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ATIVIDADE ANTIOXIDANTE *in vitro* DO EXTRATO ETANÓLICO DAS FOLHAS DE *Luehea divaricata* Mart.

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil

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ATIVIDADE ANTIOXIDANTE *in vitro* DO EXTRATO ETANÓLICO DAS FOLHAS DE *Luehea divaricata* Mart.

Leticia Priscilla Arantes

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ATIVIDADE ANTIOXIDANTE *in vitro* DO EXTRATO ETANÓLICO DAS FOLHAS DE *Luehea divaricata* Mart.

elaborada por
Leticia Priscilla Arantes

como requisito parcial para obtenção do grau de
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Santa Maria, 16 de março de 2012.

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“Não são as respostas que movem o mundo. São as perguntas.”

(Campanha publicitária canal Futura)

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 foi submetido. No mesmo constam as seções: Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas.

Os itens CONCLUSÕES e PERSPECTIVAS, encontrados no final desta 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.

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

ATIVIDADE ANTIOXIDANTE *in vitro* DO EXTRATO ETANÓLICO DAS FOLHAS DE *Luehea divaricata* Mart.

AUTORA: Leticia Priscilla Arantes
ORIENTADOR: Félix Alexandre Antunes Soares
Local e Data da Defesa: Santa Maria, 16 de março de 2012.

O estresse oxidativo tem sido relacionado a algumas desordens neurodegenerativas. As terapias atuais para essas doenças estão limitadas a atenuar os sintomas apresentados, entretanto alguns estudos têm mostrado que compostos antioxidantes podem ser capazes de prevenir ou retardar o dano oxidativo neuronal, incluindo aqueles presentes em extratos vegetais. Por esses motivos, neste trabalho foi investigada a possível atividade antioxidante do extrato etanólico das folhas de *Luehea divaricata* em cérebro de ratos *in vitro* e os possíveis mecanismos antioxidantes envolvidos. O extrato foi avaliado contra a peroxidação lipídica basal e induzida por nitroprussiato de sódio (NPS) 5 μM em cérebro de ratos através da quantificação da produção de espécies reativas ao ácido tiobarbitúrico (TBARS). Fatias de áreas do cérebro foram tratadas com NPS 100 μM e extrato para determinar a viabilidade celular através do ensaio da redução do MTT. A atividade *scavenger* do extrato foi testada contra ON, $DPPH^{\bullet}$ e $OH^{\bullet}$ através do reagente de Griess e dos ensaios de $DPPH^{\bullet}$ e de oxidação da desoxirribose, respectivamente. A capacidade quelante e redutora frente ao ferro foram determinadas através do método da orto-fenantrolina. O extrato foi analisado por HPLC quanto à presença dos ácidos gálico, clorogênico e cafeico, quercetina, rutina e kaempferol. Somente a rutina foi detectada e então usada como padrão, nas mesmas concentrações do extrato, em todos os testes. O extrato de *L. divaricata* (1-10 μg/ml) protegeu o cérebro contra peroxidação lipídica induzida, diminuiu os níveis basais de TBARS (± 50%) e manteve as células viáveis. O extrato não foi capaz de proteger a desoxirribose contra $OH^{\bullet}$ e de quelar o ferro, entretanto inibiu ON e $DPPH^{\bullet}$ em 33,14% a 20 μg/ml e 53,93% a 50 μg/ml respectivamente, e mostrou poder redutor de maneira concentração e tempo dependentes. Assim, o extrato etanólico das folhas de *L. divaricata* demonstrou propriedade antioxidante *in vitro* em baixas concentrações. Os mecanismos antioxidantes foram relacionados ao efeito *scavenger* de espécies reativas de oxigênio e de nitrogênio e à atividade redutora de metais. Esses efeitos foram similares ou superiores em comparação às mesmas concentrações de rutina, exceto na capacidade *scavenger* de ON e, portanto, outros compostos ainda não determinados no extrato parecem estar associados às atividades observadas neste trabalho. Mais estudos devem ser realizados em relação à identificação desses compostos e à atividade neuroprotetora do extrato.

Palavras-chave: Açoita-cavalo, Compostos fenólicos. Espécies reativas de nitrogênio. Espécies reativas de oxigênio. Extrato vegetal. Neuroproteção.

ABSTRACT

Master's Degree Dissertation
Graduation Program in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria

In vitro ANTIOXIDANT ACTIVITY OF THE ETHANOLIC EXTRACT OF *Luehea divaricata* Mart. LEAVES

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Place and Date of the Defense: Santa Maria, March 16 th, 2012.

Oxidative stress has been linked to some neurodegenerative disorders. Current therapies are limited to attenuate the symptoms, however some studies have shown that antioxidant compounds may be able to prevent or delay neuronal oxidative damage, including those present in plant extracts. For these reasons, this study investigated the possible antioxidant activity of the ethanolic extract of *Luehea divaricata* leaves in brain of rats *in vitro* and the possible antioxidant mechanisms involved. The extract was evaluated against basal and sodium nitroprusside (SNP) 5 μM induced lipid peroxidation in brain of rats through measurements of thiobarbituric acid reactive substances (TBARS) production. Slices of brain areas were treated with SNP 100 μM and extract to determine cellular viability by MTT reduction assay. Scavenger activity was evaluated against NO, $DPPH^{\bullet}$ and $OH^{\bullet}$ through Griess reagent, $DPPH^{\bullet}$ and deoxyribose oxidation assays, respectively. The chelating and reducing capacity for iron were determined by the orto-phenantroline method. The extract was screened by HPLC for the presence of gallic, chlorogenic, and caffeic acids, quercetin, rutin, and kaempferol. Only rutin was detected and then was used, in the same concentrations of the extract, as standard in all assays. *L. divaricata* extract (1-10 μg/ml) protected against induced lipid peroxidation, decreased basal levels of TBARS (about 50%) and maintained the cells viable. The extract was not able to protect deoxyribose against $OH^{\bullet}$ and to chelate iron, however it inhibited NO and $DPPH^{\bullet}$ in 33.14% at 20 μg/ml and 53.93% at 50 μg/ml respectively, and showed a reducing power in a concentration and time dependent manner. Therefore, *L. divaricata* ethanolic leaf extract showed antioxidant properties *in vitro* at low concentrations. The antioxidant mechanisms were related to scavenger activity on reactive oxygen and nitrogen species and metal reducing property. These effects were similar or more powerful than rutin in same concentrations in all assays, except in NO scavenger activity, and, thus, other unidentified compounds present in the extract appear to be associated with the effects observed in this study. More studies are needed regarding the identification of these compounds and the neuroprotective activity of the extract.

Keywords: Açoita-cavalo, Neuroprotection. Phenolic compounds. Plant extract. Reactive oxygen species. Reactive nitrogen species.

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Manuscrito

SUMÁRIO

1 INTRODUÇÃO

O metabolismo celular produz continuamente espécies reativas de oxigênio (EROs) como subprodutos da respiração e de algumas atividades enzimáticas (xantina oxidase e ciclooxigenases, por exemplo), como sistema de defesa em células fagocíticas e de transdução de sinal (FINKEL, 2011; HALLIWELL, 1994; PARK et al., 2004) e através de metais catalíticos presentes no organismo, como o cobre e o ferro, através da reação de Fenton ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$) (HALLIWELL; GUTTERIDGE, 1999). Essas espécies reativas também podem ser produzidas por alterações biológicas causadas por fatores endógenos, como mutações genéticas (WEI et al., 2001), ou exógenos, como cigarro (KIRKHAM; RAHMAN, 2006), radiação (TYRRELL, 2012; YOSHIDA et al., 2012) e xenobióticos (BALK et al., 2010; MOON; RICHIE; ISOM, 2010).

As EROs incluem o ânion superóxido (O_2^-), o radical hidroxil ($OH^{\bullet}$), o oxigênio singleto ($^1O_2$) e o peróxido de hidrogênio (H_2O_2) (HALLIWELL; GUTTERIDGE, 1999). O radical superóxido pode ainda reagir com o óxido nítrico (ON) formando peroxinitrito ($ONOO^-$), uma espécie reativa de nitrogênio (ERN), e consequentemente espécies reativas derivadas (WHITEMAN et al., 2004).

Geralmente, as espécies reativas podem ser neutralizadas por antioxidantes enzimáticos e não enzimáticos. Os enzimáticos incluem as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (GR), enquanto os não enzimáticos incluem moléculas endógenas, como glutationa e NADPH, e exógenas, como ácido ascórbico (vitamina C) e α-tocoferol (vitamina E) (VALKO et al., 2007).

As SOD formam um grupo de metaloenzimas que catalizam a conversão do O_2^- em H_2O_2. Em humanos, elas se expressam em três formas que diferem estruturalmente pelo metal e pelo número de subunidades: a SOD citosólica cobre-zinco (SOD1) (McCORD; FRIDOVICH, 1969), a mitocondrial manganês (SOD2) (McCORD,1976) e a extracelular cobre-zinco (SOD3) (MARKLUND, 1982). A CAT catalisa a conversão de H_2O_2 em H_2O e O_2 e é particularmente importante quando há pouca disponibilidade de glutationa (VALKO et al., 2007). A GPx constitui uma família de selênio-enzimas que detoxificam peróxidos através da oxidação de duas moléculas de glutationa. Esta pode ser posteriormente reduzida de volta pela GR a partir de elétrons do NADPH (BRIGELIUS-FLOHE, 1999; (HERBETTE; ROECKEL-DREVET; DREVET, 2007; VALKO et al., 2007). As vitaminas C e E reagem de

forma não catalítica, diretamente no radical, neutralizando-o (HALLIWELL; GUTTERIDGE, 1999).

A formação de EROs conduzida a concentrações fisiológicas é necessária para a função celular normal, mas em quantidades excessivas pode levar ao estresse oxidativo (NORDBERG; ARNÉR, 2001). O estresse oxidativo ocorre quando há um desequilíbrio entre fatores oxidantes e antioxidantes em favor dos oxidantes, causando destruição e danos irreversíveis a componentes celulares, incluindo proteínas, lipídios, DNA e RNA (HALLIWELL; GUTTERIDGE, 1999). No DNA, as espécies reativas podem lesar tanto as bases nitrogenadas quanto a desoxirribose causando mutações e câncer (BARREIROS; DAVID, 2006).

Lípidos insaturados são particularmente suscetíveis à oxidação e, por isso, a peroxidação lipídica é um marcador sensível do estresse oxidativo. A peroxidação lipídica é o resultado do ataque de espécies reativas sobre a ligação dupla de ácidos graxos insaturados, como o ácido linoleico e o ácido araquidônico, para gerar radicais lipoperóxidos altamente reativos, que iniciam uma reação em cadeia de ataque a outros ácidos graxos insaturados. A reação em cadeia leva à formação de produtos de degradação, incluindo 4-hidroxi-2,3-nonenal (HNE), acroleína, malondialdeído (MDA) e F_2-isoprostanos (BARNHAM; MASTERS; BUSH, 2004). A reação de peroxidação lipídica pode ser acelerada por ferro e cobre (HALLIWELL; GUTTERIDGE, 1984). Os efeitos globais da peroxidação lipídica são: diminuição da fluidez da membrana, aumento da permeabilidade da membrana para substâncias que normalmente só a atravessam através de canais específicos (por exemplo, K^+, Ca^{2+}) e danos a proteínas da membrana, inativando receptores, enzimas e canais iônicos (HALLIWELL, 2006).

O aumento de Ca^{2+} intracelular induzido por estresse oxidativo pode ativar a fosfolipase A_2 e liberar ácido araquidônico a partir de fosfolípidos da membrana. O ácido araquidônico livre pode, então, sofrer peroxidação lipídica e também atuar como um substrato para a síntese de eicosanóides (FAROOQUI et al., 2001). O influxo de Ca^{2+} também gera uma resposta excitotóxica, pela ativação dos receptores de glutamato, que desencadeia uma cascata de eventos conduzindo à morte celular (MATTSON; CHAN, 2003; YAMAMOTO et al., 1998).

A acroleína regula negativamente a captação de glutamato e glicose em cultura de células (LOVELL; XIE; MARKESBERY, 2000), enquanto o HNE modifica proteínas, resultando em um grande número de efeitos, incluindo a inibição do transportador de glicose neuronal tipo 3, do transportador de glutamato tipo 1 (KELLER et al., 1997) e de Na^+/K^+

ATPases (MARK et al., 1995). Ele também ativa a p38 MAPK, induzindo apoptose (TAMAGNO et al., 2003).

Dessa forma, o estresse oxidativo tem sido implicado como a causa principal de danos celulares em uma diversidade de anormalidades clínicas, incluindo aquelas relacionadas ao sistema nervoso central (HAYASHI, 2009). O dano oxidativo pode ocorrer em todo o organismo, porém o cérebro é um órgão particularmente suscetível. Isso se deve, entre outras causas, ao seu alto consumo de oxigênio e à sua concentração relativamente baixa de antioxidantes e elevada de metais de transição, como o ferro, e de ácidos graxos poliinsaturados, propiciando a ocorrência de peroxidação lipídica (HALLIWELL, 2006; REITER, 1995).

Muitas desordens neurodegenerativas têm sido relacionadas ao estresse oxidativo, por exemplo:

- Doença de Parkinson: a dopamina liberada no citoplasma do neurônio pode sofrer oxidação e gerar EROs, que lesam proteínas e lipídios (BARNHAM; MASTERS; BUSH, 2004; ZHOU; HUANG; PRZEDBORSKI, 2008). Consequentemente pode ocorrer a morte de neurônios dopaminérgicos da substância negra e o desenvolvimento de rigidez muscular, bradicinesia e tremor característicos da doença (SPILLANTINI et al., 1997).
- Doença de Alzheimer: os portadores da doença apresentam declínio cognitivo progressivo e depósitos de placas amilóides nos neurônios, cujo principal constituinte é o peptídeo beta-amilóide (Aβ) (GLENNER; WONG, 1984; KANG et al., 1987; MASTERS et al., 1985). Evidências sugerem que esse peptídeo atua como antioxidante e é secretado dentro dos neurônios como medidas compensatórias tomadas pelas células contra danos do estresse oxidativo. Porém, o estresse oxidativo não cessa e o dano neuronal se propaga (HAYASHI et al., 2007; NAKAMURA et al., 2007; SMITH et al., 2002).
- Doença de Huntington: é causada por uma expansão de poliglutamina na proteína huntingtina e herdada de forma autossômica dominante (LIN; BEAL, 2006). A toxicidade parece estar relacionada à: (1) ligação da proteína à mitocôndria e (2) alteração de genes envolvidos na função mitocondrial e na produção de EROs (LIN; BEAL, 2006; LUTHI-CARTER; CHA, 2003). Há perda de neurônios de projeção longa no córtex e estriado levando ao desenvolvimento de coréia, distúrbios psiquiátricos e demência (LIN; BEAL, 2006).

- Esclerose lateral amiotrófica: é caracterizada por fraqueza progressiva, atrofia e espasticidade do tecido muscular, refletindo a degeneração de neurônios motores da medula espinhal, córtex e tronco cerebral. Pode ser familiar, associada a mutações na SOD Cu/Zn (SOD1) (MATTIAZZI et al., 2002), ou esporádica, relacionada a anormalidades mitocondriais e à produção exacerbada de EROs. (LIN; BEAL, 2006, LIU et al., 2004).

As estratégias terapêuticas atuais para essas doenças estão limitadas a atenuar os sintomas apresentados, sem deter sua progressão. 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 *in vitro* (HALLIWELL, 2006). Há também alguns dados clínicos indicando a ação neuroprotetora de substâncias que possuem atividade antioxidante, tais como selegilina, vitamina E e *Gingko biloba* (RÖSLER et al., 1998). Por isso, diversos grupos de pesquisa têm se interessado pela descoberta e pelo estudo de substâncias potencialmente antioxidantes com atividade neuroprotetora.

Devido ao grande número de espécies vegetais e à presença de diferentes compostos antioxidantes em seus extratos, aumentam-se as chances de identificação de substâncias com atividades neuroprotetoras. Assim, muitas patologias que hoje permanecem sem um tratamento adequado, poderão vir a ser tratadas de forma mais eficiente a partir de novos e potentes fármacos de origem vegetal (SIMÕES et al., 2004).

A atividade antioxidante de extratos vegetais tem sido atribuída aos polifenóis (PESCHEL et al., 2006; SILVA, 2005), que também mostraram ter atividade antiinflamatória, antimutagênica e anticarcinogênica (KIM et al., 2012; KRIZKOVÁ et al., 2008; PEREIRA et al., 1996). 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 (WISEMAN; BALENTINE; FREI, 1997; VINSON et al., 1995). 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; RICE-EVANS; MILLER; PAGANGA, 1996).

Pesquisas têm demonstrado o efeito neuroprotetor *in vitro* e *in vivo* de diferentes extratos vegetais e seus componentes isolados. Por exemplo, o extrato de erva-cidreira (*Melissa officinalis)* apresentou atividade protetora em homogeneizado de cérebro de ratos

contra três substâncias prooxidantes (ferro, nitroprussiato de sódio e ácido 3-nitropropiônico) (PEREIRA et al., 2009). Ainda, o seu uso em pacientes com Alzheimer causou diminuição da perda cognitiva e bom efeito sedativo (SANTOS-NETO et al., 2006). 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). Em um modelo de Doença de Parkinson em roedores utilizando o agente indutor de parkinsonismo 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) e seu metabólito ativo 1-metil-4-fenilpiridínio (MPP^+), o ginseng bloqueou a perda de células da substância negra e reduziu o aparecimento de disfunção motora (VAN KAMPEN et al., 2003).

Entretanto, devido à grande diversidade, muitas espécies vegetais ainda não foram bem estudadas farmacologicamente, como, por exemplo, a *Luehea divaricata*. *Luehea divaricata* Mart., pertencente à família *Tiliaceae*, é uma árvore de grande porte natural do Brasil, Argentina e Paraguai. Também apresenta as sinonímias de *Luehea speciosa* Wild., *Brotera mediterranea* Vell. e *Luehea parvifolia* Mart. 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). As folhas são usadas tradicionalmente para tratar disenteria, leucorréia, gonorréia, reumatismo e tumores, a infusão das flores é usada contra bronquite, o caule em feridas de pele e a raiz é depurativa (TANAKA; VIDOTTI; SILVA, 2003; TANAKA et al., 2005).

Bortoluzzi et al. (2002) revelaram, na análise fitoquímica das folhas de *L. divaricata*, a presença de flavonóides, taninos catéquicos, saponinas e mucilagem e, em menor quantidade, alcalóides, óleos fixos, antocianidinas, carotenóides e polissacarídeos. Alice et al. (1995) demonstraram que antraquinonas livres, antracenosídeos, taninos gálicos, cumarinas e óleos voláteis não estão presentes nas folhas dessa espécie. 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).

Foram encontrados na literatura poucos trabalhos disponíveis sobre o potencial farmacológico dessa planta. Em um estudo, Vargas, Guidobono e Henriques (1991) relataram uma atividade genotóxica do extrato aquososo das folhas de *L. divaricata* no teste de Ames (*Salmonella*/microssomo) com ativação microssomal. Tanaka et al. (2005) demonstraram efeito citostático sem seletividade e efeito citocida seletivo do extrato metanólico das folhas em diferentes linhagens de células tumorais. Em um estudo de toxicidade conduzido por Bighetti et al. (2004), camundongos que receberam o extrato bruto hidroalcoólico de *L. divaricata*, na dose de 5,0 g/kg de peso corporal, administrado oralmente, não apresentaram

sinais de toxicidade, mostrando que o extrato pode ser considerado praticamente atóxico (LOOMIS, 1974).

Com base no contexto apresentado, este estudo foi realizado na intenção de investigar uma possível atividade antioxidante produzida pelo extrato etanólico das folhas de *Luehea divaricata* em cérebro de ratos *in vitro*, bem como os possíveis mecanismos antioxidantes envolvidos.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a possível atividade antioxidante do extrato etanólico das folhas de *Luehea divaricata* Mart. *in vitro*.

2.2 Objetivos específicos

Determinar os efeitos do extrato de *L. divaricata* sobre a peroxidação lipídica basal e induzida por nitroprussiato de sódio em cérebro total, córtex, cerebelo, hipocampo e estriado de ratos *in vitro*.

Determinar os efeitos do extrato sobre o decréscimo da viabilidade celular induzido por nitroprussiato de sódio em córtex, cerebelo, hipocampo e estriado de ratos *in vitro*.

Investigar os possíveis mecanismos antioxidantes do extrato utilizando diferentes espécies reativas e comparar os resultados obtidos com a atividade de uma substância antioxidante conhecida.

3 MANUSCRITO

In vitro ANTIOXIDANT ACTIVITY OF THE ETHANOLIC EXTRACT OF *Luehea divaricata* Mart. LEAVES

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Abstract

Oxidative stress has been implicated as a major cause of many clinical abnormalities. Thus, different research groups have been interested for new antioxidant substances, especially from natural sources. Whereby there are only few studies in literature describing pharmacological effects of *Luehea divaricata*, we investigated the antioxidant potential of its ethanolic leaf extract *in vitro*. The extract (1-10 μg/ml) was evaluated against physiological and sodium nitroprusside (SNP) 5 μM induced lipid peroxidation in brain of rats through measurements of thiobarbituric acid reactive substances (TBARS) production. Slices of brain areas were treated with SNP 100 μM and extract (1-10 μg/ml) to determine cellular viability by MTT reduction assay. Scavenger activity was evaluated against NO, $DPPH^{\bullet}$ and $OH^{\bullet}$ through Griess reagent, $DPPH^{\bullet}$ and deoxyribose oxidation assays, respectively. The chelating and reducing capacity for iron were determined by the orto-phenantroline method. *L. divaricata* extract (1-10 μg/ml) protected against induced lipid peroxidation, decreased basal levels of TBARS (about 50%) and maintained the cells viable. The extract was not able to protect deoxyribose against $OH^{\bullet}$ and to chelate iron, however it inhibited NO and $DPPH^{\bullet}$ in 33.14% at 20 μg/ml and 53.93% at 50 μg/ml respectively, and showed a reducing power in a concentration and time dependent manner. Rutin, present in the extract, was used as standard in all assays, but the extract revealed similar or more powerful effect, except in NO scavenger activity. Therefore, *L. divaricata* showed antioxidant properties *in vitro* which could be attributed to phenolic content, including rutin and other unidentified compounds.

Keywords*:* Neuroprotection. Phenolic compounds. Plant extract. Reactive oxygen species. Reactive nitrogen species.

1. Introduction

Reactive species are continuously produced during cell metabolism[1,2,3] or by biological dysfunctions caused by endogenous or exogenous factors[4,5,6,7]. Reactive oxygen species (ROS) include superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). The superoxide anion can react with nitric oxide (NO) and form peroxynitrite ($ONOO^-$), a reactive nitrogen specie (RNS), and other species derived from it[8].

The ROS formation at basal levels is necessary for normal cellular functions[9,10]. However, an imbalance between the production of reactive species and the antioxidant defense system leads to oxidative stress which causes damage to cellular components[11]. Therefore, oxidative stress has been implicated as a major cause of cellular injures, particularly in the central nervous system[11,12,13]. The brain is highly susceptible to free radical damage because of its high concentrations of unsaturated fatty acids susceptible to lipid peroxidation, high concentration of iron and low concentration of antioxidant defense[14,15].

In an attempt to protect against ROS and development of neurodegenerative disorders, different researches have been directed to the screening of new therapies using antioxidant substances, especially from natural sources[16,17]. Therapeutic properties of some plant extracts used in traditional medicine have been linked to their capacity to produce antioxidant activities. Such antioxidant properties found in plant extracts have been attributed to the polyphenols[18,19].

Luehea divaricata Mart. (*Tiliaceae*) is a plant which grows in South America [20]. The leaves are used popularly as treatment for dysentery, leucorrhea, rheumatism, blennorrhoea, and tumors[21]. A phytochemical screening of *L. divaricata* leaves reported the presence of flavonoids, tannins, saponins, and mucilage. Smaller quantities of alkaloids, fixed oils,

antocianidins, carotenoids, and polysaccharides are also present. A chemical study of a methanolic extract of the leaves describes the isolation of the triterpene 3β-*p*-hidroxibenzoil tormentic acid, and a mixture containing maslinic acid, vitexin, and glicopiranosilsitosterol[21]. Actually, there are only few studies in literature describing the pharmacological potential of this plant. They reported a genotoxic activity of the aqueous extract of *L. divaricata* leaves[22], a cytostatic effect of the methanolic extract of the leaves[21] and an antimutagenic activity of the aqueous extract of the bark[23].

Considering the fact that some disorders are related to oxidative stress and the brain is highly susceptible to ROS, we decided to investigate whether the ethanolic extract of *L. divaricata* leaves possesses an antioxidant activity in brain of rats *in vitro*, and to suggest a possible mechanism to its activity.

2. Materials and Methods

2.1. Chemicals

Gallic acid, deoxyribose, orto-phenanthroline, rutin, thiobarbituric acid (TBA), malonaldehyde-bis-dimethyl acetal (MDA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA). Sodium nitroprusside (SNP, $Na_2[Fe(CN)_5NO]\cdot 2H_2O$) was obtained from Merck (Germany) and iron sulphate ($FeSO_4$) from Reagen (Brazil). All other reagents were obtained from local suppliers.

2.2. Animals

Male Wistar rats weighing 250 (±35.36) g, approximately 3 months of age, from our own breeding colony, were maintained in an air conditioned room (20–25 ºC) under natural lighting conditions with water and food *ad libitum*. All experiments were conducted in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources of Federal University of Santa Maria, Brazil.

2.3. Plant material

Luehea divaricata leaves were collected in Santa Maria (Rio Grande do Sul, Brazil) during the flowering period.

The taxonomic identification was confirmed by the 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.

2.4. Preparation of the extract

Leaves were collected manually and macerated three times with ethanol 70% (EtOH 70%) during 3 weeks at room temperature. The extracts obtained were filtrated under vacuum and combined followed by concentration to dryness under reduced pressure rotary evaporator at 37ºC. The material was dissolved in EtOH 70% to prepare series concentrations. Rutin powder was dissolved in the same vehicle and was used as standard compound. EtOH was used for a final concentration of 3% in all assays.

2.5. Determination of total phenolic content

The total phenolic content was determined using Gallic acid as a standard phenol[24]. The reaction medium contained: 0.5 ml extract, 2.0 ml of 7.5% Na_2CO_3 and 2.5 ml of 10% Folin-Ciocalteu reagent and was incubated at 45°C for 15 minutes. Afterwards, the absorbance was measured at 765 nm. The total phenolic content was expressed as milligrams of gallic acid equivalent/g extract.

2.6. HPLC-DAD qualitative and quantitative analysis

High performance liquid chromatography (HPLC-DAD) was performed with Prominence Auto-Sampler (SIL-20A) equipped with Shimadzu LC-20AT (Shimadzu, Kyoto, Japan) reciprocating pumps connected to a DGU-20A5 degasser and a CBM-20A integrator. UV-VIS detector DAD SPD-M20A and software LC Solution 1.22 SP1 were used. Reverse phase chromatography analyses were carried out with a Phenomenex C-18 column (4.6 mm x 250 mm) packed with 5 µm diameter particles. Injection volume was 40 µl and the gradient elution was conducted as previously shown[25]. UV absorption spectra were recorded in the 200-400 nm range.

Luehea divaricata leaf extract was screened for the presence of the following polyphenolic compounds: gallic, chlorogenic, and caffeic acids, quercetin, rutin, and kaempferol. Identification of the compounds was performed by comparing their HPLC retention time and UV absorption spectrum with those of the commercial standards. Stock methanolic solutions of standards were prepared in the range of 0.0025-0.045 mg/mL. Quantification was carried out by integrating the peaks using external standard method at 327 nm wavelength for chlorogenic and caffeic acids and 365 nm for quercetin, rutin and

kaempferol. Chromatographic operations were carried out at ambient temperature and in triplicate.

2.7. Lipid peroxidation

The antioxidant effect of the *L. divaricata* leaf extract was evaluated against lipid peroxidation stimulated by SNP (5 μM) and by basal conditions through measurements of thiobarbituric acid reactive substances (TBARS) production[26,27].

Rats were killed by decapitation and the whole brain was used or dissected into cerebellum, cortex, hippocampus and striatum. Then, they were homogenized (1:10) in Tris-HCl 10 mM buffer, pH 7.4 and centrifuged at 4,000X g for 10 min at 4ºC. The low-speed supernatant (S1) fraction obtained was used for TBARS measurements.

The low-speed supernatant (S1) was pre-incubated for 1h at 37°C in a buffered medium with the *L. divaricata* leaf extract or rutin and SNP. Then, SDS 8.1%, acetic acid/HCl buffer, pH 3.5, and thiobarbituric acid 0.6% were added and incubated for 1h at 95°C. TBARS formation was determined at 532 nm and results were expressed as percentage of control.

2.8. Cellular viability

Rats were killed by decapitation and slices (0.4mm) of cerebellum, cortex, hippocampus and striatum were obtained by transversally cuts using a McIlwain chopper. Cellular viability in these areas was quantified by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[28]. SNP was used in the concentration of 100 μM to induce a decrease in mitochondrial viability. *L. divaricata* leaf

extract, rutin or vehicle and SNP (100 µM) were added directly to the slices and pre-incubated for two hours at 37°C in oxygenated PBS buffer (124mM NaCl, 5mM KH_2PO_4, 10mM Na_2HPO_4, 5mM NaH_2PO_4, 10mM Glucose). After incubation, the slices were washed twice with 0.5 ml of buffer. MTT reduction assay was performed in plates containing 500 µl of buffer, and the reaction was started by adding 0.5 mg/ml MTT. After one hour of incubation at 37°C, the medium was removed and the slices dissolved in dimethylsulfoxide (DMSO). The rate of MTT reduction was measured at 570nm. The slices were homogenized in SDS 1% and NaOH 0.1N solution and separated to carry out protein measurement according to Lowry method[29].

2.9. Free radical scavenging assays

2.9.1. Scavenging activity of NO

Nitrite content was determined by Griess reagent[30] and NO scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following equation: % inhibition = $(A_0 - A_{sample})/A_0 \times 100$, where A_0 was the absorbance of the control and A_{sample} was the absorbance in the presence of different extract concentrations. SNP (5 mM, in PBS) was incubated with *L. divaricata* leaf extract, rutin or vehicle at 25°C. After 1h, 0.5 ml of incubation solution was sampled and mixed with 0.5 ml of Griess reagent. The absorbance was measured at 550 nm. The values were compared with control to determine the percentage of inhibition of nitrite reaction with Griess reagent depicted by the *L. divaricata* leaf extract as an index of its NO scavenger activity[31].

2.9.2. DPPH• scavenging activity

The assay was performed as previously shown[32]. Briefly, DPPH• solution (85 μM) was added to a medium containing *L. divaricata* leaf extract, rutin or EtOH and the mixture was incubated for 30 minutes at 25ºC. The decrease in the absorbance measured at 518 nm depicted the scavenger activity of the *L. divaricata* extract against DPPH• radical. DPPH• scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following equation: %inhibition= $(A_0 - A_{sample})/A_0 \times 100$, where A_0 was the absorbance of the control and A_{sample} was the absorbance in the presence of different extract concentrations.

2.10. Determination of oxidative damage to deoxyribose

The deoxyribose oxidation assay was performed as previously described[33]. Briefly, oxidation was induced with H_2O_2 and $FeSO_4$ alone and in combination. The reaction medium was prepared containing the plant extract solution, deoxyribose 3mM, potassium phosphate buffer 0.05 mM, pH 7.4, $FeSO_4$ 50 μM and/or H_2O_2 500 μM. Solutions of $FeSO_4$ and H_2O_2 were made prior to use. Reaction mixtures were incubated at 37°C for 30 min and stopped by the addition of 0.8 ml of trichloroacetic acid (TCA) 2.8% followed by the addition of 0.4 ml of thiobarbituric acid (TBA) 0.6%. Next, the medium was incubated at 100°C for 20 minutes and the absorbance was recorded at 532 nm[34].

2.11. Iron chelating properties and reducing power

To examine iron chelating properties of the samples, the orto-phenantroline method was used[33]. The method is based on the reaction of free Fe^{2+} with θ-phenantroline forming a colored complex. First, the mixture containing Fe^{2+} ($FeSO_4$ 150 μM) and different concentrations of the extract or vehicle were used to form a complex. Afterwards, a solution of θ-phenantroline was added to the mixture (62.5 μg/ml equivalent to 0.25%). The formation of complexes between Fe^{2+} and the *L. divaricata* leaf extract at 30 and 60 minutes was estimated by a decrease in the color reaction at 510 nm when compared to a control solution containing Fe^{2+} and θ-phenantroline alone.

In order to examine reducing power properties of *L. divaricata* leaf extract, the same method was used. The method is based on the power of the extract in reducing the free Fe^{3+} to form Fe^{2+}. The Fe^{2+} reacts with θ-phenantroline forming a colored complex. First, the mixture containing Fe^{3+} ($FeCl_3$ 150 μM) and extract solutions in different concentrations or vehicle were used. Afterwards, a solution of θ-phenantroline was added to the mixture (62.5 μg/ml equivalent to 0.25%). The sample reducing power was estimated by an increase in the color reaction at 510 nm when compared to a control solution containing Fe^{3+} and θ-phenantroline alone in different time points (1,3, 6 and 24 hours). The values were expressed in absorbance. The solutions were prepared freshly for each experiment.

2.12. Statistical Analysis

All data are presented as means ± SEM. One-way ANOVA followed by Newman-Keuls multiple comparison test was used to evaluate differences among the concentrations of the extract. Two-way ANOVA followed by Bonferroni posttests was used to analyze

interaction among time and concentration in the data of reducing power and to compare the results of *Luehea divaricata* and the standard compound rutin. Results were considered statistically significant when $p<0.05$.

3. Results

3.1. Total phenolic content

The total phenolic content of the ethanolic extract of *L. divaricata* leaves was calculated as an equivalent of gallic acid. Results showed 647.4 ± 0.0055 mg equivalent of gallic acid/g of *L.divaricata* leaf extract.

3.2. HPLC-DAD qualitative and quantitative analysis

HPLC analysis of *Luehea divaricata* leaf extract allowed the identification of rutin at a concentration of 15.0575 ± 0.41 mg/g of extract (Figure 1) but not of gallic, chlorogenic, and caffeic acids, quercetin and kaempferol. Two unidentified two peaks were detected.

3.3. Lipid peroxidation

SNP induced a significant increase of > 200% vs. control ($p<0.05$) in TBARS levels in whole brain of rats ($p<0.05$, Fig.2A). *L. divaricata* maintained the TBARS levels at control levels starting at 5 µg/ml ($p<0.05$, Fig. 2A) and also decreased basal levels of TBARS starting at 1µg/ml (about 50%) and was more powerful than rutin in this case ($p<0.05$, Fig. 2A).

Because *L. divaricata* leaf extract was able to protect whole brain against basal and SNP-induced lipid peroxidation, we asked whether the extract antioxidant effect was dependent of the brain region evaluated.

Figure 2B-E shows the activity of *L. divaricata* in cerebellum, cortex, hippocampus, and striatum of rats respectively. Our results demonstrated that the extract significantly decreased TBARS levels generated by basal conditions and by SNP in the isolated brain structures in a similar manner starting at 1 µg/ml. The effect was concentration dependent and was more powerful than rutin in the same concentrations ($p<0.05$).

3.4. Cellular Viability

Figure 3A-D shows the cellular viabilities in different regions of rat brains. SNP 100 µM induced a significant decrease of cellular viability ($p<0.05$) in slices of cerebellum (30 %), cortex (28 %), and hippocampus (40 %), while *L. divaricata* maintained the viabilities at control levels at the concentrations of 1, 2.5, and 1 µg/ml, respectively (Figure 3A-C). The effect was not concentration dependent and was the same of rutin, except in cortex, where the standard compound did not maintain the viability at control levels in all concentrations tested. SNP group was not statistically different from control in striatum (Fig. 3D).

3.5. Free radical scavenging activity

The inhibitory effect of *L. divaricata* leaf extract on NO radicals was concentration dependent and started at 5 µg/ml (15.86% of inhibition, $p<0.05$), with a maximum inhibition of 33.14% at 20 µg/ml ($p<0.05$, Figure 4A). Rutin showed an inhibition of 27% at 5 µg/ml, 40.75% at 20 µg/ml and a maximum inhibition of 59% at 50 µg/ml ($p<0.05$, Figure 4A).

The extract significantly reduced levels of the $DPPH^{\bullet}$ radical in a concentration dependent manner ($p<0.05$, Figure 4B). This assay revealed that the extract possesses a significant $DPPH^{\bullet}$ radical scavenging activity, showing an inhibition of 9.92% at 1 µg/ml and reaching the maximum of 53.93% inhibition of the radical at 50 µg/ml. Rutin showed an inhibition of 1.47% at 1 µg/ml and was not significantly different from control and reached the maximum inhibition of 47.22% at 50 µg/ml. *L. divaricata* and rutin had a similar scavenging activity on DPPH radical with statistical difference only at 2.5 and 75 µg/ml.

3.6. Inhibition of deoxyribose oxidation

The oxidant species H_2O_2 and Fe^{2+} alone and inducing Fenton reaction ($Fe^{2+} + H_2O_2$) were able to stimulate deoxyribose oxidative degradation and increase MDA formation (about 200, 750 and 900% of control respectively). However, the protective effect of *Luehea divaricata* leaf extract and rutin, at concentrations of 1, 2.5, 5 or 10 µg/ml was not statistically different from vehicle (Fig.5). Therefore, the extract did not protect against deoxyribose degradation induced by any oxidant species tested.

3.7. Iron chelating properties and reducing power

Figure 6A shows the reducing power of *L. divaricata* leaf extract. The extract demonstrated a reducing power in a concentration and time dependent manner as indicated by a significant effect in a two-way ANOVA followed by Bonferroni posttests [$F=12.04$, $p< 0.0001$]. Moreover, one-way ANOVA showed a significant effect of the extract starting at 2.5 µg/ml ($p<0.05$) at one hour of incubation.

Figure 6B shows the reducing power of rutin. The interaction among time and concentration was not significant as indicated by a two-way ANOVA followed by Bonferroni posttests [F=0.81, p=0.6718]. A one-way ANOVA showed a significant effect of rutin starting only at 20 µg/ml ($p<0.05$) at one hour of incubation (Fig.6B). A two-way ANOVA also showed a more powerful reducing property of the extract compared to rutin at 6 hours of incubation in the concentrations of 10, 20 and 50 µg/ml and at 24 hours at 5, 10, 20 and 50µg/ml (Fig. 6A).

The chelating effect of the extract on iron (Fe^{2+}) was not significant at the concentrations of 1, 2.5, 5, 10, 20, and 50 µg/ml (data not shown).

4. Discussion

Epidemiological studies show an inverse association between the daily consumption of fruits, vegetables, some tea and wine, and the risk of degenerative and chronic diseases[36]. The protective effects have been attributed to their antioxidant compounds, such as carotenoids, vitamins and polyphenols[14,37,38], which directed considerable attention towards identification of natural substances that can be used in therapeutic. In this study, we investigated *in vitro* antioxidant activity of the ethanolic extract of *Luehea divaricata* leaves.

SNP used in this work as a pro-oxidant agent, acts through the release of NO and the generation of ferricyanide anions $[(CN)_5Fe]^{-3}$ that can react with H_2O_2 via the Fenton reaction, generating $OH^{\bullet}$[39,40]. Researchers have demonstrated the role of NO, which appears to be a neuronal messenger in the central nervous system and also a reactive specie involved in the pathophysiology of disorders such as Alzheimer's and Parkinson's diseases [13,41]. Nitric oxide can combines with O_2^- to form $ONOO^-$, a reactive specie capable of inducing lipid peroxidation in lipoproteins[42]. Cellular viability is also affected by SNP-induced oxidative

stress[43]. The production of reactive species causes damage to inner and outer mitochondrial membranes, as well as to the opening of the mitochondrial permeability transition pores, thereby inducing apoptosis[43,44].

In this work, SNP increased lipid peroxidation in brain homogenates. However, *L. divaricata* ethanolic leaf extract showed an ability to protect the whole brain against SNP-stimulated lipid peroxidation and also decreased basal lipid peroxidation in brain of rats *in vitro*. Despite literature describes some regional differences among brain areas in relation of pro-oxidant and antioxidant defenses[45,46], the extract presented the same effect in cerebellum, cortex, hippocampus and striatum. Furthermore, SNP decreased the number of viable cells in slices of cerebellum, cortex and hippocampus of rats *in vitro* and the extract showed a protective effect maintaining the cell viability.

In order to determine the antioxidant mechanisms by which the extract could be exerting its activity, we first tested the extract scavenger activity against NO and $DPPH^{\bullet}$. *L. divaricata* leaf extract effectively reduced the amount of nitrite which reacted with Griess reagent, indicating a NO scavenger activity and suggesting an inhibitory effect of RNS formation[47]. In addition, the extract showed a significant concentration dependent scavenger activity against $DPPH^{\bullet}$ radical, a reactive specie that have an electron free and a characteristic absorption, which decreases significantly on exposure to antioxidants[48]. The hydrogen donation to the free radical and its reduction to non-reactive specie is a mechanism by which the extract can remove the free radical[16].

The extract chelating and reducing activity on iron were also tested. The chelate can be formed through various covalent bonds between the metal ion and the antioxidant, preventing the first to be available to participate in oxidation-reduction reactions[49]. *L. divaricata* extract, in the concentrations tested, did not show significant Fe^{2+}-chelating activity, and did not protect deoxyribose from Fe^{2+} - induced oxidation. The extract was not

able to protect deoxyribose against H_2O_2 or hydroxyl radicals generated by the induced Fenton reaction. However, the extract was able to reduce Fe^{3+} to Fe^{2+} ions, which can be related to the presence of reducing molecules in the extract that exerts antioxidant activity by electron donation to metallic ion[50].

It is well known that the polyphenols exhibit considerable free radical scavenging activities through their reactivity as hydrogen- or electron-donating agents and their catalytic metal reducing properties[21]. *L. divaricata* leaf extract analyzed in this study showed a high content of phenolic compounds, thus, we suggest that the antioxidant activity demonstrated here could be attributed to them. In addition, the HPLC analyses identified the presence of rutin, which is a flavonoid with described antioxidant potential[50,51]. The *L. divaricata* extract showed an antioxidant activity similar or more powerful than rutin in same concentrations in all assays, except in NO scavenger activity. Therefore, the effect of the extract is possibly related to other unidentified compounds in addition to rutin.

Data from literature shows that the pharmacological properties of plant crude extracts can be lost when specific components are isolated, indicating that some of these properties may be related to the synergistic effect of various compounds. Thus, extracts can offer major advantages over the single compounds, since they offer lower cost and toxicity, and greater pharmacological activity[52,53].

In summary, our study shows an antioxidant activity of low concentrations (1-10 µg/ml) of the ethanolic extract of *L. divaricata* leaves through a protective effect against lipid peroxidation and decrease in cellular viability induced by SNP in brain of rats *in vitro*. This activity is probably due to the antioxidant potential of polyphenols present in the extract, with ROS and RNS scavenger and metal reducing properties. Further studies are needed to identify other compounds in the extract, besides rutin, that could be the responsible for the antioxidant effects depicted here.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Finkel T: Signal transduction by reactive oxygen species. *J Cell Biol* 2011; 194: 7–15.

2. Halliwell B: Free radicals, antioxidants and human disease: curiosity, cause or consequence? *Lancet* 1994; 344: 721-724.

3. Park JY, Kim EJ, Kwon KJ et al.: Neuroprotection by fructose-1,6-bisphosphate involves ROS alterations via p38 MAPK/ERK. *Brain Res* 2004; 1026: 295-301.

4. Wei YH, Lu CY, Wei CY, Ma YS, Lee HC: Oxidative stress in human aging and mitochondrial disease-consequences of defective mitochondrial respiration and impaired antioxidant enzyme system. *Chin J Physiol* 2001; 44: 1-11.

5. Kirkham P, Rahman I: Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. *Pharmacol Ther* 2006; 111: 476–494.

6. Balk R de S, Bridi JC, Portella R de L et al.: Clomipramine treatment and repeated restraint stress alter parameters of oxidative stress in brain regions of male rats. *Neurochem Res* 2010; 35: 1761–1770.

7. Tyrrell RM: Modulation of gene expression by the oxidative stress generated in human skin cells by UVA radiation and the restoration of redox homeostasis. *Photochem Photobiol Sci* 2012; 11: 135-147.

8. Whiteman M, Armstrong JS, Cheung NS et al.: Peroxynitrite mediates calcium-dependent mitochondrial dysfunction and cell death via activation of calpains *FASEB J* 2004; 18: 1395-1397.

9. Rodriguez-Marinez E, Camacho A, Maldonado PD et al.: Effect of quinolinic acid on endogenous antioxidants in rat corpus striatum. *Brain Res* 2000; 858: 436-439.

10. Santamaría A, Salvatierra-Sanchez R, Vazquez-Roman B et al.: Protective effects of the antioxidant selenium on quinolinic acid-induced neurotoxicity in rats: *in vitro* and *in vivo* studies. *J Neurochem* 2003; 86: 479-488.

11. Halliwell B, Gutteridge JMC: Oxidative stress. In: *Free Radicals in Biology and Medicine*. Oxford University Press, 1999, pp. 26-27.

12. Olanow CW: A radical hypothesis for neurodegeneration. *Trends Neurosci* 1993; 16:439–444.

13. Halliwell B: Oxidative stress and neurodegeneration: where are we now? *J Neurochem* 2006; 97: 1634–1658.

14. Reiter RJ: Oxidative processes and antioxidative defense mechanisms of the aging brain. *FASEB J* 1995; 9: 526-533.

15. Muralidhara GKS: Effect of *Centella asiatica* leaf powder on oxidative markers in brain regions of prepubertal mice *in vivo* and *in vitro* efficacy to ameliorate 3-NPA-induced oxidative stress in mitochondria. *Phytomedicine* 2008; 15: 971-984.

16. Wang H, Gao XD, Zhou GC, Cai L, Yao WB: *In vitro* and *in vivo* antioxidant activity of aqueous extract from *Choerospondias auxillaris* fruit. *Food Chem* 2008; 106: 888-895.

17. Wagner C, Fachinetto R, Dalla Corte CL et al.: Quercitrin, a glycoside form of quercetin, prevents lipid peroxidation *in vitro*. *Brain Res* 2006; 1107: 192-198.

18. Peschel W, Sánchez-Rabaneda F, Diekmann W, Plescher A, Gartzía I, Jiménez D: An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chem* 2006; 97: 137-50.

19. Silva CG, Herdeiro RS, Mathias CJ et al.: Evaluation of antioxidant activity of Brazilian plants. *Pharmacol Res* 2005; 52: 229–233.

20. Lorenzi H: *Luehea divaricata*. In: *Árvores Brasileiras: Manual de Identificação e Cultivo de Plantas Arbóreas Nativas do Brasil* . Plantarum: Nova Odessa, Brazil; 1998, pp.337.

21. Tanaka JCA, Silva CC, Dias Filho BP, Nakamura CV, Carvalho JE, Foglio MA: Constituintes químicos de *Luehea divaricata* Mart. (Tiliaceae). *Quim Nova* 2005; 28: 834-837.

22. Vargas VMF, Guidobono RR, Henriques JAP: Genotoxicity of plant extracts. *Mem Inst Oswaldo Cruz* 1991; 86: 67-70.

23. Felício LP, Silva EM, Ribeiro V et al.: Mutagenic potential and modulatory effects of the medicinal plant *Luehea divaricata* (Malvaceae) in somatic cells of *Drosophila melanogaster*: SMART/wing. *Genet Mol Res* 2001; 10: 16-24.

24. Singleton VL, Orthofer R, Lamuela-Raventos RM: Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu Reagent. *Methods of Enzymology* 1999; 299: 152-178.

25. Evaristo IM, Leitão MC: Identificação e quantificação por DAD-HPLC da fração fenólica contida em folhas de *Quercus suber* L. *Silva Lus* 2001; 9: 135-141.

26. Ohkawa H, Ohishi H, Yagi K: Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351-358.

27. Ávila DS, Gubert P, Palma A et al.: An organotellurium compound with antioxidant activity against excitotoxic agents without neurotoxic effects in brain of rats. *Brain Res Bull* 2008; 76, 114-123.

28. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65: 55-63.

29. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-275.

30. Green LC, Wanger DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum, SR: Analysis of nitrate, nitrite, [15N] nitrate in biological fluids. *Anal Biochem* 1981; 126: 131-138.

31. Marcocci L, Maguire JJ, Droy-Lefaix MT, Packer L: The nitric oxide-scavenging properties of *Ginkgo biloba* extract EGb 761. *Biochem Biophys Res Commun* 1994; 201: 748-755.

32. Cho SY, Park JY, Park EM et al.: Alternation of hepatic antioxidant enzyme activities and lipid profile in streptozotocin-induced diabetic rats by supplementation of dandelion water extract. *Clin Chim Acta* 2002; 317: 109-117.

33. Puntel RL, Nogueira CW, Rocha JBT: Krebs cycle intermediates modulate thiobarbituric acid reactive species (TBARS) production in rat brain *in vitro*. *Neurochem Res* 2005; 30: 225-235.

34. Halliwell B, Gutteridge JMC: Formation of a thiobarbituric-acid reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. *FEBS Lett* 1981; 128: 347-352.

35. Zhao Y, Zhao B: Natural antioxidants in prevention and management of Alzheimer's disease. *Front Biosci (Elite Ed*.) 2012; 4: 794-808.

36. John JH, Ziebland S, Yudkin P, Roe LS, Neil HA: Effects of fruit and vegetable consumption on plasma antioxidant concentrations and blood pressure: a randomised controlled trial. *The Lancet* 2002; 359: 1969–1974.

37. Oliveira AC, Valentim IB, Silva CA et al.: Total phenolic content and free radical scavenging activities of methanolic extract powders of tropical fruit residues. *Food Chem* v. 115, p. 469–475, 2009.

38. Sudati JH, Fachinetto R, Pereira RP et al.: *In vitro* antioxidant activity of *Valeriana officinalis* against different neurotoxic agents. *Neuroch Res* 2009; 34: 1372-1379.

39. Beal MF: Mitochondria, free radicals, and neurodegeneration. *Curr Opin Neurol* 1996; 6: 661-666.

40. Rauhala P, Khaldi A, Mohanakumar KP, Chiueh CC: Apparent role of 5 hydroxyl radicals on oxidative brain injury induced by sodium nitropruside. *Free Radic Biol Med* 1998; 24: 1065-1073.

41. Castillo J, Rama R, Davalos A: Nitric oxide-related brain damage in acute ischemic stroke. *Stroke* 2000; 31: 852-857.

42. Sah NK, Kumar S, Subramanian M, Devasagayam TP: Variation in the modulation of superoxide-induced single-strand breaks in plasmid pBR322 DNA by biological antioxidants. *Biochem Mol Biol Int* 1995; 35: 291-296.

43. Nathan C: Specificity of a third kind: reactive oxygen and nitrogen intermediates in cell signaling. *J Clin Invest* 2003; 111: 769–778.

44. Yamaguchi T, Takamura H, Matoba T, Terao J: HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Biosci Biotechnol Biochem* 1998; 62: 1201–1204.

45. Candelario-Jalil E, Mahdu, NH, Al-Dalain SM, Martinez G, Leon OS: Time-course of oxidative damage in different brain regions following transient cerebral ischemia in gerbils. *Neurosci Res* 2001; 41: 233-241.

46. Cavallini S, Marti M, Marino S et al.: Effects of chemical ischemia in cerebral cortex slices: focus on nitric oxide. *Neurochem Int* 2005; 47: 482-490.

47. Nie BM, Yang LM, Fu, SL, Jiang XY, Lu PH, Lu Y: Protective effect of panaxydol and panaxynol on sodium nitroprusside-induced apoptosis in cortical neurons. *Chem Biol Interact* 2006; 160: 225-231.

48. Sas K, Robotka H, Toldi J, Vecsei L: Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. *J Neurol Sci* 2007; 257: 221-239.

49. Lopes GK, Schulman HM, Hermes-lima M: Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. *Biochim et Biophys Acta* 1999; 1472: 142-152.

50. Alía M, Mateos R, Ramos S, Lecumberri E, Bravo L, Goya L: Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2). *Eur J Nutr* 2006; 45:19-28.

51. Kamalakkannan N, Stanely Mainzen Prince P. Rutin improves the antioxidant status instreptozotocin-induced diabetic rat tissues. *Mol Cell Biochem* 2006; 293: 211–219.

52. Pietrovski EF, Rosa KA, Facundo VA, Rios K, Marques MC, Santos AR: Antinococeptive properties of the ethanolic extract and of the triterpene 3H-6H-16H-trihidroxilup-20(29)-ene obtained from the flowers of *Combretum leprosum* in mice. *Pharmacol Bochem Behav* 2006; 83: 90-99.

53. Adebajo AC, Iwalewa EO, Obuotor EM et al.: Pharmacological properties of the extract and some isolated compounds of *Clausena lansium* stem bark: Anti-trichomonal, antidiabetic, anti-inflammatory, hepatoprotective and antioxidant effects. *J Ethnopharmacol* 2009; 122: 10-19.

Legend of Figures

Fig.1. HPLC analysis for *L. divaricata* revealed the presence of rutin (retention time ~36.20 min). Unidentified two peaks were also detected. Three independent experiments were performed and similar results obtained.

Fig.2. Effects of *L. divaricata* leaf extract on lipid peroxidation stimulated by SNP (5 μM) and by basal conditions in whole brain (A), cerebellum (B), cortex (C), hippocampus (D) and striatum (E) of rats. The homogenates of brain structures were pre-incubated with *L. divaricata* leaf extract (1, 2.5, 5 and 10 μg/ml), rutin in the same concentrations or vehicle (EtOH) and SNP. Results are expressed as percent of control ± SEM. Values are considered significantly different by one-way ANOVA, followed by Newman-Keuls Multiple Comparison Test. 100% of control corresponds to 2.045 ± 0.251 nmol MDA (A), 1.762 ± 0.181 nmol MDA (B), 1.995 ± 0.355 nmol MDA (C), 2.376 ± 0.681 nmol MDA (D) and 1.870 ± 0.379 nmol MDA (E): (*) indicates statistical difference from control (dotted line), (#) indicates statistical difference from SNP and ($^{a-c}$) indicate statistical difference among different concentrations of the extract ($p<0.05$). *L. divaricata* was compared to rutin by Two-way ANOVA followed by Bonferroni posttests and ($) indicates statistical difference among same concentrations ($p<0.05$).Vehicle was not different from control in all assays and is not represented. All experiments were performed in duplicate (n=6).

Fig.3. Protective effect of *L. divaricata* leaf extract on the decrease of cellular viability induced by SNP (100 μM) evaluated by the MTT reduction assay. Slices of cerebellum (A), cortex (B), hippocampus (C) and striatum (D) of rats were pre-incubated with *L. divaricata* leaf extract (1, 2.5, 5 and 10 μg/ml), rutin in the same concentrations or vehicle and SNP.

Results are expressed as the percentage of control (without SNP) ± SEM. Values are considered significantly different by one-way ANOVA, followed by Newman-Keuls Multiple Comparison Test. (*) indicates statistical difference from control (dotted line) and (#) indicates statistical difference from SNP ($p<0.05$). *L. divaricata* was compared to rutin by Two-way ANOVA followed by Bonferroni posttests and ($) indicates statistical difference among same concentrations ($p<0.05$).Vehicle was not different from control in all assays and is not represented. All experiments were performed in duplicate (n=6).

Fig.4. Scavenger activity of *L. divaricata* leaf extract on NO (A) and DPPH$^{\bullet}$ radical (B). NO scavenging activity was determined using the Griess reagent. SNP (5 mM) was incubated 60 min with *L. divaricata* leaf extract (1, 2.5, 5, 10, 20, 50, 75 and 100 µg/ml), rutin in the same concentrations or vehicle. For DPPH$^{\bullet}$ scavenger activity, *L. divaricata* (same concentrations), rutin or vehicle were incubated for 30 minutes with DPPH$^{\bullet}$ (85µM) and the decrease in the absorbance depicted the scavenger activity of the extract. Results are expressed as percent of inhibition in relation to control ± SEM. The mean control value is 21.07 ± 0.089 µM of nitrite (A) and 0.7859 ± 0.0228 ABS (B). ($^{a-e}$) indicate statistical difference among different concentrations and (*) indicates statistical difference from control by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test ($p<0.05$). ($) indicates statistical difference among *L. divaricata* and rutin at the same concentrations by Two-way ANOVA followed by Bonferroni posttests ($p<0.05$). Vehicle was not different from control in all experiments and is not represented. All experiments were performed in duplicate (n = 6).

Fig.5. Effect of *L. divaricata* on oxidative damage to deoxyribose induced by H_2O_2 500 µM (A) Fe^{2+} 50 µM (B) and $H_2O_2$500 µM + Fe^{2+} 50 µM (C). Results are expressed as the percentage of control ± SEM. Values are considered significantly different by one-way ANOVA, followed by Newman-Keuls Multiple Comparison Test. (*) indicates statistical difference from control (dotted line) and (#) indicates statistical difference from the oxidant compound ($p<0.05$). *L. divaricata* was compared to rutin by Two-way ANOVA followed by Bonferroni posttests. All experiments were performed in duplicate (n=6).

Fig.6. Reducing power evaluated by spectrophotometer detection of Fe^{3+} to Fe^{2+} transformation using 150 µM $FeCl_3$ and 62.5 µg/ml orto-phenantroline . (A) *L. divaricata* leaf extract (1, 2.5, 5, 10, 20 and 50 µg/ml) and (B) Rutin. The reduction was expressed in absorbance ± SEM in different times: 1 (●); 3 (▲); 6 (■) and 24 hours (◆). The mean control value is 0.0137±0.0024 ABS. (*) indicates statistical difference from control by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test ($p<0.05$). ($) indicates statistical difference among same concentrations of *L. divaricata* and rutin by Two-way ANOVA followed by Bonferroni posttests ($p<0.05$). Vehicle was not different from control in all experiments and is not represented. All experiments were performed in duplicate (n=6).

Figure 1

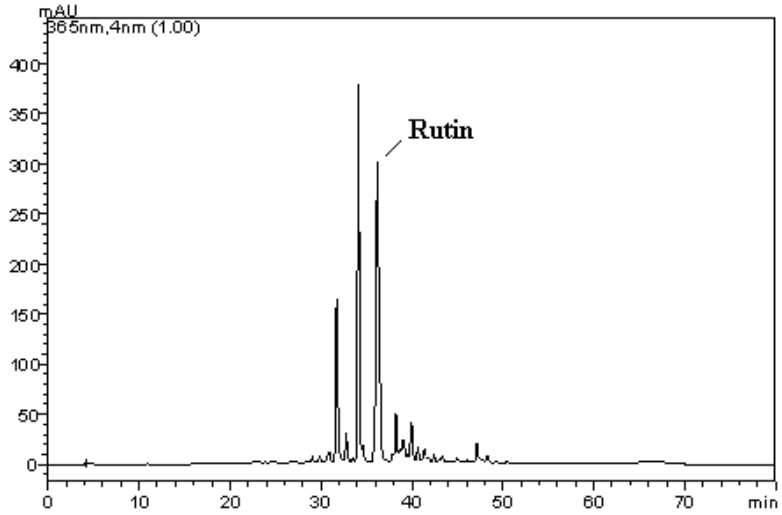
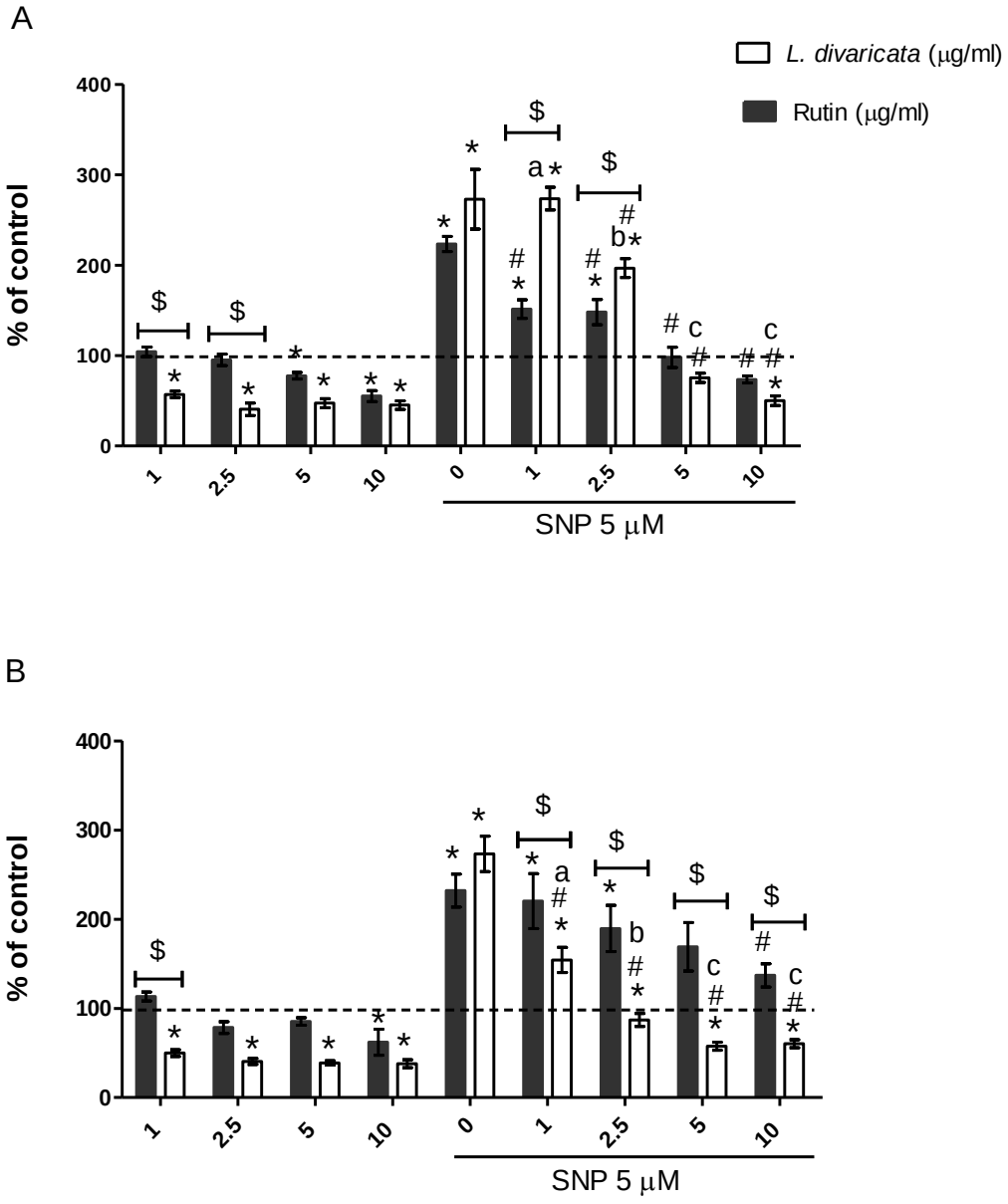
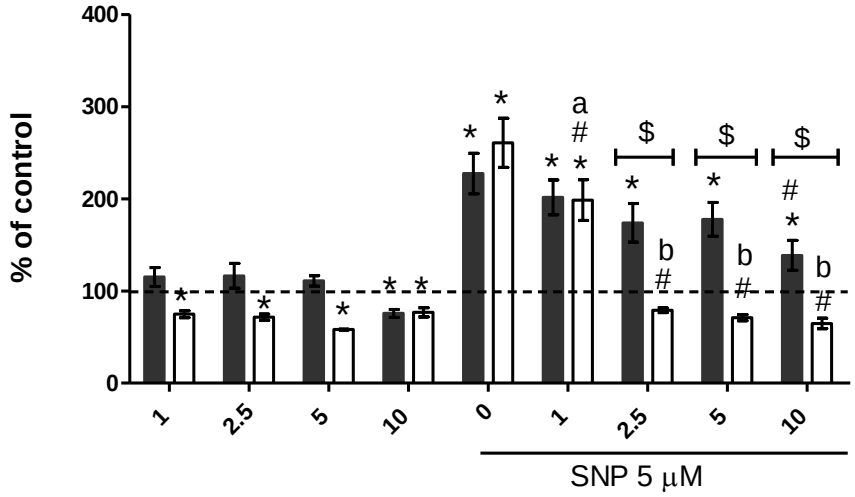

mAU
365nm,4nm (1.00)
400
350
300
250
200
150
100
50
0
Rutin
0
10
20
30
40
50
60
70
min

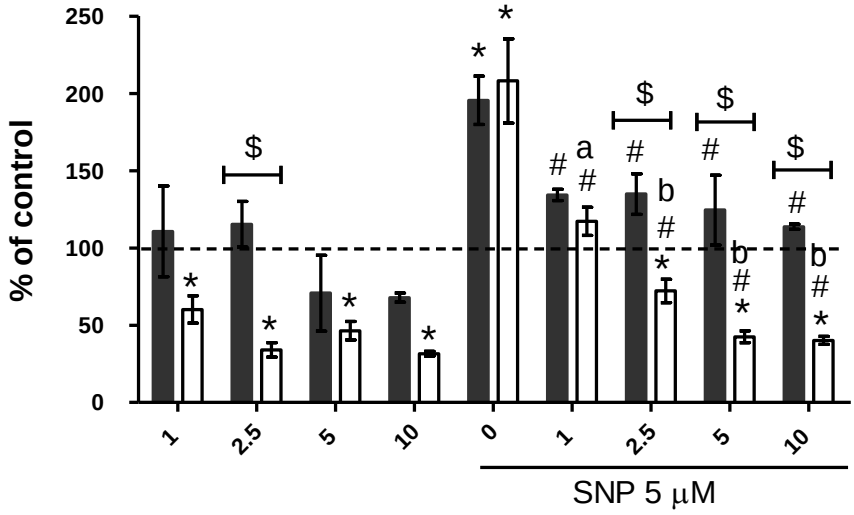
Figure 2


A
L. divaricata (μg/ml)
Rutin (μg/ml)
% of control
400
300
200
100
0
1
2.5
5
10
0
1
2.5
5
10
SNP 5 μM
B
% of control
400
300
200
100
0
1
2.5
5
10
0
1
2.5
5
10
SNP 5 μM

C


% of control
400
300
200
100
0
1
2.5
5
10
0
1
2.5
5
10
SNP 5 μM

D


% of control
250
200
150
100
50
0
1
2.5
5
10
0
1
2.5
5
10
SNP 5 μM

E

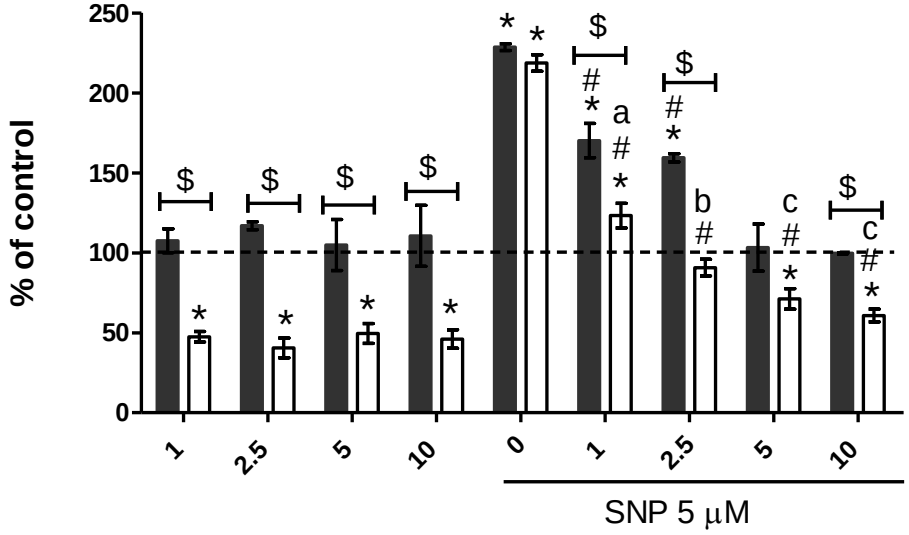
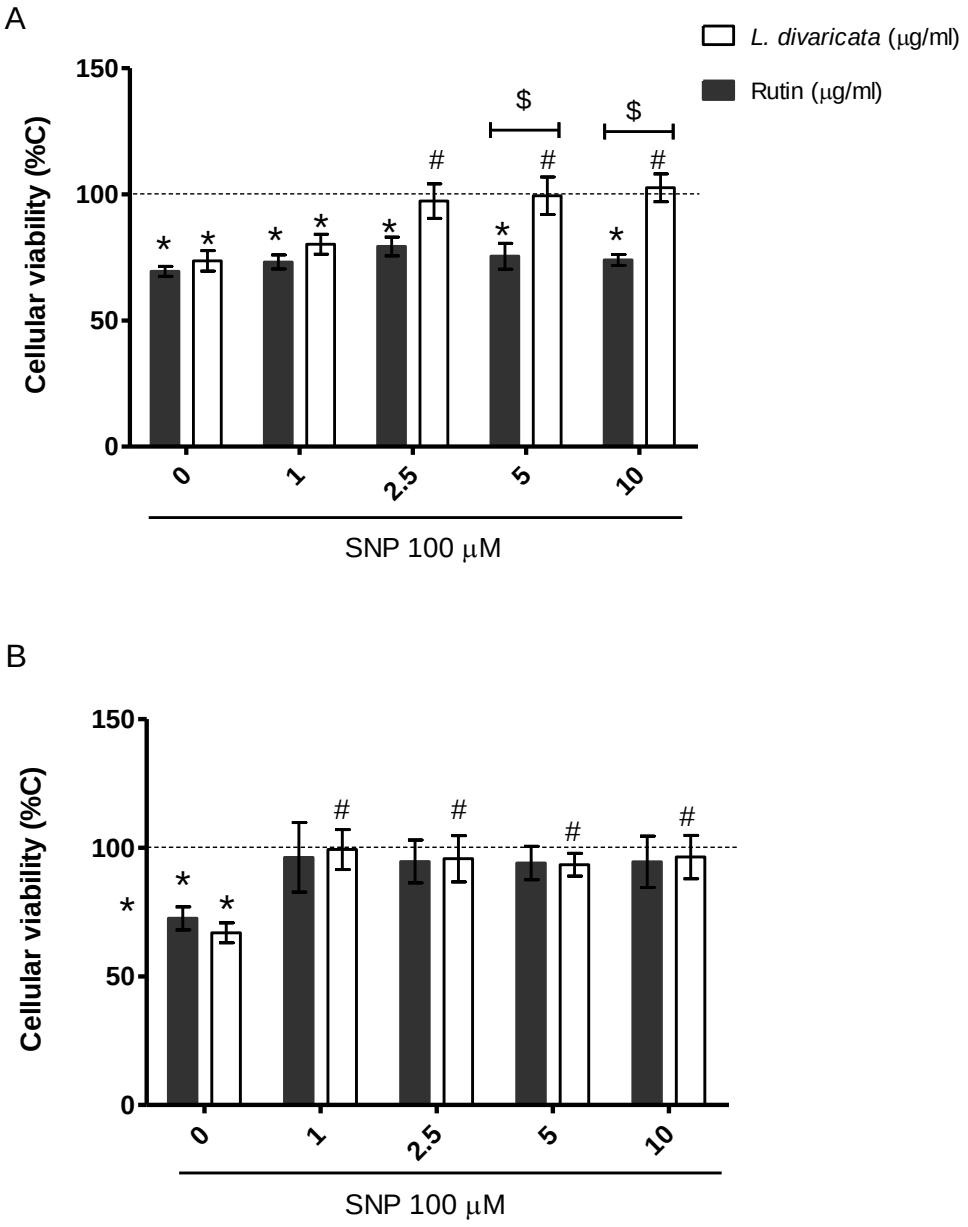
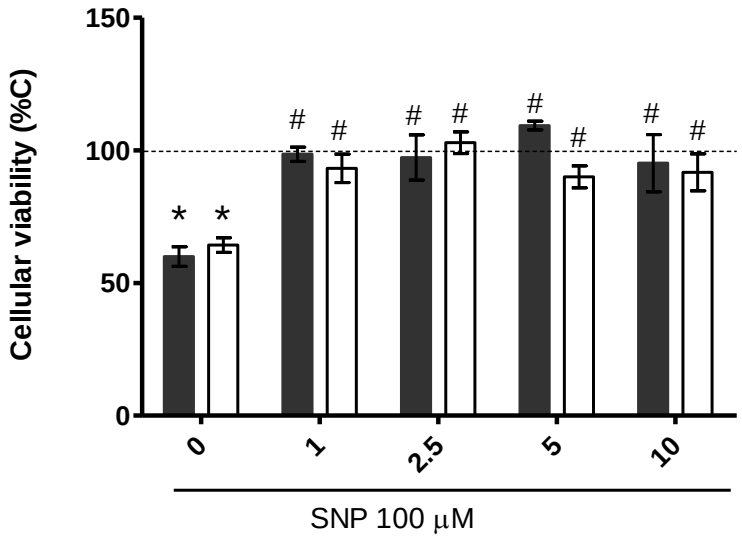


Figure 3


A
L. divaricata (μg/ml)
Rutin (μg/ml)
Cellular viability (%C)
150
100
50
0
$
$
*
#
0
1
2.5
5
10
SNP 100 μM
B
Cellular viability (%C)
150
100
50
0
*
#
0
1
2.5
5
10
SNP 100 μM

C


150
100
50
0
Cellular viability (%C)
* *
# # # # # # # #
0
1
2.5
5
10
SNP 100 μM

D

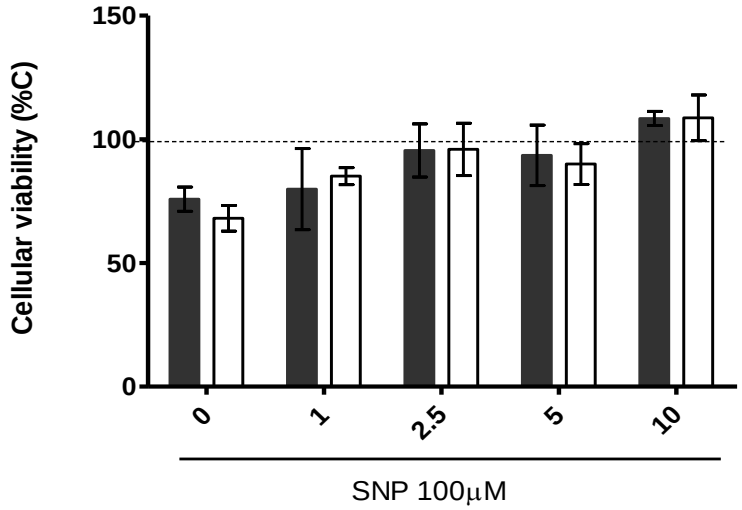

150
100
50
0
Cellular viability (%C)
0
1
2.5
5
10
SNP 100μM

Figure 4

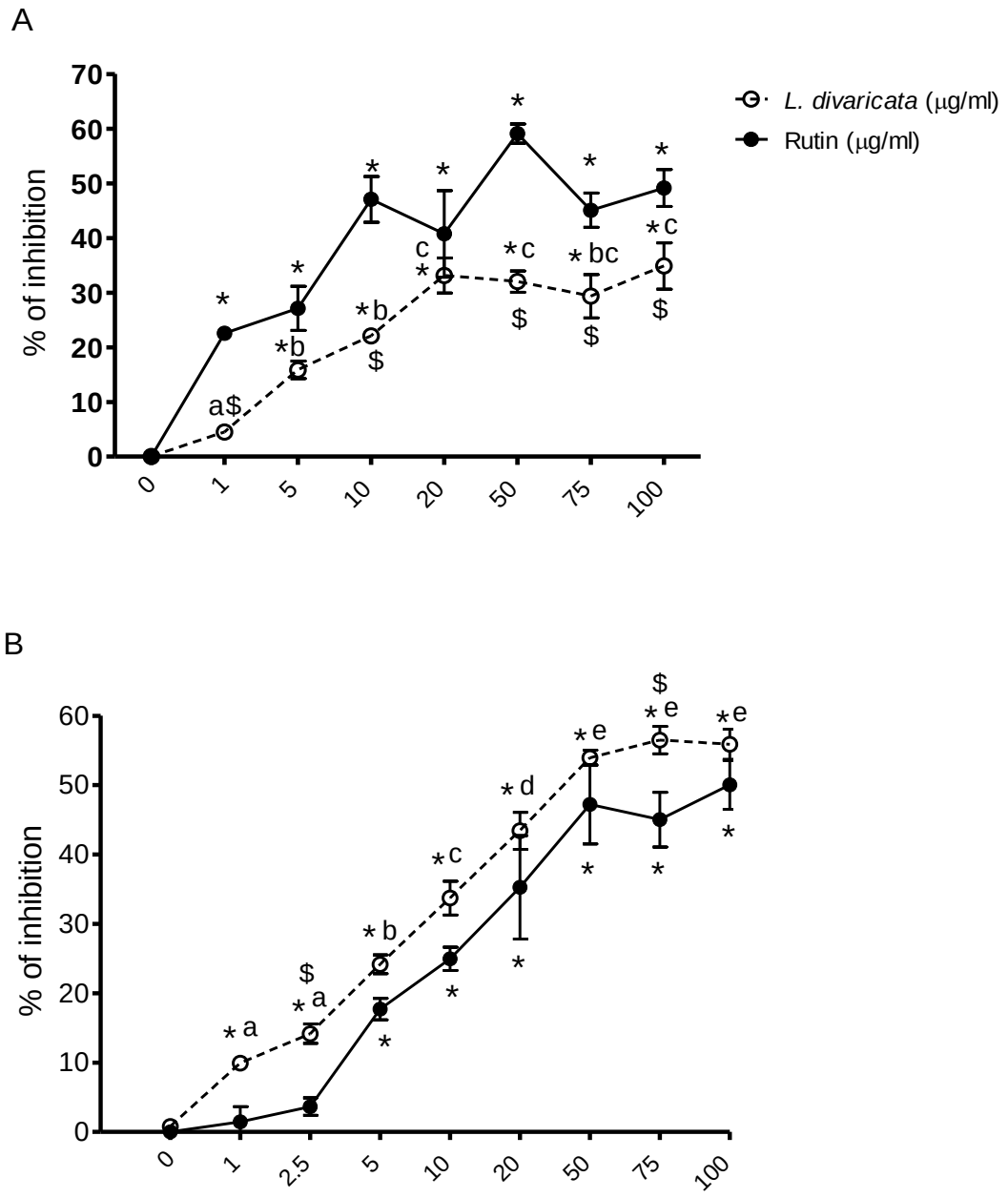

A
% of inhibition
L. divaricata (μg/ml)
Rutin (μg/ml)
0
10
20
30
40
50
60
70
0
1
5
10
20
50
75
100
B
% of inhibition
0
10
20
30
40
50
60
0
1
2.5
5
10
20
50
75
100

Figure 5

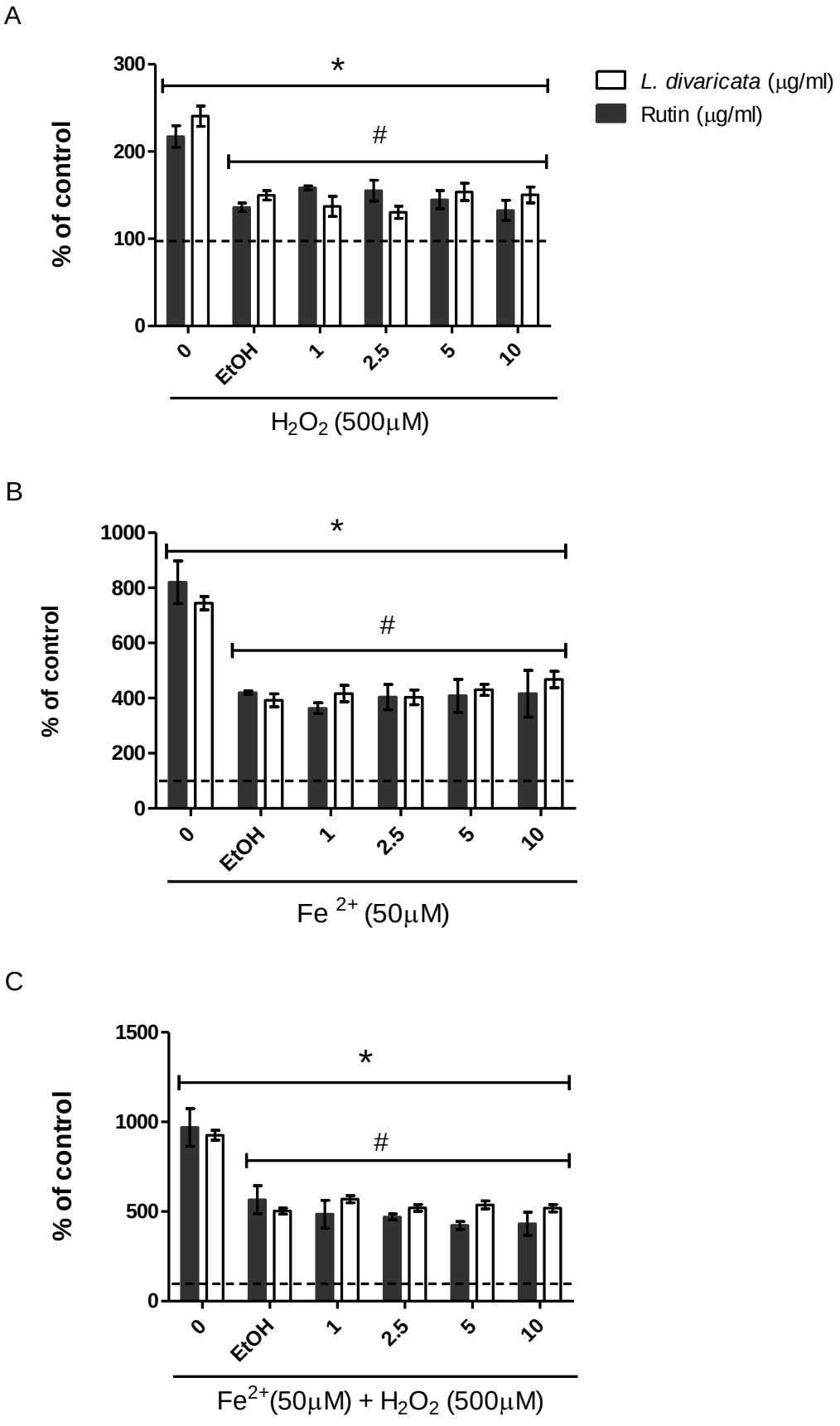
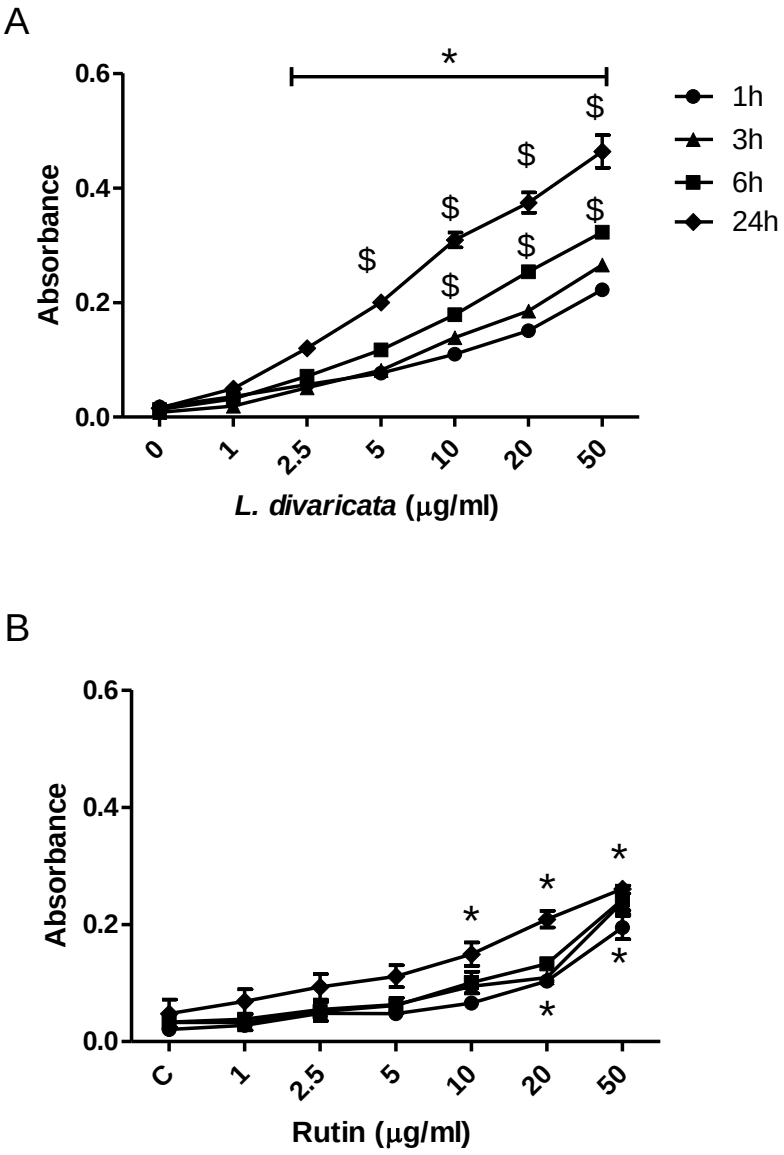

A
300
200
100
0
% of control
*
#
L. divaricata (μg/ml)
Rutin (μg/ml)
0
EtOH
1
2.5
5
10
H2O2 (500μM)
B
1000
800
600
400
200
0
% of control
*
#
0
EtOH
1
2.5
5
10
Fe 2+ (50μM)
C
1500
1000
500
0
% of control
*
#
0
EtOH
1
2.5
5
10
Fe2+(50μM) + H2O2 (500μM)

Figure 6


A
*
$
1h
3h
6h
24h
Absorbance
0.6
0.4
0.2
0.0
0
1
2.5
5
10
20
50
L. divaricata (μg/ml)
B
*
Absorbance
0.6
0.4
0.2
0.0
C
1
2.5
5
10
20
50
Rutin (μg/ml)

4 CONCLUSÕES

Os resultados apresentados neste trabalho aumentam o conhecimento do potencial farmacológico da espécie vegetal *Luehea divaricata*. Nesse estudo, foi demonstrada, pela primeira vez, a atividade antioxidante de baixas concentrações (1-10 µg/ml) do extrato etanólico das folhas de *L. divaricata*. Essa atividade pode ser observada através da redução dos níveis basais de peroxidação lipídica pelo extrato, bem como do seu efeito protetor contra a peroxidação lipídica e o decréscimo da viabilidade celular induzidos por nitroprussiato de sódio em cérebro de ratos *in vitro.*

Os mecanismos antioxidantes foram relacionados à propriedade neutralizante de espécies reativas de oxigênio e de nitrogênio e à atividade redutora de metais, exercidas, provavelmente, pelos polifenóis presentes no extrato. Neste trabalho, o extrato de *L. divaricata* não mostrou atividade quelante e não protegeu a desoxirribose do dano oxidativo gerado por Fe^{2+} e H_2O_2 isoladamente ou em conjunto. Os efeitos demonstrados foram similares ou superiores em comparação às mesmas concentrações de rutina, exceto a capacidade neutralizante de óxido nítrico, onde a rutina foi mais potente. Assim, outros compostos ainda não determinados no extrato etanólico de *L. divaricata* devem estar associados às atividades observadas em nosso estudo e precisam ser analisados.

5 PERSPECTIVAS

A partir dos resultados obtidos, mais estudos tornam-se necessários para identificar outros compostos presentes no extrato etanólico das folhas de *Luehea divaricata*, além da rutina, responsáveis pelo efeito antioxidante demonstrado neste trabalho. É necessário também investigar a atividade antioxidante e neuroprotetora *in vivo* desse extrato.

REFERÊNCIAS

ALICE, C. B. et al. Plantas medicinais de uso popular. Atlas Farmacognóstico. Canoas: Editora da ULBRA/RS, 1995.

BALK, R. S. et al. Clomipramine treatment and repeated restraint stress alter parameters of oxidative stress in brain regions of male rats. Neurochem. Res. v. 35, p. 1761–1770, 2010.

BARNHAM, K. J.; MASTERS C. L.; BUSH, A. I. Neurodegenerative diseases and oxidative stress. Nat. Rev. Drug. Discov. v. 3, p. 205-214, 2004.

BARREIRA, J. C. et al. Antioxidant activity and bioactive compounds of ten Portuguese regional and commercial almond cultivars. Food Chem. Toxicol. v. 46, p. 2230-2235, 2008.

BARREIROS, A. L. B. S.; DAVID, J. M. Estresse oxidativo: relação entre espécies reativas e defesa do organismo. Quím. nova v. 29, p. 113-123, 2006.

BIGHETTI, A. E. et al. Efeitos da administração aguda e subcrônica da *Luehea divaricata* Martus et Zuccarini. Lecta v. 22, p. 53-58, 2004.

BRIGELIUS-FLOHE, R. Tissue-specific functions of individual glutathione peroxidases. Free Rad. Biol. Med. v. 27, p. 951-965, 1999.

BORTOLUZZI, R. C. et al. Análise Química Qualitativa e Morfo-histológica de *Luehea divaricata* Mart. XVIII Simpósio de Plantas Medicinais do Brasil, Anais, Cuiabá/MT, 2002.

FAROOQUI A. A. et al. Neurochemical consequences of kainate-induced toxicity in brain: involvement of arachidonic acid release and prevention of toxicity by phospholipase A(2) inhibitors. Brain Res. Rev. v. 38, p. 61–78, 2011.

FINKEL, T. Signal transduction by reactive oxygen species. J. Cell Biol. v. 194, n. 1, p. 7–15, 2011.

GLENNER, G. G.; WONG, C. W. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem. Biophys. Res. Commun. v. 120, p. 885–890, 1984.

HALLIWELL, B. Free radicals, antioxidants and human disease: curiosity, cause or consequence? Lancet v. 344, p. 721-724, 1994.

HALLIWELL, B.; GUTTERIDGE, J. M. C. Oxidative stress. In: Free Radical Biology and Medicine. 3rd ed. Oxford University Press, p. 26-27, 1999.

HALLIWELL, B. Oxidative stress and neurodegeneration: where are we now? J. Neurochem. v. 97, p. 1634–1658, 2006.

HAYASHI, M. Oxidative stress in developmental brain disorders. Neuropathology v. 29, p. 1-8, 2009.

HAYASHI, T. et al. Lipid peroxidation and 4-hydroxy-2-nonenal formation by copper ion bound to amyloid-beta peptide. Free Rad. Biol. Med. v. 43, p. 1552-1559, 2007.

HERBETTE, S.; ROECKEL-DREVET, P.; DREVET, J. R. Seleno-independent glutathione peroxidases. More than simple antioxidant scavengers. Febs J. v. 274, n. 9, p. 2163-2180, 2007.

KANG, J. et al. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature v. 325, p. 733–736, 1987.

KASHANI, M. S. et al. Aqueous extract of lavender (*Lavandula angustifolia*) improves the spatial performance of a rat model of Alzheimer's disease. Neurosci. Bull. v. 27, n. 2, p. 99-106, 2011.

KELLER, J. N. et al. Impairment of glucose and glutamate transport and induction of mitochondrial oxidative stress and dysfunction in synaptosomes by amyloid β-peptide: role of the lipid peroxidation product 4-hydroxynonenal. J. Neurochem. v. 69, p. 273–284, 1997.

KIM, J. J. et al. Anti-inflammatory and anti-allergic effects of *Agrimonia pilosa* Ledeb extract on murine cell lines and OVA-induced airway inflammation. J. Ethnopharmacol. In press, 2012.

KIRKHAM, P.; RAHMAN, I. Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. Pharmacol. Ther. v.111, p. 476–494, 2006.

KRIZKOVÁ, L. et al. Antimutagenic *in vitro* activity of plant polyphenols: Pycnogenol and *Ginkgo biloba* extract (EGb 761). Phytother. Res. v. 22, n. 3, p. 384-388, 2008.

LIN, M. T.; BEAL, F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature v. 443, p. 787-795, 2006.

LIU, J. et al. Toxicity of familial ALS-linked SOD1 mutants from selective recruitment to spinal mitochondria. Neuron v. 43, n. 1, p. 5-17, 2004.

LOOMIS, T. A. Fundamentos de toxicologia. Zaragoza, Espanha: Acribia, 1974.

LORENZI, H. *Luehea divaricata*. In: Árvores Brasileiras: Manual de Identificação e Cultivo de Plantas Arbóreas Nativas do Brasil . Brasil: Plantarum Nova Odessa, p. 337, 1998.

LOVELL, M. A.; XIE, C.; MARKESBERY, W. R. Acrolein, a product of lipid peroxidation, inhibits glucose and glutamate uptake in primary neuronal cultures. Free Radic. Biol. Med. v. 29, p. 714–720, 2000.

LUTHI-CARTER, R.; CHA, J. H. J. Mechanisms of transcription dysregulation in Huntington's disease. Clin. Neurosci. Res. v. 3, p. 165–177, 2003.

MAHDY, H. M. et al. The effect of *Ginkgo biloba* extract on 3-nitropropionic acid-induced neurotoxicity in rats. Neurochem. Int. v. 59, n. 6, p. 770-778, 2011.

MAIER, C. M.; CHAN, P. H. Role of superoxide dismutases in oxidative damage and neurodegenerative disorders. Neuroscientist v. 8, p. 323-334, 2002.

MARK, R. et al. Amyloid β-peptide impairs ion-motive ATPase activities: evidence for a role in loss of neuronal Ca^{2+} homeostasis and cell death. J. Neurosci. v. 15, p. 6239–624, 1995.

MARKLUND, S. L. Extracellular superoxide dismutase in human tissues and human cell lines. J. Clin. Invest. v. 74, p. 1398-1403, 1984

MASTERS, C. L. et al. Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc. Natl. Acad. Sci. USA v. 82, p. 4245–4249, 1985.

MATTIAZZI, M. et al. Mutated Human SOD1 Causes Dysfunction of Oxidative Phosphorylation in Mitochondria of Transgenic Mice. J. Biol. Chem. v. 277, n. 33, p. 29626–29633, 2002.

MATTSON, M. P.; CHAN, S. L. Neuronal and glial calcium signaling in Alzheimer's disease. Cell Calcium v. 34, p. 385–397, 2003.

McCORD, J. M.; FRIDOVICH, I. Superoxide dismutase. An enxymic function for erythrocuprein (hemocuprein). J. Biol. Chem. v. 244, p. 6049-6055, 1969.

McCORD, J. M. Iron-and manganese-containing superoxide dismutases: structure, distribution, and evolutionary relationships. Adv. Exp. Med. Biol. v. 74, p. 540-550, 1976.

MOON, M. S.; RICHIE, J. P.; ISOM, H. C. Iron potentiates acetaminophen-induced oxidative stress and mitochondrial dysfunction in cultured mouse hepatocytes. Toxicol. Sci. v. 118, n. 1, p. 119-127, 2010.

NAKAMURA, M. et al. Three histidine residues of amyloid-beta peptide control the redox activity of copper and iron. Biochemistry v. 46, p. 12737-12743, 2007.

NORDBERG, J.; ARNÉR, E. S. J. Reactive oxygen species, antioxidants and the mammalian thioredoxin system. Free Radic. Biol. Med. v. 31, p. 1287-1312, 2001.

PARK, J. Y. et al. Neuroprotection by fructose-1,6-bisphosphate involves ROS alterations via p38 MAPK/ERK. Brain. Res. v. 1026, p. 295-301, 2004.

PEREIRA, M. A. et al. Effect of the phytochemicals, curcumin and quercetin upon azomethane-induced cancer and 7,12-dimethylbenz(a)anthracene-induced mammary cancer in rats. Carcinogenesis v. 17, p. 1305 – 1311, 1996.

PEREIRA, R. P. et al. Antioxidant effects of different extracts from *Melissa officinalis, Matricaria recutita* and *Cymbopogon citratus*. Neurochem. Res. v. 34, p. 973-983, 2009.

PESCHEL, W. et al. An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. Food Chem. v. 97, p. 137-150, 2006.

REITER, R. J. Oxidative processes and antioxidative defense mechanisms of the aging brain. Faseb J. v. 9, p. 526–533, 1995.

RICE-EVANS, C. A.; MILLER, N. J.; PAGANGA, G. Structure antioxidant activity relationship of flavonoids and phenolic acids. Free Radic. Biol. Med. v. 20, p. 933–956, 1996.

RÖSLER, M. et al. Free radicals in Alzheimer's dementia: currently available therapeutic strategies. J. Neural Transm. Suppl. v. 54, p. 211-219, 1998.

SANTOS-NETO, L. L. et al. The Use of Herbal Medicine in Alzheimer's Disease—A Systematic Review. Evid. Based Complement Alternat. Med. v. 3, n.4, p. 441–445, 2006.

SILVA, C. G. et al. Evaluation of antioxidant activity of Brazilian plants. Pharmacol. Res. v. 52, p. 229–233, 2005.

SIMÕES, C. M. O. et al. Farmacognosia: da planta ao medicamento. 5ª ed. Porto Alegre: Editora da UFRGS; Florianópolis: Editora da UFSC, p.13-16, 2004.

SMITH, M.A. et al. Amyloid-beta and tau serve antioxidant functions in the aging and Alzheimer brain. Free Rad. Biol. Med. v. 33, p. 1194-1199, 2002.

SPILLANTINI, M. G. et al. Alpha-synuclein in Lewy bodies. Nature v. 388, p. 839–840, 1997.

TAMAGNO, E. et al. H_2O_2 and 4-hydroxynonenal mediate amyloid β-induced neuronal apoptosis by activating JNKs and p38MAPK. Exp. Neurol. v. 180, p. 144–155, 2003.

TANAKA, J. C. A.; VIDOTTI, G. J.; SILVA, C. C. A New tormentic acid derivative from *Luehea divaricata* Mart. (*Tiliaceae*). J. Braz. Chem. Soc. v. 14, n. 3, p. 475-478, 2003.

TANAKA, J. C. A. et al. Constituintes químicos de *Luehea divaricata* Mart. (*Tiliaceae*). Quím. Nova v. 28. n. 5, p. 834-837, 2005.

TYRRELL, R. M. Modulation of gene expression by the oxidative stress generated in human skin cells by UVA radiation and the restoration of redox homeostasis. Photochem. Photobiol. Sci. v. 11, n. 1, p. 135-47, 2012

VALKO, M. et al. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell B. v. 39, p. 44–84, 2007.

VAN KAMPEN, J. et al. Neuroprotective actions of the ginseng extract G115 in two rodent models of Parkinson's disease. Exp. Neurology v. 184, p. 521–529, 2003.

VARGAS, V. M. F.; GUIDOBONO, R. R.; HENRIQUES, J. A. P. Genotoxicity of plant extracts. Mem. Inst. Oswaldo Cruz v. 86, n. 2, p. 67-70, 1991.

VINSON, J. A. et al. Plant flavonoids, especially tea flavonoids, are powerful antioxidants using an *in vitro* oxidation model for heart disease. J. Agric. Food. Chem. v. 43, p. 2800-2802, 1995.

WAGNER et al. Quercitrin, a glycoside form of quercetin, prevents lipid peroxidation *in vitro*. Brain Res. v. 1107, p. 192-198, 2006.

WEI, Y. H. et al. Oxidative stress in human aging and mitochondrial disease-consequences of defective mitochondrial respiration and impaired antioxidant enzyme system. Chin. J. Physiol. v. 44, n. 1, p. 1-11, 2001.

WHITEMAN, M. et al. Peroxynitrite mediates calcium-dependent mitochondrial dysfunction and cell death via activation of calpains. Faseb J. v. 18, p. 1395-1397, 2004.

WISEMAN, S. A; BALENTINE, D. A; FREI, B. Antioxidants in tea. Crit. Rev. Food Sci. Nutr. v. 37, p. 705-718, 1997.

YAMAMOTO, K. et al. The hydroxyl radical scavenger Nicaraven inhibits glutamate release after spinal injury in rats. Neuroreport. v. 9, p. 1655–1659, 1998.

YOSHIDA, T. et al. Mitochondrial dysfunction, a probable cause of persistent oxidative stress after exposure to ionizing radiation. Free Radic. Res. v. 46, n. 2, p. 147-153, 2012.

ZHOU, C.; HUANG, Y., PRZEDBORSKI, S. Oxidative stress in Parkinson's Disease: a mechanism of pathogenic and therapeutic significance. Ann. N. Y. Acad. Sci. v. 1147, p. 93-104, 2008.