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Aline Alves Courtes

EFEITO PROTETOR DO EXTRATO AQUOSO DE *Luehea divaricata* CONTRA OS DANOS OXIDATIVOS E COMPORTAMENTAIS INDUZIDOS PELO ÁCIDO 3-NITROPROPIÔNICO EM RATOS

Dissertação de Mestrado

URUGUAIANA
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Dissertação apresentada ao Programa de Pós-graduação *Stricto sensu* em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Bioquímica.

Orientador: Prof. Dr. Robson Luiz Puntel

Co-orientador: Prof. Dr. Félix Alexandre Antunes Soares

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ALINE ALVES COURTES

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Área de concentração: Bioprospecção Molecular

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Dedico esta dissertação aos meus amados pais, Fortunato e Tania, maiores incentivadores e fontes inesgotáveis de apoio, amor e compreensão.

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É tempo de travessia: e, se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos.

Fernando Pessoa

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioquímica
Universidade Federal do Pampa

EFEITO PROTETOR DO EXTRATO AQUOSO DE *Luehea divaricata* CONTRA OS DANOS OXIDATIVOS E COMPORTAMENTAIS INDUZIDOS PELO ÁCIDO 3-NITROPROPIÔNICO EM RATOS

Autora: Aline Alves Courtes
Orientador: Robson Luiz Puntel
Local e Data da Defesa: Uruguaiana, 25 de agosto de 2014.

A doença de Huntington (DH) é uma desordem neurodegenerativa, hereditária autossômica dominante, caracterizada por alterações motoras progressivas, distúrbios emocionais, movimentos involuntários anormais e demência, os quais podem ser atribuídos à morte de neurônios estriatais e corticais. Apesar de ser uma etiologia ainda não totalmente conhecida, tem-se sugerido que o estresse oxidativo contribua para o desenvolvimento dessa condição. Nesse contexto, o ácido 3-nitropropiônico (3-NP), um inibidor da enzima mitocondrial succinato desidrogenase (SDH), têm sido utilizado em modelos animais por desenvolver as características fenotípicas observadas na DH. De um modo geral, o efeito do 3-NP está relacionado a capacidade do mesmo em causar disfunção mitocondrial e gerar espécies reativas. Nesse cenário, a pesquisa por terapias em que se busque neutralizar os efeitos deletérios das espécies reativas são de grande importância. A *Luehea divaricata (L. divaricata)*, popularmente conhecida no Brasil como açoita cavalo contêm numerosos polifenóis, os quais poderiam atuar como agentes neuroprotetores em estudos *in vitro* e *in vivo* de doenças neurodegenerativas. Diante do exposto, buscamos nesse estudo testar a hipótese que o extrato aquoso de *L. divaricata* pode exercer efeito antioxidante e neuroprotetor frente às alterações comportamentais e oxidativas induzidas pelo 3-NP em ratos. Nossos dados demonstraram que o 3-NP induziu os sintomas da DH, uma vez que provocou mudanças de comportamento, evidenciados pela diminuição da atividade locomotora no Campo Aberto e Rota Rod; bem como alterações oxidativas evidenciadas pelo aumento dos níveis de espécies reativas de oxigênio (ROS) e peroxidação lipídica; redução nos níveis de glutationa reduzida e na atividade da acetilcolinesterase. O extrato aquoso de *L. divaricata* preveniu as alterações comportamentais e oxidativas induzidas pelo tratamento com 3-NP, sugerindo possível efeito neuroprotetor da *L. divaricata* contra a toxicidade do 3-NP, o qual pode ser devido a suas propriedades antioxidantes. Consequentemente, a planta poderia ser utilizada como um agente terapêutico para a prevenção dos sintomas da DH.

Palavras-chave: *Luehea divaricata*, Ácido 3-nitropropiônico, Doença de Huntington.

ABSTRACT

Dissertation of Master's Degree
Program of Post-Graduation in Biochemistry
Federal University of Pampa

PROTECTIVE EFFECTS OF AQUEOUS EXTRACT OF *LUEHEA DIVARICATA* AGAINST BAHAVIORAL AND OXIDATIVE CHANGES INDUCED BY 3-NITROPROPIONIC ACID IN RATS

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Advisor: Robson Luiz Puntel
Date and Place of Defense: Uruguaiana, August 25, 2014

Huntington's disease (HD) is a neurodegenerative disorder, autosomal dominant, characterized by progressive motor disorders, emotional disturbances, abnormal involuntary movements and dementia, which can be attributed to the death of striatal and cortical neurons. Although a etiology is not fully known, it has been suggested that oxidative stress contributes to the development of this condition. In this context, the 3-nitropropionic acid (3-NP), an inhibitor of the mitochondrial enzyme succinate dehydrogenase (SDH), have been used in animal models to develop the phenotypic characteristics observed in HD. In general, the effect of 3-NP associated with the same capacity to cause mitochondrial dysfunction and generating reactive species. In this scenario, the search for treatments that seek to neutralize the deleterious effects of reactive species are of great importance. *Luehea divaricata* (*L. divaricata*), popularly known in Brazil as "açoita cavalo" contain numerous polyphenols, which could act as neuroprotective agents in *in vitro* and *in vivo* neurodegenerative diseases. Given the above, this study sought to test the hypothesis that the aqueous extract of *L. divaricata* may exert antioxidant and neuroprotective effect front and behavioral changes induced by oxidative 3-NP in rats. These data demonstrate that the 3-NP induced the symptoms of HD, because changes in behavior caused evidenced by the decrease in locomotor activity in the Open Field and Rota Rod; and oxidative changes evidenced by increased levels of reactive oxygen species (ROS) and lipid peroxidation; reduction in the levels of reduced glutathione and acetylcholinesterase activity. The aqueous extract of *L. divaricata* was able to prevent the oxidative and behavioral changes induced by 3-NP treatment, suggesting the possible neuroprotective effect against 3-NP toxicity, which may be due to its antioxidant properties. Consequently, this plant could be used as a potential therapeutic for the prevention of HD-like simptoms.

Keywords: *Luehea divaricata*, 3-Nitropropionic acid, Huntington's disease.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

3-NP - Ácido 3-nitropropiônico

AChE - Acetilcolinesterase

ATP - Adenosina trifosfato

Ca^{2+} - Íons Cálcio

CAG - Citosina adenina guanina

DCF - 2,7-diclorofluoresceína

DH - Doença de Huntington

GPx – Glutationa peroxidase

GSH - Glutationa reduzida

GSSG - Glutationa oxidada

HAP1 - Huntingtina associada a proteína 1

H_2O_2 – Peróxido de Hidrogênio

MDA - Bis malonaldeído (dimetil acetal)

NO – Óxido nítrico

NOS - Óxido nítrico sintase

L. divaricata - *Luehea divaricata*

IP - Intraperitoneal

$ONOO^-$ - Peroxinitrito

$O_2^{\bullet -}$ - Ânion Superóxido

Poli Q - Poliglutaminas

ROS - Espécies reativas de oxigênio

SDH - Succinato desidrogenase

SOD – Superóxido dismutase

TBA - Ácido Tiobarbitúrico

TBARS - Substâncias reativas ao ácido tiobarbitúrico

TCA - Ácido tricloroacético

SUMÁRIO

APRESENTAÇÃO

No item INTRODUÇÃO consta uma revisão sucinta da literatura sobre os temas trabalhados nesta dissertação.

A metodologia realizada e os resultados obtidos que fazem parte desta dissertação estão apresentados no item MANUSCRITO sob a forma de um manuscrito redigido em inglês conforme as normas do periódico ao qual será submetido. No mesmo constam as seções: Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas.

Os itens CONCLUSÕES e PERSPECTIVAS, encontrados no final da dissertação, apresentam conclusões gerais sobre os resultados do manuscrito presente neste trabalho e as perspectivas para futuros trabalhos.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO desta dissertação.

1 INTRODUÇÃO

A doença de Huntington (DH) é uma patologia neurodegenerativa, autossômica dominante, caracterizada por alterações motoras progressivas, distúrbio emocional, movimentos involuntários anormais, morte neuronal, demência e perda de peso (RAMASWAMY et al., 2007; ROSS et al., 2014). Descrita em 1872, pelo médico norte americano George Huntington, o qual identificou as características clínicas da doença e o padrão de transmissão familiar (BATES, 2005). A mutação gênica causadora da DH está localizada no braço curto do cromossomo 4, que codifica a proteína huntingtina, resultando em uma expansão da sequência de nucleotídeos citosina, adenina e guanina (CAG codifica o aminoácido glutamina) (KROBITSCH & KAZANTSEV, 2011; WEIR et al. 2011). Resultando em uma proteína mutante com uma sequência de poliglutaminas (poli Q) no terminal amínico da proteína huntingtina, podendo exceder 55 repetições, considerando que um indivíduo sem a doença apresenta menos de 35 repetições (DAMIANO et al., 2013; CHIANG et al., 2012).

Muitas proteínas têm sido descritas como possuidoras de inter-relações com a huntingtina, foi identificado uma proteína chamada HAP1 (huntingtina associada a proteína 1) que se liga fortemente a huntingtina devido à repetição expandida de poliglutaminas desta proteína (VONSATTEL, 2008) (Figura 1). À medida que estas repetições aumentam, a ligação torna-se mais intensa devido à formação inespecífica de pontes de hidrogênio. Este aumento na intensidade da ligação também pode ocorrer com outras proteínas presentes no citoplasma neuronal. A proteína HAP1 é encontrada largamente no tecido cerebral, com marcada preferência pelos núcleos da base, sugeriu-se que ela seria responsável pela seletividade regional no cérebro comprometido pela DH (ROSS et al., 2014).

Quando existe um grande número de repetições CAG (mais de 40), a doença de Huntington apresenta penetrância completa e pode ocorrer antes dos 20 anos de idade, sendo chamada de DH juvenil, “acinética-rígida” ou variante de Westphal. É responsável por cerca de 7% dos casos de Doença de Huntington (NANCE & MYERS, 2001).

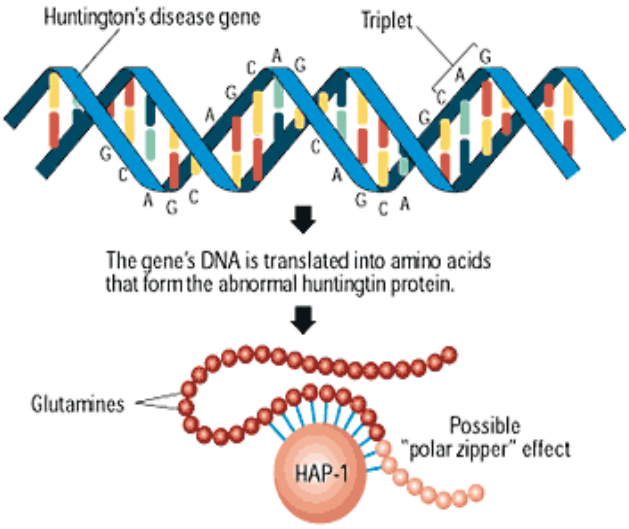

FIGURA 1 – Alterações na proteína huntingtina na DH. Huntington's disease gene: Gene da Doença de Huntington. Triplet: triplicar. The gene's DNA is translated into amino acids that form the abnormal huntingtin protein: Os genes do DNA são traduzidos em aminoácidos que formam a proteína huntingtina anormal. Fonte: Tunez et al., 2010.

A proteína huntingtina mutante é expressa durante toda a vida em pacientes com a DH. Na maioria dos casos surge apenas na idade adulta, entre os 35 e 50 anos de idade (ANDREWS & BROOKS, 1998). Ao longo do tempo a doença progride e torna-se fatal entre 15 a 20 anos após o aparecimento dos sintomas (ROSS et al., 2014). Possui prevalência de 5-10 casos para cada 100 mil habitantes na Europa e América do Norte (HO et al., 2001; ROSS & TABRIZI, 2011).

Neuropatologicamente caracteriza-se por disfunção e degeneração no estriado e no córtex cerebral, ocorrendo também em outras regiões como cerebelo, tálamo, núcleo subtalâmico e hipocampo (SANDHIR & MEHROTRA, 2013; CHAKRABORTY et al., 2014). Os neurônios mais afetados no estriado são os neurônios espinhosos médios GABAérgicos, que correspondem a aproximadamente 95% do número total de neurônios estriatais (HAN et al., 2010). Com a progressão da patologia, os neurônios espinhosos médios que se projetam para o globo pálido interno (via direta) e neurônios piramidais corticais também são afetados. A degeneração tardia dos neurônios da via direta é responsável pelo desenvolvimento de bradicinesia e rigidez em estágios terminais da doença (BROUILLET et al., 1999; ZUCCATO et al., 2010).

Os movimentos anormais da DH acredita-se que sejam causados pela perda da maioria dos corpos celulares dos neurônios secretores de GABA no núcleo caudado e no putâmen e dos neurônios secretores de acetilcolina (Ach) em muitas

partes do cérebro. Evidências sugerem que as manifestações coreiformes da DH podem ser causadas por déficits na síntese de acetilcolina em neurônios do estriado (VONSATTEL, 2008). As terminações axonais dos neurônios gabaérgicos normalmente causam inibição do globo pálido e da substância negra. A perda da inibição parece permitir descargas espontâneas de atividade do globo pálido e da substância negra que causa os movimentos de distorção (HAN et al., 2010). A demência na DH provavelmente não resulta da perda dos neurônios GABA, mas da perda dos neurônios secretores de Ach, talvez especialmente localizados nas áreas de pensamento do córtex cerebral (lobo frontal) (SOROLLA, et al 2008).

Apesar de vários danos bioquímicos, moleculares, fisiológicos e anatômicos terem sido extensivamente descritos, os mesmos não foram totalmente esclarecidos. No entanto, inúmeras pesquisas apresentadas nas últimas décadas, sugerem diversas hipóteses sobre o mecanismo molecular envolvido nesta doença (RANGONE et al., 2004; SOROLLA et al., 2008).

Diferentes estudos bioquímicos revelaram a existência de grandes defeitos no metabolismo energético dos pacientes com DH caracterizados pela disfunção mitocondrial (MIRANDOLA et al., 2010; KROBITSCH & KAZANTSEV, 2011). As mitocôndrias desses pacientes são afetadas por disfunções na cadeia transportadora de elétrons, onde os complexos II, III e IV são alterados, levando a uma diminuição significativa na oxidação de succinato e na síntese de ATP (WALKER, 2007).

A disfunção mitocondrial é a principal fonte geradora de espécies reativas de oxigênio (EROS). Essas EROS desencadeiam excitotoxicidade (Figura 2), a qual induz a entrada maciça de íons de cálcio (Ca^{2+}), a partir do meio extracelular, que passam da mitocôndria para o citoplasma, resultando na ativação da óxido nítrico sintase neuronal (NOS) ou óxido nítrico sintase tipo I, com posterior liberação de óxido nítrico (NO). Por sua vez, o óxido nítrico é transformado em peroxinitrito ($ONOO^{-}$) depois de reagir com o ânion superóxido ($O_2^{\bullet -}$) da cadeia transportadora de elétrons (PÉREZ-DE LA CRUZ & SANTAMARÍA, 2007; DE MOURA et al., 2010). Esses eventos criam um desequilíbrio entre os sistemas oxidantes e antioxidantes caracterizados pela produção excessiva de EROS como $O_2^{\bullet -}$, peróxido de hidrogênio (H_2O_2), $ONOO^{-}$ e diminuição no sistema antioxidante, tanto enzimático (superóxido dismutase, SOD; glutationa peroxidase, GPx) e não enzimático (glutationa reduzida, GSH). Este desequilíbrio está associado ao estresse oxidativo

(oxidação de proteínas, peroxidação lipídica), a danos celulares e a morte neuronal, desempenhando um papel crucial no processo neurodegenerativo da DH, auxiliando na intensificação do efeito tóxico da huntingtina mutante (HALLIWELL, 2006; UTTARA et al., 2009; WEIR, 2011).

Desta forma a huntingtina mutante pode formar agregados protéicos citoplasmáticos, bem como inclusões nucleares no córtex e estriado, sendo altamente tóxica e responsável por causar disfunção neuronal, a qual está diretamente envolvida nos sintomas clínicos da doença (RANGONE et al., 2004). Todos esses eventos associados podem afetar proteínas nucleares e citoplasmáticas que regulam fatores de transcrição, a sobrevivência, a neurogênese, a sinalização da apoptose, a função mitocondrial, a proteólise, os neurotransmissores e o transporte axonal (BATES, 2005; ADAM & JANKOVIC, 2008).

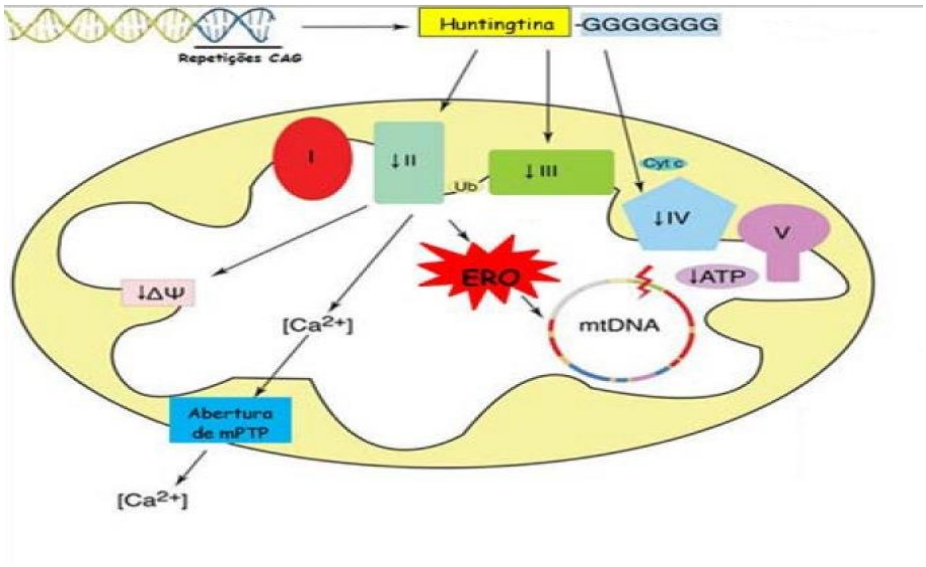

FIGURA 2 – Mecanismo de disfunção mitocondrial na DH. A proteína huntingtina mutante altera a função mitocondrial através da diminuição da atividade dos complexos II, III e IV da cadeia respiratória. Causando a diminuição do potencial de membrana mitocondrial, consequentemente, abertura do poro de transição com liberação de íons cálcio, devido alterações no complexo II, assim como produção de EROS, as quais podem promover dano oxidativo ao DNA mitocondrial (Adaptado de De Moura et al., 2010).

Clinicamente observa-se na DH, coreia progressiva, declínio cognitivo (principalmente da capacidade intelectual e de memória) e distúrbios psiquiátricos

(ROOS et al., 2014). A fase precoce caracteriza-se por alterações moderadas na execução dos movimentos, dificuldades na resolução de problemas, irritabilidade e depressão (GIL-MOHAPEL & REGO, 2011). Os movimentos involuntários dos músculos tornam-se mais graves e os pacientes perdem gradualmente a capacidade de movimento, fala e deglutição em fases avançadas da doença. A morte geralmente ocorre devido a complicações respiratórias infecciosas, cardiovasculares ou até mesmo por quedas, engasgos e suicídio (VONSATTEL, 2008; ROOS et al., 2010; DAMIANO et al., 2013).

Os critérios usados para o diagnóstico da DH incluem: histórico familiar de DH, déficit motor progressivo associado à coreia ou rigidez, bem como alterações psiquiátricas com demência progressiva, sem outra causa definida (RAMASWAMY et al., 2007; ROSS & TABRIZI, 2011). Os indivíduos que apresentam estes sintomas são submetidos ao teste genético, de forma a avaliar a presença da mutação associada à DH e confirmar o diagnóstico (GIL-MOHAPEL & REGO, 2011).

É uma enfermidade incurável, cuja progressão não pode ser interrompida, sendo que o tratamento é puramente sintomático (ADAM & JANKOVIC, 2008). A terapia farmacológica, com drogas bloqueadoras dos receptores dopaminérgicos, como as fenotiazinas ou o haloperidol, pode controlar a discinesia e alguns dos distúrbios comportamentais. Todavia, esses fármacos podem induzir um quadro de discinesia tardia superposta ao distúrbio crônico, devendo ser utilizados apenas se absolutamente necessários (WALKER, 2007).

Desta forma, modelos animais que induzam as características da DH, são extremamente valiosos para elucidar mecanismos patológicos, anomalias e testar possíveis estratégias terapêuticas para minimizar as alterações da doença. Assim, o ácido 3-nitropropiônico (3-NP) vem sendo utilizado em modelos animais por induzir diversas características clínicas e neuropatológicas semelhantes às observadas na DH (BROUILLET, 2014; CHAKRABORTY et al., 2014; MURALIDHARA, 2014).

O ácido 3-NP é uma toxina natural sintetizada por algumas espécies de fungos (*Aspergillus flavus, Astragalus arthrinium*) e plantas (*Indigofera endecapylla*) (LUDOLPH et al., 1991; TUNEZ et al., 2010). Entre os anos de 1950 a 1960, o ácido 3-NP foi relacionado a episódios de envenenamento em mamíferos no oeste dos Estados Unidos. Posteriormente, aproximadamente 100 casos de envenenamento com 3-NP foram reportados na China, associados ao consumo de cana-de-açúcar contaminada com o fungo *Arthrinium* (LUDOLPH et al., 1991). Tais intoxicações

foram responsáveis por causar encefalopatia aguda em adultos e crianças, seguida por casos de distonia e discinesia associados à degeneração do putamen (HE et al., 1995).

Estudos em animais de laboratório levaram a caracterização anatomopatológica da toxicidade do 3-NP. Sabe-se que ele é capaz de atravessar a barreira hematoencefálica podendo causar dano no sistema nervoso central, após ser administrado sistematicamente por via subcutânea ou intraperitoneal (ip) (BORLOGAN et al., 1997). Administrações agudas do ácido 3-NP produzem lesões com perda neuronal mais difusa (TUNEZ et al., 2010), com diminuição da atividade motora, que pode ser seguida por episódios de hiperatividade e movimentos anormais (tremores, movimentos de cabeça, rigidez e elevação de cauda, movimentos em círculo) (LUDOLPH et al., 1991; NAM et al., 2005; TSANG et al., 2009).

As projeções neuronais principalmente afetadas pelo ácido 3-NP são os neurônios espinhais GABAérgicos no estriado (HAN et al., 2010). Estudos sobre o efeito inibitório do 3-NP sobre a succinato desidrogenase (SDH) indicaram que a inibição da enzima é similar a outras regiões do cérebro apesar do estriado ser a principal área afetada pela toxina (ALEXI et al., 1998; BROUILLET et al., 1999).

O mecanismo primário de neurotoxicidade induzido pelo 3-NP envolve a inibição irreversível da enzima mitocondrial succinato desidrogenase, responsável pela oxidação do succinato a fumarato no Ciclo de Krebs e principal constituinte do complexo II da cadeia transportadora de elétrons (MIRANDOLA et al., 2010). Nesse contexto o 3-NP é conhecido por interferir na cascata de transporte de elétrons causando déficit energético, disfunção mitocondrial e consequentemente prejuízo na fosforilação oxidativa, (THANGARAJAN et al., 2014) depleção nos níveis de adenosina trifosfato (ATP), geração de espécies reativas de oxigênio, excitotoxicidade entre outros (SANDHIR & MEHROTRA, 2013; BROUILLET, 2014) (Figura 3).

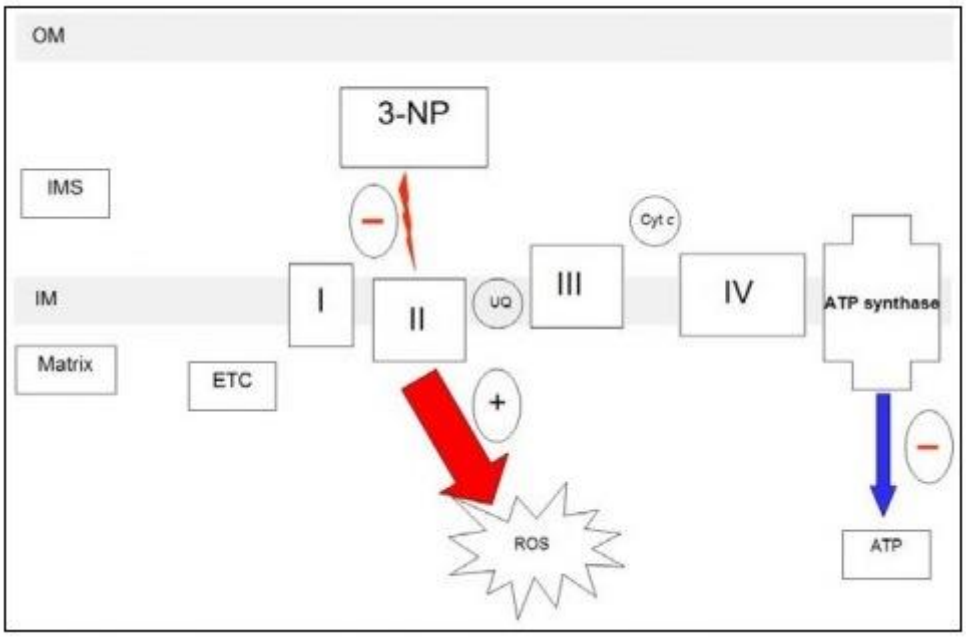

FIGURA 3 – Representação dos efeitos do 3-NP na cadeia transportadora de elétrons (ETC). 3NP inibe a enzima succinato desidrogenase (complexo II). IM: membrana interna; IMS: espaço intermembranas; OM: membrana externa. Complexo I: NADH desidrogenase; Complexo III: citocromo bc1 ou citocromo c redutase; Complex IV: citocromo c oxidase; Complex V: ATP sintase.
Fonte: Tunez et al., 2010.

Sabe-se que o 3-NP causa uma depleção, nos níveis de ATP produzido pelo déficit no metabolismo energético que diminui a atividade da enzima Na^+, K^+-ATPase e causa despolarização da membrana plasmática, liberando o bloqueio pelos íons Mg^{2+} nos receptores N-metil-D-aspartato (NMDA) com consequente influxo de Ca^{2+} e íons sódio (Na^{2+}) (PÉREZ-DE LA CRUZ & SANTAMARÍA, 2007; MIRANDOLA et al., 2010). Sob essas condições os neurônios tornam-se mais sensíveis a níveis basais de glutamato, levando-os a morte neuronal (ALEXI et al., 1998). Além de causar aumento de espécies reativas de nitrogênio e EROS, derivadas do NO (através da estimulação da NOS) estudos também relataram dano oxidativo ao DNA e níveis elevados de marcadores de estresse oxidativo como produtos da peroxidação lipídica (SANDHIR et al., 2010). O mecanismo de morte neuronal induzido pela toxina também está relacionado com aumentos nas concentrações de Ca^{2+} intracelulares e ativação de caspases e calpaínas (TUNEZ et al., 2010), resultando em morte celular tanto por necrose como apoptose (Figura 4).

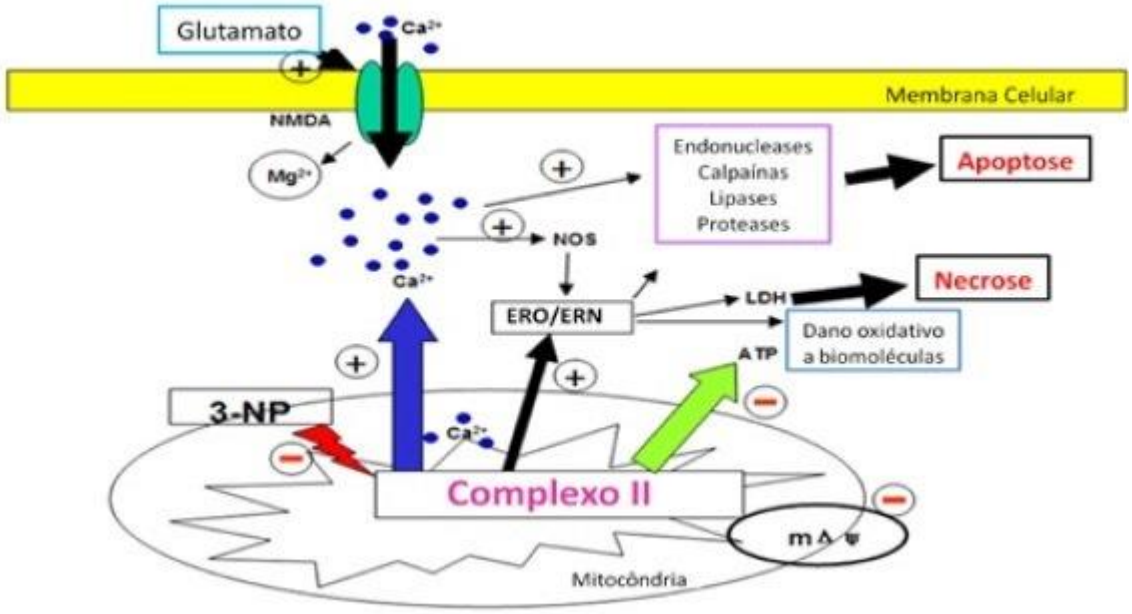

FIGURA 4 – Mecanismos de neurotoxicidade induzidos pelo 3NP.
Fonte: Tunez et al., 2010.

Desta forma, a utilização do ácido 3-NP em modelos animais, pode ser uma ferramenta valiosa para avaliar o efeito de novas terapias e outras anomalias manifestadas na DH (TUNEZ et al., 2010), auxiliando na investigação de mecanismos patológicos e na descoberta de novos agentes neuroprotetores.

Estratégias terapêuticas destinadas a prevenir ou retardar a degeneração neuronal podem ser uma escolha para o tratamento de doenças neurogenerativas (UTTARA et al., 2009; KIM et al., 2012). Dentre as várias estratégias terapêuticas, uma das maneiras mais utilizadas, é aumentar ou fortalecer a defesa endógena contra o estresse oxidativo (HUANG & ZHANG, 2010). Há evidências de que compostos que atuam removendo radicais livres ou evitando a sua formação têm sido capazes de prevenir ou retardar o dano oxidativo neuronal (HALLIWELL, 2006; BARREIRA et al., 2008). Há também alguns dados clínicos indicando a ação neuroprotetora de substâncias que possuem atividade antioxidante tais como selegina, vitamina E e *Gingko biloba* (ROSLER et al., 1998). Assim, há um interesse crescente em antioxidantes naturais, principalmente polifenóis, presentes em plantas medicinais e alimentos que possam impedir a neurotoxicidade associada a

diferentes neurotoxicantes (PEREIRA et al., 2011; MARTINS et al., 2012; COLLE et al., 2013).

Os polifenóis incluem os flavonóides, os triterpenos e os taninos, e são metabólitos secundários das plantas. Estudos demonstraram que estes compostos são mais efetivos que as vitaminas C e E em proteger as células contra o dano causado por espécies reativas (VINSON et al., 1995; WISEMAN et al., 1997). Os mecanismos pelos quais os polifenóis têm sido relacionados à atividade antioxidante são basicamente: atividade neutralizante de radicais livres, atividade quelante de íons metálicos, doação de hidrogênio e ação como substrato para espécies reativas (BARREIRA et al., 2008; JAVED et al., 2012; PARK et al., 2014) .

Estudos têm demonstrado o efeito neuroprotetor *in vitro* e *in vivo* de diferentes extratos vegetais. Por exemplo, o extrato de erva-cidreira (*Melissa officinalis*) apresentou atividade protetora em homogeneizado de cérebro de ratos contra três substâncias pró-oxidantes (ferro, nitroprussiato de sódio e ácido 3 nitropropiônico) (PEREIRA et al., 2009). O extrato de lavanda (*Lavandula augustifolia*) também mostrou efeito benéfico em modelo de Doença de Alzheimer em ratos, revertendo a diminuição da aprendizagem espacial (KASHANI et al., 2011).

Neste contexto, devido à grande diversidade, muitas espécies vegetais ainda não foram estudadas farmacologicamente, como a *Luehea divaricata (L. divaricata)*. A *Luehea divaricata* Mart., pertence à família *Tiliaceae,* é uma árvore de grande porte, natural da América do Sul. No Brasil, pode ser encontrada em diversos estados, desde o Rio Grande do Norte até o Rio Grande do Sul e é popularmente conhecida como Açoita-Cavalo (ALICE et al., 1995; LORENZI, 1998). Na medicina popular é utilizada para tratar disenteria, leucorréia, reumatismo, gonorréia, tumores, bronquites, feridas de pele entre outros (LORENZI, 2000; BIGHETTI et al., 2004; TANAKA et al., 2005).

Tanaka et al. (2005) revelaram, na análise fitoquímica das folhas de *L. divaricata*, a presença de flavonóides, taninos catéquicos, saponinas e mucilagem. Além disso, alcaloides, óleos fixos, antocianidinas, carotenóides e polissacarídeos foram identificados no extrato bruto das folhas de *L. divaricata* (BORTOLUZZI et al., 2002). O estudo químico do extrato bruto metanólico das folhas revelou a presença de ácido 3b-p-hidroxibenzoil-tormêntico, ácido maslínico, vitexina e glicopiranosilsitosterol (TANAKA et al., 2005).

Entretanto, não existem estudos na literatura que descrevam o potencial antioxidante da planta, não obstante, nenhum deles correlacionando o consumo do chá das folhas de *L. divaricata* com doenças neurodegenerativas. Dados prévios, contudo, têm relatado a atividade genotóxica do extrato aquoso de folhas de *L. divaricata* (VARGAS et al., 2001), um efeito citostático do extrato metanólico das folhas e uma atividade antimutagênica do extrato aquoso da casca (FELÍCIO et al., 2011). Além disso, em um estudo de BIGHETTI et al. (2004), verificou-se que camundongos tratados com extrato bruto hidroalcoólico de *L. divaricata*, na dose de 5,0 g/kg de peso corporal, administrado por via oral, não demonstrou sinais de toxicidade, de modo que o extrato pode ser considerado praticamente não tóxico (LOOMIS, 1974).

Com base no exposto, nossa pesquisa foi motivada por dados anteriores que apoiam a busca por novas estratégias terapêuticas as quais potencializam as defesas antioxidantes e/ou evitam o estresse oxidativo, a fim de retardar a progressão da DH. Principalmente devido ao alto consumo de chás de plantas medicinais, tradicionalmente utilizados pela população, normalmente preparados por infusão das folhas em água quente, contendo elevados níveis de polifenóis, os quais podem atuar como antioxidantes com atividade neuroprotetora (SOROLLA et al, 2008; MARINHO et al, 2013).

Considerando: a) o crescente interesse em antioxidantes naturais, principalmente polifenóis, presentes em plantas medicinais e alimentares; b) ao fato que não há estudos os quais relatem as possíveis propriedades antioxidantes do extrato aquoso de *L. divaricata*; c) a falta de evidências sobre o efeito protetor de *L. divaricata* em modelos experimentais de neurotoxicidade/neuropatologia; d) o envolvimento do estresse oxidativo em desordens neurodegenerativas induzidas pelo ácido 3-NP; propomos nesse trabalho testar a hipótese que o extrato aquoso de *L. divaricata* pode ajudar a prevenir as doenças mediadas pelo ácido 3-NP, em um modelo experimental da Doença de Huntington em ratos.

2 OBJETIVOS

2.1 Objetivo Geral

O presente estudo teve por objetivo testar a hipótese que o extrato aquoso de *L. divaricata* pode exercer efeito antioxidante e neuroprotetor, *in vivo,* frente às alterações comportamentais e oxidativas induzidas pelo ácido 3-nitropropiônico em ratos.

2.2 Objetivos Específicos

- ✓ Determinar *in vivo* os efeitos da administração aguda do ácido 3-NP por via intraperitoneal sobre a atividade locomotora e exploratória de ratos, bem como os efeitos do co-tratamento com extrato aquoso de *L. divaricata* sobre estes parâmetros comportamentais;

- ✓ Investigar *ex-vivo* os efeitos do tratamento com o extrato de *L. divaricata* contra a neurotoxicidade induzida pelo 3-NP, nas porções do cérebro (córtex e estriado), através de parâmetros bioquímicos.

3 MANUSCRITO

Os resultados que fazem parte desta dissertação estão representados sob a forma de um manuscrito científico, o qual se encontra aqui organizado. O referido estudo será submetido à revista Brain Research Bulletin, e está apresentado de acordo com as normas desta revista.

Protective Effects of Aqueous Extract of *Luehea divaricata* against Behavioral and Oxidative Changes Induced by 3-Nitropropionic Acid in Rats

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ABSTRACT

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease. 3-nitropropionic acid (3-NP), an inhibitor of the mitochondrial enzyme succinate dehydrogenase has been found to effectively produce HD like symptoms. *Luehea divaricata (L. divaricata)*, popularly known in Brazil as "Açoita Cavalo" contains numerous polyphenols, which may act as neuroprotective agents in several *in vitro* assays and *in vivo* neurodegenerative diseases. The propose of this study to test the hypothesis that the aqueous extract of *L. divaricata* could prevent behavioral and oxidative alterations induced by 3-NP in rats, used as an experimental model of HD. For that, 25 adult Wistar male rats divided in 5 groups [(1) Control, (2) *L. divaricata* (1000 mg/kg), (3) 3-NP, (4) *L. divaricata* (500 mg/kg) + 3-NP and (5) *L. divaricata* (1000 mg/kg) + 3-NP] were used. Groups 3, 4 and 5 received, during 10 days, the aqueous extract through intragastric gavage. From eighth day, groups 2, 4 and 5 received 20 mg/Kg 3-NP during 3 consecutive days. At day 10, parameters of locomotor activity (Open Field and Rota Rod), and biochemical evaluations (estimation of ROS formation using (2',7'-

dichlorofluorescein diacetate (DCFH-DA), lipid peroxidation as TBARS, levels of GSH, GSSG and activity of acetylcholinesterase in cortex and striatum) were performed. 3-NP caused symptoms-like DH (i.e. caused behavioral changes, evidenced by decreased locomotor activity on Open Field and Rota Rod; oxidative damage by increased levels of reactive oxygen species (ROS) and lipid peroxidation, decrease levels of GSH and acetylcholinesterase activity). The aqueous extract of *L. divaricata* was able to prevent the oxidative and behavioral changes induced by 3-NP treatment, suggesting a possible neuroprotective effect of *L. divaricata* against 3-NP toxicity, which may be due to its antioxidant properties.

Keywords: *Luehea divaricata*, 3-Nitropropionic acid, Huntington's disease.

1. INTRODUCTION

Huntington's disease (HD) is an autosomal dominant, progressive neurodegenerative disorder, characterized by motor dysfunction, emotional disturbances, abnormal involuntary movements, dementia and weight loss [14, 61, 62]. The neuropathological changes include progressive neuronal degeneration and atrophy affecting principally the striatum and cortex [38, 64, 17]. This disorder is thought to be caused by an expanded trinucleotide CAG sequence in exon 1 of the huntingtin gene (Htt), which encodes a stretch of glutamines in the huntingtin protein [50, 82]. Formation of Htt aggregates and alteration of overall gene expression profiles have also been reported in peripheral tissues [5, 13, 57]. Moreover, there is compelling evidence that mutant Huntingtin alters mitochondrial trafficking and function [56, 66, 68].

3-Nitropropionic acid (3-NP) is a natural neurotoxin produced by some species of fungi (*Aspergillus flavus, Astragalus arthrinium*) and plants (*Indigofera endecapylla*) [44, 73] that has been used to induce HD-like symptoms in animal models [12, 17, 51]. The mechanism by which 3-NP induce neurotoxicity involves the irreversible inhibition of succinate dehydrogenase (SDH) [4, 49, 31], which results in mitochondrial dysfunction, as evidenced by energy failure and oxidative stress [2, 72, 81]. Animals present motor-behavioral disorders, such as in gait, ability to balance over a narrow beam, foraging or exploratory behaviors, cognition, anxiety or depression [11, 53, 74]. Thus, the 3NP induces HD-like symptoms, similarly as a phenotypic model, can be a valuable tool to evaluate the effect of new therapies and other abnormalities manifested in HD [18].

So, therapeutic strategies aimed to prevent or delay neuronal degeneration might be a reasonable choice for the treatment of neurodegenerative disease [29, 32, 64, 75]. Accordingly, there is a growing interest in natural antioxidants, namely polyphenols, present in medicinal and dietary plants that might prevent neurotoxicity associated to different neurotoxicants [59, 47, 15, 20]. In this context, *Luehea divaricata* Mart. (Tiliaceae) (*L. divaricata*), popularly known in South America as "açoita cavalo" [40, 41], contain numerous polyphenols. Indeed, this plant has been already used in folk medicine to treat dysentery, leucorrhea, rheumatism, blennorrhoea, tumors, bronchitis and skin wounds, among others [42, 7, 71]. A phytochemical screening of *L. divaricata* leaves reported the presence of flavonoids, tannins, saponins, and mucilage. Additionally, alkaloids, fixed oils, antocianidins, carotenoids, and polysaccharides are also present in the crude extract of *L. divaricata* [71]. However, there are not studies in literature describing the antioxidant potential of this plant, associated with the consumption of tea of leaves of *L. divaricata*. Of particular importance, it was previously reported a genotoxic activity of the aqueous extract of *L. divaricata* leaves [76], a cytostatic effect of the methanolic extract of the leaves and an antimutagenic activity of the aqueous extract of the bark [22].

Based on the exposed, our research was motivated by previous data that support the rationale search for therapeutic strategies that either potentiate antioxidant defenses or avoid oxidative stress generation, in order to delay HD progression, due on the fact that this plant is traditionally used by the population and present high content of polyphenols and flavonoids when prepared by infusion of the leaves in hot water [69, 46, 67].

Altogether, and considering: a) the growing interest in natural antioxidants, especially polyphenols, present in medicinal and food plants; b) the putative antioxidant properties of *L. divaricata* extract; c) the lack of evidence concerning the potential protective effect of *L. divaricata* in experimental models of neurotoxicity/neuropathology; d) the involvement of oxidative stress in neurodegenerative disorders induced by 3-NP, we propose in this study to test the hypothesis that the aqueous extract of *L. divaricata* could prevent disorders induced by 3-NP in rats, in an experimental model of HD.

2. MATERIALS AND METHODS

2.1 Chemicals

3-Nitropropionic acid, tiobarbituric acid (TBA), malonaldehyde-bisdimethylacetal (MDA), 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO,USA). All other reagents were obtained from local suppliers.

2.2 Plant Material

The leaves of *Luehea divaricata* Mart. (family Tiliaceae), were used as the plant material and were collected in Santa Maria (Rio Grande do Sul, Brazil). The collection of the leaves of *L. divaricata* was carried out during the flowering period, which occurs in December. The taxonomic identification was confirmed by Department of Industrial Pharmacy of the Federal University of Santa Maria and registered under the number 225 in the Herbarium of the Industrial Pharmacy Department.

2.3 Preparation of the extract

The leaves were dried for five days in a kiln with controlled temperature (40ºC). Aqueous extract was obtained by decoction for 10 minutes in distilled water at 100ºC. The resulting extract was then filtered by using a filter paper to remove particles in suspension. *L. divaricata* at 500 mg/kg and 1000 mg/kg were chosen to treat experimental animals based in previous pilot experiment, which demonstrated none toxic effect of the extract. Of particular importance, literature data are not conclusive regarding *L. divaricata* therapeutic dose in animal experiments [7].

2.4 Animals

All experiments were conducted using male adult Wistar rats (200–250 g) from our own breeding colony. Animals were housed in cages (5 rats per cage) with free

access to food and water. They were kept in a 12-h light/12-h dark cycle, with lights on at 7:00 a.m., in an air-conditioned room (22 ± 2º C). Commercial diet and tap water were supplied *ad libitum*. Animal care and all experimental procedures were conducted in compliance with the Committee on Care and Use of Experimental Animal Resources, from the Federal University of Santa Maria, Brazil (CEUA/UFSM 102/2014). All efforts were made to minimize the number of animals used and their suffering.

2.3 3-NP Induced Neurotoxicity

3-NP was diluted in buffered saline (pH 7.4) and administered intraperitoneally (i.p.) at a dose of 20 mg/kg once a day, for a period of 3 days to induce HD-like signs. The 3-NP dose was chosen based in a preliminary study in which were observed biochemistry alterations characteristic of 3-NP neurotoxicity, but with some modifications, once that the dose of 25 mg/kg was changed to 20 mg/kg [15].

2.4 Treatment

Twenty five animals were divided into five groups with five animals each.

Group 1 (Control): received pre-treatment with distilled water for 7 days, by intragastric gavage.

Group 2 (*L. divaricata*): received daily, during 7 days, the aqueous extract at a concentration of 1000 mg/kg via intragastric gavage.

Group 3 (3-NP): received pre-treatment with distilled water for 7 days, by intragastric gavage.

Group 4 (*L. divaricata*+3-NP): received daily, during 7 days, the aqueous extract at a concentration of 500 mg/kg via intragastric gavage.

Group 5 (*L. divaricata*+3-NP): received daily, during 7 days, the aqueous extract at a concentration of 1000 mg/kg via intragastric gavage.

On the eighth day, the groups 3, 4 and 5 received the administration of 20 mg/kg 3-NP via i.p. [15] for 3 consecutive days, while groups 1 and 3 received only saline (also via i.p). During the administration of 3-NP, rats continued to receive the aqueous extract by intragastric gavage, which result in 10 days of treatment.

All the behavioral parameters were observed on day 10, 3 h after the last 3-NP administration. At the end of the behavioral analyses, rats were euthanized, in a total of 6 h after the last 3-NP administration, the brain was removed and the cortex and the striatum were dissected. A portion of the cortex and striatum were homogenized (1:10) in 10mM Tris- buffer (pH 7.4) and centrifuged at 2.500 rpm for 12 min. The low-speed supernatant fraction obtained was used for biochemical analyses.

2.5 Behavioral Evaluations

2.5.1 Open Field

Animals were individually placed at the center of the open field apparatus (45 cm X 45 cm X 30 cm, divided into 9 squares). Spontaneous ambulation (number of segments crossed with the four paws) and exploratory activity (expressed by the number of rearing on the hind limbs) were recorded for 5 min [9].

2.5.2 Rota rod task

The integrity of motor system was evaluated using the Rota rod test. Briefly, the Rota rod apparatus consists of a rod 30 cm long and 3 cm in diameter that is subdivided into three compartments by discs from 24 cm in diameter. The rod rotates at a constant speed of 10 rpm. The animals were given a prior training session before the initialization of any therapy to acclimate them to Rota rod apparatus. The latency for first fall and number of falls of from the rod were noted. The cut-off time was 120 s [65].

2.6 Biochemical Analysis

2.6.1 Estimation of ROS formation

2’-7’-Dichlorofluorescein (DCF) levels were determined as an index of the reactive species production by the cellular components [52]. Aliquots (20 µL) of homogenate of brain structures (cortex and striatum) were added to a medium

containing 2.460 µL Tris–HCl buffer (10 mM, pH 7.4) and 20 µL 2'-7'-dichlorofluorescein diacetate DCFH-DA (0.1 mM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm, and both slit widths used were at 1.5 nm). DCF levels were determined using a standard curve of DCF, and results were corrected by the protein content.

2.6.2 Thiobarbituric acid reactive substances (TBARS) levels determination

Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al. (1979) [55]. An aliquot (200 µL) of homogenate of brain structures (cortex and striatum) was mixed with 500 µL thiobarbituric acid (TBA, 0.6%), 200 µL sodium dodecyl sulphate (SDS, 8.1%), and 500 µL acetic acid (500 mM, pH 3.4) and incubated at 100ºC for 1 h. TBARS levels were measured at 532 nm using a standard curve of malondialdehyde (MDA), and the results were reported as nmol MDA/mg protein.

2.6.3 Fluorimetric assay of reduced (GSH) and oxidized glutathione (GSSG)

For measurement of GSH and GSSG levels we used the method previously described by Hissin and Hilf (1976) [28]. Briefly, 400 µL of homogenate each of brain structures (cortex and striatum) were mixed to 200 µL trichloroacetic acid (TCA, 13%). Resulting mixtures were centrifuged at 4ºC at 13.000 rpm for 10 min. For GSH measurement, 100 µL of the supernatant was diluted in 1.800 µL of phosphate - EDTA buffer (sodium phosphate 100 mM and EDTA 5 mM, pH 8) and 100 µL of O-Phthalaldehyde (OPT,1 mg/mL). The mixtures were incubated at room temperature for 15 min and their fluorescent signals were recorded in the RF-5301 PC Shimadzu spectrofluorometer (Kyoto, Japan) at 420 nm of emission and 350 nm of excitation wavelengths. For measurement of GSSG levels, a 250 µL of the supernatant was incubated at room temperature with 100 µL of N-ethylmaleimide (NEM, 0.04 M) for 30 min at room temperature, and after that, 140 µL of the mixture, were added to 1.760 µL of sodium hydroxide (NaOH, 0.1 N) buffer, following of added 100 µL OPT and incubated for 15 min, using the procedure outlined above for GSH assay.

2.6.4 Acetylcholinesterase (AChE) activity

AChE activity was determined according to the method of Hissin and Hilf (1976) [28], with some modifications. In brief, we used 875 µL of the reaction mixture, containing potassium phosphate buffer (0.1 M, pH 8), 50 µL 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 10 mM), 25 µL of homogenate of each brain structures (cortex and striatum) and 50 µL acetylthiocholine iodide (9 mM). Change in absorbance was monitored for 2 min at 412 nm.

2.6.5 Protein determination

The protein content was determined as described previously Lowry et al., (1951) [43], using bovine serum albumin (BSA) as standard.

2.7 Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by multiple comparison test of Newman–Keuls when appropriate. Data are expressed as means ± SEM. Values of $p<0.05$ were considered significant.

3. RESULTS

3.1 Behavioral alterations

Locomotor and exploratory activities in the open field were significantly decreased by 3-NP (Fig. 1A and B, respectively), while treatment with *L. divaricata* 500 and 1000 mg/Kg partially restore both parameters ($p<0.05$, Fig. 1A and 1B). Statistical analysis of motor performance in the rota rod task demonstrated that 3-NP caused a significant reduction of latency and number of falls of rod when compared to control group, whereas, *L. divaricata* 500 and 1000 mg/Kg significantly prevents against 3-NP-induced changes in the latency and partially restore the number of falls in the rota rod task ($p<0.05$, Fig. 2A and B).

3.2 Biochemical alterations

Figure 3 shows that animals treated with 3-NP present a significant increase ($p<0.05$) in DCF oxidation, an index of the ROS formation, both in cortex and striatum, when compared with control group (Fig. 3A and B, respectively). *L. divaricata* completely prevents ROS formation in cortex (Fig. 3A), while its effect on striatum was partial (Fig. 3B).

In addition, 3-NP significantly increases lipid peroxidation, measured by TBARS production, in cortex when compared to the control group ($p<0.05$, Fig. 4A). *L. divaricata,* at both concentrations, completely prevent against 3-NP-induced TBARS levels in cortex ($p<0.05$). Striatal TBARS levels were not modified by 3-NP and/or *L. divaricata* treatment (Fig. 4B).

Administration of 3-NP caused a markedly decrease in reduced glutathione (GSH) levels in rat's cortex and striatum ($p<0.05$, Fig. 5A and B). Similarly, 3-NP significantly decreased the oxidized glutathione (GSSG) levels ($p<0.05$, Fig. 6A and B). Treatment with *L. divaricata* (at 500 and 1000 mg/Kg) partially prevent the 3NP-induced depletion of GSH in cortex ($p<0.05$, Fig. 5A), while only at 1000 mg/Kg its partially prevents against 3-NP-induced GSH depletion in striatum ($p<0.05$, Fig. 5B). Similar results of *L. divaricata* (at 1000 mg/Kg) were found regarding GSSG levels ($p<0.05$, Fig. 6A and B).

Figure 7A shows that the administration of *L. divaricata*, either alone or combined to 3-NP, significantly decreased activity of acetylcholinesterase ($p<0.05$) in cortex, being the 3-NP without effect *per se*. However, different from cortex, the striatal activity of acetylcholinesterase was significantly inhibited by 3-NP, and was not changed by *L. divaricata* (500 and 1000 mg/Kg) treatment ($p<0.05$, Fig. 7B).

4. DISCUSSION

In the present study we tested the hypothesis that the aqueous extract of *L. divaricata* could prevent disorders in an experimental model of HD induced by 3-NP [73] in rats. Accordingly, our results demonstrate that *L. divaricata* treatment protected against behavioral (improved locomotor and rotarod performance) and

oxidative (decreased ROS formation in cortex and striatum, reduced lipid peroxidation in cortex and improving GSH and GSSG level in cortex and striatum) changes induced by 3-NP.

The administration of 3-NP, in rats, for 3 consecutive days caused impairment in the motor system, which characterized by decreased the motor and grip strength performance, suggesting that the effects of 3-NP most probably mimic either the juvenile onset or late stages of HD-like behavior [10, 21, 80]. These data are supported by previous data, where was found that 3-NP affects the motor system causing behavioral deficits [36, 6, 70, 48]. Alterations in locomotor and motor behavioral could be due to its specific action on striatum and cortex which controls body movements. Besides, studies indicated that abnormal behavioral symptoms in early HD patients are due to primarily either dysfunction of cholinergic interneurons in striatal circuits or cell loss within the lateral striatum, ventral pallidum, and entopedoncular nucleus [60, 27]. Researchers also confirmed 3-NP-induced lesions and oxidative damage in cortex and hippocampus, which would also be related to the deficit in motor performance [37, 64].

Pre-treatment with *L. divaricata* significantly attenuated behavioral alterations (locomotor as well as rotarod performance) following 3-NP, suggesting its therapeutic potential. Previous studies reports also reconfirm that antioxidant treatments significantly restored the behavioral changes and oxidative defense level in 3NP-treated animals. By using other plant species, Kumar and Kumar (2009) [35] reported that the root extract of *Withania somnifera*, characterized by high antioxidant content, reverses motor dysfunction caused by 3-NP in rats. So, therapy with antioxidants, polyphenols principally, protect *in vivo* against oxidative damage in childhood-onset hydrocephalus in rats and it was found to be effective in improving learning and memory in senescence- accelerated mice including Alzheimer transgenic mice. Thus considering the presented results, the use of *L. divaricata* aqueous extract could be considered as a therapeutic strategy for the treatment and/or search for new drugs to treat/prevent human HD-like simptoms [1, 34, 78, 15].

Moreover, evidence suggests the involvement of oxidative stress in 3-NP neurotoxicity that includes a rapid increase in ROS production in cells neuronal [8, 39] and hydroxyl free radicals, lipid peroxidation and impaired antioxidant defense in the brain [2, 34, 45, 63]. Accordingly, in these study we found a pro-oxidant effect of the 3-NP, which caused an increased in ROS production, as measured *via* DCF

oxidation, and in lipid peroxidation, both in cortex and striatum. These changes were significantly restored by pre-treatment with *L. divaricata* extract, suggesting neuroprotective action due to its antioxidant effect. In fact, many studies indicate that the antioxidant activities of aqueous extracts of plants are benefits to the treatment of several diseases by the presence of numerous polyphenols, especially flavonoids [33, 30, 54, 58], which are much more effective than vitamins C and E in protecting cells from free radical damage [77, 59]

Alterations in the antioxidant defense system were also observed in this study, as evidenced by a decrease in concentration of GSH in 3-NP-treated rats. GSH, a non-enzymatic antioxidant, plays an important role in reduction of ROS in brain that interacts directly to detoxify certain ROS (e.g., hydroxyl radical). Diminished GSH status has been linked with normal aging as well as with neurodegenerative diseases [3, 16, 25]. Decreased GSH level, as observed in this study, might be due to enhanced utilization of this antioxidant to scavenge free radicals, clearly suggesting the role of oxidative stress in this neurodegenerative process. Treatment with *L. divaricata* significantly prevents 3-NP-induced GSH consumption. Accordingly, antioxidants have been shown to protect nervous system against variety of toxins [15, 19, 23]. Of particular importance, Muralidhara (2013) [51], demonstrated the efficacy of fish oil and quercetin in combination to enhance GSH levels in 3-NP treated animals. Surprisingly, we found a decrease in the glutathione pool (GSH + GSSG) in animals treated with 3-NP, which was partially prevented by *L. divaricata* treatment. Thus, more studies are necessary to better clarify the effect of 3-NP on glutathione synthesis pathway, which could directly reflect on the pool of glutathione under our experimental conditions.

Finally, we found that aqueous extract *L. divaricata* showed an inhibitory effect on acetylcholinesterase activity, which could be due to tannins and alkaloids presents in the aqueous extract. Indeed, previous studies reported that condensed tannins, as procyanidins, present in the extract of *Lotus seedpod* and alkaloids present in the methanol extract of *Berberis darwinii*, were able to cause inhibition of AChE activity [79, 26, 24]. Meanwhile, more studies are necessary to better understand the significance of AChE inhibition by plant extracts.

5 CONCLUSION

In conclusion, our study shows that the aqueous extract of *L. divaricata* was able to prevent oxidative and behavioral changes induced by treatment with 3-NP. Consequently, this plant could be used as a potential therapeutic for the prevention of HD-like simptoms. However, more studies are necessary to identify the main components of the aqueous extract of *L. divaricata* and also to evaluate its pharmacological use *in vivo* as a putative adjuvant of the HD treatment.

Conflicts of interest statement

All authors report no conflict of interest.

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Legend of Figures

Figure 1: Effects of 3-NP (20 mg/Kg, i.p., 3 days) and/or *Luehea divaricata* (LD) (500 and 1000 mg/Kg, by gavage, 10 days) treatment on locomotor and exploratory activities. (A) number of crossing in the open field; (B) number of rearing in the open field. Each bar represents means ± S.E.M. (n=5). (*) indicates statistic difference from control group and (#) indicates statistic difference from 3-NP group by one-way ANOVA, followed by Newman Keuls's test for post-hoc comparison ($p<0.05$).

Figure 2: Effects of 3-NP (20 mg/Kg, i.p., 3 days) and/or *Luehea divaricata* (LD) (500 and 1000 mg/Kg, by gavage, 10 days) treatment on latency to the first fall motor performance of rats in the rota rod task. Each bar represents means ± S.E.M. (n=5). (*) indicates statistic difference from control group and (#) indicates statistic difference from 3-NP group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$).

Figure 3: Effects of 3-NP (20 mg/Kg, i.p., 3 days) and/or *Luehea divaricata* (LD) (500 and 1000 mg/Kg, by gavage, 10 days) treatment on ROS formation in cortex (A) and striatum (B) of treated rats. Data are expressed as nmol DCF/mg. Each bar represents means ± S.E.M. (n=5). (*) indicates statistic difference from control group and (#) indicates statistic difference from 3-NP group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$).

Figure 4: Effects of 3-NP (20 mg/Kg, i.p., 3 days) and/or *Luehea divaricata* (LD) (500 and 1000 mg/Kg, by gavage, 10 days) treatment on TBARS levels in cortex (A) and striatum (B). Data are expressed as nmol MDA/mg of tissue. Each bar represents means ± S.E.M. (n=5). (*) indicates statistic difference from control group and (#) indicates statistic difference from 3-NP group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$).

Figure 5: Effects of 3-NP (20 mg/Kg, i.p., 3 days) and/or *Luehea divaricata* (LD) (500 and 1000 mg/Kg, by gavage, 10 days) in levels of GSH in cortex (A) and striatum (B) of treated rats. Data are expressed as nmol GSH/mg of tissue. Each bar represents means ± S.E.M. (n=5). (*) indicates statistic difference from control group and (#)

indicates statistic difference from 3NP group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$).

Figure 6: Effects of 3-NP (20 mg/Kg, i.p., 3 days) and/or *Luehea divaricata* (LD) (500 and 1000 mg/Kg, by gavage, 10 days) in levels of GSSG in cortex (A) and striatum (B) of treated rats. Data are expressed as nmol GSSG/mg of tissue. Each bar represents means ± S.E.M. (n=5). (*) indicates statistic difference from control group and (#) indicates statistic difference from 3NP group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$).

Figure 7: Effects of 3-NP (20 mg/Kg, i.p., 3 days) and/or *Luehea divaricata* (LD) (500 and 1000 mg/Kg, by gavage, 10 days) on the activity levels of acetylcholinesterase in cortex (A) and striatum (B) of treated rats. Data are expressed as % of control. Each bar represents means ± S.E.M. (n=5). (*) indicates statistic difference from control group and (#) indicates statistic difference from 3NP group by one-way ANOVA, followed by Newman Keuls's post-hoc test ($p<0.05$

Figure 1

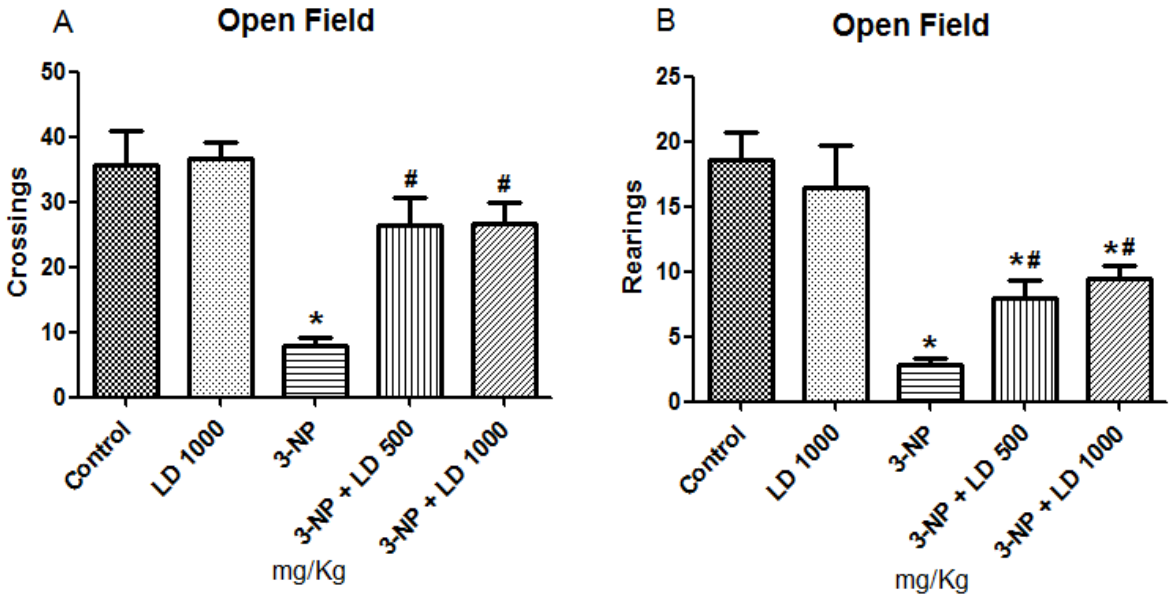


Figure 2

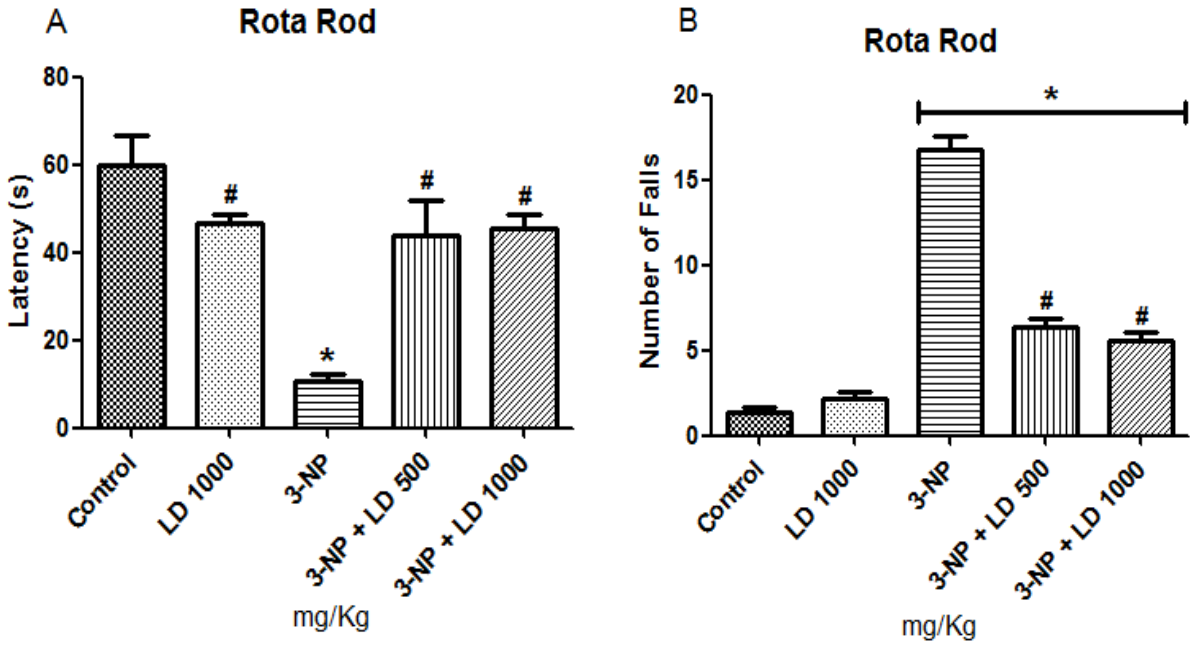


Figure 3

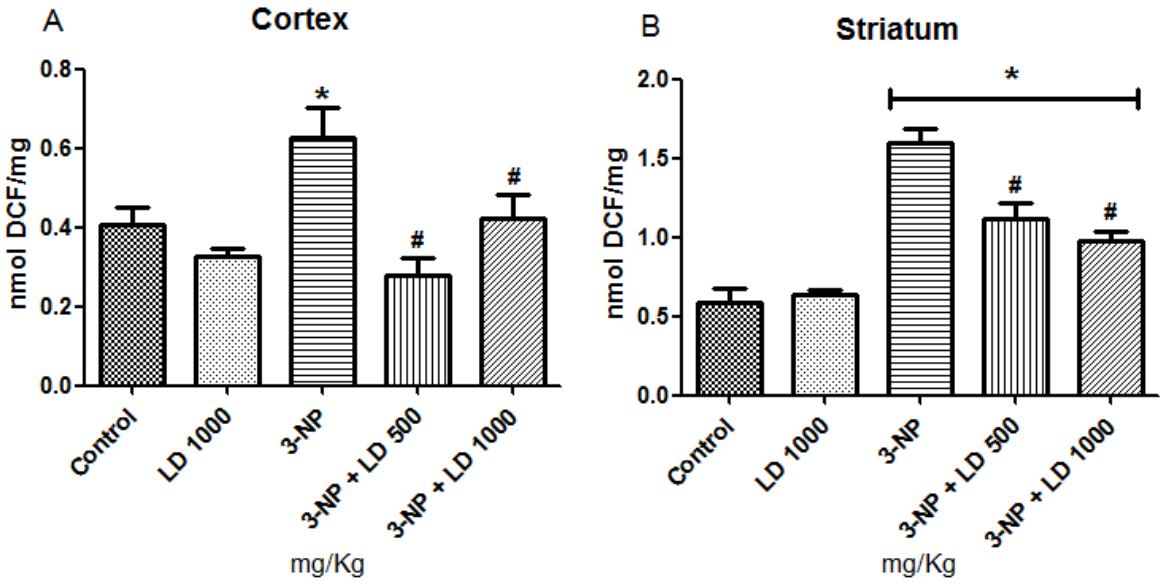


Figure 4

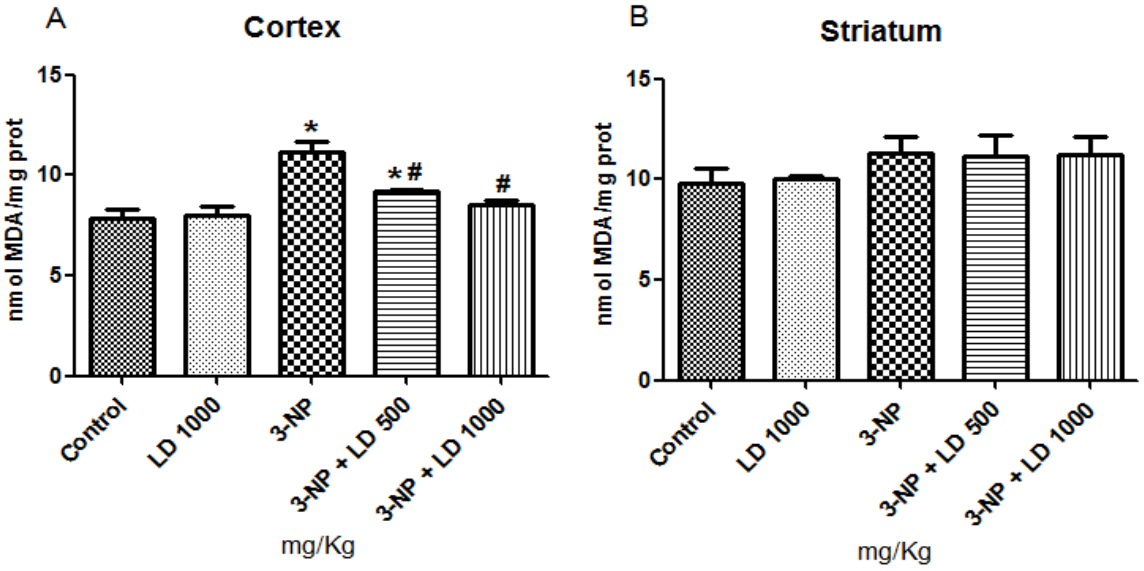


Figure 5

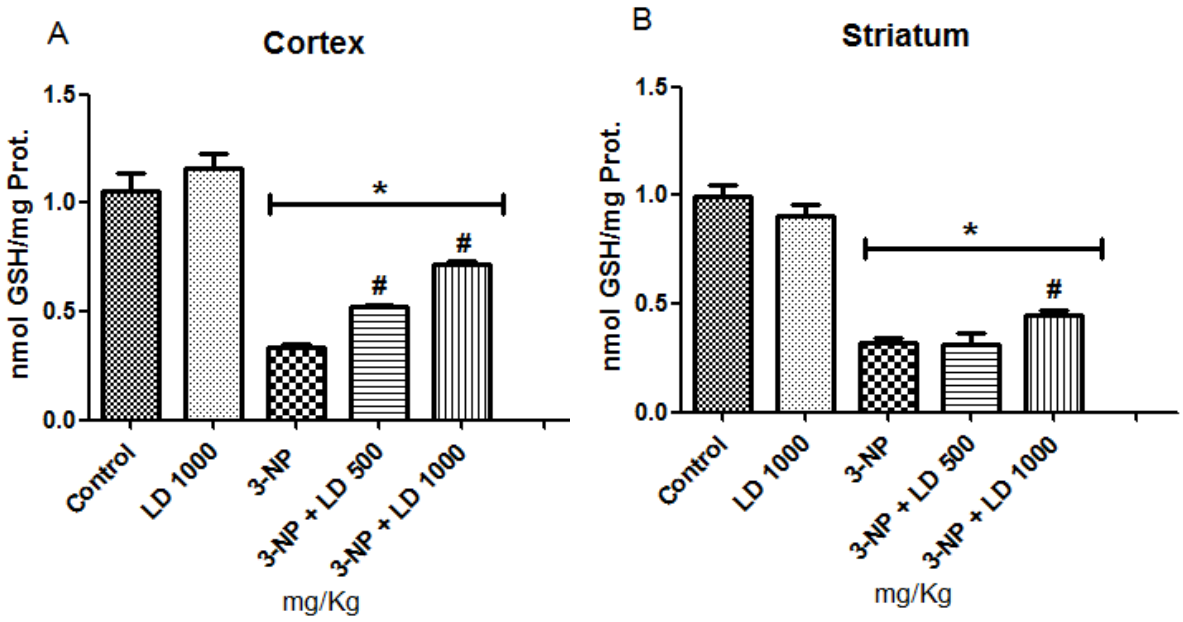


Figure 6

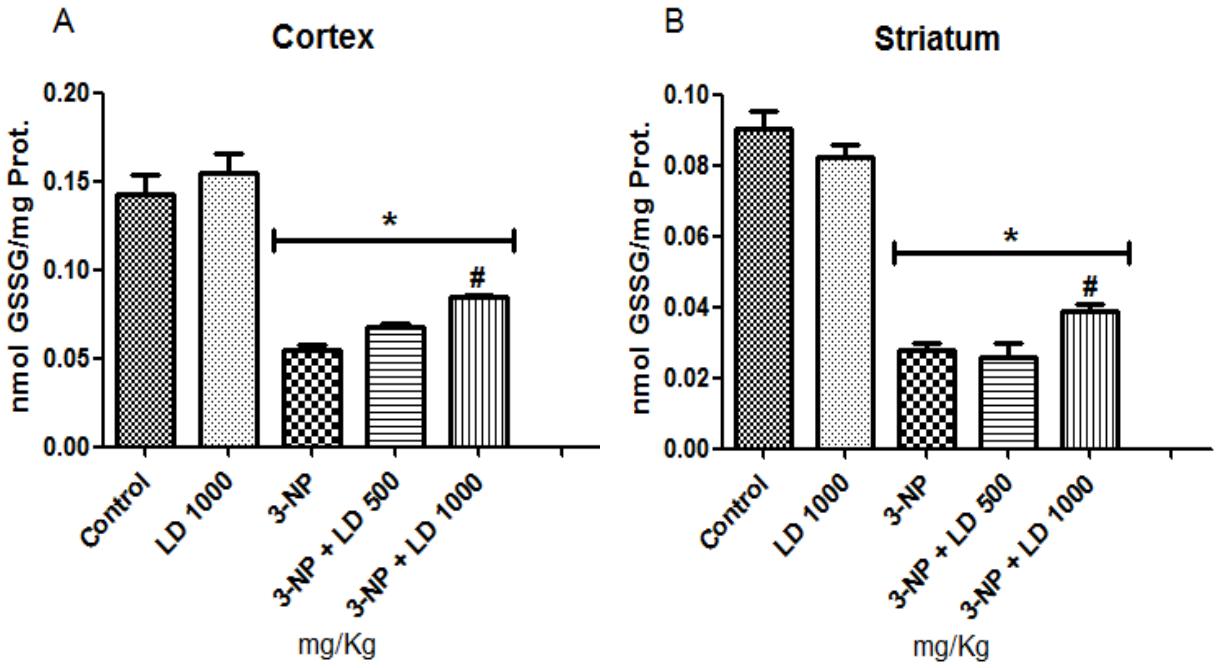
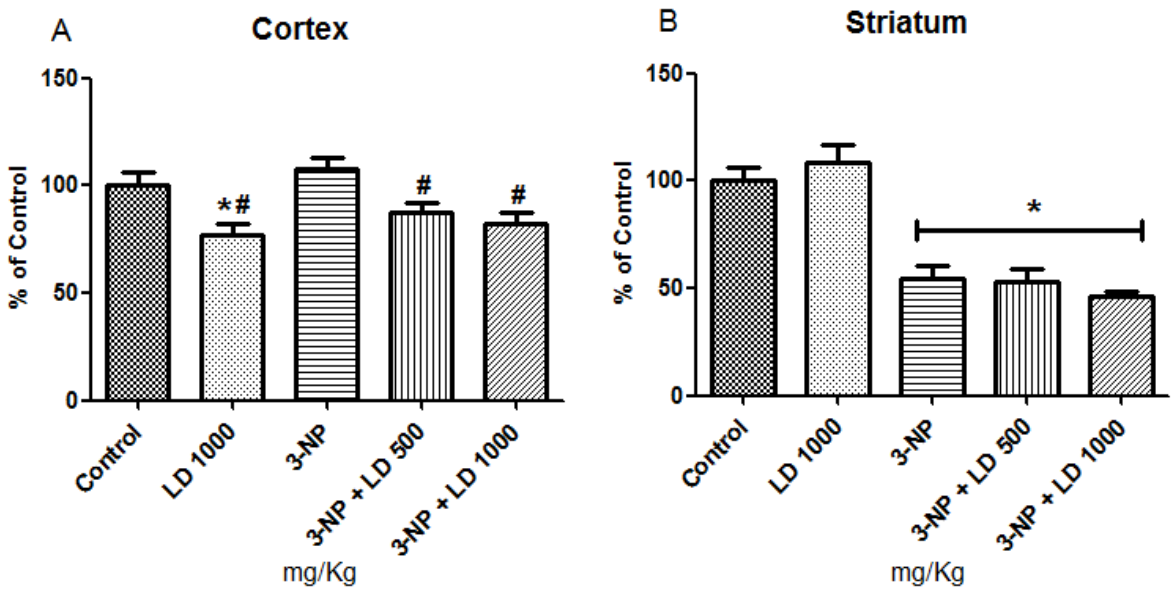


Figure 7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3-NP + LD 1000
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4 CONCLUSÕES

Os resultados apresentados neste trabalho aumentam o conhecimento do potencial antioxidante da espécie vegetal *Luehea divaricata*. Nesse estudo, foi demonstrado que o extrato aquoso de *L. divaricata* foi capaz de evitar a oxidação e as alterações comportamentais induzidas pelo tratamento com 3-NP. Ou seja, foi eficaz na prevenção de sintomas da DH induzidos pela administração de 3-NP. Consequentemente, a planta poderia ser utilizada como um agente potencial para a prevenção de diversas doenças neurológicas associadas com danos oxidativos. É importante ressaltar que os extratos aquosos de plantas tendem a apresentar maior capacidade antioxidante, que é a preparação utilizada pela população em geral.

5 PERSPECTIVAS

A partir dos resultados obtidos, as perspectivas para trabalhos posteriores são:

- Identificar os principais componentes do extrato aquoso de *L. divaricata,* responsáveis pelo efeito antioxidante demonstrado neste estudo;
- Avaliar a sua utilização farmacológica *in vivo* como um coadjuvante no tratamento sintomático de outras desordens neurodegenerativas;
- Investigar o possível efeito da *L. divaricata* contra o dano mitocondrial induzido pelo 3-NP.

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