercetin, revealed by HPLC-DAD analysis. MEPC displayed antioxidant activity against ABTS radicals (IC_{50} = 68.5 μg/mL) and MDA (74%). MEPC and alkaloid PC-1 demonstrated an anti-edematogenic effect in Cg-induced paw edema in 2 and 4 h, and also significantly reduced mechanical hyperalgesia, cold response to acetone in mice, at 3 and 4 h after injection, as well as leukocyte migrationin the pleurisy model. No toxicity was detected by MEPC. For the first time, *P. crocea* was evaluated for its antioxidant, systemic anti-inflammatory, and anti-hyperalgesic activities.

KEY WORDS: antioxidant; anti-hyperalgesic; croceaine A; douradina; edema; pleurisy.

INTRODUCTION

Palicourea crocea (Sw.) Roem. and Schult., known as "douradina", "douradão," and "douradão-do-campo," is used by Ribeirinhos in the North Araguaia microregion, Mato Grosso, Brazil, to make infusions or decoctions for general infections and anti-inflammatory (edema) [1], and is not considered a toxic plant [2–6]; however, some species in this genus are highly lethal, *i.e.*, *P. marcgravii* St. Hil. in Brazil contains a toxic organofluorine compound and is a very poisonous plant due to its acute toxicity and palatability [7–12]. Chemical studies of this species reported the isolation of alkaloids such as croceaines A and B, psychollatine, 3,4-dihydro-1-(1-β-glucopyranosyloxy-1, 4a, 5, 7a-tetrahydro-4-methoxycarbonylcyclopenta [c] pyran-7-yl)-β-carboline-N-2-oxide, brachycerine, and palicroceaine [13–15]. Studies with leaves have

[1] Faculty of Biological and Environmental Sciences, Federal University of Grande Dourados UFGD, Dourados, Mato Grosso do Sul State, Brazil
[2] Faculty of Health Sciences, Federal University of Grande Dourados UFGD, Dourados, Mato Grosso do Sul State, Brazil
[3] Chemistry, State University of Mato Grosso do Sul UEMS, Dourados, Mato Grosso do Sul State, Brazil
[4] Chemistry, State University of Maringá UEM, Maringá, Paraná, Brazil
[5] To whom correspondence should be addressed at Faculty of Biological and Environmental Sciences, Federal University of Grande Dourados UFGD, Dourados, Mato Grosso do Sul State, Brazil. E-mail: aneliseformagio@ufgd.edu.br

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