**UNIVERSIDADE FEDERAL DE SANTA MARIA**
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**Larissa Finger Schäffer**

**INFLUÊNCIA DE *Harpagophytum procumbens* SOBRE PARÂMETROS COMPORTAMENTAIS E MOLECULARES EM UM MODELO DE DISCINESIA OROFACIAL EM RATOS**

Santa Maria, RS
2017

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Tese apresentada ao Curso de Pós-Graduação em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como Requisito parcial para obtenção do título de **Doutor em Farmacologia.**

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Orientadora: Profª. Drª. Caroline Wagner
Co-orientadora: Profª. Drª. Roselei Fachinetto

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**Aprovado em 18 de fevereiro de 2017:**

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Santa Maria, RS
2017

*Dedico esta tese aos meus pais, Josemar e Silvana Schäffer pelo apoio incondicional em todos os momentos, principalmente nos de incerteza, sem a dedicação de vocês eu não teria conseguido alcançar este grande sonho.*

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# RESUMO

## INFLUÊNCIA DE *Harpagophytum procumbens* SOBRE PARÂMETROS COMPORTAMENTAIS E MOLECULARES EM UM MODELO DE DISCINESIA OROFACIAL EM RATOS

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A flufenazina é um antipsicótico típico utilizado para o tratamento da esquizofrenia. No entanto, o seu uso crônico tem sido relacionado com o aparecimento de efeitos colaterais extrapiramidais, como a discinesia tardia (DT). Estudos sugerem o envolvimento da neuroinflamação, bem como o estresse oxidativo (EO), como possíveis causas para o aparecimento da DT, o que também pode levar à neurodegeneração dopaminérgica. O *Harpagophytum procumbens* (HP) é um fitoterápico utilizado na clínica devido principalmente aos seus efeitos anti-inflamatórios. Desta forma o objetivo deste trabalho foi avaliar a influência do *HP* sobre parâmetros comportamentais, bioquímicos e moleculares em modelo de discinesia orofacial (DO) induzida por flufenazina em ratos. Primeiramente analisamos várias frações de HP por cromatografia líquida de alta eficiência e realizamos o teste de scavenger do radical DPPH, a fim de eleger uma fração que apresentasse maior quantidade de harpagosídeo e que tivesse uma boa atividade antioxidante *in vitro*, sendo então eleita a fração de acetato de etila de *H. procumbens* (EAF HP). Desta forma, verificamos o efeito da EAF HP (10; 30 e 100 mg/kg- 21dias) sobre a DO induzida por flufenazina (25 mg/kg dose única) em ratos através da análise de parâmetros comportamentais motores. Além disso, realizamos análises bioquímicas sorológicas, parâmetros de EO (no fígado, rim, córtex e estriado), citocinas pró-inflamatórias (no estriado e córtex), imunoreatividade da tirosina hidroxilase (TH), do transportador de dopamina (DAT), do receptor dopaminérgico $D_2$ ($DRD_2$), do glutamato descarboxilase (GAD) e da ciclooxigenase 2 (COX 2) (no estriado). A administração crônica de flufenazina aumentou significativamente os movimentos de mascar no vazio (MMVs) em todos tempos analisados (2, 7, 14 e 21 dias) e esse aumento foi inibido pela EAF HP, especialmente na dose de 30 mg/kg, nos dias 7, 14 e 21. A flufenazina diminuiu a locomoção e atividade exploratória, no entanto EAF HP não foi capaz de proteger contra esta alteração. A flufenazina induziu EO identificado por alterações na atividade da catalase e nos níveis de de oxigênio/nitrogênio (ER) no córtex e estriado. Além disso, aumentou todas as citocinas pro-inflamatórias, e apresentou tendência em aumentar os níveis de COX 2. Contudo, não modificou a imunoreatividade da TH, DAT, $DRD_2$ e GAD. Os efeitos da flufenazina foram reduzidos pela administração da EAF HP especialmente no estriado, estrutura que está relacionado com o controle motor. Além disso, esta fração se mostrou segura, pois nenhuma das doses testadas causou alteração nos parâmetros bioquímicos no soro e de EO no fígado e rins dos animais. Os nossos resultados sugerem o envolvimento do EO e da neuroinflamação induzida pela flufenazina no desenvolvimento da DO em ratos e aponta a EAF HP como um agente terapêutico promissor para o tratamento de movimentos involuntários orais.

**Palavras-chave:** Garra do diabo. Flufenazina. Discinesia tardia. Neuroinflamação. Estresse oxidativo.

# ABSTRACT

## INFLUENCE OF *Harpagophytum procumbens* ON BEHAVIORAL AND MOLECULAR PARAMETERS IN A MODEL OF OROFACIAL DYSKINESIA IN RATS

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ADVISOR: Caroline Wagner

Fluphenazine is a typical antipsychotic used for the treatment of schizophrenia. However, its chronic use has been related to the appearance of extrapyramidal side effects, such as tardive dyskinesia (TD). Studies suggest the involvement of neuroinflammation as well as oxidative stress (OS), as possible causes for the onset of DT, which can also lead to dopaminergic neurodegeneration. *Harpagophytum procumbens* (HP) is a herbal medicine used in the clinic mainly due to its anti-inflammatory effects. Thus, the objective of this study was to evaluate the influence of HP on behavioral, biochemical and molecular parameters in fluphenazine-induced orofacial dyskinesia (OD) model in rats. First, we analyzed several fractions of HP by high performance liquid chromatography and performed the radical-scavenging activity DPPH assay. In order to choose a fraction that presented greater amount of harpagoside and that had a good antioxidant activity in vitro, where we chose the ethyl acetate fraction of *H. procumbens* (EAF HP). Thus, we verified the effect of EAF HP (10, 30 and 100 mg / kg-21 days) on fluphenazine-induced OD (25 mg / kg single dose) in rats through motor behavioral parameters. In addition, we performed biochemical serological analyzes, OS parameters (in liver, kidney, cortex and striatum), proinflammatory cytokines (in striatum and cortex), immunoreactivity of tyrosine hydroxylase (TH), dopamine transporter (DAT), dopamine receptor subtype $D_2$ ($DRD_2$), glutamate decarboxylase (GAD) and cyclooxygenase 2 (COX 2) (in striatum). Chronic administration of fluphenazine significantly increased vacuos chewing movements (VCMs) at all analyzed times (2, 7, 14 and 21 days) and this increase was inhibited by EAF HP, especially at the dose of 30 mg / kg, on days 7, 14 and 21. Fluphenazine decreased locomotion and exploratory activity, however EAF HP did not protect against this change. Fluphenazine induced OS identified by changes in catalase activity and levels of reactive oxygen / nitrogen species (RS) in the cortex and striatum. In addition, it increased all proinflammatory cytokines, and showed a tendency to increase COX 2 levels. However, it did not modify the immunoreactivity of TH, DAT, $DRD_2$ and GAD. The effects of fluphenazine were reduced by administration of HP EAF especially in the striatum, which structure is related to motor control. Moreover, this fraction proved to be safe, because none of the doses tested showed a change in the biochemical parameters in serum and OS in the liver and kidneys of animals. Our results suggest the involvement of OS and neuroinflammation fluphenazine-induced in the development of OD in rats and points to EAF HP as a promising therapeutic agent for the treatment of oral involuntary movements.

**Key-words:** Devil's claw. Fluphenazine. Tardive dyskinesia. Neuroinflammation. Oxidative stress.

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**Artigo**

**Manuscrito**

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# LISTA DE ABREVIATURAS

| | |
|---|---|
| ANVISA | - Agência Nacional de Vigilância Sanitária |
| AP-1 | - Proteína ativadora 1 |
| CLAE | - Cromatografia líquida de alta eficiência |
| COX 2 | - Cicloxigenase 2 |
| DAT | - Transportador de dopamina |
| DO | - Discinesia orofacial |
| DT | - Discinesia tardia |
| EAF HP | - Fração de acetato de etila de *H. procumbens* |
| EO | - Extresse oxidativo |
| ER | - Espécies reativas de Oxigênio e/ou Nitrogênio |
| GABA | - Ácido gama- aminobutírico |
| GAD | - Glutamato descarboxilase |
| GDNF | - Fator neurotrófico derivado de células gliais |
| GHS-R1A | - Receptor de grelina do tipo 1A |
| $H_2O_2$ | - Peróxido de hidrogênio |
| IL-1 β | - Interleucina 1 β |
| IL-2 | - Interleucina 2 |
| IL-6 | - Interleucina 6 |
| iNOS | - Síntese de óxido nítrico induzível |
| MAO | - Monoamino oxidase |
| MMVs | - Movimentos de mascar no vazio |
| MP | - Manuscrito em preparação |
| MPTP | - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| MS | - Manuscrito em submissão |
| NF-Kb | - Fator de necrose tumoral Kb |
| OMS | - Organização Mundial da Saúde |
| PG | - Prostaglandina |
| ROS | - Espécies reativas de oxigênio |
| SNC | - Sistema nervoso central |
| TH | - Tirosina hidroxilase |
| TNF-α | -Fator de necrose tumoral α |

# SUMÁRIO

**APRESENTAÇÃO**

No item **INTRODUÇÃO**, está descrita uma revisão sucinta sobre os temas trabalhados nesta tese, enquanto no item **REFERECIAL TEÓRICO**, a temática é abordada com mais profundidade.

Os resultados que fazem parte desta tese estão apresentados sob a forma de um artigo já publicado e um manuscrito submetido, os quais se encontram no item **RESULTADOS**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigo e manuscrito e representam a íntegra deste estudo. O artigo está disposto na forma que foi publicado na edição da revista científica *Neurochemical Researche* e o manuscrito está na forma que foi submetido para a revista *Molecular and Cellular Neuroscience.*

O item **DISCUSSÃO** apresenta uma discussão englobando os dois trabalhos.

O item **CONCLUSÕES** encontra-se no final dessa tese, e apresenta comentários sobre o artigo e o manuscrito contidos nesse trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se às citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO** dessa tese.

# 1 INTRODUÇÃO

A esquizofrenia é uma doença psiquiátrica grave que afeta cerca de 1% da população mundial (FRANGOU, 2008; VAN; KAPUS, 2009; KIRKBRIDE et al., 2012). Os antipsicóticos são os fármacos mais efetivos para o tratamento da esquizofrenia, principalmente os chamados antipsicóticos típicos ou clássicos, como a flufenazina e o haloperidol, os quais têm como mecanismo de ação o bloqueio de receptores dopaminérgicos, principalmente os do subtipo $D_2$. No entanto, a eficácia clínica dos antipsicóticos típicos tem sido comprometida pelos vários efeitos colaterais extrapiramidais que tem afetado os pacientes, dentre os quais se destaca a discinesia tardia (DT) (KANE; SMITH, 1982; ANDREASSEN; JORGENSEN, 2000).

A DT é uma síndrome extrapiramidal caracterizada pela presença de movimentos involuntários, repetitivos e não-intencionais localizados principalmente na região orofacial (boca, face, língua) (ANDREASSEN; JORGENSEN, 2000; KHOUZAM, 2015). A prevalência da DT em pacientes que fazem uso de antipsicóticos é cerca de 20 - 40%, porém este índice pode variar de acordo com a idade e o gênero do paciente, além do tempo e da classe de antipsicótico utilizada (KANE; SMITH, 1982; YASSA; JESTE, 1992; PATTERSON et al., 2005; LERNER et al., 2015).

Os mecanismos moleculares relacionados à fisiopatologia da DT em humanos ou discinesia orofacial (DO) em roedores permanecem incertos e várias hipóteses têm sido propostas (ANDREASSEN; JORGESSEN, 2000). Entre elas, podemos citar a hipótese da hipersensibilidade dopaminérgica, diminuição da atividade do sistema gabaérgica, excitotoxicidade glutamatérgica e a predisposição genética (CASEY, 2000; LENCZ et al., 2009; RIZOS et al., 2009; ALABED et al., 2010; BHIDAYASIRI; BOONYAWAIROJ, 2011). No entanto, recentemente, o papel do estresse oxidativo (EO) e especialmente da neuroinflamação ganhou espaço na literatura para tentar explicar a etiologia da DT. A produção de radicais livres (LOHR et al., 2003) e a sinalização inflamatória (BISHNOI et al., 2008a,d) por antipsicóticos levando a danos celulares e então uma possível anormalidade estrutural neuronal podem ser os fatores chave na patogênese da DT (LIU et al., 2012; LERNER et al., 2015).

Há muitos anos se têm o conhecimento desse transtorno de movimento sério, porém até hoje não existe uma abordagem terapêutica padrão para ser utilizada nos pacientes que apresentam DT. Neste contexto, o *Harpagophytum procumbens* é

uma planta nativa do sul da África, comercializada no Brasil como fitoterápico, popularmente conhecida como garra do diabo sendo utilizado na clínica principalmente no tratamento de doenças articulares devido aos seus potentes efeitos anti-inflamatórios (LAUDAHN; WALPER, 2001; WEGENER; LUPKE, 2003; WARNOCK et al., 2007). Contudo, alguns estudos demonstram a possível ação do HP ou de seus constituintes no SNC, onde este parece atuar nas células neuronais modulando o sistema dopaminérgico (SUN et al., 2012) o sistema GABAérgico (MAHOMED; OJEWOLE, 2006) e também parece apresentar atividade antioxidante em tecidos neuronais (BHATTACHARYA; BHATTACHARYA, 1998, SCHAFFER et al., 2013).

Tendo em vista que os poucos fármacos que são utilizados na prática médica para o tratamento da DT, tem eficácia clínica questionável e que o HP parece atuar no SNC modulando vias relacionadas com o aparecimento da DT, resolvemos testar a influência do HP no modelo de DO induzida por flufenazina em ratos, bem como verificar qual o possível mecanismo de ação do HP neste modelo experimental.

## 1.1 OBJETIVOS

### 1.1.1 Objetivo geral

Avaliar a influência do *Harpagophytum procumbens* no SNC em modelo de discinesia orofacial induzida por flufenazina em ratos.

### 1.1.2 Objetivos específicos

➢ Elucidação química da planta e a eleição da fração mais adequada para o tratamento *in vivo*;
➢ Induzir os distúrbios motores extrapiramidais através da administração de flufenazina;
➢ Avaliar o desenvolvimento de distúrbios do movimento através da quantificação de DO e da atividade locomotora e exploratória nos animais;
➢ Avaliar o possível papel neuroprotetor da fração de *H. procumbens* no desenvolvimento dos distúrbios do movimento;

➢ Avaliar parâmetros bioquímicos no soro e de estresse oxidativo no rim e fígado dos animais tratados com a fração de *H. procumbens* e/ou flufenazina;

➢ Investigar o envolvimento do processo de estresse oxidativo e da neuroinflamação na DO, bem como verificar o possível efeito protetor da fração *H. procumbens* sobre esses parâmetros.

➢ Avaliar a possível alteração provocada pela flufenazina e/ou HP sobre circuitos dopaminérgicos estriatais, incluindo TH, $DRD_2$ e DAT;

➢ Verificar se o tratamento com a fração de HP e/ou a flufenazina alteram a imunoreatividade da glutamato descarboxilase.

## 1.2 JUSTIFICATIVA

Considerando que o uso crônico de antipsicóticos pode causar efeitos extrapiramidais, muitas vezes debilitantes, como a DT; que o mecanismo pelo qual aparecem esses efeitos não está bem esclarecido; que existem estudos relacionando esses efeitos com o aumento de marcadores inflamatórios e de EO no SNC e que não há existência de fármacos seguros para o tratamento dessas desordens. Torna-se interessante avaliar a influência do HP (uma planta já comercializada como fitoterápico no Brasil devido à sua ação anti-inflamatória e que já está relatada a sua atividade antioxidante, bem como sua ação no SNC) no modelo de DO induzida por flufenazina em ratos.

## 2 REFERÊNCIAL TEÓRICO

2.1 ESQUIZOFRENIA

A esquizofrenia é uma doença psiquiátrica grave, crônica, incapacitante, com etiologia desconhecida, que afeta cerca de 1% da população mundial. Esta doença tem conseqüências negativas não só para os que estão acometidos por ela, mas também seus familiares e a sociedade no geral. Os pacientes apresentam risco significativamente aumentado de cometerem suicídio e freqüentemente sofrem desvantagens socioeconômicas (MUESER; McGURK, 2004; VAN; KAPUS, 2009; KIRKBRIDE et al., 2012).

Esta doença é caracterizada por apresentar sintomas positivos (tais como: alucinações e delírios), sintomas negativos (tais como: retraimento social apático, pobreza de expressão, redução de interesse e desmotivação) e déficits cognitivos (tais como: déficit de atenção, aprendizagem, memória e processamento de informações), resultando em uma desorganização comportamental. Esses sintomas aparecem geralmente no final da segunda década de vida do paciente e o início precoce dos mesmos determina, na maioria dos casos, um maior prejuízo funcional. Cabe salientar que esses sinais e sintomas podem se manifestar de forma diferente entre os indivíduos (FRANGOU, 2008; URFER-PARNAS et al., 2010; RETHELYI, 2011).

A neurotransmissão alterada é um mecanismo fisiopatológico chave que acaba explicando os diferentes sintomas da esquizofrenia, onde essas alterações ocorrem principalmente devido ao aumento e/ou desbalanço da atividade dopaminérgica no sistema nervoso central (SNC) acometendo mais especificamente as vias dopaminérgicas mesolímbica e mesocortical (Figura 1). Assim, os sintomas positivos estão relacionados com a hipótese dopaminérgica da esquizofrenia onde os eventos psicóticos ocorreriam devido a esse aumento da neurotransmissão dopaminérgica mesolímbica (GUILLIN et al., 2007; FRANGOU, 2008). Esta relação foi comprovada por estudos tomográficos que mostraram o aumento na liberação de dopamina durante o episódio psicótico agudo (LARUELLE et al., 1996). Entretanto no córtex cerebral ocorre uma diminuição de neurotransmissão dopaminérgica e essa condição acaba levando ao aparecimento dos sintomas negativos e cognitivos nos pacientes esquizofrênicos (GUILLIN et al., 2007; ABI-DARGHAM et al., 2002).

Figura 1: Principais vias dopaminérgicas
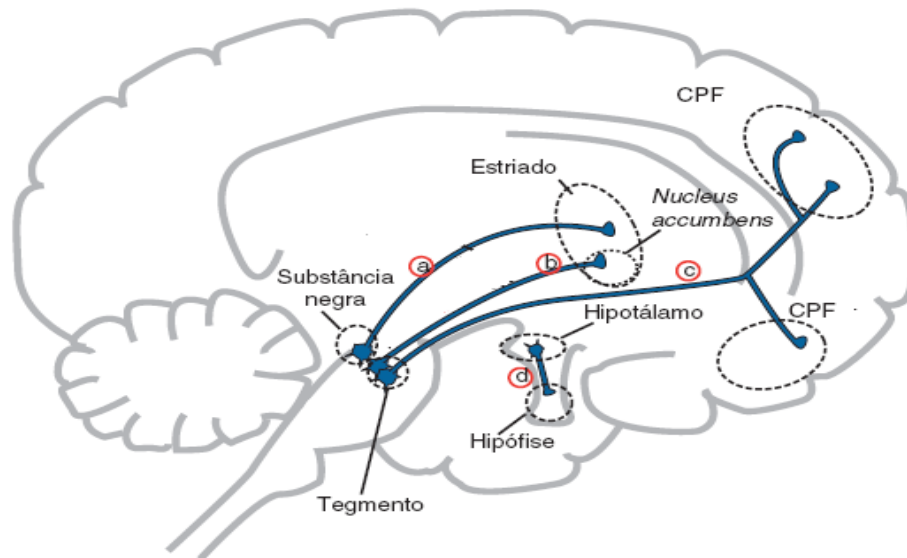


(a) Via nigroestriatal se projeta da substância negra para o estriado, faz parte do sistema nervoso extrapiramidal e controla a função motora e o movimento.
(b) Via mesolímbica projeta-se da área tegmental ventral do mesencéfalo para o nucleus accumbens, uma parte do sistema límbico do cérebro que está envolvida em muitos comportamentos, como sensação de prazer, euforia intensa produzida por uso abusivo de substâncias psicoativas, bem como delírios e alucinações da psicose.
(c) Via mesocortical se projeta da área tegmental ventral do mesencéfalo, enviando axônios para áreas do córtex pré-frontal (CPF) estando relacionada com sintomas cognitivos e afetivos da esquizofrenia.
(d) Via dopaminérgica tuberoinfundibular, projeta-se do hipotálamo para a adeno-hipófise e controla a secreção de prolactina.

Fonte: (STAHL, 2014), com modificações.

As causas que levam o paciente a ter esquizofrenia ainda são desconhecidas. Existem algumas teorias na literatura para o desenvolvimento da esquizofrenia, entre elas, podemos destacar a susceptibilidade genética (HARRISON; WEINBERGER, 2005), complicações no neurodesenvolvimento (AKIL; WEINBERGER, 2000), uso de drogas (SEMPLE et al., 2005), entre outros fatores ambientais (SPAUWEN; VAN, 2006; LARSON et al., 2011). Contudo, acredita-se que a esquizofrenia é de origem multifatorial, onde fatores genéticos ligados a fatores ambientais parecem ser importantes para o desenvolvimento da esquizofrenia. Estudos demonstram que indivíduos que possuem parentes em primeiro grau com esquizofrenia possuem um risco aumentado em desenvolver a doença (HARRISON; WEINBERGER, 2005; BALE et al., 2011; NAGAI et al., 2011). Eventos ocorridos durante o desenvolvimento cerebral, especialmente no período pré-natal, tais como, desnutrição maternal, infecções pré-natais, complicações

obstétricas podem acarretar no desenvolvimento de um cérebro fetal anormal, através de mudanças na sinapse. Além disso, fatores ambientais após o nascimento até a juventude, tais como, experiências psicológicas negativas, traumas, abuso físico e sexual, uso de drogas, podem influenciar esse processo, desencadeando as primeiras manifestações clínicas da doença (FRANGOU, 2008; LARSON et al., 2011).

## 2.2 ANTIPSICÓTICOS

Os antipsicóticos, ou também chamados neurolépticos, são os fármacos mais efetivos para o tratamento da esquizofrenia. Eles atuam na diminuição dos sintomas da esquizofrenia permitindo assim, na maioria das vezes, a reintegração do paciente ao convívio social (VAN; KAPUR, 2009; FRANGOU, 2008). Os antipsicóticos podem ser amplamente divididos em três grupos: antipsicóticos de primeira geração ou típicos (ex: haloperidol e flufenazina), antipsicóticos de segunda geração (ex: risperidona e olanzapina) e terceira geração (ex: aripiprazol) ou atípicos (HORACEK et al., 2006). Os antipsicóticos típicos são classificados entre eles de acordo com sua estrutura química, enquanto os antipsicóticos atípicos são classificados de acordo com suas propriedades farmacológicas (PARK et al., 2013).

Os antipsicóticos típicos, descobertos na década de 1950, têm como mecanismo de ação o bloqueio de receptores dopaminérgicos, principalmente os do subtipo $D_2$. No entanto, a eficácia clínica dos antipsicóticos típicos tem sido comprometida pelos vários efeitos colaterais extrapiramidais decorrentes do bloqueio de $RDD_2$ na via nigroestriatal. É importante ressaltar que na esquizofrenia a via nigroestriatal não é afetada. Entretanto, a ação farmacológica dos antipsicóticos não apresenta seletividade apenas para os $DRD_2$ da via mesolímbica e acaba por atingir todas as vias dopaminérgicas, impedindo assim a ligação da dopamina também na via nigroestriatal, causando assim, efeitos motores, como a Discinesia tardia (DT) (Figura 2) (RANG et al., 2012; STAHL, 2014).

Figura 2: Ação dos antipsicóticos na via dopaminérgica nigroestriatal
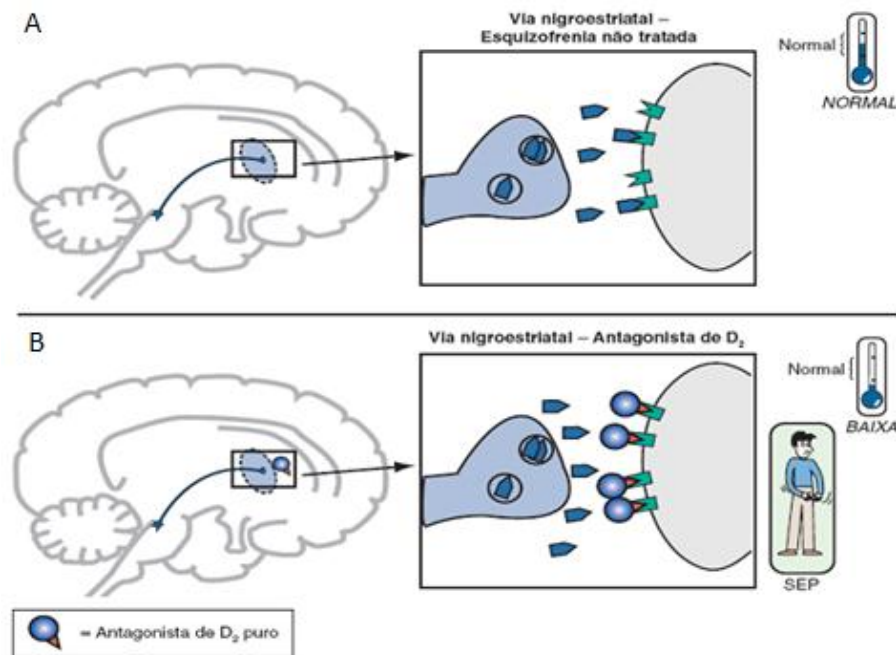


(A) Via dopaminérgica nigroestriatal funcionando normalmente no paciente esquizofrênico não tratado;
(B) Via dopaminérgica nigroestriatal hipofuncional após o tratamento com antagonistas de receptor $D_2$, levando ao aparecimento de sintomas extrapiramidais como a discinesia tardia.

Fonte: (STAHL, 2014), com modificações.

Enquanto os antipsicóticos típicos são potentes antagonistas de receptores $D_2$, os quais estão relacionados com seus graves efeitos adversos ligados ao desenvolvimento de movimentos involuntários, os antipsicóticos atípicos, desenvolvidos na década de 1980, são menos potentes em antagonizar os receptores $D_2$, tendo seu mecanismo relacionado com a ação em outros receptores, tais como receptores de serotonina 5-$HT_2A$ o que contribui também com sua eficácia contra os sintomas negativos e cognitivos. Desta forma, vários estudos demonstram, que os pacientes que usam antipsicóticos atípicos apresentam um menor índice de desenvolver DT (LEUCHT et al., 2003; MELTZER; SUMIYOSHI, 2008), porém essa informação é conflitante na literatura, onde alguns estudos apontam que o uso de antipsicóticos atípicos não diminuiu a incidência e prevalência de DT (MARSSHALL et al., 2002; DE LEON, 2007; WOODS, 2010). Por fim, os antipsicóticos atípicos são responsáveis pelo aparecimento de desordens metabólicas, como o *diabetes mellitus* tipo 2, ganho de peso, aumento dos níveis de triglicerídeos e colesterol (HENDERSON, 2002; VAN; KAPUR, 2009; MIRRON et al., 2014), além do inconveniente de possuírem custo muito elevado se comparados aos típicos. Desta

forma, os antipsicóticos típicos, como o haloperidol e a flufenazina, continuam sendo largamente empregados no tratamento da esquizofrenia.

2.3 DISCINESIA TARDIA

O uso prolongado de antipsicóticos típicos, especialmente, aumenta o risco para o desenvolvimento de desordens do movimento, tais como a DT. A DT é uma síndrome extrapiramidal grave, de natureza iatrogênica, incapacitante e potencialmente permanente, caracterizada pela presença de movimentos involuntários, repetitivos e não-intencionais localizados principalmente na região orofacial (boca, face, língua), podendo acometer também a musculatura do tronco, membros superiores e inferiores (ANDREASSEN; JORGENSEN, 2000; FRANGOU, 2008; KHOUZAM, 2015). Dentre os movimentos orofaciais podemos destacar a mastigação no vazio, protrusão de língua, movimentos de franzir e de piscar os olhos (Figura 3). Além disso, alguns pacientes podem apresentar a discinesia respiratória, que são alterações nos padrões rítmicos levando a hiper e hipoventilação podendo ser fatal (SAMIE et al., 1987). A descrição original da DT foi publicada por Schonecker em 1957, cerca de cinco anos após o início do tratamento antipsicótico na psiquiatria.

Figura 3: Discinesia Tardia.



(1) Mulher tratada com numerosos antipsicóticos por pelo menos 15 anos desenvolveu discinesia tardia típica orofacial-bucolingual
(2) Homem com torcicolo e protrusão da língua decorrentes do uso crônico com antipsicótico. Fonte: Imagens adaptadas de http://e-medicaltextbook.blogspot.com.br/2008/07/schizophrenia-2.html

Hoje, sabe-se que a DT não é apenas um efeito secundário, mas sim uma doença que aparece durante o tratamento médico. Ela tem o seu próprio campo de evolução, o qual pode ser visto nas diferentes formas clínicas. É considerada um distúrbio motor socialmente estigmatizado que têm sido associado a uma má qualidade de vida e que por vezes torna o paciente debilitado (BHIDAYASIRI; BOONYAWAIROJ, 2011; LERNER; MIODOWNIK, 2011). Além disso, essa desordem motora pode ser irreversível, persistindo após a retirada do antipsicótico indicando que os mesmos produzem mudanças na função cerebral que não estão relacionadas com a presença do fármaco (CRANE, 1973; GIAZER et al.,1993; KANE, 1995).

A prevalência da DT em pacientes que fazem uso de antipsicóticos é cerca de 20–40%, porém este índice aumenta principalmente com a idade e com o tempo de utilização da medicação antipsicótica (KANE; SMITH, 1982; YASSA; JESTE, 1992; GLAZER et al., 1993; PATTERSON et al., 2005; LERNER et al., 2015). Já foi descrito que nos primeiros 4-5 anos de tratamento com antipsicóticos, a incidência cumulativa de DT aumenta linearmente, juntamente com a duração da exposição ao fármaco (KANE et al., 1988). Esses dados foram suportados em outras pesquisa que demonstraram que o risco de DT aumentou até 25% após 5 anos de tratamento com antipsicótico, 49% após 10 anos e 68% após 25 anos (CHAKOS et al., 1996; GLAZER et al., 1993; WOENER et al., 1998; LERNER et al., 2015).

A etiologia da DT é complexa, multifatorial, e ainda não completamente compreendida (KHOUZAM, 2015). Para melhor entendimento dos possíveis mecanismos relacionados com a DT em humanos, alguns modelos animais são utilizados. Nos roedores a discinesia é denominada discinesia orofacial (DO). Estudos evidenciam que o tratamento sub-crônico e crônico com antipsicóticos típicos é capaz de induzir o aparecimento de movimentos orais espontâneos em roedores e esses movimentos são chamados de movimentos de mascar no vazio (MMVs) (WADDINGTON, 1990; TURRONE et al., 2002; LISTER et al., 2014), sendo que antipsicóticos atípicos, como a risperidona, parecem não induzir esses movimentos em roedores (PEROZA et al., 2016). Os MMVs, quantificados em roedores, são amplamente utilizados como modelos de DT sendo considerados os movimentos mais análogos aos encontrados na DT possuindo várias similaridades com as alterações encontradas em humanos (ANDREASSEN; JORGENSEN, 2000; CASEY, 2000; TURRONE et al., 2002; BLANCHET et al., 2012). Desta forma, várias

hipóteses têm sido propostas baseadas em estudos em roedores e humanos para explicar este efeito colateral desagradável e, por vezes incapacitante.

A hipótese da hipersensibilização dopaminérgica (Figura 4) é a hipótese mais antiga (sugerida em 1970) e popular para explicar o desenvolvimento da DT após uso crônico de antipsicóticos (KLAWANS, 1970). Segundo esta hipótese, o bloqueio crônico dos receptores $D_2$ pelos antipsicóticos principalmente em locais relacionados com o controle motor, como a via dopaminérgica nigroestriatal, é seguido por um aumento compensatório no número de receptores dopaminérgicos, receptores estes, que provavelmente respondem a menores níveis de dopamina levando a um estado hiperdopaminérgico e a manifestações clínicas como, por exemplo, a DT (KLAWANS; RUBOVITS, 1972; BURT et al., 1977; RUBINSTEIN et al., 1990). Além disso, este aumento da atividade dos neurônios dopaminérgicos está associado com o aumento de síntese e liberação de dopamina na fenda sináptica (ZIVKOVIC et al., 1975) e consequente aumento na atividade da tirosina hidroxilase (TH), a enzima limitante na síntese de catecolaminas (MOORE, 1987).

Neste contexto, uma das principais formas de retirada da dopamina da fenda sináptica e, portanto da redução da neurotransmissão dopaminérgica, ocorre através da recaptação de dopamina via transportador de dopamina (DAT) (BECKMAN; QUICK, 1998; KAHLIG; GALLI, 2003). Este é responsável por limitar a extensão, duração e área da ativação do receptor de dopamina tendo um papel crucial em doenças que alteram a plasticidade neuronal dopaminérgica (AMARA; SONDERS, 1998; MILLER et al., 1999). Vale ressaltar que o sistema dopaminérgico nigroestriatal é a região que apresenta maior quantidade neste tipo de transportador (GIROS; CARON, 1993).

Figura 4: Hipótese da hipersensibilidade dopaminérgica
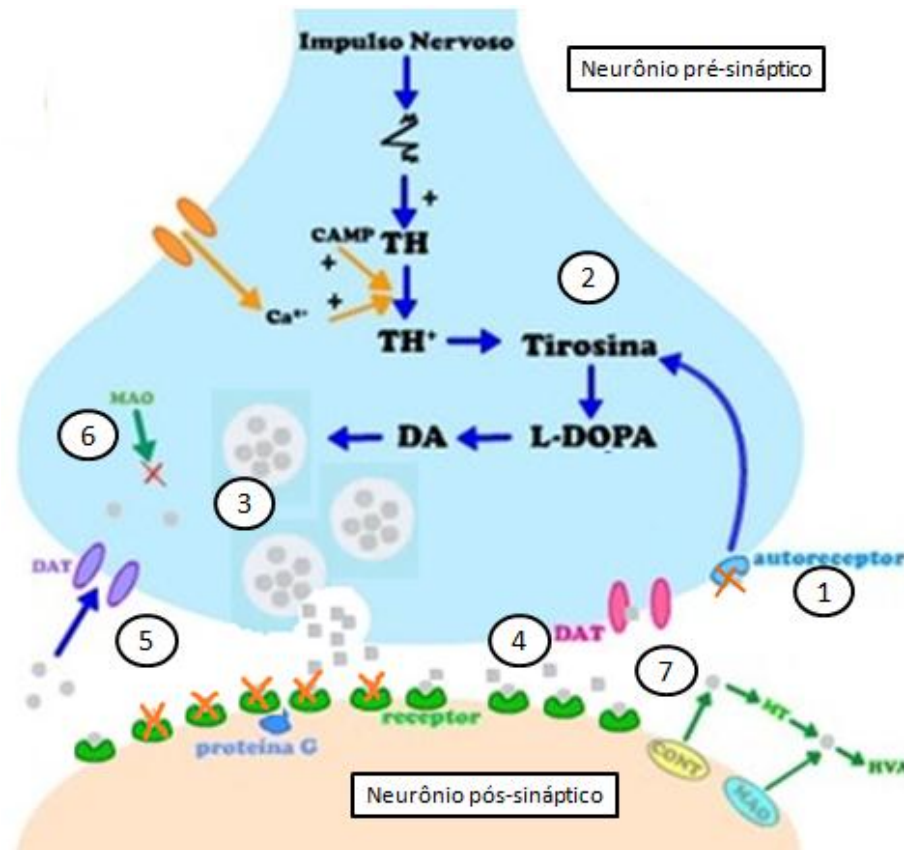


(1) O bloqueio dos receptores $D_2$ pré-sinapticos na via dopaminérgica nigroestriatal (2) pode causar o aumento na síntese de dopamina a partir da L-tirosina (3) e seu armazenamento em vesículas sinápticas. (4) A liberação da dopamina na fenda sináptica agirá em receptores ainda não bloqueados e hipersensível à dopamina. (5) A dopamina é então recaptada do meio extracelular via transportador de dopamina (6) e ocorre o seu metabolismo pela enzima monoamino oxidase (MAO) (7) ou então pelo catecol- o – metiltransferase (COMT).

Fonte: Figura adaptada de https://www.youtube.com/watch?v=hcJQkCC2Rzk

No entanto, a hipótese da hipersensibilidade dopaminérgica não pode explicar completamente os achados clínicos dessa desordem do movimento, pois a DT geralmente não aparece em todos os usuários que utilizam fármacos que bloqueiam os receptores de dopamina (FENTON, 2000; GLAZER, 2000). Desta forma, a hipótese dopaminérgica como o principal mecanismo molecular de DT tem sido questionada por vários estudos, sendo que outros fatores podem estar relacionados com o aparecimento DT (KLAWANS; RUBOVITS, 1972; WOLFARTH; OSSOWSKA, 1989; WADDINGTON, 1990; ANDREASSEN; JORGENSEN, 2000; LOHR et al., 2003). Entre as demais hipóteses neuroquímicas para se explicar o aparecimento da DT se encontram a disfunção dos neurônios GABAérgicos, excitotoxicidade glutamatérgica e a predisposição genética (MILLER; CHOUINARD, 1993;

ELKASHEF; WYATT, 1999; ANDREASSEN; JORGENSEN, 2000; LIOU et al., 2007; LENCZ; MALHOTRA, 2009; RIZOS et al., 2009; BHIDAYASIRI; BOONYAWAIROJ, 2011). Porém, especialmente, o papel do estresse oxidativo (EO), bem como, a ativação de marcadores pró-inflamatórios e consequentes anormalidades estruturais no cérebro desses pacientes tem ganhado espaço na literatura para tentar explicar a fisiopatologia da DT (BISHNOI et al., 2008; LERNER et al., 2015).

### 2.3.1 Hipótese do estresse oxidativo

Esta hipótese é suportada por estudos clínicos que demonstraram que a utilização crônica de fármacos antipsicóticos é capaz de induzir o EO e causar dano estrutural no SNC (PALL et al., 1987; LOHR et al., 1990; TSAI et al., 1998). Além disso, outros estudos também relatam que o tratamento com substâncias antioxidantes, tais como a vitamina E (EGAN et al., 1992; DABIRI et al., 1994), vitamina B6 (LERNER et al., 2007) e piracetam (LIBOV et al., 2007) podem atenuar o desenvolvimento da DT em pacientes com esquizofrenia.

O estado de estresse oxidativo (EO) pode ser definido como o desequilíbrio entre a formação e remoção de agentes oxidantes no organismo, decorrente da geração excessiva de espécies reativas de oxigênio/nitrogênio (ER) e/ou diminuição de antioxidantes endógenos (HALLIWELL, 1992; DAWSON; DAWSON, 1996; KOHEN; NYSKA, 2002; BERG, 2004; VALKO, 2007). Desta forma, o acúmulo de ER decorrente do EO podem causar danos à estrutura de lipídios, proteínas e DNA (DUFFY et al., 1998; FINKEL; HOLBROOK, 2000; KOHEN; NYSKA, 2002; HALLIWELL; GUTTERIDGE, 2007; SILVA; COUTINHO, 2010) com consequente alteração funcional e prejuízo das funções vitais em diversos tecidos e órgãos (HALLIWELL, 1992), sendo relacionado com diversas patologias, entre elas, os distúrbios neurológicos e inflamatórios (YUTING et al., 1991; HALLIWELL; GUTTERIDGE, 2007; HAYASHI, 2009).

A indução do EO pelo uso de antipsicóticos pode ocorrer principalmente pelo aumento da liberação de dopamina após o bloqueio crônico do receptor dopaminérgico pré-sinaptico e consequente aumento de sua auto-oxidação e metabolização pela enzima monoamino oxidase (MAO), podendo levar ao aumento na produção de peróxido de hidrogênio ($H_2O_2$) (ROGOZA et al., 2004), por exemplo, o qual pode provocar danos nos neurônios dopaminérgicos (SPINA; COHEN, 1989).

É importante ressaltar que o cérebro é um órgão conhecido por ser vulnerável ao EO devido a sua elevada quantidade de ácidos graxos poli-insaturados, do seu alto consumo de oxigênio e por conter maior quantidade de metais de transição do que outros órgãos, tais como o ferro. Já foi descrito que pacientes com DT apresentaram um aumento no níveis de marcadores de peroxidação lipídica no líquor comparado com pacientes que não possuem DT (LOHR et al., 1990). Além disso, o cérebro tem baixos níveis de enzimas antioxidantes como a catalase e a glutationa peroxidase, o que facilita ainda mais o estabelecimento de um estado de EO nas células cerebrais (REITER, 1995; DUFFY; MURPHY, 1998; SMITH et al., 2013).

Alguns estudos demonstram que animais que desenvolvem MMVs apresentam alterações nos parâmetros de estresse oxidativo em estriado especialmente (NAIDU et al., 2003; BURGER et al., 2003, 2005), além disso, também existem relatos na literatura do papel protetor dos antioxidantes contra a DO induzida por antipsicóticos em roedores (BURGER et al., 2005; COLPO et al., 2007; FACHINETO et al., 2007a; TREVIZOL et al., 2011; BUSANELLO et al., 2012; PEROZA et al., 2013).

## 2.3.2 Hipótese da neuroinflamação

A resposta inflamatória no SNC compreende um grande espectro de respostas celulares complexas e integradas, principalmente devido à ativação de células gliais, tais como a microglia e os astrócitos. A microglia são células residentes no SNC que são geralmente as primeiras a serem ativadas em resposta a danos nos tecidos ou infecções cerebrais (STERTZ et al., 2013). Estas células pequenas têm várias funções descritas, incluindo: reconhecimento de patógenos, fagocitose, apresentação de antígenos e remodelação de sinapse (PARK et al., 2012). Como resultado da ativação dessas células gliais ocorre a liberação de mediadores inflamatórios (citocinas, por exemplo), que podem potencialmente contribuir para a disfunção neuronal e progressão de patologias no SNC. (KHANSARI et al., 2009). Além disso, em resposta a alterações no ambiente tecidual, as células microgliais podem ser ativadas pela alteração da sua morfologia e função (MARSHALL et al., 2013).

As citocinas estão envolvidas na regulação da comunicação entre as células do sistema imunológico, portanto altos níveis de citocinas no SNC indicam ativação da resposta inflamatória (TANSEY, 2010), onde a expressão persistente de duas citocinas pró-inflamatórias principais, o fator de necrose tumoral α (TNF α) e interleucina-1β (IL-1β) compromete diretamente a plasticidade e a sobrevivência das células neuronais (ROTHWELL; LUHESHI, 2000; ALLAN; ROTHWELL, 2001).

Segundo Barth e colaboradores (2009), em células neuronais, o TNF-α e Il-1β estimulam uma reorganização redox-dependente transitória do citoesqueleto de actina e induzem a formação de ER, onde a presença persistente de ER intracelulares provoca danos oxidativos (carbonilação) à actina ocorrendo à perda da plasticidade celular.

Godoy e colaboradores (2010) demonstraram que níveis aumentados de citocinas, especialmente IL-1β, podem alterar a viabilidade neuronal com consequente perda de neurônios dopaminérgicos. Além disso, esse aumento significativo de citocinas em certas regiões do cérebro pode influenciar diretamente a transmissão sináptica, com consequente mudança comportamental, o que pode ajudar a explicar o desenvolvimento de DT (MILLER et al., 2009, HAROON et al., 2012).

Há relatos na literatura que o tratamento prolongado com antipsicóticos típicos pode causar neuroinflamação (PATERSON et al., 2006; BISHNOI et al., 2008 a,b; LAU et al., 2013) e segundo Bishnoi e colaboradores (2008a) o aumento de marcadores inflamatórios no cérebro está correlacionado com o desenvolvimento comportamental da DO. Além disso, estudo realizado por Rapaport e Lohr (1994), demonstra que indivíduos com esquizofrenia que desenvolveram DT, tinham níveis aumentados de interleucina 2 (IL-2) comparados com pacientes sem DT. Em estudo recentemente publicado pelo nosso grupo foi demonstrado uma correlação positiva entre os MMVs e o aumento de citocinas pró-inflamatórias (IL1β, IL6, TNF-α e INF-γ) no estriado de ratos tratados com haloperidol, sugerindo a relação da neuroinflamação com o desenvolvimento da DO induzida por haloperidol (PEROZA et al., 2016). Em relação ao tratamento da DO, Naidu e Kulkarni (2001) mostraram que a indometacina (um inibidor de ciclooxigenase não seletivo) reverte os MMVs induzidos pelo haloperidol, sugerindo o envolvimento de prostaglandinas na DO.

Por fim, as espécies reativas de oxigênio também são capazes de induzir a ativação e a expressão de determinados genes e fatores de transcrição

pró-inflamatórios. O TNF-α e o fator nuclear kB (NF-Kb) são fatores de transcrição que são produzidos diretamente em resposta ao EO (AGGARWAL et al., 2002). O aumento destes fatores de transcrição, juntamente com outras citocinas pode conduzir à iniciação da cascata de sinalização celular que leva à morte celular (AGGARWAL, 2000; AGGARWAL et al., 2002).

Assim, se torna importante ressaltar que o EO pode ser conseqüência ou também causa da neuroinflamação, desta forma, podemos sugerir que pelo menos em partes, a neuroinflamação e o EO, podem estar relacionados com a DT causada pelo uso crônico de antipsicóticos.

## 2.3.3 Tratamentos utilizados na discinesia tardia

Como a DT é uma desordem iatrogênica, o melhor meio de tratamento (mas não possível) seria a prevenção. No entanto, os pacientes necessitam de tratamento com agentes antipsicóticos de forma contínua, e estes fármacos são, na maioria das vezes, o melhor tratamento para distúrbios psiquiátricos de longo prazo, como a esquizofrenia. Desta forma, os pacientes e suas famílias devem ser aconselhados sobre o risco de DT no momento da escolha da terapêutica farmacológica, onde sempre deve ser priorizada a menor dose eficaz do fármaco mais seguro. O paciente deve ser monitorado de perto quanto às características de DT e, se possível, reduzir a dose quando as características são detectadas pela primeira vez e / ou considerar a mudança para um fármaco com um menor risco de DT (JANKELOWITZ, 2013).

Há muitos anos se têm o conhecimento desse transtorno de movimento debilitante, porém até hoje não existe uma abordagem terapêutica padrão para a utilização nos pacientes que apresentam essa desordem. O fato de que o surgimento dos antipsicóticos atípicos reduz a propensão ao desenvolvimento da DT, acabou por diminuir também a importância da busca de um tratamento padrão para esse transtorno. Contudo, alguns estudos vêm demonstrando que o uso de antipsicóticos típicos é ainda muito elevado e que mesmo os antipsicóticos atípicos não diminuíram significativamente a prevalência da DT na clínica, como era esperado (MARSSHALL et al., 2002; DE LEON, 2007; WOODS, 2010; CLOUD et al., 2014), tornando este transtorno do movimento um desafio para a comunidade científica.

Em vista da fisiopatologia proposta para a DT, as intervenções terapêuticas têm tentado modular a atividade de inúmeros neurotransmissores tais como, a dopamina, GABA, acetilcolina, norepinefrina e serotonina. Vários artigos têm tentado rever todos os agentes testados para o tratamento da DT (AIA et al., 2011; ALABED et al., 2011; JANKELOWITZ, 2013; RANA et al., 2013; CLOUD et al., 2014; KHOUZAM, 2015). Muitos agentes apresentaram eficácia em estudos clínicos pequenos, cegos e randomizados, porém nota-se que faltam estudos controlados maiores a fim de definir a abordagem de tratamento. Entre os agentes testados podemos destacar o baclofeno (GERLACH et al., 1978; ALABED et al., 2011), amantadina (PAPPA et al., 2010), levetiracetam (WOODS et al., 2008), piracetam (LIBOV et al., 2007), clonazepam (THAKER et al., 1990), propranolol (SCHRODT et al., 1982), melatonina (SHAMIR et al., 2011) e vitamina B6 (LERNER et al., 2007), onde estes abordam as mais diversas hipóteses para o aparecimento da DT.

Contudo é importante salientar, que os poucos fármacos que são utilizados na prática médica para o tratamento da DT, tem eficácia clínica questionável, principalmente porque promovem outros efeitos adversos associados à sua utilização ou não se têm comprovação científica clínica da sua eficácia.

## 2.3.4 Produtos naturais e a discinesia orofacial

O uso terapêutico de produtos naturais por diferentes culturas tem sido extensivamente documentado, sendo que em 1985 a Organização Mundial da Saúde (OMS) estimou que 65% da população do mundo contaram com medicamentos tradicionais derivados de plantas para os seus cuidados primários de saúde (FARNSWORTH et al., 1985). Além disso, 80% da população dos países em desenvolvimento utilizam práticas tradicionais na atenção primária, e desse total, 85% dessas práticas utilizam as plantas medicinais ou preparações destas, para os cuidados com a saúde, demonstrando assim a grande utilização dos produtos naturais pela população mundial. Vale salientar, que se encontra em expansão o uso de plantas medicinais, bem como medicamentos fitoterápicos no mundo inteiro (WHO, 2002).

As propriedades terapêuticas das plantas medicinais são atribuídas a diferentes constituintes químicos isolados de seus extratos. Sendo a atividade antioxidante uma das propriedades bastante buscada, pois tem mostrado efeito benéfico em várias doenças degenerativas, câncer, *Diabetes mellitus,* doenças

cardiovasculares, doenças neurológicas e doenças inflamatórias (HEO; LEE, 2004; QUINE; RAGHU, 2005; KAMALAKKANNAN; PRINCE, 2006; DUARTE et al., 2010; RODRIGO et al., 2010; PARK et al., 2012; RODRIGO et al., 2013; JAYASENA et al., 2013; CAROCHO; FERREIRA, 2013).

Um dos principais constituintes responsáveis pela ação antioxidante presentes nas plantas medicinais, são os compostos fenólicos, tais como flavonóides, que são compostos químicos presentes em diversas plantas, frutos, sementes, grãos e em algumas bebidas como vinho e cerveja, sendo mais de 8000 diferentes estruturas já identificadas e descritas (PIETTA, 2000). Vale salientar, que a potente atividade antioxidante dos flavonóides se dá principalmente pela atividade de *scavenger* de radicais livres, diminuindo assim o dano oxidativo e consequentemente a morte celular (DUGAS et al., 2000). Devido à crescente compreensão do potencial benéfico destes compostos fenólicos, tanto na manutenção da saúde quanto na doença humana, o número de pesquisas utilizando plantas medicinais que tenham na sua composição compostos fenólicos, tem aumentado nos últimos anos (COLPO et al., 2007; PEREIRA et al., 2008; SUDATI et al., 2009; BOLIGON et al., 2009; KADE et al., 2008; BONFANTI et al., 2013; PEROZA et al., 2013).

Em modelos de discinesia orofacial utilizando roedores, produtos naturais com capacidade antioxidante tem demonstrado efeito protetor contra as desordens de movimento (NAIDU et al., 2003 a; b; BISHNOI et al., 2007; COLPO et al., 2007; TREVIZOL et al., 2011). Em trabalhos prévios do nosso grupo, a *Bauhinia forficata* (PEROZA et al., 2013) e o resveratrol (BUSANELLO et al., 2012) demonstraram efeito benéfico em diminuir os MMVs induzidos por antipsicóticos. Contudo, a atividade antioxidante parece não ser a única responsável por esse efeito protetor, pois só uma parte dos estudos consegue atribuir a diminuição da DO ao efeito antioxidante do composto e outros trabalhos não conseguem demonstrar essa relação. Desta forma, podemos acreditar que os compostos presentes nas plantas podem estar exercendo outros efeitos farmacológicos, como por exemplo, tendo atividade anti-inflamatória ou até mesmo ação direta no sistema dopaminérgico.

Assim, percebemos a necessidade de mais pesquisas acerca dos mecanismos farmacológicos pelos quais esses compostos presentes nos produtos naturais têm a sua ação na DO, como propósito de elucidar seus potenciais efeitos, podendo aplicar futuramente em estudos clínicos ajudando na melhora dessa desordem do movimento.

## 2.4 *HARPAGOPHYTUM PROCUMBENS*

O *Harpagophytum procumbens* (HP) (Figura 5) é uma planta perene pertencente à família Pedaliceae, nativa do sul da África, comumente encontrada no deserto do Kalahari e estepes da Namíbia (GERICKE, 2002; ANDERSEN et al., 2004; GRANT et al., 2007; MNCWANGI et al., 2012). Essa planta é popularmente conhecida como garra do diabo devido às características semelhantes a uma garra na extremidade do seu fruto (Figura 5A) (GERICKE, 2002; MAHOMED; OJEWOLE, 2004). Porém, é nas raízes tuberosas onde são encontrados os seus principais componentes bioativos, sendo estes responsáveis pela sua ação terapêutica (WEGENER, 2000; GERICKE, 2002; GRANT et al., 2007).

Figura 5: *Harpagophytum procumbens*



(A) Fruto do *Harpagophytum procumbens;*
(B) Flores e folhas do *H. procumbens*,
(C) "Curandeira" de Molopo na África demonstrando as raízes de *H. procumbens*

Fonte: (MNCWANGI et al., 2012).

Os constituintes característicos do HP são os glicosídeos iridóides, principalmente o harpagosídeo, harpagogídeo, procumbídeo, 8-coumaroil harpagídeo e o verbascosídeo (GERICKE, 2002; ABDELOUAHAB; HEARD, 2007; ABDELOUAHAB; HEARD, 2008; GEORGIEV et al., 2013). Sendo que o principal constituinte da planta para a sua ação farmacológica é o harpagosídeo (Figura 6), podendo ser encontrado em cerca de 3% de todos constituintes. No entanto, sabe-se que a ação do HP não se dá exclusivamente devido a presença deste

constituinte, podendo estar associada também à outros glicosídeos iridóides e demais componentes presentes na planta como os flavonóides (KASZKIN et al., 2004; BRIEN et al., 2006), os quais poderão antagonizar ou potencializar sua ação terapêutica (FIEBICH et al., 2001; ANUATE et al., 2010; MNCWANGI et al., 2012; GEORGIEV et al., 2013).

Figura 6: Estrutura química do harpagosídeo
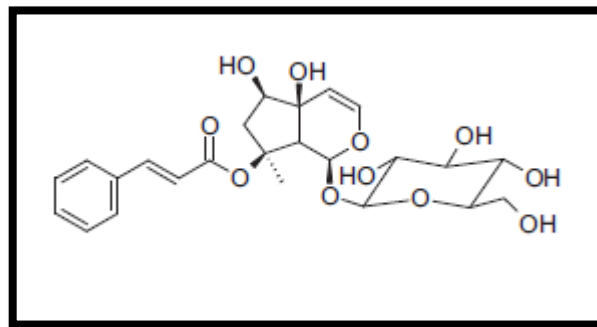


Fonte: (MNCWANGI et al., 2012).

O HP tem recebido atenção especial na literatura devido principalmente aos seus potentes efeitos anti-inflamatórios observados em estudos *in vitro*, *in vivo* e estudos clínicos (WEGENER; LUPKE, 2003; MAHOMED; OJEWOLE, 2004; KUNDU et al., 2005; HUANG et al., 2006; WARNOCK et al., 2007; FIEBICH et al., 2012; WACHSMUTH et al., 2011). O mecanismo pelo qual o HP exerce seu efeito anti-inflamatório ainda não está bem elucidado. Estudos *in vitro* já demonstraram que esta planta ou seu constituinte principal, o harpagosídeo, tem capacidade de suprimir a produção de citocinas inflamatórias (TNF-α, IL-1β e IL-6) (FIEBICH et al., 2001; INABA et al., 2010; HASEEB et al., 2016) e prostaglandinas (PG)$E_2$ (FIEBICH et al., 2001); suprimir a atividade da cicloxigenase 2 (COX 2) e da síntese de óxido nítrico induzível (iNOS) através da inibição da ativação do NF-κB (HUANG et al., 2006); bloquear a Proteína Ativadora 1 (AP-1), diminuindo a liberação de interleucinas e do TNF- α (FIEBICH et al., 2012); e suprimir a expressão de IL-6 (HASEEB et al., 2016). Devido a sua ação terapêutica, o HP foi então liberado como medicamento fitoterápico em 2011 pela Agência Nacional de Vigilância Sanitária (ANVISA) MS: 1.1860.0035, sendo então indicado para o tratamento da artrite, artrose, tendinites, e como tratamento auxiliar da gota.

Além da ação anti-inflamatória, outros efeitos vêm sendo atribuídos ao HP podendo o mesmo desempenhar um papel terapêutico numa série de condições, incluindo uma potente ação analgésica (LIM et al., 2014; PARENTI et al., 2015), propriedades antidiabéticas em ratos (MAHOMED; OJEWOLE, 2004), efeito uterotônico e espasmogênico em musculatura uterina de mamíferos (MAHOMED; OJEWOLE, 2009), inibição da colinesterase *in vitro* (GEORGIEV et al., 2012), efeito antiplasmodial *in vitro* de alguns componentes extraídos da planta (CLARKSON et al., 2003) e mais recentemente foi relatado o efeito anti-obesidade, via modulação do receptor de grelina do tipo 1A (GHS-R1A) (TORRES-FUENTERS et al., 2014).

De particular importância existem poucos estudos na literatura sobre a ação do *H. procumbens* no SNC. Sun e colaboradores (2012) demonstraram a ação benéfica do harpagosídeo (2, 6, 18 mg/kg/dia) no sistema dopaminérgico, onde este foi capaz de atenuar a neurodegeneração dopaminérgica e as desordens do movimento induzidas por 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) em camundongos através do aumento do fator neurotrófico derivado de células gliais (GDNF). Em outro estudo, Mahomed e Ojewole, (2006) relataram a atividade anticonvulsivante do *H. procumbens* (50-800 mg/kg via intraperitoneal) em camundongos, frente a diferentes indutores de convulsão, usando anticonvulsivantes de referência para as comparações de seus efeitos. Em estudo realizado por Bhattacharya e Bhattacharya (1998), o *H. procumbens* foi capaz de aumentar a atividade da catalase e da glutationa peroxidase, além de diminuir a peroxidação lipídica já existente no córtex e estriado de ratos. Além disso, em estudo publicado pelo nosso grupo, o extrato bruto e diferentes frações de *H. procumbens* (em especial a fração acetato de etila) foram capazes de proteger contra o dano oxidativo induzido por diferentes agentes pró-oxidantes ($Fe^{2+}$ e Nitroprussiato de Sódio) em córtex de ratos *in vitro*, além de proteger contra a perda da viabilidade celular (SCHAFFER et al., 2013).

Vale ressaltar que o efeito antioxidante do *H. procumbens*, pode estar relacionado com o efeito terapêutico da planta, uma vez que o estresse oxidativo pode estar relacionado à patogênese de doenças inflamatórias crônicas (LIANG et al., 2008). Por sua vez, agentes que são capazes de interferir com a geração e/ou ação dos radicais livres em tecidos biológicos são, portanto, de interesse terapêutico (GRANT et al., 2009).

**3 RESULTADOS**

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo cientifico e de manuscrito, os quais se encontram organizados nesta seção. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no próprio artigo e manuscrito. O artigo está disposto na forma que foi publicado na edição da revista cientifica *Neurochemical Research* e o manuscrito está na forma que foi submetido para a publicação na revista científica *Molecular and Cellular Neuroscience.*

3.1 ARTIGO PUBLICADO – FRAÇÃO ACETATO DE ETILA DE *HARPAGOPHYTUM PROCUMBENS* REDUZ MOVIMENTOS DE MASCAR NO VAZIO INDUZIDOS PELA FLUFENAZINA E ESTRESSE OXIDATIVO EM CÉREBRO DE RATOS

**Artigo**

*Harpagophytum procumbens* ETHYL ACETATE FRACTION REDUCES FLUPHENAZINE-INDUCED VACUOUS CHEWING MOVEMENTS AND OXIDATIVE STRESS IN RAT BRAIN

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CrossMark

**ORIGINAL PAPER**

# *Harpagophytum Procumbens* Ethyl Acetate Fraction Reduces Fluphenazine-Induced Vacuous Chewing Movements and Oxidative Stress in Rat Brain

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Roselei Fachinetto[1,2] · Caroline Wagner[1,5]

**Abstract** Long-term treatment with fluphenazine is associated with manifestation of extrapyramidal side effects, such as tardive dyskinesia. The molecular mechanisms related to the pathophysiology of TD remain unclear, and several hypotheses, including a role for oxidative stress, have been proposed. *Harpagophytum procumbens* is an herbal medicine used mainly due to anti-inflammatory effects, but it also exhibits antioxidant effects. We investigated the effect of ethyl acetate fraction of *H. procumbens* (EAF HP) in fluphenazine-induced orofacial dyskinesia by evaluating behavioral parameters at different times (vacuous chewing movements (VCM's) and locomotor and exploratory activity), biochemical serological analyses, and biochemical markers of oxidative stress of the liver, kidney, cortex, and striatum. Chronic administration of fluphenazine (25 mg/kg, intramuscular (i.m) significantly increased the VCMs at all analyzed times (2, 7, 14, and 21 days), and this was inhibited by EAF HP (especially at a dose of 30 mg/kg). Fluphenazine decreased locomotion and exploratory activity, and EAF HP did not improve this decrease. Fluphenazine induced oxidative damage, as identified by changes in catalase activity and ROS levels in the cortex and striatum, which was reduced by EAF HP, especially in the striatum. In the cortex, EAF HP was protective against fluphenazine-induced changes in catalase activity but not against the increase in ROS level. Furthermore, EAF HP was shown to be safe, since affected serum biochemical parameters or parameters of oxidative stress in the liver and kidney. These findings suggest that the *H. procumbens* is a promising therapeutic agent for the treatment of involuntary oral movements.

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## Introduction

Fluphenazine is a typical antipsychotic utilized for the treatment of schizophrenia. Its main mechanism of action is blockage of dopamine receptors, especially the $D_2$ subtype, and it is effective for the treatment of positive symptoms (such as hallucinations and delusions) of schizophrenic patients [1]. However, it is well established that chronic use of typical antipsychotics can lead to the onset of extrapyramidal symptoms, such as tardive dyskinesia (TD) in humans and orofacial dyskinesia (OD) in rodents [2, 3].

Dyskinesia is characterized by a variety of hyperkinetic movements, which are irregular, repetitive, and involuntary. It can occur in various regions of the body but mainly affects the facial region [4]. There are some hypotheses in the literature for the onset of TD, but its pathophysiology is not fully elucidated. A few effective treatments are available, and there is no known cure [3]. The dopamine receptor super-sensitivity hypothesis is widely accepted to explain the appearance of dyskinesia [5]. Importantly, it has been proposed that antipsychotic drugs cause a compensatory increase in dopamine metabolism owing to the intense blockade of dopamine receptors by antipsychotics, causing an increase in the formation of free radicals, leading to neuroinflammation and even cell death [3, 6–11]. Thus, oxidative stress may play an important role in the development of TD. Importantly, studies of our group and others have demonstrated a protective role of antioxidants in antipsychotic-induced OD in rodents [3, 12–17].

*Harpagophytum procumbens,* popularly known as devil's claw, is native to southern Africa and is a perennial plant belonging to the family Pedaliceae. The main bioactive components, iridoid glycosides, are found in the roots of these plants. Among them, we highlight harpagoside, which has been listed as the main bioactive component responsible for the therapeutic action of the plant [18–21]. *H. procumbens* has received special attention in the literature owing to its potent anti-inflammatory effects observed in vitro, in vivo and clinically [22–28]. However, the mechanism by which *H. procumbens* exerts its anti-inflammatory effect has not been elucidated and other effects of this plant have been reported in the literature [23, 29–35].

Of particular importance are a few studies on the action of *H. procumbens* in the central nervous system (CNS). Sun et al. [36] demonstrated the beneficial action of harpagoside on the dopaminergic system, where it was able to attenuate dopaminergic neurodegeneration and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced movement disorders in mice by increasing glia derived neurotrophic factor (GDNF). Bhattacharya and Bhattacharya [37] demonstrated an increase in the activity of antioxidants enzymes in brain of rats treated only with *H. procumbens*. Moreover, in a recent study published by our group, the crude extract and various fractions of *H. procumbens* (especially the ethyl acetate fraction) protected against oxidative damage of rat brain in vitro, and protect against loss of cell viability [34]. Thus, the antioxidant effect of the plant may be related to its therapeutic effect, since oxidative stress may be associated with the pathogenesis of chronic inflammatory diseases [38].

Thus, the objective of this study was to analyze the effect of *H. procumbens* on behavioral and oxidative stress parameters in an OD model induced by fluphenazine in rats.

## Materials and methods

### Drugs and preparation of fractions

All reagents were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Fluphenazine enantate (Flufenan®) was obtained from a commercial pharmacy. *H. procumbens* powder was obtained commercially from Quimer Comercial LTD (São Paulo, Brazil). To obtain the *H. procumbens* fractions, the powdered roots of *H. procumbens* were added to 70 % ethanol and allowed to stand at room temperature for a week with daily shaking. After filtration, the extract was evaporated under reduced pressure to remove the ethanol. The extract was then re-suspended in water and partitioned successively with chloroform, ethyl acetate, and n-butanol (3 × 200 ml for each solvent), as previously described by Schaffer et al. [34].

### Quantification of harpagoside by high performance liquid chromatography-diode array detection (HPLC–DAD)

Reverse phase chromatographic analyses were carried out under gradient conditions using $C_{18}$ column (4.6 mm × 250 mm) packed with 5 µm diameter particles. Isocratic elution was used with a binary mobile phase composed of distillate water: methanol (50:50, v/v), following the method described by Babili et al. [39] with slight modifications. Crude extract and fractions were tested at concentrations of 10 mg/mL. The injection volume was 50 µl and the wavelength was 278 nm. All samples and the mobile phase were filtered through 0.45 µm membrane filter (Millipore, Billerica, MA, USA) and then degassed by

ultrasonic bath prior to use. A stock solution of harpagoside standard reference was prepared in the HPLC mobile phase at a concentration range of 0.010–0.250 mg/mL. The calibration curve for harpagoside was $Y = 23719x + 1248.3$ ($r = 0.9995$). All chromatography operations were carried out at ambient temperature and in triplicate.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Boligon et al. [40]. LOD and LOQ were calculated as 3.3 and 10 $\sigma$/S, respectively, where $\sigma$ is the standard deviation of the response and S is the slope of the calibration curve.

### Radical-scavenging activity-2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant activity of the extracts was evaluated by monitoring their ability to quench the stable free radical DPPH as described by Choi et al. [41] with minor modifications. Free radical scavenging capacity (FRSC) of plant extracts was calculated as their half-maximal inhibition concentration (IC 50) values. Six different concentrations of each extract dissolved in distilled water (10, 50, 100, 150, 200, and 250 µg/ml) were mixed with 1.0 ml (0.3 mM) DPPH. Distilled water (1.0 ml) plus plant extract solution was used as a blank. The absorbance was measured at 518 nm after 30 min of reaction at room temperature. DPPH was prepared daily and protected from light. Relative activities were calculated from the calibration curve of L-ascorbic acid standard solutions obtained under the same experimental conditions. Scavenging or inhibitory capacity in percent (IC%) was calculated using the equation: $IC\% = 100 - [(Abs_{sample} - Abs_{blank}) \times 100/ Abs_{control}]$ where $Abs_{sample}$ is the absorbance obtained in the presence of different extract concentrations and $Abs_{control}$ is that obtained in the absence of extracts. Tests were carried out in triplicate.

### Animals

Adult male Wistar rats (220–270 g) were housed under standard laboratory conditions and maintained on a 12:12 h light–dark cycle with free access to food and water. Animals were acclimatized to laboratory conditions before the test. All experiments were performed in accordance with the guidelines of the National Council of Control of Animal Experimentation (CONCEA). This protocol was approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria under process number 109/2014.

### Experimental design

Male Wistar rats were divided into eight groups (Fig. 1). For induction of OD, fluphenazine (25 mg/kg) was administered intramuscularly (i.m.) in a single dose (groups 5, 6, 7, and 8), and for controls (groups 1, 2, 3, and 4), the vehicle of fluphenazine (vegetable oil, 1 ml/kg) was administered in the same manner [13, 42]. The ethyl acetate fraction of *H. procumbens* (EAF HP) was administered to the plant control groups (2, 3, and 4) as well as the treatment groups (6, 7 and 8) as a daily intraperitoneal (i.p.) injection at 10, 30, or 100 mg/kg, along with administration of fluphenazine. In the control group (1), vehicles for fluphenazine and EAF HP were administered. In the control fluphenazine group (5), fluphenazine and the vehicle of EAF HP were administered. Fraction was administered i.p. because previous studies had reported that much of the constituents of *H. procumbens* are degraded by stomach acid [43, 44].



| | Treatment | n |
|---|---|---|
| 1 | Control | 5 |
| 2 | EAF *H.procumbens* (10mg/kg, i.p. 21 days) | 5 |
| 3 | EAF *H. procumbens* (30mg/kg, i.p. 21 days) | 5 |
| 4 | EAF *H. procumbens* (100mg/kg, i.p. 21 days) | 5 |
| 5 | Fluphenazine (25mg/kg, i.m. 1 days) treated | 10 |
| 6 | Fluphenazine ((25mg/kg, i.m. 1 days) + EAF *H.procumbens* (10mg/kg, i.p. 21 days) | 10 |
| 7 | Fluphenazine ((25mg/kg, i.m. 1 days) + EAF *H.procumbens* (30mg/kg, i.p. 21 days) | 10 |
| 8 | Fluphenazine ((25mg/kg, i.m. 1 days) + EAF *H.procumbens* (100mg/kg, i.p. 21 days) | 10 |

**Fig. 1** Experimental design. Experimental groups: rats were divided into eight groups: 1, 2, 3, and 4 were the controls groups, and 5, 6, 7, and 8 were treatment groups. Fluphenazine and its vehicle were administered on day 1, and different doses of EAF HP and its vehicle were administered for 21 consecutive days. The behavioral tests were performed 2, 7, 14, and 21 days after the onset of plant and fluphenazine treatment (@ Quantify the VCMs; # Open Field test). After 21 days of treatment the animals were sacrificed, and the serum, liver, kidney, and cerebral structures (cortex and striatum) were dissected to carry out the ex vivo analysis

Behavioral assessment was performed on day 2 (24 h after administration of drugs), 7, 14, and 21 of the experiment. After the last behavioral analysis, animals were fasted for 5 h and then sacrificed. Blood was collected for biochemical analysis, the brain was dissected (cortex and striatum), and the liver and kidney were removed for ex vivo analysis (Fig. 1).

## Behavioral analysis

### Quantification of vacuous chewing movements (VCMs)

Behavior measurement of VCMs was assessed before the treatment with fluphenazine, plant, or its vehicle (basal evaluation), as previously described [13, 45]. The effects of treatments on behavior were assessed on day 2 (24 h after the first administration of the drugs), 7, 14, and 21 as written in Fig. 1. To quantify the VCMs, the rats were placed individually in glass cages (20 × 20 × 19 cm) containing one movable mirror under the floor to permit the observation of VCMs when the animals were away from the observer. The VCMs were recorded for 6 min after a 6 min acclimation period, according to the previously published method [13, 45–49]. VCM was defined as a single mouth opening on the vertical plane not directed toward physical material. If VCMs occurred during a period of grooming, they were not taken into account. After each test, the cages were cleaned with 30 % alcohol solution to eliminate possible odors and prevent the next animal from smelling the previous one. Experimenters were always blind to treatments.

### Open field test

To evaluate possible changes in spontaneous locomotor and exploratory activity caused by treatment with fluphenazine and/or EAF HP, rats were placed in the center of an open field arena, divided into hundred parts [50]. The number of lines crossed (locomotor activity) and the frequency of rearing (exploratory activity) were measured for 5 min. In order to ensure that continue exposure to the apparatus did not influence locomotor and exploratory activity of the animals, this behavioral analysis was performed only on the last day of treatment (21 days).

## Biochemical serological analyses

Serum levels of glucose, total cholesterol, triglycerides, creatinine, urea, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase were measured. The analyses were performed using a commercial kit (Bioclin®, San Ramon, CA, USA) and the protocol of each technique was performed as described in the instructions submitted by the manufacturer.

## Biochemical analyses of tissue

### Tissue preparations

The cerebral structures (cortex and striatum) and the liver were dissected and homogenized in 10 volumes (w/v) of 10 mM Tris–HCl, pH 7.4 and then centrifuged (3000 rpm for 10 min). The same procedure was performed for the kidney except it was homogenized in 5 volumes (w/v) of buffer. The resulting supernatants were used in the biochemical assays described below.

### Lipid peroxidation assay

To measure the effect of EAF HP on lipid peroxidation, thiobarbituric acid reactive species (TBARS) were determined as described by Ohkawa et al. [51]. In brief, samples were incubated at 100 °C for 1 h in a medium containing 8.1 % sodium dodecyl sulfate, 1.4 M acetic acid, pH 3.4, and 0.6 % thiobarbituric acid. The pink chromogen produced in the reaction was measured spectrophotometrically at 532 nm. Results are expressed as nmol of malondialdehyde (MDA)/mg of protein.

### Thiols levels

Total thiols and non-protein-thiols content in samples was determined as described by Ellman [52]. For the non-protein thiol groups (NPT), the samples of S1 were precipitated with 200 μl of 10 % trichloroacetic acid followed by centrifugation. The colorimetric assay was carried out in phosphate buffer 1 M, pH 7.4. The reaction was measured spectrophotometrically at 412 nm. Results are expressed as μmol/mg of protein.

### 2′, 7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation

DCFH-DA assay was used to estimate reactive oxygen species (ROS) levels in samples. Supernatants were incubated with DCFH-DA as described by Pérez-Severiano et al. [53]. Fluorescence of the samples was determined at 488 nm for excitation and 520 nm for emission. The result was defined as the difference between the fluorescence at 30 and 15 min of the reaction and expressed as DCFH-DA oxidation/mg of protein.
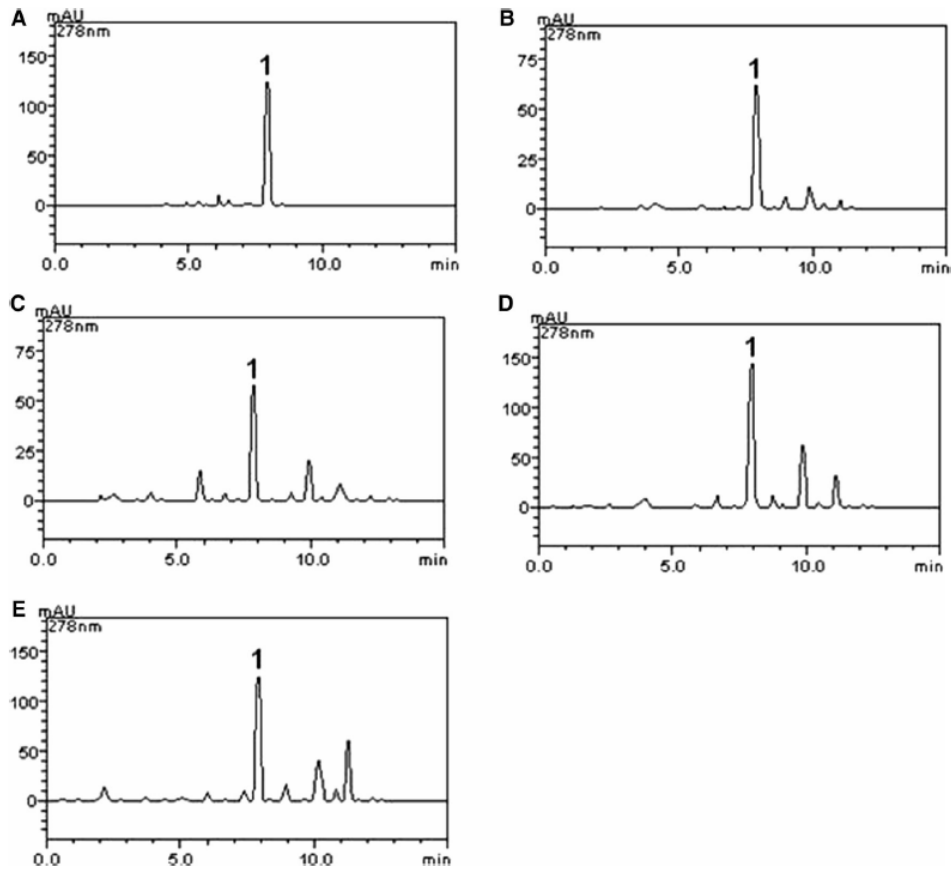
**Fig. 2** Chromatograms at 278 nm of the iridoid glycoside harpagoside (peak 1) in *Harpagophytum procumbens*. **a** Harpagoside standard, **b** crude extract, **c** chloroform fraction, **d** ethyl acetate fraction, and **e** butanolic fraction

**Table 1** Determination of harpagoside in *Harpagophytum procumbens* crude extract and fractions

| H. procumbens | Harpagoside | |
|---|---|---|
| | mg/g of dry fraction | % |
| Crude extract | 16.07 ± 0.02 a | 1.60 |
| Chloroform fraction | 15.89 ± 0.03 a | 1.58 |
| Ethyl acetate fraction | 35.73 ± 0.04 b | 3.57 |
| Butanolic fraction | 30.16 ± 0.09 c | 3.01 |
| LOD (µg/mL) | 0.013 | |
| LOQ (µg/mL) | 0.042 | |

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters, differ statistical by Tukey test at $p < 0.01$

*LOD* limit of detection, *LOQ* limit of quantification

### Catalase activity

Catalase activity was quantified by measuring the disappearance of $H_2O_2$ at 240 nm [54] and expressed in mmol $H_2O_2$/mg protein/min.

### Protein quantification

The total protein content in supernatants homogenates was determined by the method of Lowry [55] using bovine serum albumin as standard.

### Statistical analysis

Data are presented as mean ± SEM and were statistically analyzed by one-way analysis of variance (ANOVA),

**Table 2** Biochemical serological analysis of rats treated with fluphenazine (Flu, 25 mg/kg/i.m) or its vehicle followed by treatment with different doses of EAF HP (10, 30, or 100 mg/kg/day, i.p) or saline for 21 consecutive days

| Measurements | Control | EAF HP 10 mg/kg | EAF HP 30 mg/Kg | EAF HP 100 mg/Kg | Flu | Flu + EAF HP 10 mg/Kg | Flu + EAF HP 30 mg/Kg | Flu +EAF HP 100 mg/Kg |
|---|---|---|---|---|---|---|---|---|
| Glucose | 126.6 ± 8.08 | 114.7 ± 2.34 | 126.0 ± 4.79 | 125.5 ± 6.61 | 118.3 ± 3.70 | 122.9 ± 5.22 | 127.6 ± 8.16 | 119.9 ± 4.94 |
| Cholesterol | 97.46 ± 4.57 | 91.26 ± 4.39 | 92.93 ± 9.79 | 87.11 ± 4.13 | 96.64 ± 4.36 | 93.78 ± 4.32 | 90.77 ± 5.84 | 95.17 ± 6.17 |
| Triglycerides | 194.4 ± 44.7 | 177.3 ± 17.87 | 118.3 ± 18.90 | 148.2 ± 14.21 | 191.6 ± 23.20 | 181.7 ± 27.30 | 148.7 ± 21.30 | 168.0 ± 13.46 |
| Urea | 43.87 ± 0.95 | 40.97 ± 1.29 | 42.69 ± 1.65 | 46.39 ± 2.17 | 38.65 ± 0.94 | 39.63 ± 1.59 | 38.55 ± 1.11 | 39.29 ± 2.11 |
| Creatinine | 0.449 ± 0.03 | 0.467 ± 0.02 | 0.514 ± 0.02 | 0.467 ± 0.02 | 0.471 ± 0.01 | 0.489 ± 0.02 | 0.476 ± 0.01 | 0.427 ± 0.02 |
| AST | 38.36 ± 3.46 | 34.66 ± 4.45 | 35.22 ± 3.88 | 35.57 ± 4.11 | 50.46 ± 3.74 | 41.02 ± 4.29 | 41.79 ± 3.44 | 41.59 ± 3.22 |
| ALT | 58.31 ± 4.62 | 52.90 ± 7.06 | 52.81 ± 8.72 | 60.52 ± 4.02 | 64.28 ± 6.99 | 61.49 ± 7.41 | 58.59 ± 8.04 | 61.28 ± 5.44 |
| LDH | 1.146 ± 0.08 | 1.176 ± 0.15 | 1.232 ± 0.07 | 0.970 ± 0.08 | 1.330 ± 0.05 | 1.149 ± 0.10 | 1.264 ± 0.08 | 1.116 ± 0.10 |

Data (mean ± SEM) were analyzed using one-way ANOVA followed by post hoc Tukey

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactate dehydrogenase
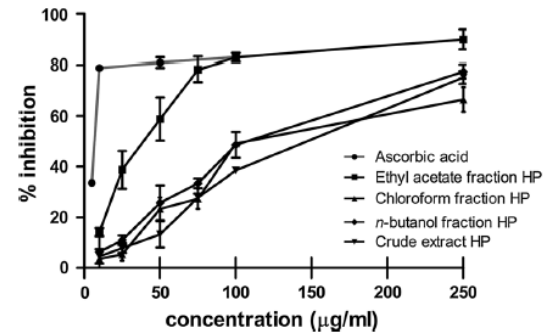


**Fig. 3** Effects of crude extract and fractions of *H. procumbens* in the DPPH test. The results are expressed as the percentage of inhibition, and ascorbic acid was used as a positive control. Data show mean ± SEM values average from three independent experiments performed

followed by a post hoc test when appropriate. The results were considered statistically significant when $p < 0.05$.

## Results

### HPLC analysis

HPLC fingerprinting of *H. procumbens* crude extract and fractions revealed the presence of harpagoside (retention time- tR = 7.93 min; peak 1) (Fig. 2; Table 1). However, the ethyl acetate fraction had the highest concentration of this constituent (Table 2) and therefore, we chose this fraction for the other experiments in vivo.

### DPPH radical-scavenging activity of *H. procumbens* crude extract and fractions

Crude extract, the *n*-butanol fraction, and the chloroform fraction inhibited DPPH with similar potency, IC 50 values were 135.5 ± 3.26, 114.4 ± 17.35, and 158.8 ± 8.55, respectively. The ethyl acetate fraction exhibited greater potency (IC 50: 38.21 ± 7.95; $p < 0.001$) than the other fractions and extracts (Fig. 3).

### Effects of EAF HP on fluphenazine-induced vacuous chewing movements (VCMs) in rats

Treatment with fluphenazine significantly increase the number of VCMs at all times analyzed compared to control (24 h: $p < 0.01$, Fig. 4a; 7 days: $p < 0.001$, Fig. 4b; 14 days: $p < 0.001$, Fig. 4c and 21 days: $p < 0.001$, Fig. 4d). In addition, co-treatment with EAF HP at doses of 30 mg/kg (day 7: $p < 0.001$, Fig. 4b; day 14: $p < 0.001$, Fig. 4c and day 21: $p < 0.01$, Fig. 4d) and 100 mg/kg
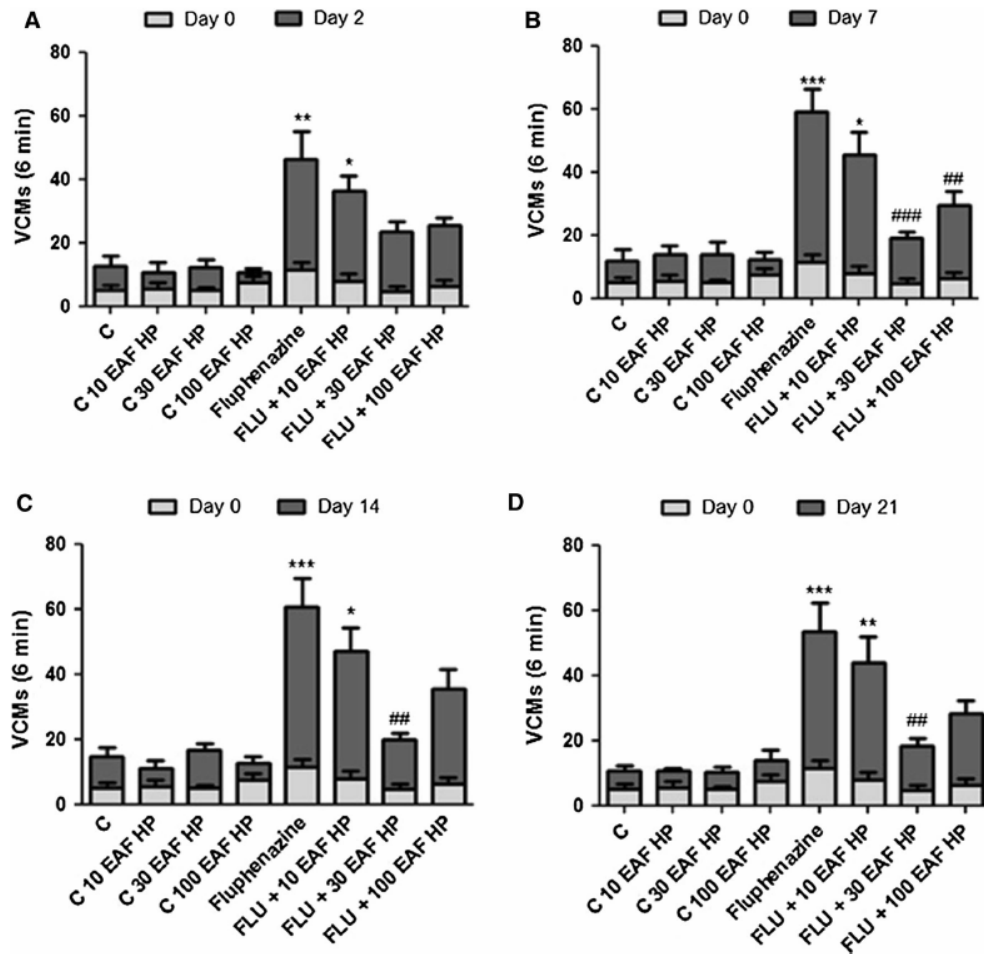
**Fig. 4** Effects of EAF HP on vacuous chewing movements (VCMs) induced by fluphenazine in rats. Quantification of VCMs was performed before drug administration (baseline, day 0). After administration of fluphenazine and EAF HP, quantification of VCMs was performed on days 1 (**a**), 7 (**b**), 14 (**c**) and 21 (**d**). Values of number of VCMs are presented as mean $\pm$ SEM. One-way ANOV followed by Tukey test. $*p < 0.05$, $**p < 0.01$, $***p < 0.0$ represents significant differences compared to control; $^{#}p < 0.0$ $^{##}p < 0.01$, $^{###}p < 0.001$ represents significant differences compar to fluphenazine

(only on day 7: $p < 0.01$, Fig. 4b) significantly reduced fluphenazine-induced VCMs compared to the fluphenazine group (Fig. 4). Furthermore, administration of EAF HP alone at the tested doses did not affect the number of VCMs (Fig. 4).

### Effects of EAF HP and/or fluphenazine on locomotor and exploratory activity in rats

The open field test revealed a significant decrease in the number of crossings (Fig. 5a) and rearings (Fig. 5b) in animals treated with fluphenazine compared to those in the control group ($p < 0.001$). The decrease in locomotor and

exploratory activity by fluphenazine was not affected l co-treatment with different doses of EAF HP. In additio EAF HP treatment alone did not affect locomotor activi or exploratory behavior of the animals (Fig. 5).

### Effects of EAF HP and/or fluphenazine on biochemical serological analysis and oxidative stress parameters in rats

Fluphenazine and/or EAF HP at all doses (10, 30, ai 100 mg/kg/day) did not affect biochemical paramete analyzed in the serum of animals (glucose, total cholester triglycerides, creatinine, urea, aspartate aminotransferas

**Fig. 5** Effects of EAF HP and/or fluphenazine on the open field test in rats. **a** Number of crossings in 5 min. **b** Number of rearings in 5 min. Values of number of crossings and rearings are presented as mean ± SEM. One-way ANOVA followed by Tukey test. *$p < 0.05$ Represents significant differences compared to control

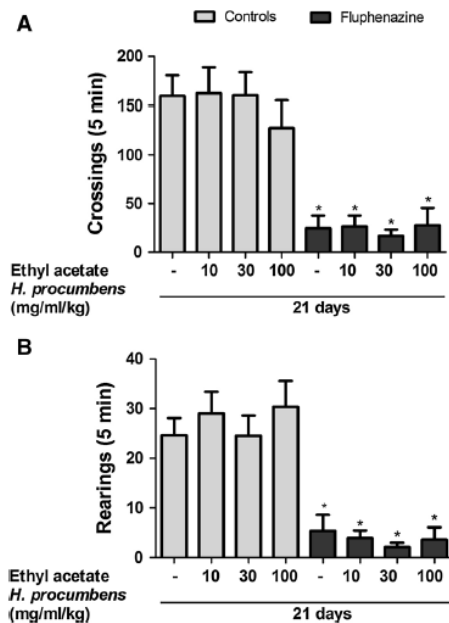alanine aminotransferase, and lactate dehydrogenase; Table 2). Similarly, the treatments did not significantly alter any parameters of oxidative stress (TBARS levels, DCFH-DA oxidation, catalase activity and protein or non-protein thiol levels) in the liver (Fig. 6) and kidney (Fig. 7).

In striatum (Fig. 8) and cortex (Fig. 9), treatment significantly altered DCFH-DA oxidation and catalase activity. Fluphenazine significantly increased DCFH-DA oxidation in the cortex (Fig. 9b) and striatum (Fig. 8b) compared to control. Co-treatment with 30 mg/kg EAF HP protected only the striatum against the increase in ROS production (Fig. 8b). In the cortex, co-treatment with EAF HP at any dose was not protective against increased DCFH oxidation induced by fluphenazine (Fig. 9b).

Catalase activity in the striatum decreased significantly in the fluphenazine group compared to that in the control group (Fig. 8e). Co-treatment with 100 mg/kg EAF HP significantly increased the activity of catalase, and the effect of EAF HP was dose-dependent (Fig. 8e). Cerebral cortex, catalase activity significantly increased in the fluphenazine group compared to that in the control group (Fig. 9e). Co-treatment with EAF HP at all three doses maintained the catalase activity to similar levels as the control in the striatum (Fig. 8e) and cortex (Fig. 9e), and it

was significantly different from that in the fluphenazine group.

Lipid peroxidation, as determined by the TBARS test, and the levels of protein and non-protein thiol were not affected by fluphenazine treatment and co-treatment with EAF HP in the striatum (Fig. 8) and cortex (Fig. 9). In addition, EAF HP alone did not affect oxidative stress parameters in any tissue analyzed).

## Discussion

Typical antipsychotics (e.g., fluphenazine and haloperidol) are the class of drugs most commonly used for the treatment of schizophrenia, but its chronic use can lead to the appearance of undesirable side effects, which are often debilitating and irreversible, as in TD [2, 5, 56]. The main problem is that the mechanism by which these drugs cause extrapyramidal effects is not well understood, and therefore, there are no safe drugs available to treat these effects [57]. A possible relationship between the appearance of these effects and increased oxidative stress markers in the brain has been reported, and it has been proposed that antioxidant substances could be beneficial to reduce involuntary movements [3, 5].

*Harpagophytum procumbens*, known as devil's claw, is a plant of African origin with numerous therapeutic effects, including mainly anti-inflammatory [18–20, 22, 26, 58] and antioxidant action [34, 37, 59]. Its main constituent, the harpagoside, is responsible for its pharmacological action [21, 60]. Thus, quantification of the bioactive component in the crude extract and fractions of the species is important. Here, HPLC analysis of the extracts showed that the ethyl acetate fraction presented the highest amount of harpagoside (Fig. 2; Table 1). Our results are in agreement with other authors who performed a quantification of harpagoside in *H. procumbens* extracts [61, 62]. Here, the EAF HP was also more potent than other fractions in the scavenger radical DPPH assay. The DPPH assay is widely used as a tool for in vitro evaluation of extracts and fractions, and its results may indicate the presence of phenolic compounds and flavonoids in these natural products [63, 64]. Notably, in a previous study by our group, we found that EAF HP reduced oxidative stress induced by different pro-oxidants and prevented the loss of cell viability in vitro [34].

In the present study, EAF HP (rich in phenolic compounds according to Schaffer et al. [34], and harpagoside, as shown by HPLC) was tested against involuntary oral movements induced by fluphenazine in rats. Our investigations revealed that fluphenazine significantly increased the number of VCMs, and EAF HP, particularly at doses of 30 mg/kg ($p < 0.001$ day 7; $p < 0.01$ day 14; $p < 0.01$ day 21) and 100 mg/kg ($p < 0.01$ day 7) was

**Fig. 6** Effects of EAF HP and/or fluphenazine on oxidative stress parameters in liver. **a** TBARS levels, **b** DCFH oxidation, **c** Proteic thiol levels, **d** Non-proteic thiol levels, **e** Catalase activity. Values of number of VCMs are presented as mean ± SEM. One-way ANOVA followed by Tukey test

protective against the occurrence of fluphenazine-induced involuntary movements (Fig. 4). Importantly, 30 mg/kg completely reduced the number of VCMs starting the seventh day of treatment, *i.e.*, none of the co-treated animals with this dose showed an increase in the number of VCMs relative to control. We believe that the effect presented by different doses (10, 30–100 mg/Kg) of EAF HP can be attributed to the hormesis phenomenon presented by some natural products [65, 66], since the best results in reducing VCMs were related to intermediate dose of EAF HP (30 mg/kg). The term hormesis have been used to describe the phenomenon where a specific chemical is able to induce biologically opposite effects at different doses [67]. It is worth mentioning that the effect of EAF HP in

decreasing VCMs is achieved only after continuous administration of the fraction, because we also tested the effect of a single dose (administered on 1st day of experiment) after 21 days of treatment with fluphenazine and we did not obtain significant protective effect of EAF HP (data not shown). These VCMs, quantified in rodents, are considered analogous to the involuntary orofacial movements of TD [68, 69]. Taken together with the biochemistry analyses (mainly by DCFH-DA oxidation and catalase activity), the plant effect seems to involve, at least in part, antioxidant activity in the brain.

The hypothesis that oxidative stress is involved in the onset of TD is supported by the following findings: (1) blocking dopamine $D_2$ autoreceptors and postsynaptic
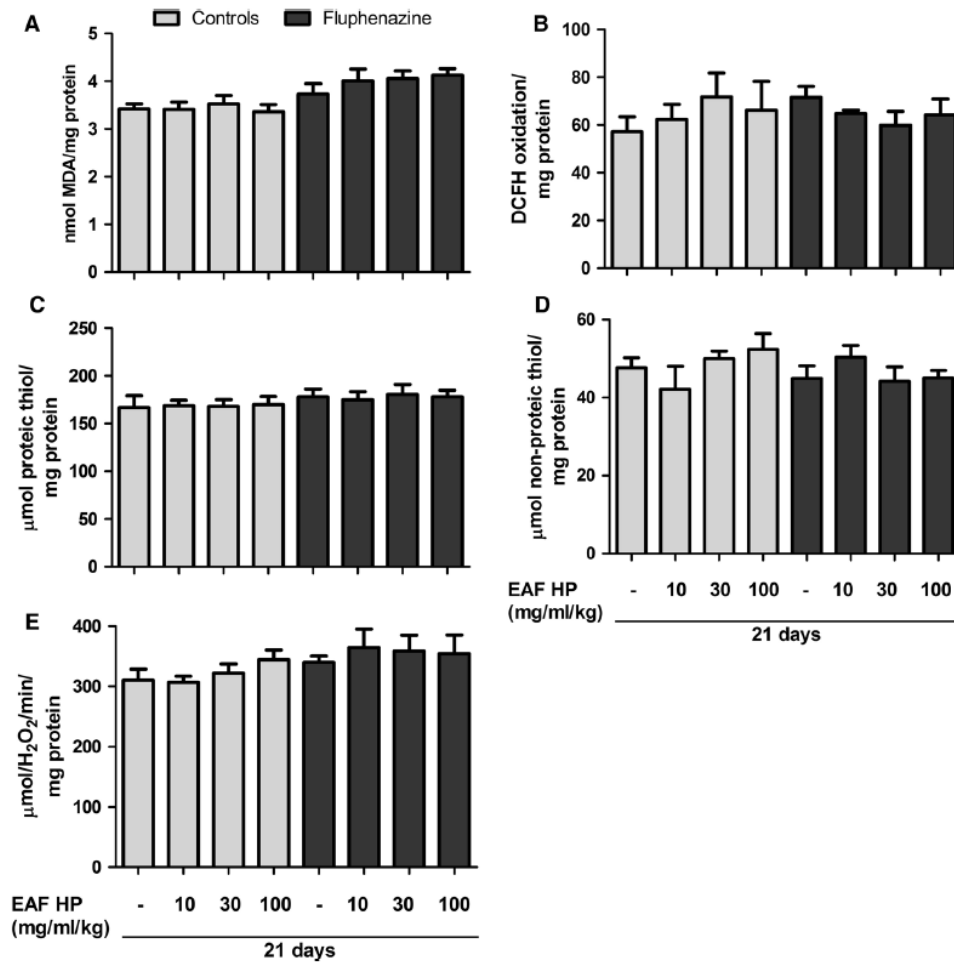
**Fig. 7** Effects of EAF HP and/or fluphenazine on oxidative stress parameters in Kidney. **a** TBARS levels, **b** DCFH oxidation, **c** Proteic thiol levels, **d** Non-proteic thiol levels, **e** Catalase activity. Values of number of VCMs are presented as mean ± SEM. One-way ANOVA followed by Tukey test

receptors in the striatum and other brain regions induces a compensatory increase in dopamine synthesis. This increase leads to subsequent metabolism of monoamine oxidase enzyme, resulting in an increase in the release of hydrogen peroxide ($H_2O_2$), which can cause damage to dopaminergic neurons [5, 70–72]; (2) the appearance of TD in patients using typical antipsychotics is associated with increased ROS levels and reduced antioxidant enzymes in CNS and plasma [73–77]; (3) antioxidant compounds are able to diminish the appearance of involuntary movements induced by antipsychotics [14, 16, 17, 78–81]; (4) other hypotheses for the appearance of TD include dopaminergic hypersensitivity, GABAergic failure with consequent glutamatergic excitotoxicity, nigrostriatal neuroinflammation,

genetic abnormalities, and other risk factors, such as age, which, interestingly, can also lead to oxidative stress [3, 5].

In our study, we found a plant protective effect in the restoration of catalase activity in the striatum of animals co-treated with EAF HP (Fig. 8e). This antioxidant effect may have contributed to the protection against the increase in the VCMs induced by fluphenazine (Fig. 4). Catalase can catalyze extremely quickly the degradation of hydrogen peroxide into water and oxygen. Its activity is easily modified by oxidative insults [82, 83]. Thus, an increase in dopamine metabolism by monoamine oxidase may be lead to increased release of hydrogen peroxide, and EAF HP mediated increases in catalase activity may decrease the formation of ROS in an attempt to maintain the redox state
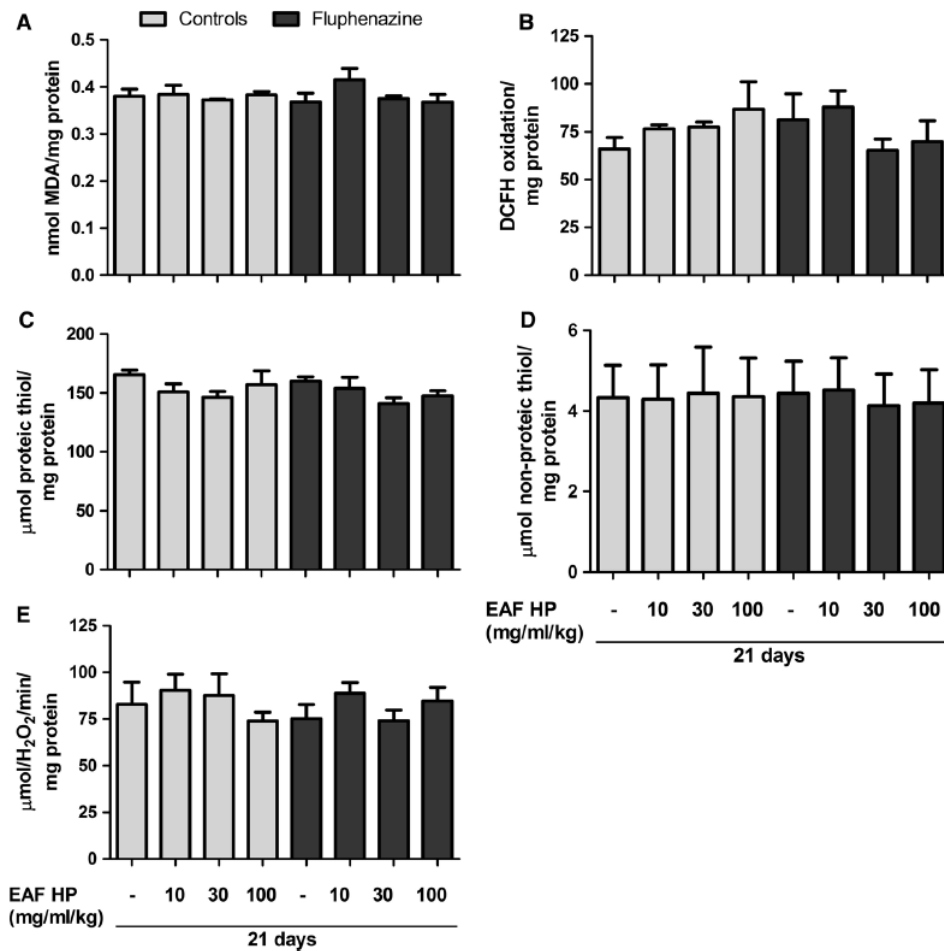
**Fig. 8** Effects of EAF HP and/or fluphenazine on oxidative stress parameters in striatum. **a** TBARS levels, **b** DCFH oxidation, **c** Proteic thiol levels, **d** Non-proteic thiol levels, **e** Catalase activity. Values of number of VCMs are presented as mean ± SEM. One-way ANOVA followed by Tukey test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ represents significant differences compared to control; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ represents significant differences compared to fluphenazine

in the striatum. In addition, the results of DCFH-DA, a probe that can quantify $H_2O_2$, corroborated these hypotheses. Fluphenazine treatment increased ROS production ($H_2O_2$); and EAF HP was protective in striatum. This antioxidant effect presented by HP EAF may have contributed to the protection against the increase in the VCMs induced by fluphenazine (Fig. 4). These results are in agreement with Bhattacharya and Bhattacharya [37], which demonstrated a dose-dependent increase in the activity of catalase in brain of rats treated only with *H. procumbens* extract for 14 days.

Similar to *H. procumbens*, other antioxidant compounds have been shown antipsychotic-induced decreases in catalase activity [10, 15, 81]. However, these studies also demonstrated antipsychotics-induced changes in other markers of oxidative damage, such as lipid peroxidation (TBARS test) and/or levels of thiols, which were not observed in our study [14, 15, 81]. In some studies, including from our group, oxidative stress markers were not altered in brain areas related to movement control (like striatum) [13, 45, 48, 84], suggesting there may be additional mechanisms involved in the development of VCMs.

We observed alterations in ROS status in cortex (Fig. 9b). In fact, fluphenazine increased catalase activity and DCFH-DA oxidation, and EAF HP was protective only against the modification in catalase activity (Fig. 9e). These results were not related to behavior results, since the cortex is not a brain area directly related to involuntary
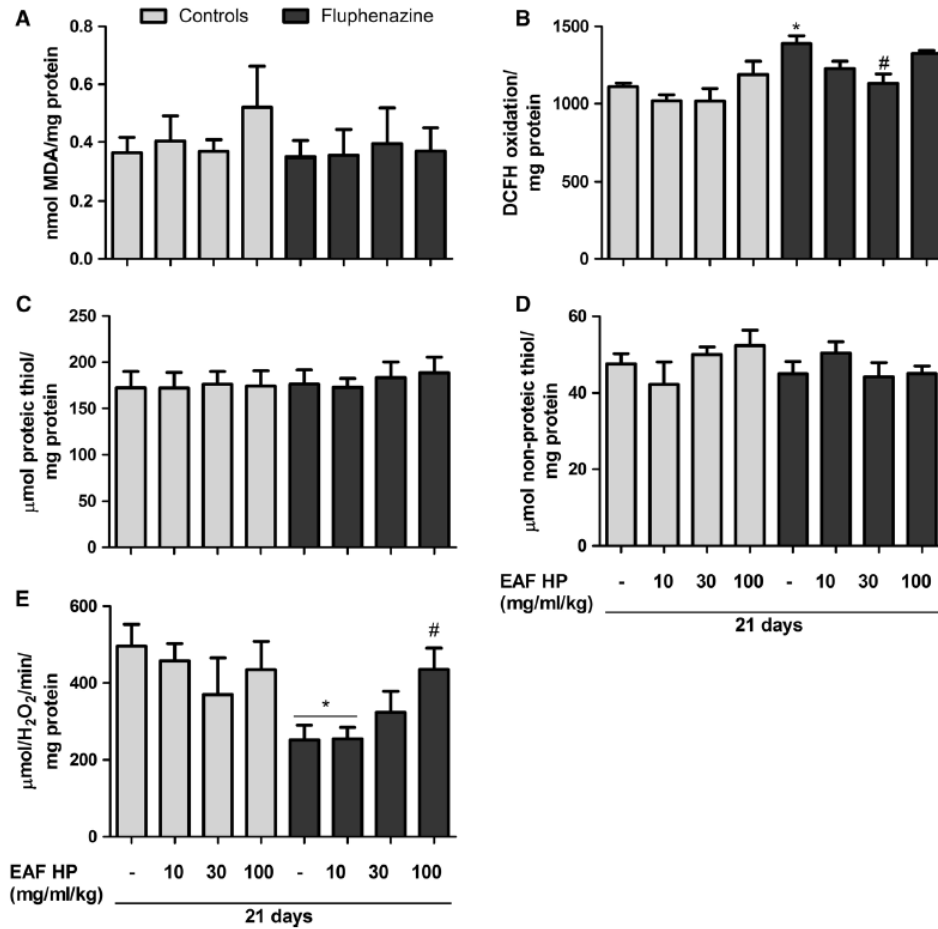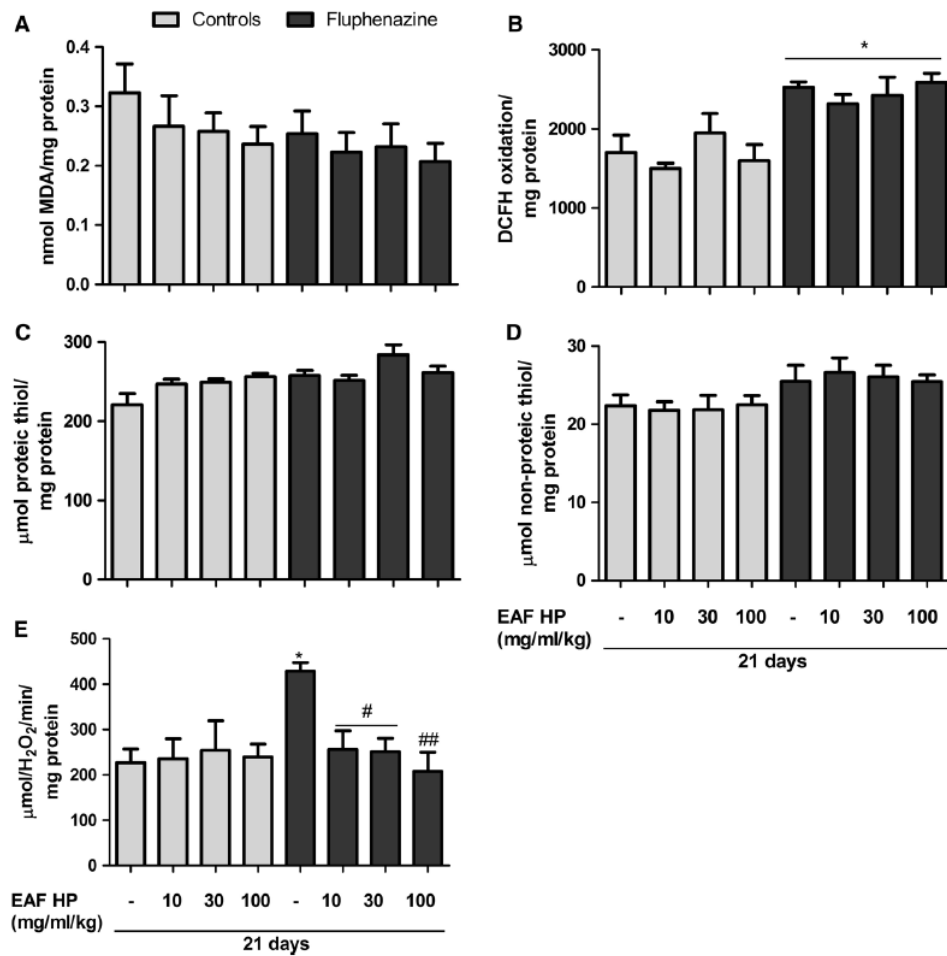
**Fig. 9** Effects of EAF HP and/or fluphenazine on oxidative stress parameters in cortex. **a** TBARS levels; **b** DCFH oxidation; **c** Proteic thiol levels; **d** Non-proteic thiol levels; **e** Catalase activity. Values of number of VCMs are presented as mean ± SEM. One-way ANOVA followed by Tukey test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ represents significant differences compared to control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ represents significant differences compared to fluphenazine

movement. Taken together, our results suggested that EAF HP could exert antioxidant effects in different areas of the brain.

Dopamine receptor antagonists, such as fluphenazine, commonly decrease locomotor activity by blocking the dopaminergic pathway in the nucleus accumbens [85, 86]. In our study, we evaluated locomotor and exploratory activity in the open field test. Fluphenazine treatment, as expected, significantly decreased locomotor and exploratory activity relative to that in the control, as demonstrated by the number of crossings and rearings, respectively. Co-treatment with EAF HP did not reverse the effect of fluphenazine on locomotor activity and exploratory (Fig. 5).

Similarly, in previous studies, the strong effect of antipsychotics on locomotor activity was hardly reversed by antioxidants treatment [16, 17].

We also evaluated serum biochemical serum parameters for toxicity and oxidative stress parameters in the kidney and the liver, since this new fraction had only previously been tested in vitro. None of the groups showed any significant changes in serum, liver, or kidney, with this treatment protocol. Notably, none of the animals exhibited any signs of toxicity by the plant, such as weight loss (data not shown).

In addition to its antioxidant effect, as described in this article and another study [34], EAF HP may have

additional effects on the CNS. It has been reported that prolonged treatment with typical antipsychotics may cause neuroinflammation [8, 9, 11], and according to Bishnoi et al. [9, 10], an increase in inflammatory markers in the brain was correlated with the behavioral development of OD. Since *H. procumbens* has been used for the treatment of diseases with inflammatory origin, it follows that it could also be used to protect against fluphenazine-induced increase in VCMs by decreasing neuroinflammation. It is known that ROS are generated in acute and chronic inflammatory conditions, thereby causing high cytotoxicity and, tissue damage. In addition, it was show that, harpagoside neuroprotective effects on the dopaminergic system in a rodent model of MPTP-induced parkinsonism [36]. Thus, HP may possibly act in different molecular pathways to decrease VCMs in rats.

In conclusion, EAF HP was protective against fluphenazine-induced VCMs and decreases in oxidative damage in the brain. Although there is no prevention or safe treatment for TD, our results suggest that the *H. procumbens* is a promising therapeutic agent for the treatment of involuntary oral movements. However, further studies should be conducted to examine the other protective mechanisms of fraction the CNS.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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3.2 MANUSCRITO EM SUBMISSÃO – *HARPAGOPHYTUM PROCUMBENS* REDUZ CITOCINAS PRÓ-INFLAMATÓRIAS E COX-2 EM CÉREBRO DE RATOS TRATADOS COM ANTIPSICÓTICO: O PAPEL NA DISCINESIA OROFACIAL

**Manuscrito**

*Harpagophytum procumbens* REDUCES PRO-INFLAMMATORY CYTOKINES AND COX 2 IN THE BRAIN OF RATS TREATED WITH ANTIPSYCHOTIC: ROLE IN OROFACIAL DYSKINESIA

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**ABSTRACT**

Prolonged use of typical antipsychotics increases the risk for the development of movement disorders such as tardive dyskinesia (TD). Studies suggest the involvement of neuroinflammation, as well as imbalances in several neurotransmitters as possible causes for the onset of TD. Harpagophytum procumbens (HP) is a phytotherapeutic used in the clinic mainly due to its anti-inflammatory effects. Thus, we evaluated the effect of HP on inflammatory, dopaminergic, and GABAergic markers in a fluphenazine-induced orofacial dyskinesia (OD) model in rats. For the treatment, we used the ethyl acetate fraction of HP (EAF HP) whose actions have already been described in the central nervous system (CNS). Thus, we measured the effect of EAF HP (10, 30 and 100 mg / kg) on fluphenazine-induced OD (25 mg / kg single dose) in rats by quantifying vacuos chewing movements (VCMs). In addition, we measured pro-inflammatory cytokines in the striatum and cortex and immunoreactivity of cyclooxygenase 2 (COX 2), tyrosine hydroxylase (TH), dopamine receptor subtype $D_2$ ($DRD_2$), dopamine transporter (DAT) and glutamate decarboxylase (GAD) in the striatum. Chronic administration of fluphenazine significantly increased VCMs 21 days post-treatment, and this increase was strongly inhibited by 30 mg/kg EAF HP. Fluphenazine also induced neuroinflammation, as identified by increased pro-inflammatory cytokines in the cortex and striatum and by the tendency to increase COX 2 immunoreactivity in the striatum. Our results demonstrated for the first time that HP can act as an anti-inflammatory agent, *in vivo*, in the CNS. However, fluphenazine and EAF HP did not alter immunoreactivity of dopaminergic or GABAergic markers. Our results suggest the involvement of fluphenazine-induced neuroinflammation in the development of OD in rats and points to EAF HP as a promising therapeutic agent for the treatment of involuntary oral movements because of its modulation of inflammation in the CNS.

Key words: tardive dyskinesia, neuroinflammation, fluphenazine , devil's claw

**INTRODUCTION**

Tardive dyskinesia (TD) is a severe extrapyramidal syndrome, incapacitating and potentially permanent, which may occur after months or years of treatment with antipsychotic drugs such as fluphenazine and haloperidol. TD occurs in 20-40% of schizophrenic patients who use typical antipsychotics (Glazer et al. 1993; Khouzam, 2015). It is characterized by the presence of involuntary, repetitive, and unintentional movements located mainly in the orofacial region (mouth, face, and tongue), which sometimes persists after withdrawal of the antipsychotic. In rodents, TD is called orofacial dyskinesia (OD) (Andreassen and Jorgensen, 2000; Frangou, 2008; Revuelta et al. 2012).

The etiology of TD and OD is complex, multifactorial, and not completely understood (Khouzam, 2015). Multiple hypotheses have been proposed, including dopaminergic hypersensitization due to the overexpression of $D_2$ receptors in the striatum, in response to dopamine block antipsychotic-mediated, reduction of gabaergic neurotransmission in the basal ganglia, excitotoxicity caused by increased glutamatergic transmission and genetic predisposition (Casey, 2000; Lencz et al. 2009; Rizos et al. 2009; Alabed et al. 2010; Bhidayasiri and Boonyawairoj, 2011). Recently, the role of oxidative stress (OE), especially neuroinflammation, in the pathophysiology of TD has gained traction in the literature. Antipsychotic-induced activation of free radicals (Lohr et al. 2003) and inflammatory signaling leading to cellular damage and possible neuronal structural abnormalities could be key factors in the pathogenesis of TD (Lerner et al. 2015; Schaffer et al. 2016). Studies have shown that increased pro-inflammatory factors, such as cytokines in regions responsible for motor control, such as striatum, may be correlated with behavioral development of OD. (Rapaport and Lohr,1994; Bishnoi et al. 2008a, 2008b; Peroza et al. 2016) Furthermore, the persistent increase of two major pro-inflammatory cytokines, tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) directly compromises the plasticity and survival of neuronal cells (Rothwell and Luheshi, 2000; Allan and Rothwell, 2001).

Some drugs and compounds have already been tested as treatments for this undesirable side effect, but so far, no effective treatment has been found.

(Woods et al. 2010; Alabed et al. 2011; Khouzam, 2015). In this context, *Harpagophytum procumbens* (HP), popularly known as devil's claw, is a perennial plant native to Southern Africa with numerous therapeutic effects, the most notable being its potent anti-inflammatory action, indicated mainly for the treatment of inflammatory diseases of the joints (Wegener and Lupke, 2003; Mahomed and Ojewole, 2004; Kundu et al. 2005; Huang et al. 2006; Warnock et al. 2007; Fiebich et al. 2012; Wachsmuth et al. 2011). In addition to its anti-inflammatory action, other benefits have been attributed to HP. Of particular importance studies demonstrate its the action or its bioactive components in the CNS. Sun and collaborators (2012) demonstrated the beneficial action of harpagoside (the main constituent of the plant) on the dopaminergic system in a model of neurodegeneration in rodents. Mahomed and Ojewole (2006) demonstrated the anticonvulsive effect of aqueous extract of HP root , suggesting its action in the GABAergic systems. In addition to the possible effects on the dopaminergic and GABAergic system, data in the literature also demonstrate the antioxidant action of HP in the CNS (Bhattacharya and Bhattacharya, 1998). In a study published by our group, the crude extract and different fractions of HP (especially the ethyl acetate fraction of HP – called EAF HP) were able to protect against oxidative damage and loss of cell viability in rat brain *in vitro*, and protect against loss of cell viability (Schaffer et al. 2013). Since this plant is linked to some hypothesis of the pathophysiology of TD, we decided to test HP in a model of OD induced by fluphenazine in rats. The EAF HP reduced involuntary movements induced by the antipsychotic, as well as showed antioxidant effects *in vivo* through the modulation of the catalase enzyme in the striatum (Schaffer et al. 2016). However, we believe that other mechanisms are involved in the protective effect exercised by HP in the central nervous system. Thus, the aim of this study was to evaluate the influence of HP on inflammatory, dopaminergic and GABAergic system in a fluphenazine-induced OD model in rats.

## 2 MATERIAL AND METHODS

### 2.1 Drugs and preparation of fractions

All reagents were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Fluphenazine enantate (Flufenan®) was obtained from a commercial pharmacy. *H. procumbens* powder was obtained commercially from Quimer Comercial LTD (São Paulo, Brazil). To obtain the HP fractions, the powdered roots of *H. procumbens* were added to 70% ethanol and allowed to stand at room temperature for a week with daily shaking. After filtration, the extract was evaporated under reduced pressure to remove the ethanol. The extract was then re-suspended in water and partitioned successively with ethyl acetate (3 x 200 ml of solvent) as previously described by Schaffer et al. (2013).

### 2.2 Animals

Adult male Wistar rats (220–270 g) were housed under standard laboratory conditions and maintained on a 12:12 h light–dark cycle with free access to food and water. Animals were acclimatized to laboratory conditions before the test. All experiments were performed in accordance with the guidelines of the Nacional Council of Control of Animal Experimentation (CONCEA). This protocol was approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria under process number 109/2014.

### 2.3 Experimental design

Male Wistar rats were divided into eight groups (Fig 1). For induction of OD, fluphenazine enanthate (25 mg/kg) was administered intramuscularly (i.m.) in a single dose (groups 5, 6, 7, and 8). For controls (groups 1, 2, 3, and 4), the vehicle of fluphenazine (vegetable oil, 1ml/kg) was administered in the same manner (Van Kampen and Stoessl, 2000; Fachinetto et al. 2007b). EAF HP was administered to the plant control groups (2, 3, and 4) as well as the treatment groups (6, 7 and 8) as a daily intraperitoneal (i.p.) injection at 10, 30, or 100 mg/kg, for 21 days. In the control fluphenazine group(5), fluphenazine and the vehicle of EAF HP (saline solution, 1ml/kg) were administered. In the control

group (1), vehicles for fluphenazine and EAF HP were administered. The EAF HP was administered i.p. because previous studies reported that many of the constituents of *H. procumbens* are degraded by stomach acid (Soulimani et al. 1994; Chrubasik et al. 2000). Behavioral assessment was performed on day 21 of the experiment. After the last behavioral analysis, animals were sacrificed. The brain was quickly removed and the cortex and striatum were dissected. The samples were stored at -80°C until processed.

| Group | Treatament (mg/kg) | n |
|---|---|---|
| 1 → | Control | 5 |
| 2 → | EAF *HP* 10mg/kg, (i.p. 21 days) per se | 5 |
| 3 → | EAF *HP* 30mg/kg (i.p. 21 days) per se | 5 |
| 4 → | EAF *HP* 100mg/kg (i.p. 21 days) per se | 5 |
| 5 → | Flu (25mg/kg, i.m. 1 days) treated | 10 |
| 6 → | Flu (25mg/kg, i.m. 1 days) + EAF *HP* 10mg/kg (i.p. 21 days) | 10 |
| 7 → | Flu (25mg/kg, i.m. 1 days) + EAF *HP* 30mg/kg (i.p. 21 days) | 10 |
| 8 → | Flu (25mg/kg, i.m. 1 days) + EAF *HP* 100mg/kg (i.p. 21 days) | 10 |

8 GROUPS

**Figure 1:** Experimental groups: Rats were divided into 8 groups: 1, 2, 3 and 4 are the controls groups and 5, 6, 7 and 8 treatment groups. Fluphenazine and its vehicle were administered on day 1, different doses of EAF HP and its vehicle were administered 21 consecutive days. HP: *H. procumbens*; Flu: Fluphenazine; i.p: intraperitoneal; i.m: intramuscular.

## 2.3.1 Behavioral testing

## 2.3.1.1 Quantification of vacuous chewing movements (VCMs)

Behavior measurement of VCMs was assessed before the treatment with fluphenazine or its vehicle (basal evaluation), as previously described (Fachinetto et al. 2007a, 2007b; Schaffer et al. 2016). The effect of treatments on behavior was examined 21 days after the fluphenazine injection. To evaluate the increase in the number of VCMs resulting from treatment, the delta (Δ) was calculated, which is the difference between the number of VCMs the end of the experiment and the number of VCMs at baseline (for each animal).To quantify the VCMs, the rats were placed individually in glass cages (20×20×19 cm) containing one movable mirror under the floor to permit the observation of VCMs when the animals were away from the observer. The VCMs were

recorded for 6 min after a 6 min acclimation period, according to the previously published method (FACHINETTO et al., 2007a, 2007b; PEROZA et al., 2013, 2016). VCM was defined as a single mouth opening on the vertical plane not directed toward physical material. If VCMs occurred during a period of grooming, they were not taken into account. After each test, the cages were cleaned with 30% alcohol solution to eliminate possible odors and prevent the next animal from smelling the previous one. Experimenters were always blind to treatments.

## 2.3.2 Biochemical Assays

### 2.3.2.1 Cytokines measurements

The cytokines quantification in striatum and cortex was assessed by ELISA using commercial kits (eBIOSCIENCE, San Diego, USA) for interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ), according to the manufacturer's instructions. Results were expressed as pg/mg of protein.

### 2.3.2.2 Western Blotting analyze

The striatum was homogenized in 700 μL of lysis buffer (4% SDS, 2 mM EDTA, 50 mM Tris, 0.5 mM $Na_2VO_4$, 2 μg/mL aprotinin, 0.1 mM benzamidine, 0.1 mM PMSF), boiled for 6 minutes and then centrifuged at 8.000 rpm at 4ºC for 10 minutes. The supernatant was used to determine protein concentration by Lowry method (1951). Then, it was added to the samples 10% glycerol and 8% 2-mercaptoethanol. The proteins (60 μg for the striatum) were resolved by 10% SDS-PAGE and transferred on to nitrocellulose membrane (Millipore, USA). The proteins on the membrane were stained with a ponceau solution (0.5% ponceau plus 5% glacial acetic acid in water), as a loading control (Romero-Calvo et al. 2010).  After staining, the membranes were dried, scanned, and quantified. Membranes were then processed using the SNAP ID system (Millipore, USA), blocked with 1% bovine serum albumin, incubated overnight either with an anti- DAT (Dopamine transporter, 1:1000; Millipore; AB2231) or anti-TH (Tyrosine hydroxylase, 1:1000; Millipore; AB152) or anti-D2

(Dopamine receptor D2, 1:5000; Millipore; AB5084P) or anti-GAD (Glutamate decarboxylase, 1:10.000, Milipore; AB5406) anti-COX 2 (Cyclooxygenase 2, 1:1000; Millipore; AB5118). After, the membranes were incubated with alkaline phosphatase-coupled secondary antibody (1:3000; Millipore) for 1 hour. The reaction was determined by a colorimetric assay using nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrate (De Freitas et al. 2016). The membranes were dried, scanned and quantified. Finally, all values were normalized using ponceau quantification.

### 2.3.2.3 Protein Quantification

The total protein content in homogenates was determined by the method of Lowry (1951), using bovine serum albumin as standard.

### 2.4 Statistical Analyses

Data in text and figures are expressed as means ± SEM and were statistically analyzed by one-way analysis of variance (ANOVA), followed by a post hoc test when appropriate. The results were considered statistically significant when $p < 0.05$.

### 3 RESULTS

### 3.1 Effects of EAF HP on fluphenazine-induced VCMs in rats.

Treatment with 25 mg/kg of fluphenazine significantly increased the number of VCMs, compared to the control group on day 21 ($p < 0.001$, Fig 2). In addition, co-treatment with EAF HP at doses of 30 mg/kg or 100 mg/kg significantly reduced the fluphenazine-induced increase in VCMs compared to the fluphenazine group (day 21 $p < 0.001$ and 0.01 respectively, Fig 2). Administration of EAF HP alone at all tested doses did not affect the number of VCMs (Fig 2).
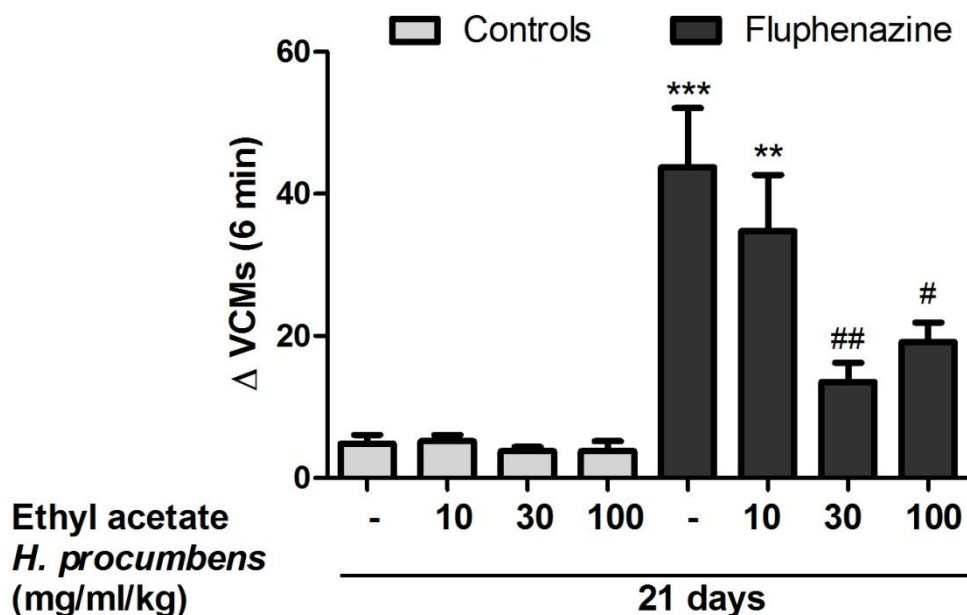
**Figure 2:** Effects of EAF HP on fluphenazine-induced VCMs in rats. Values of number of VCMs are presented as mean ± SEM. One-way ANOVA followed by Tukey test. **(p<0.01), ***(p<0.001) Represents significant differences compared to control; #(p<0.05), ##(p<0.01), Represents significant differences compared to fluphenazine.

## 3.2 Effects of EAF HP and/or fluphenazine on cytokines levels in striatum and cortex of rats.

In the striatum, the fluphenazine treatment significantly increased the levels of pro-inflammatory cytokines: IL-1β (p <0.001; Fig 3A), IL-6 (p <0.001; Fig 3B), TNF-α (p <0.001; Fig 3C) and IFN-γ (p <0.001; Fig 3D), compared to control animals. Co-treatment with higher doses of EAF HP reduced the levels of pro-inflammatory cytokines in the striatum: IL-1β (30 mg/kg; p <0.05; Fig 3A), IL-6 (30 mg/kg; 100mg/kg; p <0.05; Fig 3B), TNF- α (30 mg/kg; p<0.01; 100 mg/kg; p <0.05; Fig 3C), and INF-γ (30 mg/kg; p<0.001; 100 mg/kg; p <0.01; Fig 3D). Furthermore, the highest doses of EAF HP (30 mg/kg and 100 mg/kg) alone reduced IL-1β (p< 0.05; Fig 3A), IL-6 (p<0.05; Fig 3B), TNF-α (p <0.01; Fig 3C) and IFN-γ (p <0.001; Fig 3D).

**Figure 3:** Effects of EAF HP and/or fluphenazine on levels of pro-inflammatory cytokines: IL-1β (A), IL-6 (B), TNF-α (C) and IFN-γ (D) in striatum of rats. Data are expressed as means ± SEM. One-way ANOVA followed by Tukey test. *($p<0.05$), **($p<0.01$), ***($p<0.001$) Represents significant differences compared to control; #($p<0.05$), #($p<0.01$), ###($p<0.001$) Represents significant differences compared to fluphenazine.

Similarly to the striatum, fluphenazine treatment significantly increased the levels of pro-inflammatory cytokines in the cortex compared to control animals: IL-1β ($p <0.01$; Fig 4A), IL-6 ($p <0.01$; Fig 4B), and TNF-α ($p <0.001$; Fig 4C). Levels of INF-γ did not change after fluphenazine treatment (Fig 4D). Co-treatment with EAF HP did not significantly alter levels of cytokines in the cortex, compared with fluphenazine group (Fig 4). However, the highest doses of EAF HP alone reduced INF-γ (30 mg/kg and 100 mg/kg; $p< 0,001$; Fig 4D) and IL-1β (100 mg/kg; $p<0.05$; Fig 4A).
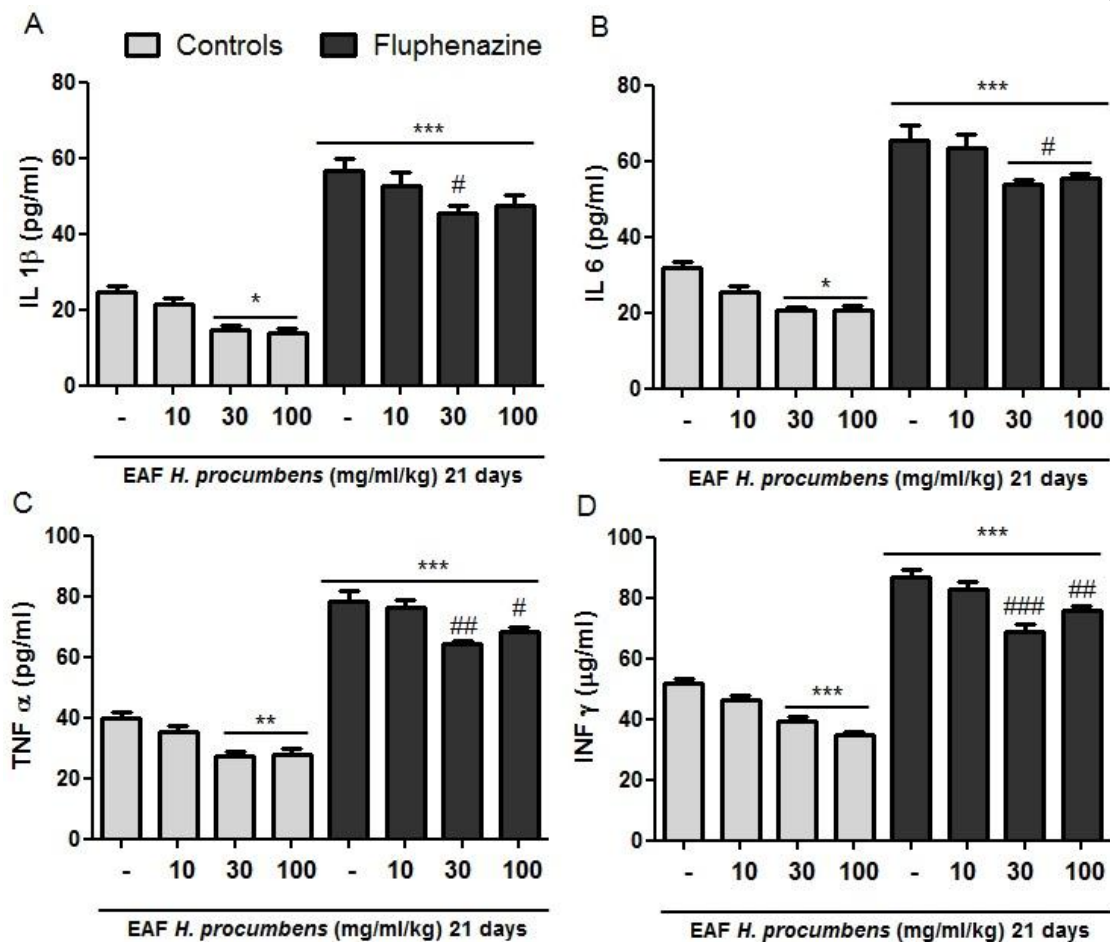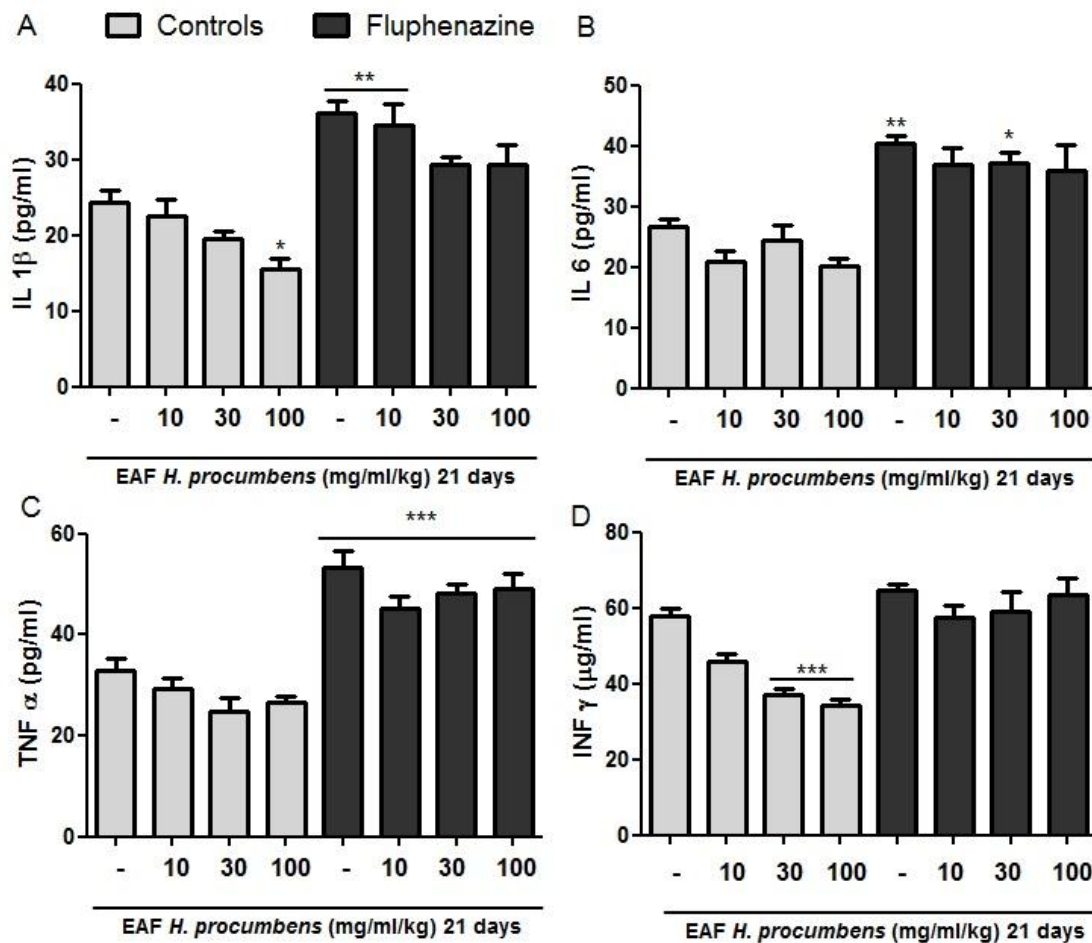
**Figure 4:** Effects of EAF HP and/or fluphenazine on levels of pro-inflammatory cytokines: IL-1β (A), IL-6 (B), TNF-α (C) and IFN-γ (D) in cortex of rats. Data are expressed as means ± SEM. One-way ANOVA followed by Tukey test. *($p<0.05$), **($p<0.01$), ***($p<0.001$) Represents significant differences compared to control; #($p<0.05$), ##($p<0.01$), ###($p<0.001$) Represents significant differences compared to fluphenazine.

## 3. Effects of EAF HP and/or fluphenazine on cyclooxygenase (COX-2) immunoreactivity in striatum of rats.

Due to previous studies that have shown that HP is able to modulate COX-2 activity *in vitro* (Huang et al. 2006), we decided to test the COX-2 levels in the striatum of these animals. Post hoc analysis revealed that administration of fluphenazine led to no significant increase in the immunoreactivity of COX 2 in striatum compared to the control group (Fig 5). However, there was a trend for the COX-2 immunoreactivity to increase in striatum in the fluphenazine group. In co-treated EAF HP groups, statistical analysis revealed a significant decrease in the immunoreactivity of COX 2 with a dose of 100 mg/kg when compared to the fluphenazine group (p <0.05; Fig 5). It is worth mentioning that co-treatment with 30 mg/kg showed a tendency to decrease the

immunoreactivity of COX-2 in the striatum of these animals. In addition, EAF HP alone at the tested doses did not affect the immunoreactivity of COX 2 (Fig 5).
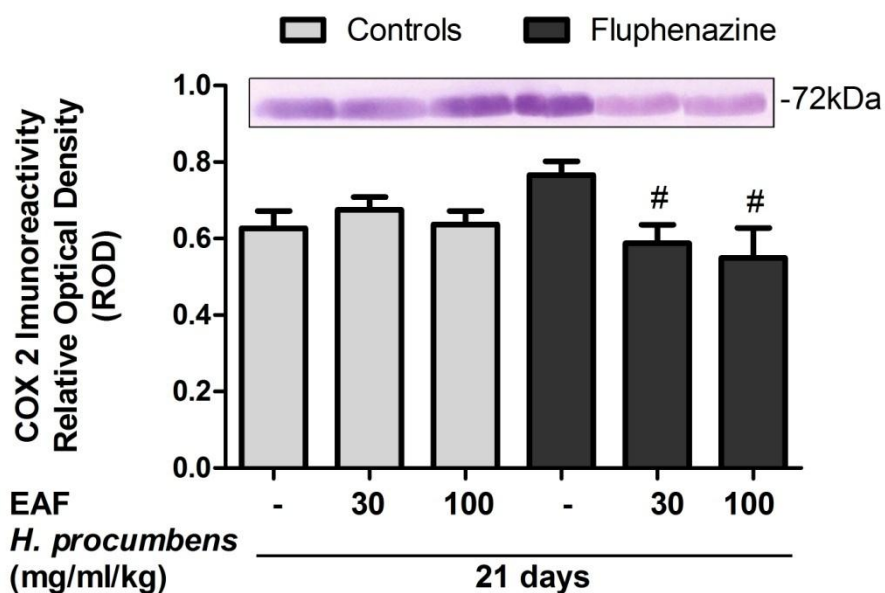


**Figure 5:** Western blot analysis of cyclooxygenase 2 (COX 2) in striatum rats treated with flufenazine and/or EAF HP. COX 2 immunoreactivity on day 21 were analyzed by relative optical density (ROD). Data are expressed as means ± SEM. One-way ANOVA followed by Tukey test.[#]($p<0.05$), Represents significant differences compared to fluphenazine.

## 5. Effects of EAF HP and/or fluphenazine on tyrosine hydroxylase (TH), dopamine receptor D$_2$ (DRD$_2$) and dopamine transporter (DAT) immunoreactivity in striatum of rats.

Due to the positive results obtained with the treatment of 30 and 100 mg/kg of EAF HP, we also tested the effect of fluphenazine and/or EAF HP (30 and 100 mg/kg) on the immunoreactivity of important dopaminergic system proteins in the striatum, such as TH, DRD$_2$ and DAT. Western blotting analysis revealed no change in TH (Fig 6A), DRD$_2$ (Fig 6B) or DAT (Fig 6C) immunoreactivity in any experimental group. Thus, neither, fluphenazine nor treatment with EAF HP changed the immunoreactivity of these relevant dopaminergic markers.
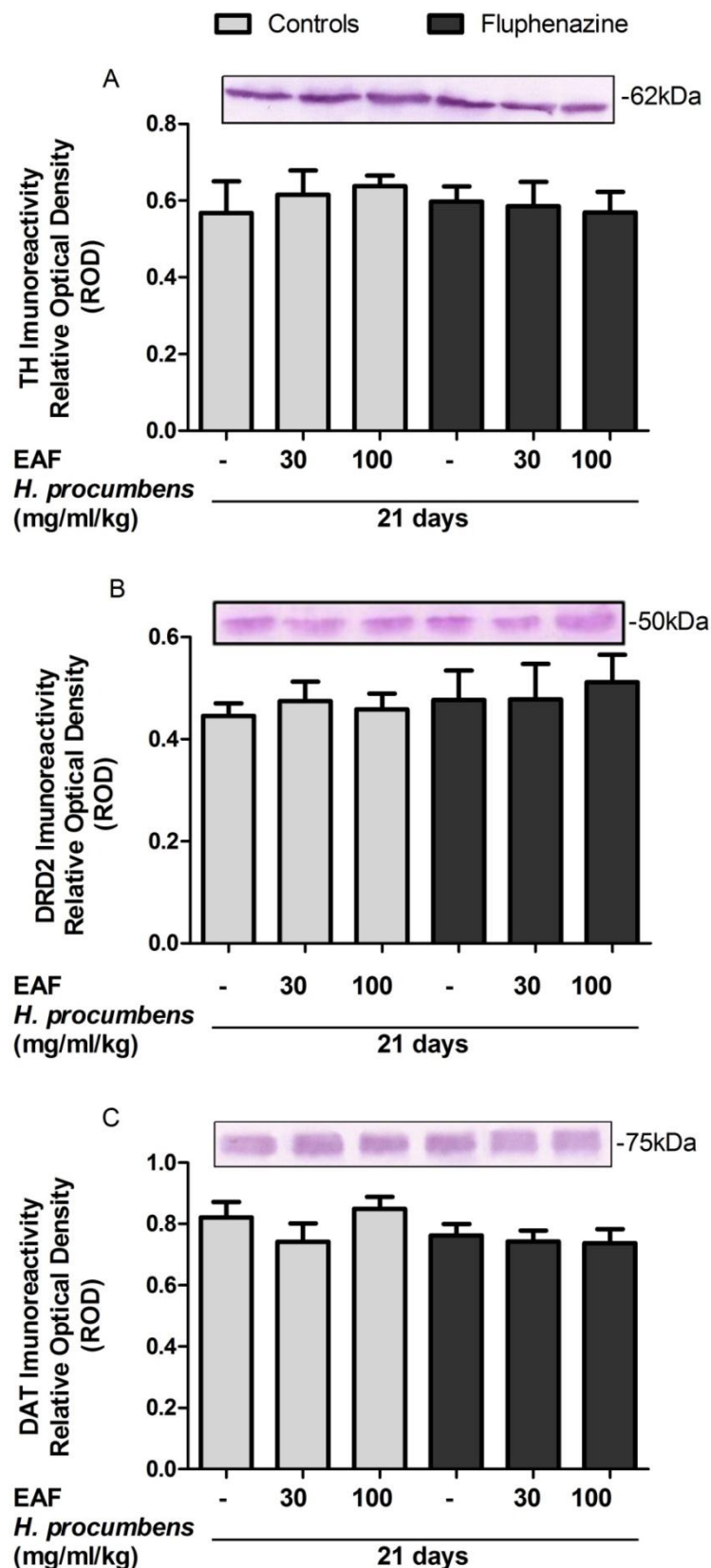
**Figure 6:** Western blot analysis of tyrosine hydroxylase (TH), dopamine transporter (DAT) and dopamine receptor D2 (DRD$_2$) in striatum rats treated with flufenazine and/or EAF HP. (A) TH immunoreactivity, (B) DRD$_2$ immunoreactivity and (C) DAT immunoreactivity on day 21 were analyzed by relative optical density (ROD). Data are expressed as means ± SEM. One-way ANOVA followed by Tukey test.

## 6. Effects of EAF HP and/or fluphenazine in glutamate decarboxylase (GAD) immunoreactivity in striatum of rats

We also tested the possible involvement of the GABAergic system through the quantification of GAD immunoreactivity. Western blot analysis did not show changes in striatal GAD immunoreactivity in any experimental group (Fig 7).
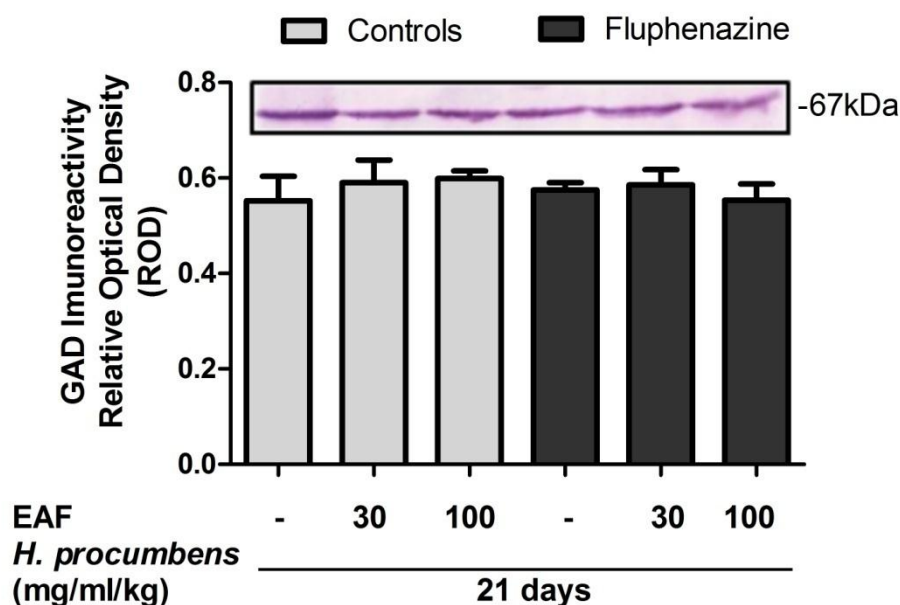


**Figure 7:** Western blot analysis of glutamate decarboxylase (GAD) in striatum rats treated with flufenazine and/or EAF HP. GAD immunoreactivity on day 21 were analyzed by relative optical density (ROD). Data are expressed as means ± SEM. One-way ANOVA followed by Tukey test.

## DISCUSSION

The pathogenesis of TD due to the chronic use of antipsychotics remains enigmatic, although hypotheses have been proposed to describe the biochemical mechanisms involved. The lack of knowledge about the causes of TD has made it difficult to find a treatment (Soares and Fernandez, 2007; Tarsy and Baldessarini, 2006). In the present study, we elucidated potential mechanisms for the beneficial effect of HP in significantly reducing involuntary orofacial movements.in rats with OD. We tested the EAF HP because it contains larger amounts of phenolic compounds, flavonoids (Schaffer et al.

2013) and harpagoside (the active chemical constituent of the plant) (Schaffer et al. 2016) than other extracts and fractions of HP.

Initially, we examined the effect of the EAF HP on fluphenazine-induced orofacial movements in rats. As expected, 21 days after administration fluphenazine enanthate caused a significant increase in the number of VCMs. Co-treatment with the EAF HP was effective in reducing the number of VCMs, with the most pronounced reduction at a dose of 30 mg/kg. These results are consistent with a study recently published by our group (Schaffer et al. 2016), in which we demonstrated the involvement of oxidative stress in the OD in rats treated with fluphenazine, as well as the effectiveness of the antioxidant EAF HP in decreasing oral movements in these animals. In fact, the antioxidant effect of the plant seems to be involved, at least in parts, with the beneficial effect it demonstrates in this model, mainly because of its antioxidant effect demonstrated in the striatum of rats treated with fluphenazine and co-treated with HP.

However, knowing that reactive oxygen species are capable of inducing the activation and expression of pro-inflammatory genes and transcription factors, and sometimes the inflammatory process may be which leads to oxidative stress in tissue (Aggarwal, 2000; Aggarwal et al., 2002), we decided to analyze the possible involvement of neuroinflammation in OD. The inflammatory response in the central nervous system comprises a large spectrum of complex and integrated cellular responses, mainly due to the activation of glial cells, such as microglia and astrocytes. This activation may indicative the presence of pathogens, tissue injury or neurotoxins, which result in the release of pro-inflammatory mediators (cytokines, for example), which may potentially contribute to neuronal dysfunction and progression of pathologies in the CNS (Khansari et al. 2009; Park et al. 2011). Cytokines are involved in the regulation of communication between cells of the immune system. Therefore, high levels of cytokines in the CNS indicate activation of the inflammatory response (Tansey, 2010), and the persistent expression of two major pro-inflammatory cytokines, TNF-α and IL-1β directly compromise the plasticity and survival of neuronal cells (Rothwell and Luheshi, 2000; Allan and Rothwell, 2001).

One recent hypothesis links the onset of TD with neuroinflammation. Studies have shown that chronic treatment with typical antipsychotics leads to

the increase of cytokines and other pro-inflammatory factors (Paterson et al. 2006; Bishnoi et al., 2008a, 2008b; Liu et al., 2012) in the CNS, where they can mediate cytotoxic effects (Aggarwal, 2000; Aggarwall et al., 2002; Bishnoi et al., 2008), being related to neurodegenerative diseases, due to the chronic production of cytokines (Gonzalez-Scarano and Baltuch, 1999; Nakajima and Kohsaka, 2001; Kuno et al. 2005).

Here, we analyzed IL-1β, IL-6, TNF-α and INF-γ in the striatum and in the cerebral cortex of fluphenazine-treated and/or co-treated with EAF HP animals. Fluphenazine caused an increase in all pro-inflammatory cytokines, both in the striatum and in the cortex. Co-treatment with EAF HP protected against this increase of cytokines, an effect that was more pronounced in the striatum. It is noteworthy that the dose that had the most effect in decreasing cytokines in the striatum was 30 mg/kg of EAF HP, being this dose that decreased in a more pronounced way the MMVs. These results suggest a possible link between fluphenazine-induced OD and inflammatory mediators. There are some studies demonstrating the relationship between TD/OD and neuroinflammation caused by the use of antipsychotics. Bishnoi et al. (2008a) demonstrated that chronic administration of haloperidol leads to oxidative stress by increasing inflammatory mediators such as TNF-α, and these neuronal changes may be associated with antipsychotic-induced OD. In addition, a study of schizophrenic patients showed that patients with TD had lower IL-2 levels than patients without TD (Rapaport and Lohr, 1994). Of particular importance, our group demonstrated a positive correlation between VCMs and the increase pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, and INF-γ) in the striatum of haloperidol-treated rats, supporting the relationship between neuroinflammation and haloperidol-induced OD (Peroza et al. 2016).

In addition to increased cytokine immunoreactivity, treatment with fluphenazine showed a tendency to increase striatal COX-2 immunoreactivity. Treatment with EAF HP also reduced COX-2 immunoreactivity in this brain structure (Fig. 5). COX-2 is a limiting enzyme for the production of prostaglandins related to tissue inflammation and is expressed in the CNS in neurons and glial cells. The increase in the activity of this enzyme plays a crucial role in the intense inflammatory responses that lead to neuronal death, and eventually neurodegenerative processes. In fact, compounds or drugs that

modulate the activity of this enzyme have shown neuroprotective effects in some studies (Liu and Hong, 2003; Teismann et al. 2003; Minghety, 2004). Naidu and Kulkarni (2001), showed that indomethacin (a nonselective cyclooxygenase inhibitor) reverses haloperidol-induced VCMs, suggesting the involvement of prostaglandins in OD.

The anti-inflammatory effect exerted by the EAF HP in the CNS can present great magnitude, mainly with respect to the neuronal viability, since it is well described that increased levels of cytokines can alter the plasticity and neuronal viability of dopaminérgicos neurons, for example (Godoy et al. 2010). In addition, TNF-α and IL-1β can induce reactive oxygen and/or nitrogen species formation (RS), which leads to the alteration of neuronal plasticity due to intracellular accumulation of RS, thereby impairing the cellular cytoskeleton (Barth et al. 2009). This could explain, at least in par, the antioxidant effect (Schaffer et al. 2016) of HP found in this model in previous study. In addition, the significant increase of cytokines in certain regions of the brain may directly influence synaptic transmission, with consequent behavior change (Miller et al. 2009; Haroon et al. 2012), which may help to explain the development of TD.

The potent anti-inflammatory activity of HP in joint diseases, is well described in the literature; however, there are several contradictions in the literature on the mechanism by which it presents the anti-inflammatory action. Studies have shown that this plant or its main constituent, harpagoside, has the ability to suppress the production of inflammatory cytokines (TNF-α, IL-1β, and IL-6) and the prostaglandin E2 (Fiebich et al. 2001); as well as suppress the activities of COX-2 and inducible nitric oxide synthesis (iNOS) by inhibiting NF-κB activation (Huang et al. 2006); block Activator Protein 1 (AP-1), decreasing the liberation of interleukins and TNF-α (Fiebich et al. 2012) and suppress IL-6 expression in chondrocytes stimulated with IL-1β (Haseeb et al. 2016). However, no study has analyzed the changes in pro-inflammatory markers in the CNS as a result of HP administration. Our study is the first to demonstrate this anti-inflammatory effect in an experimental model *in vivo*, showing that HP protected against fluphenazine-induced neuroinflammation, especially in the striatum. Notably, EAF HP also demonstrated anti-inflammatory effect *per se* in the two brain structures analyzed (striatum and cortex), demonstrating its direct action in the CNS. However, we observed that EAF HP did not restore pro-

inflammatory factors to control levels, which suggests that it may be acting in other VCM-related pathways, such as by directly sequestering ER and increasing antioxidant defenses, an effect already demonstrated by HP (Schaffer et al. 2013, 2016).

We also analyzed important markers of the dopaminergic system, since the use of typical antipsychotics antagonizes dopaminergic subtype $D_2$ receptor in the mesolimbic pathway, which improve psychotic symptons. However, $D_2$ receptor blockade in the nigrostriatal pathway is associated with development of side effects, such as TD (Soares and Fernandez, 2007; Tarsy and Baldessarini, 2006). An older hypothesis for the etiology of TD is dopaminergic hypersensitivity. According to this hypothesis, chronic blockade of $D_2$ receptors mainly in the nigrostriatal pathway would induce a compensatory increase in the number of dopaminergic receptors, which are probably responsive to lower levels of dopamine leading to hyperdopaminergic state and clinical manifestations of TD (Andreassen and Jorgensen, 2000). However, there are conflicting studies in the literature on the effect of antipsychotics on changes in the dopaminergic system. Marchese et al. (2002) demonstrated that treatment with haloperidol was able to decrease immunostaining of TH and DAT in the striatum. Similarly, Zhang et al. (2007) showed that administration of haloperidol decreased the immunoreactivity of TH in the striatum. However, Salvatore et al. (2000) showed that administration of haloperidol increased the state of phosphorylation of TH in the striatum of rats. Studies from our group have shown that animals that develop OD in response to treatment with haloperidol and fluphenazine also have reduced levels of dopamine reuptake (via DAT) (Fachinetto et al. 2007a, 2007b). In addition, it has been described in the literature that harpagoside attenuated dopaminergic neurodegeneration, protecting against the decrease of DAT (Sun et al., 2012). However, in the present experimental model in rats, neither fluphenazine nor HP EAF altered TH (Fig 6A), $DRD_2$ (Fig 6B) or DAT (Fig 6C) immunoreactivity in the striatum. It seems that the changes in dopaminergic markers are closely related to treatment time. Hakansson et al. (2004) showed that after haloperidol administration (14 hours) there is an increase in TH phosphorylation in the striatum of mice. However, administration of haloperidol for 14 days does not affect the levels of striatal TH, measured 24 h after the last injection.

GABAergic hypofunction in the basal ganglia is described as a possible mechanism underlying the pathophysiology of TD (Fibiger and Loyd, 1984). Furthermore, Mahomed and Ojewole (2006) demonstrated the anticonvulsant effect of aqueous extract of HP root, suggesting modulation of the GABAergic system. In addition, administration of GABA-mimetic drugs or those that positively modulate the GABAergic receptor inhibits the occurrence of OD in animals (Gao et al. 1994; Peixoto et al. 2003; Bishnoi et al. 2008c). For these reasons, we analyzed the involvement of GABAergic system in HP treatment. However, treatment with fluphenazine and/or EAF HP did not change striatal immunoreactivity of GAD (Fig 7), the limiting enzyme for the synthesis of GABA.

In conclusion, our work demonstrated that HP has an anti-inflammatory effect in the CNS, modulating cytokines and the COX-2 enzyme in OD model fluphenazine-induced in rats. This suggests HP may be a promising therapeutic agent for the treatment of involuntary oral movements. In addition, our results support the hypothesis of the involvement of neuroinflammation in orofacial dyskinesia, since fluphenazine significantly increased VCMs and pro-inflammatory markers in the CNS of the animals.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgment:

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## 4 DISCUSSÃO

Na presente tese, a influência do HP no modelo da DO induzida por flufenazina em ratos foi avaliada por parâmetros comportamentais e análises bioquímicas e moleculares. Isso vem em acordo com a necessidade de se encontrar um tratamento padrão e eficaz para a DT, além da busca por elucidação dos mecanismos envolvidos no aparecimento dessa desordem do movimento em alguns pacientes que usam antipsicótico.

No primeiro momento, resolvemos analisar o extrato bruto e diferentes frações de HP, a fim de eleger um extrato que tivesse em sua constituição uma maior quantidade de compostos fenólicos/flavonóides e harpagosídeo para então ser testado posteriormente em modelo experimental *in vivo*. Em trabalho prévio do nosso grupo, foi analisado o extrato bruto e diferentes frações de HP, entre elas, a fração acetato de etila foi a mais potente em proteger contra o dano oxidativo induzido por ferro ou nitroprussiato de sódio em córtex de ratos *in vitro*, além de proteger contra a perda da viabilidade celular. Esse efeito foi então atribuído a maior quantidade de compostos fenólicos e flavonóides presentes nesta fração (SCHAFFER et al., 2013). Aqui, nós testamos a capacidade *scavenger* do radical DPPH pelo extrato bruto e diferentes frações de HP. Como resultado a fração acetato de etila de HP (FAE HP) foi a fração que teve maior potência em seqüestrar esse radical, demonstrando mais uma vez a potente atividade antioxidante *in vitro* desta fração [Fig 3 -.artigo publicado (AP)]. Além disso, verificamos a presença e a quantidade de harpagosídeo presente nos diferentes extratos de HP através da análise por cromatografia líquida de alta eficiência (CLAE), onde a FAE HP foi a que demonstrou maior quantidade deste constituinte (Fig 2D-AP).

O harpagosídeo tem sido proposto como principal constituinte do HP, sendo que este, por sua vez, teria ação central na atividade terapêutica desta planta. A quantidade de harpagosídeo presente na raiz da planta é usada para determinar a qualidade dos tubérculos secos fornecidos para a comercialização, sendo reconhecido pela Farmacopéia Européia (2004) não menos que 1.2% de harpagosídeo presente nos tubérculos de HP, calculado com referência à planta seca (STEWART; COLE, 2005; BRIEN et al., 2006; ABDELOUAHAB; HEARD, 2007; MNCWANGI et al., 2012; GEORGIEV et al., 2013). Contudo, existem estudos que vêm demonstrando que outros componentes presentes no HP teriam grandes

influências em sua ação terapêutica, como os constituintes fenólicos e flavonóides. De fato, a presença do harpagosídeo no HP é importante para a obtenção dos efeitos anti-inflamatórios desta planta, porém este constituinte possivelmente estaria agindo de forma sinérgica com demais componentes presente no HP para conseguir induzir o seu efeito inibitório máximo contra principalmente a inflamação (LOEW et al., 2001; KASZKIN et al., 2004; GEORGIEV et al., 2013). Relatos na literatura demonstram que extratos sem a presença do harpagosídeo foram capazes de inibir a indução da iNOS porém em menor proporção do que quando testado com a presença do harpagosídeo, demonstrando mais uma vez o possível sinergismo realizado pelos constituintes do HP. Vale salientar, que neste mesmo estudo o harpagosídeo foi testado isoladamente e não obteve resultados inibitórios na indução da iNOS (KASZKIN et al., 2004). Estes dados suportam a eleição da FAE HP para os testes posteriores, uma vez que se acredita na necessidade de vários constituintes presentes no HP para que a planta obtenha seus efeitos farmacológicos.

Nos testes comportamentais em ratos, como esperado, o tratamento com flufenazina causou um significativo aumento no número de MMVs em todos os tempos analisados e uma diminuição da atividade locomotora e exploratória, em comparação com animais controle. O co-tratamento com a FAE HP foi eficaz na redução do número de MMVs, de forma mais pronunciada na dose de 30 mg/kg, diminuindo aos níveis dos animais controles nas avaliações nos dias 7, 14 e 21 (Fig 3-AP). Contudo, não foi capaz modificar a diminuição da atividade locomotora e exploratória induzida pela flufenazina (Fig 4-AP). Sabe-se que a diminuição da locomoção está relacionada com o bloqueio dopaminérgico no núcleo accumbens, enquanto os MMVs estão relacionados com o bloqueio dopaminérgico, ou diminuição da dopamina, no neoestriado (KELLEY et al., 1989; SALAMONE et al., 1998), desta forma parece que a FAE HP age em locais anatômicos diferentes no SNC.

A patogênese da DT devido ao uso crônico de antipsicóticos ainda permanece enigmática, desta forma algumas hipóteses vêm sendo discutidas na literatura para elucidar os mecanismos bioquímicos envolvidos na DT. No nosso estudo, depois de verificarmos a eficácia do EAF HP em diminuir a DO induzida por flufenazina em ratos, resolvemos analisar os possíveis mecanismos que poderiam estar envolvidos com este efeito protetor.

O papel dos radicais livres e o EO na DT tem sido extensamente discutido na literatura, onde a possível indução do dano oxidativo, decorrente do bloqueio crônico dos neurônios dopaminérgicos pelos antipsicóticos, especialmente no estriado, poderiam estar levando a morte celular. Sendo importante salientar que outras hipóteses para o aparecimento da DT, as quais incluem: hipersensibilidade dopaminérgica, excitotoxicidade glutamatérgica, neuroinflamação nigroestriatal, e outros fatores de risco, tais como idade, interessantemente também podem levar ao EO, podendo este ser um fator importante na patogênese do desenvolvimento desses movimentos involuntários. Com isso, a nossa primeira linha de raciocínio foi analisar o possível envolvimento do EO na eficácia da FAE HP em diminuir os MMVs induzidos pela flufenazina.

Vários estudos demonstram a relação possível do co-tratamento com antioxidantes e a redução do dano oxidativo induzido por antipsicóticos, suportando a hipótese do EO para o desenvolvimento da DT e DO (BURGER et al., 2005; NADE et al., 2010; BISCHNOI et al., 2011; PATHAN et al., 2011; LISTER et al., 2014). Contudo, nosso grupo relatou que DO induzida pelo tratamento crônico com antipsicóticos (tanto haloperidol quanto a flufenazina) não estaria diretamente relacionado com alterações nos parâmetros de EO analisados em nossos estudos (TBARS, níveis de ER e Superóxido dismutase) (FACHINETTO et al., 2007a, b). Aqui, resolvemos analisar tanto marcadores de dano celular como na técnica de TBARS (peroxidação lipídica) e níveis de ER, quanto marcadores antioxidantes como a atividade da enzima catalase e os níveis dos tióis (protéico e não-proteico). Como resultado das análises, nós verificamos alterações entre os grupos apenas na atividade da catalase e nos níveis de ER. No estriado, a flufenazina induziu a elevação nos níveis de ER e a FAE HP foi capaz de proteger contra esse aumento (Fig 8 – AP). Por sua vez, esse aumento nos níveis de ER, pode estar relacionado com uma diminuição na atividade da catalase identificada no estriado, demonstrando prejuízo nas defesas antioxidantes, sendo que o co-tratamento com a FAE HP foi capaz de restaurar a atividade da catalase de maneira dependente de dose aos níveis do controle. Esse resultado está de acordo com outros trabalhos que demonstram que os antipsicóticos típicos são capazes de diminuir a atividade de enzimas antioxidantes endógenas (como a catalase) em várias regiões do cérebro de ratos (TSAI et al., 1998; PARIKH et al., 2003; ABÍLIO et al., 2004), bem como no fluído cérebroespinal de pacientes tratados com haloperidol (LOHR; BRACHA,

1988). Desta forma, no presente estudo, sugere-se que o EO está relacionado, pelo menos em partes, com o efeito protetor do EAF HP em diminuir a DO nos ratos.

No córtex, a flufenazina também induziu alterações na atividade da catalase e nos níveis de ER, porém a FAE HP protegeu apenas contra a alteração na atividade da catalase (Fig 9-AP). As alterações demonstradas no córtex provavelmente não estão interligadas com os resultados comportamentais, uma vez que o córtex não é uma área cerebral diretamente relacionada com os efeitos extrapiramidais. Tomados em conjunto, os nossos resultados sugerem que FAE HP poderia exercer efeitos antioxidantes em diferentes áreas do cérebro.

Como nossa segunda hipótese para os efeitos protetores da EAF HP encontrados *in vivo*, resolvemos verificar marcadores inflamatórios no estriado e córtex dos animais, uma vez que já é bem descrito a potente atividade anti-inflamatória do HP (WEGENER; LUPKE, 2003; MAHOMED; OJEWOLE, 2004; KUNDU et al., 2005; HUANG et al., 2006; WARNOCK et al., 2007; FIEBICH et al., 2011; WACHSMUTH et al., 2011) e que o tratamento prolongado com antipsicóticos típicos pode causar neuroinflamação (PATERSON et al., 2006; BISHNOI et al., 2008a, b; LAU et al., 2013). O haloperidol, por exemplo, é capaz de induzir a expressão de TNF-α, NF kB e outros fatores celulares que respondem diretamente ao EO (POST et al., 1998, 2002). Essas citocinas e fatores pró-inflamatórios acabam por mediar efeitos citotóxicos no SNC, podendo assim induzir a cascata de sinalização celular que leva à morte celular (AGGARWAL, 2000; AGGARWAL et al., 2002) estando assim relacionadas com as doenças neurodegenerativas (GONZALEZ-SCARANO; BALTUCH, 1999; NAKAJIMA; KOHSAKA, 2001; KUNO et al., 2005). Além disso,o EO pode ser causa ou também conseqüência de processos inflamatórios, onde o sistema imunológico pode contribuir para o processo patogênico gerando ER e promovendo a neuroinflamação, via citocinas liberadas por linfócitos e microglia (MAN et al., 2016).

Aqui, nós analisamos a IL1β, IL 6, TNF-α e INF-γ no estriado e no córtex cerebral dos animais. A flufenazina causou aumento de todas citocinas pro-inflamatórias, tanto no estriado quanto no córtex (Fig 3 e 4- MS), além disso, houve uma tendência em aumentar a imunoreatividade da COX 2 no estriado (Fig 5-MS). O co-tratamento com FAE HP foi capaz de proteger contra esse aumento de citocinas de forma mais pronunciada no estriado e também foi capaz de diminuir a imunoreatividade da COX 2 nesta estrutura cerebral. Vale ressaltar que a dose que

mais teve efeito em diminuir as citocinas no estriado foi a de 30 mg/kg de FAE HP, sendo esta dose que diminuiu de forma mais pronunciada os MMVs (Fig 2-MS), esses dados sugerem uma ligação da DO induzida pela flufenazina com os mediadores inflamatórios, estando assim de acordo com estudos na literatura que demonstram essa ligação, porém utilizam o haloperidol como antipsicótico para induzir a DO (BISHNOI et al., 2008 a,d). Ainda em relação à DO, Naidu e Kulkarni (2001) mostraram que a indometacina (um inibidor não seletivo da ciclooxigenase) reverte os MMVs induzidos pelo haloperidol, sugerindo o envolvimento de prostaglandinas em DO.

Os mecanismos pelos quais o HP promove a sua ação anti-inflamatórios no organismo não estão totalmente esclarecidos. Estudos já descreveram a sua ação via inibição da ativação do NF-κB o que diminuiria seletivamente a COX 2 (HUANG et al., 2006), via bloqueio da AP-1 diminuindo assim a liberação de interleucinas e do TNF- α (FIEBICH et al., 2012). Em estudo mais recente, o harpagoside suprimiu a expressão de IL-6 em condrócitos estimulados com IL-1β, entretando não foi capaz de inibir a ativação do NF-kB (HASEEB et al., 2016). Contudo, não existe um mecanismo de ação exato, pois ocorrem várias divergências na literatura, que são justificadas na maioria das vezes pela insuficiência na caracterização química da planta e pelo uso de modelos de estudo e métodos de extração diferentes (CLARKSON et al., 2006; FIEBICH et al., 2012). Vale salientar que não existem trabalhos que tenham analisado as alterações nos marcadores pro-inflamatórios no SNC induzidos pelo *H. procumbens*. O nosso estudo foi o primeiro a demonstrar esse efeito anti-inflamatório em modelo experimental *in vivo*, protegendo contra a neuroinflamação induzida pela flufenazina, sendo esse efeito especialmente pronunciado no estriado cerebral. Notavelmente, a EAF HP demonstrou efeito anti-inflamatório *per se* nas duas estruturas cerebrais analisadas (estriado e córtex), demonstrando a sua ação direta no SNC. Contudo, no córtex o co-tratamento com a FAE HP não conseguiu proteger contra o aumento das citocinas pró-inflamatórias induzidas pela flufenazina. Esse resultado pode estar relacionado com a falta de ação da FAE HP em proteger contra o aumento dos níveis das ER induzidos pela flufenazina (Figura 9- AP), sendo isto uma conseqüência da ausência da atividade anti-inflamatória da fração no córtex (Figura 4-MS), isso suporta a hipótese que a formação de ER poderia estar sendo uma conseqüência da atividade inflamatória no SNC neste modelo de DO.

As células microgliais são uma fonte predominante de várias citocinas, isto é, a IL-1β, TNF-a e o IFN-γ, que podem então induzir uma amplo espectro de reações inflamatórias. A ativação de microglia e astrócitos em resposta a estímulos internos e externos ou insultos pode aumentar ainda mais a liberação de substâncias citotóxicas, citocinas pró-inflamatórias, ER e aminoácidos excitatórios, causando assim lesões neuronais adicionais no cérebro (LIU et al., 2015). Ainda, a COX- 2 é outro importante marcador inflamatório, pois é a enzima limitante na produção de prostaglandinas sendo expressa em neurônios e células gliais. Estudos vêm demonstrando que os compostos ou fármacos que modulam a atividade desta enzima têm mostrado efeitos neuroprotetores (LIU; HONG, 2003; TEISMANN et al., 2003; MINGHETY, 2004).

Esse efeito protetor anti-inflamatório no SNC exercido pela FAE HP pode apresentar grande magnitude, principalmente no que se refere à viabilidade neuronal, pois já está bem descrito que níveis aumentados de citocinas podem alterar a plasticidade e a viabilidade neuronal com consequente perda de neurônios dopaminérgicos, por exemplo (GODOY et al., 2010). E, em células neuronais, o TNF-α e Il-1β podem induzir a formação de ER o que acaba levando a alteração da plasticidade neuronal devido ao acumulo intracelular das ER prejudicando assim, o citoesqueleto celular (BARTH et al., 2009). Além disso, o aumento significativo de citocinas em certas regiões do cérebro pode influenciar diretamente a transmissão sináptica, com consequente mudança de comportamento (MILLER et al., 2009, HAROON et al., 2012), o que pode ajudar a explicar o desenvolvimento de DT, bem como o efeito neuroprotetor da FAE HP neste modelo experimental.

Ainda resolvemos analisar importantes marcadores dopaminérgicos, TH, RDD$_2$ e DAT, uma vez que a mais antiga hipótese para o aparecimento da DT é a possível hipersensibilidade dopaminérgica e que já foi descrito na literatura que o harpagosídeo atenuou a neurodegeneração dopaminérgica, protegendo contra a diminuição do DAT (SUN et al., 2012).

Alguns estudos suportam essa hipótese demonstrando que o bloqueio crônico dos receptores dopaminérgicos levaria à hipersensibilização gradual dos receptores da dopamina ainda não bloqueados pelo antipsicótico (MARSDEN; JENNER, 1980; RUBINSTEIN et al., 1990), aumentando também a atividade da tirosina hidroxilase (TH), com consequente aumento na liberação de dopamina (MOORE, 1987; SALVATORE et al., 2000), como já mencionado. Além disso, estudos mostram um

aumento na densidade de receptores $D_2$ estriatais (BURT et al., 1977; GINOVART et al., 2009) sendo que esses receptores $D_2$ estariam em um estado de alta afinidade pela dopamina (SAMAHA et al., 2008, 2007). Entretanto, existem algumas controvérsias quanto a essa hipótese, onde essa teoria em humanos é menos forte. Em estudo *post mortem* foi demonstrado que o número de receptores $D_2$ é semelhantes entre pacientes com DT e sem DT (CROW et al., 1982) e ainda existem autores relatando que em alguns modelos animais, a administração do antipsicótico diminuiu a atividade da TH (MARCHESE et al., 2002; ZHANG et al., 2007), bem como a atividade do DAT (MARCHESE et al., 2002). Cabe salientar que se ocorresse um aumento na liberação de dopamina a principal forma de sua retirada da fenda sináptica seria através da sua recaptação via transportador de dopamina (DAT) (BECKMAN; QUICK, 1998; KAHLIG; GALLI, 2003). Fachineto e colaboradores (2007a,b) já demonstraram que os animais que desenvolvem DO, em resposta ao tratamento crônico com haloperidol e flufenazina, apresentam níveis reduzidos de recaptação de dopamina (via DAT). Aqui, no presente modelo experimental em ratos, a flufenazina nem a FAE HP foram capazes de alterar de forma significativa a imunoreatividade de TH (Fig 6A-MS) DRD$_2$ (Fig 6B-MS) e DAT (Fig 6C-MS) no estriado.

Além do envolvimento do sistema dopaminérgico, a hipofunção GABAérgica nos núcleos da base é descrita como um possível mecanismo subjacente a fisiopatologia da DT (FIBIGER; LLOYD, 1984). Animais tratados com antipsicóticos apresentam uma diminuição na atividade da glutamato descarboxilase (GAD), enzima limitante para a síntese de GABA (GERLACH; CASEY, 1988). Pesquisas demonstram que a administração de drogas GABA-miméticas ou que modulem positivamente o receptor GABAérgico inibem o aparecimento de DO em animais (PEIXOTO et al., 2003; BISHNOI et al., 2008c). Clinicamente, pacientes discinéticos apresentam uma redução na concentração de GABA no líquido cefalorraquidiano (GUNNE et al., 1984; THAKER et al., 1990). Desta forma, resolvemos analisar a imunoreatividade do GAD (enzima limitante para a produção de GABA) e verificar se os efeitos protetores da FAE HP no presente estudo *in vivo* podem estar relacionados com uma modulação GABAérgica, uma vez que já existe relatos na literatura que o *H. procumbens* apresenta atividade anticonvulsivante (MAHOMED; OJEWOLE, 2006). No entanto, o tratamento com flufenazina e FAE HP também não alterou a imunoreatividade da GAD estriatal (Fig. 7).

Assim, tomando em conjunto os resultados encontrados nessa tese, acreditamos que o efeito da FAE HP em reduzir o MMVs induzidos pela flufenazina está relacionado principalmente por seu efeito anti-inflamatório no SNC. Em resposta ao antagonismo crônico exercido pelo antipsicótico (dano tecidual), a microglia e astrócitos podem aumentar a produção e liberação de citocinas inflamatórias, iniciando pela liberação de IL-1β, o qual irá ativar outras citocinas como TNF-α e IL-6, aumentando a geração de ER no estriado especialmente. Além disso, a diminuição da atividade da catalase provocada pela flufenazina pode levar a um desequilíbrio no estado redox celular, contribuindo assim para o EO e a ativação das citocinas pró-inflamatórias, podendo assim resultar em lesão neuronal ou futuramente uma perda neuronal. Vale salientar que muitos pacientes persistem os movimentos involuntários mesmo com a retirada do antipsicótico, dando a entender que o efeito do fármaco provoca mudanças significativas na função cerebral que não estão relacionadas com a presença do mesmo.

# 5 CONCLUSÕES

5.1 CONCLUSÕES PARCIAIS

- ➤ A FAE HP foi a fração que apresentou maior quantidade de harpagosídeo e melhor atividade antioxidante *in vitro.*

- ➤ A FAE HP foi capaz de proteger contra o aumento de MMV induzidos por flufenazina, porém não alterou os efeitos do antipsicótico na atividade locomotora e exploratória em ratos.

- ➤ As doses de FAE HP testadas neste trabalho parecem não exercer alterações periféricas relacionadas aos parâmetros bioquímicos analisados no soro e de EO analisados no rim e fígado dos animais.

- ➤ A flufenazina alterou os marcadores de EO (atividade da catalase e níveis de ER) no estriado e córtex e a FAE HP foi capaz de exercer efeito antioxidante, nas maiores doses testadas;

- ➤ A flufenazina aumentou as citocinas pró-inflamatórias no estriado e córtex cerebral e o co-tratamento com FAE HP exerceu efeito anti-inflamatório apenas no estriado. Esta fração apresentou também efeito *per se* na diminuição de citocinas pró-inflamatórias no estriado e córtex. Além disso, foi capaz de diminuir os níveis de COX 2 no estriado dos animais co-tratados com FAE HP.

- ➤ O sistema dopaminérgico não parece estar envolvido nos efeitos protetores da FAE HP, pois não foi encontrada nenhuma alteração nos ensaios de imunodetecção realizados para a TH, $DRD_2$ e DAT.

- ➤ A flufenazina nem o co-tratamento com a FAE HP foram capazes de alterar a glutamato descarboxilase neste modelo de DO, demonstrando que possivelmente o sistema gabaérgico não está relacionado com os efeitos benéficos exercidos pela FAE HP.

➢ O EO e a neuroinflamação parecem estar relacionados com o aparecimento da DO neste modelo induzido por flufenazina em ratos.

## 5.2 CONCLUSÃO GERAL

Este estudo demonstrou o efeito protetor da EAF HP em diminuir os MMVs induzidos pela flufenazina, sendo que esse efeito parece estar relacionado com a atividade antioxidante e anti-inflamatória desta fração. Além disso, é importante ressaltar que os resultados do presente estudo fornecem evidências da relação do desenvolvimento da DO induzida por flufenazina com os mediadores inflamatórios e estresse oxidativo em ratos, reforçando assim o envolvimento da neurotoxicidade na fisiopatologia da DO.

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**ANEXO**

**ANEXO A – CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA**

**UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM**

**CARTA DE APROVAÇÃO**

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

**Título do Projeto:** "Efeito do Harpagophytum procumbens sobre parâmetros comportamentais, bioquímicos e moleculares em modelo de discinesia orofacial induzida por flufenazina em ratos."

**Número do Parecer: 109/2014**

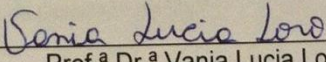**Pesquisador Responsável:** Prof.ª Dr.ª Roselei Fachinetto

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

**OBS:** Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

**DATA DE APROVAÇÃO:** 30/09/2014.

Santa Maria, 30 de setembro de 2014.

Prof.ª Dr.ª Vania Lucia Loro
Vice-Coordenadora da Comissão de Ética no Uso de Animais- UFSM

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