

UNIVERSIDADE FEDERAL DE SANTA MARIA  
CENTRO DE CIÊNCIAS NATURAIS E EXATAS  
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:  
BIOQUÍMICA TOXICOLÓGICA

Catiuscia Molz de Freitas

**EFEITO DA SILIMARINA E SILIBININA SOBRE AS ALTERAÇÕES  
COMPORTAMENTAIS E NEUROQUÍMICAS INDUZIDAS POR 6-  
HIDROXIDOPAMINA EM CAMUNDONGOS**

Santa Maria, RS  
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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do título de **Doutor em Ciências Biológicas: Bioquímica Toxicológica**.

Orientadora: Prof<sup>a</sup>. Dr<sup>a</sup>. Roselei Fachinetto

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**Aprovado em 11 de outubro de 2018:**

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Santa Maria, RS  
2018

## **AGRADECIMENTOS**

Agradeço a Deus, que nas suas mais diferentes formas de manifestação, está sempre presente guiando meus passos.

À minha família, em especial aos meus pais, José e Alice, à minha Vó Jandira e minha tia Malu meus maiores exemplos de vida, pelo amor, compreensão, por estarem sempre ao meu lado, dando-me toda força e incentivo que precisei.

À minha orientadora, Prof<sup>a</sup> Roselei Fachinetto, por me orientar desde a graduação. Agradeço pela orientação, conhecimento compartilhado, pela confiança depositada, pela paciência dispensada e pelo apoio dado à realização deste trabalho.

Aos meus colegas de laboratório, Alcindo, Ana Paula, Bárbara, Camila, Caroline, Elizete, Getúlio, Janaína, Jeane, Larissa, Talita, por todos esses anos de convivência, pelos momentos de alegria, companheirismo, amizade e ajuda nos experimentos. A vocês minha gratidão e amizade.

Às minhas amigas Carol, Clarissa e Luísa pela amizade, companhia, risadas, conselhos, incentivo e pela motivação nos momentos de desânimo.

Aos professores da banca por dedicarem um pouco do seu tempo na análise desta tese, contribuindo com preciosas sugestões para o seu engrandecimento.

Aos demais professores do Programa de Pós-Graduação em Bioquímica Toxicológica, que contribuíram de alguma forma para minha formação.

Ao CNPq, FAPERGS e a CAPES pela bolsa de estudos e pelos recursos financeiros concedidos.

Enfim, agradeço à Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica pela possibilidade de realização deste curso.

*“A maior recompensa para o trabalho do homem não é o que ele ganha com isso,  
mas o que ele se torna com isso.”*

*John Ruskin*

## RESUMO

### EFEITO DA SILIMARINA E SILIBININA SOBRE AS ALTERAÇÕES COMPORTAMENTAIS E NEUROQUÍMICAS INDUZIDAS POR 6-HIDROXIDOPAMINA EM CAMUNDONGOS

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ORIENTADORA: Roselei Fachinetto

A doença de Parkinson (DP) é uma doença neurodegenerativa incapacitante que afeta cerca de 1-2% da população mundial. A levodopa é o tratamento mais utilizado para atenuar os sintomas da DP, entretanto a longo prazo este fármaco leva a flutuações na resposta clínica e ao surgimento de discinesias, fazendo-se necessário a busca por novas alternativas farmacológicas. Nesse sentido, a silimarina, isolada das sementes de *Silybum marianum* e a silibinina, seu constituinte majoritário, são substâncias biotivas que tem demonstrado efeitos antioxidantes, neuroprotetores. Desta forma, o objetivo deste estudo foi investigar os efeitos da silimarina e da silibinina nas alterações comportamentais e neuroquímicas induzidas por 6-hidroxidopamina (6-OHDA) em camundongos, além de avaliar o efeito do co-tratamento com silimarina e levodopa. Primeiramente, os animais receberam uma injeção intracerebroventricular (i.c.v.) de 6-OHDA (60 µg) ou veículo (salina contendo 0,05% de ácido ascórbico). Após 7 dias iniciou-se o tratamento com diferentes doses de silimarina (10, 30 ou 100 mg/kg) por via intraperitoneal (i.p.) durante 7 dias. As avaliações comportamentais foram realizadas nos dias 8 e 15 do período experimental. A 6-OHDA causou prejuízo motor nos animais, o qual foi acompanhado por uma redução na imunoreatividade da tirosina hidroxilase (TH) e um aumento na ERK1/2 fosforilada no estriado e na substância negra. O tratamento com silimarina recuperou a coordenação motora dos animais sem efeito na ERK1/2 fosforilada. Na substância negra a silimarina na dose de 30 mg/kg restaurou as alterações na TH induzidas pela 6-OHDA. Além disso, foi avaliado o efeito da silibinina no modelo da 6-OHDA, onde os animais receberam uma injeção i.c.v. de 6-OHDA ou veículo. Após 7 dias iniciou-se o tratamento com diferentes doses de silibinina (50 ou 100 mg/kg, i.p.) durante 7 dias. A 6-OHDA induziu uma disfunção motora que foi acompanhada por uma redução na imunoreatividade da TH no estriado. A silibinina recuperou o equilíbrio motor dos animais avaliado no teste da caminhada sobre a barra como também modificou os níveis da TH. Na sequência investigou-se o efeito do co-tratamento com silimarina e levodopa no modelo da 6-OHDA. Sete dias após a administração de 6-OHDA ou veículo iniciou-se o tratamento com silimarina (30 mg/kg, i.p.) e/ou levodopa (25 mg/kg, i.p.) mais benserazida (10 mg/kg, i.p.) por 28 dias. O tratamento com silimarina recuperou a atividade locomotora dos animais, embora o co-tratamento com silimarina não tenha potencializado o efeito da levodopa no dano motor causado pela 6-OHDA. Além disso, o tratamento concomitante com silimarina e levodopa parece antecipar o surgimento da discinesia orofacial, observado por um aumento no número nos movimentos de mascar no vazio (MMVs). No 36º dia administraram-se apomorfina (1 mg/kg) e avaliou-se a locomoção e a estereotipia dos animais. No entanto, não foram observadas diferenças significativas na locomoção e na estereotipia dos animais. Os resultados sugerem que o efeito da silimarina no modelo da 6-OHDA possivelmente se deve ao sinergismo de todos os seus componentes e não apenas a ação de seu principal constituinte, a silibinina. Assim, a silimarina poderia vir a ser um agente terapêutico promissor no tratamento inicial para os sintomas parkinsonianos leves. No entanto, a silimarina deve ser melhor estudada antes de ser utilizada como um adjuvante à terapia com levodopa, tendo em vista que a combinação de ambos os tratamentos parece precipitar o surgimento da discinesia em camundongos.

**Palavras-chave:** *Silybum marianum*. Doença de Parkinson. Sistema dopaminérgico. Tirosina hidroxilase. Levodopa.

## ABSTRACT

### EFFECT OF SILYMARIN AND SILIBININ IN BEHAVIORAL AND NEUROCHEMICAL ALTERATIONS INDUCED BY 6-HYDROXYDOPAMINE IN MICE

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Parkinson's disease (PD) is a disabling neurodegenerative disease that affects about 1-2% of the world's population. L-dopa is the most used treatment to attenuate the symptoms of PD; however, in the long term this drug leads to fluctuations in clinical response and the emergence of dyskinesias, it becoming necessary the search for new therapeutic strategies. In this sense, silymarin, isolated from the seeds of *Silybum marianum* and silibinin, its major constituent, are bioactive substances that have demonstrated antioxidant and neuroprotective effects. Thus, the objective of this study was to investigate the effects of silymarin and silibinin on the behavioral and neurochemical alterations induced by 6-hydroxydopamine (6-OHDA) in mice, in addition, we evaluated the effect of co-treatment with silymarin and L-dopa. Firstly, the animals received an intracerebroventricular (i.c.v.) injection of 6-OHDA (60 µg) or vehicle (saline containing 0.05% ascorbic acid). After 7 days, was started the treatment with different doses of silymarin (10, 30 or 100 mg/kg) by via intraperitoneal (i.p.) for 7 days. Behavioral evaluations were performed on days 8 and 15 of the experimental period. 6-OHDA caused a motor impairment in the animals, which was accompanied by a reduction in tyrosine hydroxylase (TH) immunoreactivity and an increase in phospho-ERK1/2 in both striatum and substantia nigra. Silymarin treatment recovered the motor coordination of the animals without changing in the phospho-ERK1/2. In the substantia nigra, silymarin at the dose of 30 mg/kg restored changes in TH induced by 6-OHDA. In addition, it was evaluated the effect of silibinin on the 6-OHDA model, where the animals received an i.c.v. injection of 6-OHDA or vehicle. After 7 days it was started the treatment with different doses of silibinin (50 or 100 mg/kg, i.p.) during 7 days. 6-OHDA induced a motor dysfunction that was accompanied by a reduction in TH immunoreactivity in the striatum. Silibinin recovered the motor balance of the animals evaluated on the beam walk test as well as modified the TH levels. In the sequence, it was investigated the effect of co-treatment with silymarin and L-dopa on the 6-OHDA model. Seven days after administration of 6-OHDA or vehicle was started the treatment with silymarin (30 mg/kg, i.p.) and/or L-dopa (25 mg/kg, i.p.) plus benserazide (10 mg/kg, i.p.) for 28 days. Treatment with silymarin recovered the locomotor activity of the animals; although the co-treatment with silymarin did not potentiate the effect of L-dopa on motor damage caused by 6-OHDA. Furthermore, concomitant treatment with silymarin and L-dopa seems to anticipate the emergence of dyskinesia, observed by an increase on the number of vacuous chewing movements (VMCs). On the day 36, apomorphine (1 mg/kg) was administered and locomotion and stereotypy of the animals were evaluated. However, no significant differences were observed in locomotion and in stereotypy of animals. The results suggest the effect of silymarin in the 6-OHDA model is possibly due to the synergism of all its components and not only the action of its major constituent, silibinin. Thus, silymarin could be a promising therapeutic agent in the initial treatment for mild parkinsonian symptoms. However, silymarin should be better studied before it is used as an adjunct to L-dopa therapy, since their association seems to precipitate the emergence of dyskinesia in mice.

**Keywords:** *Silybum marianum*. Parkinson's disease. Dopaminergic system. Tyrosine hydroxylase. L-dopa.

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## LISTA DE ABREVIATURAS

3-MT	3-metoxitiramina
5-HT	Serotonina
6-OHDA	6-hidroxidopamina
AADC	Aminoácido aromático descarboxilase
Ach	Acetilcolina
AMPc	Adenosina monofosfato cíclico
AKT	Proteína quinase B
ATP	Adenosina trifosfato
Bcl -2	Célula B de linfoma 2
CAT	Catalase
COMT	Catecol-O-metiltransferase
DA	Dopamina
DOPAC	Ácido 3,4-diidroxifenilacético
DP	Doença de Parkinson
ERK1/2	Quinases Reguladas por Sinais Extracelulares (do inglês <i>extracellular signal-regulated kinases</i> )
EROs	Espécies reativas de oxigênio
GLU	Glutamato
GPI	Parte interna do globo pálido
GPx	Glutathione peroxidase
H <sub>2</sub> O <sub>2</sub>	Peróxido de hidrogênio
HVA	Ácido homovanílico
Levodopa	L-3,4-dihidroxifenilalanina
LRRK2	Quinase repetida 2 rica em leucina (do inglês <i>leucine-rich repeat kinase 2</i> )
MAO	Monoaminoxidase
MFB	Feixe prosencefálico medial (do inglês <i>medial forebrain bundle</i> )
MPP <sup>+</sup>	1-metil-4-fenilpiridínio
MPTP	1-metil-4-fenil-1,2,3,6-tetrahidropiridina
mTOR	Alvo da rapamicina em mamíferos
NA	Noradrenalina
NF-κB	Fator Nuclear kappa B
NMDA	N-metil-D-aspartato
OMS	Organização Mundial da Saúde
PINK1	Quinase 1 induzida por PTEN (fosfatase homóloga à tensina) (do inglês <i>PTEN-induced putative kinase 1</i> )
SN	Substância negra
SNC	Sistema nervoso central
SNpc	Parte compacta da substância negra
SNpr	Parte reticulada da substância negra
SUS	Sistema Único de Saúde
SOD	Superóxido dismutase
TDA	Transportador de dopamina
TH	Tirosina hidroxilase
TNF	Fator de necrose tumoral

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## APRESENTAÇÃO

No item **INTRODUÇÃO** está descrito uma exposição sucinta sobre as ideias essenciais, situando o assunto em um contexto global, trabalhadas nesta tese. No item **REFERENCIAL TEÓRICO** será apresentada uma breve revisão literária da temática que fundamenta esse trabalho, bem como destacando as pesquisas básicas que explicam ou compreendem o objeto do estudo.

Os **RESULTADOS** que fazem parte desta tese estão apresentados sob a forma de um artigo, um manuscrito submetido para publicação e um manuscrito em preparação, os quais se encontram no item ARTIGO e MANUSCRITOS. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências, encontram-se nos próprios ARTIGO e MANUSCRITOS e representam a íntegra deste estudo. O artigo está disposto na forma que foi publicado na edição da Revista Científica *Food and Chemical Toxicology*, o manuscrito 1 está disposto seguindo as normas exigidas pela Revista Científica *Journal of Neural Transmission*, à qual está submetido para publicação e o manuscrito 2 se encontra em preparação e está disposto seguindo as normas exigidas pela Revista Científica *Behavioural Brain Research*.

O item **DISCUSSÃO** apresenta uma interpretação de todos os dados encontrados e que fazem parte desta tese.

O item **CONCLUSÃO**, encontrada no final desta tese, apresenta os comentários gerais sobre este trabalho.

As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, REFERENCIAL TEÓRICO e DISCUSSÃO** desta tese.

## 1 INTRODUÇÃO

A doença de Parkinson (DP) é considerada a segunda doença neurodegenerativa mais prevalente e caracteriza-se, principalmente, pela perda progressiva e seletiva de neurônios dopaminérgicos da substância negra (KALIA; LANG, 2015). Clinicamente, a DP é caracterizada pelos sintomas motores como bradicinesia, tremor de repouso, rigidez e anormalidades posturais (KLOCKGETHER, 2004). Os pacientes também podem apresentar sintomas não motores tais como deficiências cognitivas (VERBAAN et al., 2007), dificuldade de aprendizado (SCHMITT-ELIASSEN et al., 2007) e déficits de atenção (BRONNICK et al., 2006).

Atualmente não existem terapias eficazes para curar ou capazes de interromper a progressão da DP. A levodopa (L-3,4-dihidroxi-fenilalanina) é o fármaco mais efetivo para atenuar os sintomas motores da DP (ALACHKAR et al., 2010). No entanto, o tratamento prolongado com este fármaco leva ao desenvolvimento de complicações motoras como flutuações na resposta clínica e movimentos involuntários anormais (discinesias) (NAGATSU; SAWADA, 2009), comprometendo, desta forma, sua eficácia clínica (AHLISKOG; MUENTER, 2001). Isso reforça a necessidade do desenvolvimento de novas estratégias terapêuticas que possam ser utilizadas como adjuvantes no tratamento da DP.

Neste contexto, a silimarina é uma mistura de flavonoides obtida das sementes do fruto da planta *Silybum marianum*, conhecida popularmente como cardo de leite (SVOBODOVÁ et al., 2016). Seu extrato contém aproximadamente 70% a 80% de flavonolignanas (complexo de silimarina) e aproximadamente 20 a 30% de compostos polifenólicos (COMELLI et al., 2007). A silibinina é o constituinte majoritário da silimarina, correspondendo a cerca de 50 a 70% (HACKETT et al., 2013).

Há muitos anos a silimarina vem sendo utilizada para o tratamento de doenças hepáticas de diferentes etiologias (THAKUR, 2002) principalmente devido às suas propriedades antioxidantes (SONG et al., 2006) e de regeneração de tecidos (ABENAVOLI et al., 2010). Além da capacidade hepatoprotetora, dados da literatura têm demonstrado que a silimarina/silibinina também apresentam propriedades antioxidantes (de OLIVEIRA et al., 2015), anti-apoptóticas (MANNA et al., 1999; YANG et al., 2018), anti-inflamatórias (GUPTA et al., 2000; STOLF et al., 2018) e neuroprotetoras (LU et al., 2009; RAZA et al., 2011; FERNANDES et al., 2018).

Embora, o efeito neuroprotetor da silimarina/silibinina seja em grande parte devido as suas propriedades antioxidantes (GALHARDI et al., 2009), essas substâncias podem agir modulando outras vias de sinalização celular assim como enzimas (HADDADI; NAYEBI; BROOSHGHALAN, 2018; de OLIVEIRA et al., 2015; PEREZ et al., 2014) envolvidas nos processos neurodegenerativos conferindo assim, uma neuroproteção adicional.

Vários modelos animais vêm sendo utilizados no estudo da DP, dentre eles pode-se citar o modelo da 6-hidroxidopamina (6-OHDA) (LINDGREN et al., 2007; PADOVAN-NETO et al., 2009; SMITH et al., 2012; RIBEIRO et al., 2013). Esta neurotoxina, um análogo da dopamina (DA), possui alta afinidade pelo transportador de catecolaminas devido a sua semelhança química com estas (BOVÉ; PERIER, 2012), produzindo degeneração dos neurônios dopaminérgicos no sistema nervoso central (SNC) (BLUM et al., 2001). A fim de encontrar substâncias com potencial terapêutico que possam agir como adjuvantes no tratamento da DP vários compostos têm sido testados no modelo da 6-OHDA (ZHANG et al., 2012; PASBAN-ALIABADI et al., 2013; RIBEIRO et al., 2013).

Dessa forma, com a utilização de um modelo animal tipo parkinsonismo induzido por 6-OHDA, pretende-se avaliar neste trabalho os possíveis efeitos modulatórios da silimarina e de seu constituinte majoritário, a silibinina, bem como investigar se a associação da silimarina com a levodopa, poderia levar a melhora dos sintomas associados as alterações comportamentais e neuroquímicas induzidas por 6-OHDA.

## 2 REFERENCIAL TEÓRICO

### 2.1 DOENÇA DE PARKINSON

#### 2.1.1 Epidemiologia

A DP é uma doença neurodegenerativa crônica e progressiva de distribuição universal que atinge todos os grupos étnicos e classes socioeconômicas (BOVÉ; PERIER, 2012). É considerada a segunda doença neurodegenerativa mais comum, afetando cerca de 1 a 2% da população mundial acima de 65 anos (SUBRAMANIAM; CHESSELET, 2013). Estima-se que ela atinge cerca de 10 milhões de pessoas em todo o mundo (EUROPEAN PARKINSON'S DISEASE ASSOCIATION, 2018).

A incidência da DP na população em geral é de 14 a cada 100.000 pessoas, enquanto que naquelas com idade superior a 65 anos a incidência aumenta para 160 a cada 100.000 pessoas (ASCHERIO; SCHWARZSCHILD, 2016). Nos Estados Unidos da América estima-se que surjam 59 mil casos/ano (LAU; BRETELER, 2006; WIRDEFELT et al., 2011). Além disso, estudos têm demonstrado que a incidência da DP é maior em homens do que em mulheres. As explicações para a preponderância masculina incluem efeitos protetores do estrogênio, maior frequência de exposição ocupacional à toxinas, bem como de trauma cranioencefálico em homens, além de ter sido encontrados genes de susceptibilidade recessiva no cromossomo X (TAYLOR; COOK; COUNSELL, 2007; WOOTEN et al., 2004).

No Brasil, não há estudos de prevalência e incidência da DP em grande escala, o que não permite que haja um panorama evidente em relação à sua epidemiologia. No entanto, levando em consideração a população brasileira acima de 65 anos de idade, estima-se que pelo menos 200 mil pessoas tenham a doença e que 36 mil novos casos surjam por ano no país (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2013; SANTOS, 2015). Um estudo realizado na cidade de Bambuí, em Minas Gerais, encontrou uma prevalência de 3,3% em pessoas com idade acima de 65 anos (BARBOSA et al., 2006).

### 2.1.2 Fisiopatologia da DP

A DP foi descrita inicialmente, em 1817, pelo médico inglês James Parkinson em sua monografia intitulada “*Essay on the Shaking Palsy*” (“Ensaio da paralisia agitante”) (DAUER; PRZEDBORSKI, 2003). Mais tarde, em 1875, o neurologista francês Jean Martin Charcot sugeriu o nome *maladie de Parkinson* (doença de Parkinson) para o distúrbio caracterizado por tremor em repouso, rigidez, imobilidade facial característica e distúrbios de marcha e postura (MENESES; TEIVE, 1996). Além disso, sabe-se que a DP tem sua gênese relacionada ao sistema dopaminérgico.

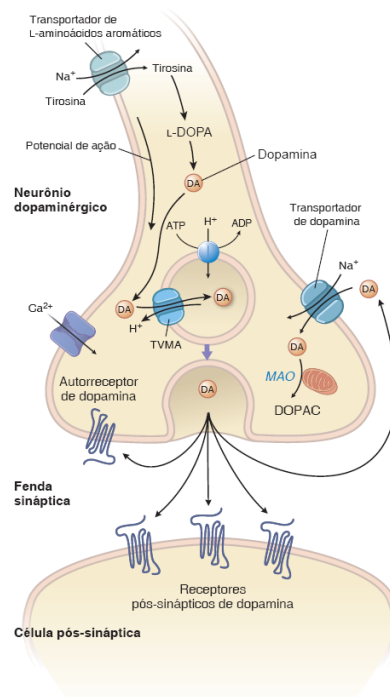
O sistema dopaminérgico possui como neurotransmissor a DA, cujo precursor da síntese é o aminoácido tirosina. Sua hidroxilação, formando dopa, é mediada via enzima tirosina hidroxilase (TH), sendo esta a etapa limitante na biossíntese da DA (STANDAERT; ROBERSON, 2012). A etapa posterior dessa biossíntese é determinada pela aminoácido aromático descarboxilase (AADC), que catalisa a reação de remoção do grupo carboxila da levodopa, tendo como produto final a DA. A DA é então liberada na fenda sináptica em resposta ao estímulo nervoso que leva a um aumento de cálcio citosólico e despolarização do neurônio pré-sináptico (Figura 1) (STANDAERT; WALSH, 2014).

A DA presente no espaço sináptico pode agir em receptores dopaminérgicos do tipo D<sub>1</sub> ou D<sub>2</sub>, exercendo suas ações celulares. Esta catecolamina estando no espaço sináptico pode ser transportada para o interior dos neurônios pré-sinápticos pelo transportador de dopamina (TDA), levando a redução dos níveis de DA extracelular (AMARA; KUHAR, 1993). Além disso, a ligação da DA em receptores pré-sinápticos levam à redução na atividade da TH e conseqüentemente a redução na sua síntese (STANDAERT; ROBERSON, 2012).

Após suas ações a DA pode ser novamente armazenada em vesículas sinápticas ou ser metabolizada. O metabolismo ocorre principalmente através da atividade da enzima monoaminoxidase (MAO) e da catecol-O-metiltransferase (COMT) formando como principais metabólitos o ácido homovanílico (HVA), o ácido 3,4-dihidroxifenilacético (DOPAC) e a 3-metoxitiramina (3-MT) (STANDAERT; WALSH, 2014).



Figura 1 - Neurotransmissão dopaminérgica



Fonte: (adaptado de STANDAERT; WALSH, 2014).

Os neurônios dopaminérgicos originam-se, em sua maior parte, em áreas distintas do cérebro, e apresentam projeções divergentes. O maior trato da DA no cérebro é a via nigroestriatal, que contém cerca de 80% da DA do cérebro. Essa via projeta-se dos corpos celulares para a parte compacta da substância negra (SNpc) até as terminações que inervam o estriado e está envolvida na estimulação do movimento voluntário (STANDAERT; WALSH, 2014).

O estriado é a principal estrutura de influxo dos núcleos da base, que são compostos além do estriado (caudado e putâmen) pelo o globo pálido, a substância negra e o núcleo subtalâmico (BLANDINI et al., 2000). O estriado recebe influxo glutamatérgico excitatório de muitas áreas do córtex cerebral. Contém neurônios de projeção que expressam receptores dopaminérgicos do tipo D<sub>1</sub> e D<sub>2</sub>, assim como interneurônios que usam acetilcolina (ACh) como um neurotransmissor (STANDAERT; ROBERSON, 2012).

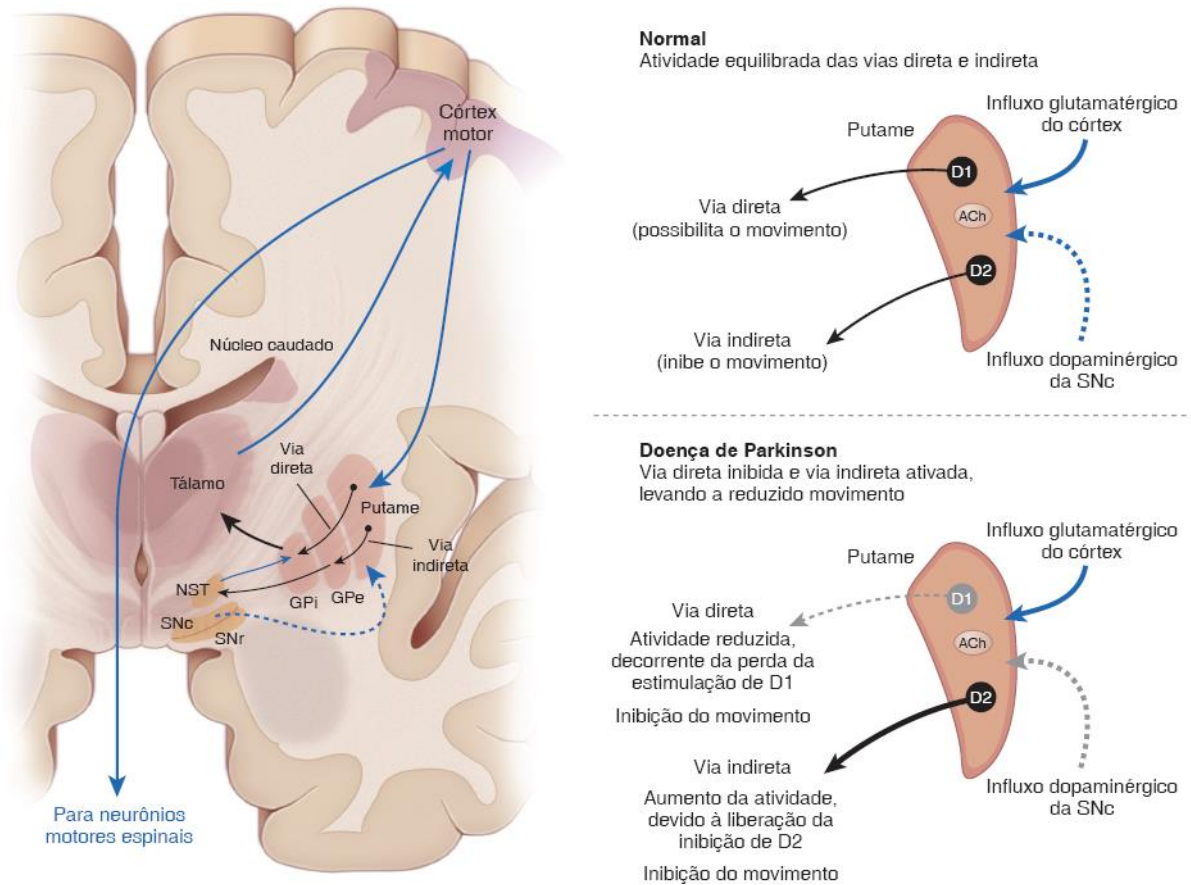
A inervação do estriado estende-se ao longo de dois trajetos, a via direta e indireta. Os neurônios estriatais que originam a via direta expressam principalmente o receptor dopaminérgico do tipo D<sub>1</sub> acoplado à proteína G<sub>s</sub> que leva a um aumento na produção de adenosina monofosfato cíclico (AMPc) (excitatório) enquanto os que

constituem a via indireta exprimem o receptor dopaminérgico do tipo D<sub>2</sub> acoplado à proteína G<sub>i</sub> que leva a uma diminuição na produção de AMPc (inibitório). Assim, a DA liberada no estriado aumenta a atividade da via direta e reduz a da via indireta, aumentando a excitabilidade dos neurônios motores superiores e possibilitando o movimento (STANDAERT; ROBERSON, 2012). Alterações nesse sistema resulta em anormalidades do movimento, que são características da DP.

As principais características fisiopatológicas da DP são a perda progressiva de neurônios dopaminérgicos pigmentados localizados na SNpc e a presença de inclusões citoplasmáticas proteináceas, denominadas corpúsculos de Lewy, que são principalmente formadas por agregados de  $\alpha$ -sinucleína (DAUER; PRZEDBORSKI, 2003; SPILLANTINI et al., 1998). A perda desses neurônios na SNpc resultam em uma diminuição dos níveis de DA no estriado (BLANDINI et al., 2000).

Na DP a morte dos neurônios dopaminérgicos da SNpc geram alterações na atividade das vias direta e indireta dos núcleos da base; a via direta está menos ativa, enquanto a via indireta apresenta um aumento na atividade. Dessa forma, como efeito final há um aumento do efluxo inibitório provenientes da parte reticulada da substância negra (SNpr) e da parte interna do globo pálido (GPi) (via indireta) para o tálamo, reduzindo a excitação do córtex motor, o que conduz aos sintomas motores típicos observados na DP (Figura 2) (BLANDINI et al., 2000; STANDAERT; ROBERSON, 2012).

Figura 2 - Efeito da DP sobre as vias dopaminérgicas que regulam o movimento



Fonte: (adaptado de STANDAERT; WALSH, 2014). (ACh: acetilcolina; D<sub>1</sub> e D<sub>2</sub>: receptores dopaminérgicos; GPi, segmento interno do globo pálido; GPe, segmento externo do globo pálido; SNc, parte compacta da substância negra; SNr, parte reticulada da substância negra; NST, núcleo subtalâmico).

O aparecimento dos sintomas motores na DP ocorre quando a morte dos neurônios dopaminérgicos atinge aproximadamente 50 a 60% na SNpc e há uma redução de cerca de 70 a 80% dos níveis de DA no estriado (DAUER e PRZEDBORSKI, 2003). No entanto, acredita-se que o processo neurodegenerativo na DP inicie mais ou menos 20 anos antes da manifestação dos sintomas motores clássicos, o que é denominado período pré-clínico da doença. Nesse período, o paciente pode apresentar sintomas não-motores como ansiedade, depressão, constipação, dano olfatório e distúrbio comportamental do sono (CUMMINGS, 1992; RICHARD, 2005; SAVICA; ROCCA; AHLSSKOG, 2010). Os sintomas motores clássicos da DP são: bradicinesia, tremor em repouso, rigidez e anormalidades posturais (KLOCKGETHER, 2004).

A bradicinesia é caracterizada pela dificuldade em iniciar o movimento, pobreza e lentidão de movimentos e está relacionada à dificuldade de programação e execução dos movimentos (KLOCKGETHER, 2004). Esse é considerado o sintoma que mais incapacita o paciente, pois os movimentos habituais e automáticos estão reduzidos, comprometendo as atividades diárias. Além disso, pode-se observar que os passos tornam-se lentos e curtos (LIMONGI, 1995).

O tremor em repouso é o sintoma inicial em cerca de 60 a 70% dos pacientes e ocorre principalmente nos membros superiores mas também pode afetar as pernas e menos frequentemente a cabeça, não sendo necessariamente incapacitante por ser atenuado pelo movimento voluntário (MENESES e TEIVE, 1996). A rigidez muscular pode ser definida como uma maior resistência da articulação ao movimento passivo (DAUER e PRZEDBORSKI, 2003) e a uma capacidade reduzida de relaxar os músculos dos membros (KLOCKGETHER, 2004). A anormalidade postural se caracteriza pela instabilidade, devido à perda do reflexo postural. Em estágios mais avançados da doença o paciente está predisposto a perda do equilíbrio e as quedas podem se tornar frequentes (MORRIS et al., 2000).

É importante ressaltar que a evolução da doença, a gravidade e a progressão dos sintomas variam de um paciente para outro (LEES; HARDY; REVESZ, 2009).

### **2.1.3 Etiologia**

A etiologia da DP ainda é desconhecida. Ela vem sendo considerada como uma doença multifatorial resultado da interação entre diversos fatores como a idade, predisposição genética e fatores ambientais. A idade é considerada um fator de risco para o desenvolvimento e para a progressão da DP por afetar diversos processos celulares que predispõem a neurodegeneração. A acumulação de danos somáticos relacionados a idade combinado com a falha de mecanismos compensatórios podem levar a uma aceleração da DP com a idade (HINDLE, 2010).

O envelhecimento também está associado com o aparecimento de disfunção mitocondrial, aumento na produção de radicais livres e aumento do estresse oxidativo (MAGLIORE; COPPEDÉ, 2009). Além disso, juntamente com o processo de envelhecimento é observado uma diminuição na expressão da tirosina hidroxilase (TH), uma redução no número de neurônios pigmentados na SN e na densidade de

receptores dopaminérgicos (RUDOW et al., 2008). Todas essas mudanças relacionadas à idade podem favorecer o aparecimento da DP (HINDLE, 2010).

A DP atribuída a fatores genéticos aparece em 5% a 10% dos pacientes, e tem sido associada a mutações em diversos genes que codificam proteínas como  $\alpha$ -sinucleína, LRRK2, parkina, DJ-1 e PINK1 (JAIN; WOOD; HEALY, 2005). A  $\alpha$ -sinucleína é uma proteína localizada no terminal pré-sináptico que está envolvida na plasticidade neuronal (CLAYTON; GEORGE, 1998) e uma mutação nesta proteína tem sido associada a DP familiar com formação de corpúsculos de Lewy, enquanto que uma mutação na parkina está relacionada a DP de início precoce, antes dos 40 anos e as alterações patológicas incluem degeneração seletiva de neurônios pigmentados na SN e, geralmente, falta de corpúsculos de Lewy (HATTORI et al., 2003).

As proteínas DJ-1 e PINK1 têm demonstrado um papel em proteger neurônios dopaminérgicos do estresse oxidativo e/ou do dano mitocondrial. No entanto, essa função é perdida quando ocorre uma mutação podendo levar a degeneração de neurônios nigrais (JAIN; WOOD; HEALY, 2005; YANG et al., 2005). Mutações na LRRK2 também é uma das causas de DP familiar de início precoce (ZIMPRICH et al., 2004). Entretanto, não se conhece os mecanismos pelos quais a forma mutante desta proteína induz neurodegeneração (OLANOW, 2007).

Além disso, fatores externos ou comportamentais como exposição a neurotoxinas ambientais (pesticidas e herbicidas, químicos industriais), que afetam o complexo I mitocondrial ou causam estresse oxidativo (TANNER et al., 2011); e lesões cerebrais traumáticas que podem causar um dano da barreira hematoencefálica, inflamação cerebral, interrupção da função mitocondrial e acumulação de  $\alpha$ -sinucleína no cérebro (RUGBJERG et al., 2008) podem contribuir para o aumento da incidência da DP. Somado a isso, fatores dietéticos que podem alterar o equilíbrio oxidativo no cérebro ou servir como veículo para neurotoxinas ambientais associados a uma predisposição genética aumentam o risco de desenvolver DP idiopática (CHEN et al., 2007).

A neuropatologia da DP não está restrita exclusivamente à perda de DA na via nigroestriatal, alterações em outros neurotransmissores como noradrenalina (NA), serotonina (5-HT) e acetilcolina (Ach) contribuem para o desenvolvimento dos sintomas observados na DP (DAUER; PRZEDBORSKI, 2003).

### 2.1.4 Diagnóstico da DP

Até o momento não existem exames laboratoriais, marcadores biológicos ou estudos de imagem que confirmem o diagnóstico definitivo da DP (OGAWA et al., 2018). O diagnóstico é baseado em critérios clínicos e exame físico minucioso. Os critérios do banco de cérebros da Sociedade de doença de Parkinson do Reino Unido (do inglês *UK Parkinson's Disease Society Brain Bank*) são os mais utilizados para o diagnóstico clínico da DP (BRASIL, 2010).

Segundo a Sociedade de doença de Parkinson do Reino Unido o paciente terá o diagnóstico da DP se apresentar bradicinesia, um dos critérios do item I e pelo menos três critérios do item III (Tabela 1) (BRASIL, 2010):

Tabela 1 – Critérios para o diagnóstico clínico da DP

<b>I – Critérios para diagnóstico da DP</b>
a. Rigidez muscular
b. Tremor de repouso 4-6 Hz
c. Instabilidade postural não causada por distúrbios visuais, vestibulares, cerebelares ou proprioceptivos.
<b>II – Critérios de exclusão para DP</b>
a. História de acidente vascular cerebral de repetição
b. História de trauma craniano grave
c. História definida de encefalite
d. Crises oculogíricas
e. Tratamento prévio com antipsicóticos
f. Remissão espontânea dos sintomas
g. Quadro clínico estritamente unilateral após 3 anos
h. Paralisia supranuclear do olhar
i. Sinais cerebelares
j. Sinais autonômicos precoces
k. Demência precoce
l. Liberação piramidal de Babinski
m. Tumor cerebral ou hidrocefalia comunicante
n. Resposta negativa à levodopa
o. Exposição ao 1-metil-4-fenil-1,2,3,6-tetraidropiridina (MPTP)
<b>III – Critérios de suporte positivo para o diagnóstico da DP</b>
a. Início unilateral
b. Presença de tremor em repouso
c. Doença progressiva
d. Persistência da assimetria dos sintomas
e. Boa resposta à levodopa
f. Presença de discinesias induzida pela levodopa
g. Resposta à levodopa por 5 anos ou mais
h. Evolução clínica de 10 anos ou mais

Fonte: (adaptado de HUGHES et al., 1992).

### 2.1.5 Tratamentos disponíveis

Atualmente não existe terapia farmacológica que seja eficaz na prevenção, na cura ou capaz de retardar a progressão da DP. As abordagens terapêuticas atuais são apenas paliativas e consistem em reposição de DA, inibição de sua degradação ou utilização de agonistas dopaminérgicos para tratamento sintomático das manifestações clínicas da doença (STANDAERT; WALSH, 2014). A escolha do medicamento mais adequado deve levar em consideração fatores como o estágio da doença, a sintomatologia presente, a idade do paciente, os medicamentos em uso e os custos dos mesmos, a ocorrência de efeitos adversos, além da presença de comorbidades (BRASIL, 2010).

De acordo com o Protocolo Clínico e Diretriz Terapêutica do Sistema Único de Saúde (SUS) para pessoas com DP, publicado pela portaria nº 228, de 10 de maio de 2010, os medicamentos disponíveis no SUS para o tratamento da DP são: levodopa/carbidopa, levodopa/benserazida, bromocriptina, pramipexol, amantadina, biperideno, triexifenidil, selegilina, tolcapona e entacapona.

Os agonistas dopaminérgicos como a bromocriptina e o pramipexol agem nos receptores dopaminérgicos, principalmente do tipo D<sub>2</sub> (STANDAERT; ROBERSON, 2012) e podem ser utilizados em monoterapia para o controle dos sintomas motores na fase inicial da doença (BRASIL, 2010). Esses medicamentos são particularmente eficazes no tratamento de pacientes jovens que possuem maior risco de aparecimento de discinesias ou em associação a levodopa em pacientes com flutuações motoras (PINTER; POGARELL; OERTEL, 1999; PARKINSON STUDY GROUP, 2000). A principal limitação ao uso dos agonistas dopaminérgicos é sua tendência em induzir efeitos indesejáveis, que podem incluir náuseas, hipotensão, sedação excessiva e alucinações (STANDAERT; WALSH, 2014).

A isoenzima MAO do subtipo B (MAO-B) é a forma predominante no estriado e é a responsável pela maior parte do metabolismo oxidativo da DA no cérebro (STANDAERT; ROBERSON, 2012). Assim, a selegilina, um inibidor da enzima MAO-B que age diminuindo a degradação da DA, é eficaz no tratamento sintomático da DP e geralmente é bem tolerada em pacientes jovens com DP inicial ou leve (PARKINSON STUDY GROUP, 1989). No entanto, em pacientes com DP avançada ou com disfunção cognitiva, a selegilina pode acentuar os efeitos motores e cognitivos,

além de causar insônia, hipotensão e confusão mental (STANDAERT; ROBERSON, 2012).

A entacapona e tolcapona, inibidores da COMT são eficazes no controle sintomático da doença quando usados em associação com a levodopa. A tolcapona atua por inibição central e periférica da COMT, no entanto está associado à hepatotoxicidade (STANDAERT; WALSH, 2014). Já a entacapona inibe a COMT periférica e geralmente é administrada junto com cada dose de levodopa. A inibição dessa enzima reduz o metabolismo da levodopa resultando em uma manutenção prolongada dos seus níveis séricos e, portanto, uma resposta clínica mais prolongada (STANDAERT; ROBERSON, 2012).

Os anticolinérgicos biperideno e triexifenidil, úteis no tratamento da DP inicial ou como adjuvante à levodopaterapia, agem diminuindo o desequilíbrio da neurotransmissão de Ach/DA por antagonizar os receptores muscarínicos de Ach (STANDAERT; ROBERSON, 2012). No entanto, causam uma variedade de efeitos adversos anticolinérgicos, que podem incluir boca seca, retenção urinária e comprometimento da memória e cognição (STANDAERT; WALSH, 2014).

A amantadina é um antagonista dos receptores de glutamato (Glu) do tipo N-metil-D-aspartato (NMDA) que diminuiu a excitabilidade córtico-estriatal e o desequilíbrio da neurotransmissão de Glu/DA e tem sido usada como terapia inicial na DP leve ou em associação à levodopa em pacientes que apresentam flutuações dependentes da dose e discinesias (GREENAMYRE; O'BRIEN, 1991; VERHAGEN METMAN et al., 1998). Entretanto não há estudos avaliando seus benefícios a longo prazo, além disso, produzem efeitos adversos como confusão mental e alucinações visuais (MOREIRA et al., 2007).

A levodopa, introduzida como terapia de reposição de DA no início dos anos 60, ainda hoje, é o fármaco mais efetivo no controle dos sintomas motores da DP (COTZIAS; PAPAVALIOLIS; GELLENE, 1969; KATZENSCHLAGER; LEES, 2002). Seu mecanismo de ação consiste em sua conversão a DA pela AADC, aumentando assim os níveis de DA cerebral (ALACHKAR et al., 2010). Na prática clínica, a levodopa é administrada juntamente com inibidores periféricos da AADC, como a carbidopa ou benserazida, que não penetram bem o SNC. Essa inibição da AADC periférica promove o aumento da biodisponibilidade central da levodopa e reduz a incidência de efeitos hipotensores e gastrointestinais indesejáveis decorrentes da sua metabolização periférica (STANDAERT; ROBERSON, 2012).



Nos estágios iniciais da DP, a terapia com levodopa é altamente efetiva. No entanto, o tratamento prolongado com este fármaco leva ao aparecimento de complicações motoras como flutuações na resposta clínica e o desenvolvimento de movimentos involuntários anormais, conhecidos como discinesias (NAGATSU; SAWADA, 2009), comprometendo sua eficácia clínica. Flutuações motoras referem-se a respostas motoras flutuantes à administração de levodopa, com encurtamento da duração do seu efeito e interrupção de sua ação, levando a uma situação “resposta-falta de resposta” (fenômeno conhecido como liga/desliga) ao medicamento (AHLKOG; MÜENTER, 2001).

A discinesia induzida pela levodopa apresenta uma fenomenologia clínica complexa e geralmente afeta a face, pescoço e membros, mas pode afetar qualquer grupo muscular esquelético (NUTT, 1990). É importante salientar que aproximadamente 50% dos pacientes tratados com levodopa desenvolvem discinesia cerca de cinco anos após o início do tratamento e esse percentual se eleva para cerca de 95% após quinze anos de terapia com esse medicamento, apresentando assim, um impacto significativo na qualidade de vida dos pacientes (AHLKOG; MÜENTER, 2001). Dessa forma, levando em consideração que a levodopa é amplamente utilizada para o tratamento da DP, torna-se importante a busca por novas estratégias terapêuticas que visem minimizar os efeitos adversos da levodopa como as flutuações motoras e a discinesia.

## 2.2 PRODUTOS NATURAIS

Há séculos o homem busca, na natureza, recursos para atender suas necessidades básicas, entre as quais o uso de produtos naturais como remédio para o tratamento das enfermidades humanas (CRAGG; NEWMAN, 2013). Índícios do uso de plantas medicinais foram encontrados nas civilizações mais antigas, sendo considerada uma das práticas mais remotas utilizadas pelo homem para cura, prevenção e tratamento de doenças, fazendo com que a flora se constitua como importante fonte de compostos biologicamente ativos (ANDRADE et al., 2007).

O uso terapêutico de produtos naturais vem sendo praticado ao longo de séculos por diferentes culturas, fundindo hoje os conhecimentos populares das mais variadas origens e agregando novas descobertas científicas (OKIGBO; EME; OBOGU, 2008). Segundo a Organização Mundial da Saúde (OMS) cerca de 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 (WHO, 2002).

Atualmente a busca por novos medicamentos tem despertado o interesse tanto de cientistas, como da indústria farmacêutica em desenvolver pesquisas com o objetivo de descobrir novos princípios ativos e também aprimorar as descobertas de novas atividades farmacológicas de substâncias já conhecidas e oriundas de plantas. O estudo sistemático de compostos naturais é fundamental para a comprovação de seus benefícios terapêuticos e dos mecanismos bioquímicos subjacentes, além de identificar os possíveis riscos à saúde devido às ações tóxicas (CAMPOS et al., 2011).

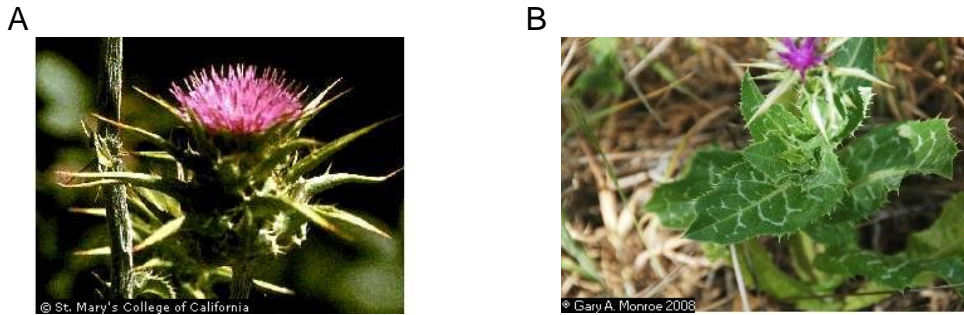
As propriedades terapêuticas das plantas medicinais são atribuídas a um ou mais princípios ativos presentes entre as centenas de substâncias originárias do seu metabolismo primário ou secundário, componentes que são conhecidos como fitocomplexos (GURIB-FAKIM, 2006). Nos últimos anos tem crescido os estudos com constituintes ativos de plantas, ervas e extratos como uma forma alternativa de tratamento e prevenção de doenças neurodegenerativas (AHMAD, et al., 2005; KHAN et al., 2010; MURATA et al., 2010; SINGHAL et al., 2011), uma vez que os tratamentos atualmente disponíveis tratam apenas os sintomas, não alterando o curso ou a progressão dessas doenças (CAMPOS et al., 2011).

Dessa forma, percebe-se a necessidade de mais pesquisas acerca dos potenciais usos assim como dos possíveis mecanismos farmacológicos das plantas medicinais com o propósito de elucidar seus potenciais efeitos, colaborando para a melhora da qualidade de vida dos pacientes.

### **2.2.1 *Silybum marianum* L. Gaertn.**

*Silybum marianum* é uma planta pertencente à família Asteraceae, encontrada nas regiões do Mediterrâneo e norte da África (ZHU et al., 2013; KROLL; SHAW; OBERLIES, 2007). Essa planta é um cardo robusto de um à três metros de altura que cresce em solos rochosos com capítulo floral de coloração roxa (Figura 3A). As folhas possuem manchas brancas características ao longo dos nervos e que, quando quebradas, expelem um líquido branco leitoso (Figura 3B), por isso é conhecida popularmente como cardo-de-leite ou cardo-mariano (LUPER, 1998).

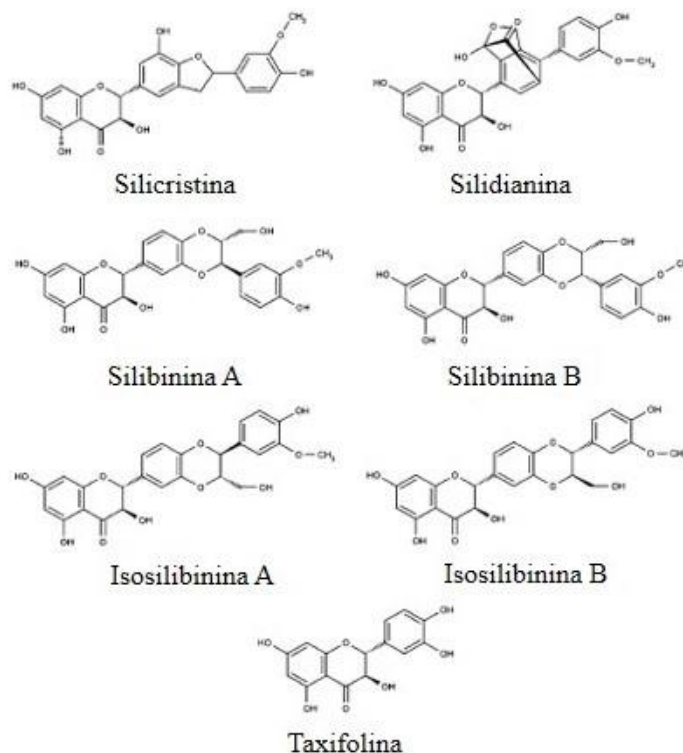
Figura 3 - *Silybum marianum* L. Gaertn. (Cardo mariano)



Fonte: (adaptado de USDA, NRCS, 2018). A – Flor; B – Folhas.

A partir dessa planta extrai-se a silimarina, uma mistura de flavonoides polifenólicos obtido das suas sementes que contém aproximadamente 70% a 80% de flavonolignanas (complexo de silimarina) e 20-30% de uma fração quimicamente indefinida, compreendendo principalmente compostos poliméricos e polifenólicos oxidados (LUPER, 1998; MORAZZONI; BOMBARDELLI, 1995). O complexo de silimarina consiste principalmente de silibinina (60-70%), do isômero isosilibinina (5%), silicristina (20%) e silidianina (10%) (Figura 4) (DIXIT et al., 2007; KREN; WALTEROVA, 2005). Dentre as substâncias minoritárias presentes no extrato estão também os flavonoides quercetina e taxifolina (KREN; WALTEROVA, 2005). A silibinina, uma mistura de dois diastereoisômeros A e B na proporção de aproximadamente 1:1, foi o primeiro membro da família de compostos naturais chamada flavonolignanas a ser descoberta em 1959 (BIEDERMAN et al., 2014) e é o constituinte predominante da silimarina (HACKETT et al., 2013).

Figura 4 - Estrutura química dos principais componentes do complexo da silimarina



Fonte: (adaptado de ZHU et al., 2013).

Desde a antiguidade, extratos de *Silybum marianum* têm sido utilizados no tratamento de doenças hepáticas e problemas do trato gastrointestinal (FLORA et al., 1998). Atualmente, preparações contendo silimarina são comercializadas na forma de cápsulas (Legalon®) e utilizadas no tratamento da cirrose, hepatite crônica e doenças associadas ao consumo de álcool, além de atuarem como protetores contra uma série de hepatotoxinas (THAKUR, 2002; FLORA et al., 1998). Em modelos animais, têm mostrado proteção contra lesões hepáticas induzidas por toxinas como tetracloreto de carbono, galactosamina, tioacetamida, álcool, paracetamol, benzopireno e endotoxinas bacterianas (SURAI, 2015).

O potencial benéfico da silimarina e da silibinina no tratamento de doenças hepáticas ainda não é totalmente compreendido, mas acredita-se que está associado às suas propriedades antioxidantes (SONG et al., 2006) e de regeneração de tecidos (ABENAVOLI et al., 2010). Um estudo de Muezes et al. (1990) demonstrou que a silimarina aumentou a atividade da enzima superóxido dismutase (SOD) em eritrócitos

e linfócitos e diminuiu a concentração de malondialdeído em pacientes com doença hepática alcoólica crônica.

As propriedades antioxidantes da silimarina e da silibinina tem sido demonstradas em diversos modelos *in vitro* e *in vivo* de estresse oxidativo induzido por dano hepatocelular (COMELLI et al., 2007). Ambas exercem proteção contra a peroxidação lipídica, restauram e/ou potencializam enzimas antioxidantes como a SOD, catalase (CAT) e glutathiona peroxidase (GPx), além de afetarem a liberação e expressão de citocinas pro-inflamatórias como interleucinas e o fator de necrose tumoral (TNF) (MANSOUR; HAFEZ; FAHMY, 2006; PRADEEP et al., 2007; SONG et al., 2006; SCHUMANN et al., 2003).

Assim como outros flavonoides, a silimarina é rapidamente absorvida, sendo sua absorção oral em humanos em torno de 23 a 47% e o pico de concentração plasmática é atingido entre 4 a 6 horas (DIXIT et al., 2007). Estudos em roedores demonstraram uma baixa biodisponibilidade após a administração oral de silibinina, apresentando uma biodisponibilidade em torno de apenas 0,95% (WU et al., 2007). No entanto, a biodisponibilidade da silimarina no cérebro não é conhecida (BORAH et al., 2013), apesar desta ter a habilidade de atravessar a barreira hematoencefálica (WANG et al., 2002).

Após a administração oral do extrato de *Silybum marianum*, as flavonolignanas são rapidamente absorvidas e eliminadas com uma meia-vida no soro de aproximadamente 6 horas (LORENZ et al., 1984; JAVED et al., 2011). Como agentes terapêuticos, a silimarina e silibinina são bem tolerados, com um bom perfil de segurança, sendo reportados na literatura poucos efeitos adversos nas preparações à base de *Silybum marianum*, como sintomas gastrointestinais, dermatológicos e enxaqueca (JACOBS et al., 2002). Além disso, com diferentes doses e modos de administração em camundongos, ratos, coelhos e cães, tanto a silimarina quanto a silibinina demonstram baixa toxicidade mesmo em grandes doses, e não apresentam efeitos secundários, com valores altos de dose letal 50 (LD50) (DIXIT et al., 2007; FLORA et al., 1998).

Atualmente, a silimarina e a silibinina têm estado em destaque devido às suas múltiplas atividades benéficas que vão além da sua atividade hepatoprotetora e antioxidante (GAZAK; WALTEROVA; KREN, 2007). Estas incluem ações anticarcinogênicas e quimioprotetoras por inibir a angiogênese e modular reguladores da sinalização e ciclo celular (AHMAD et al., 1998; ZI; AGARWAL, 1999),

cardioprotetora (ÁGOSTON et al., 2003) e hipocolesterolêmica (SKOTTOVÁ; KRECMAN; ŠIMANEK, 1999). Esses compostos também têm demonstrado propriedades anti-apoptótica (MANNA et al., 1999; YANG et al., 2018), anti-inflamatórias (GUPTA et al., 2000; STOLF et al., 2018) e neuroprotetora contra doenças neurodegenerativas como doença de Alzheimer (LU et al., 2009), DP (SINGHAL et al., 2011) e isquemia cerebral (RAZA et al., 2011).

Vale ressaltar que em diferentes modelos animais de DP induzido por toxinas, a administração de silimarina protegeu contra a perda neuronal dopaminérgica (SINGHAL et al., 2011) e aumentou os níveis de DA e serotonina (5-HT) no hipocampo e regiões corticais do cérebro (LU et al., 2010). Interessantemente, também tem sido relatada sua capacidade em inibir *in vitro* a enzima MAO-B em cérebro de ratos (MAZZIO et al., 1998; de OLIVEIRA et al., 2015). Além disso, foi demonstrado que a silibinina pode ativar a via de sinalização Akt/mTOR, regular negativamente o marcador inflamatório NF- $\kappa$ B e positivamente o marcador anti-apoptótico, Bcl-2 na isquemia cerebral (WANG et al., 2012).

## 2.3 MODELOS ANIMAIS DE DP

Os modelos animais tem sido ferramentas importantes para estudar os mecanismos de diversas patologias e alvos promissores para intervenções terapêuticas (GERLACH; RIEDERER, 1996). Modelos experimentais da DP que utilizam neurotoxinas buscam elucidar mecanismos fisiopatológicos da degeneração dos neurônios dopaminérgicos, além de contribuir para o desenvolvimento de estratégias terapêuticas destinadas a tratar os sintomas, uma vez que esses modelos replicam características patológicas e fenotípicas da doença (BLANDINI; ARMENTERO, 2012; BOVÉ; PERIER, 2012). Estudos pré-clínicos utilizam modelos genéticos ou neurotoxinas como o MPTP, rotenona e 6-OHDA em roedores para a indução de algumas características clínicas da DP.

### 2.3.1 MPTP

O MPTP é uma toxina altamente lipofílica que atravessa a barreira hematoencefálica rapidamente (DAUER; PRZEDBORSKI, 2003). No cérebro é convertida em seu metabólito tóxico, 1-metil-4-fenilpiridínio (MPP<sup>+</sup>) que prejudica a

fosforilação oxidativa pela inibição do complexo I da cadeia transportadora de elétrons levando a morte de neurônios dopaminérgicos, devido a redução dos níveis de adenosina trifosfato (ATP) e aumento da produção de espécies reativas de oxigênio (EROs) (NICKLAS; VYAS; HEIKKILA, 1985).

Essa toxina é usualmente administrada por via subcutânea, intraperitoneal ou intramuscular. A sua toxicidade replica os principais sinais clínicos encontrados na DP: tremor, rigidez, acinesia e instabilidade postural (BEAL, 2001). No entanto, esse modelo tem sido criticado frequentemente por não reproduzir a morte progressiva dos neurônios dopaminérgicos observadas em pacientes com DP (BLESA et al., 2012).

### **2.3.2 Rotenona**

A rotenona é extraída de plantas leguminosas e comumente usada como inseticida e pesticida (HISATA, 2002). Ela é altamente lipofílica e ultrapassa facilmente a barreira hematoencefálica. Na mitocôndria, a rotenona impede a fosforilação oxidativa pela inibição do NADH-ubiquinona (BLESA et al., 2012).

O método de administração da rotenona pode ser por infusão intrajugular ou subcutâneo (BOVÉ; PERIER, 2012). Seu efeito neurotóxico ainda é muito discutido mas sabe-se que ela leva a formação de inclusões positivas de ubiquitina e  $\alpha$ -sinucleína nas células nigrais, morfologicamente similares aos corpúsculos de Lewy na DP (BETARBET et al., 2000). Entretanto seu uso em modelos de neuroproteção é limitado devido à grande variabilidade na sensibilidade dos animais frente a sua lesão (BEAL, 2001).

### **2.3.3 Paraquat**

O paraquat é um dos herbicidas mais usados no mundo. O seu uso para induzir DP teve origem na sua similaridade estrutural com o MPP<sup>+</sup> (DAUER; PRZEDBORSKI, 2003). A toxicidade do paraquat parece ser mediada pela formação de EROs e geração de estresse oxidativo (BLESA et al., 2012).

A administração sistêmica de paraquat em animais leva a degeneração de neurônios dopaminérgicos acompanhado por inclusões contendo  $\alpha$ -sinucleína (DAUER; PRZEDBORSKI, 2003). No entanto, uma desvantagem é a falta de

reprodutibilidade desse modelo, enquanto alguns animais demonstram perda de neurônios dopaminérgicos, outros não reproduzem essa neurotoxicidade (BOVÉ; PERIER, 2012).

#### **2.3.4 Modelos genéticos**

Mutações genéticas na DP são raras e representam apenas cerca de 10% de todos os casos da doença (DAUER; PRZEDBORSKI, 2003). Modelos animais dessas mutações ( $\alpha$ -sinucleína e LRRK2) são importantes pois representam potenciais alvos terapêuticos.

Duas mutações no gene da  $\alpha$ -sinucleína (A53T, A30P) tem sido usadas para criar camundongos transgênicos com o intuito de estudar o papel de agregados de  $\alpha$ -sinucleína na DP (BLESA et al., 2012). No entanto, esse modelo falha em não causar a morte de neurônios dopaminérgicos, desde que esses neurônios parecem ser resistentes a neurotoxicidade induzida por  $\alpha$ -sinucleína (DAUER; PRZEDBORSKI, 2003).

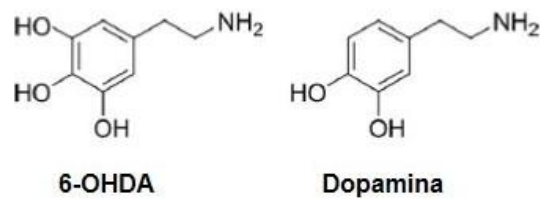
Mutações no gene LRRK2 mostraram causar uma forma dominante de DP (ZIMPRICH et al., 2004). Modelos de LRRK2 em camundongos transgênicos são utilizados para estudar a relação da mutação da LRRK2 com a DP, no entanto, mostram apenas níveis mínimos de neurodegeneração (BLESA et al., 2012).

#### **2.3.5 6-OHDA**

A 6-OHDA é uma neurotoxina estruturalmente análoga à DA (Figura 5) com alta afinidade pelo transportador de catecolaminas, utilizada há mais de 50 anos na produção de modelos experimentais da DP (UNGERSTEDT, 1968, BOVÉ; PERIER, 2012). O modelo da 6-OHDA é uma ferramenta útil para replicar a perda de neurônios dopaminérgicos no SNC, pois é considerado de baixa complexidade e baixo custo, além de possuir boa reprodutibilidade (BLESA et al., 2012).



Figura 5 – Comparação entre as estruturas químicas da 6-OHDA e DA



Fonte: (adaptado de Bóve; Perier, 2012).

Essa neurotoxina não atravessa a barreira hematoencefálica e, portanto deve ser injetada diretamente no cérebro para desencadear a morte neuronal no SNC (UNGERSTEDT, 1968). A 6-OHDA pode ser injetada nos ventrículos, através de uma injeção intracerebroventricular induzindo uma lesão bilateral, ou pode ser injetada diretamente na SN ou no corpo do estriado em apenas um dos hemisférios cerebrais induzindo assim, uma lesão unilateral (BOVÉ; PERIER, 2012). A amplitude da lesão é dependente da dose de 6-OHDA injetada e do local de injeção (BOVÉ; PERIER, 2012). Diferentes modelos utilizando 6-OHDA em roedores têm sido desenvolvidos a fim de se obter um grau de variação na neurodegeneração, do massivo ao moderado (CARMAN; GAGE; SHULTS, 1991; LEE; SAUER; BJORKLUND, 1996; RODRÍGUEZ et al., 2001). No entanto, uma desvantagem dessa toxina é que ela não produz degeneração extra-nigral ou inclusões do tipo corpúsculos de Lewy (DAUER; PRZEDBORSKI, 2003).

Após injeção local, essa neurotoxina é captada pelas moléculas transportadoras de DA e NA e se acumula no citosol (SCHOBER, 2004). No entanto, afim de proteger os neurônios noradrenérgicos do dano neuronal e seletivamente lesionar neurônios dopaminérgicos frequentemente é administrado um inibidor seletivo da recaptção da NA, como por exemplo a desmipramina ou a imipramina antes da administração de 6-OHDA (BOVÉ; PERIER, 2012).

As injeções de 6-OHDA produzem uma degeneração prolongada dos neurônios nigro-estriatais (SCHOBER, 2004). Os sinais de degeneração celular começam nas primeiras 24 horas da injeção intracerebroventricular, ficando estável após 7 dias de lesão (RODRÍGUEZ et al., 2001).

O mecanismo de toxicidade da 6-OHDA envolve a geração de estresse oxidativo desencadeado pela produção de EROs através de mecanismos enzimáticos

e não-enzimáticos (CHOI et al., 1999; SOTO-OTERO et al., 2000): a oxidação da 6-OHDA pela enzima MAO gera peróxido de hidrogênio ( $H_2O_2$ ) (BLUM et al., 2001). Além disso, essa neurotoxina sofre auto-oxidação, gerando  $H_2O_2$ , EROs e quinonas (KUMAR; AGARWAL; SETH, 1995). Os aumentos nos níveis de EROs e outras espécies reativas resultam na depleção de enzimas antioxidantes, amplificando a neurotoxicidade resultando, assim, em dano neuronal (BLUM et al., 2001).

A 6-OHDA pode, além de acentuar o estresse oxidativo, induzir a neurotoxicidade alterando a função mitocondrial, através da inibição da atividade do complexo I e IV da cadeia respiratória mitocondrial (GLINKA; YODIM, 1995). Devido aos déficits metabólicos gerados pelo bloqueio desses complexos enzimáticos, os neurônios não podem exercer normalmente suas funções fisiológicas e como resultado final ocorre a morte neuronal (GLINKA; TIPTON; YODIM, 1996). Esses dois mecanismos de toxicidade não estão necessariamente ligados, mas parecem agir sinergicamente durante a degeneração neuronal (SCHOBBER, 2004).

Além da esperada lesão neuronal e perda do conteúdo dopaminérgico, modelos experimentais fornecem importantes informações acerca das alterações comportamentais (SAMPAIO et al., 2017). A avaliação do comportamento motor de animais pode envolver a análise da atividade locomotora, coordenação motora e equilíbrio (MEREDITH; KANG, 2006). A lesão com 6-OHDA mimetiza várias características da degeneração de neurônios observadas nos pacientes com DP, sendo muito utilizada como modelo experimental para o estudo de tratamentos com potencial neuroprotetor, uma vez que ela reproduz a perda de neurônios dopaminérgicos e produz disfunções motoras detectáveis por testes comportamentais (CAI et al., 2016; MACHADO-FILHO et al., 2014). Dessa forma, a fim de encontrar substâncias com potencial terapêutico que possam agir como adjuvantes no tratamento da DP vários compostos têm sido testados no modelo da 6-OHDA (ZHANG et al., 2012; PASBAN-ALIABADI et al., 2013; RIBEIRO et al., 2013).

Dessa forma, considerando que a DP é um distúrbio incapacitante que reduz a qualidade de vida do paciente e os tratamentos atualmente disponíveis apresentam eficácia relativa, temporária e com o desenvolvimento de inúmeros efeitos adversos, torna-se relevante a busca por novas estratégias terapêuticas.

### 3 OBJETIVOS

#### 3.1 OBJETIVO GERAL

O objetivo geral deste estudo consistiu em investigar o efeito da silimarina e do seu principal constituinte, a silibinina em um modelo de parkinsonismo induzido por 6-OHDA em camundongos.

#### 3.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito da silimarina nas alterações comportamentais e bioquímicas induzidas por 6-OHDA;
- Investigar o efeito da silibinina nas alterações motoras e na imunorreatividade da TH induzidas por 6-OHDA;
- Verificar o efeito do co-tratamento com silimarina e levodopa nas alterações comportamentais induzidas por 6-OHDA.

#### 4 ARTIGO E MANUSCRITOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo científico e manuscritos os quais se encontram aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências, encontram-se nos próprios artigo e manuscritos. O **artigo** está disposto na forma que foi publicado na edição da Revista Científica **Food and Chemical Toxicology**. O **manuscrito 1** está disposto na forma como foi submetido para a Revista Científica **Journal of Neural Transmission**. O **manuscrito 2** se encontra em preparação e está disposto seguindo as normas exigidas pela Revista Científica **Behavioural Brain Research**.

## 4.1 ARTIGO

**Artigo****SILYMARIN RECOVERS 6-HYDROXYDOPAMINE-INDUCED MOTOR DEFICITS IN MICE**

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Food and Chemical Toxicology 118 (2018) 549–556

DOI: <http://dx.doi.org/10.1016/j.fct.2018.05.062>



## Silymarin recovers 6-hydroxydopamine-induced motor deficits in mice

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### 1. Introduction

Silymarin is a complex of flavonolignans isolated from the seeds of the milk thistle plant *Silybum marianum* (L.) Gaertn. (*Carduus marianus* L., Asteraceae), commonly used to treat liver diseases of different etiologies (Pepping, 1999). Currently, it is commercially available as food supplement in capsules form (Zeng et al., 2016) and it has been considered very safe with few reports on the adverse effects (Jacobs et al., 2002). Furthermore, previous studies have reported that silymarin crosses the blood–brain barrier and reaches the central nervous system (Nencini et al., 2007). This herbal medicine has shown antioxidant (de Oliveira et al., 2015; Singhal et al., 2011), anti-inflammatory (Gupta et al., 2000), anti-apoptotic (Manna et al., 1999), neuroprotective effects (Li et al., 2017; Perez et al., 2014) and inhibitory activity on monoamine oxidase (MAO) *in vitro* (de Oliveira et al., 2015). Of particular importance, studies have been carried out giving attention to protective effects of silymarin in different experimental models of neuronal injury (Baluchnejadmojarad et al., 2010; Murata et al., 2010; Perez et al., 2014; Raza et al., 2011).

6-hydroxydopamine (6-OHDA) is a neurotoxin that exhibits high affinity for the dopamine transporter and thus it is able to damage the dopaminergic neurons (Luthman et al., 1989). The 6-OHDA model has been used as a tool for replicating Parkinson's disease-like (PD-like) behavioral, biochemical, and pathological alterations (Blandini et al., 2008). In this context, several compounds have been tested in animal models of 6-OHDA aiming to find agents with therapeutic potential which could act as adjuvant in the treatment of Parkinson's disease (PD) symptoms (Afshin-Majd et al., 2015; Zhang et al., 2012, 2016).

Parkinson's disease (PD) is a neurodegenerative disease caused by progressive and selective degeneration of dopaminergic neurons of the *substantia nigra pars compacta* resulting in depletion of dopamine in the striatum (Fiorentini et al., 2013; Schapira, 2008). This disease is characterized by severe motor symptoms, including bradykinesia, resting tremor, rigidity and postural abnormalities (Lang and Lozano, 1998). Although the etiopathogenesis of PD is still not established, several

studies have demonstrated the involvement of dopaminergic and oxidative mechanisms (Miller et al., 2009). Moreover, the enzyme monoamine oxidase-B (MAO-B) and regulators of cellular signaling pathways as extracellular signal-regulated kinase 1/2 (ERK1/2) have been implicated in neurodegenerative diseases including PD (Kulich and Chu, 2001; Lieu et al., 2013).

Due a dopamine deficit observed in patients with PD, the most effective treatment is the pharmacological dopamine replacement with L-3,4-dihydroxyphenylalanine (L-dopa); however the long-term use of L-dopa causes undesirable adverse effects as motor fluctuations, abnormal involuntary movements known as dyskinesia (Ahlskog and Muenter, 2001; Obeso et al., 2000) and hallucinations (Aarsland et al., 1999; Moskovitz et al., 1978). Thus, these problems with the actual therapy stimulate the search for the development of new therapeutic strategies that could be used as adjuvant in the treatment of PD.

Based on the mentioned evidence, the present study aims to investigate the effects of silymarin on behavioral and biochemical alterations induced by 6-OHDA in mice.

### 2. Materials and methods

#### 2.1. Animals

One hundred male Albino Swiss mice weighing 25–35g and aged from 2 months, from breeding colony of experimental house of UFMS (Universidade Federal de Santa Maria). The animals were housed in polycarbonate cages (44 cm × 30 cm) with 4–5 animals per cage, with controlled temperature (22 ± 2°C) and under a 12 h light/dark cycle (lights on at 7:00 a.m.). Food and water were provided *ad libitum*. All experiments were performed in accordance with the guidelines of the National Council of Control of Animal Experimentation (CONCEA). All protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria under process number 4398020415/2015. All efforts were made to minimize animal suffering and to reduce the number of animals used.

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<https://doi.org/10.1016/j.fct.2018.05.062>

Received 9 March 2018; Received in revised form 8 May 2018; Accepted 26 May 2018  
Available online 28 May 2018

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## 2.2. Drugs

6-OHDA was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in 0.05% ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 0.9% NaCl (Tanaka et al., 2006). Vehicle consisted of 0.05% ascorbic acid plus 0.9% NaCl. Silymarin (powder) was commercially acquired in a local magistral pharmacy and its phytochemical profile was characterized by HPLC (de Oliveira et al., 2015). Pargyline, clorgyline, and kynuramine were obtained from Sigma-Aldrich (St. Louis, MO, USA).

## 2.3. Experimental design

Initially, the mice were randomly divided into two groups: control group (n = 40) and 6-OHDA group (n = 60).

Thirty minutes prior to 6-OHDA injection, all animals were treated with imipramine (25 mg/kg, intraperitoneally (i.p.)) to protect noradrenaline-containing neurons. After anesthesia with ketamine:xilazine (100 mg/kg; 10 mg/kg, i.p.), mice from 6-OHDA group received a single intracerebroventricular (i.c.v.) injection of 60 µg 6-OHDA (dissolved in 3 µL of physiological solution containing 0.05% ascorbic acid) into the right cerebral ventricle via a 10 µL Hamilton microsyringe (Tanaka et al., 2006). Control animals received the same volume of the vehicle (3 µL of physiological solution containing 0.05% ascorbic acid). The needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye and 1 mm posterior to a line drawn through the anterior base of the eyes (used as external reference) (Ribeiro et al., 2013). The solutions were injected into the lateral ventricle, at the following coordinates from bregma: anteroposterior (AP) = -0.1 mm, mediolateral (ML) = 1 mm, and dorsoventral (DV) = -2.4 mm (Laursen and Belknap, 1986). The injections were performed at a rate of 1 µL/min (Carvalho et al., 2013). The needle was left in place for additional 4 min before slowly retracting it (Carvalho et al., 2013). 6-OHDA normally causes a mortality rate of about 50%, which is observed within 24 h after 6-OHDA administration (Ribeiro et al., 2013). In the present study, four animals from control group died probably due to the anesthesia and in 6-OHDA group, twenty-eight animals died. Animals that survived the first 24 h after the injection did not present detectable signs of systemic toxicity during the experimental period.

One week after 6-OHDA administration mice were randomly assigned to one of eight groups: (I) control (n = 9); (II) silymarin 10 mg/kg (n = 9); (III) silymarin 30 mg/kg (n = 9); (IV) silymarin 100 mg/kg (n = 9); (V) 6-OHDA (n = 7); (VI) 6-OHDA + silymarin 10 mg/kg (n = 7); (VII) 6-OHDA + silymarin 30 mg/kg (n = 9); (VIII) 6-OHDA + silymarin 100 mg/kg (n = 9).

Mice received different doses of silymarin (10, 30 and 100 mg/kg, i.p.) or vehicle (physiological solution containing 1% ethanol) for 7 succeeding days. The i.p. route to the silymarin administration was chosen to increase its bioavailability. Based on results found in behavioral assessments, we selected two doses (30 and 100 mg/kg) of silymarin for the biochemical analysis.

The behavioral parameters were measured on days 8 and 15 of experimental protocol (from 8:00 a.m. to 10:00 a.m.). Four observers evaluated the behavior of mice. A different person distributed the animals to the observers to assure that the blind observation.

## 2.4. Behavioral testing

### 2.4.1. Rotarod test

The effect of the treatments on fine motor coordination was evaluated on the rotarod apparatus as previously described with some modification (Godoy et al., 2004). Twenty-four hours before the experiments, the animals were trained in the rotarod apparatus (8 rpm constant speed) (Insight Equipamentos, Ribeirão Preto, Brazil) throughout a session of 10 trials; each trial starts with the mouse being placed in the apparatus and ends when the mouse falls off the rod or

after reaching the cut-off time of 60 s. A resting time of 60 s was allowed between each trial. On the day 8 and 15 of the experimental period, the latency to first fall and the number of falls were evaluated on the rotarod with a stop watch up to 240 s.

### 2.4.2. Beam walk

Beam Walk test was performed to verify balance and motor performance. The animals were placed on a thin (1.5 cm × 75 cm) wooden bar elevated 90 cm from the floor, on the end of the beam it was attached a dark goal box with an opening on one end large enough for the mice to pass through. The time to cross the bar was recorded until reaching cut-off time of 120 s (Irintchev et al., 2005).

### 2.4.3. Open field test

To measure locomotor activity, mice were placed in the center of an open field arena (44 × 44 × 44 cm), divided into sixteen squares (4 rows of 4). The spontaneous locomotor (number of segments crossed with the four paws) and exploratory activities (expressed by the number of rearing on the hind limbs) were counted during 5 min without habituation session (Busanello et al., 2011).

## 2.5. Tissue preparation and biochemical assays

After the last behavioral test, on 15th day of experimental period, mice were euthanized by cervical dislocation and the brains were rapidly dissected and put on ice. The striatum and region containing the substantia nigra were separated and immediately frozen on powdered dry ice and thereafter stored at -80 °C.

### 2.5.1. Western blotting analyzes

The striatum and the region containing the substantia nigra were homogenized in lysis buffer (4% SDS, 2 mM EDTA, 50 mM Tris, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, 2 µg/mL aprotinin, 0.1 mM benzamide, 0.1 mM PMSF). Samples were boiled for 6 min and centrifuged at 4.000 g at 4 °C for 10 min. The supernatant was used to determine the protein concentration by Lowry method (Lowry et al., 1951). Then, the samples (40 µg for the striatum and 80 µg for the region containing the substantia nigra) were mixed with 10% glycerol and 8% 2-mercaptoethanol and resolved by 10% SDS-PAGE. The samples were transferred into nitrocellulose membrane (Millipore, USA). Proteins on the membrane were stained with a ponceau solution, as a loading control (Romero-Calvo et al., 2010). After staining, the membranes were dried and scanned for quantification. Membranes were then blocked with 1% bovine serum albumin and incubated overnight either with an anti-TH (1:10.000; Millipore; AB152) or anti-ERK1/2 (1:5.000; Cell Signaling; 4370). After, the membranes were incubated with alkaline phosphatase-coupled secondary antibody for 1 h (1:10.000; Millipore). 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.

### 2.5.2. Determination of MAO activity

Monoamine oxidase (MAO) activity was determined by measuring the kynuramine oxidation to 4-hydroxyquinoline (de Freitas et al., 2016). The striatum and the region containing the substantia nigra were homogenized in assay buffer (16.8 mM Na<sub>2</sub>HPO<sub>4</sub>, 10.6 mM KH<sub>2</sub>PO<sub>4</sub>, 3.6 mM KCl, pH 7.4). Brain homogenates, containing 0.25 mg of protein to region containing the substantia nigra and 0.15 mg of protein to striatum, were pre-incubated at 37 °C with 250 nM pargyline (selective MAO-B inhibitor) and 250 nM clorgyline (selective MAO-A inhibitor) for 20 min, for MAO-A and MAO-B activity estimation. The reaction was started by the addition of 600 µM kynuramine in the reaction mixture and then incubated at 37 °C for 30 min. The reaction was stopped with 10% trichloroacetic acid (TCA). The samples were

centrifuged at 500g for 8 min, and the supernatant was used to estimate the MAO A and B activity. Then, 1 mL of the supernatant was mixed with 1 mL of 1 N NaOH. The product of reaction was measured spectrofluorimetrically at 315 nm for excitation and 380 nm for emission. Results were expressed in nmol of 4-HQ per milligram of protein per minute (Matsumoto et al., 1985).

## 2.6. Statistical analysis

Statistical analysis was carried out using Statistica software version 7.0. All values are expressed as mean  $\pm$  standard error of mean. Data were statistically analyzed by unpaired *t*-test for behavioral tests on day 8. Kruskal-Wallis followed by *post-hoc* Dunn's test or one-way ANOVA followed by *post-hoc* Student-Newman-Keuls were performed for behavioral tests on day 15 when appropriate. For measuring TH and phospho-ERK1/2 immunoreactivity in striatum and substantia nigra one-way ANOVA followed by *post-hoc* Student-Newman-Keuls was performed. Differences were considered statistically significant with  $p < 0.05$ .

## 3. Results

### 3.1. Silymarin attenuates behavioral alterations caused by 6-OHDA on rotarod test

The effect of 6-OHDA and silymarin on fine motor coordination was evaluated by rotarod test. 6-OHDA administration induced a significant motor impairment in comparison with control group observed by a decrease on latency to first fall and an increase on number of falls on day 8 ( $p < 0.05$ ; Fig. 1A and B). Afterwards, control and 6-OHDA treated animals were subdivided into four another groups and treated with vehicle or different doses of silymarin (10, 30 or 100 mg/kg). Statistical analysis on day 15 revealed that treatment with 100 mg/kg of silymarin significantly increased the latency to fist fall ( $p < 0.05$ ;

Fig. 1C) and diminished the number of falls ( $p < 0.05$ ; Fig. 1D) on the rotarod in those animals previously treated with 6-OHDA when compared with 6-OHDA group. In addition, no statistical differences were found in silymarin treatment *per se* when compared with control group.

### 3.2. Silymarin alters the behavioral response induced by 6-OHDA on beam walk test

6-OHDA increased the latency to cross the bar in comparison with control group on beam walk test ( $p < 0.05$ ; Fig. 2A). *Post-hoc* analysis demonstrated that silymarin at all doses evaluated decreased the latency to cross the bar in those animals previously treated with 6-OHDA ( $F(7,60) = 4.48$ ;  $p < 0.05$ ; Fig. 2B). Furthermore, silymarin *per se* did not cause alteration on latency to cross the bar.

### 3.3. Silymarin recovers the behavioral alterations induced by 6-OHDA in open field test

Fig. 3A and B shows that 6-OHDA administration caused a marked decrease in spontaneous locomotor and exploratory activities ( $p < 0.05$ ), represented by the number of crossings and rearings, respectively, in the open field test after 6-OHDA administration on day 8. One-way ANOVA showed that the treatment with silymarin at all doses tested was effective in restoring the locomotor ( $F(7,60) = 4.55$ ;  $p < 0.05$ ; Fig. 3C) and exploratory behavior ( $F(7,60) = 3.57$ ;  $p < 0.05$ ; Fig. 3D) in those animals that received 6-OHDA. Treatment with silymarin *per se* did not modify the locomotor and exploratory activity of animals.

### 3.4. Silymarin does not alter the effect of 6-OHDA on the immunoreactivity of TH and phospho-ERK1/2 in the striatum

It was observed a significant decrease in TH immunoreactivity in the striatum of mice that received 6-OHDA compared with the control

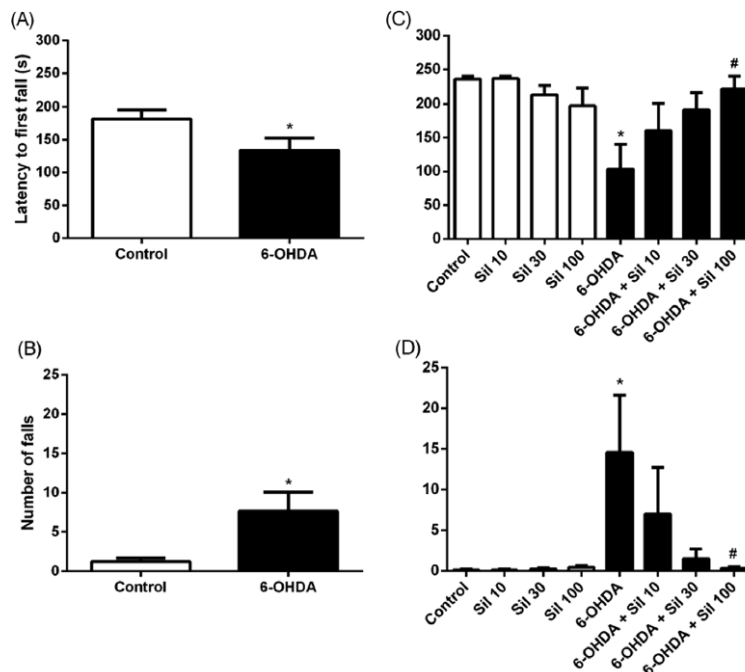


Fig. 1. Effect of 6-OHDA administration in mice on rotarod test. Latency to first fall (A) and number of falls (B) in the rotarod test on day 8. Data were analyzed by unpaired *t*-test. \* $p < 0.05$  compared with control group. Effect of silymarin on latency to first fall (C) and number of falls (D) in the rotarod test after 6-OHDA administration on day 15. Data are expressed as means  $\pm$  standard error of mean ( $n = 7-9$ ). \* $p < 0.05$  compared with control group; # $p < 0.05$  compared with 6-OHDA group (Kruskal-Wallis followed by Dunn's test) (Sil 10 - silymarin 10 mg/kg; Sil 30 - silymarin 30 mg/kg; Sil 100 - silymarin 100 mg/kg; 6-OHDA - 6-hydroxydopamine).



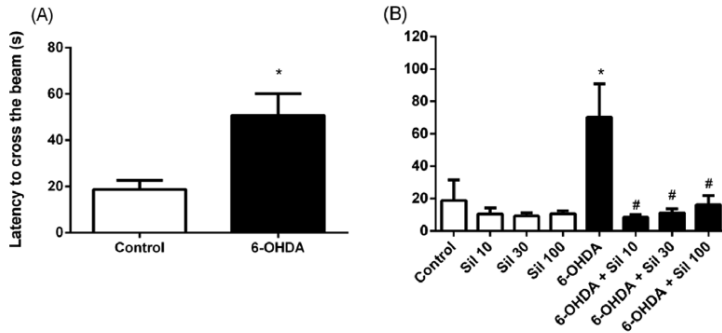


Fig. 2. Effect of 6-OHDA administration in mice on the beam walk test (A) on day 8. Data were analyzed by unpaired *t*-test. \**p* < 0.05 compared with control group. Effect of silymarin on the beam walk test (B) after 6-OHDA administration on day 15. Data are expressed as means  $\pm$  standard error of mean (*n* = 7–9). \**p* < 0.05 compared with control group; #*p* < 0.05 compared with 6-OHDA group (One-way ANOVA followed by Newman-Keuls test) (Sil 10 - silymarin 10 mg/kg; Sil 30 - silymarin 30 mg/kg; Sil 100 - silymarin 100 mg/kg; 6-OHDA - 6-hydroxydopamine).

group ( $F(5,24) = 6.71$ ;  $p < 0.05$ ; Fig. 4A). However, the treatment with silymarin was not able to recover the alterations caused by 6-OHDA.

With regard to ERK1/2 signaling, western blot analyses showed that 6-OHDA administration caused an increase in phospho-ERK1/2 immunoreactivity in striatum when compared with the control group ( $F(5,18) = 3.59$ ;  $p < 0.05$ ; Fig. 4B). However, the treatment with silymarin did not modify striatal phospho-ERK1/2 levels in those animals that received 6-OHDA. Silymarin treatment alone does not change the immunoreactivity of phospho-ERK1/2.

### 3.5. Silymarin modifies the effect of 6-OHDA on TH, but not on phospho-ERK1/2 immunoreactivity in the region containing the substantia nigra

When TH immunoreactivity was evaluated in the region containing the substantia nigra, it was observed a significant decrease in TH

immunoreactivity in the animals that received 6-OHDA compared with control group ( $F(5,24) = 3.01$ ;  $p < 0.05$ ; Fig. 5A). The treatment with 30 mg/kg of silymarin recovered the decrease caused by 6-OHDA. Furthermore, treatment with silymarin *per se* did not cause alterations in TH immunoreactivity in substantia nigra.

Moreover, 6-OHDA administration induced a significant increase in the phospho-ERK1/2 in the region containing the substantia nigra when compared with the control group ( $F(5,12) = 2.20$ ;  $p < 0.05$ ; Fig. 5B); however treatment with silymarin did not alter phosphorylated ERK1/2 levels in animals that received 6-OHDA. Furthermore, treatment with silymarin *per se* did not cause alterations in phospho-ERK1/2 immunoreactivity in substantia nigra.

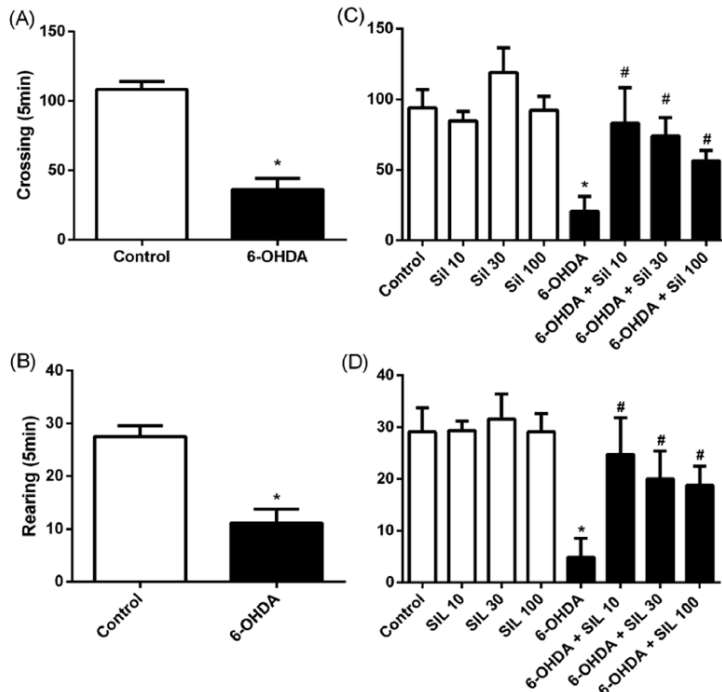


Fig. 3. Effect of 6-OHDA administration in mice in open field test. Number of crossing (A) and number of rearing (B) in the open field test on day 8. Data were analyzed by unpaired *t*-test. \**p* < 0.05 compared with control group. Effect of silymarin on number of crossing (C) and number of rearing (D) in the open field test after 6-OHDA administration on day 15. Data are expressed as means  $\pm$  standard error of mean (*n* = 7–9). \**p* < 0.05 compared with control group; #*p* < 0.05 compared with 6-OHDA group (One-way ANOVA followed by Newman-Keuls test) (Sil 10 - silymarin 10 mg/kg; Sil 30 - silymarin 30 mg/kg; Sil 100 - silymarin 100 mg/kg; 6-OHDA - 6-hydroxydopamine).

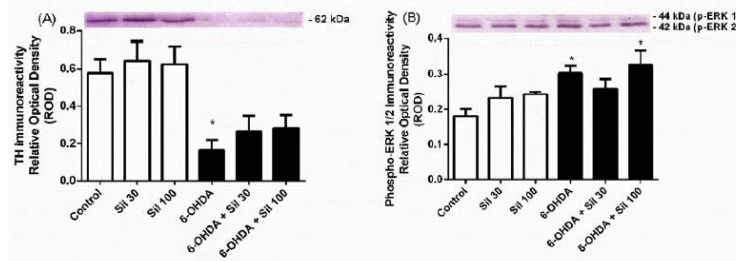


Fig. 4. Western blot analysis of TH (A) and phospho-ERK1/2 (B) in the striatum of mice treated with silymarin after 6-OHDA administration. Data are represented by relative optical density (ROD). Data are expressed as means ± standard error of mean (n = 3–5). \*p < 0.05 compared with control group (One-way ANOVA followed by Newman-Keuls test) (Sil 30 – silymarin 30 mg/kg; Sil 100 – silymarin 100 mg/kg; 6-OHDA – 6-hydroxydopamine).

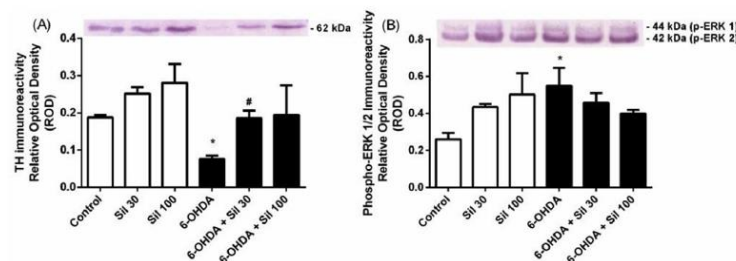


Fig. 5. Western blot analysis of TH (A) and phospho-ERK1/2 (B) in the region containing the substantia nigra of mice treated with silymarin after 6-OHDA administration. Data are represented by relative optical density (ROD). Data are expressed as means ± standard error of mean (n = 3–5). \*p < 0.05 compared with control group; #p < 0.05 compared with 6-OHDA group (One-way ANOVA followed by Newman-Keuls test) (Sil 30 – silymarin 30 mg/kg; Sil 100 – silymarin 100 mg/kg; 6-OHDA – 6-hydroxydopamine).

3.6. 6-OHDA and silymarin does not change the MAO activity in the striatum and the region containing the substantia nigra

With regard to MAO activity, both 6-OHDA administration as well as treatment with silymarin did not alter the MAO-A or MAO-B activity neither in the striatum nor in substantia nigra compared with the control group (Fig. 6).

OHDA-induced motor impairment in mice and if its effects could be related with changes in the immunoreactivity of TH and phospho-ERK1/2 as well as MAO activity. 6-OHDA administration caused a motor impairment, which was accompanied by a decrease in TH levels and an increase in phospho-ERK1/2 immunoreactivity in both striatum and substantia nigra region. Silymarin reversed 6-OHDA-induced motor impairment without changing the alterations caused by 6-OHDA in TH and phospho-ERK1/2 immunoreactivity in striatum. In the substantia nigra the treatment with 30 mg/kg of silymarin improved TH levels without recovering 6-OHDA-induced changes in phospho-ERK1/2 levels. The effect of silymarin and/or 6-OHDA on motor improvement

4. Discussion

In the present study, it was investigated if silymarin could recover 6-

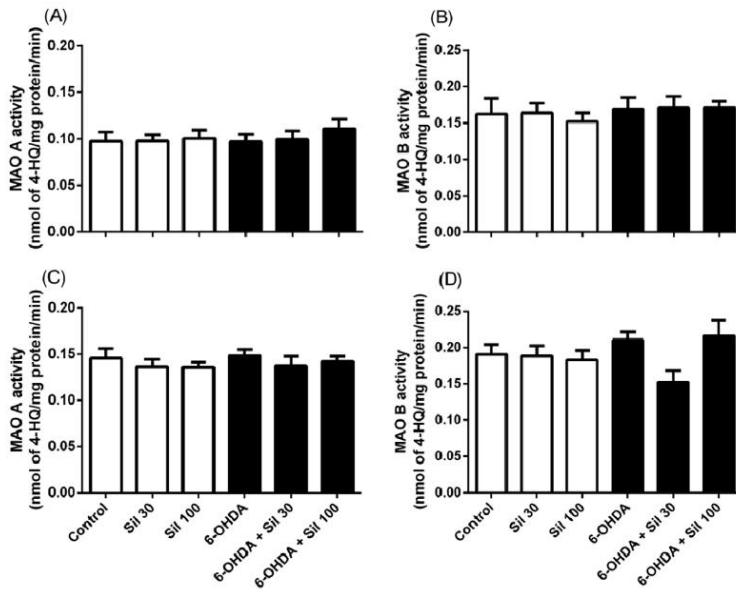


Fig. 6. MAO-A (A) and MAO-B (B) activity in striatum and MAO-A (C) and MAO-B (D) activity in the region containing the substantia nigra of mice treated with silymarin after 6-OHDA administration. Data are expressed as means ± standard error of mean (n = 7). One-way ANOVA followed by Newman-Keuls test (Sil 30 – silymarin 30 mg/kg; Sil 100 – silymarin 100 mg/kg; 6-OHDA – 6-hydroxydopamine).

seems not to involve changes in MAO activity.

Silymarin was chosen for this study due to its beneficial effects observed *in vitro* (de Oliveira et al., 2015; Podder et al., 2012; Wang et al., 2002) and *in vivo* studies (Hou et al., 2010; Murata et al., 2010; Singhal et al., 2011). Silymarin has showed protective effects against some models of neuronal injury such as cerebral ischemia (Raza et al., 2011), lipopolysaccharide induced neurotoxicity (Wang et al., 2002) and Alzheimer's Disease (Murata et al., 2010) which could be useful to slow down progression of the neurodegenerative diseases. Of particular importance, other researchers investigated the effects of silymarin on 6-OHDA and MPTP models (Baluchnejadmojarad et al., 2010; Perez et al., 2014), however, the majority of them used pre- (Baluchnejadmojarad et al., 2010; Haddadi et al., 2013, 2014) or concomitant (Perez et al., 2014) treatment with silymarin in a single dose (Baluchnejadmojarad et al., 2010) or 5 days of treatment (Haddadi et al., 2013, 2014; Perez et al., 2014). The present study was the first where the treatment with silymarin was started seven days after 6-OHDA administration where the PD-like symptoms are already present, investigating also the possible pharmacological target. The present experimental design intent to mimic what happens clinically in patients, where the treatment usually begins after the onset of symptoms (Reagan-Shaw et al., 2008).

In the literature, there is little information regarding the effects of silymarin on motor coordination in experimental models of PD. Thus, firstly we investigated the effects of different doses of silymarin (10, 30 or 100 mg/kg) on behavioral alterations caused by 6-OHDA in mice. The doses of silymarin were selected based on the allometric conversion of drug doses from humans to mice (Reagan-Shaw et al., 2008) being the dose of 100 mg/kg used to mice similar to that used orally as food supplement for humans. As the intraperitoneal route presents a higher bioavailability than by oral route, it was also tested the doses of 10 and 30 mg/kg.

In order to evaluate the balance and motor coordination of the animals we performed the rotarod test. This is a recognized test used to evaluate neurological deficits in rodents, usually following pharmacological treatments, brain injuries or genetic manipulations (Rogers et al., 2001). Our data demonstrate that 6-OHDA administration caused an impairment on fine motor coordination observed by a decrease in latency to first fall and an increase in the number of falls on the rotarod apparatus. These findings are in accordance with previous studies, which reported 6-OHDA induced motor imbalance as an early PD-like symptom (Carvalho et al., 2013; Deumens et al., 2002). Silymarin at a dose of 100 mg/kg reversed these alterations in the rotarod test, which could represent a neurorescue effect of this compound on dopaminergic neurons against 6-OHDA toxicity (Geed et al., 2014). Likewise, we also analyzed the balance and motor performance of animals on the beam walk test. The animals whose received 6-OHDA needed a longer time to cross the beam, which indicates an impairment in balance and/or motor coordination. The treatment with silymarin reduced the latency to cross the beam at all doses tested, suggesting a protective effect of this flavonoid complex in the 6-OHDA-induced toxicity. These findings are consistent with studies from literature, where flavonoids as catechin, baicalein and rutin have been shown to attenuate motor deficits in neurotoxin-induced animal model of PD (Gao et al., 2015; Khan et al., 2012; Teixeira et al., 2013).

The open-field test is based on the natural rodent exploratory instincts in a novel place, and has been widely used to evaluate locomotor and exploratory activities in response to a novel environment (Rubin et al., 2004). As a result, the animals whose received 6-OHDA exhibited a loss in locomotor activity in open field test. Interestingly, the treatment with silymarin at all doses tested enhanced the locomotion (crossing and rearing) of animals in comparison with 6-OHDA group, suggesting that silymarin treatment might has neurorescue action against dopaminergic toxicity caused by 6-OHDA in mice brain. Corroborating our results, early reports have indicated protective effects of silymarin in improving motor deficits induced by maneb and paraquat in mouse (Singhal et al., 2011) as well as silibinin, the major active

constituent of silymarin, in MPP<sup>+</sup>-induced dopaminergic neurotoxicity in rats (Geed et al., 2014). It is important to emphasize that in patients with hepatic diseases which is the main clinical use of silymarin, it is required a long-term of consumption to silymarin exert its pharmacological effects (Zeng et al., 2016). However, the present data show that silymarin had benefits on behavioral alterations induced by 6-OHDA with a short-term treatment.

Further, we investigated if the effects of 6-OHDA and silymarin in behavioral alterations were associated with changes in the immunoreactivity of TH and phospho-ERK1/2 as well as MAO activity. In this sense, TH, the rate-limiting enzyme in catecholamine biosynthesis, is an indicator of dopamine (DA) production (Lima et al., 2012) and the reduction in its levels in the substantia nigra is a hallmark feature of neuronal loss that occurs in PD (Olanow and Tatton, 1999). 6-OHDA administration caused a reduction in TH immunoreactivity in both the striatum and in the substantia nigra, suggesting that this model was effective in causing damage to dopaminergic neurons since the behavioral deficits observed in mice could be associated with a decreased in TH levels and consequently of dopamine. In fact, literature data have demonstrated that the dose of 60 µg reduced the dopamine levels in about 90% (Nowak et al., 2011). However, the treatment with silymarin was not able to recover the decrease caused by 6-OHDA in striatal TH immunoreactivity. Specifically to substantia nigra, we observed that silymarin at a dose of 30 mg/kg recovered the decrease in TH immunoreactivity caused by 6-OHDA. Previous studies showed that silibinin, the main component of the silymarin, attenuated the loss of dopaminergic neurons induced by MPP<sup>+</sup> in substantia nigra of rats (Jung et al., 2014). Data of literature suggest that 6-OHDA initially causes a lesion in dopaminergic terminals which are in the striatum while nigral cell bodies are secondarily affected by this neurotoxin which leads to the loss of dopaminergic neurons in the substantia nigra slowly over weeks after the administration of 6-OHDA (Blandini et al., 2008; Deumens et al., 2002; Sauer and Oertel, 1994). Our results showed a reduction of about 50% in TH levels in substantia nigra, suggesting that the effect of silymarin may be linked with the degree of injury induced by 6-OHDA and with different brain areas.

In this sense, ERKs 1 and 2, c-Jun N-terminal kinase (JNK) and p38 MAP kinase are members of mitogen-activated protein kinases (MAPKs) family and are important regulators of cellular signaling pathways (Kulich and Chu, 2001). While, it has been demonstrated that ERK activation leads to favorable responses such as differentiation and neuroplasticity, evidences suggest ERK activation may play a role in neurotoxicity, particularly in the context of oxidative insults (Chu et al., 2004; Kulich et al., 2007; Kuperstein and Yavin, 2002). In our experimental design, 6-OHDA administration increased the phosphorylated ERK1/2 in both striatum and substantia nigra, suggesting that sustained activation of ERK pathways may contribute to mechanisms of 6-OHDA-dependent neuronal cell death (Kulich and Chu, 2001). Data of literature have demonstrated an increase in levels of phospho-ERK in substantia nigra tissue from patients with PD supporting the hypothesis that ERK activation can contribute to neuronal injury in neurodegenerative diseases (Perry et al., 1999; Zhu et al., 2002). However, the treatment with silymarin did not reduce the effect of 6-OHDA in phospho-ERK levels. Additional studies are needed to explore other pathways responsible for the behavioral improvement observed in animals treated with silymarin.

An enzyme considered as an important target for neurodegenerative diseases is the MAO which catalyzes the oxidative deamination of biogenic amines (Youdim and Bakhle, 2006). The two MAO isoforms are distributed throughout all tissues, the inhibition of MAO-A can provide protection against apoptosis (Ou et al., 2006) while the inhibition of MAO-B is related a therapeutic role in the treatment of PD (Youdim and Bakhle, 2006). It has been showed that an increase in MAO-B activity is associated with increases in the production of this reactive oxygen species (ROS) (Adams and Oduunze, 1991). This increase in ROS and consequently of oxidative stress contribute to a

decrease in neuronal mitochondrial function, deterioration dopaminergic neuron viability, and ultimately leads to cell death and motor impairment (Koppula et al., 2012; Mallajosyula et al., 2008; Prajapati et al., 2017). In this way, there are studies demonstrating the neuroprotective effects of the MAO-B inhibitors (Carradori et al., 2016; Finberg et al., 1998; Magyar and Szende, 2004). Moreover, a recent study of our group demonstrated the capacity of silymarin in decreasing the MAO activity *in vitro* (de Oliveira et al., 2015). However, in this study the effect of silymarin on motor impairment induced by 6-OHDA seems not to be related to alterations in MAO activity. Thus, it is suggested that the metabolism of constituents present in silymarin or its cerebral availability may explain the lack of effects of silymarin *per se* in MAO activity, since the necessary concentrations to inhibit MAO activity *in vitro* could be not reaching in cerebral tissue *in vivo*.

Taken together, our findings demonstrated that silymarin recovered the behavioral alterations caused by 6-OHDA, suggesting a potential therapeutic effects in parkinsonian symptoms. However, the exact mechanism of silymarin protection in this experimental model has not been completely elucidated but seems to involve, at least in part, the recovery of TH immunoreactivity in the substantia nigra. Further studies should be performed for whole understanding of the silymarin effects.

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fct.2018.05.062>.

#### Acknowledgments

This study was supported by the FAPERGS (PqG- 2080-2551/13-5-1) and CNPq (Universal- 475210/2013-1). We also acknowledge fellowships from CNPq (R.F. and F.A.A.S.), CAPES (C.M.F, B.N.K., A.P.C.C., L.F.S. and C.P.B.) and FAPERGS (E.M.R. and J.P.S.).

#### Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.fct.2018.05.062>.

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4.2 MANUSCRITO 1

**Manuscrito 1 submetido para publicação**

**EFFECT OF SILIBININ ON BEHAVIORAL AND TYROSINE HYDROXYLASE  
ALTERATIONS INDUCED BY 6-HYDROXYDOPAMINE IN MICE**

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**Acknowledgments:** This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, CAPES/PROEX (process number: 88882.182134/2018-01; support number: 0737/2018), CNPq (475210/2013-1) and FAPERGS (PqG - 2080-2551/13-5-1). We also acknowledge fellowships from CNPq (R.F.) and CAPES (C.M.F., B.N.K., G.N.B., A.P.C.C., J.B.R., T.R.).

## ABSTRACT

Silibinin is a flavonoid which is the major active constituent of silymarin and has showed neuroprotective and antioxidant effects. Parkinson's disease (PD) is a chronic neurodegenerative disorder, characterized by motor symptoms associated to dopaminergic system degeneration. However, it remains unclear whether silibinin could improve 6-hydroxydopamine (6-OHDA)-induced neurotoxicity. Thus, the aim of the current study was to evaluate the effect of silibinin in 6-OHDA-induced parkinsonism model. Mice received an intracerebroventricular injection of 6-OHDA (60 $\mu$ g) or vehicle. On day 8, treatment was started with silibinin (50 or 100 mg/kg, i.p.) for 7 days. In 15<sup>th</sup> day, the behavioral symptoms were evaluated by the tests of rotarod, beam walk and open field. Moreover, it was investigated the immunoreactivity of striatal tyrosine hydroxylase (TH). 6-OHDA induced a motor dysfunction, which was accompanied by a decrease in TH immunoreactivity in striatum. Silibinin treatment for seven days recovered the motor balance of animals evaluated in beam walk test. In the open field and rotarod tests, 6-OHDA caused a severe motor impairment, which was not restored by silibinin. Furthermore, silibinin at dose of 100 mg/kg recovered the alterations caused by 6-OHDA in TH immunoreactivity. These findings suggest that silibinin presents a benefit effect in recovering the motor equilibrium of mice in 6-OHDA-induced parkinsonism reinforcing its efficacy in neurodegenerative diseases.

**Keywords:** Parkinson's disease. *Silybum marianum*. 6-OHDA model. Tyrosine hydroxylase. Motor activity.



## Introduction

Parkinson's disease (PD) is a debilitating neurological disease with slowly progression, resulting from dopaminergic cell loss in the nigrostriatal pathway, with a corresponding decrease in striatal dopamine content (Meissner et al. 2011). The cause of dopaminergic cell death in PD remains unknown, but some events such as oxidative stress, mitochondrial dysfunction, excitotoxicity and apoptosis processes may contribute to neuronal degeneration in PD (Miller et al. 2009).

Nowadays, the PD symptoms can be treated using dopamine (DA) replacement strategies as L-3,4-dihydroxyphenylalanine (L-dopa) and dopaminergic agonists. The chronic treatment with these drugs results in severe motor adverse effects (dyskinesia and motor fluctuation) without ameliorate the progression of dopaminergic degeneration (Ahlskog and Muentner 2001; Meissner et al. 2011). In this way, it becomes necessary to investigate new therapeutic strategies as the use of natural compounds that could alleviate parkinsonian symptoms and simultaneously present neuroprotective property.

6-OHDA model is largely used to study PD due its similarity with the human pathology since this neurotoxin causes lesion in the nigrostriatal pathway inducing motor impairment in animals (Blandini and Armentero 2012). Studies have been demonstrated the potential neuroprotective effects of several compounds in 6-OHDA animal models (Chaturvedi et al. 2006; Khan et al. 2012) and drugs clinically used to treat PD also promote behavioral recovery in this model (Francardo et al. 2011).

Silibinin (silybin) is a flavonoid which is the major active constituent of silymarin, a flavonoid complex obtained from seeds of *Silybum marianum* and used as hepatoprotective in the treatment of liver diseases (Kren and Walterova 2005). It has been reported that silibinin presents neuroprotective (Lu et al. 2010) and anti-inflammatory effects (Kren and Walterova 2005). It has been demonstrated the beneficial effects of silibinin in several disease models including ischemic stroke (Wang et al. 2012) and amyloid beta (A $\beta$ ) peptide-induced memory impairment (Lu et al. 2009). Moreover, previous studies of our group demonstrated that silymarin recovers motor deficits induced by 6-OHDA in mice (de Freitas et al. 2018) and inhibits the enzyme monoamine oxidase (MAO) activity *in vitro* (de Oliveira et al. 2015). However, there are no studies investigating if silibinin could protect against 6-OHDA toxicity in mice.

Thus, the present study aims to evaluate the possible efficacy of silibinin in recovering the locomotor deficits induced by 6-OHDA and alterations in TH immunoreactivity in the striatum of mice.

## Materials and methods

### Animals

Male Albino Swiss mice weighing 25-35g and aged from 2 months, from breeding colony of experimental house of UFSM, were kept in cages of 4-5 animals each, with controlled temperature ( $22\pm 2$  °C) and under a 12 h light/dark cycle (lights on at 7:00 a.m.). Food and water were provided *ad libitum*. 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 4398020415/2015.

### Drugs

6-OHDA was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and was dissolved in 0.05% ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) plus 0.9% NaCl (sodium chloride) (Sigma-Aldrich, St. Louis, MO, USA). Vehicle consisted of 0.05% ascorbic acid plus 0.9% NaCl. Silibinin was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and was dissolved in 0.9% NaCl.

### Experimental design

#### *6-OHDA lesion*

Initially, the mice were randomly divided into two groups: control group and 6-OHDA group. Thirty minutes prior to 6-OHDA injection, all animals received imipramine (25 mg/kg (w/v), via intraperitoneal (i.p.)) to protect noradrenaline-containing neurons. After anesthesia with ketamine:xylazine (10:1 (v/v) equivalent to 100 mg/kg:10 mg/kg, i.p.), mice from 6-OHDA group received a single intracerebroventricular (i.c.v.) injection of 60 µg 6-OHDA (dissolved in 3 µL of physiological solution containing 0.05% ascorbic acid) into the right

cerebral ventricle via a 10  $\mu$ L Hamilton microsyringe (Tanaka et al. 2006). Control animals received the same volume of the vehicle (3  $\mu$ L of physiological solution containing 0.05% ascorbic acid). The needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye and 1 mm posterior to a line drawn through the anterior base of the eyes (used as external reference) by the method of free hand (Ribeiro et al. 2013). The injections were performed at a rate of 1  $\mu$ L/min (Carvalho et al. 2013). The needle was left in place for additional 4 min before slowly retracting it (Carvalho et al. 2013).

6-OHDA normally causes a mortality rate of about 50%, which is observed within twenty-four hours after 6-OHDA administration (Ribeiro et al. 2013). In the present study, five animals from control group died probably due the anesthesia and in 6-OHDA group, eighteen animals died.

The time necessary to mice recover from anesthesia was approximately 3–4 h. The animals were kept in a well-ventilated room in cages until they gained full consciousness. Food was kept inside the cages for the first week so that animals could easily access. Animals that survived the first 24 hours after the injection did not present detectable signs of systemic toxicity during the experimental period. In addition, the present procedure did not cause alterations in body weight of animals (data not shown).

In the 6-OHDA group, only animals that showed consistent motor damage evaluated in behavioral tests (rotarod, open field and beam walk) seven days after 6-OHDA administration were selected for the subsequent treatment and tests.

### *Treatment*

In the day of the lesion, the mice were randomly assigned to one of six groups to avoid chose the animals after knowing its behavioral response on day 8: (I) control (n=6); (II) silibinin 50 mg/kg (n=6); (III) silibinin 100 mg/kg (n=6); (IV) 6-OHDA (n=8); (V) 6-OHDA + silibinin 50 mg/kg (n=6); (VI) 6-OHDA + silibinin 100 mg/kg (n=8).

Mice received different doses of silibinin (50 or 100 mg/kg, i.p., 10 mL/kg body weight) or its vehicle (0.9 % NaCl, i.p., 10 mL/kg body weight) for 7 succeeding days. The doses of silibinin were selected based in previous studies (Jung et al. 2014). The behavioral parameters were measured on days 8 and 15 of the experimental period in a behavior room (from 8:00 a.m. to 11:00 a.m.). Four observers evaluated the behavior of mice. The experimental design is schematized in figure 1.

## Behavioral testing

### *Rotarod test*

The effect of the 6-OHDA and/or silibinin on fine motor coordination was tested on the rotarod apparatus as previously described (Godoy et al. 2004). Twenty-four hours before the experiments, the animals were trained in the rotarod apparatus (8 rpm constant speed) in a session of 10 trials; each trial starts with the mouse being placed in the apparatus and ends when the mouse falls off the rod or after reaching the cut-off time of 60 s. A resting time of 60 s was allowed between each trial. On the day 8 and 15 of the experimental period, it was evaluated the latency to first fall from the rotarod up to a maximal observation time of 240 s (de Freitas et al. 2018).

### *Open field test*

The spontaneous locomotor activity was assessed in an open field arena performed in a sound attenuated room on day 8 and 15 of experimental period. Mice were placed in the center of the open field (44×44×44 cm), divided into sixteen equal areas (Busanello et al. 2011). The number of crossing (lines crossed with four paws) was counted during 5 min.

### *Beam Walk test*

To verify the effect of 6-OHDA and/or silibinin on balance and motor performance, the beam walk test was evaluated on day 8 and 15 of experimental period (de Freitas et al. 2018). The time to cross a thin wooden bar (1.5 cm× 75 cm) elevated 90 cm from the floor was evaluated during 120 s.

## Tissue preparation and biochemical assays

On 15<sup>th</sup> day, after the last behavioral test, mice were euthanized by cervical dislocation and the brains were rapidly dissected and put on ice. The striatum was separated and immediately frozen on powdered dry ice and thereafter stored at -80°C.

### *Western Blotting analyzes*

The striatum was homogenized in lysis buffer (4% SDS, 2 mM EDTA, 50 mM Tris, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, 2µg/mL aprotinin, 0.1 mM benzamidine, 0.1 mM PMSF). Samples were boiled for 6 minutes and centrifuged at 4.000g at 4 °C for 10 minutes. The supernatant was used to determine protein concentration by Lowry method. Then, 40µg of sample was mixed with 10% glycerol (v/v) and 8% (v/v) 2-mercaptoethanol and resolved by 10% SDS-PAGE. The samples were transferred into nitrocellulose membrane (Millipore, USA). SeeBlue Pre-stained Protein Standard (Novex; catalog #LC5602) was used for molecular weight guidance and Ponceau staining served as the loading control (Romero-Calvo et al. 2010). After staining, the membranes were dried and scanned for quantification. Optical density of Ponceau staining between the 64-50kDa marker (approximately 0.5 cm above and below the band) was considered the loading control for TH. Membranes were then blocked with 10% non-fat dried milk for 1 hour at room temperature and incubated overnight either with an anti-TH (1:10.000; Millipore; catalog# AB152). After, the membranes were incubated with alkaline phosphatase-coupled secondary antibody for 1 h (1:10.000; Millipore; catalog # 12-448). 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 and scanned for quantification. All values were normalized using ponceau quantification.

### Statistical analysis

Data were statistically analyzed by unpaired t test for behavioral tests on day 8. Kruskal-Wallis followed by *post-hoc* Dunn's test or one-way ANOVA followed by *post-hoc* Student-Newman-Keuls were performed for behavioral tests on day 15. One-way ANOVA followed by *post-hoc* Student-Newman-Keuls was performed for TH immunoreactivity in striatum. All values are expressed as mean + SEM. Differences were considered statistically significant with  $p < 0.05$ .

## Results

### Effect of silibinin on 6-OHDA-induced changes in rotarod test

The results of the rotarod test are presented in Fig. 2 6-OHDA decreased the latency for the first fall in comparison with control group on day 8 of the experimental period ( $p < 0.05$ ; Fig. 2A). Then, the control and 6-OHDA groups were subdivided and treated with 50 or 100 mg/kg of

silibinin for 7 days. Silibinin treatment was not able to recover motor damage caused by 6-OHDA (Fig. 2D). In addition, no statistically differences were found in silibinin treatment *per se* when compared with control group.

#### Effect of silibinin on 6-OHDA-induced changes in locomotor activity

Fig. 3 shows the effect of 6-OHDA and silibinin treatment on the locomotor activity represented by number of crossings in the open field. 6-OHDA decreased the number of crossings on day 8 of the experimental period ( $p < 0.05$ ; Fig. 2B). On day 15, the animals that received 6-OHDA showed a decrease on the locomotor activity compared to control group [ $F(5,34) = 8.165$ ,  $p < 0.05$ ; Fig 2E]. However, the treatment with silibinin did not recover the impairment on locomotor activity caused by 6-OHDA. Furthermore, silibinin at dose of 100 mg/kg *per se* caused a reduction on locomotor activity when compared with control group ( $p < 0.05$ ; Fig 2E).

#### Effect of silibinin on 6-OHDA-induced changes in beam walk test

The effect of 6-OHDA and silibinin on balance and motor coordination was evaluated by beam walk test. 6-OHDA increased the latency to cross the bar compared with control group on day 8 of the experimental period ( $p < 0.05$ ; Fig. 2C). Statistical analysis on day 15 revealed that treatment with silibinin decreased the latency to cross the bar in those animals previously treated with 6-OHDA when compared with 6-OHDA group [ $F(5,34) = 8.230$ ,  $p < 0.05$ ; Fig. 2F]. Furthermore, silibinin *per se* did not cause alteration on latency to cross the bar.

#### Effect of silibinin on 6-OHDA-induced changes in the striatal TH immunoreactivity

There was a significant decrease in TH immunoreactivity in the striatum of mice that received 6-OHDA compared with the control group ( $p < 0.05$ ). The treatment with 100 mg/kg of silibinin recovered the decrease in TH levels caused by 6-OHDA [ $F(5,22) = 10.99$ ,  $p < 0.05$ ; Fig.3]. Moreover, silibinin *per se* did not modify the TH immunoreactivity in the striatum.

## Discussion

In the present study, it was examined whether silibinin could recover behavioral changes and the alterations in TH immunoreactivity induced by 6-OHDA. 6-OHDA administration caused a motor impairment, which was accompanied by a decrease in TH immunoreactivity in the striatum. The treatment with silibinin for 7 days recovered the motor balance of animals on beam walk test at both doses tested. Furthermore, the treatment with silibinin at dose of 100 mg/kg ameliorated the alterations caused by 6-OHDA in TH immunoreactivity in striatum.

Clinical features of Parkinson's disease include motor impairments, such as bradykinesia, resting tremor, rigidity and postural instability and also non-motor symptoms like cognitive and psychiatric disorders (Thomas and Beal 2007). Moreover, deterioration in posture and balance have been reported in the clinic at stage 2–3 (mild–moderate PD symptoms) using the Hoehn and Yahr scale (Truong et al. 2006). These symptoms can be reproduced, at least in part, in *in vivo* models. 6-OHDA is a catecholaminergic neurotoxin that has been used to produce PD symptoms *in vivo* (Bove and Perier 2012). The selective loss of dopaminergic neurons induced by 6-OHDA administration leads to alterations in the regulation and function of dopaminergic system and the behavioral deficits observed are linked to the neuronal dysfunction degree (Ogura et al. 2005). Furthermore, the 6-OHDA icv injections has to advantage of reproduce a DA-neuron loss profile similar to reported in PD (Rodriguez et al. 2001).

Data of literature have demonstrated the neuroprotective effects of silymarin in parkinsonism models (Perez et al. 2014; Singhal et al. 2011) and its capacity to inhibit the MAO activity *in vitro* (de Oliveira et al. 2015). The major constituent of silymarin is silibinin, which has presented neuroprotective effects in models of memory deficits (Lu et al. 2009) , and stroke (Wang et al. 2012). A recent work of our group showed that silymarin reversed 6-OHDA-induced motor impairment without recovering the alterations caused by 6-OHDA in TH and phospho-ERK1/2 immunoreactivity (de Freitas et al. 2018). In the present study, it was evaluated the possible efficacy of silibinin in recovering the locomotor deficits caused by 6-OHDA, aiming to investigate if the effects of silymarin in this model are due to presence of its major constituent.

The present results demonstrate that 6-OHDA caused a loss in the balance and motor coordination in mice evaluated in the rotarod test, indicating impairment in motor performance of animals. Moreover, a decrease in the spontaneous locomotor activity was observed after 6-OHDA administration, evaluated in the open field test. The treatment with silibinin at doses of 50 and 100 mg/kg was not able to recover the behavioral alterations in both tests. Silibinin at dose of 100 mg/kg decreased *per se* the number of crossing in the open field test. Regarding

the beam walk test, the animals that received 6-OHDA showed a longer time to cross the bar, indicating loss of the mice equilibrium function. Silibinin improved the equilibrium of mice in the beam walk test. Considering the results obtained in behavioral tests, we suggest silibinin could be acting selectively in pathways responsible by controlling the equilibrium.

In this way, as distinct motor symptoms have particular neural basis, silibinin treatment could be affecting differently the behavioral responses being beam walk test more sensible to its effects than rotarod and open field test. Moreover, it has been assumed that several motor symptoms observed in the PD are caused by striatal dopamine depletion; however, it is known that other neural structures and neurochemical systems are also involved in the motor impairments, including prefrontal cortical areas and the cerebellum, as well as serotonergic, glutamatergic, and cholinergic systems (Moustafa et al. 2016). This would to explain the lack of effect of silibinin on behavioral alterations induced by 6-OHDA observed in rotarod and open field tests despite showing a significant recovery of motor balance of animals in beam walk test.

In PD patients, motor dysfunction is associated with the extent of dopamine loss (Damier et al. 1999). Thus, TH evaluation is widely accepted as a marker of dopaminergic neuronal damage, because this enzyme catalyzes the formation of L-dopa, the rate-limiting step in the biosynthesis of dopamine (Haavik and Toska 1998). The present study showed that 6-OHDA administration induced dopaminergic toxicity in mice, which was observed behaviorally and biochemically by the decrease of the TH immunoreactivity in striatum. These results are in agreement with those obtained by other authors, which demonstrated that 6-OHDA-induced motor dysfunction is associated with a lesion of dopaminergic terminals, expressed by the reduction of TH levels (Chaturvedi et al. 2006). Here, the treatment with silibinin at a dose of 100 mg/kg significantly recovered the alterations caused by 6-OHDA in striatal TH immunoreactivity. Our results suggest that silibinin could be modulating the cell viability of dopaminergic neurons as evidenced by the increase in TH immunoreactivity in striatum. Corroborating our data, a previous study showed that silibinin attenuated the loss of dopaminergic neurons induced by MPP<sup>+</sup> in substantia nigra of rats (Jung et al. 2014).

Collectively, the results of the present study demonstrate that treatment with silibinin recovered the motor balance of animals in the beam walk test which could be associated with a recovery of TH immunoreactivity in the striatum. However, further studies are necessary to elucidate which mechanisms are related with the effect of silibinin in this parkinsonism model.

**Ethical approval:** All procedures performed in studies involving animals were in accordance



with the ethical standards of the institution or practice at which the studies were conducted.

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

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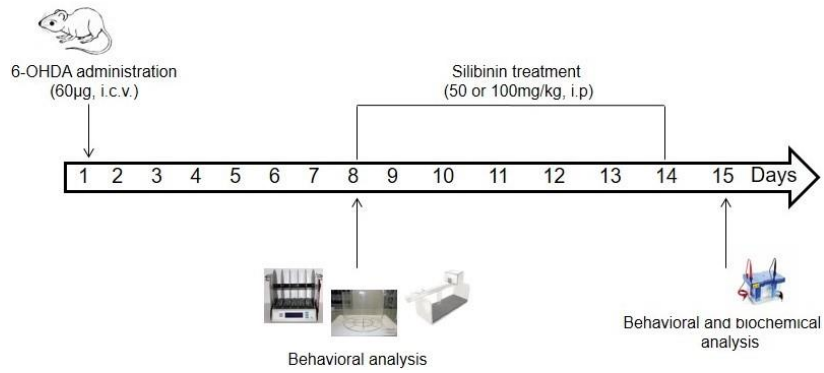
## FIGURE LEGENDS

**Fig. 1** Experimental design. Day 1 – 6-OHDA administration (60 $\mu$ g, i.c.v.); day 8 – behavioral analysis (rotarod, open field and beam walk tests); day 8 until day 14 – treatment with 50 or 100 mg/kg of silibinin (i.p.); day 15 – behavioral (rotarod, open field and beam walk tests) and TH immunoreactivity analysis.

**Fig. 2** Effect of 6-OHDA administration in mice on latency to first fall in the rotarod test (A), number of crossing in the open field test (B) and latency to cross the beam walk test (C) on day 8. Data are expressed as mean + SEM (n=18-22). Data were analyzed by unpaired t test. \*p < 0.05 compared with control group. Effect of silibinin on latency to first fall (D), number of crossing (E) and latency to cross the beam (F) after 6-OHDA administration on day 15. Data are expressed as mean + SEM (n=6-8). \*p < 0.05 compared with control group; #p < 0.05 compared with 6-OHDA group (Kruskal-Wallis followed by Dunn's test or One-way ANOVA followed by Newman-Keuls test) (Sil 50 - silibinin 50 mg/kg; Sil 100 – silibinin 100 mg/kg; 6-OHDA – 6-hydroxydopamine).

**Fig. 3** Western blot analysis of TH immunoreactivity in the striatum of mice treated with silibinin after 6-OHDA administration. Data are represented by relative optical density (ROD). Data are expressed as mean + SEM (n=4-5). \*p < 0.05 compared with control group; #p < 0.05 compared with 6-OHDA group (One-way ANOVA followed by Newman-Keuls test) (Sil 50 - silibinin 50 mg/kg; Sil 100 – silibinin 100 mg/kg; 6-OHDA – 6-hydroxydopamine).

**FIGURE 1:**



**FIGURE 2:**

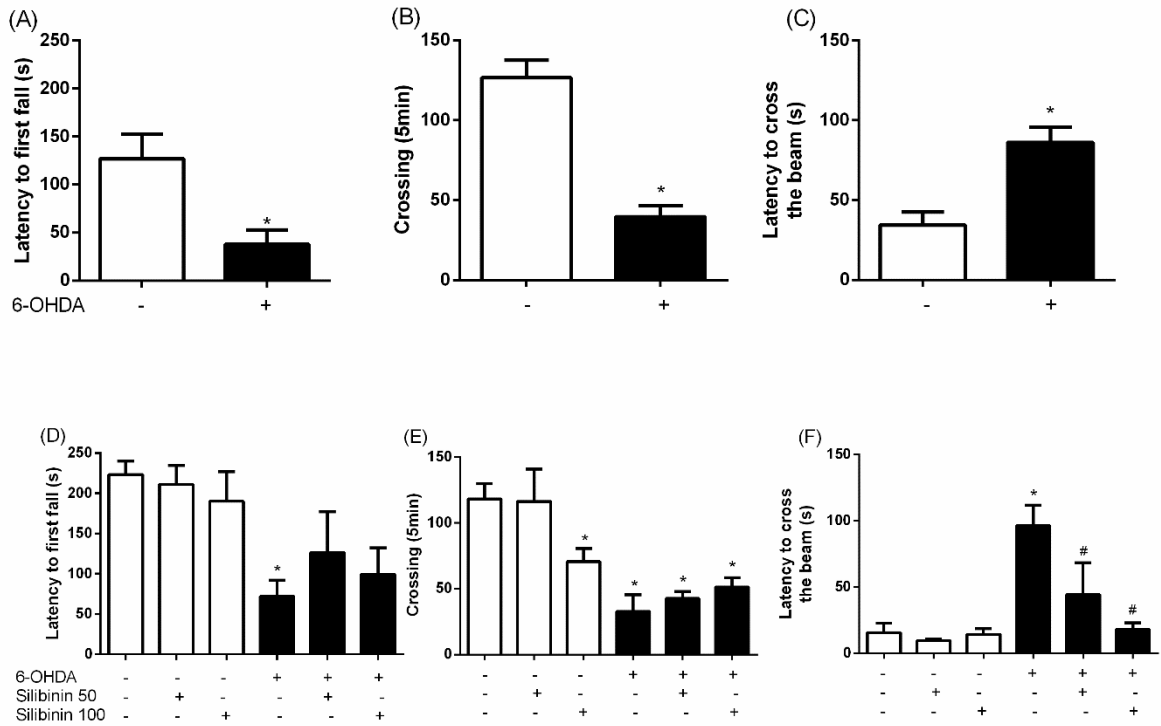
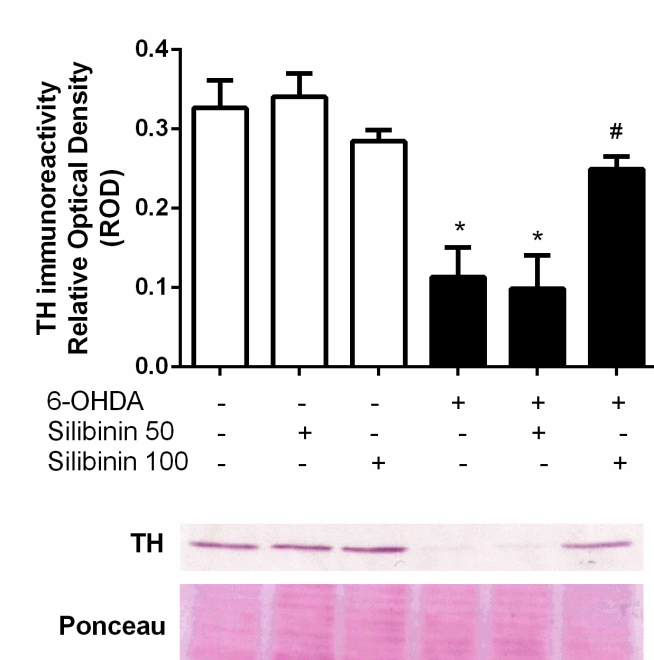


FIGURE 3:



### 4.3 MANUSCRITO 2

**Manuscrito 2 em preparação  
(dados preliminares)**

**EFFECT OF CO-TREATMENT WITH SILYMARIN AND L-DOPA IN A 6-OHDA-  
INDUCED PARKINSONISM MODEL IN MICE**

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**EFFECT OF CO-TREATMENT WITH SILYMARIN AND L-DOPA IN A 6-OHDA-  
INDUCED PARKINSONISM MODEL IN MICE**

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## Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra, leading to the development of motor symptoms. The most effective treatment is L-dopa; however, long-term treatment leads motor complications such as dyskinesias. Silymarin extracted from the seeds of the *Silybum marianum* has demonstrated neuroprotective effects and could be an alternative as adjuvant to L-dopa therapy. So, this study investigate the effects of co-treatment with silymarin and L-dopa in a 6-hydroxydopamine (6-OHDA) model of parkinsonism in mice. Mice received an intracerebroventricular injection of 6-OHDA (60 µg) or vehicle. Seven days after, the treatment was started with silymarin (30 mg/kg, via intraperitoneal) and/or L-dopa (25 mg/kg) plus benserazide (10 mg/kg) via intraperitoneal for 28 days. On days 8, 15, 22, 29 and 36 of experimental period locomotor activity and orofacial dyskinesia were evaluated. 6-OHDA induced a motor impairment and silymarin treatment recovered the locomotor activity of animals, although co-treatment with silymarin did not potentiate the effect of L-dopa on motor damage caused by 6-OHDA. Furthermore, the concomitant treatment with silymarin and L-dopa seems to precipitate the emergence of orofacial dyskinesia. Despite apomorphine (administered on day 36 of experimental period) has caused changes on behavioral responses as crossing, grooming, time of rotation, percentage of animals turning and Straub tail reaction, these did not be significant. However, a significant alteration on time of immobility of animals was observed. In conclusion, our findings demonstrate that silymarin should be better studied prior to its use as an adjuvant in L-dopa therapy for the treatment of Parkinson's disease since their association seems to precipitate the emergence of dyskinesia.

**Keywords:** Parkinson's disease. *Silybum marianum*. Dyskinesia. Apomorphine.

## 1. Introduction

Parkinson's disease (PD) is a slowly progressive neurodegenerative disorder affecting 1% of the population over 50 years old [1]. Clinically, the symptoms that define PD are tremor, muscle rigidity and slowness in initiating and carrying out voluntary movements [2]. This disorder is characterized by a severe loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [3], which causes the appearance of majority of symptoms in PD [4]. However, the exact cause and mechanisms involved in neuronal cell death in PD remain unclear.

L-dopa is still the most effective treatment to motor symptoms of PD, however, its effectiveness diminishes as the severity of the clinical symptoms increases due to progression of the underlying neurodegeneration [5]. Furthermore, the long-term use of L-dopa results in the development of motor complications such as motor fluctuations ('on-off' phenomenon) and dyskinesias, compromising its clinical efficacy [6]. One of the current approaches is to find a suitable therapeutic adjuvant to reduce the dose and frequency of L-dopa therapy, which could alleviate PD symptoms and avoid and/or reduce the occurrence of dyskinesias leading to clinical improvements.

Silymarin, a multicomponent extract obtained from the seeds of *Silybum marianum*, is a mixture of several flavolignans and used clinically as hepatoprotective agent without notable adverse effects [7, 8]. Several studies have shown that silymarin has a wide-ranging of pharmacological actions as anticarcinogenic [9], anti-inflammatory [10], antioxidant [11], neuroprotective effects [12] and inhibitory activity on monoamine oxidase (MAO) *in vitro* [13]. Additionally, studies have demonstrated the protective effects of silymarin in experimental models of PD [14, 15]. Of particular importance, a recent work of our group showed that silymarin recovered the behavioral alterations in a 6-OHDA-induced parkinsonism model [16]. However, to our knowledge, there are no data showing the possible pharmacological or adverse effects of combined treatment with silymarin and L-dopa.

Therefore, the objective of present study was to investigate the effects of co-treatment with silymarin and L-dopa in a 6-OHDA-induced parkinsonism model considering pharmacological and adverse effects.

## 2. Materials and methods

### 2.1 Animals

Male Albino Swiss mice aged from 2 months and weighing 25-35g, from breeding colony of experimental house of UFSM, were kept in cages of 4-5 animals each, with controlled temperature ( $22\pm 2$  °C) and under a 12 h light/dark cycle (lights on at 7:00 a.m.). Food and water were provided *ad libitum*. All protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria under process number 9144181016 and are in accordance with the guidelines of the National Council of Control of Animal Experimentation (CONCEA). Every possible effort was made to limit animal's suffering as well as to keep their number to a minimum.

## 2.2 Drugs

6-OHDA was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and was dissolved in 0.05% ascorbic acid and 0.9% NaCl (sodium chloride). Vehicle consisted of 0.05% ascorbic acid and 0.9% NaCl. Silymarin (China), imipramine (India) and benserazide (powder; China) were commercially acquired in a local magistral pharmacy. The phytochemical profile of silymarin was characterized by HPLC [13]. L-dopa was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

## 2.3 Experimental design

The experiment was divided into 3 sets with all experimental group. We conducted the experiment in this way because we previously observed a high mortality of mice in the group treated with 6-OHDA [16]. At the first set, we allocated 50 animals distributed into the groups. The second set contained 33 animals and the last contains 17. Thus, the experimental design described below contains the final number of animals in each group after the three repetitions of the experimental design. We only observed death in the group treated to 6-OHDA and they occurred maximally at 48 hours after 6-OHDA administration.

In each set of the experiment, mice initially were randomly divided in two groups: control and 6-OHDA groups. Thirty minutes prior to 6-OHDA injection, animals were treated with imipramine (25 mg/kg, via intraperitoneal (i.p.)) to protect noradrenaline-containing neurons. After anesthesia with ketamine:xylazine (10:1; equivalent to 100 mg/kg:10 mg/kg, i.p.), mice from 6-OHDA group received a single intracerebroventricular (i.c.v.) infusion of 60 µg 6-OHDA (dissolved in 3 µL physiological solution containing 0.05% ascorbic acid) into the

right cerebral ventricle via a 10  $\mu$ L Hamilton microsyringe [17]. Control animals received the same volume of the vehicle (3  $\mu$ L physiological solution containing 0.05% ascorbic acid) as described by De Freitas et al [16]. The needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye and 1 mm posterior to a line drawn through the anterior base of the eyes (used as external reference) by method of free hand [18]. The injections were performed at a rate of 1  $\mu$ L/min [19]. The needle was left in place for additional 4 min before slowly retracting it [19]. Animals that survived the early hours after the injection did not present detectable signs of systemic toxicity during the experimental period.

One week after 6-OHDA administration the mice were assigned to one of eight groups: (I) control (n=6); (II) silymarin 30 mg/kg (n=7); (III) L-dopa (n=6); (IV) L-dopa + silymarin 30 mg/kg (n=5); (V) 6-OHDA (n=9); (VI) 6-OHDA + silymarin 30 mg/kg (n=9); (VII) 6-OHDA + L-dopa (n=7); (VIII) 6-OHDA + L-dopa + silymarin 30 mg/kg (n=10).

Silymarin was dissolved in physiological solution containing 1% ethanol. Benserazide was dissolved in physiological solution (0.9% NaCl) and L-dopa was dissolved in physiological solution containing 0.1% ascorbic acid. Silymarin (30 mg/kg) was co-administered with benzerazide (10 mg/kg) 15 min prior to injection of L-dopa (25 mg/kg). The drugs were injected by via intraperitoneal at the volume of 10 ml/kg for 28 days. Control animals received saline containing 0.1% ascorbic acid instead of L-DOPA or saline containing 1% ethanol instead of silymarin. The doses of silymarin, benserazide and L-dopa were selected based in previous studies [16, 20].

## *2.4 Behavioral testing*

The behavioral parameters were measured on days 8, 15, 22, 29 and 36 of experimental protocol (from 8:00 a.m. to 11:00 a.m.). Four observers evaluated the behavior of mice. A different person distributed the animals to the observers to assure that the observation would be blind.

### *2.4.1 Open Field Test*

To measure locomotor activity mice were placed in the center of an open field arena (44 $\times$ 44 $\times$ 44 cm), divided into sixteen squares (4 rows of 4). The spontaneous locomotor activity (number of segments crossed with the four paws) and exploratory activities (expressed by the number of rearing on the hind limbs) was counted during 5 min with no habituation time [21].

#### 2.4.2 Quantification of vacuous chewing movements (VCMs)

Behavior measurement of VCMs was assessed before the treatment with 6-OHDA, silymarin, L-dopa or its vehicle (basal evaluation), as previously described [22, 23]. The effect of treatments on behavior were assessed on day 8 (7 days after the 6-OHDA administration), 15, 22, 29 and 36 as depicted in Fig. 1. To quantify the occurrence of VCMs, mice were placed individually in glass cages (20 cm x 20 cm x 19 cm) containing one mirror under the floor to allow the quantification when the animals were away from the observer. The number of VCMs, defined as single mouth openings in the vertical plane not directed towards physical material, were counted. The time of grooming on day 36 was also quantified. The behavioral parameters were recorded for 6 min after a 6 min adaptation period [21].

#### 2.5 Apomorphine test

In the day 36 of experimental period, after the behavioral analyses animals received a subcutaneous injection of apomorphine (a direct dopaminergic agonist) 1 mg/kg in a volume of 10 mL/kg [24]. Then it was evaluated the locomotor activity and stereotyped behavior.

##### 2.5.1 Open Field

To assess the locomotor activity after apomorphine administration the animals were placed in the center of an open field arena and the number of crossing was accounted during 20 min, as described above.

##### 2.5.2 Stereotypy

To evaluate changes in stereotyped behavior, immediately after the open field test, mice were placed individually in glass cages (20 cm x 20 cm x 19 cm). A video tape was recorded during 5 min. Thereafter, the total time of stereotypy was counted by a blind experimenter. The following parameters were counted: time of rotation, percentage of animals with turning behavior, percentage of animals with straub tail, immobility time and time of grooming, according to the previously published method [25].

## 2.6 Statistical analysis

Statistical analysis was carried out using Statistica software version 7.0. Data were statistically analyzed by unpaired t-test for behavioral tests on day 8. One-way analysis of variance (ANOVA) followed by Duncan's multiple range tests was performed for the others behavioral evaluations when appropriate. All data are expressed as mean  $\pm$  standard error of the mean (SEM). The results were considered statistically significant when  $p < 0.05$ .

## 3. Results

### 3.1 Effect of co-treatment with silymarin and L-dopa in the open field test

6-OHDA administration caused a decrease in spontaneous locomotor activity of animals ( $p < 0.05$ ; Fig. 2A), represented by the number of crossings in open field on day 8. Statistical analysis on day 15 revealed that silymarin, L-dopa and the co-treatment significantly improved the locomotor activity in those animals that received 6-OHDA [ $F(7,50) = 4.148$ ,  $p < 0.05$ , Fig. 2B]. On days 22 and 36 of experimental period, the group treated with silymarin showed a significant increase in the number of crossings ( $p < 0.05$ ) whereas the L-dopa and co-treated groups did not differ significantly from 6-OHDA group (Fig. 2C and E). On day 29, silymarin, L-dopa and co-treated groups did not show significant difference compared with 6-OHDA group (Fig. 2D).

We observed a significant reduction in exploratory activity in those animals previously treated with 6-OHDA in comparison with control group on day 8 ( $p < 0.05$ ; Fig. 3A). On day 15, L-dopa treatment significantly increased the number of rearings compared with 6-OHDA group [ $F(7,50) = 5.276$ ,  $p < 0.05$ , Fig. 3B]. On days 22, 29 and 36 of experimental period silymarin, L-dopa and co-treated groups did not differ significantly from 6-OHDA group (Fig. 3C, D and E).

Moreover, silymarin and/or L-dopa alone did not modify the locomotor and exploratory activity of animals.

### 3.2 Effect of co-treatment with silymarin and L-dopa on VCMs number and time of grooming

When evaluated the orofacial dyskinesia, represented by the number of VCMs, a significant effect was observed in the co-treated group with silymarin and L-dopa compared with control group (days 22 and 36) and with 6-OHDA group (day 22) [ $F(7,50) = 2.295$ ,  $p < 0.05$ , Fig. 4C] and [ $F(7,50) = 2.065$ ,  $p = 0.05$ , Fig. 4E] on days 22 and 36, respectively. Silymarin and L-dopa groups did not produce alteration on VCMs number during the experimental period. Silymarin and/or L-dopa alone did not alter the number of VCMs in the period analyzed.

Regarding the time of grooming evaluated on day 36 of experimental period was observed that the silymarin and L-dopa co-treatment showed a significant increase compared with control group [ $F(7,50) = 2.741$ ,  $p < 0.05$ , Fig. 5].

### *3.3 Effect of apomorphine administration in locomotor activity, immobility and stereotypy*

The apomorphine test was performed at the end of the behavioral assessment to gain insight possible modulation of dopaminergic sensitivity. All groups received an injection of apomorphine and it was evaluated the locomotor activity in an open field arena. The treated groups did not differ significantly from control group on the number of crossings evaluated during 20 min after apomorphine administration (Fig. 6A).

The administration of apomorphine increased the time of immobility in mice that received 6-OHDA in comparison to control group ( $p < 0.05$ ; Fig. 6B). L-dopa and co-treated groups showed a significant reduction on time of immobility compared with 6-OHDA group [ $F(7,47) = 2.731$ ;  $p < 0.05$ ; Fig. 6B].

A trend towards increased in total time of rotation also occurred in 6-OHDA and L-dopa groups, although no post-hoc comparison involving any treated groups reached statistical significance (Fig. 6D). Moreover, L-dopa group showed a higher percentage of animals with apomorphine-induced turning behavior (Fig. 6E).

In addition, no statistical differences were found among the groups when evaluated time of grooming and straub tail reaction after apomorphine administration (Fig. 6C and F).

## **4. Discussion**



In the present study, the effects of co-treatment with silymarin and L-dopa in a 6-OHDA model of parkinsonism was investigated considering pharmacological and adverse effects. 6-OHDA induced a motor impairment and silymarin treatment recovered the locomotor activity of animals, although the co-treatment with silymarin did not potentiate the effect of L-dopa on motor damage caused by 6-OHDA. Furthermore, the concomitant treatment with silymarin and L-dopa seems to precipitate the emergence of orofacial dyskinesia, observed by an increase on the number of VCMs when co-administered. When dopaminergic sensitivity was tested by injecting apomorphine it was not possible to affirm that a dopaminergic sensibilization occurred.

Silymarin is a polyphenolic flavonoid that has demonstrated different effects in several *in vitro* [13] and *in vivo* [14, 26] studies. Recently, the beneficial effect of silymarin was reported in a 6-OHDA-induced parkinsonism model [16]. However, of our knowledge, this is the first study showing the co-treatment with silymarin and L-dopa in a parkinsonism model and investigating the effect of silymarin for a period of 30 days.

Nowadays, the treatment with L-dopa is the most effective noninvasive therapy for Parkinson's disease [20]. However, chronic L-dopa administration causes abnormal involuntary movements known as dyskinesias [27]. The appearance of dyskinesia represents a challenge to treatment of PD because it can be severe enough to warrant reducing the L-dopa dose below optimal therapeutic levels. Thus, the search for new therapeutic strategies that could be used as an adjuvant to L-dopa therapy to alleviate the PD symptoms is relevant.

Our first aim was to evaluate the effect of co-treatment with silymarin and L-dopa in locomotor activity of animals. According to the results, the animals whose received 6-OHDA exhibited a loss in locomotor activity in open field test, observed by a reduction on number of crossing and rearing. Silymarin treatment recovered the locomotion of animals. These results are in agreement with those obtained by other authors, which also demonstrated that this compound was effective in restore motor activity in parkinsonism models [16, 28, 29]. However, the co-treatment with silymarin did not potentiate the effect of L-dopa on motor damage caused by 6-OHDA. Data of literature has demonstrated that quercetin, a flavonoid potentiated L-dopa reversal of drug-induced catalepsy [2]. Despite the fact of silymarin and L-dopa co-treatment did not have showed a synergistic effect on motor damage induced by 6-OHDA, silymarin alone showed a beneficial effect, suggesting it could be useful in early stages of PD when there is a moderate lesion.

Furthermore, we investigated if the co-treatment with silymarin and L-dopa could have effect on involuntary oral movements, represented by VCMs. It is known that with prolonged

L-dopa treatment many patients develop motor complications such as dyskinesia [30]. Dyskinesia is characterized by a variety of hyperkinetic movements, repetitive, and involuntary. It can occur in various regions of the body but mainly affects the facial region [27]. L-dopa-induced dyskinesia is common and difficult to treat.

One of the main risk factors for the development of dyskinesia in PD patients is the severity of disease at the initiation of L-dopa pharmacotherapy [31]. In our study treatment with L-dopa (25 mg/kg) combined with the dopa-decarboxylase inhibitor benserazide (10 mg/kg) for 28 days did not induce orofacial dyskinesia; however the co-treatment with silymarin and L-dopa seems to precipitate the emergence of orofacial dyskinesia, observed by an increase on the number of VCMs. It is important to emphasize that incidence and severity of dyskinesia induced by L-dopa is related to extent of nigrostriatal lesion, since pronounced striatal dopamine denervation is required for the animals to exhibit dyskinesia on L-dopa treatment [32, 33]. Thus, the absence of dyskinesia in the group treated L-dopa could be linked with the degree of injury induced by 6-OHDA. Additionally, data of literature have reported that the sensitivity to L-dopa differ between the PD models, since striatally lesioned mice required a three- to fourfold higher dose to reach a severity of dyskinesia comparable to animals lesioned in medial forebrain bundle (MFB) [32].

Moreover, we initially hypothesized that silymarin treatment could be causing a modulation of dopaminergic sensitivity. Data of literature have demonstrated that the selective loss of DA neurons leads to an increase in sensitivity of postsynaptic DA receptors as a compensatory response due to the deficiency of dopamine [34]. Drugs that increase dopaminergic function in the brain cause pattern of behavior which includes increased locomotion and repetitive activity, considered as stereotypy [35]. Therefore, after the last behavioral evaluation the animals received an injection of apomorphine in order to verify a possible sensitization response to silymarin and/or L-dopa treatment.

Despite of apomorphine (administered on day 36 of experimental period) has caused changes on behavioral responses as crossing, grooming, time of rotation, percentage of animals turning and Straub tail reaction, these did not be significant. However, a significant alteration on time of immobility of animals was observed. It is known that the behavioral effects are closely linked to the degree of neuronal dysfunction [36] and apomorphine is a reliable marker for the nigrostriatal dopamine depletion. Apomorphine-induced behavioral is only possible when the lesion is complete or nearly complete [37], which could explain the lack of effect of apomorphine in locomotor activity and stereotyped behavior of animals treated with 6-OHDA.

In conclusion, we demonstrate that silymarin should be better studied prior to its use as an effective adjuvant in L-dopa therapy for the treatment of Parkinson's disease since the association of silymarin with L-dopa seems precipitate the emergence of VCMs. Further investigation into the mechanisms of action of silymarin is needed to determine whether it can be co-administered with L-dopa.

### **Acknowledgments**

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, CAPES/PROEX (process number: 88882.182134/2018-01; support number: 0737/2018), CNPq (475210/2013-1) and FAPERGS (PqG - 2080-2551/13-5-1). We also acknowledge fellowships from CNPq (R.F.) and CAPES (C.M.F., B.N.K., G.N.B., A.P.C.C., J.B.R., T.R.).

**Declarations of interest:** none

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## FIGURE LEGENDS

**Fig. 1** Experimental design. Day 1 – Imipramine (25 mg/kg, i.p.) prior to 6-OHDA administration (60 $\mu$ g, i.c.v.); day 8 until day 35 – treatment with silymarin (30 mg/kg, i.p.) and/or L-dopa (25 mg/kg, i.p.) + benserazide (10 mg/kg, i.p.); day 8, 15, 22, 29 and 36 – behavioral analysis (open field and VCM); day 36 – administration of apomorphine and behavioral analysis (open field, time of immobility and stereotypy). Circle - open field test; triangle – VCM quantification; rhombus – time of immobility; square – stereotypy.

**Fig. 2** Effect of 6-OHDA administration in mice in open field test. Number of crossing (A) in the open field test on day 8. Data were analyzed by unpaired t test. \* $p < 0.05$  compared with control group. Effect of silymarin and/or L-dopa on number of crossing in the open field test after 6-OHDA administration on days 15, 22, 29 and 36. Data are expressed as means  $\pm$  standard error of mean (n=5-10). \* $p < 0.05$  compared with control group; # $p < 0.05$  compared with 6-OHDA group (One-way ANOVA followed by Duncan's multiple range tests).

**Fig. 3** Effect of 6-OHDA administration in mice in open field test. Number of rearing (A) in the open field test on day 8. Data were analyzed by unpaired t test. \* $p < 0.05$  compared with control group. Effect of silymarin and/or L-dopa on number of rearing in the open field test after 6-OHDA administration on days 15, 22, 29 and 36. Data are expressed as means  $\pm$  standard error of mean (n=5-10). \* $p < 0.05$  compared with control group; # $p < 0.05$  compared with 6-OHDA group (One-way ANOVA followed by Duncan's multiple range tests).

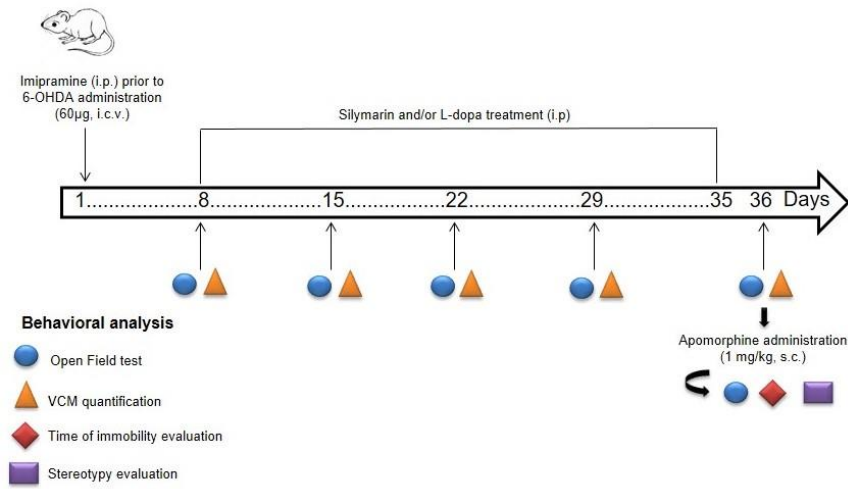
**Fig. 4** Effect of 6-OHDA administration in mice on VCMs number on day 8 (A). Data were analyzed by unpaired t test. \* $p < 0.05$  compared with control group. Effect of silymarin and/or L-dopa on VCMs number after 6-OHDA administration on days 15, 22, 29 and 36. Data are expressed as means  $\pm$  standard error of mean (n=5-10). \* $p < 0.05$  compared with control group; # $p < 0.05$  compared with 6-OHDA group (One-way ANOVA followed by Duncan's multiple range tests).

**Fig. 5** Effect of silymarin and/or L-dopa at time of grooming after 6-OHDA administration on day 36. Data are expressed as means  $\pm$  standard error of mean (n=5-10). \* $p < 0.05$  compared with control group (One-way ANOVA followed by Duncan's multiple range tests).

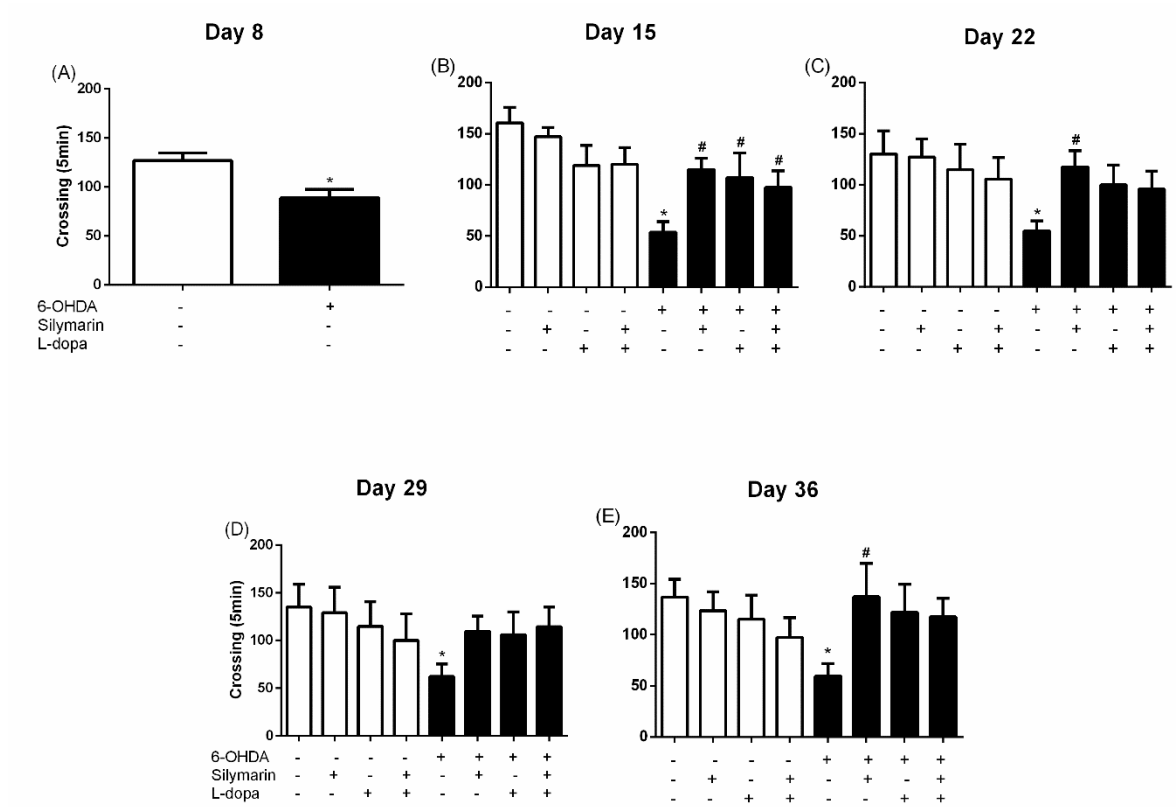
**Fig. 6** Effect of silymarin and/or L-dopa after administration of 1 mg/kg apomorphine (s.c.) on day 36. (A) Number of crossing; (B) Time of immobility; (C) time of grooming; (D) time of rotation; (E) Percentage of animals with turning behavior; (F) Percentage of animals with straub tail. Data are expressed as means  $\pm$  standard error of mean (n=5-8). \*p < 0.05 compared with control group; #p < 0.05 compared with 6-OHDA group (One-way ANOVA followed by Duncan's multiple range tests).



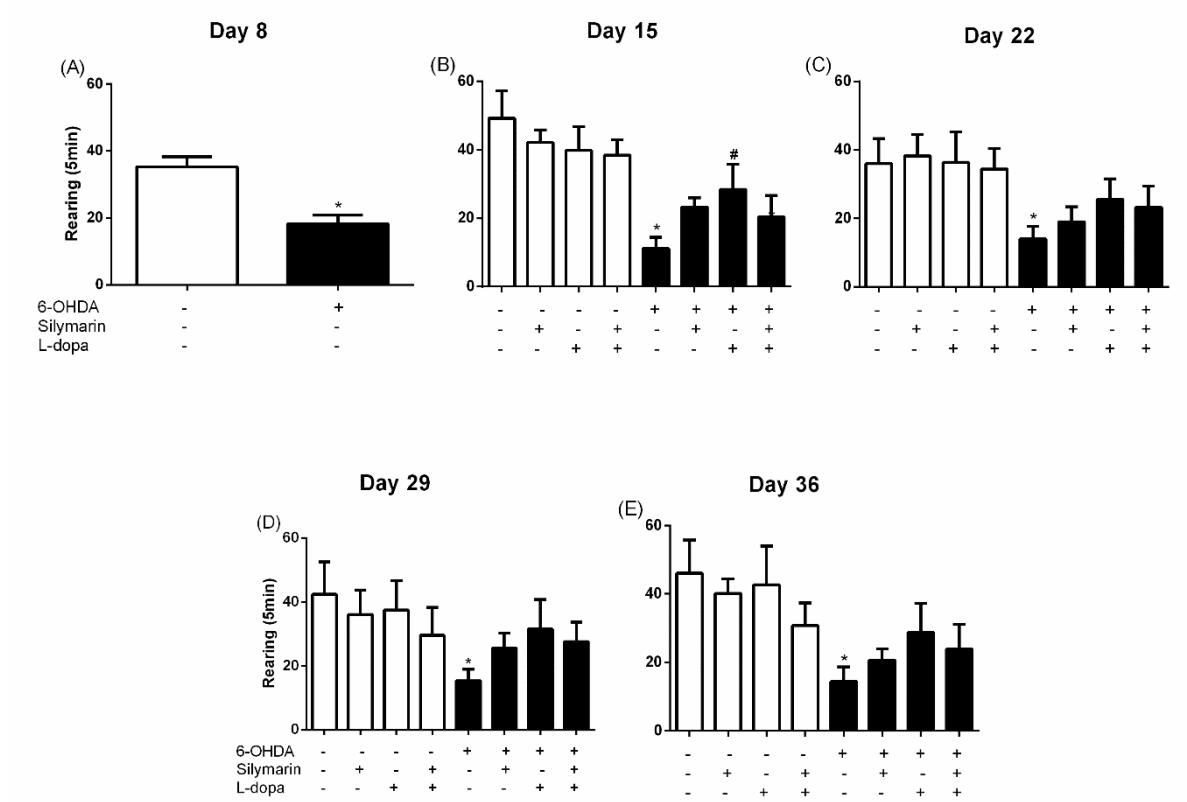
**FIGURE 1:**



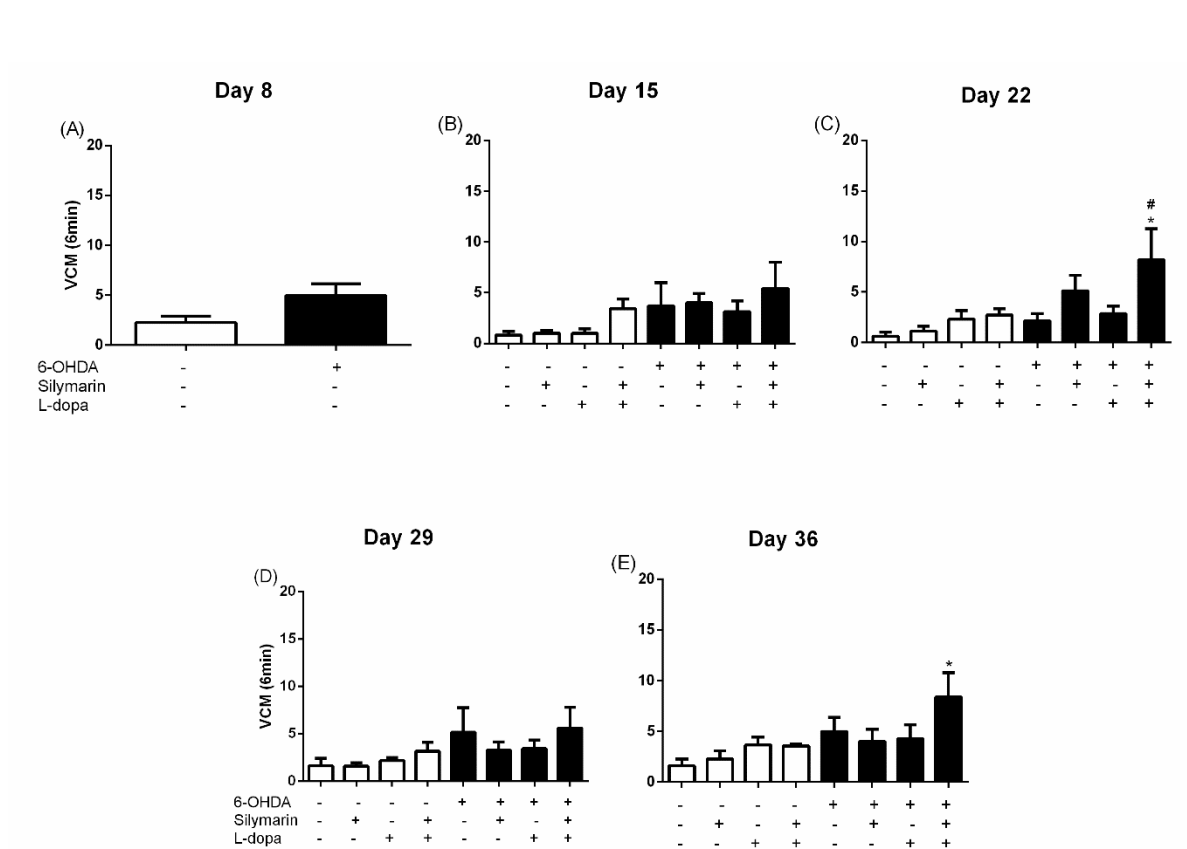
**FIGURE 2:**



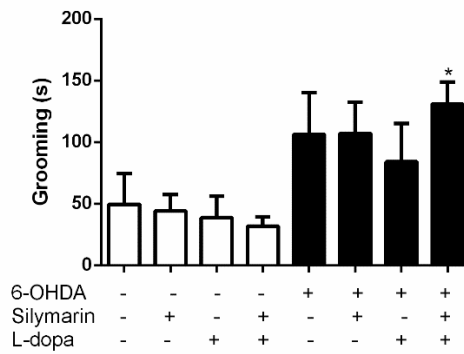
**FIGURE 3:**



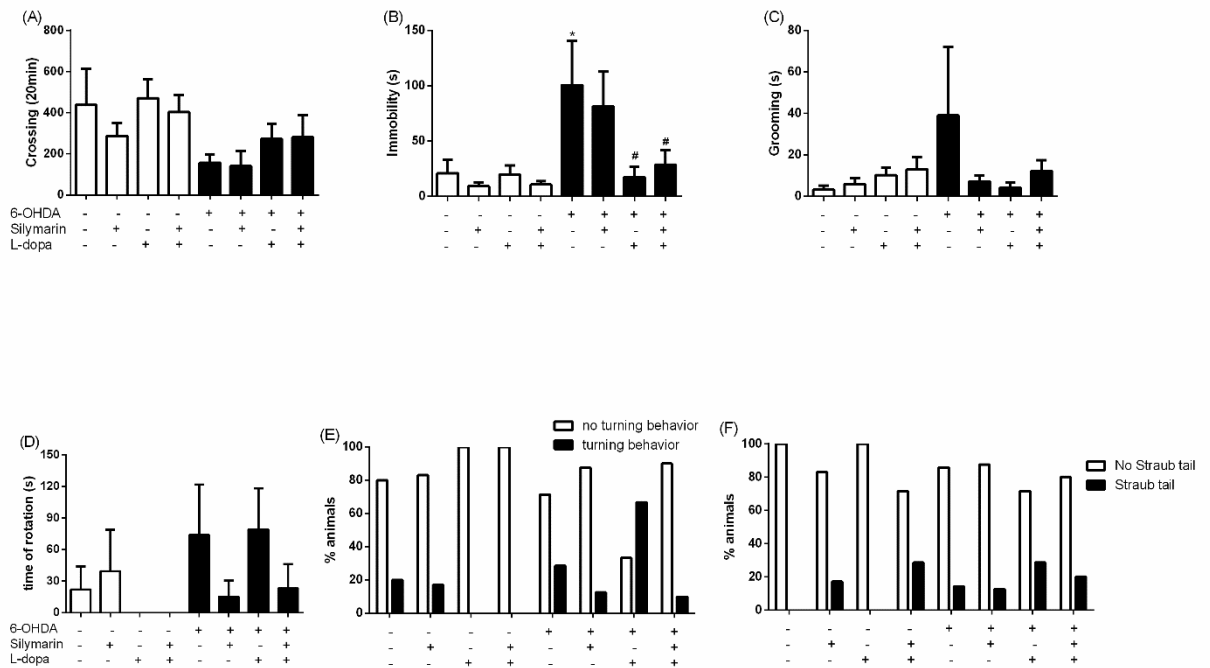
**FIGURE 4:**



**FIGURE 5:**



**FIGURE 6:**



## 5 DISCUSSÃO

A DP é a segunda doença neurodegenerativa mais comum e está relacionada à perda dos neurônios dopaminérgicos da SNpc, e conseqüentemente diminuição da concentração de DA na área de projeção destes neurônios (SCHOBER, 2004). Essa depleção de DA desencadeia uma modificação funcional complexa nos núcleos da base, o que leva aos sintomas motores típicos da doença: tremores, rigidez muscular e bradicinesia (BLANDINI; ARMENTERO; MARTIGNONI, 2008). Além destes sintomas também pode ocorrer a acinesia, alterações da marcha e instabilidade postural com quedas frequentes e falta de expressão facial (AGID, 1991). O tratamento clássico da DP tem sido fundamentalmente sintomático, direcionado para o controle dos sinais motores por meio do aumento do tônus dopaminérgico.

Neste contexto, a levodopa continua sendo amplamente utilizada como terapia farmacológica, uma vez que ela melhora os sintomas da doença, reduzindo a morbidade dos pacientes com DP. No entanto, esta terapia apresenta eficácia temporária, uma vez que a perda dopaminérgica na DP é progressiva e este fármaco depende de que haja ainda funcionalidade dopaminérgica, além de apresentar uma série de reações adversas inclusive motoras (NAGATSU; SAWADA, 2009). Com a cronicidade do uso da L-dopa, podem ser observados efeitos adversos como discinesias, fenômeno liga/desliga, estes, devidos à curta meia-vida da levodopa, caracterizados por flutuações na concentração da droga no plasma, e conseqüente exacerbação dos sinais parkinsonianos (CONTIN; MARTINELLI, 2010). Além disso, sintomas psiquiátricos como agitação, alucinações visuais, psicoses, paranoia, hipersexualidade, entre outros também podem ser observados com o uso crônico de levodopa (AHLISKOG, 2011). Dessa forma, torna-se relevante a busca de novas estratégias terapêuticas que possam ser utilizadas como adjuvantes no tratamento da DP.

O primeiro objetivo do nosso estudo foi investigar os efeitos da silimarina nas alterações comportamentais e bioquímicas induzidas por 6-OHDA em camundongos.

A silimarina foi escolhida para este estudo devido aos seus efeitos benéficos antioxidantes e da capacidade de inibir a atividade da MAO-B, observados em estudos *in vitro* (de OLIVEIRA et al., 2015; PODDER et al., 2012; WANG et al., 2002) e *in vivo* (HOU et al., 2010; MURATA et al., 2010; SINGHAL et al., 2011). A silimarina tem

mostrado efeitos protetores contra alguns modelos de lesão neuronal, como isquemia cerebral (RAZA et al., 2011), neurotoxicidade induzida por lipopolissacarídeos (WANG et al., 2002) e doença de Alzheimer (MURATA et al., 2010), o que poderia ser útil para retardar a progressão das doenças neurodegenerativas.

De particular importância, outros estudos investigaram os efeitos da silimarina no modelos de 6-OHDA e MPTP (BALUCHNEJADMOJARAD; ROGHANI; MAFAKHERI, 2010; PEREZ et al., 2014), no entanto, a maioria deles usou pré-tratamento (BALUCHNEJADMOJARAD; ROGHANI; MAFAKHERI, 2010; HADDADI; NAYEBI; BROOSHGHALAN, 2013; HADDADI et al., 2014) ou tratamento concomitante (PEREZ et al., 2014) com silimarina, em dose única (BALUCHNEJADMOJARAD; ROGHANI; MAFAKHERI, 2010) ou 5 dias de tratamento (HADDADI; NAYEBI; BROOSHGHALAN, 2013; HADDADI et al., 2014; PEREZ et al., 2014). O presente estudo foi o primeiro em que o tratamento com silimarina foi iniciada sete dias após a administração de 6-OHDA, onde os sintomas parkinsonianos já estão presentes, investigando também o possível alvo farmacológico.

Para avaliar o equilíbrio e a coordenação motora do animais realizamos o teste de rotarod. Nossos dados demonstraram que a administração de 6-OHDA causou um prejuízo na coordenação motora dos animais, observada por uma diminuição latência para a primeira queda e um aumento no número de quedas no rotarod. A silimarina reverteu essas alterações no teste do rotarod, o que poderia representar um efeito benéfico deste composto contra a toxicidade da 6-OHDA (GEED et al., 2014). Da mesma forma, também avaliou-se o equilíbrio e desempenho motor dos animais no teste da caminhada sobre a barra. Os animais que receberam 6-OHDA precisaram de mais tempo para atravessar a barra, o que indica uma deficiência no equilíbrio e/ou na coordenação motora. O tratamento com silimarina reduziu a latência para atravessar a barra em todas as doses testadas, sugerindo um efeito protetor deste complexo na toxicidade induzida por 6-OHDA. Esses resultados são consistentes com estudos da literatura, onde flavonoides como catequina, a baicaleína e a rutina demonstraram atenuar os déficits motores em modelos animais de DP induzido por neurotoxina (GAO et al., 2015; KHAN et al., 2012; TEIXEIRA et al., 2013).

O tratamento com silimarina recuperou a atividade locomotora dos animais que receberam 6-OHDA. Corroborando com nossos resultados, estudos anteriores tem mostrado efeitos protetores da silimarina sobre os déficits motores induzidos por maneb e paraquat em camundongos (SINGHAL et al., 2011), bem como da silibinina,

principal constituinte da silimarina, na neurotoxicidade dopaminérgica induzida por MPP<sup>+</sup> em ratos (GEED et al., 2014).

Além disso, investigamos se os efeitos da 6-OHDA e da silimarina nas alterações comportamentais estavam associadas a modificações na imunorreatividade da TH e da ERK1/2 fosforilada, bem como na atividade da MAO. Neste sentido, a TH, enzima limitante da biossíntese das catecolaminas, é um indicador da produção de DA (LIMA et al., 2012) e a redução em seus níveis na substância negra é uma característica de perda neuronal que ocorre na DP (OLANOW; TATTON, 1999). A 6-OHDA causou uma redução na imunorreatividade da TH no estriado e na SN, sugerindo que este modelo foi eficaz em causar danos aos neurônios dopaminérgicos desde os déficits comportamentais observados podem estar associados a diminuição dos níveis de TH e, conseqüentemente, de DA. No entanto, o tratamento com silimarina não recuperou a diminuição causada pela 6-OHDA na imunorreatividade da TH no estriado. Especificamente para a SN, observamos que a silimarina na dose de 30 mg/kg recuperou a diminuição na imunorreatividade TH causada pela 6-OHDA, sugerindo que o efeito da silimarina pode estar ligado ao grau de lesão induzida por 6-OHDA e com diferentes áreas do cérebro. Dados da literatura sugerem que a 6-OHDA inicialmente causa uma lesão nos terminais dopaminérgicos no estriado enquanto que os corpos celulares nigrais são secundariamente afetados por esta neurotoxina, o que levaria à uma perda de neurônios dopaminérgicos na SN lentamente ao longo de semanas após a administração de 6-OHDA (BLANDINI; ARMENTERO; MARTIGNONI, 2008; DEUMENS; BLOKLAND; PRICKAERTS, 2002; SAUER; OERTEL, 1994).

Embora tenha sido demonstrado que a ativação da ERK leva a respostas favoráveis, como diferenciação e neuroplasticidade, evidências sugerem que a sua ativação pode ter um papel na neurotoxicidade, particularmente no contexto de insultos oxidativos (CHU et al., 2004; KULICH et al., 2007; KUPERSTEIN; YAVIN, 2002). Nesse estudo a administração de 6-OHDA aumentou a imunorreatividade da ERK1/2 fosforilada tanto no estriado como na SN, sugerindo que ativação de vias da ERK pode contribuir para os mecanismos de morte celular neuronal dependente de 6-OHDA (KULICH; CHU, 2001). Entretanto, o tratamento com silimarina não mostrou efeito nas alterações causadas pela 6-OHDA nos níveis da ERK1/2 fosforilada. Dados da literatura mostram que a ativação da ERK1/2 no estriado está associada com a perda de TH (FRANCARDO et al., 2011). Assim, os níveis elevados da ERK1/2

fosforilada coincidem com níveis reduzidos de TH no estriado após o tratamento com silimarina, o que poderia explicar a falta de efeito da silimarina na imunorreatividade de ERK1/2.

Uma enzima considerada como um alvo importante para as doenças neurodegenerativas é a MAO a qual catalisa a desaminação oxidativa de aminas biogênicas (YOUUDIM; BAKHLE, 2006). As duas isoformas da MAO estão distribuídas em todos os tecidos, sendo que a inibição da MAO-A pode fornecer proteção contra a apoptose (OU; CHEN; SHIH, 2006), enquanto a inibição da MAO-B está relacionada a um papel terapêutico no tratamento da DP (YOUUDIM; BAKHLE, 2006). Um recente estudo do nosso grupo demonstrou a capacidade da silimarina em diminuir a atividade da MAO *in vitro* (de OLIVEIRA et al., 2015). No entanto, nesse estudo o efeito da silimarina no prejuízo motor induzido por 6-OHDA parece não estar relacionado a alterações na atividade da MAO. Acredita-se que o metabolismo dos constituintes presentes na silimarina ou a sua disponibilidade cerebral poderia explicar a falta de efeitos da silimarina *per se* na atividade da MAO, desde que as concentrações necessárias para inibir a atividade da MAO *in vitro* poderiam não ser atingidas no tecido cerebral *in vivo*.

Embora a neuropatologia da DP é baseada na perda celular na via nigrostriatal, outros eventos como o estresse oxidativo, disfunção mitocondrial, apoptose e inflamação estão envolvidos na degeneração neuronal observada nessa doença (MILLER et al., 2009). Neste contexto, apesar da silimarina não ter recuperado os parâmetros bioquímicos avaliados nesse trabalho, dados da literatura tem mostrado que este complexo de flavonoides apresenta propriedades antioxidantes (SINGHAL et al., 2011) e anti-inflamatórias (GUPTA et al., 2000) e estes fatores poderiam estar envolvidos na recuperação comportamental observada nesse estudo. Além disso, pode-se considerar que o principal componente da silimarina, a silibinina, poderia ter participação em sua ação contra a 6-OHDA.

Então, o segundo objetivo do trabalho foi avaliar se os efeitos da silimarina poderiam ser devido à presença de seu principal constituinte, a silibinina. Neste segundo trabalho investigou-se a possível eficácia da silibinina em recuperar os danos locomotores induzidos pela 6-OHDA em camundongos.

No entanto, o tratamento com diferentes doses de silibinina (50 ou 100 mg/kg) por 7 dias não recuperou as alterações comportamentais induzidas pela 6-OHDA nos testes do rotarod e do campo aberto. Em relação ao teste da caminhada sobre a barra,

os animais que receberam 6-OHDA precisaram de um tempo maior para atravessar a barra, indicando uma perda da função de equilíbrio e a silibinina recuperou o equilíbrio dos animais nesse teste, sugerindo que este composto poderia estar agindo diferentemente nas vias responsáveis pelo controle do equilíbrio.

Nesse sentido, como sintomas motores distintos tem base neuronais particulares, a silibinina pode estar afetando diferentemente as respostas comportamentais, sendo o teste da caminhada sobre a barra mais sensível aos seus efeitos que o teste do rotarod e do campo aberto. Além disso, sabe-se que vários sintomas motores observados na DP são causados pela depleção de dopamina no estriado; entretanto, se tem conhecimento que outras estruturas neuronais e sistemas neuroquímicos também estão envolvidos nos prejuízos motores, incluindo as áreas do córtex pré-frontal e o cerebelo, bem como o sistema serotoninérgico, glutamatérgico e colinérgico (MOUSTAFA et al., 2016). Isso poderia explicar, em partes, a falta de efeito da silibinina nas alterações comportamentais induzidas pela 6-OHDA observada nos testes do campo aberto e do rotarod, apesar de mostrar uma recuperação significativa do equilíbrio dos animais no teste de caminhada sobre a barra.

A administração de 6-OHDA induziu a toxicidade dopaminérgica nos animais, a qual foi observada de forma comportamental e bioquimicamente pela diminuição da imunorreatividade da TH no estriado. O tratamento com silibinina recuperou as alterações causadas pela 6-OHDA na imunorreatividade da TH. Estes resultados sugerem que a silibinina poderia estar modulando a viabilidade celular dos neurônios dopaminérgicos como evidenciado pelo aumento da TH. Corroborando com nossos dados, um estudo anterior mostrou que a silibinina atenuou a perda de neurônios dopaminérgicos induzida por MPP<sup>+</sup> na SN de ratos (JUNG et al., 2014). Além disso, vale ressaltar que a dose de silibinina que recuperou os níveis de TH são muito maiores que a quantidade de silibinina presente na dose de 100 mg/kg de silimarina utilizada no primeiro trabalho, o que poderia explicar o fato da silibinina ter recuperado os níveis de TH e a silimarina não ter mostrado efeito nos níveis da TH no estriado.

Interessantemente, o complexo da silimarina parece ser melhor que a silibinina na recuperação dos sintomas motores induzidos por 6-OHDA, sugerindo que os efeitos da silimarina nesse modelo de parkinsonismo possivelmente se deve ao sinergismo de todos os seus componentes e não apenas a ação de seu principal constituinte.



Levando em consideração que a silimarina recuperou as alterações comportamentais induzidas pela 6-OHDA, sugerindo um efeito terapêutico potencial nos sintomas parkinsonianos, resolvemos investigar os efeitos desse composto em associação com a levodopa. Dessa forma, o terceiro objetivo desse trabalho foi avaliar o possível efeito do co-tratamento com silimarina e levodopa no modelo de parkinsonismo induzido por 6-OHDA.

O tratamento com silimarina recuperou o prejuízo motor dos animais induzidos pela administração intracerebroventricular de 6-OHDA. No entanto, o co-tratamento com silimarina não potencializou o efeito da levodopa sobre o dano motor. Apesar do co-tratamento com silimarina e levodopa não ter mostrado um efeito sinérgico, a silimarina sozinha mostrou um efeito benéfico sobre o prejuízo motor causado pela 6-OHDA tanto no tratamento a curto prazo (7 dias) quanto no tratamento a longo prazo, sugerindo assim, que ela poderia ser útil nos estágios iniciais da DP.

Além disso, investigamos se o co-tratamento com silimarina e levodopa poderia ter efeito sobre os movimentos orais involuntários, representado pelos movimentos de mascar no vazio (MMVs). Sabe-se que, com o tratamento prolongado com levodopa, muitos pacientes desenvolvem complicações motoras, como a discinesia (NAGATSU; SAWADA, 2009). A discinesia é caracterizada por uma variedade de movimentos hiperkinéticos, repetitivos e involuntários. Pode ocorrer em várias regiões do corpo, mas afeta principalmente a região facial (JENNER, 2004). A discinesia induzida por levodopa é comum e difícil de tratar.

Um dos principais fatores de risco para o desenvolvimento de discinesia em pacientes com DP é a gravidade da doença no início da farmacoterapia com levodopa (KOSTIC et al., 2002). Em nosso estudo, o tratamento com levodopa não induziu discinesia nos animais, entretanto, o co-tratamento com silimarina e levodopa parece precipitar o aparecimento da discinesia orofacial. É importante enfatizar que a incidência e gravidade da discinesia induzida por levodopa está relacionada com a extensão da lesão nigroestriatal, uma vez que é necessária uma pronunciada depleção de DA no estriado para que os animais exibam discinesia (DARMOPIL et al., 2008; LUNDBLAD et al., 2004). Assim, a ausência de discinesia no grupo tratado com levodopa poderia estar relacionada com o grau de lesão induzido pela 6-OHDA. Além disso, dados da literatura têm relatado que a sensibilidade à levodopa difere entre os modelos de PD, uma vez que os animais que receberam injeção de 6-OHDA no estriado necessitaram de uma dose três a quatro vezes de levodopa maior para atingir

uma severidade de discinesia comparável a animais lesionados no feixe prosencefálico medial (MFB) (LUNDBLAD et al., 2004).

Além disso, inicialmente hipotetizamos que o tratamento com silimarina poderia estar causando uma modulação da sensibilidade dopaminérgica. Dados da literatura demonstraram que a perda seletiva de neurônios DA leva a um aumento na sensibilidade de receptores dopaminérgicos pós-sinápticos como uma resposta compensatória devido à deficiência de DA (NEVE; KOZLOWSKI; MARSHALL, 1982). Sabe-se que fármacos que aumentam a função dopaminérgica no cérebro causam um padrão de comportamento que inclui aumento da locomoção e estereotipia (DICKINSON; CURZON, 1983). Portanto, após a última avaliação comportamental, os animais receberam uma injeção de apomorfina para verificar uma possível resposta de sensibilização ao tratamento com silimarina e/ou levodopa.

Apesar da apomorfina (administrada no dia 36 do período experimental) ter causado mudanças nas respostas comportamentais como o número de cruzamentos, tempo de comportamento de limpeza, tempo de rotação, porcentagem de animais com comportamento de giro e porcentagem de animais com reação de cauda de Straub, estas mudanças não foram significativas. No entanto, foi observada uma alteração significativa no tempo de imobilidade dos animais. Sabe-se que os efeitos comportamentais estão intimamente ligados ao grau de disfunção neuronal (SCHWARTING et al., 1991) e a apomorfina é um marcador confiável para a depleção de dopamina nigroestriatal. O comportamento induzido por apomorfina só é possível quando a lesão está completa ou quase completa (PRZEDBORSKI et al., 1995), o que poderia explicar a falta de efeito da apomorfina na atividade locomotora e no comportamento estereotipado dos animais tratados com 6-OHDA.

## 6 CONCLUSÕES ESPECÍFICAS

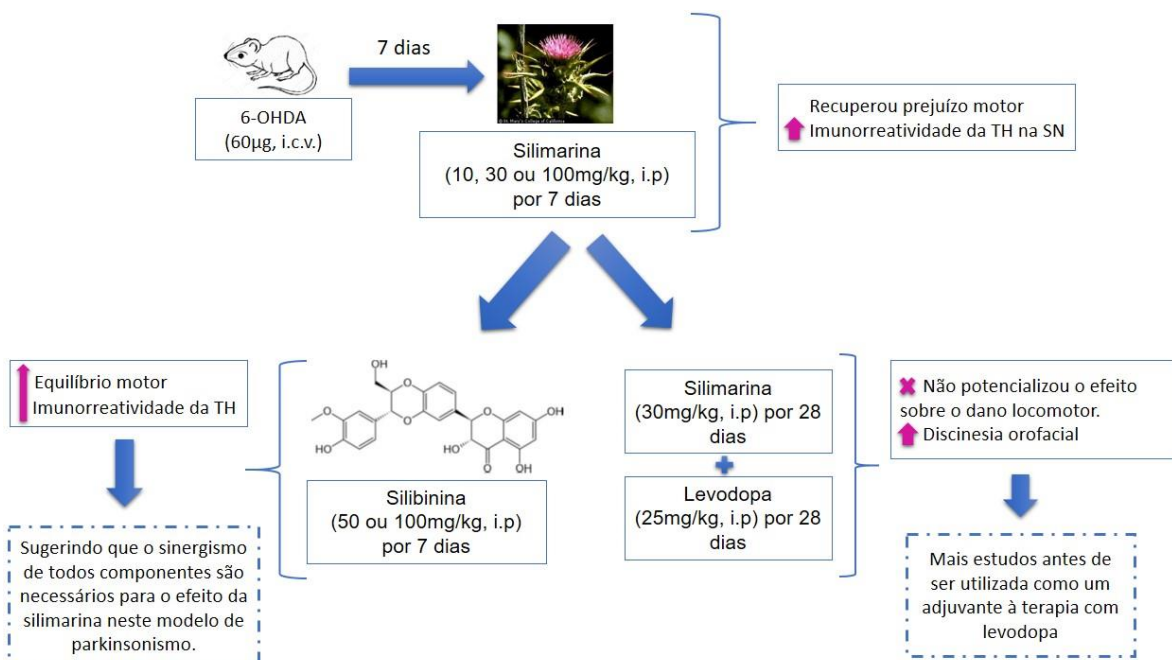
De acordo com os resultados apresentados nesta tese podemos concluir que:

- A silimarina recuperou as alterações comportamentais induzidas pela 6-OHDA, sugerindo um potencial efeito terapêutico nos sintomas parkinsonianos. O efeito da silimarina neste modelo parece envolver a modulação da imunorreatividade da TH na substância negra.
- A silibinina recuperou o equilíbrio motor dos animais no teste da caminhada sobre a barra, o qual pode estar associado com a recuperação da imunorreatividade da TH no estriado.
- O co-tratamento com silimarina não potencializou o efeito da levodopa sobre o dano motor causado pela 6-OHDA. Além disso a associação da silimarina com levodopa parece antecipar o aparecimento da discinesia orofacial.

## 7 CONCLUSÃO FINAL

Esse estudo demonstrou que a silimarina tem um efeito positivo sobre os sintomas do tipo parkinsonianos induzidos pela 6-OHDA. Assim, a silimarina poderia vir a ser um agente terapêutico potencial no tratamento dos sintomas parkinsonianos leves. No entanto, este composto deve ser melhor estudado antes de ser utilizado como um adjuvante à terapia com levodopa, já que a associação de silimarina com levodopa parece antecipar o aparecimento da discinesia.

Figura 6 – Principais achados do trabalho



Fonte: Autora

## 8 PERSPECTIVAS

Com base nos resultados obtidos no presente trabalho, temos como perspectivas:

- ✓ Investigar os efeitos do co-tratamento com silimarina e levodopa em marcadores dopaminérgicos: imunorreatividade da TH e de receptores dopaminérgicos e níveis de dopamina e seus metabólitos no estriado e na substância negra.
- ✓ Verificar o efeito do co-tratamento com silimarina e levodopa na imunorreatividade da ERK1/2 e da FosB/ $\Delta$ FosB.
- ✓ Realizar testes de toxicidade para avaliar o perfil de segurança tanto da silimarina quanto da silibinina.

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## ANEXO A - CERTIFICADO DE APROVAÇÃO DE PROJETO NA COMISSÃO DE ÉTICA NO USO DE ANIMAIS 2015



Comissão de Ética no Uso de Animais

da  
Universidade Federal de Santa Maria

### CERTIFICADO

Certificamos que a proposta intitulada "Estudo dos efeitos neuroprotetores da silimarina e da silibinina em um modelo de parkinsonismo induzido por 6-hidroxidopamina em camundongos.", protocolada sob o CEUA nº 4398020415, sob a responsabilidade de **Roselei Fachinetto e equipe; Alcindo Busanello; Ana Paula Chiapinotto Ceretta; Bárbara Nunes Krum; Catiúscia Molz de Freitas; Getúlio Nicola Bresssan; Larissa Finger Schaffer** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 02/06/2015.

We certify that the proposal "Study of neuroprotective effects of silymarin and silibinin in a model of parkinsonism induced by 6-OHDA in mice", utilizing 10 Heterogenics mice (10 males), protocol number CEUA 4398020415, under the responsibility of **Roselei Fachinetto and team; Alcindo Busanello; Ana Paula Chiapinotto Ceretta; Bárbara Nunes Krum; Catiúscia Molz de Freitas; Getúlio Nicola Bresssan; Larissa Finger Schaffer** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 06/02/2015.

Finalidade da Proposta: [Pesquisa \(Acadêmica\)](#)

Vigência da Proposta: de [05/2015](#) a [10/2017](#)

Área: [Fisiologia E Farmacologia](#)

Origem: [Biotério Central UFSM](#)

Espécie: [Camundongos heterogênicos](#)

sexo: [Machos](#)

idade: [2 a 2 meses](#)

N: [10](#)

Linhagem: [Swiss](#)

Peso: [25 a 30 g](#)

Resumo: A doença de Parkinson (DP) consiste num distúrbio motor incapacitante que afeta cerca de 1-2% da população mundial. O aumento da população idosa prevista para os próximos anos implicará em um aumento na incidência da DP na população mundial, fazendo-se cada vez mais necessária a busca por novas alternativas farmacológicas que apresentem menos efeitos adversos que os fármacos atualmente disponíveis e que ofereçam uma perspectiva de não só frear a progressão da doença, mas que também possam agir na prevenção da ocorrência desta. Nesse sentido a silimarina, isolada das sementes de *Silybum marianum* e a silibinina, componente ativo da silimarina são flavonoides antioxidantes que apresentam efeitos anti-inflamatórios e neuroprotetores. Assim, o presente estudo visa investigar os efeitos neuroprotetores da silimarina e da silibinina em um modelo de parkinsonismo induzido por 6-hidroxidopamina em camundongos. Para isso, camundongos com dois meses de idade pesando entre 25-35g receberão uma injeção intracerebroventricular (i.c.v.) de 60 µg de 6-hidroxidopamina ou veículo para indução do parkinsonismo. Após 14 dias os animais serão subdivididos em grupos que receberão diariamente durante sete dias diferentes doses de silimarina (10, 30 ou 100 mg/kg) ou silibinina (1, 3 ou 10mg/kg) por via intraperitoneal. A avaliação comportamental será realizada nos dias 0, 14 e 22 do período experimental, onde será avaliada a atividade locomotora, a marcha, a catalepsia, a [jascinesia] e a caminhada sobre a barra, e também o peso corporal dos animais. No 22º dia os animais serão mortos por deslocamento cervical. O córtex, substância negra e o estriado serão utilizados para análise de parâmetros oxidativos (oxidação da diclorofluoresceína, níveis de tióis totais e nitro-protéicos e níveis de peroxidação lipídica), parâmetros dopaminérgicos (imunoreatividade da tirosina hidroxilase e do transportador de dopamina; atividade da enzima monoaminoxidase), atividade da Na<sup>+</sup>/K<sup>+</sup>-ATPase, expressão da proteína alfa-sinucleína, além do papel da apoptose e da inflamação (caspase 3, caspase 9, Bax, Bcl-2, NF-κB, Nrf-2). Acredita-se que a silimarina e a silibinina possam ser úteis no tratamento da doença de Parkinson e através deste projeto espera-se elucidar o seu possível mecanismo de ação no modelo de parkinsonismo em roedores, o que pode futuramente auxiliar no tratamento de pacientes com doença de Parkinson.

Local do experimento: Laboratório do Departamento de Fisiologia e Farmacologia (Sala 5209)

Santa Maria, 12 de setembro de 2016



*Comissão de Ética no Uso de Animais*

da \_\_\_\_\_  
*Universidade Federal de Santa Maria*

Profa. Dra. Daniela Bitencourt Rosa Leal  
Coordenadora da Comissão de Ética no Uso de Animais  
Universidade Federal de Santa Maria

Prof. Dr. Denis Brook Rosenberg  
Vice-Coordenador da Comissão de Ética no Uso de Animais  
Universidade Federal de Santa Maria

## ANEXO B - CERTIFICADO DE APROVAÇÃO DE PROJETO NA COMISSÃO DE ÉTICA NO USO DE ANIMAIS 2017



Comissão de Ética no Uso de Animais

da

Universidade Federal de Santa Maria

### CERTIFICADO

Certificamos que a proposta intitulada "Avaliação do efeito da silimarina na discinesia orofacial induzida por l-dopa em um modelo de parkinsonismo em camundongos", protocolada sob o CEUA nº 9144181016, sob a responsabilidade de **Roselei Fachinnetto e equipe; Bárbara Nunes Krum; Camila Parcianello Saccol; Catiúscia Molz de Freitas; Getúlio Nicola Bressan; Janaína Paola Schwerz; Juliane Reckziegel** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 19/01/2017.

We certify that the proposal "Evaluation of sylimarin effects on L-dopa-induced orofacial dyskinesia in a parkinsonism model in mice", utilizing 100 Heterogenics mice (100 males), protocol number CEUA 9144181016, under the responsibility of **Roselei Fachinnetto and team; Bárbara Nunes Krum; Camila Parcianello Saccol; Catiúscia Molz de Freitas; Getúlio Nicola Bressan; Janaína Paola Schwerz; Juliane Reckziegel** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 01/19/2017.

Finalidade da Proposta: [Pesquisa \(Acadêmica\)](#)

Vigência da Proposta: de [11/2016](#) a [12/2017](#)

Área: [Farmacologia](#)

Origem: [Biotério Central UFSM](#)

Espécie: [Camundongos heterogênicos](#)

sexo: [Machos](#)

idade: [2 a 2 meses](#)

N: [100](#)

Linhagem: [Swiss](#)

Peso: [25 a 30 g](#)

Resumo: A doença de Parkinson (DP) consiste num distúrbio motor incapacitante que afeta cerca de 1-2% da população mundial. A levodopa é o agente farmacológico mais utilizado no tratamento desta doença, no entanto, o tratamento prolongado leva ao desenvolvimento da discinesia, a qual não possui fisiopatologia completamente esclarecida. Nesse sentido a silimarina, isolada das sementes de *Silybum marianum* é um composto de flavonóides antioxidantes que apresentam efeitos anti-inflamatórios e neuroprotetores. Assim, este estudo tem como finalidade avaliar o efeito da silimarina na discinesia orofacial e nas alterações bioquímicas induzidas por levodopa em um modelo de parkinsonismo em camundongos. Para isso os camundongos receberão uma injeção intracerebroventricular (i.c.v.) de 60 µg de 6-hidroxidopamina ou veículo para indução do parkinsonismo. Após 7 dias os animais serão subdivididos em grupos que receberão diariamente por via intraperitoneal (i.p.) veículo ou levodopa 25 mg/kg + benserazida 10 mg/kg e/ou silimarina (30 mg/kg/dia, i.p.) por 21 dias. As avaliações comportamentais (atividade locomotora, parâmetros de discinesia orofacial e caminhada sobre a barra) serão realizadas nos dias 7, 14, 21 e 28. Após as análises comportamentais serão analisados os parâmetros dopaminérgicos (tirosina hidroxilase, do transportador de dopamina, do receptor D2, atividade da monoaminoxidase), além de parâmetros apoptóticos (caspase 3, 9, Bax e Bcl-2) e inflamatórios (COX-2 e NF- $\kappa$ B), bem como a proteínas da via das MAPKs (proteínas quinases ativadas por mitógenos) (p38 e ERK1/2) em estriado e na região contendo a substância negra. Acredita-se que a silimarina possa ser útil no tratamento da discinesia induzida por levodopa e através deste projeto pretendemos elucidar o seu possível mecanismo de ação para que no futuro possa ser utilizado em pacientes com movimentos involuntários.

Local do experimento: Laboratório de Neurotoxicidade e Comportamento do Departamento de Fisiologia e Farmacologia

Santa Maria, 18 de abril de 2017



*Comissão de Ética no Uso de Animais*

da

*Universidade Federal de Santa Maria*

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