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AVALIAÇÃO DA ATIVIDADE FARMACOLÓGICA CENTRAL DO EXTRATO HIDROETANÓLICO (EHPA), FRAÇÃO ACETATO DE ETILA (FAE) E FRAÇÃO ETANÓLICA (FET) DA PLANTA *POLYGALA ALTOMONTANA* EM RATOS

Recife
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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia, da Universidade Federal de Pernambuco, como parte dos requisitos parciais para obtenção do título de mestre em Bioquímica e Fisiologia.

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“Se quiser triunfar na vida, faça da perseverança a sua melhor amiga; da experiência, o seu conselheiro; da prudência, o seu irmão mais velho; e da esperança, o seu anjo da guarda.” (ADDISON, 2018).

RESUMO

O gênero vegetal *Polygala* é o maior representante da família *Polygalaceae*, apresentando diversas propriedades farmacológicas e ações no sistema nervoso central. Estudos fitoquímicos da espécie *Polygala altomontana* revelaram a presença de compostos cumarínicos, flavonoides e estirilpironas, representando uma excelente ferramenta farmacológica para estudar sua ação central em modelos animais. Neste estudo avaliamos os efeitos do extrato hidroetanólico (EHPA) e frações acetato de etila (FAE) e etanólica (FET) de *P. altomontana* em ratos. Também investigamos a possível participação dos receptores benzodiazepínicos/$GABA_A$, adenosina e opióides nas ações centrais do EHPA. Todos os protocolos experimentais utilizados neste estudo foram aprovados pelo Comitê de Ética no Uso de Animais. Ratos Wistar adultos machos foram tratados por via oral com EHPA, FAE e FET (50, 100 ou 300 mg/Kg), 1 h antes dos testes da caixa de transição claro-escuro, campo aberto, roda giratória, convulsão induzida por pentilenotetrazol (PTZ), sono induzido por quetamina ou isoflurano. Outros grupos de ratos previamente canulados intracerebroventricularmente (i.c.v.) por estereotaxia receberam por via v.o. EHPA 300 mg/Kg/dia, por 3 dias consecutivos, seguido da administração i.c.v. do veículo ou estreptozotocina (ETZ 0,1 mg/4 µL) e, após 2 dias, a memória foi avaliada no teste da esquiva inibitória. No último protocolo experimental, os animais foram pré-tratados com antagonistas de receptores benzodiazepínicos, o flumazenil (FLU 5 mg/Kg, i.p), antagonista de receptores de adenosina, a cafeína (CAF 3 mg/Kg, i.p) ou antagonistas de receptores opioides, a naltrexona (NTX 2,5 mg/Kg, s.c) seguido da administração v.o. com EHPA (300 mg/Kg), e 1 h após, o comportamento foi avaliado no campo aberto. Na caixa de transição claro-escuro, a FAE 300 mg/Kg reduziu o número de transições entre os dois compartimentos. No campo aberto, o EHPA 300 mg/Kg reduziu a movimentação central, periférica e total, além do número de comportamentos de levantar. EHPA 50 mg/Kg aumentou a movimentação central. No teste do PTZ, EHPA 300 mg/Kg e FET 50 mg/Kg diminuíram a letalidade dos animais em 25 e 30 %, respectivamente. No teste da quetamina, a FAE e FET aumentaram a duração total do sono, enquanto no teste do isoflurano, EHPA, FAE e FET aumentaram a duração total do sono. Na esquiva inibitória, a ETZ reduziu a latência para descida da plataforma, efeito totalmente prevenido pelo tratamento repetido com EHPA. Por fim, o FLU, CAF ou NTX bloquearam totalmente os efeitos do EHPA na movimentação central, periférica e total, bem como no número de levantar, no teste do campo aberto. Nossos resultados sugerem que a *P. altomontana* é uma planta com ações do tipo ansiolítica, hipnosedativo, anticonvulsivante e

promnésica. Este estudo também fornece evidências para o envolvimento dos receptores benzodiazepínicos/$GABA_A$, adenosina e opióides nas ações centrais da *P. altomontana*, pelo menos para suas ações sedativas.

Palavras-chave: *P. altomontana*. Ansiolítico. Hipnosedativo. Promnésico. $GABA_A$. Adenosina. Opióides. Ratos.

ABSTRACT

The genus *Polygala* is the main representative of plant family *Polygalaceae* that shows several pharmacological properties, among them neuropharmacological actions. The phytochemical study of *P*. altomontana revealed the presence of coumarins, flavonoids, styryl-2-pyrones and dihydrostyryl-2-pyrones compounds that represent an excellent pharmacological tool to study its central action in animal models. In this study, we evaluated the effects of the hydroethanolic extract (HE), ethyl acetate (EA) and ethanolic (ET) fractions of *P. altomontana* in several animal behavioral models. The possible participation of benzodiazepine/$GABA_A$, adenosine and opioids receptors in the profile of central action of this plant also was investigated. Adult male Wistar rats were orally treated with HE, EA and ET (50, 100 or 300 mg/Kg), 1 h before the light-dark, open field, rotarod, pentylenetetrazole (PTZ)-induced convulsion, ketamine or isoflurane-induced hypnosis. Other animals groups previously cannulated intracerebroventricularly (i.c.v.) by stereotaxy received via v.o. HE 300 mg/Kg/day, by 3 consecutive days, followed by i.c.v. administration of vehicle or streptozotocin (STZ 0.1 mg/4 μL) and, 2 days latter, the memory was evaluated in the inhibitory avoidance test. In the last experimental protocol, the animals were pretreated with benzodiazepine receptor antagonist, flumazenil (FLU 5 mg/Kg, i.p), adenosine receptor antagonist, caffeine (CAF 3 mg/Kg, i.p) or opioid receptor antagonist,naltrexone (NTX 2.5 mg/Kg, s.c) followed by HE (300 mg/Kg, v.o.), and 1 h after, the behavior was evaluated in the open field test. In the light-dark transition test, EA 300 mg/Kg reduced the number of transitions between the two compartments. In the open field, HE 300 mg/Kg reduced the central, peripheral and total movements, as well the number of rearings. HE 50 mg/Kg increased the central movements. In the PTZ-test, HE 300 mg/Kg and ET 50 mg/Kg decreased lethality rate in 25 and 30%, respectively. In the k*etamine-induced hypnosis test*, EA and ET increased the total sleep time, while in the isoflurane-*induced hypnosis* test, HE, EA and ET increased the total sleep time. In the rotarod test, latency for the first fall and the total number of falls of the rotarod were not altered by any treatments. In the inhibitory avoidance test, STZ reduced the step-down latency time, an effect totally prevented by repeated treatment with HE. Phytochemical analysis revealed the major presence of aurapten and isoquercetin compounds, suggesting that these active substances could be responsible by central actions seen for *P. altomontana*. Finally, the pretreatment with FLU, CAF or NTX totally blocked the effects of HE on the central, peripheral and total movements, as well as the number of rearings, in the open field test. The present work suggests that *P. altomontana* is an

herbal medicine which possesses anxiolytic, hypnosedative, anticonvulsant and promnestic actions. Our data provides evidence for the involvement of benzodiazepine/$GABA_A$, adenosine and opioids receptors in sedative effects promoted by HE.

Keywords: *Polygala altomontana*. Anxiolytic. Hypnosedativo. Promnestic. $GABA_A$. Adenosine. Opioids. Rats.

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1 INTRODUÇÃO

O uso das plantas medicinais como agentes psicoativos para tratar doenças relacionadas ao sistema nervoso central (SNC) data de tempos muito remotos. Pelo mundo inteiro estão distribuídas inúmeras espécies vegetais com supostas atividades psicoativas, de forma que as plantas medicinais sempre representaram, na história da humanidade, uma ferramenta para a origem de um vasto repertório de substâncias com propriedades farmacológicas distintas, as quais podem subsidiar a descoberta de modernos medicamentos para uso na psiquiatria (Garcia-Garcia et al., 2008; Gomes et al., 2009; Sarris et al., 2011).

Nos últimos anos, transtornos psiquiátricos como ansiedade e depressão, bem como os distúrbios do sono vêm aumentando em taxas expressivas na população mundial, em parte devido às mudanças ocorridas no contexto cultural, econômico e social, exigindo das pessoas maior capacidade de adaptação ao novo ritmo de vida. Por estas e outras razões, o ser humano se tornou cada vez mais vulnerável a problemas de saúde mental advindas das excessivas demandas físicas e psicológicas. Embora estas doenças representem patologias totalmente distintas e independentes, uma alta prevalência de pacientes exibe uma sobreposição destas em comorbidade, dificultando o tratamento. Dentro deste cenário, a falta de um tratamento adequado ou o subtratamento acarreta em diversos problemas de âmbito sócio-econômico. Apesar da vasta quantidade de medicamentos disponíveis atualmente na clínica, existem ainda vários tipos de psicopatologias que não respondem ao tratamento convencional. Além disso, a maior parte dos medicamentos utilizados atualmente, como benzodiazepínicos e antidepressivos, está associada a um grande número de efeitos indesejáveis ou à lentidão para alívio da sintomatologia, o que interfere negativamente na aderência do paciente ao tratamento medicamentoso. Outra doença que vem se tornando cada vez mais um grande problema de saúde pública no mundo é a Doença de Alzheimer (DA), a principal causa de demência entre idosos. Trata-se de uma doença neurodegenerativa, crônica e progressiva. Infelizmente os medicamentos aprovados atualmente para o tratamento da DA limitam-se ao retardo na evolução natural da doença, permitindo apenas uma melhora temporária do estado funcional do paciente (El Gaamouch et al., 2016; Scheltens et al., 2016). Portanto, o interesse na busca de compostos como fonte de novos medicamentos para o tratamento de doenças do SNC deriva das necessidades

terapêuticas e da grande diversidade tanto em suas estruturas químicas, quanto em suas atividades biológicas. Diante desta perspectiva, os metabólitos secundários provenientes de plantas medicinais representam a maior fonte geradora de substâncias biologicamente ativas (Liu et al., 2015).

O gênero de planta *Polygala*, pertencente à família Polygalaceae, contém 600 espécies vegetais (Fenner et al., 2005), 19 das quais são encontradas no estado de Santa Catarina, Brasil. Muitas das espécies de *Polygala* são utilizadas na medicina popular como anestésicos, agentes anti-inflamatórios e para o tratamento de doenças intestinais, renais e do sistema nervoso central (De Campos et al., 1997; Pinheiro et al., 1998; El Sayah et al., 1999; Weinhold et al., 2008). Uma investigação fitoquímica deste gênero revelou a ocorrência de uma grande variedade de metabólitos secundários importantes, entre eles as xantonas (Pinheiro et al., 1998; Cristiano et al., 2003; Chaturvedi et al., 2005; Lin et al., 2005), saponinas (Zhang et al., 1997; Mitaine-Offer et al., 2003; Jia et al., 2004) oligossacarídeos (Ikeya et al., 2004; Li et al, 2005), flavonóides (Rao e Raman, 2004; Pizzolatti et al., 2008), cumarinas (Hamburger et al., 1984 e 1985; Pizzolatti et al., 2000 e 2002) e estirilpironas (Pizzolatti et al., 2000 e 2004). Estas substâncias são apontadas como as responsáveis por diversas ações centrais atribuídas aos extratos e frações das espécies deste gênero, entre elas se destacando as propriedades neuroprotetora, ansiolítica, hipnosedativa, anticonvulsivante e antidepressiva.

Polygala altomontana representa uma nova espécie de planta encontrada nos estados do Paraná e Santa Catarina (Ludtke et al., 2013). Estudos fitoquímicos desta espécie vegetal revelaram a presença de compostos cumarínicos, estirilpironas, dihidroestirilpironas e flavonoides (Tizziani et al., 2017). Por ser uma das poucas espécies deste gênero a sintetizar estes grupos de compostos, esta planta representa uma excelente ferramenta para estudar sua ação em modelos animais, já que, até o presente momento, não há relatos na literatura sobre os possíveis efeitos centrais desta espécie vegetal em modelos animais de psicopatologias.

1.1 Objetivos

1.1.1 Geral:

Investigar a atividade farmacológica central da planta *Polygala altomontana* na tentativa de se buscar novas alternativas terapêuticas para o tratamento da ansiedade,

convulsão, distúrbios do sono, bem como no défict de memória. Para tal finalidade, os seguintes objetivos específicos foram estabelecidos:

1.1.2 Específicos:

- ✓ Avaliar o perfil de atividade farmacológica central do extrato hidroetanólico provenientes da planta *P. altomontana* nas doses de 50, 100 e 300 mg/Kg em ratos;
- ✓ Avaliar o perfil de atividade farmacológica central da fração acetato de etila provenientes da planta *P. altomontana* nas doses de 50, 100 e 300 mg/Kg em ratos;
- ✓ Avaliar o perfil de atividade farmacológica central da fração etanólica provenientes da planta *P. altomontana* nas doses de 50, 100 e 300 mg/Kg em ratos;
- ✓ Investigar se os receptores benzodiazepínicos/$GABA_A$, opióides e adenosina estão envolvidos nas ações centrais do EHPA na dose de 300 mg/Kg.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 Plantas medicinais no cenário de tratamento de transtornos psiquiátricos

As plantas medicinais vêm sendo utilizadas em diversas partes do mundo no tratamento de várias doenças, dentro de um contexto cultural na medicina popular, como única opção pelas populações mais carentes, como alternativa terapêutica nos países ricos e desenvolvidos, e como fonte geradora de novas moléculas biológicas, prototípicas ou não, pela indústria farmacêutica mundial. Na área de saúde psiquiátrica, em 1998, nos Estados Unidos, estimou que 43 % da população que sofria de transtornos de ansiedade usavam alguma forma de terapia complementar (Eiseberg et al., 1998), sendo as plantas medicinais as mais populares (Astin, 1998; Wong et al., 1998). No Brasil, país com a maior biodiversidade mundial (estimada em torno de 20 % do total mundial), as plantas superiores respondem por aproximadamente 24 % da biodiversidade (Guerra et al., 2001). Com o seu grande patrimônio genético representado pelos seus cinco biomas florestais principais, associado à rica diversidade étnico-cultural envolvendo grupos indígenas, europeus e africanos, o ecossistema nacional brasileiro possui inúmeras, talvez incontáveis, espécies de plantas que ainda carecem de estudos em busca das mais diversas atividades biológicas.

Atualmente, aproximadamente 40 % dos medicamentos disponíveis na terapêutica foram desenvolvidos de fontes naturais, sendo 25 % a partir de plantas medicinais, 13 % de microorganismos e 3 % de animais (Calixto, 2001). A OMS estimou que aproximadamente 80 % da população mundial utilizam diretamente da natureza as plantas medicinais como principal fonte de tratamento em seus cuidados básicos de saúde. Além disso, 25 % de todos os medicamentos prescritos pelos médicos na medicina atual são obtidos a partir de ervas de diferentes formas. Alguns deles são produzidos diretamente a partir de extratos de plantas e outros são produzidos artificialmente para proporcionar efeitos semelhantes a medicamentos à base de plantas (Ernst, 2005).

No Brasil, a atenção básica de saúde e O Sistema Único de Saúde (SUS) têm poucas experiências registradas envolvendo ações de plantas medicinais até o final da primeira década do século XXI. Isso poderia estar relacionado à introdução da Política

Nacional sobre Práticas Integrativas e Complementares, e a Política Nacional de Plantas Medicinais, em 2006, que foram passos decisivos para a introdução do uso de plantas medicinais e fitoterápicas no SUS (Antonio et al., 2014).

2.2 O gênero *Polygala* no cenário de investigação neurofarmacológica

O gênero vegetal *Polygala* é o maior representante dos 19 gêneros que compõem a família *Polygalaceae* que inclui aproximadamente 600 espécies de ampla distribuição geográfica (América Central, América do Sul, África e Ásia) (Marques, 1996). O gênero *Polygala* inclui plantas herbáceas distribuídas em todo o mundo, especialmente em áreas tropicais e temperadas, com exceção da Nova Zelândia e zonas Árticas e Antárticas (Marques e Peixoto, 2007). Na Paraíba, por exemplo, estima-se a ocorrência de 20 espécies da família Polygalaceae, sendo o gênero *Polygala* predominante com 55 % das espécies. Em Pernambuco, destaca-se a espécie *Polygala lancifolia St. Hill*. em campos de restingas, *Polygala corisoides St. Hill*. em vegetações rasteiras próximas de praias e *Polygala longicaulis H. B. K.* nas zonas de savanas (Coelho et al., 2008). Entretanto, as informações publicadas sobre a família *Polygalaceae* no nordeste do Brasil são raras e esporádicas.

Do ponto de vista químico, apenas 29 espécies da família *Polygalaceae* foram estudadas e, entre estas, 16 apresentaram propriedades farmacológicas, com destaque as atividades anti-inflamatórias, antifúngicas, tripanomicida, antitumoral e hipoglicemiante (Kako et al., 1997; El Sayah et al., 1999). Em relação às ações no sistema nervoso central, algumas espécies do gênero *Polygala* foram descritas apresentar atividades neuroprotetora (*P. japonica*, *P. paniculata*, *P. tenuifolia*, *P. glomerata* e *P. tricornus*) (Park et al., 2002; Ikeya et al., 2004; Farina et al., 2005; Franco et al., 2007; Li et al., 2012; Li et al., 2014), neuritogênica (*P. molluginifolia*) (Naidu et al., 2007), ansiolítica, sedativa ou anticonvulsivante (*P. sabulosa, P. tenuifolia*) (Duarte et al., 2007; Duarte et al., 2008; Kawashima et al., 2004; Yao et al., 2010;Lee et al., 2013), mnemônica, antipsicótica (*P. tenuifolia*) (Chung et al., 2002; Karakida et al., 2007; Li et al., 2008), antidepressiva (*P. japonica*, *P. paniculata*, *P. sabulosa* e *P. tenuifolia*) (Cheng et al., 2006; Li et al., 2006; Capra et al., 2010; Hu et al., 2010; Bettio et al., 2011) e capacidade de reduzir sinais de abstinência a opióides (*P. telephioides*) (Egashira et al., 2006). A espécie *P. tenuifolia* vem se destacando pelo seu amplo estudo por diversos grupos de pesquisa, além do maior número de atividades farmacológicas identificadas

até o momento. Os estudos fitoquímicos e de bioatividade mostraram a presença de diversas classes de metabólitos secundários que são apontados como os responsáveis pelas ações centrais atribuídas aos extratos e frações de cada espécie acima descrita. Os principais compostos identificados até o presente momento foram: saponinas, esteróides, flavonóides livres e glicosilados, cumarinas, ésteres de ácidos cinâmicos com oligossacarídeos e polissacarídeos, dihidroestirilpironas, estirilpironas e espinasterol.

Figura 1 – Atividades biológicas (ação central) e compostos isolados de espécies de plantas do gênero *Polygala.*

PLANTA	AÇÃO CENTRAL	COMPOSTOS BIOATIVOS
P. caudata	Neuritogênica	Euxantonas, xantonas
P. glomerata	Neuroprotetora	Xantonalignóides
P. japonica	Neuroprotetora, mnemônica, Antidepressiva	Saponinas triterpênicas Saponinas
P. molluginifolia	Antinociceptiva	5,3',4'-trihidroxi-6',6'-dimetilpirano[2',3':7,6]isoflavona (isoflavona)
P. paniculata	Neuroprotetora Antinociceptiva Antidepressiva	Xantonas
P. sabulosa	Antinociceptiva Ansiolítica, hipnosedativa, anticonvulsivante Antidepressiva	α-espinasterol (esterol), escopoletina (cumarina) Estirilpironas, dihidroestirilpironas (estéril-lactonas) Escopoletina (cumarina)
	Neuroprotetora	Quercetina, apigenina e 3-O-β-D-glicopiranosil-quercetina (flavonóides)
P. telephioides	Diminuição da abstinência a opióides	Ésteres de oligossacarídeos, benzofenona C-glicosídeos
P. tenuifolia	Antipsicótica Neuroprotetora, mnemônica Anti-estresse, ansiolítica, sedativa Antidepressiva	Poligalasaponinas (saponinas triterpenóides) Tenuigenina = senegenina (isoprenóide), poligalasaponinas, tenuifoilina, ácido poligalácico (saponinas triterpenóides), ésteres de oligossacarídeos e oligossacarídeos acilados, tenuifolisídeo A (éster de oligossacarídeo), ácido sinápico (ácido carboxílico fenilpropanóide) Ácido-3,4,5-trimetoxicinâmico (ácido cinâmico), poligalasaponinas (saponinas triterpenóides) 3,6'-disinapoil-sacarose (Oligossacarídeo)
P. tricornis	Neuroprotetora	5-formilfurano-2-il) metil-4-hidroxi-2-metilenobutanoato

Fonte: O Autor (2016)

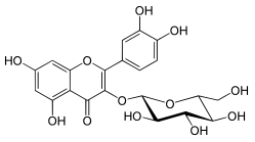
2.3 *Polygala altomontana* e aspectos fitoquímicos

A planta *Polygala altomontana* Lüdtke, Boldrini & Miotto é uma das 39 espécies do gênero Polygala L. descrita para a região sul do Brasil e encontrada em

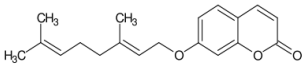
campos de alta altitude dos estados do Paraná e Santa Catarina. É um subarbusto prostrado com uma altura entre 16-47 cm, com caule folioso, quadrangular, glabro. As folhas são opostas e raramente alternas. Apresenta flores com carena cristada, carnosas, glandulosas, predominantemente brancas, mas podem ser rosa ou lilás. Além disso, têm corola persistente, fruto estreitamente alado, estilete e estigma persistentes no fruto (Ludtke et al., 2013).

A partir desta espécie de planta foi possível o preparo do extrato bruto hidroetanólico (EHPA), que por sua vez foi submetido ao fracionamento cromatográfico, fornecendo a Fração acetato de etila (FAE) e Fração etanólica (FET) (Tizziani et al., 2017). Estudos fitoquímicos da fração acetato de etila desta espécie de planta revelaram a presença de compostos denominados estirilpironas, dihidroestirilpironas, além de α-espinasterol e cumarinas. Até o presente momento, foram isoladas da fração acetato de etila da *P. altomontana*, uma dihidroestiril-2-pirona, quatro estiril-2-pironas, uma cumarina e um esteróide (Figura 2). A planta *P. altomontana* é a segunda espécie do gênero estudada até o presente momento, além da espécie *P. sabulosa*, cuja rota bioquímica produz estiril-2-pironas e dihidroestiril-2-pironas com ausência de xantonas (Pizzolatti et al., 2000). A partir de outra coluna cromatográfica foi possível o isolamento do flavonoide isoquercetina (Figura 2), composto majoritário presente na fração etanólica da planta *P. altomontana.*

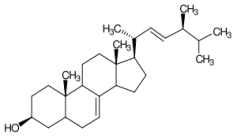
Figura 2 – Compostos isolados das frações acetato de etila e etanólica da planta *Polygala altomontana*.



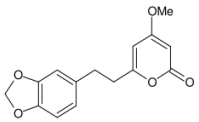
Quercetina-3-O-β-D-glucopiranosídeo



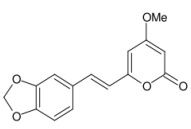
6-metoxi-7-preniloxicumarina



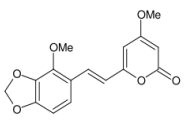
α-espinasterol



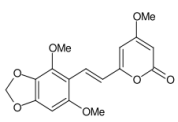
4-metoxi-6-(11,12-metilenodioxi-dihidroestiril)-2-pirona



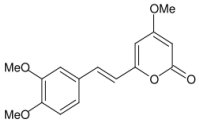
4-metoxi-6-(11,12-metilenodioxiestiril)-2-pirona



4-metoxi-6-(11,12-metilenodioxi-10-metoxiestiril)-2-pirona



4-metoxi-6-(11,12-metilenodioxi-10,14-dimetoxiestiril)-2-pirona



4-metoxi-6-(11,12-dimetoxiestiril)-2-pirone

Fonte: O Autor (2016)

2.3.1 Estirilpironas e dihidroestirilpironas

As estiril-pironas ou estiril-lactonas são compostos que possuem como característica molecular básica um anel γ-, δ-, ζ-lactona conectado a um fragmento estiril ou dihidroestiril. Acredita-se que a biossíntese desta classe de compostos seja resultante da rota metabólica do ácido chiquímico (Herderich et al., 1997). O ácido chiquímico é conhecido como uma espécie química essencial para o metabolismo secundário dos vegetais, pois a partir dele é sintetizada a maioria dos constituintes com núcleos benzênicos. Assim, ao precursor ácido cinâmico são incorporadas duas moléculas de malonil-CoA, sobre ação da enzima estirilpirona-sintase, formando o policetídeo como intermediário-chave, que, por sua vez, sofre lactonização, resultando nas estiril-pironas. É postulado que a síntese destes compostos nesta espécie de planta seja devido a um desvio metabólico da biossíntese de xantonas (Herderich et al., 1997).

Duarte et al., 2007, mostrou que o extrato bruto e a fração de acetato de etila obtida da *Polygala sabulosa* promoveram um efeito anticonvulsivante, hipnosedativo e ansiolítico em roedores. Essas ações centrais foram atribuídas aos princípios ativos presentes: as dihidroestiril-2-pironas e estiril-2-pironas.

2.3.2 Aurapteno

O aurapteno (7-geraniloxicumarina), uma cumarina natural, está associada a um amplo espectro de atividades farmacológicas, entre elas antiparasitária (Napolitano et al., 2004), antitumorais (Murakami et al., 1997; Tanaka et al., 1998; Tanaka et al., 2008), anti-inflamatória (Curini et al., 2006; Yan et al., 2013) e atividade reguladora sobre o metabolismo lipídico hepático (Nagao et al., 2010; Takahashi et al., 2011). Já em relação ao as informações são escassas, limitando-se apenas a uma possível atividade neuroprotetora. Okuyama et al., em 2016, sugeriu o potencial do aurapteno como agente neuroprotetor para Doença de Parkinson (DP), visto que este composto foi capaz de suprimir a ativação microglial e subseqüente morte de células neuronais dopaminérgicas na região da substância negra de ratos no modelo de DP induzidos por LPS. Furukawa et al., 2012 mostrou que o aurapteno tem a capacidade de induzir a ativação de ERK1/2 e CREB em cultura de neurônios e células PC12, que é um sistema modelo para estudos sobre proliferação e diferenciação neuronal. Além disso, é capaz

de induzir o crescimento de neuritos a partir de células PC12, sugerindo como um agente neurotrófico no tratamento de doenças neurodegenerativas. Genovese e colaboradores, em 2009, também mostraram num estudo *in vivo*, uma atividade neuroprotetora do aurapteno em um modelo de convulsões induzidas por electrochoque máximo em ratos.

2.3.3 α-espinasterol

O α-espinasterol é um esterol encontrado em uma variedade de plantas, como folhas de espinafre, alfafa, pepino, sementes de abóbora, entre outras (Yannai, 2012). Socała et al., 2015,mostrou que o α-espinasterol, atua como antagonista de receptor vanilóides TRPV1, exercendo efeitos anticonvulsivantes, em três modelos distintos de convulsões agudas em camundongos. Além disso, estes mesmos autores mostroram que o α-espinasterol (em doses de 1 e 2 mg/Kg) diminuiu o tempo de imnobilidade em camundongos avaliados no teste de natação forçada, sugerindo um ação do tipo antidepressiva. Além disso, a coadministração de uma dose ineficaz de α-espinasterol (0,5 mg/Kg) com uma dose ineficaz de um outro antagonista de TRPV1 - capsazepina (50 ug) produziu um efeito sinérgico na diminuição da imobilidade no teste de natação forçada, podendo representar uma nova abordagem terapêutica para o desenvolvimento de antidepressivos. No entanto, este composto mostrou-se desprovido de efeitos do tipo ansiolíticos em animais avaliados nos testes da caixa claro-escuro e labirinto em cruz elevado (Socała, 2016).

2.3.4 Isoquercitina

A isoquercetina (Quercetina-3-*O*-β-D-glicopiranosídeo) é uma das principais formas glicosiladas do flavonoide quercetina (3,5,7,3′,4′-pentahidroxiflavonoide) e é comumente encontrado em frutas, vegetais, cereais e várias bebidas derivadas de plantas, como chás e vinhos (Hasumura et al., 2004). A isoquercitina está presente em diversos tranquilizantes naturais, e provavelmente com outros flavonoides, são os responsáveis por partes das propriedades farmacológicas (Loscalzo et al., 2009). Estudos *in vivo* com isoquercetina evidenciam suas potenciais atividades antioxidantes (Li et al., 2011), anti-inflamatórias (Morikawa et al., 2003), anticarcinogênicas (Nishimura et al., 2010; Shimada et al., 2010; Kuwata et al., 2011; Morita et al., 2011; Fujii et al., 2013), cardioprotetoras (Emura et al., 2007; Motoyama et al., 2009;

Gasparotto Junior et al., 2012), antidiabéticas (Panda e Kar, 2007) e antialérgicas (Fernandez et al., 2005; Rogerio et al., 2007; Makino et al., 2009). Em relação as suas atividades neurofarmacológicas descritas, foram sugeridos possíveis efeitos antidepressivos e sedativos em modelos animais de comportamento (Butterweck et al., 2000; Loscalzo et al., 2009).

2.4 Psicopatologias e os receptores $GABA_A$, opióides e de adenosina

Os transtornos de ansiedade são amplamente aclamados como doenças psiquiátricas de preocupação global que são capazes de comprometer o bem-estar do ser humano (Fajemiroye et al., 2014). Cerca de 500 milhões de pessoas no mundo sofrem de transtorno de ansiedade (Kaviani et al., 2008). A ansiedade é uma situação emocional que contém o sentimento de medo ou preocupação (Farahini et al., 1999), sendo caracterizado por alterações cognitivas, somáticas, emocionais e comportamentais (Seligman et al., 2001). Aproximadamente 4-6% da população sofre de várias formas de transtornos de ansiedade com sintomas como pressão arterial elevada, freqüência cardíaca elevada, sudorese, fadiga, sensação desagradável, tensão, irritabilidade e inquietação (Mendlowicz e Stein, 2000; Smith et al., 2008). Estes sintomas constituem um impacto negativo para o paciente, para as famílias e para a sociedade, que na ausência de tratamento, os pacientes progridem para a depressão e às vezes contemplam o suicídio.

A incidência dos transtornos de ansiedade aumenta mundialmente em taxas expressivas, em parte em decorrente do estresse da vida cotidiana, consumo de substâncias de abuso, entre outros fatores. O desenvolvimento de novos agentes terapêuticos capazes de controlar a ansiedade constitui um grande desafio para os laboratórios de pesquisa e para a indústria farmacêutica na atualidade, considerando a grande prevalência mundial dessas doenças. A falta de um tratamento adequado ou o subtratamento dos estados de ansiedade acarreta em diversos problemas de âmbito sócio-econômico. Apesar dos grandes investimentos e da vasta quantidade de medicamentos ansiolíticos disponíveis atualmente na clínica, existem ainda vários tipos de transtornos de ansiedade que não respondem ao tratamento convencional. Além disso, a utilização de drogas ansiolíticas está frequentemente associada a um grande número de efeitos indesejáveis, como a sedação, amnésia anterógrada, tolerância, abuso, dependência e síndrome de abstinência, vistos para os ansiolíticos benzodiazepínicos, e

aqueles comuns à buspirona e aos antidepressivos como complicações gastrintestinais, fadiga, insônia, sedação, agitação e disfunções sexuais (Doghramji e Jangro, 2016). Além disso, a latência retardada de três a quatro semanas para o aparecimento de seus benefícios terapêuticos (clínicos) leva à busca de medicamentos mais potentes, eficazes e/ou com maior especificidade (Machado-Vieira et al., 2008). Desta forma, a identificação de medicamentos capazes de controlar os sintomas da ansiedade, mas que apresentem pouco ou nenhum efeito colateral e com melhor aderência ao uso pelos pacientes, é sem dúvida, de grande interesse científico na atualidade. Outra doença que vem se tornando cada vez mais um grande problema de saúde pública no mundo é a Doença de Alzheimer (DA). Trata-se de uma doença neurodegenerativa multifacetada do sistema nervoso central que afeta mais de 37 milhões de pessoas em todo o mundo. A DA leva a uma disfunção cognitiva, caracterizada pela perda progressiva da memória e demais habilidades cognitivas (Wang et al., 2012; Hritcu et al., 2014). Pacientes que apresentam esta doença tornam-se incapazes de desempenhar atividades da vida diária e de cuidar de si mesmo, passando a depender de um cuidador. Podem apresentar, muitas vezes, sintomas psiquiátricos, juntamente com o declínio cognitivo (Wuwongse et al., 2010), entre eles os transtornos de ansiedade e distúrbios do sono. Estudos recentes têm destacado uma forte relação entre a ansiedade com a DA (Caraci et al., 2010). Em destaque, elas aumentam a gravidade do declínio cognitivo em doentes com DA (Wuwongse et. al., 2010). Infelizmente, os medicamentos aprovadas atualmente para o tratamento DA é meramente paliativo, permitindo apenas uma melhora temporária do estado funcional do paciente. Desta forma, as plantas medicinais representam uma grande ferramenta do ponto de vista farmacológico como potencial identificação de futuros medicamentos que possam controlar ou retardar doenças neurodegenerativas como a DA (Russo et al., 2013).

Existe um número razoável de estudos utilizando animais de laboratório indicando o potencial de várias plantas brasileiras para melhorar os processos cognitivos (Galvão et al., 2002; Da Silva et al., 2004; Marques et al., 2004; Kennedy et al., 2004). Os índios brasileiros indicaram apenas duas plantas para melhorar a memória (Ficus Anthelmintica e Tabernaemontana heterophylla), e as mesmas não possuem estudos fitoquímicos, o que torna inviável qualquer correlação (Schultes, 1993). O Ginkgo biloba é uma das plantas mais utilizadas na forma de fitoterápico para o tratamento da demência e outros problemas cognitivos, cujos princípios ativos são os flavonóides e lactonas diterpênicas (Heinrich et al., 2004). O efeito antioxidante de muitas plantas

pode contribuir para o efeito terapêutico em algumas categorias. Sabe-se que as doenças neurodegenerativas apresentam como característica o dano ocasionado por processos oxidativos (Harman, 1994; Giasson et al., 2002). Na medida em que podem prevenir ou diminuir danos resultantes do processo oxidativo, antioxidantes também poderiam contribuir para a propriedade adaptogena de algumas plantas (Panossian et al.,1999).

2.4.1 Receptores $GABA_A$

O ácido gama-aminobutírico (GABA) é o principal neurotransmissor inibitório presente em neurônios do sistema nervoso central de mamíferos e capaz de ativar uma série de subtipos de receptores farmacologicos e estruturalmente diferentes: receptores ionotrópicos ($GABA_A$ e $GABA_C$) e receptores metabotrópicos ($GABA_B$) (Olsen e Tobim, 1990). Os primeiros estão envolvidos na transmissão sináptica rápida, enquanto os últimos regulam os efeitos neuromoduladores do GABA (Goudet et al., 2009). Os receptores $GABA_A$ são formados por um pentâmero das subunidades α (α1 a α6) e β (β1 a β3), com uma ou mais subunidades γ (γ1 a γ3), ρ (ρ1 a ρ3), δ, ε ou θ. As possíveis combinações dessas subunidades são inúmeras, mas as isoformas reais dos receptores $GABA_A$ presentes no cérebro são limitadas (Rudolph e Knoflach, 2011).

A fisiopatologia dos transtornos de ansiedade não é tão clara e ainda precisa ser estabelecida, entretanto, evidências atuais indicam anormalidades em diversos sistemas de neurotransmissão, entre eles se destacando o sistema GABAérgico (Nutt, 2002). O sistema GABA tem sido implicado no mecanismo terapêutico de ação de vários agentes psicotrópicos com atividade ansiolítica. A maioria dos estudos relataram déficits corticais ou subcorticais de GABA em estruturas relevantes para o circuito de medo em vários transtornos de ansiedade distintos (Goddard, 2016). Estudos genéticos sobre as complexidades das funções do receptor $GABA_A$ mostram que os déficits gabaérgicos causam anormalidades comportamentais e endócrinas que estão associadas a distúrbios psiquiátricos, como ansiedade e depressão (Earnheart et al., 2007; Reynolds, 2008; Shen et al., 2010; Luscher et al., 2011). A expressão de ansiedade envolve diferentes neurotransmissores, todos os quais interagem e são modulados pela retransmissão sináptica local e distante. O papel do neurotransmissor inibitório GABA tem sido considerado como central para a regulação da ansiedade e este sistema neurotransmissor é o alvo dos benzodiazepínicos usados para tratar transtornos de ansiedade (Lydiard, 2003). Em doses baixas, os BZDs têm efeitos ansiolíticos e anticonvulsivantes. Em

doses maiores, os BZDs produzem sedação, amnésia e, finalmente, inconsciência. Portanto, o efeito das BZDs está claramente relacionado com a dose, mas parece haver um teto além do qual o aumento da dose não aumenta o efeito (Hall et al., 1988).

Estudos recentes mostram que o tempo das atividades neuronais durante processos cognitivos é controlado pela inibição gabaérgica no córtex frontal, portanto, moldando o fluxo de informação em circuitos corticais (Constantinidis et al., 2002). O envolvimento crítico da sinalização muscarínica cortical na cognição e doença de Alzheimer, combinado com o papel central da inibição gabaérgica na memória de trabalho, nos leva a supor que o sistema GABA poderia ser um substrato celular chave para a sinalização muscarínica na cognição e memória (Zhong et al., 2003). Kumar et al., 2017, mostrou que o bloqueio farmacológico dos receptores GABA afetou significativamente as funções cognitivas de aprendizagem e memória em ratos com infusão de ETZ, avaliados no labirinto Morris e no teste de reconhecimento de objetos. Além disso, a redução nas subunidades do receptor GABA-A na região do hipocampo foi relatada pertubar a memória para localizar objetos em camundongos (Prut et al., 2010).

A epilepsia é uma das doenças neurológicas mais graves e comuns encontradas na prática clínica. Os principais sistemas de neurotransmissores envolvidos na fisiopatologia da epilepsia são o GABA, o glutamato e seus respectivos receptores, desempenhando um papel importante na iniciação, manutenção e interrupção das crises convulsivas (Meldrum e Rogawsk, 2007; Sierra-Paredes e Sierra-Marcuno, 2007). Acredita-se que a epilepsia seja devida a um desequilíbrio entre as redes excitatórias mediadas pelo glutamato e as inibitórias gabaérgicas, através das alterações na função e composição do receptor ionotrópico, a atividade do segundo mensageiro alterada pelo cálcio ou falha nas atividades anticonvulsivantes endógenas e neuroprotetoras (Boison, 2007). Sabe-se que o diazepam medeia sua ação anticonvulsivante, aumentando a ação do GABA nos receptores $GABA_A$ pela modulação alostérica positiva (White et al., 2007). Estudos pré-clínicos mostram o efeito neuroprotetor de diversos compostos de origem natural em modelos animais de convulsão, similar a ação do diazepam, e atribuem este efeito a atividade em receptores gabaérgicos (Figueiredo et al., 2011; Citraro et al., 2016; Ataee et al., 2016).

Existe um consenso geral de que a neurotransmissão mediada por GABA desempenha um papel particularmente importante nas respostas emocionais induzidos pelo medo, no entanto, deve-se reconhecer que o GABA não é o único neurotransmissor

importante na modulação de respostas relacionadas à ansiedade, outros neurotransmissores têm sido implicados, incluindo peptídeos opióides e a adenosina (Davis e Myers, 2002; Walker et al., 2003), descritos na sequência.

2.4.2 Receptores opióides

Os opióides são substâncias utilizadas na clínica para o tratamento e controle de dores moderadas e severas. A sua eficácia depende da capacidade de imitar os peptídeos endógenos nos receptores opióides (Wei e Loh, 2002). Existem três famílias de peptídeos opióides endógenos (β-endorfina, encefalinas e dinorfinas) e três famílias de receptores opióides (μ, δ e κ), que compõem o chamado sistema opioide endógeno cerebral (Samadi et al., 2006; Benarroch, 2012). Todos os receptores estão associados às subunidades Go ou Gi e medeiam as ações inibitórias. Devido às suas diferenciações celulares e distribuições regionais, os agonistas ligam-se aos diferentes receptores opióides provocando efeitos farmacológicos diferentes (Vanderah, 2010).

Embora o sistema opióide seja mais reconhecido pelo seu papel na antinocicepção, estudos mostram evidências envolvendo os opióides em várias patologias, entre elas no abuso de drogas, epilepsias, transtornos do movimento e demência (Colasanti et al., 2011). Nos últimos anos, estudos de neuroimagem vêm mostrando a participação do sistema opióide na regulação do afeto e transtornos afetivos, baseado em evidências que mostram a liberação de opióides endógenos duarnte experiências afetivas (Zubietta et al., 2003; Kennedy et al., 2006; Koepp et al., 2009), embora ainda permaneça desconhecida a inter-relação entre os opióides e a ansiedade (Colasanti et al., 2011). Após administração de agonistas do receptor μ-opióide ratos apresentaram um efeito ansiolítico (Millan e Duka, 1981; Asakawa et al., 1998; Koks et al., 1999; Zarrindast et al., 2008). A administração de morfina, agonista receptores opioides, também induz efeitos ansiolíticos (Shin et al., 2003), podendo modular a expressão do receptor receptor GABA / benzodiazepina cerebral (Bartlett et al., 1994). Portanto, sugere-se que os receptores opióides μ podem modular o comportamento ansiolítico, que é regulado pela transmissão sináptica mediada por GABA (Sasaki et al., 2002).

Os receptores opióides possuem padrões únicos de distribuição dentro do cérebro humano, tipicamente exibindo alta densidade na substância cinzenta, e são preferencialmente encontrados nas estruturas cerebrais límbicas (Hiller et al., 1987;

Blackburn et al., 1988). A distribuição dos receptores opióides nessas áreas tem um papel crítico nos comportamentos cognitivos e emocionais. O sistema opióide é conhecido por seu papel na neurodegeneração, comprometimento cognitivo, disfunção comportamental, produção de amilóide β e hiperfosforilação de proteínas τ (Lengauer, 2007; Anthony et al., 2010) .O receptor opióide mu, em ratos ou camundongos , desempenha um papel positivo na aprendizagem e memória, aumentando a potencialização a longo prazo (LTP) em neurónios CA3 do hipocampo (Jamot et al., 2003). Enquanto que a ativação do receptor opióide kappa do hipocampo prejudica a aprendizagem espacial (Daumas et al., 2005). Sugerindo que os opioides e seus receptores poderiam desempenhar um papel na aprendizagem e na memória, mas a função exata não é clara.

Em relação ao envolvimento do sistema opioide na epileptogênese ainda é controverso. Panuccio et al., 2009, mostrou que os receptores mu-opioides são capazes de modular os eventos da rede gabaérgica e glutamatérgica gerados no ACC (córtex cingulado anterior) durante a aplicação do convulsivante 4AP (4-aminopiridina). Por sua vez, os receptores opioides Kappa têm atuado como anticonvulsivantes em modelos de epilepsia, especificamente, os agonistas de receptores opiáceos Kappa inibem convulsões e neurotoxicidade agudas induzidas por pilocarpina em camundongos (Przewlocka et al., 1994; Bausch et al., 1998). Já em relação aos receptores opioides delta, Chu Sin Chung e coloboradores, em 2015, demonstram pela primeira vez que a ativação de receptores opioides delta induzida pelo SNC80 induz crises epilépticas através da inibição direta dos neurônios gabaérgicos do prosencéfalo, confirmando os efeitos pró-convulsivos do agonista delta e capacidade de modulação da rede gabaérgica em camundongos normais.

2.4.3 Receptores de adenosina

A adenosina é um nucleosídeo formado pela união de uma base nitrogenada adenina ligada a um grupamento ribose. Atuando como um neuromodulador, a adenosina desempenha um papel complexo em múltiplos processos fisiológicos e fisiopatológicos no SNC, entre eles na regulação do sono (Rétey et al., 2006; Elmenhorst et al., 2007), epilepsias (Boison, 2010), transtorno de pânico (Lam et al., 2005), ansiedade generalizada (Alsene et al., 2003), Doença de Alzheimer (Albasanz et al., 2008), Parkinson (Hurley et al., 2000) e esquizofrenia (Urigüen et al., 2009). A

regulação dessas funções ocorre através de quatro subtipos de receptores de adenosina (AR), denominados A1, A2A, A2B e A3, que pertencem à superfamília do receptor acoplado à proteína G (GPCR). Os receptores A1 e A3 se acoplam principalmente à proteína Gi que leva à inibição da adenilato ciclase e à diminuição dos níveis de adenosina 5'-monofosfato (AMPc), enquanto que os receptores A2A e A2B sinalizam-se através da proteína Gs, levando à ativação da adenilato ciclase e ao aumento da produção de AMPc (Fredholm et al., 2001; Fredholm et al., 2005).

Os receptores A_1 (A_1R) são altamente expressos no córtex cerebral, cerebelo, hipocampo e corno dorsal da medula espinhal (Cunha, 2005; Sebastião e Ribeiro, 2009; Costenla, et al., 2010), enquanto que os receptores A_2A (A_2AR) são altamente expressos no bulbo olfatório e neurônios GABAérgicos do núcleo caudado, putâmen, núcleo accumbens e tubérculo olfatório (Sebastião e Ribeiro, 2009). Já os receptores A_2B e A_3 apresentam níveis relativamente baixos de distribuição no SNC (Sebastião e Ribeiro, 2009). Além disso, esses receptores têm afinidades diferentes para a adenosina, com o A_1R com maior afinidade, o A_2AR com menor afinidade e os receptores A_2B e A_3 têm uma afinidade muito menor (Dunwiddie e Masino, 2001). Essas afinidades, juntamente com a expressão cerebral diferenciada de A_1R e A_2ARs, desempenham um papel fundamental nessas ações dos receptores no cérebro (Stockwell et al., 2017).

Os A_1Rs foram descritos como neuroprotetores, enquanto que os A_2ARs como neurodegenerativos (Cunha, 2005), sugerindo que isto ocorra em grande parte devido aos efeitos inibitórios de A_1Rs e efeitos excitadores de A_2ARs. O antagonismo de receptores A_2AR mostrou-se promissor tanto na pesquisa pré-clínica como clínica em relaçãoa sua atividade neuroprotetora em doenças neurodegenerativas. Os antagonistas não seletivos dos receptores de adenosina, como a cafeína, também mostraram um papel neuroprotetor em modelos pré-clínicos de acidente vascular cerebral isquêmico, epilepsia e doença de Parkinson (Stockwell et al., 2017). Coelho e colaboradoress, em 2014, mostraram que ratos que superexpressam receptores A_{2A} no hipocampo, córtex e estriado exibem comportamento do tipo depressivo e comportamento exploratório alterado (Coelho et al., 2014, Frontiers in Psychiatry).

A ação primária da cafeína é bloquear os receptores de adenosina, entretanto isso pode levar a efeitos secundários importantes em muitas classes de neurotransmissores, incluindo noradrenalina, dopamina, serotonina, acetilcolina, glutamato e GABA (Daly, 1993), que estão envolvidos na formação, consolidação e recuperação dos processos de aprendizagem e de memória (Paulsen e Moser, 1998;

Buhot et al., 2000; Myhrer, 2003; Meilandt et al., 2004; Kamei et al., 2005; Simonyi et al., 2005; Dringenberg e Kuo, 2006; Cole e McNally, 2007). Além disso, em modelos de roedores, a ativação do receptor A_1 foi associada a interrupções de aprendizagem e memória, enquanto o bloqueio seletivo do receptor mostrou melhora em várias tarefas comportamentais (Normile et al., 1991; Homayoun et al., 2001; Vollert et al., 2013).

Como foi visto, os receptores de adenosina exercem efeitos neuromoduladores em todo o cérebro, afetando tanto processos fisiológicos normais como aprendizagem e memória (Zhou et al., 2009; Wei et al., 2011) mas também está envolvida em neuropatologias, a exemplo, epilepsia (Boison, 2005) e doença de Parkinson (Schwarzschild et al., 2006).

A adenosina é considerada também uma molécula com propriedades anticonvulsivantes, onde disfunções do sistema neuromodulador à base de adenosina pode contribuir para a epileptogênese (Boison, 2008). Um grande número de evidências experimentais enfatiza que os receptores de adenosina podem desempenhar um papel na epilepsia e podem representar um alvo terapêutico promissor (Pagonopoulou et al., 2006; Jacobson e Gao, 2006; Boison, 2008). Estudos usando ratos adultos mostraram que a ativação dos receptores A1 tem um efeito anticonvulsivante nas convulsões do córtex cerebral e hipocampo (Zeraati et al., 2006; Hosseinmardi et al., 2007; Namvar et al., 2008) e os antagonistas seletivos do receptor A1 exacerbam convulsões no hipocampo em Vitro (Etherington e Frenguelli, 2004), assim como os receptores A2A têm efeitos proconvulsivos na região do córtex cerebral (Zeraati et al., 2006) e os antagonistas dos receptores A2A mostram ser potentes neuroprotetores em diferentes modelos animais (Jones et al., 1998; Monopoli et al., 1998; Schwarzchild et al., 2003) . Estudos de expressão gênica mostrou que os efeitos anticonvulsivantes da estimulação de baixa frequência, foram acompanhados de um aumento na expressão do receptor de adenosina A1 e uma diminuição do receptor de adenosina A2A (Jahanshahi et al., 2009).

O papel da adenosina e de seus receptores na ansiedade ainda não está claro. Em humanos, altas doses de cafeína, um antagonista de receptores de adenosina, tendem a aumentar os níveis de ansiedade (Greden, 1974; Green et al., 1996; Sicard et al., 1996), enquanto doses baixas de cafeína tendem a reduzir (Lieberman et al., 2002; Haskell et al., 2005). Já animais de laboratório avaliados nos testes da caixa claro-escuro e labirinto em cruz elevado exibiram comportamento do tipo ansiogênios em camundongos knockout de receptores A1 e receptor A2A (Ledent et al., 1997;

Johansson et al., 2001; Giménez-Llort et al., 2002; Bilbao et al., 2006). Portanto, há motivos para considerar os receptores A1 e A2A como alvos possíveis para o desenvolvimento de novos fármacos.

3 RESULTADOS

3.1 Artigo 1 - Involvement of benzodiazepine/$GABA_A$, adenosine, and opioids receptors in the psychopharmacological actions of *Polygala altomontana* (Polygalaceae) in rats

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Running title: *Psychopharmacological effects of Polygala altomontana in Wistar rats.*

Abstract:

Introduction: The genus *Polygala* is the main representative of the plant family *Polygalaceae* that shows several pharmacological properties, among them neuropharmacological actions. The phytochemical study of the *P*. altomontana revealed the presence of coumarins, flavonoids, styryl-2-pyrones, and dihydrostyryl-2-pyrones compounds that represent an excellent pharmacological tool to study its central action in animal models. **Objective:** In this study, we evaluated the effects of the hydroethanolic extract (HE), ethyl acetate (EA), and ethanolic (ET) fractions of the *P*. *altomontana* in several animal behavioral models. The possible participation of benzodiazepine/$GABA_A$, adenosine, and opioids receptors in the profile of central action of this plant was also investigated. **Materials and methods**: Adult male Wistar rats were orally treated with HE, EA, or ET (50, 100 or 300 mg/Kg), 1 h prior to the tests of light-dark box, open field, rotarod, pentylenetetrazole (PTZ)-induced convulsion, ketamine or isoflurane-induced hypnosis. Other groups previously intracerebroventricularly cannulaed (i.c.v.) by stereotaxy received v.o. HE 300 mg/Kg/day, by three consecutive days, followed by i.c.v. administration of vehicle or streptozotocin (STZ 0.1 mg/4 μL) and, 2 days later, the memory was evaluated in the inhibitory avoidance test. In the last experimental protocol, the animals were pretreated with flumazenil (FLU 5 mg/Kg, i.p), caffeine (CAF 3 mg/Kg, i.p) or naltrexone (NTX 2.5 mg/Kg, s.c) followed by HE (300 mg/Kg, v.o.), and 1 later, the behavior was evaluated in the open field test. **Results:** In the light-dark transition test, EA 300 mg/Kg reduced the number of transitions between the two compartments. In the open field, HE 300 mg/Kg reduced the central, peripheral, and total movements, as well the number of rearings. HE 50 mg/Kg increased the central movements. In the k*etamine*-induced hypnosis test, EA and ET increased the total sleep time, while in the isoflurane-*induced hypnosis*, HE, EA and ET increased the total sleep time. The latency for the first fall and the total number of fall of the rotarod were not altered by any treatments. In the PTZ-test, HE 300 mg/Kg and ET 50 mg/Kg decreased lethality rate in 25 and 30%, respectively. In the inhibitory avoidance test, STZ reduced the step-down latency time, an effect totally prevented by repeated treatment with HE, but not by EA or ET. Phytochemical analysis revealed in the HE the main presence of aurapten (4.93 mg/g of HE), α-spinasterol (0.28 mg/g), styryl-2-pirone-4 (0.21 mg/g), styryl-2-pirone-5 (0.11 mg/g), styryl-2-pirone-6 (0.15 mg/g) and styryl-2-pirone-7 (0.20 mg/g). From ET

fraction was isolated isoquercetin (6.25 mg/g of ET) and guaijaverin (3.0 mg/g) compounds, suggesting that these substances of the *P. altomontana* could be responsible by its psychoactive actions. Finally, the pretreatment with FLU, CAF or NTX totally blocked the effects of HE in the central, peripheral, and total movements, as well as the number of rearings in the open field test. **Conclusion:** The present work suggests that *P. altomontana* is an herbal medicine which possesses anxiolytic, hypnosedative, anticonvulsant, and promnestic actions that can be attributed to the presence of the aurapten and isoquercetin compounds. Our data provides evidence for the involvement of benzodiazepine/$GABA_A$, adenosine, and opioids receptors in sedative effects promoted by HE.

Keywords: *Polygala altomontana*. Anxiolytic. Hypnosedativo. Promnestic. $GABA_A$. Adenosine. Opioids. Rats.

1 Introduction

The anxiety disorders and imsomnia are among the most prevalent chronic illnesses that are highly disabling. Both medical illnesses are common in individuals with dementia (Seignourel et al., 2008) and have affecting many people from all around the world, increasing the severity of cognitive decline in Alzheimer's Disease (AD) patients (Wuwongse et al., 2010).

AD is a chronic and progressive neurodegenerative disorder that represents the main cause of human dementia and leads to cognitive decline, behavioral alterations e.g. anxiety, depression, insomnia, personality change, and ultimately, death (Zufferey et al., 2017). The number of cases of DA is expected to grow significantly in the current decade due to the increasing numbers of the elderly at risk in the population (Rocca et al., 2011). Unfortunately the drugs currently approved for the treatment of AD are limited to the delay in the natural evolution of the disease, allowing only a temporary improvement of their symptoms. Co-occurring anxiety disorders and sleep disorder in the context of AD are associated with a more chronic and treatment refectory course (Wilson et al., 2011). Current medications to treat these comorbid psychiatry conditions or non-cognitive symptoms of DA as benzodiazepines or antidepressants have several significant limitations, notably a time lag for therapeutic response, a low rates of efficacy accompanied by important side effects such as cognitive impairment, sexual dysfunctions, severe withdrawal syndrome, abuse, and dependence (O'brien, 2005; Khawam et al., 2006). These questions have attracted considerable scientific attention to the development of new drugs due to social, health, and economic burden imposed to the general population (Griebel et al., 2013; Murrough et al., 2015).

Therefore, the interest in the search for compounds as a source of new drugs for the treatment of CNS diseases derives from the therapeutic needs and great diversity both in their chemical structures and in their biological activities. From this perspective, secondary metabolites obtained from medicinal plants represent the largest source of biologically active substances for the development of standardized phytomedicines with proven effectiveness in neuropsychiatry practice (Zhang, 2004; Pieters et al., 2005; Gurib-Fakim, 2006; Farzaei et al., 2016). *Polygala altomontana* represents a new species of plant of south region of Brazil, found in the states of Paraná and Santa Catarina. Phytochemical studies of this plant species revealed the presence of coumarin, styrylpirones, dihydrostyrylpirones, and flavonoids compounds (Tizziani et al., 2018a; Tizziani, 2018b). As this plant is one of the few species of the genus *Polygala* to

synthesize these groups of compounds, *P. altomontana* represents an excellent tool to study its action in animal models of psychopathology. Thus, the present work investigated for the first time the psychopharmacological effects of the hydroethanolic extract (HE), ethanolic (ET), and ethyl acetate (EA) fractions from *P. altomontana* in rats evaluated in several well validated behavior animal models. Additionally, the underlying mechanism(s) of action for HE focusing the involvement of opioids, adenosine, and $GABA_A$ receptors using *in vivo approaches* was investigated to better understand the central actions of *P. altomontana* in rats.

2 Material and methods

2.1 Animals

Adult male Wistar rats (300-350 g) were used for behavioral evaluation. Animals were maintained in a 12-h light–dark cycle (lights on at 7:00 a.m.) at constant room temperature (23 ± 2 °C). Rats were housed in groups of 4 per cage, and had free access to food and water, except during the experiments. All animals were allowed to adapt to the laboratory conditions for at least two hours before the beginning of the experiments. Each behavioral test was conducted during the light phase of the cycle (between 8:00 and 13:00 a.m.). All experiments were conducted in accordance with international standards of animal welfare recommended by the Brazilian Society of Neuroscience and Behavior (Act 1992) and approved by the local Committee for Animal Use and Care in Research (number 0004/2016). The minimum number of animals and duration of observations required to obtain consistent data were employed.

2.2 Plant material

P. altomontana Lüdtke, Boldrini & Miotto was collected in Bom Jardim da Serra (Santa Catarina, Brazil), in October 2014, and identified by Prof. Dr. Rafael Trevisan. A voucher specimen (number 55189) was deposited in the Flower Herbarium of the Department of Botany, Federal University of Santa Catarina (Florianópolis, SC, Brazil).

2.3 Extraction and isolation

The dried and powdered whole plant (477 g) was extracted by exhaustive maceration (three times for seven days each time) with 1 L of 96º GL EtOH at room temperature. The extracts resulting from the maceration process were combined, filtered and concentrated under reduced pressure at 50 ºC to yield the hydroalcoholic extract HE (86 g). An aliquot of the HE (71 g) was chromatographed by SiO_2-CC eluting with hexane-ethyl acetate solutions, yielding 150 fractions. The fractions obtained were then

processed by chromatography or crystallization to afford the following isolated compounds: 350 mg of auraptene (7-geranyloxycoumarin) **(1**), 20 mg of α-spinasterol **(2**), 10 mg of dihydrostyryl-2-pyrone (**3**), 15 mg of 4-methoxy-6-(11,12-methylenedioxystyryl)-2-pyrone (**4**), 8 mg of 4-methoxy-6-(11,12-methylenedioxy-10-methoxystyryl)-2-pyrone (**5**), 11 mg of 4-methoxy-6-(11,12-methylenedioxy-10,14-dimethoxystyryl)-2-pyrone (**6**) and 14 mg of 4-methoxy-6-(11,12-dimethoxystyryl)-2-pyrone (**7**). From another chromatographic column using an aliquot of the ET (40 g) and the solvent mixture ethyl acetate/ethanol, starting from 100% AcOEt to 100% EtOH, it was possible to isolate 5 mg of the flavonoid quercetin (**8**) 250 mg of isoquercetin (quercetin-3-*O*-β-D-glucopyranoside) (**9**) and 120 mg of guaijaverin (quercetin-3-*O*-α-L-arabnoside) (**10**). Compounds **1**-**10** were identified by comparison of their spectral data (IR, NMR and HRMS) with those reported in the literature and had previously described for this species (Tizziani et al., 2018a; Tizziani, 2018b).

FIGURE 1

2.4 Drugs and solvents

Diazepam (DZP; Santisa Lab. Farmac. S/A, SP, Brazil), isoflurane (Instituto BioChimico Ind. Farmac. LTDA, RJ, Brazil), ketamine hydrochloride (Sespo Ind. e Com. LTDA, SP, Brazil), xylazine hydrochloride (Sespo Ind. e Com. LTDA, SP, Brazil), pentylenetetrazole (PTZ; Sigma – Aldrich Brasil LTDA, SP, Brazil), streptozotocin (SZT; Cayman Chemical, Michigan, USA), caffeine hydrochloride (CAF; Cayman Chemical, Michigan, USA), flumazenil (FLU; Tocris, Ellisville, MO, USA), naltrexone (NTX; Cayman Chemical, Michigan, USA). PTZ, CAF, FLU, and NTX were dissolved in saline (0.9% NaCl) immediately before of the administration in a volume of 1 mL/Kg. HE, EA and ET fractions of *P. altomontana* were freshly suspended in 10 % Tween-80 and tap water, before each pharmacological test. SZT was dissolved in a citrate buffer 0.01 M (0.295 g of sodium citrate and 0.9 g of NaCl in 100 mL; pH = 4.5) to the final concentration of 0.1 mg/mL and injected directly into the lateral ventricles (i.c.v.) of rats as described in the following section. Dosing levels of each drug were chosen from our preliminary studies as well derived from *in vivo* behavioral studies realized by others authors (Olayiwola et. al., 2013; Zakaria et. al.,2014; Ozsoy et al., 2015).

2.5 Treatments

In experiment 1, rats received through an intragastric cannula (*per os route*, p.o) vehicle, HE, EA, or ET fractions of *P. altomontana* (50, 100 or 300 mg/Kg) in a

constant volume of 1 ml/Kg. Other animals were treated by the same route and volume with the standard anxiolytic diazepam (DZP 10 mg/Kg, p.o.) being used as the positive control drug. One hour later, each animal was sequentially evaluated in the light-dark box test, open-field, rotarod, and pentylenetetrazol (PTZ)-induced convulsions.

In experiment 2, rats were p.o. treated with vehicle, DZP (10 mg/Kg), HE, EA, or ET fractions of *P. altomontana* (300 mg/Kg) and 1 h later, the sleep was induced with ketamine or isoflurane as described in the following section.

In experiment 3, each rat was flat-positioned on a stereotaxic apparatus and implanted with a stainless-steel guide cannula of 7 mm (22 gauge) under ketamine plus xylazine (v/v; 1 mL/Kg body weight) anesthesia. The cannula was directed to one of the lateral brain ventricles and fixed to the skull with screws and dental polyacrylic cement. The site of cannula implantation was counterbalanced across all groups to control for possible lateralization effects. Coordinates for implantation were 1.5 mm lateral and 0.8 mm posterior to the bregma, and 2.5 mm vertically from the skull surface, according to the coordinates of Paxinos and Watson (1986). One day later, each rat was daily p.o. treated with vehicle or HE (300 mg/Kg/day) by three days and, 1 h after the last treatment, received SZT (0,1 mg/site) in a volume of 4 μl. Two day later, the memory of each rat was evaluated in the step down inhibitory avoidance task.

In experiment 4, rats were divided into groups and treated intraperitoneally (i.p.) with vehicle, FLU (5 mg/Kg), or CAF (3 mg/Kg), 20 min before of the oral administration of vehicle or HE 300 mg/Kg. Other groups received subcutaneously (s.c.) vehicle or NTX (2.5 mg/Kg) followed by the oral administration of vehicle or HE 300 mg/Kg. One hour later, each rat was individually evaluated in the open-field test.

2.6 Behavioral tests

2.6.1 Light-dark transition test

The putative anxiolytic-like activity of extract and fractions from the *P. altomontana* was assessed using the light-dark transition test, as proposed by Crawley and Goodwin (1981). This test is based on the natural aversion of rodents to white and brightly illuminated spaces, being widely validated to measure anxiety in rodents. The apparatus consisted of a box made of Plexiglas with overall dimensions of 80 x 40 x 20 cm (length, width, height). The box was divided in two compartments by a barrier with a doorway (12 x 8 cm) through which rat could cross from one black chamber that was not illuminated to a white chamber that was brightly illuminated with a 700 lux light source. Each rat was individually placed in the middle of the white compartment facing

the opposite side of a doorway. The behavior of the animals was registered during a 5 minutes period for measuring two parameters: total time spent in the lighted compartment and total number of transitions between the two compartments. Immediately after being tested in the light-dark transition test, animals were individually evaluated in the open-field test.

2.6.2 Open-field test

The open-field test serves as a powerful tool for investigating drugs with potencial anxiolytic, psychostimulants, and sedatives effects (Carlini and Mendes, 2011). The equipment consist in a transparent Plexiglas arena (100 x 100 x 40 cm), it had a black Plexiglas floor divided by white lines in teen squares (10 cm x 10 cm). Animals were individually placed in the center of the open field and the number of peripheral, central and total squares crossed as well as total rearing behavior performed were recorded during a 5-min test. The rearing parameter and the total squares crossed was used as indicative of a sedative effect (Royce, 1977).

2.6.3 Rotarod test

An automated rota-rod apparatus (Insight® model EFF 411) was used, it consisted of an acrylic box (530 cm x 400 cm x 410 cm) with an 8 cm diameter cylinder, installed transversely 20 cm from the base and was maintained in rotation for 12 rpm through a motor. The box is divided into four compartments of 10 cm wide, allowing the analysis of four animals simultaneously. Each rat was subjected to the rota-rod test twice. In the first session (training session), the animals were preselected among those that stayed for at least 1 min on the rotating bar in a session of 2 min 24 h before of the experiments. The second session (test session) was performed after the treatments. The latency for the first fall of the rota-rod (in s) and the total number of falls during the session were recorded during the period of 1 min. This test allows the evaluation of whether the treatments promote motor incoordination in the animals by sedation and/or muscle relaxation (Dunham and Myia, 1957).

2.6.4 Pentylenetetrazol (PTZ)-induced convulsions

PTZ at 80 mg/Kg was administered i.p. to groups of rats pretreated with vehicle, DZP, HE, EA, or ET of *P. altomontana*. The latency and duration (in s) of the first convulsive episode (clonic or tonic/clonic convulsion) as well as the lethality ratio were recorded. Convulsion severity was registered using a Convulsive Reactivity Scale proposed by Czuczwar and Frey (1986) as it follows: stage 0: no change in behavior; stage 1: ear and facial twitching; stage 2: isolated myoclonic jerks; stage 3: clonus of the

forelimbs, neck, and/or head; stage 4: clonus of the forelimbs, neck, and/or head with rearing, and falling; stage 5: GCS (no tonic phase) beginning with running, followed by loss of righting reflex. Each animal was assigned a score for the most severe seizure presented.

2.6.5 Ketamine-induced hypnosis

To evaluate the potentiation of the ketamine hypnosis, ketamine hydrochloride was administered i.p. at the dose of 100 mg/Kg, 1 h after the different treatments. The latency to the loss of righting reflex (in s) and the duration of sleep (in min) were recorded for each animal (Carlini et al., 1986; Mimura e Namiki et al., 1990; Rabbani et al., 2008; Singh et al. 2011; Vanzella et al., 2012; Zapata-Morales et al., 2016), with a cutoff of 2 h to evaluate the effects of vehicle, DZP, HE, EA, and ET.

2.6.6 Isoflurane-induced hypnosis

Groups of rats were treated p.o. with the different preparations of *P. altomontana* or vehicle and 1 h later the animals were placed in an isoflurane (5 mL, for 5 min of saturation) saturated glass cage (30 x 20 cm) as described and adapted from Vieira (2001) and Duarte et al. (2007). The latency to the loss of righting reflex and the duration of sleep (in s) were recorded using a stopwatch. Sleeping-time was measured by the loss of the righting reflex, with the recovery of this reflex being considered the hypnosis endpoint as previously described by (Carlini et al., 1986). DZP 10 mg/Kg i.p. was used as the positive drug control (standard anxiolytic/hypnosedative compound) in both assays (ketamine and isoflurane-induced sleep).

2.6.7 Step-down inhibitory avoidance task

The apparatus consists of a box with the dimensions (30 x 26 x 21 cm) composed by three steel walls and an acrylic one, with a platform (8.0 x 1.5 x 21 cm) at its right end and a square base (metal grid 23 x 21 cm) on the left side containing a series of stainless steel bars (1 mm in diameter) spaced apart by 1 cm and connected to a shock generator and a scrambler (Insight Ltda., Ribeirão Preto, SP, Brazil). For the evaluation of the work (WM), short (SM) and long duration (LM) memories, the animals were submitted to the test session for 5 s (WM), 90 min (SM) and 24 h (LM) after the session (Bianchin et al., 1999; Barros et al., 2000, 2001, 2002, 2005).

2.7 Statistical analysis

All results are presented as mean ± S.E.M. and analyzed by a one-way analysis of variance (ANOVA) followed by the post-hoc Student Newman-Keuls' test for multiple comparisons., with treatment being the independent variable. All of the

statistical analysis was carried out using Graphpad-Prism version 5.0. Differences between treated and control groups were considered statistically significant when $P \leq 0.05$.

3 Results

3.1 Effects of the treatment with HE, EA, and ET of Polygala almontana

3.1.1 Light-dark transition test

EA 300 mg/Kg reduced the number of transitions between the two compartments of the light-dark box test ($F_{(3,36)}$=2.75, $P \leq 0.05$), while HE, ET or DZP did not elicit any significant change in this behavioral parameter in relation to the control group ($P>0.05$), as shown in Figure 2A. The time spent in the light compartiment was not significantly modified by any of the treatments in relation to the control group ($P>0.05$) (Figure 2B).

FIGURE 2

3.1.2 Open-field test

HE 300 mg/Kg reduced the total ($F_{(3,28)}$=7.19, $P \leq 0.01$; Figure 3A), central ($F_{(3,28)}$=9.87, $P \leq 0.0001$; Figure 3B) and peripheral moviments (data not shown) in the open field test, while HE 50 mg/Kg only increased the central movements in relation to the control group ($F_{(3,28)}$=9.87, $P \leq 0.0001$; Figure 3B). Both doses of HE (100-300 mg/Kg) reduced the number of rearings behaviors ($F_{(3,28)}$=7.06, $P \leq 0.001$). DZP 10 mg/Kg reduced the total moviments ($t_{(22)}$=4.35, $P \leq 0.01$) (Figure 3A), peripheral movements ($t_{(22)}$=4.86, $P \leq 0.01$) (data not shown) and the number of rearing behaviors ($t_{(22)}$=3.37, $P \leq 0.01$) (Figure 3C). Both EA and ET fractions were not able to promote any significant changes in the behavioral parameters evaluated in the open field test ($P>0.05$; Figure 3A-3C).

FIGURE 3

3.1.3 Rotarod test

In the rotarod test, latency for the first fall and the total number of falls of the rotarod were not altered by any treatments ($P>0.05$) (data not shown).

3.1.4 Pentylenetetrazol (PTZ)-induced convulsions

As shown in Table 1, HE, EA, or ET (50 to 300 mg/Kg), did not alter the latency of the first convulsive episode ($P>0.05$), but HE 300 mg/Kg showed a tendency towards a reduction in the duration of the first convulsion (F = 2.42, $P = 0.08$). DZP 10 mg/Kg showed a tendency towards an increasing the latency of the first convulsive episode ($t_{(22)}$=1.85, $P = 0,07$) and reducting the duration of the first convulsion ($t_{(22)}$=1.89, P =

0,07). Regarding to lethality, HE 300 mg/Kg and ET 50 mg/Kg reduced the lethality in 25% and 30%, respectively, while DZP 10 mg/Kg reduced the lethality in 75% (Table 1).

TABLE 1

3.1.5 Ketamine or isoflurane-induced hypnosis

Oral treatment with HE, EA, or ET at doses of 300 mg/Kg, 1 h before the ketamine injection did not modify the latency to induce sleep (*P>0.05*) (data not shown). However, EA ($t_{(16)}$=7.62, *P ≤0.001*) or ET ($t_{(15)}$=2.74, *P≤0.05*), but not HE (*P>0.05*), significantly increased the total duration of the hypnosis, a sedative effect similar to that produced by DZP ($t_{(15)}$=3.38, *P≤0.01*) (Figure 4A). None of treatments with the fractions of *P. altomontana* changed the latency to induced sleep, 1 h before the isoflurane injection (*P>0.05*) (data not shown), but all fractions significantly increased the duration of the hypnosis in a similar way to DZP (HE: $t_{(18)}$=2.74, *P≤0.05;* EA: $t_{(18)}$=3.76, *P≤0.01*; ET: $t_{(18)}$=2.58, *P≤0.05*; DZP: $t_{(18)}$=4.80, *P≤0.001*) (Figure 4B).

FIGURE 4

3.1.6 Step-down inhibitory avoidance task

The animals group treated with Veh + STZ significantly reduced the latency time to step-down inhibitory avoidance on 5 s (WM: $t_{(16)}$=10.40, *P*≤0.001), 1.5 h (SM: $t_{(16)}$=6.74, *P*≤0.001) and 24 h (LM: $t_{(16)}$=9.28, *P*≤0.001) after the training session, when compared to the control group (Veh + PBS). Repeated treatment with HE 300 mg/Kg *per se* did not interfere in the latency time to step-down from the platform (*P>0.05*, data not shown). However, HE significantly increased the latency time to step-down inhibitory avoidance in comparison to the group Veh + STZ on 5 s (WM: $t_{(19)}$=2.97, *P*≤0.01), 1.5 h (SM: $t_{(19)}$=2.85, *P*≤0.01) and 24 h (LM: $t_{(19)}$=2.89, *P*≤0.01) after the training session (Figure 5). The repeated administration of EA or ET not produced any significant change on the STZ-induced effects in this test (*P>0.05*) (data not shown).

FIGURE 5

3.2 Effects of flumazenil (FLU), caffeine (CAF), or naltrexone (NTX) on the open-field performance of rats orally treated with HE

HE 300 mg/Kg decreased the central, peripheral, and total movement, as well as the number of rearing behaviors in the open field test (Figure 6A-C). FLU, which had no effect *per se*, completely blocked these HE-induced effects in the open field test (central moviment: F=3.96, *P≤0.05*; peripheral movement: F=7.89, *P=0.05*; Total movement: F=7.99; *P≤0.05*; number of rearings: F=11.97, *P≤0.05*) (Figure 6A). CAF,

which had no effect *per se*, completely blocked the HE-induced effects on central (F=15.78, *P≤0.001*), peripheral (F=9.04, *P≤0.001*) and total movements (F=11.25, *P≤0.001*), as well as the number of rearing behaviors (F=15.22, *P≤0.001*) (Figure 6B). NTX, which had no effect *per se*, completely blocked all HE-induced alterations on central (F=3.34, *P≤0.05*), peripheral (F=4.83, *P≤0.05*) and total movements (F=5.22, *P≤0.05*), as well as the number of rearing behaviors (F=5.22, *P≤0.01*) (Figure 6C).

FIGURE 6

4 Discussion

In the present study, it has been showed the effects of oral treatment with HE, EA, or ET fractions of *P. altomontana* plant on rat behavior submitted to different behavioral tests, with the psychopharmacological actions promoted by *P. altomontana* compared with those of DZP, a benzodiazepine used as a standard anxiolytic drug. The possible anxiolytic-like effects was investigated using the light-dark box test and open-field tests, while that the central depressor effect was assessed using the open-field, k*etamine or isoflurane*-induced hypnosis and rotarod tests. PTZ-induced convulsion test was used to investigate the anticonvulsant potential of the *P. altomontana*. Aiming to elucidate an underlying mechanism of action by which *P. altomontana* induces its central actions, we investigated the possible involvement of benzodiazepine/$GABA_A$ receptors, adenosine, and opioids on their depressant action in rats using *in vivo approaches*. In parallel to this biological study, a phytochemical analysis of secondary metabolism phytoconstituents was undertaken.

In the light-dark box test, our results showed that EA 300 mg/Kg reduced the number of transitions between light and dark side, while HE, ET or DZP did not elicit any significant change in this behavioral parameter. These data suggest that EA fraction obtained from *P. altomontana* could have sedative properties, since the total number of transitions in this test is considered as an index of locomotor activity. As any animal model is a simplification of reality, false positive or an isolated result may occur in this test. For example, tested drugs that increase the general locomotor activity, the animal would also increase the number of transitions between the two sides of the light-dark box, wrongly suggesting a possible anxiolytic effect (Bourin and Hascoet, 2003). Therefore, other tests that assess behavior are required, such as the open field test. In open-field test, an increase in the central locomotion or time spent in the central region can be interpreted as an anxiolytic-like effect (Prut and Belzung, 2003). In our current study, HE increased the central movement at a dose of 50 mg/Kg and decreased central

movement at a dose of 300 mg/Kg, suggesting an anxiolytic and sedative action profile, depending on the extract dose used. According to Carlini and Mendes (2011), a reduction in the vertical activity and movement are related to sedation. This hypothesis is confirmed by the reduction of all of the other behavioral parameters evaluated in this test: total, peripheral movement, and the number of rearing when the animals were treated with HE at a dose of 300 mg/Kg. In fact, most anxiolytic agents become hypnotic at high doses. This can be seen with herbal products commercially known as *Valeriana officinales, Kava-kava,* and *Passiflora incarnata L.*, that have an anxiolytic and hypnotic effect in animal models and humans (Soulimani et al., 1997; Garrett et al., 2003; Murphy et al., 2010).

Another method used in the current study to evaluate the central activity was the rotarod test, a widely used method in the investigation of motor activity, since it can detect physical deficiency caused by pharmacological agents, such as muscle relaxants and CNS depressants (Sen and Chaudhuri, 1992; Pultrini et al., 2006). An animal under normal conditions could maintain its equilibrium for an indefinite period of time, while a decrease in the permanency time of the animals in the rotarod bar is used as motor impairment index (Dunham and Miya, 1957; Martínez-Vázquez et al., 2012). In our study, no difference has been observed between the treated and control groups in this test, showing that *P. altomontana* is not able to promote motor impairment in the dose range used in the present work. Thus, the decrease in motor performance cannot be attributed to the central effects of *P. altomontana*. This information is extremely important, since benzodiazepines that are widely used to treat patients with anxiety disorders, have muscle relaxation as one of their side effects (Hollister, 1993).

PTZ is one of the main drugs that are experimentally used to induce seizures in rodents and it has been widely used as a pharmacological tool in the preclinical screening of new drugs with anticonvulsant properties. The PTZ-test was chosen in the present study to evaluate a possible anticonvulsant activity for HE, EA, and ET fractions of *P. altomontana*. Drugs with anticonvulsant action such as DZP increase the seizure threshold, reduce the number and severity of seizures, as well as mortality (Löscher, 2011). It has been shown that oral administration of HE 300 mg/Kg or ET 50 mg/Kg reduced mortality by 25% and 30% respectively, indicating partial protection against PTZ-induced seizures. In addition, HE 300 mg/Kg decreased the duration of the 1st generalized convulsive episode, suggesting potential anticonvulsant effect. Convulsions experimentally induced with PTZ may be suppressed either by

pharmacological agents that increase GABA-mediated synaptic inhibition or glutamate receptor antagonists (McNamara, 2006). Thus, HE action may involve both GABAergic action and an antagonistic action on glutamate receptors.

The central depressant effect of a particular drug under study, when its hypnotic effect is not evident *per se*, can be investigated through the potentiation test of ketamine-induced hypnosis through the synergistic inhibitory interaction between the latter and the test substance in the CNS (Mimura et al., 1990; Lancel, 1999; Fauth et al., 2002). Several evidences have demonstrated the ability of plant extracts to potentiate hypnosis induced by CNS depressant drugs. This effect has been attributed to an action in central mechanisms involved in the regulation of sleep (N'Gouemo et al., 1994; Chindo et al., 2003), reinforcing the utility of this test in the investigation of the central properties of plants in laboratory animals (Lu, 1998; Carpendo et al., 1994; Gamaniel et al., 1998). CNS depressant drugs generally reduce latency and /or increase sleep duration induced by hypnotic drugs. In this study, it has been shown that EA and ET 300 mg/Kg increased the total duration of sleep induced by ketamine, suggesting a central depressant effect. The potentiation of sleep by a particular substance in the ketamine-induced sleep test may be due to pharmacokinetic changes, which in turn could increase the plasma levels of ketamine and consequently an increase in the duration of sleep could be observed. This would be avoided with the use of inhalational gas isoflurane as a sleep inducing agent, serving to certify the hypnosedative properties observed for *P. altomontana.*

To clarify this hypothesis, it has been performed the isoflurane-induced sleep test. This test follows the same principles of ketamine-induced hypnosis, differing as to the sleep inducer, in this case isoflurane. In addition to its rapid pharmacological screening, the major advantage of this test is the fact that isoflurane is not metabolised by the cytochrome P450 hepatic enzyme system (Medrado, 2002). Isoflurane is widely used in clinical surgery as an anesthetic by inhalation, leading to rapid induction and recovery (Olsen and Li, 2011; Cao et al., 2012, Palmer, 2013). Our results showed that HE, EA or ET increased sleep duration by potentiating isoflurane-induced hypnosis. Plate et al. (2005) showed that rats under isoflurane anesthesia (up to 75 seconds of exposure) did not have their CYP2E1 or P450 reduction activity affected. Hence, we can suggest that HE has sedative properties, since, excluding a pharmacological interaction of HE with isoflurane at the hepatic level, minimizing the induction of the enzymatic activity by the anesthetic.

The step-down test is an experimental model used in the study of mechanisms involved in memory processes and has been widely used to investigate substances with therapeutic potential for dementia (El-Sherbiny *et al.*, 2003; Saxena *et al.*, 2007 ; Sharma *et al.*, 2010). In this test, we used STZ as a substance that induces neurotoxicity and cognitive deficit (Mayer et al., 1990). A larger latency to step-down from platform in this test indicates an improvement in the memory, while the inverse is valid for drugs that impair the processes involved in learning and memory (Singh and Goel, 2015). In our study, we chose the step-down test to evaluate the preventive treatment with HE 300 mg/Kg (a daily dose for three consecutive days) against STZ-induced memory impairment. It was observed that this treatment promoted an increase in the latency time for platform descent when compared to the STZ group, suggesting that the extract was able to prevent STZ-induced injury in short and long term working memory.

The phytochemical and chemotaxonomic study of *P. altomontana* has recently been reported (Tizziani et al, 2018a). The EA was subjected to chromatographic fractionation resulting in the isolation of eight compounds, all new in the species, a coumarin "aurapten (7-geraniloxycoumarin)" (4.93 mg/g), a sterol "α-spinasterol" (0.28 mg/g), one dihydrostyryl-2-pirone-3 (0.14 mg/g), styryl-2-pyrones known as styryl-2-pirone-4 (0.21 mg/g), styryl-2-pirone-5 (0.11 mg/g), styryl-2-pirone-6 (0.15 mg/g) and styryl-2-pirone-7 (0.20 mg/g). From ET fraction was possible to isolate more three compounds, affording the flavonoids quercetin (0.12 mg/g), isoquercetin (quercetin-3-*O*-β-D-glucopyranoside) (6.25 mg/g) and guaijaverin (quercetin-3-*O*-α-L-arabnoside) (3.0 mg/g). Consequently, the major compound observed in HE was aurapten (4.93 mg/g) while in the ET fraction was isoquercetin (6.25 mg/g) and guaijaverin (3.0 mg/g) (Tizziani, 2018b). Although there is no consensus on the compounds that are putatively involved in the neuropharmacological activity of *P. altomontana*, these compounds could be responsible for their central effects, especially the major compounds aurapeten and isoquercetin.

Isoquercitin is a flavonoid present in several natural tranquilizers and possibly combined with other flavonoids, it represents one of the substances responsible for the neuropharmacological properties described in literature. In rats, isoquercetin (30 mg/Kg i.p) significantly reduced the number of rearing behavior in the hole board test, suggesting possible sedative effects (Loscalzo et al., 2009). Thus, we could propose that the anxiolytic, sedative, anticonvulsive, and even neuroprotective effects promoted by

HE could be due to a possible action of isoquercetin on GABA-A receptors. Another substance, auraptene, is associated with important pharmacological activities. Furukawa et al., 2012, showed that aurapten has the ability to induce the activation of ERK1/2 and CREB in culture of neurons and PC12 cells and neurite growth from PC12 cells, suggesting neurotrophic /neuroprotective activity. Genese and collaborators, 2009, in vivo, showed a neuroprotective activity of auraptene in a model of convulsions induced by maximal electroshock in rats. Recently, Ghanbarabadi et al., 2016, showed the memory enhancing effect and neuroprotective activity of auraptene against brain ischemia in a rat model of vascular dementia. In the current study, it is important to mention that during the capillary electrophoresis procedure, the substance umbelliferone appears as a hydrolysis product of auraptene. Umbelliferone is not part of the biosynthesis of *P. altomontana*, appearing only after acid hydrolysis (Tizziani et al., 2018b). However, it is possible that this hydrolysis occurs *in vivo* and is responsible for the central effects of *P. altomontana*. In the literature it has been found that umbelliferone exhibits a range of pharmacological activities. Umbelliferone potentiated the anticonvulsant activity of phenobarbital and valproate in rats in an animal electroshock model (Zagaja, 2015). Bagheri, 2015, has shown that *Ferula asafoetida*, a medicinal plant native of Iran, has protective and treatment effects on D-galactose and NaNo2-induced memory impairment in rats and these beneficial effects may be due to the presence of umbelliferone. Subramaniam and Ellis (2013) demonstrated the neuroprotective ability of umbelliferone to protect against neurotoxicity induced by MPTP (neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) in rats. In addition, it has also been suggested that coumarin derivatives are capable of interacting at the benzodiazepine binding site in the GABA-A receptor complex, modulating GABA-induced chloride currents and participating in the modulation of neuroprotective activity (Singhuber, 2011).

Depressants drugs exert their effects through a number of different neuro-pharmacological mechanisms. So far, the mechanism of action that underlies the central actions promoted by HE had never been studied. Thus, this study investigated, for the first time, three possible neurotransmitters systems that are widely involved in the modulation of anxiety and sedative processes, among them the benzodiazepine, opioid and adenosine receptors. The actions of the benzodiazepines and some other pharmacological compounds used to treat anxiety disorders, insomnia, and epilepsy are mediated by an enhancement of GABAergic neuronal inhibition (Whiting, 2006; Riss et

al., 2008). Consequently, aiming to elucidate the possible participation of $GABA_A$ receptors in the central action mechanism of *P. altomontana*, we used flumazenil (5 mg/Kg, ip.), a competitive benzodiazepine antagonist of $GABA_A$ receptors (Hammers, 2004). Our results showed that the sedative effect of HE 300 mg/Kg was completely blocked by pretreatment with flumazenil, suggesting that HE contains substances that may have action at the benzodiazepine binding site of the receptor complex $GABA_A$, justifying the contribuition to the central inhibitory depressants effects. Sollozo-Dupont (2015) investigated the participation of $GABA_A$ receptors in the central effects of another herbal known as *Montanoa tomentosa.* Using flumazenil (5 mg/Kg), the authors demonstrated a blockage of the anxiolytic effects of this plant. *Montanoa tomentosa* possesses flavonoids (Quijano et al., 1982) that present high affinity for the benzodiazepine binding site in the $GABA_A$ receptor complex (Marder and Paladini, 2002, Wasowski and Marder , 2011). It is known that the inhibitory action of GABA consists on the opening of ionic channels permeable to chloride ions of the GABA-A receptor, allowing higher chloride influx into the cell, making membrane less susceptible to polarization (Gottesmann, 2002). In a similar way, we suggest that the sedative effect of HE is mediated by GABAergic mechanism, since it is known that GABAergic transmission produces deep sedation in rodents (Gottesmann, 2002).

In addition to GABAergic receptors, evidence indicates that endogenous opioids play a key role in controlling stress and anxiety (Olson et al., 1996). Vaccarino and Kastin (2001) demonstrated that activation of the endogenous opioid system is one of the physiological responses evoked during exposure to stressful stimuli. To investigate the involvement of opioid receptors in the HE actions, it was used NTX, a non-selective opioid receptor antagonist with high affinity for mu receptors (Wentland, 2009). NTX crosses the blood-brain barrier and exhibits central opioid blocking effects (Brown and Goldberg, 1985). In a study realized by Olayiwola et al. (2013), NTX inhibited the rearing induced by the extract and fractions of *S. cayennensis*, partially blocking the opioid receptors in mice. In addition, in this same study, flumazenil also blocked the effect of the methanol extract and butanol fraction. In our study, pre-treatment with naltrexone reversed the increase in movement (central, peripheral, and total) and rearing HE-induced, suggesting that opioid receptors are also involved on the depressant effect promoted by HE.

In this work, it is further investigated the influence of adenosine receptors on the central actions of HE. Adenosine is involved in homeostasis of the central nervous

system, as in the regulation of homeostatic processes of sleep (Reichert et al., 2016), the activation of its receptors is associated with cerebral neuroprotection (Von Lubitz et al., 1994; Jacobson et al, 1995), and also a complex role in many disorders of the nervous system, such as epilepsy, anxiety, Alzheimer's, and Parkinson's disease (Correa e Font, 2008; Gomes et al, 2011; Rahman, 2009; Yamada et al., 2014). Current evidence indicates that the pathophysiology of anxiety and other affective disorders includes abnormalities of the neurobiology of noradrenaline, serotonin, gabaergic, and glutamatergic transmission (Nutt, 2002), which may be indirectly influenced by caffeine, a non-selective adenosine receptor antagonist (Smith et al., 2006). In the current study, pre-treatment with caffeine (3 mg/Kg, i.p) reversed the sedative effect of HE 300 mg/Kg. Surprisingly caffeine promoted increased central and total movements in the open field test when compared to the control group. These data suggest that caffeine, in addition to blocking the sedative effect of HE, provoked some kind of pharmacological interaction with HE, since the treatment with caffeine alone did not show significant differences in comparison to the control group. One of the hypotheses suggested would be that caffeine and HE in a synergistic interaction could be facilitating the release of neurotransmitters or that the blockade of the adenosine receptors would favor the binding in another molecular target that would lead to opposite actions, that is, stimulating.

Future studies are necessary in order to clarify which compounds from *P. altomontana* are more effective and elucidate the type of relationship that exists between their structures and neuropharmacological activities.

5 Conclusion

In conclusion, the results showed strong evidence that HE is capable of producing anxiolytic, hypnosedative, and anticonvulsive as well to reverse the STZ-induced memory impairment in rats. These central effects were not accompanied by motor impairment. Phytochemical analysis revealed the major presence of aurapten and isoquercetin, suggesting that these active substances could be responsible, at least in part, for the central actions seen for *P. altomontana*. Finally, our findings supports the involvement of benzodiazepine/$GABA_A$, adenosine and opioids receptors in sedative effects promoted by HE.

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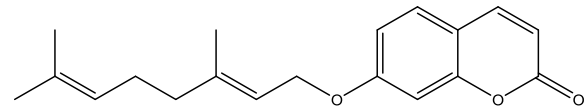
FIGURE 1 – CHEMICAL STRUCTURES OF COMPOUNDS ISOLATED FROM *POLYGALA ALTOMONTANA*.



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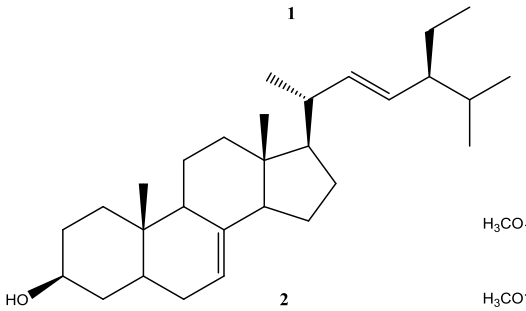

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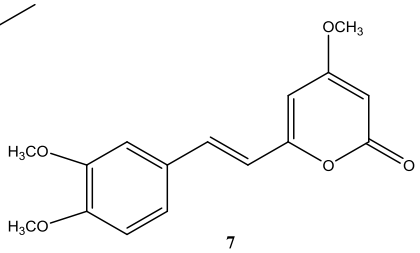

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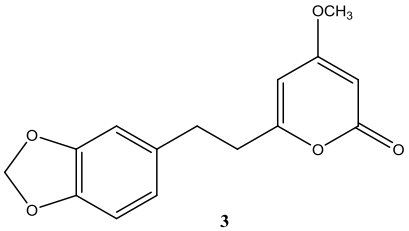

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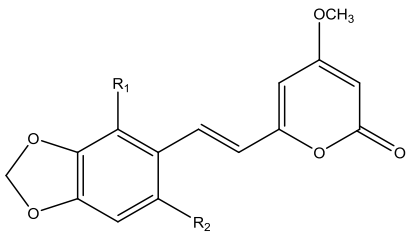
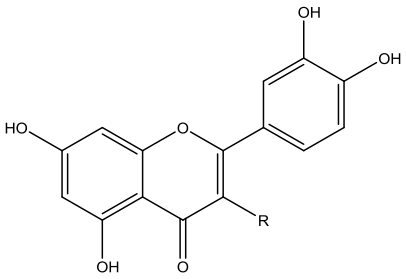


	R_1	R_2
4	H	H
5	OCH_3	H
6	OCH_3	OCH_3



	R
8	OH
9	O-Glucose
10	O-Arabinose

FIGURE 2 - EFFECT OF HE, EA, ET (50-300 MG/KG V.O.) FROM *P. ALTOMONTANA* OR DZP (10 MG/KG V.O.) IN THE LIGHT-DARK TRANSITION BOX TEST.

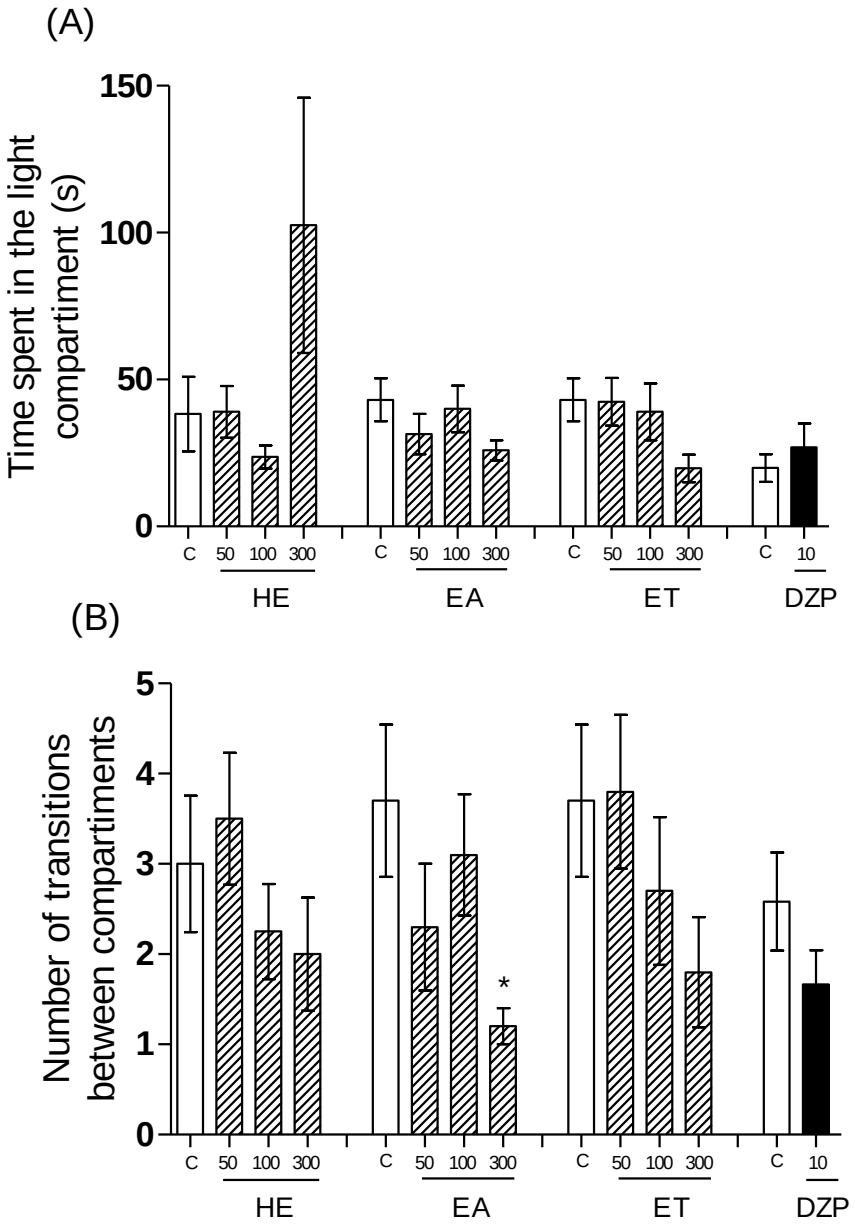


Figure 2 - Effect of HE, EA, ET (50-300 mg/Kg, v.o.) from *P. altomontana* or DZP (10 mg/Kg, v.o.) on the total time in the ligh compartment in the light-dark transition box test (A) and number of transitions between the compartments in the light-dark transition box test (B). Each value represents the mean ± SEM of 8-10 rats. Data analyzed by a one-way ANOVA followed by the Student Newman-Keuls test. Data from the positive control group (DZP) were analysed using the unpaired two-tailed Student t-test. *P≤0.05 as compared to control group (C).

FIGURE 3 - EFFECT OF HE, EA, ET (50-300 MG/KG V.O.) FROM *P. ALTOMONTANA* OR DZP (10 MG/KG V.O.) IN THE OPEN FIELD TEST.

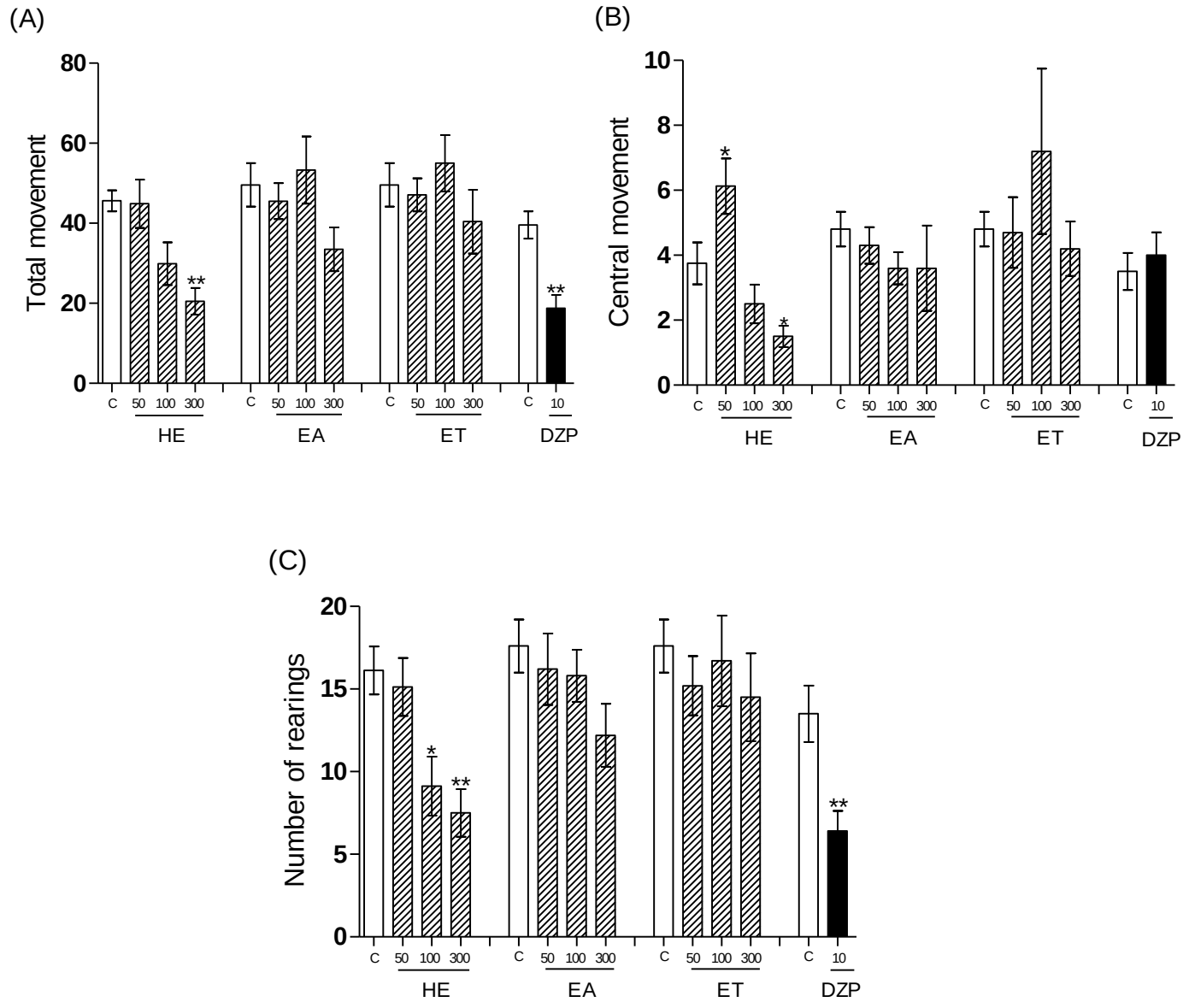


Figure 3 - Effect of HE, EA, ET (50-300 mg/Kg, v.o.) from *P. altomontana* or DZP (10 mg/Kg) on the total movement (A), central movement (B) and number of rearings behaviors (C) evaluated in the open field test. Each value represents the mean ± SEM of 8-10 rats. Data analyzed by one-way ANOVA followed by the Student Newman-Keuls test. Data from the positive control group (DZP) were analyzed using the unpaired two-tailed Student t-test. *P≤0.05 and **P≤0.01 as compared to the respective control group (C).

FIGURE 4 - EFFECT OF HE, EA, ET (50-300 MG/KG V.O.) FROM *P. ALTOMONTANA* OR DZP (10 MG/KG V.O.) ON THE SLEEPING-TIME.

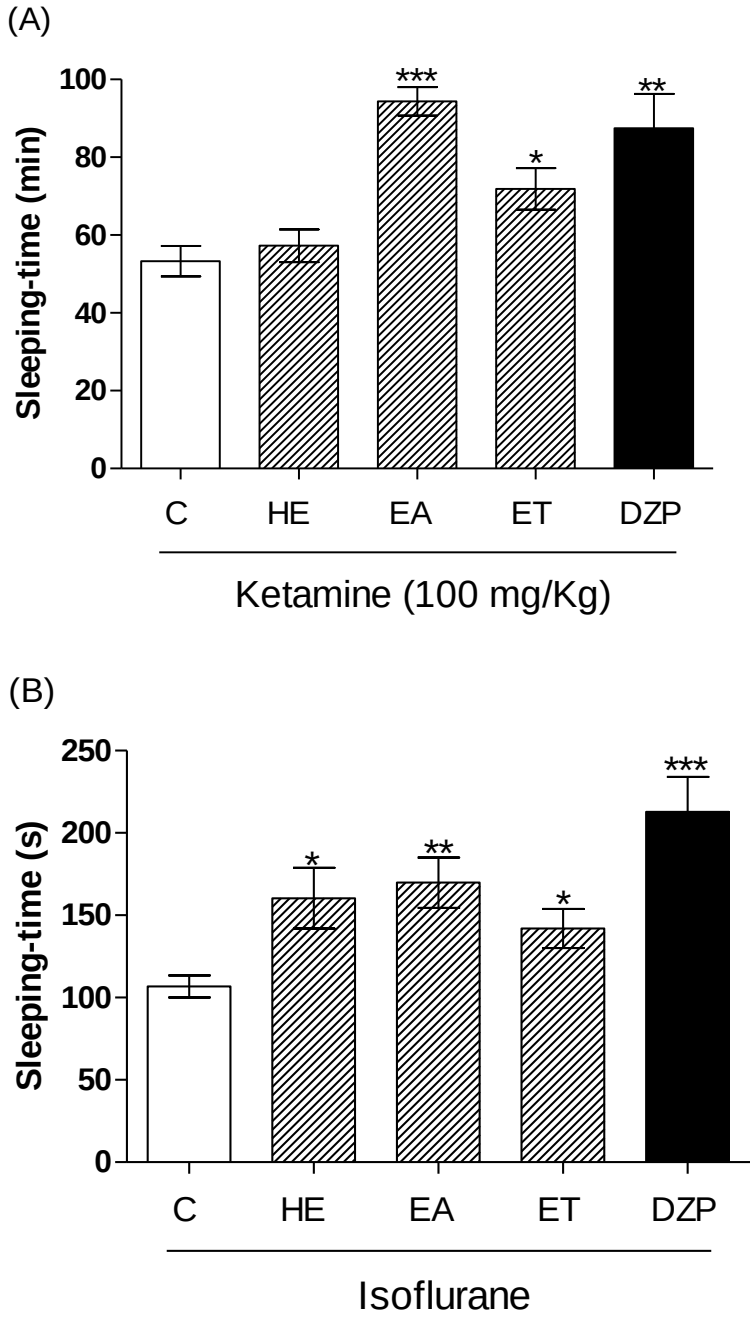


Figure 4 - Effect of HE, EA, ET (300 mg/Kg) from *P. altomontana* or DZP (10 mg/Kg) on the sleeping-time in the ketamine-induced sleep (A) and isoflurane-induced sleep tests (B). Each value represents the mean ± SEM of 8-10 rats. Data analyzed using the unpaired two-tailed Student t-test. *P≤0.05, **P≤0.001 and ***P≤0.0001 as compared to control group (C).

FIGURE 5 - EFFECT OF HE (300 MG/KG V.O.) FROM *P. ALTOMONTANA* IN THE INHIBITORY AVOIDANCE TEST.

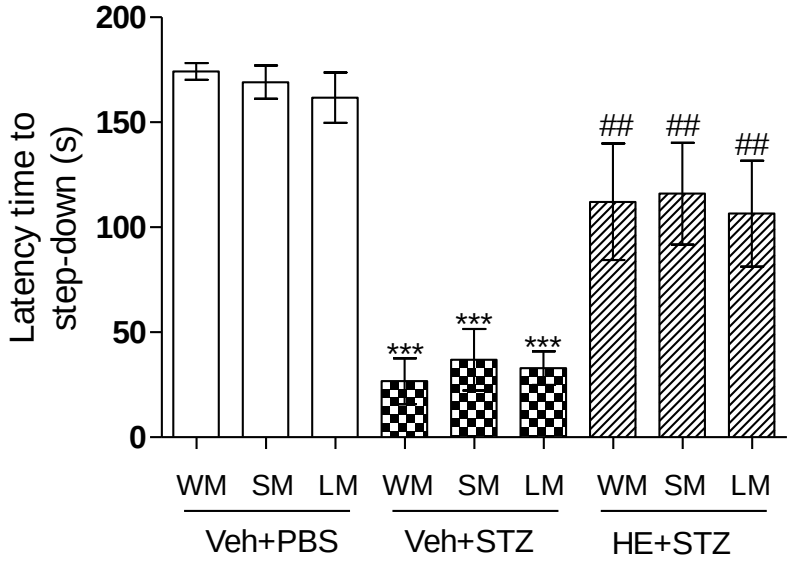


Figure 5 - Effect of HE (300 mg/Kg, v.o.) from *P. altomontana* on the latency time to step-down in the inhibitory avoidance test. Each value represents the mean ± SEM of 8-10 rats. Data analyzed by a one-way ANOVA followed by the Student Newman-Keuls test. ***P≤0.0001 as compared to control group (Veh + PBS), # #P≤0.001 as compared to the group (Veh + STZ). Abbreviations: work (WM), short (SM) and long duration (LM) memories.

FIGURE 6 – EFFECT OF FLUMAZENIL (FLU, 5MG/KG, I.P), CAFFEINE (CAF, 3MG/KG, I.P) OR NALTREXONE (NTX, 2.5 MG/KG, S.C) ON THE OPEN FILED TEST PERFORMACE OF RATS TREATED WITH HE (300 MG/KG, V.O.) FROM *POLYGALA ALTOMONTANA*.

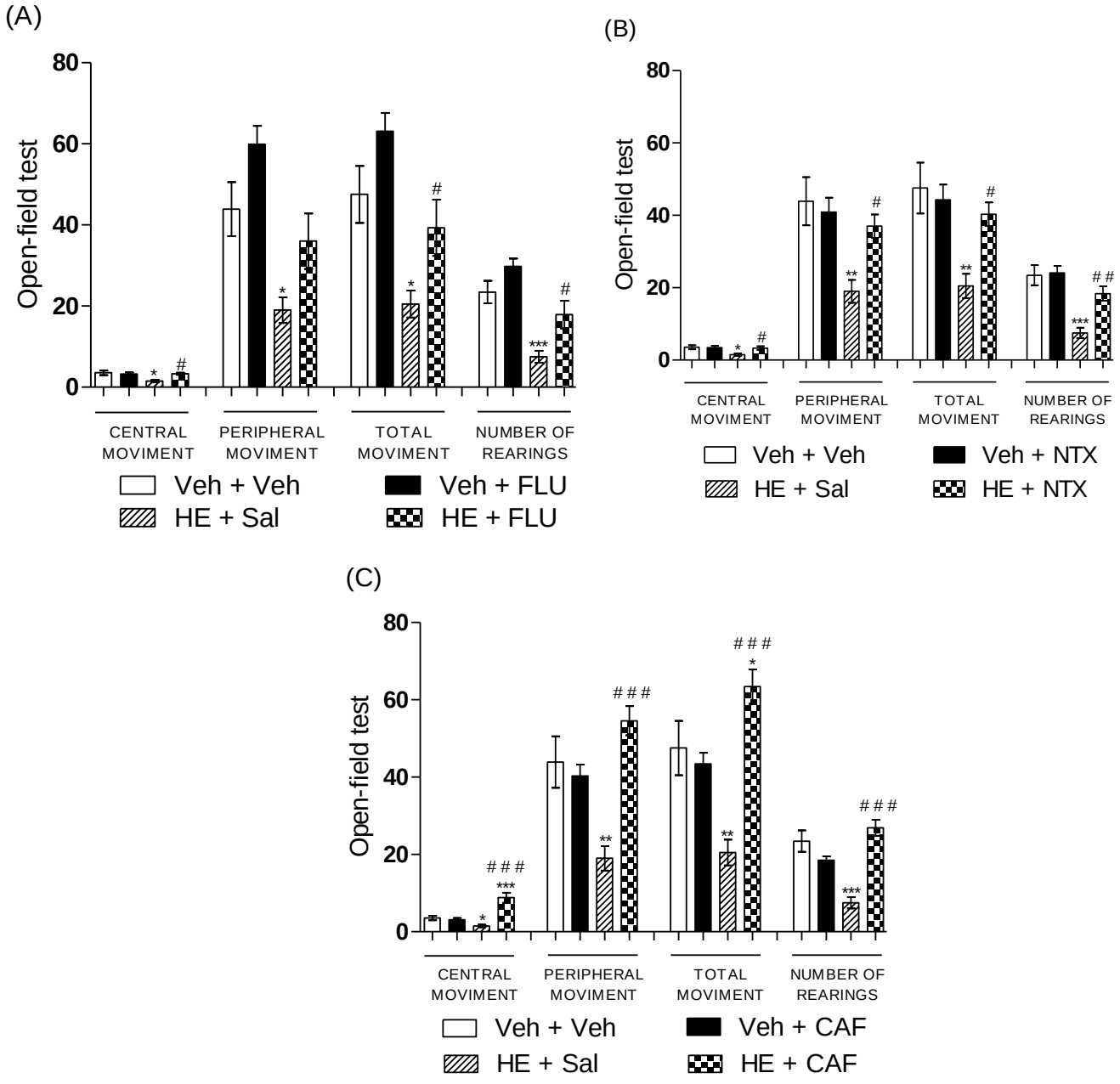


Figure 6 - Effect of flumazenil (FLU, 5 mg/Kg, i.p.), caffeine (CAF, 3 mg/Kg, i.p.) or naltrexone (NTX 2.5 mg/Kg s.c.) on the open-field test performance of rats treated with HE (300 mg/Kg, vo) from *P. altomontana.* Each value represents the mean ± SEM of 8-10 rats. Data analyzed by a one-way ANOVA followed by the Student Newman-Keuls test. *P≤0.05, **P≤0.001, ***P≤0.0001 as compared to control group (vehicle + vehicle), #P≤0.05, ##P≤0.001 and ###P≤0.0001 as compared to the respective HE + saline group.

TABLE 1 – EFFECT OF ORAL TREATMENT WITH HE, EA, ET (50-300 MG/KG), OR DZP (10 MG/KG) ON THE BEHAVIOR OF RATS EVALUATED IN THE PTZ-INDUCED SEIZURE TEST.

Drug/Dose (mg/Kg)	Latency to first convulsion (min)	Duration of the first convulsion (s)	Severity	Lethality
Vehicle	73,88±7,51	25,38±6,07	28,13±13,09	100%
HE 50	67,13±3,84	32,25±7,61	14,38±4,24	100%
HE 100	103,00±33,58	33,38±6,87	20,13±12,47	87,5%
HE 300	68,50±6,26	13,00±1,52^{T}	8,37±1,70	75%
Vehicle	76,50±5,66	19,10±3,40	21,00±6,15	100%
EA 50	85,90±19,81	18,80±1,55	10,30±1,53	80%
EA 100	113,90±42,69	18,80±1,58	10,90±1,79	80%
EA 300	67,50±8,30	13,40±1,82	16,00±2,77	80%
Vehicle	76,50±5,66	19,10±3,40	21,00±6,15	100%
ET 50	772,50±471,30	13,40±2,97	11,20±3,55	70%
ET 100	187,7±60,65	18,30±2,11	14,10±2,27	90%
ET 300	67,70±6,95	13,80±1,68	17,20±3,14	90%
Vehicle	70,33±5,27	26,25±4,96	21,50± 8,99	100%
DZP 10	407,8±178,4^{T}	15,92±2,56^{T}	15,25±5,83	25%

Table 1 - Each value represents the mean ± SEM of 8-10 rats. Data analyzed by a one-way ANOVA followed by the Student Newman-Keuls test. Data from the positive control group (DZP) were analyzed using the unpaired two-tailed Student t-test. *P≤0.05 as compared to control group. 0.05 <^{T}P <0.1 as compared to control group. Abbreviations: HE = hydroethanolic extract of *P. altomontana*; EA = ethyl acetate fraction of *P. altomontana*; ET = ethanolic fraction of *P. altomontana*; DZP = diazepam.

4 CONSIDERAÇÕES FINAIS

Os presentes resultados mostram que o EHPA obtido da *P. altomontana* promove um efeito ansiolítico, hipnosedativo, anticonvulsivante e promnésico, que não foram acompanhados de prejuizo motor. Representando uma excelente ferramenta na busca por tratamentos alternativos para a ansiedade, convulsão, distúrbios do sono e défict de memória. Estudo fitoquimico da *P. Altomontana* revelou a presença marjoritária do aurapteno (7-geraniloxicumarina) e isoquercetina (quercetina-3-O-β-D-glicopiranosídeo), sugerindo esses princípios ativos responsáveis pelas ações centrais da *P. Altomontana*. Na investigação dos mecanismos de ação, nossos dados dão suporte para o envolvimento dos receptores benzodiazepínicos/$GABA_A$, adenosina e opióides nos efeitos sedativos promovidos pelo EHPA.

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