

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

*Centella asiática* **REDUZ PEROXIDAÇÃO LIPÍDICA  
INDUZIDA POR ÁCIDO QUINOLÍNICO E  
NITROPRUSSIATO DE SÓDIO *IN VITRO* EM REGIÕES  
DO CÉREBRO DE RATO**

**DISSERTAÇÃO DE MESTRADO**

**Naiani Ferreira Marques**

**Santa Maria, RS, Brasil  
2014**

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**por**

**Naiani Ferreira Marques**

Dissertação apresentada ao curso de Mestrado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do grau de **Mestre em Bioquímica Toxicológica.**

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*Centella asiática* **REDUZ PEROXIDAÇÃO LIPÍDICA INDUZIDA POR  
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REGIÕES DO CÉREBRO DE RATO**

elaborada por

**Naiani Ferreira Marques**

Como requisito parcial para obtenção do grau de  
**Mestre em Bioquímica Toxicológica**

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*“A mente que se abre a uma nova idéia jamais volta ao seu tamanho original.”*

*Albert Einstein*

## RESUMO

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

### *Centella asiática* **REDUZ PEROXIDAÇÃO LIPÍDICA INDUZIDA POR ÁCIDO QUINOLÍNICO E NITROPRUSSIATO DE SÓDIO *IN VITRO* EM REGIÕES DO CÉREBRO DE RATO**

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ORIENTADORA: ROSELEI FACHINETTO

Local e data de defesa: Santa Maria, 21 de Agosto de 2014

O estresse oxidativo está envolvido em várias patologias incluindo as doenças neurológicas. A *Centella asiática* é uma planta medicinal que tem sido muito utilizada para o tratamento de distúrbios neurológicos na medicina Ayurvédica. O objetivo do presente estudo foi avaliar o potencial antioxidante de diferentes extratos de *C. asiática in vitro*. Foi quantificado por cromatografia líquida de alta eficiência (CLAE) o conteúdo fenólico da infusão e das frações: acetato de etila, *n*-butanólica e diclorometano. Além disso, analisou-se a capacidade dos extratos de *C. asiática* como *scavenger* do radical DPPH<sup>•</sup>, bem como, a capacidade antioxidante total através da redução do molibdênio (VI) (Mo<sup>6+</sup>) a molibdênio (V) (Mo<sup>5+</sup>). Finalmente, determinou-se o efeito dos extratos na peroxidação lipídica induzida por ácido quinolínico (AQ) e nitroprussiato de sódio (NPS), em diferentes regiões do cérebro de rato (córtex, estriado e hipocampo). A análise por CLAE revelou que flavonóides, glicosídeo triterpeno, taninos e ácidos fenólicos estavam presentes nos extratos de *C. asiática*. O teor de compostos fenólicos demonstrou que a fração acetato de etila é rica nestes compostos, seguida da fração diclorometano, da *n*-butanólica e por fim da infusão. Além disso, com as primeiras análises também verificamos um maior potencial antioxidante da fração acetato de etila como *scavenger* de radical DPPH<sup>•</sup>. Em acordo com as análises *in vitro*, a fração acetato de etila apresentou o maior efeito antioxidante através da diminuição da peroxidação lipídica induzidas por AQ em córtex (IC<sub>50</sub>=11,82), estriado (IC<sub>50</sub>=13,91) e hipocampo (IC<sub>50</sub>=13,55) de cérebro de rato. Por outro lado, quando o agente pró-oxidante foi NPS, a potência de infusão, das frações de acetato de etila e diclorometano não foram diferentes significativamente para córtex e hipocampo, sendo destacada uma maior diferença de ação no estriado, entre a infusão (IC<sub>50</sub>=16,12), a acetato de etila (IC<sub>50</sub>= 13,57) e a diclorometano (IC<sub>50</sub>= 11,05) em relação a fração butanólica (IC<sub>50</sub> = 47,94). Em conclusão, os resultados encontrados demonstraram que a infusão e demais frações de *C. asiática* apresentam capacidade antioxidante *in vitro*, a qual está relacionada ao seu conteúdo fitoquímico. Assim, o potencial terapêutico de *C. asiática* em doenças neurológicas, poderia ser associado com a sua atividade antioxidante.

**Palavras-chave:** *Centella asiática*, antioxidante, ácido quinolínico, nitroprussiato de sódio, peroxidação lipídica.



## ABSTRACT

Dissertation of Master's degree  
Post-Graduate Course in Biological Sciences: Toxicological Biochemistry  
Federal University of Santa Maria, RS, Brazil

### ***Centella asiatica* AND ITS FRACTIONS REDUCES LIPID PEROXIDATION INDUCED BY QUINOLINIC ACID AND SODIUM NITROPRUSSIDE *IN VITRO* IN RAT BRAIN REGIONS**

AUTHOR: NAIANI FERREIRA MARQUES  
ADVISOR: ROSELEI FACHINETTO

Place and date of the defense: Santa Maria, August 21, 2014

The oxidative stress is involved in several diseases, including neurological diseases. *Centella asiatica* is a medicinal plant which has long been used to treat neurological disturbances in Ayurvedic medicine. The aim of this study was to evaluate the antioxidant potential of different extracts of *C. asiatica in vitro*. Were quantified by High Performance Liquid Chromatograph (HPLC) the present compounds and examined the phenolic content of the infusion and fractions: ethyl acetate, *n*-butanol and dichlorometane. Furthermore, the ability of *C. asiatica* extracts as *scavenger* of DPPH<sup>•</sup>, as well as, the antioxidant capacity it was analyzed, through the reduction of molybdenum (VI)(Mo<sup>6+</sup>) to molybdenum (V)(Mo<sup>5+</sup>). Finally, we determined the effect of the extracts on lipid peroxidation induced by quinolinic acid (QA) and sodium nitroprusside (SNP) in different regions of the rat brain (cerebral cortex, striatum and hippocampus). HPLC analysis showed that flavonoids, triterpene glycosides, tannins and phenolic acids were present in extracts of *C. asiatica*. The content of phenolic compounds showed that the ethyl acetate fraction is rich in these compounds, followed by dichloromethane fraction of butanol and of infusion. Moreover, with the first analyzes it was also found a higher antioxidant potential of ethyl acetate fraction as DPPH<sup>•</sup> radical *scavenger*. In agreement with *in vitro* assays, the ethyl acetate fraction showed the highest antioxidant effect by decreasing lipid peroxidation induced by AQ in cerebral cortex (IC<sub>50</sub> = 11.82), striatum (IC<sub>50</sub> = 13.91) and hippocampus (IC<sub>50</sub> = 13.55) from rat brain. However, when the pro-oxidant agent was NPS, potency of infusion, the ethyl acetate fraction and dichloromethane were not significantly different to the cortex and hippocampus, which highlighted a greater difference of action in the striatum between the infusion (IC<sub>50</sub> = 16.12), the ethyl acetate (IC<sub>50</sub> = 13.57) and dichloromethane (IC<sub>50</sub> = 11.05) regarding the butanol fraction (IC<sub>50</sub> = 47.94). In conclusion, the results demonstrated that the infusion of *C. asiatica* and other fractions exhibit antioxidant capacity *in vitro*, which is related to their phytochemical content. Thus, the therapeutic potential in neurological diseases of *C. asiatica*, could be associated with its antioxidant activity.

**Keywords:** *Centella asiatica*, antioxidant, quinolic acid, sodium nitroprusside, lipid peroxidation.

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## LISTA DE ABREVIATURAS

**RL** - Radical livre  
**EROs** – Espécies Reativas ao Oxigênio  
**ERNs** – Espécies Reativas ao Nitrogênio  
**MDA** – Malondialdeído  
**SOD** – Superóxido dismutase  
**GPx** – Glutathione peroxidase  
**GRd** – Glutathione reductase  
**CAT** – Catalase  
**XO** – Xantina oxidase  
**XD** – Xantina desidrogenase  
**PUFAs** – Ácidos graxos poliinsaturados  
**AA** – Ácido aracdônico  
**AQ** – Ácido Quinolínico  
**NPS** – Nitroprussiato de Sódio  
**NMDA** – Receptor N-metil-D-aspartato  
**ATP** – Adenosina trifosfato  
**SNC** – Sistema Nervoso Central  
**PTZ** – Pentilenotetrazol  
**GABA** – Ácido Gama-aminobitúrico  
**MPTP** – 1-metil-4-fenil-1,2,3,6-tetraidropiridina  
**HPLC** – High Performance Liquid Chromatography  
**TBA** – Ácido Tiobarbitúrico  
**GAE** – Equivalente de Ácido Gálico  
**AG** – Ácido Gálico

## SUMÁRIO

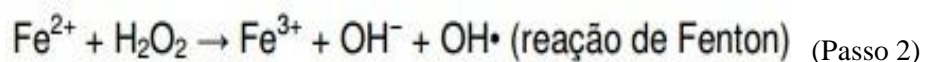
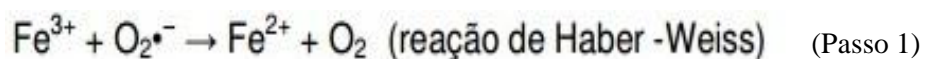
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# 1 INTRODUÇÃO

## 1.1 Espécies reativas, defesas antioxidantes

A homeostase redox *in vivo* é mantida por um equilíbrio entre os níveis oxidantes e antioxidantes, sendo que um desequilíbrio entre essas moléculas pode causar o que no geral é denominado estresse oxidativo[2]. Essa condição é bem relacionada com inúmeras patologias, onde as espécies reativas podem contribuir para o agravamento produzindo alterações na membrana celular (peroxidação lipídica e oxidação de proteínas), além de mutações no DNA[3,4]. Um radical livre (RL) é definido como qualquer átomo, grupo de átomos ou moléculas capazes de existir sob forma independente e que contém um ou mais elétrons desemparelhados na camada de valência [5]. Compostos que são igualmente reativos, porém não possuem um elétron não pareado na última camada de valência são denominados espécies reativas de oxigênio (EROs) ou espécies reativas de nitrogênio (ERNs)[6].

As principais EROs são o radical ânion superóxido ( $O_2^{\bullet-}$ ), o radical hidroxila ( $OH^{\bullet}$ ) e o peróxido de hidrogênio ( $H_2O_2$ ) e como ERNs o óxido nítrico ( $NO^{\bullet}$ ) e o peróxido de nitrito ( $ONOO^-$ ), sendo que  $H_2O_2$  não possui elétrons desemparelhados e não é classificados como radical livre[7]. O  $H_2O_2$  é formado durante o metabolismo mitocondrial em condições fisiológicas [8] e, em altas concentrações, pode reagir nas reações de Halbert-Weiss e de Fenton (Figura 1) e gerar o radical  $OH^{\bullet}$ , que é altamente reativo [1].



**Figure 1** – Reação de Haber-Weiss e reação de Fenton [1]

O radical  $O_2^{\cdot -}$  é gerado por reações de auto-oxidação do oxigênio na cadeia transportadora de elétrons mitocondrial [9]. Pode reagir com outros radicais, como por exemplo o  $NO^{\cdot}$  para a formação de  $ONOO^-$ . Também é capaz de inativar enzimas com centro ferro-enxofre, liberando ferro que pode reagir em cadeia na reação de Haber-weiss e reação de Fenton e gerar  $OH^{\cdot}$  [10].

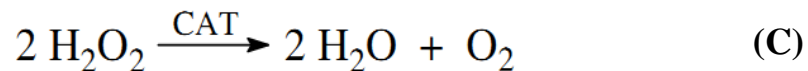
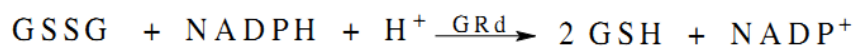
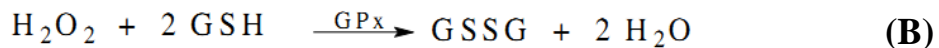
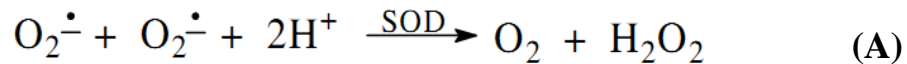
O radical  $OH^{\cdot}$  é capaz de reagir com qualquer biomolécula presente no seu local de formação sendo bem descrito o dano causado por essa espécie reativa ao DNA, gerando bases purínicas e pirimídicas modificadas[11]. Este radical livre também é importante no processo de peroxidação lipídica, sendo o responsável direto pela fase de iniciação com a retirada do átomo de hidrogênio de fosfolipídios de membrana que conduz a formação de malondealdeído (MDA).

O  $H_2O_2$ , como dito antes, não é considerado um RL porque não possui um elétron desemparelhado, porém é considerado um ERO por estar diretamente envolvido em reações que geram RL. Pode ser gerado por dismutação do radical  $O_2^{\cdot -}$  e ação de enzimas como, por exemplo, a xantina oxidase [11].

Para a manutenção de um estado redox ideal para o organismo, existem as defesas antioxidantes, essas podem ser endógenas ou não. Exemplos de defesas antioxidantes endógenas são as enzimas responsáveis pelo metabolismo dos EROs, como a catalase e a glutathiona peroxidase por exemplo, com a sua conversão em espécies estáveis. Dentre estas enzimas está a superóxido dismutase (SOD) responsável pela conversão do radical  $O_2^{\cdot -}$  em  $H_2O_2$  e  $O_2$  (Figura 2A). Existem três tipos diferentes de SOD no organismo, as que contêm cobre e zinco no sítio ativo (Cu, Zn-SOD) e são encontradas no citosol, peroxissomas, lisossomas e espaço intermembranas da mitocôndria[12]. E a SOD que contém manganês (Mn-SOD) no sítio ativo que é encontrada na matriz mitocondrial.

A glutathiona peroxidase (GPx) (Figura 2B) é responsável pela degradação de hidroperóxidos orgânicos e que pode usar selênio como cofator (citosol e mitocôndria) [1,13]. A GPx apresenta-se acoplada a outra enzima importante, a glutathiona redutase (GRd) que é responsável por catalisar a reação de conversão da glutathiona oxidada (GSSG) a glutathiona reduzida (GSH), usando NADPH como co-enzima [14,15].

A catalase (CAT) (Figura 2C) é responsável essencialmente pela metabolização de  $\text{H}_2\text{O}_2$  e é expressa em vários tecidos, mas de forma heterogênea, pois tem alta atividade no fígado, sangue e rins [16] e em menor proporção no tecido cardíaco, pulmão e cérebro [16].



**Figura 2** – Reações catalisadas pelas principais enzimas antioxidantes endógenas. (A) superóxido dismutase; (B) glutathiona peroxidase e glutathiona redutase e (C) catalase.

Além das enzimas antioxidantes, outros fatores também são importantes na defesa do organismo contra a produção exacerbada de EROs. A proteção não enzimática é constituída basicamente de moléculas presentes no organismo como a glutathiona, um tripeptídeo que atua como co-fator das enzima GPx, como mostrado anteriormente. Além disso, a coenzima Q ou ubiquinona, a qual desempenha um papel central na cadeia respiratória mitocondrial, regulando a permeabilidade de membrana diminuindo a oxidação de proteínas e DNA, também inibe a iniciação da peroxidação lipídica[17,18].

O ácido úrico derivado do metabolismo das purinas e é produzido pela oxidação de hipoxantina a xantina pela xantina oxidase (XO) e xantina desidrogenase (XD)[19], bem como o  $\alpha$ -tocoferol que é um antioxidante lipossolúvel que atua bloqueando a etapa de propagação da peroxidação lipídica dos ácidos graxos poliinsaturados das membranas e lipoproteínas [20].

Em sistemas biológicos cujo pH é 7,4, 99,95% da vitamina C encontra-se na forma de ascorbato, que é a forma que atua como antioxidante, ao doar um  $\text{H}^{\cdot}$  ou  $[\text{H}^+ + \text{e}^-]$  para um radical e atua eficientemente sobre o radical ânion  $\text{O}_2^{\cdot-}$ , os radicais  $\text{OH}^{\cdot}$  e peroxila ( $\cdot\text{OOH}$ ) e  $\text{H}_2\text{O}_2$  [21].



As propriedades antioxidantes dos carotenóides, como o  $\beta$ -caroteno estão associadas com sua capacidade de capturar RL e outras espécies, em baixas concentrações e em baixa pressão parcial de oxigênio, tais como aquelas da maioria dos tecidos sob condições fisiológicas[22]. Por fim, algumas proteínas de transporte de metais de transição, como a transferrina e a ceruloplasmina são responsáveis pelo transporte de ferro e cobre, respectivamente, no plasma sanguíneo também são importantes para regulação das espécies reativas [13].

## 1.2 Estresse oxidativo e Doenças relacionadas

O estresse oxidativo tem sido relacionado com a progressão de muitas doenças que vão desde o câncer, diabetes, doenças crônicas cardiovasculares e renais, até doenças neurodegenerativas como Alzheimer e Parkinson [23,24,25,26,27,28]. Neste trabalho focaremos especialmente os danos do estresse oxidativo no cérebro, especialmente em regiões específicas como córtex, estriado e hipocampo.

O cérebro é extremamente sensível a danos oxidativos por várias razões. Por um lado, o cérebro consome uma quantidade excessiva de oxigênio (cerca de 20%), particularmente quando se considera o fato de o mesmo ser responsável por apenas 2% do peso do corpo [29]. É sabido que o ferro se acumula no cérebro de indivíduos mais velhos, e íons de ferro que são liberados após dano cerebral podem catalisar reações de geração de RL[30].

Diversos modelos animais de doenças que atingem especialmente o cérebro, como é o caso das doenças neurodegenerativas, demonstram que a redução do estresse oxidativo melhora os sintomas. Em modelos de parkinsonismo induzido com reserpina podemos observar que o tratamento com antioxidantes conhecidos, como o ácido gálico ou o resveratrol diminui os movimentos de mascar no vazio [31,32]; este efeito também é observado em modelos de discinesia tardia [33,34,35,36]; doença de Alzheimer [37,38,39,40] e doença de Huntington [41,42,43].

Por fim, as membranas neuronais são ricas em ácidos graxos poliinsaturados (PUFAs), particularmente o ácido araquidônico (AA), ácido docosahexaenóico (DHA) e ácido eicosapentaenóico (EPA)[44]. Estes PUFAs são particularmente vulneráveis ao estresse oxidativo devido à presença de insaturações em sua estrutura química. Por estas razões, as

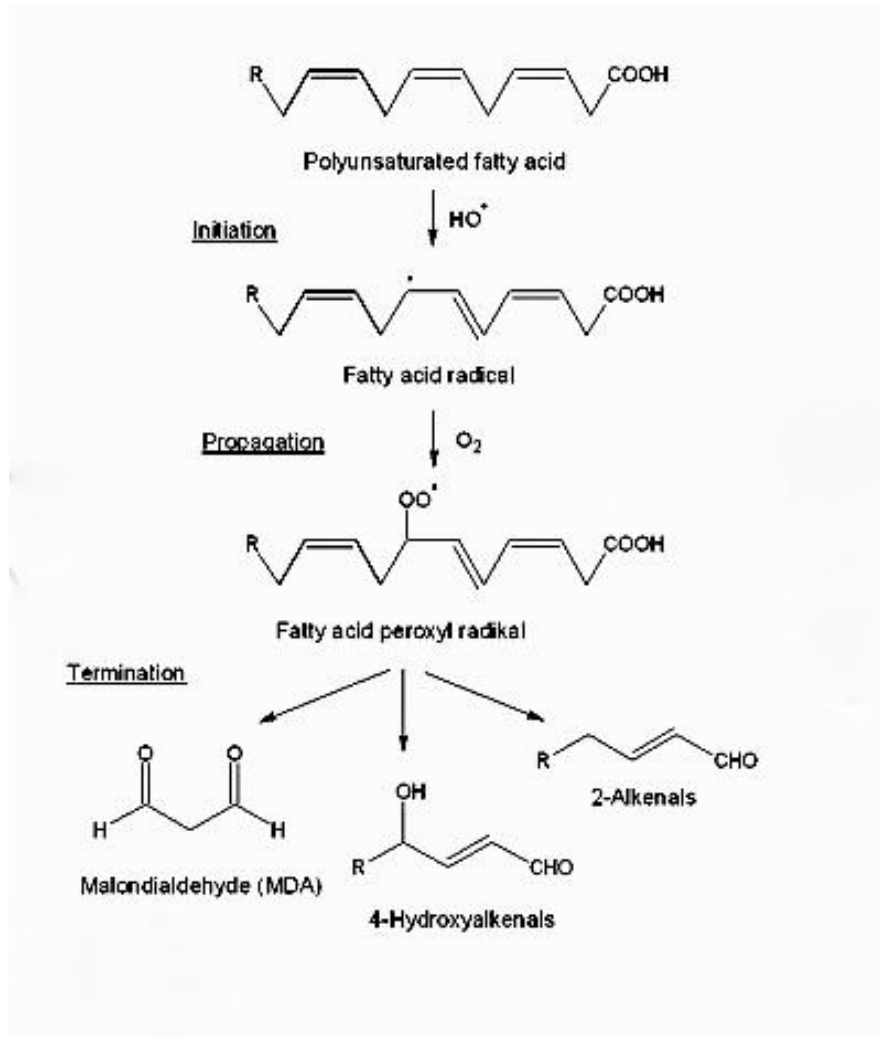
células neuronais são mais susceptíveis ao dano oxidativo, quando comparado com outros tecidos corporais [45].

### 1.3 Peroxidação lipídica e danos oxidativos

É conhecido que os PUFAs formam facilmente peróxidos na presença de oxigênio, o que induz a produção de RL, que atacam os fosfolípidos de membrana e iniciam o processo de peroxidação lipídica. Quando os radicais livres reagem com esses ácidos graxos insaturados modificam os lipídeos e a membrana celular perde suas características arquitetônicas, tornando-se menos fluída, criando-se verdadeiras fendas iônicas que alteram sua permeabilidade seletiva, o que favorece a entrada e saída indiscriminada de metabólitos e detritos da célula, provocando sua ruptura e lise com necrose [46].

A peroxidação lipídica mediada por EROs segue um mecanismo em cadeia, isto é, um aumento de radicais livres pode oxidar ambas as moléculas de lipídios em membranas biológicas e lipoproteínas de baixa densidade. As reações primárias de peroxidação lipídica incluem a retirada do átomo de hidrogênio por espécies reativas ( $\text{OH}^\bullet$  por exemplo) formando radicais lipídicos, etapa denominada iniciação; a reação desses radicais lipídicos com oxigênio forma radicais lipídicos peroxil, que é capaz de reagir com um lipídio estável de membrana, essa é a etapa de propagação; e, por fim, quando os intermediários formados reagem entre si formando produtos estáveis é a etapa de terminação (peroxil-peroxil, por exemplo). A peroxidação lipídica pode causar mudanças na organização de membrana, e perda funcional por modificação de proteínas e de DNA [47,48].

A peroxidação lipídica pode ser catalisada por íons ferro, por conversão de hidroperóxidos lipídicos (LOOH) em radicais altamente reativos (alcoxila,  $\text{LO}^\bullet$  e peroxila,  $\text{LOO}^\bullet$ ), que por sua vez, iniciam nova cadeia de reações, denominada ramificação [49]. O radical  $\text{OH}^\bullet$  é frequentemente reconhecido como a espécie iniciadora e a mais importante da peroxidação lipídica [50]. Entretanto, estudos recentes indicam que o ferro também desempenha papel determinante na iniciação deste processo, sendo necessária uma relação equimolar  $\text{Fe}^{+++} : \text{Fe}^{++}$  no meio, para que ocorra a peroxidação lipídica [51,52].



**Figura 3** - Etapas da peroxidação lipídica [53]

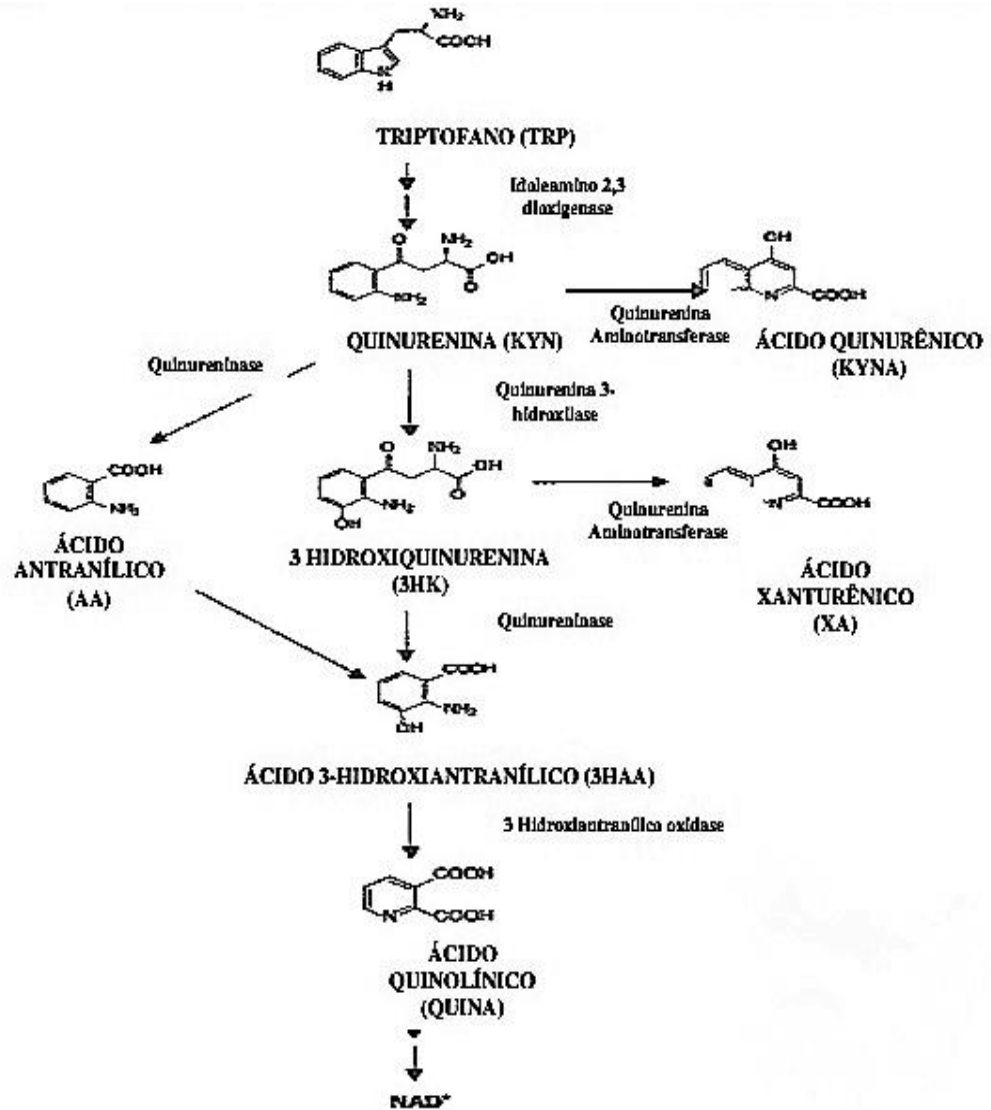
Reações envolvendo os vários intermediários entre si levam a novos produtos, por exemplo, MDA reage com o grupo amina de purinas, e o hidroxinonenal reage com guanosina, entre outras reações. Estudos que visam investigar o envolvimento da peroxidação lipídica podem ser feitos utilizando uma técnica de determinação indireta das substâncias reativas ao ácido tiobarbitúrico, como o MDA, que forma um produto de cor rosa que pode ser quantificado espectrofotometricamente [54].

Na literatura, é possível encontrar vários estudos onde agentes oxidantes específicos são usados em modelos de neurotoxicidade para investigar o efeito de agentes antioxidantes sobre a peroxidação lipídica. Em nosso estudo, utilizamos o ácido quinolínico (AQ), um agonista de receptores NMDA de glutamato e o nitroprussiato de sódio (NPS), um doador de íons  $\text{NO}^\bullet$ .

## 1.4 Agentes oxidantes

### 1.4.1 Ácido quinolínico

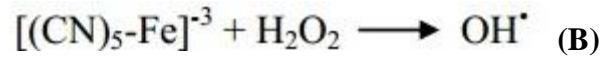
O ácido quinolínico (2, 3-piridina dicarboxílico, AQ) é um metabolito endógeno e neuroativo da via das quinureninas responsável pelo metabolismo do triptofano (Figura 4) [55]. O AQ é capaz de produzir um padrão de alterações neuroquímicas e neurodegenerativas pelo seu efeito agonista em receptores NMDA de glutamato, levando ao aumento da concentração de cálcio intracelular e a depleção de ATP, disfunção mitocondrial, estresse oxidativo e dano celular. O glutamato é o principal neurotransmissor excitatório do sistema nervoso central (SNC) e quando encontrado em altas concentrações estabelece um estado de excitotoxicidade, que por sua vez está envolvido na fisiopatologia de várias doenças neurodegenerativas como doença de Parkinson, doença de Alzheimer e doença de Huntington [56]. Além da atividade agonista em receptores NMDA, o AQ também modula o transporte de glutamato sendo capaz de inibir a captação de glutamato em cultura de astrócitos [57]. Em relação à produção de EROs, sabe-se que é capaz de formar complexos com o Ferro (II), gerando especialmente a produção de radicais  $\text{OH}^{\bullet}$ , molécula responsável pelo processo de iniciação da peroxidação lipídica e também quebra da cadeia de DNA[58]. Em estudos *in vitro*, a capacidade de induzir peroxidação lipídica depende da formação de complexos com o Fe (II) , já que homogenatos tratados com potentes queladores como a deferoxamina na concentração de  $10\mu\text{M}$ , o quinolinato é incapaz de produzir a peroxidação lipídica [59]. Assim conclui-se que em homogenatos de cérebro, o AQ não age diretamente na formação do dano lipídico, mas sim através da modulação da peroxidação lipídica através da interação com o Fe (II)[59].



**Figura 4** – Esquema adaptado da via das Kinureninas, metabolismo do triptofano e formação de ácido quinolínico [60].

#### 1.4.2 Nitroprussiato de Sódio

O Nitroprussiato de Sódio ( $[\text{NO-Fe}(\text{CN})_5]$ ) é um complexo coordenado de íons  $\text{Fe}^{++}$ , cinco íons cianeto e um cátion  $\text{NO}^+$ , o qual em meio aquoso é capaz de liberar  $\text{NO}^{\bullet}$  [61]. O  $\text{NO}^{\bullet}$  por sua vez pode reagir com o ânion  $\text{O}_2^{\bullet-}$  formando  $\text{ONOO}^-$  [62]. O NPS também é capaz de gerar o ânion ferricianeto ( $[(\text{CN})_5\text{-Fe}^{-3}]$ ), que ao reagir com  $\text{H}_2\text{O}_2$  e, via reação de Fenton, leva à formação de radicais  $\text{OH}^{\bullet}$  (Figura 5).



**Figura 5** – Formação de peroxinitrito (A) e formação de radical hidroxila a partir de NPS [13].

Por fim, estudos tem relacionado o óxido nítrico a fisiopatologia de severas patologias neurológicas como trauma, epilepsia, doença de Parkinson e doença de Alzheimer [63]. Assim como ocorre com o AQ, existem vários trabalhos demonstrando o potencial antioxidante de extratos de plantas medicinais em proteger o cérebro contra o dano oxidativo induzido com NPS [34,64,65,66].

### 1.5 *Centella asiática*

As plantas medicinais e outros compostos naturais têm sido amplamente estudados com a perspectiva de utilização como tratamento adjuvante em patologias que envolvem danos oxidativos. Nos últimos anos, é crescente o interesse dos grupos de pesquisa em estudar essas plantas usadas na medicina popular devido ao acúmulo de evidências destacando o grande potencial terapêutico de plantas medicinais como a *Centella asiática* [67].

A *Centella asiática* é uma planta perene psicoativa pertencente à família *Apiaceae* que tem sido utilizado por vários anos na medicina Ayurvédica indiana como um antioxidante e anti-inflamatório [68]. No século XIX, seus extratos foram incorporados a farmacopéia indiana, sendo recomendada para cicatrização e inúmeros problemas de pele como hanseníase, lúpus, úlceras varicosas, eczema, psoríase e também diarreia, febre, amenorreia e doenças do trato urinário feminino [69]. É encontrada em toda a Índia, cresce em lugares úmidos até uma altitude de 1800 metros e é também encontrada em todos os países tropicais e subtropicais, onde cresce em áreas pantanosas. Não possui cheiro nem gosto característico, vive em torno da água, tem forma de leque com folhas verdes pequenas com flores com tons roxos até o rosa ou brancas e tem uma pequena fruta oval [70] (Figura 6). Todas as partes da

planta são usadas para fins medicinais, acreditando-se na cultura Ayurvédica que a *C. asiática* teria poder de purificar o sangue, tratar pressão arterial, melhorar a memória e promover a longevidade. Já na medicina ocidental, os extratos alcoólicos da planta foram amplamente usados no século XX como tratamento da pele em pacientes com Lepra [69]. No Brasil é conhecida com os nomes populares de Cairuçu-asiático, Centella, codagem e pata-de-mula. A importância medicinal deste extrato está associada ao seu conteúdo bioativo de triterpenos: ácido asiático, asiaticosídeo, ácido madecássico, e madecassosídeo [71].

Os efeitos dos extratos de *C. asiática* no SNC são relatados em vários estudos e em diferentes modelos experimentais. Entre as ações benéficas está a modulação da depressão atuando nos níveis de monoaminas e corticosterona em cérebro de camundongos e resultados promissores em testes de nado forçado e tempo de imobilidade [72]. Também foi observado a melhora na resposta a exposição ao pentilenotetrazol (PTZ) e estímulo elétrico em modelo de convulsão, acredita-se que a capacidade de aumento nos níveis de GABA explique esse efeito de *C. asiática* [73]. Neste contexto, sugerem que um estudo dos mecanismos neuroprotetores de *C. asiática* mostrou um aumento da fosforilação de AMP cíclico, o qual foi observado em cultura de células de neuroblastoma expressando placas  $\beta$ -amilóide e em células embrionárias de rato, sugerindo um mecanismo para a melhora da memória [74].

Adicionalmente, estudos anteriores demonstraram que a *C. asiática* protege significativamente o cérebro dos efeitos neurotóxicos do 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) em modelo de parkinsonismo em ratos [75] e melhora déficits de memória em modelo usando D-galactose em camundongos [76]. Também se sabe que os componentes ativos de *C. asiática* são capazes de causar melhora no dano isquêmico em ratos [77] e também no dano oxidativo induzido com  $H_2O_2$  e rotenona em cultura de células SH-SY5Y de neuroblastoma [78]. Os triterpenos madecassosídeo e asiaticosídeo juntos em extrato padronizado possuem efeito ansiolítico em modelo de estresse por imobilização em ratos [79], enquanto que o asiaticosídeo isolado tem valor terapêutico em modelo de neurotoxicidade na formação de placas  $\beta$ -amilóide [80]. O madecassosídeo, além de proteger contra o dano isquêmico, também é capaz de produzir efeitos benéficos em modelo de doença de Parkinson induzido com MPTP [81,82].

O potencial antioxidante de *C. asiática* também é bem relatado na literatura, o tratamento por via oral com 50 mg/kg/dia com o extrato bruto metanólico de *C. asiática* durante 14 dias aumenta significativamente a atividade das enzimas antioxidantes, como a superóxido dismutase (SOD), catalase e glutatona peroxidase (GPx) em camundongos portadores de linfoma [83].



**Figura 6** – *Centella asiática*.

Fonte: <http://earthmedicineinstitute.com/more/library/medicinal-plants/centella-asiatica/>

Entretanto, os mecanismos envolvidos nesses efeitos ainda não estão bem esclarecidos e a participação dos compostos fenólicos e outros componentes presentes nos diferentes extratos da planta podem ser essenciais para os efeitos medicinais da planta. Em nosso estudo nós analisamos a presença dos compostos fenólicos em quatro diferentes extratos de *C.asiática* (infusão, fração *n*-butanólica, fração acetato de etila e fração diclorometano) e analisamos o potencial antioxidante desses extratos como *scavenger* do radical DPPH<sup>•</sup>, na redução de molibdênio (MO<sup>6+</sup> a Mo<sup>5+</sup>) e por fim a ação sobre a peroxidação lipídica induzida com dois agentes oxidantes diferentes (AQ e SNP) em três diferentes regiões do cérebro de rato (córtex, estriado e hipocampo). Assim demonstrando as possíveis diferenças em relação a produção de EROs/ERNs por mecanismos diferentes em diferentes regiões do cérebro relacionadas com as principais patologias neuronais e também a relação entre as diferentes extrações e diferentes componentes da planta envolvidos.



## 2 OBJETIVOS

### 2.1 Objetivo Geral

O objetivo geral do presente estudo foi avaliar o potencial antioxidante de diferentes extratos de *C. asiática in vitro*.

### 2.2 Objetivos Específicos

Considerando os aspectos já mencionados, os objetivos específicos deste trabalho compreendem:

- Determinar o perfil fitoquímico da infusão e das frações *n*-butanol, acetato de etila e diclorometano de *C. asiática*;
- Avaliar o potencial antioxidante da infusão e das frações *n*-butanol, acetato de etila e diclorometano de *C. asiática*;
- Quantificar os compostos fenólicos na infusão e nas frações de *C. asiática*;
- Investigar a capacidade *scavenger* de radical DPPH<sup>•</sup> da infusão e das frações *n*-butanol, acetato de etila e diclorometano;
- Analisar o efeito das frações de *C. asiática* sobre a peroxidação lipídica induzida com AQ em córtex, estriado e hipocampo de ratos;
- Analisar o efeito das frações de *C. asiática* sobre a peroxidação lipídica induzida com NPS em córtex, estriado e hipocampo de ratos.

### **3 RESULTADOS**

Os resultados que fazem parte dessa dissertação estão apresentados na forma de artigo científico. Os itens Material e Métodos, Resultados, Discussão e Referências encontram-se no artigo que está disposto de acordo com as recomendações do periódico *Neurochemical Research*, ao qual será submetido.

### 3.1 Artigo Científico

*Centella asiática* e suas frações reduz a peroxidação lipídica induzida por ácido quinolínico e nitroprussiato de sódio em regiões do cérebro de ratos

*Centella asiatica* AND ITS FRACTIONS REDUCES LIPID PEROXIDATION INDUCED BY QUINOLINIC ACID AND SODIUM NITROPRUSSIDE IN RATS BRAIN REGIONS

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***Centella asiatica* AND ITS FRACTIONS REDUCES LIPID PEROXIDATION INDUCED BY QUINOLINIC ACID AND SODIUM NITROPRUSSIDE IN RATS BRAIN REGIONS**

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## ABSTRACT

Oxidative stress has been implicated in many pathologies including neurological disturbance. *Centella asiatica* is a popular medicinal plant which has long been used to treat neurological disturbances in Ayurvedic medicine. In the present study, we quantified of compounds by HPLC-DAD and examined the phenolic content of infusion, ethyl acetate, *n*-butanolic and dichlorometane fractions. Furthermore, we analyzed the ability of the extracts from *C. asiatica* to scavenge the DPPH radical as well as total activity antioxidant through the reduction of molybdenum (VI) ( $\text{Mo}^{6+}$ ) to molybdenum (V) ( $\text{Mo}^{5+}$ ). Finally, we examined the antioxidant effect of extracts on different oxidant agents, quinolinic acid (QA) and sodium nitroprusside (SNP), in brain regions (cerebral cortex, striatum and hippocampus). The HPLC analysis revealed that flavonoids, triterpene glycoside, tannins, phenolics acids were present in the extracts of *C. asiatica* and also the phenolic content assay demonstrated that ethyl acetate fraction is rich in this compounds. Besides, the ethyl acetate fraction presented the highest antioxidant effect by decreasing the lipid peroxidation in brain regions induced by QA. By the other hand, when the pro-oxidant agent was SNP, the potency of infusion, ethyl acetate and dichlorometane fractions was equivalent. Thus, the therapeutic potential of *C. asiatica* in neurological diseases could be associated to its antioxidant activity.

**Keywords:** *Centella asiatica*, antioxidant, quinolinic acid, sodium nitroprusside, lipid peroxidation

## INTRODUCTION

Reactive species (RS) are formed in cellular conditions by several mechanisms including autoxidation of unstable biomolecules such as dopamine and activation of neutrophils or nitric oxide synthases whose produce nitric oxide [1]. Oxygen metabolism also leads to the production of small quantities of reactive oxygen species (ROS), such as superoxide radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $OH^\cdot$ ) [2]. In physiological conditions, the production of free radicals and other reactive species are kept in an equilibrium state by antioxidant defence system [3]. These species are maintained at low, but measurable, concentrations in the cells, through a balance between their rates of production removal by antioxidants [4].

Oxidative stress occurs when cellular antioxidant defense mechanisms fail to counterbalance and control the endogenous production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [5, 6]. Therefore, it has been related to numerous pathologies where ROS production can contribute to worsen the symptoms by causing alterations in the cell membrane (lipid peroxidation and protein oxidation) and DNA mutations [4,7]. In the mid-1950s, Denham Harman articulated a 'free-radical theory' of ageing, speculating that endogenous oxygen radicals were generated in cells and resulted in a pattern of cumulative damage. Particularly, oxidative stress has been implicated as a major cause of cellular injuries in the central nervous system [8], as Alzheimer's disease [9], cognitive deficits [10], Parkinson's disease [11], and Huntington's disease [12].

In this context, different neurotoxic agents have been used to induce oxidative stress *in vitro* [13, 14,15,16] and *in vivo* [17,18,19] models, such as quinolinic acid (QA) and sodium nitroprusside (SNP). QA is a major metabolite of the kynurenine pathway of tryptophan metabolism [20] and is an endogenous glutamate agonist with relative selectivity to N-methyl-D-aspartate (NMDA) receptor [21]. The activation of NMDA receptor leads to an increase in intracellular  $Ca^{2+}$  concentration, leading to ATP depletion, mitochondrial dysfunction, oxidative stress and cell damage [22]. Sodium nitroprusside (SNP) is a nitric oxide donor, which in turn is a reactive nitrogen species that reacts with oxygen to form other reactive species in aqueous medium [23] contributing to oxidative stress.

Medicinal plants and other natural compounds have been largely studied as alternative or adjuvant in the treatment of pathologies involving oxidative damage. *Centella asiatica* is a psychoactive therapeutic plant belonging to the family Apiaceae that has been used for several

years in Indian Ayurvedic medicine with antioxidant and antiinflammatory properties [24]. Previous reports demonstrated that *C. asiatica* significantly protected the brain from neurotoxic effects of MPTP [25] and avoid memory deficits against D-galactose-induced senescence in mice [26]. In addition, different bioactive component of *C. asiatica* improves neurological damages such as asiatic acid, asiaticoside, madecassoside and madecassic acid, all these triterpenes have effective mechanisms against free radical generation. Several studies have showed that asiatic acid ameliorates ischemic damage [27] and H<sub>2</sub>O<sub>2</sub>-induced injury in SH-SY5Y cells [24]; anxiolytic effects of standardized extract compound of madecassoside and asiaticoside against chronic immobilization stress [28]; asiaticoside has been found to have therapeutic value against  $\beta$ -amyloid neurotoxicity [29]; madecassoside has neuroprotective effect on focal reperfusion ischemic injury and early stage of Parkinson's disease induced by MPTP in rats [30, 31]. However, the mechanisms involved in pharmacological properties of *C. asiatica* are not well understood.

Thus, the aim of the present study was compare different extracts of *C. asiatica* against oxidative damage induced by QA and SNP in brain regions (cerebral cortex, striatum and hippocampus) verifying the importance of the composition of the extracts to *C. asiatica* action in specific brain structure.

## **MATERIAL AND METHODS**

### *Animals*

Male adult rats ( $\pm 2$  months old), weighing 280–320g, were obtained from a local breeding colony (Animal House, UFSM, Brazil). The animals were kept in a room, with free access to food and water, on a 12 h light/dark cycle, controlled temperature ( $22 \pm 2^\circ\text{C}$ ). The animals were used according to the guidelines of the National Council to Control of Animal Experimentation, Brazil.

### *Chemicals reagents*

Thiobarbituric acid (TBA) was purchased from Merck (Brazil). Quinolinic acid (QA; 2, 3-pyridine dicarboxylic acid), Tris-HCl, malonaldehydebis-(dimethyl acetal) (MDA), Folin and Ciocalteu's phenol reagent, sodium nitroprusside, madecassoside, quercetin, quercitrin, rutin, catechin, epicatechin and kaempferol were obtained from Sigma (St. Louis, MO, USA). Acetonitrile, formic acid, gallic acid, chlorogenic acid, rosmarinic acid and caffeic acid purchased from Merck (Darmstadt, Germany). The extract of *C.asiatica* was obtained from Pharma Nostra Comercial (Anápolis, GO, Brazil).

### *Preparation of infusion and fractions*

The infusion of *C.asiatica* was prepared by dissolving 2 mg/mL of powder in boiling distilled water which was filtered after 10 minutes. To obtain the different fractions, the aqueous extract was evaporated under reduced pressure to remove the water. Aqueous extract was then re-suspended in water and partitioned successively with dichloromethane, ethyl acetate and n-butanol (3 x 200 ml for each solvent) [32].

### *Quantification of compounds by HPLC-DAD*

Chromatographic separation was performed with a reversed phase using C<sub>18</sub> column (4.6 mm x 250 mm) packed with 5 µm diameter particles. Mobile phase was water containing 1% formic acid (A) and acetonitrile (B), and the composition gradient was: 20% of B until 0 min and changed to obtain 35%, 45%, 65%, 80%, 90% and 20% of B at 15, 30, 35, 40, 45 and 60 min, respectively [33] with slight modifications. *C. asiatica* infusion, dichloromethane fraction, ethyl acetate fraction and butanolic fraction were analyzed dissolved in water at a concentration of 10 mg/mL. The presence of the following compounds was investigated, gallic acid, chlorogenic acid, caffeic acid, rosmarinic acid, madecassoside, catechin, epicatechin, quercetin, quercitrin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of



the commercial standards. The flow rate was 0.7 ml/min, injection volume 50 $\mu$ l and the wavelength were 206 nm for madecassoside, 254 nm for gallic acid, 280 nm for catechin and epicatechin, 327 nm for caffeic, rosmarinic and chlorogenic acids, and 366 nm for quercetin, quercitrin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45  $\mu$ m membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030 – 0.250 mg/ml for kaempferol, quercetin, quercitrin, rutin, catechin, epicatechin and madecassoside; and 0.030 – 0.250 mg/ml for gallic, caffeic, rosmarinic and chlorogenic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 600 nm). Calibration curve for gallic acid:  $Y = 13629x + 1195.8$  ( $r = 0.9993$ ); catechin:  $Y = 12407x + 1259.6$  ( $r = 0.9997$ ); epicatechin:  $Y = 12547x + 1193.4$  ( $r = 0.9991$ ); caffeic acid:  $Y = 11758x + 1359.2$  ( $r = 0.9996$ ); chlorogenic acid:  $Y = 14061x + 1325.3$  ( $r = 0.9995$ ); rosmarinic acid:  $Y = 12658x + 1195.3$  ( $r = 0.9998$ ); madecassoside:  $Y = 13628x + 1273.8$  ( $r = 0.9995$ ); rutin:  $Y = 12845x + 1065.7$  ( $r = 0.9999$ ); quercetin:  $Y = 13560x + 1192.6$  ( $r = 0.9991$ ), quercitrin:  $Y = 13719x + 1256.7$  ( $r = 0.9993$ ) and kaempferol:  $Y = 14253x + 1238.9$  ( $r = 0.9997$ ). All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10  $\sigma/S$ , respectively, where  $\sigma$  is the standard deviation of the response and S is the slope of the calibration curve [34].

#### *Determination of total phenolic compounds*

For the total phenolic determination, the extracts were mixed with 1.25 ml of 10 % Folin-Ciocalteu's reagent (v/v), which was followed by the addition of 1.0 ml of 7.5 % sodium carbonate ( $\text{NaCO}_3$ ) as previously described [35]. The reaction mixture was incubated at 45°C for 15 min, and the absorbance was spectrophotometrically measured at 765 nm. Galic acid (GA) was used as standard for phenolic compounds and results are showed as GA equivalent (GAE).

### *DPPH radical scavenging method*

The radical scavenging activity of the compounds was determined as previously described [36]. Each extract was tested at concentrations of 10, 20, 50, 100 and 150 µg/mL. gallic acid was used as a control. DPPH<sup>·</sup> (dilluted in ethanol) was added to final concentration of 0.15 mM and allowed to react at room temperature during 30 min in dark conditions. The absorbance was spectrophotometrically measured at 518 nm.

### *Spectrophotometric quantitation of antioxidant capacity*

A sample solution aliquot of *C. asiatica* infusion and fractions in water was combined in a vial with reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH [37]. The extracts were tested at concentrations of 10, 20, 50, 100 and 150 µg/mL. Thus, they were capped and incubated in a water bath at 95°C for 90 min and the absorbance was measured at 695 nm against a blank control. Gallic acid was used as a positive control.

### *Preparation of brain homogenates*

Animals were sacrificed by decaptation on the day of the experiments. The encephalic tissue (whole brain) was quickly removed and placed on ice-cold. Striatum, hippocampus and cortex were removed, weighed and immediately homogenized in Tris-HCl 10 mM (1:10), pH=7.4. The homogenate was centrifuged for 10 min at 3,000 x g to yield a pellet, which was discarded, and the low speed supernatant was used for *in vitro* analysis.

### *Prepare solutions*

QA or SNP solutions were prepared to obtain final concentrations of 1 mM [14] and 5  $\mu$ M [35,38] respectively. QA (Sigma) was dissolved in 0.1 M phosphate buffer (pH 7.4) and neutralized with 1 N NaOH for the solutions stock. SNP was dissolved in distilled water just before the experiment. Oxidizing agents were added to the reaction just before the pre-incubation.

### *Lipid peroxidation induced by QA or SNP*

The potential to prevent lipid peroxidation *in vitro* by *C. asiatica* was determined by thiobarbituric acid reactive substances (TBARS) production according to the method described earlier [39]. Analyses were performed in brain structures by mixing 200 $\mu$ L of S1 for 1h at 37°C, with pro-oxidant agents (QA 1 mM or SNP 5  $\mu$ M) in the presence or absence of different concentrations of *C.asiatica*. TBARS formation was determined spectrophotometrically at 532 nm, using malondialdehyde (MDA) as standard.

### *Statistical analysis*

The results were statistically analyzed by one-way ANOVA followed by a post hoc test when appropriate. The results were considered statistically significant when  $p < 0.05$ .

## RESULTS

### *HPLC analysis*

HPLC fingerprinting of *C.asiatica* infusion and fractions revealed the presence of the gallic acid ( $t_R = 6.61$  min; peak 1), catechin ( $t_R = 9.18$  min; peak 2); madecassoside ( $t_R = 11.45$  min; peak 3), chlorogenic acid ( $t_R = 14.86$  min; peak 4), caffeic acid ( $t_R = 18.03$  min; peak 5), rosmarinic acid ( $t_R = 21.34$  min; peak 6), epicatechin ( $t_R = 24.57$  min; peak 7), rutin ( $t_R = 32.15$  min; peak 8), quercitrin ( $t_R = 36.20$  min; peak 9), quercetin ( $t_R = 37.12$  min; peak 10) and kaempferol ( $t_R = 43.79$  min; peak 11) (Figure 1 and Table 1). The HPLC analysis revealed that flavonoids (quercetin, quercitrin, rutin and kaempferol), triterpene glycoside (madecassoside), tannins (catechin and epicatechin) and phenolics acids (gallic, chlorogenic, rosmarinic and caffeic acids) are present in the extract of *C. asiatica*.

The ethyl acetate fraction displayed the highest percentage of the main constituents identified in comparison to other fractions, *n*-butanol fraction and infusion showed high presence of rutin and quercetin respectively. The highest percentage of madecassoside was found in dichloromethane fraction while in butanolic fraction it was not found (Table 1).

### *Total phenolic compounds of Centella asiatica infusion and fractions*

The quantification of phenolic compounds showed that ethyl acetate (EA) fraction of *C.asiatica* presented the highest quantity of GAE/mg of extract followed by dichloromethane fraction > *n*-butanol fraction > infusion extract (Table 2). Phenolic compounds are usually presented in higher polar fractions [40] as ethyl acetate fraction. These results corroborate with HPLC analysis of the ethyl acetate fraction.

### *DPPH<sup>·</sup> assay*

The ability of *C. asiatica* extracts in quenching the stable free radical DPPH<sup>·</sup> was showed in control (GA) at concentrations of 10, 50, 100 and 150µg/mL (Figure 2). The inhibitory potency (IC<sub>50</sub>) of DPPH radical by different extracts of *C.asiatica*, at same concentrations of control, was in the following order: ethyl acetate > dichloromethane > infusion (Table 3, p<0.05). By the other hand, the *n*-butanolic fraction and infusion did not show statistical difference in DPPH<sup>·</sup> scavenger assay.

### *Total antioxidant activity*

The curve of GA was determined using the concentrations of 5, 10, 20 and 50µg/mL represented in Figure 3 as the letters a, b, c and d. All concentrations of *n*-butanolic and dichlorometane fraction showed total antioxidant activity similar to GA at 5 µg/mL. Infusion also presented total antioxidant activity similar to GA at 5 µg/mL at 10, 20, 50 and 100µg/mL. In addition, the total antioxidant activity of the infusion at 150 µg/mL, ethyl acetate at 20-50 µg/mL, *n*-butanolic at 50-150 µg/mL and dichloromethane at 50-150 µg/mL were similar to GA at 10 µg/mL. Furthermore the total antioxidant activity of the ethyl acetate fraction at 100 and 150µg/mL were similar to control at 20 and 50µg/mL, respectively.

### *Centella asiatica on lipid peroxidation*

QA increased lipid peroxidation when compared with basal conditions (p<0.0001), as well as SNP (p<0.0001) in brain regions. Infusion (Figure 4) of *C. asiatica* or its fractions (butanolic, Figure 5; ethyl acetate, Figure 6 or dichloromethane, Figure 7) significantly inhibited QA(A,B,C) –or SNP (D,E,F)- induced TBARS formation in cortex, striatum and hippocampus homogenates. However, the inhibitory potency varied according to brain region, extract preparation and oxidant agent used which are demonstrated in Table 4.

The order of inhibitory potency (Table 4) obtained according with the extract from *C.asiatica* and brain region used was: in the cerebral cortex, striatum and hippocampus to QA-induced TBARS: ethyl acetate > *n*-butanolic > dichloromethane > infusion; for SNP-induced in the cerebral cortex was ethyl acetate > dichloromethane > infusion > *n*-butanolic, in the striatum was dichloromethane > ethyl acetate > infusion > *n*-butanolic and in the hippocampus was infusion > dichloromethane > ethyl acetate > *n*-butanolic.

## DISCUSSION

The brain tissue is a target to oxidative damage due to its high  $\text{Ca}^{+2}$  trafficking across neuronal membranes [41,42], high oxygen demand, high content of unsaturated fatty acids, rapid oxidative metabolic activity, and little endogenous antioxidant potential and insufficient neuronal cell repair capacity comparatively to its necessity [43]. In the present study, we show a significant increase in TBARS production in cerebral cortex, striatum and hippocampus when exposed to QA and SNP *in vitro*. It was also demonstrated that infusion and different fractions of *C.asiatica* have antioxidant action and avoid lipid peroxidation induced by QA and SNP.

QA is a neurotoxic agent that can produce excitotoxicity and decrease cellular viability through free radical formation [44, 45] promoting an increase in extracellular glutamate levels that triggers oxidative stress via over-stimulation of NMDA receptors [46]. Furthermore, QA inhibits glutamate uptake in astrocytes [47]. However in *in vitro* studies, the ability to induce lipid peroxidation depends on the formation of complexes with Fe (II) [48]. In homogenates treated with potent chelators such as deferoxamine concentration of 10 $\mu\text{M}$ , the quinolinato is unable to produce lipid peroxidation. Recent studies showed the improvement of lipid peroxidation induced by QA with *V. officinalis* extract [49], red and white Ginger extracts [50] emphasizing beneficial effects of medicinal plants on neurotoxicity. These data are in agreement with our results since we show that *C.asiatica* extracts can reduce the increase in MDA levels in different brain structures induced by QA *in vitro*. Nevertheless, this protection is dependent of the oxidant agent used, antioxidant extract and brain structure since we observed that ethyl acetate fraction had the highest inhibitory potentials in all brain regions tested when it was used QA to induce lipid peroxidation. This effect is probably due to ethyl acetate constituents since this fraction has the highest quantity of phenolic content (Table 2)

in comparison to the other fractions which was confirmed by HPLC analysis that demonstrated higher quantities of flavonoids, tannins and phenolics acids when compared with infusion, *n*-butanolic and dichloromethane fraction (Table 1). This result is in agreement with previous studies whose have been demonstrating that the solvents used to obtain ethyl acetate fraction can extract a greater amount of antioxidant compounds [32, 51] particularly phenolic acids which are prominently present in the ethyl acetate fraction. Besides, literature data show that phenolic acids are able to decrease MDA levels and ROS production [52, 53, 54, 55].

About the mechanism of its pharmacological action, it is known that phenol and triterpenes present in the extract have high antioxidant potentials due to its capacity in chelated iron, preventing the formation of (OH $\cdot$ ) in Fenton reaction [56]. In addition, the antioxidant activities of flavonoids are believed to be associated with their chemical structure and the two hydroxyl groups in the catechol B-ring [57], these groups can act by allowing donation of hydrogen stabilizing radical species [58,59]. The triterpenes of *C.asiatica*, as madecassoside and asiaticoside, also have decrease MDA levels production in substantia nigra pars compacta in a model of Parkinson's disease induced by MPTP [31, 60]. Wanasuntronwong (2012) showed the importance of synergism between madecassoside and asiaticoside in ansiolytic effects and stimulation of glutamic acid decarboxylase (GAD), an enzyme involved in the synthesis of GABA and demonstrate similar activity of diazepam, an agonist of GABA A receptor [61]. These findings corroborate with our study and suggest possible mechanism for the action of *C.asiatica* extracts in lipid peroxidation induced by QA in brain besides the effect of reaction iron, this effect could be observed in future *in vivo* studies. In previous studies we found that agonists of GABA-ergic receptors (such as muscimol) reverts neuronal damage caused by activation of NMDA and cell death in hippocampal cells [61,62].

SNP exposure causes cytotoxicity via either release of cyanide and/or nitric oxide (NO $\cdot$ ) and rapidly releases NO $\cdot$  in tissue preparations, which in turn produces peroxynitrite (ONOO $-$ ) and superoxide anion radical (O $2^{\cdot-}$ ), thus leading to lipid peroxidation [17,64,65,66]. In cerebral cortex, ethyl acetate fraction had a highest potency in inhibiting TBARS induced by SNP. However, in striatum we observed that dichloromethane fraction had better ability to protect from TBARS formation while in hippocampus, the infusion of *C.asiatica* had the better activity. Besides literature data suggesting madecassoside as a determining fator to *C.asiatica* action *in vivo*[67] we were unable to demonstrate it since,

ethyl acetate fraction presented the better antioxidant activity in the majority of our experiments and the highest concentration of madecassoside was in dichloromethane extract.

Additionally, using a total antioxidant activity assay, we demonstrated that the ethyl acetate fraction presented a greater antioxidant activity when compared with other fractions, which is probably due to the presence of phenolic content (Table 2). Similarly, the effect of antioxidant on DPPH radical scavenging is involved with their capacity to donate a hydrogen atom, and in the present study, we also demonstrated that the ethyl acetate fraction has the higher capacity in to remove DPPH radical in comparison with the other fractions (Figure 2, Table 3).

## CONCLUSIONS

In conclusion, all extracts of *C.asiatica* tested in this study were able to prevent lipid peroxidation in brain induced by two well-known pro-oxidant agents, QA and SNP. Also, *C.asiatica* presented DPPH scavenger activity and reduced of molybdenum (VI) to molybdenum (V). In part, these effects can be related to their phenolic content, including the presence of flavonoids since ethyl acetate fraction presented the best antioxidant activities and the highest content of flavonoids, tannins and phenolics acids, when compared to infusion, *n*-butanolic and dichloromethane fractions. These results are interesting because this plant could be used as a potential agent for the prevention of various neurological diseases associated with oxidative damage. However, additional studies are necessary to investigate the exact mechanism responsible for protective effect of *C.asiatica* on lipid peroxidation, and, its effects on *in vivo* models of oxidative stress.



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## **CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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### 3.1.1 Legends of Figures

**Fig. 1** Representative high performance liquid chromatography profile of *Centella asiatica* infusion (a), dichloromethane fraction (b), ethyl acetate fraction (c) and butanolic fraction (d). Gallic acid (peak 1), catechin (peak 2), madecassoside (peak 3), chlorogenic acid (peak 4), caffeic acid (peak 5), rosmarinic acid (peak 6), epicatechin (peak 7), rutin (peak 8), quercitrin (peak 9), quercetin (peak 10) and kaempferol (peak 11).

**Table 1** Composition of *Centella asiatica* infusion, dichloromethane, ethyl acetate fraction and butanolic fractions.

**Table 2** Total phenolic compounds of *Centella asiatica* infusion and fractions.

**Fig. 2** Effects of different concentrations of infusion, ethyl acetate, *n*-butanolic and dichloromethane fractions from *Centella asiatica* on DPPH<sup>•</sup> assay. The results are expressed as percentage of inhibition and GA was used as a positive control. Data show means  $\pm$  SEM values averages from 3 independent experiments performed in duplicate.

**Table 3** IC<sub>50</sub> ( $\mu$ g/ml) values of extracts from *Centella asiatica* obtained on DPPH<sup>•</sup> assay.

**Fig. 3** Total antioxidant activity of the extracts from *Centella asiatica* were measured by the phosphomolybdenum assay. Bars marked with letters differentes (a-d) represent the effect of control and gallic acid and equivalent concentrations of the same *C.asiatica*. Data are expressed as absorbance means  $\pm$  SEM of 3 individual experiments performed in duplicate. Means were compared by Bonferroni's test at  $p < 0.05$ .

**Fig. 4** Effect of infusion from *Centella asiatica* on lipid peroxidation induced by QA (1mM) (A, B and C) or SNP (5  $\mu$ M) (D, E and F) in cerebral cortex (A, D), striatum (B, E) and hippocampus (C, F) of rats. Data are expressed as mean  $\pm$  SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when  $p < 0.05$ . Significant differences are marked as (\*)  $p < 0.05$  or (\*\*)  $p < 0.0001$  when compared to QA or SNP group and marked as (<sup>#</sup>)  $p < 0.05$  or (<sup>##</sup>)  $p < 0.0001$  when compared to control group.

**Fig. 5** Effect of ethyl acetate fraction from *Centella asiatica* on lipid peroxidation induced by QA (1mM) (A, B and C) or SNP (5  $\mu$ M) (D, E and F) lipid peroxidation in cerebral cortex (A, D), striatum (B, E) and hippocampus (C, F) of rats. Data are expressed as mean  $\pm$  SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when  $p < 0.05$ . Significant differences are marked as (\*)  $p < 0.05$  or (\*\*)  $p < 0.0001$  when compared to QA or SNP group and marked as (<sup>#</sup>)  $p < 0.05$  or (<sup>##</sup>)  $p < 0.0001$  when compared to control group.

**Fig. 6** Effect of *n*-butanolic fraction from *Centella asiatica* on lipid peroxidation induced by QA (1mM) (A, B and C) and SNP (5  $\mu$ M) ( D, E and F) lipid peroxidation in cerebral cortex (A, D), striatum (B, E) and hippocampus (C, F) of rats. Data are expressed as mean  $\pm$  SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when  $p < 0.05$ . Significant differences are marked as (\*)  $p < 0.05$  or (\*\*)  $p < 0.0001$  when compared to QA or SNP group and marked as (<sup>#</sup>)  $p < 0.05$  or (<sup>##</sup>)  $p < 0.0001$  when compared to control group.

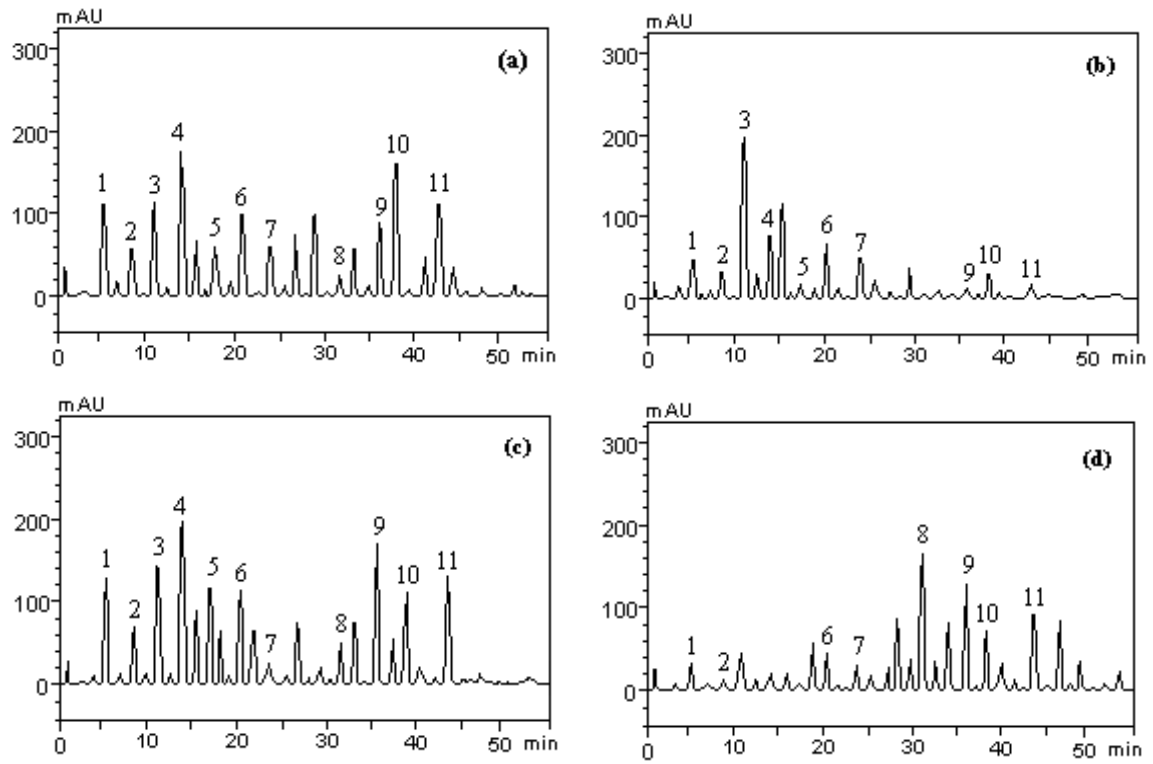
**Fig. 7** Effect of dichlorometane fraction from *Centella asiatica* on lipid peroxidation induced by QA (1mM) (A, B and C) or SNP (5  $\mu$ M) ( D, E and F) lipid peroxidation in cerebral cortex (A, D), striatum (B, E) and hippocampus (C, F) of rats. Data are expressed as mean  $\pm$  SEM (n = 3) and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated. Differences were considered significant when  $p < 0.05$ . Significant differences are marked as

(\*)  $p < 0.05$  or (\*\*)  $p < 0.0001$  when compared to QA or SNP group and marked as (<sup>#</sup>)  $p < 0.05$  or (<sup>##</sup>)  $p < 0.0001$  when compared to control group.

**Table 4** IC<sub>50</sub> ( $\mu\text{g/mL}$ ) values for infusion, ethyl acetate, *n*-butanolic and dichloromethane fractions from *Centella asiatica* of TBARS production induced by QA (1 mM) and SNP (5  $\mu\text{M}$ ) in rat brain regions homogenate.

### 3.1.2 Figures

**Figure 1**



**Table 1**

Compounds	Infusion	Dichloromethane	Ethyl acetate	<i>n</i> -butanolic	LOD	LOQ
	mg/g	mg/g	mg/g	mg/g	µg/mL	µg/mL
Gallic acid	6.91 ± 0.02 <sup>a</sup>	2.89 ± 0.01 <sup>a</sup>	7.39 ± 0.01 <sup>a</sup>	1.46 ± 0.03 <sup>a</sup>	0.024	0.079
Catechin	4.07 ± 0.03 <sup>b</sup>	1.76 ± 0.02 <sup>b</sup>	4.16 ± 0.01 <sup>b</sup>	0.61 ± 0.01 <sup>b</sup>	0.007	0.023
Madecassoside	7.15 ± 0.03 <sup>a</sup>	14.07 ± 0.01 <sup>c</sup>	9.03 ± 0.03 <sup>c</sup>	-	0.032	0.105
Chlorogenic acid	11.83 ± 0.01 <sup>c</sup>	6.51 ± 0.02 <sup>d</sup>	15.14 ± 0.01 <sup>d</sup>	-	0.013	0.042
Caffeic acid	4.13 ± 0.02 <sup>b</sup>	0.81 ± 0.01 <sup>e</sup>	7.27 ± 0.03 <sup>a</sup>	-	0.035	0.115
Rosmarinic acid	6.28 ± 0.01 <sup>ad</sup>	4.19 ± 0.01 <sup>f</sup>	7.08 ± 0.01 <sup>a</sup>	2.79 ± 0.02 <sup>c</sup>	0.026	0.083
Epicatechin	4.27 ± 0.01 <sup>b</sup>	2.90 ± 0.03 <sup>a</sup>	1.39 ± 0.01 <sup>e</sup>	1.52 ± 0.01 <sup>a</sup>	0.021	0.070
Rutin	1.64 ± 0.03 <sup>e</sup>	-	2.83 ± 0.02 <sup>f</sup>	12.30 ± 0.03 <sup>d</sup>	0.042	0.138
Quercitrin	6.13 ± 0.01 <sup>d</sup>	0.72 ± 0.01 <sup>e</sup>	13.48 ± 0.01 <sup>g</sup>	8.69 ± 0.01 <sup>e</sup>	0.019	0.063
Quercetin	10.26 ± 0.02 <sup>c</sup>	1.68 ± 0.02 <sup>b</sup>	7.12 ± 0.01 <sup>a</sup>	5.42 ± 0.02 <sup>f</sup>	0.028	0.091
Kaempferol	7.29 ± 0.01 <sup>a</sup>	0.89 ± 0.03 <sup>e</sup>	8.16 ± 0.01 <sup>c</sup>	7.11 ± 0.02 <sup>g</sup>	0.015	0.049

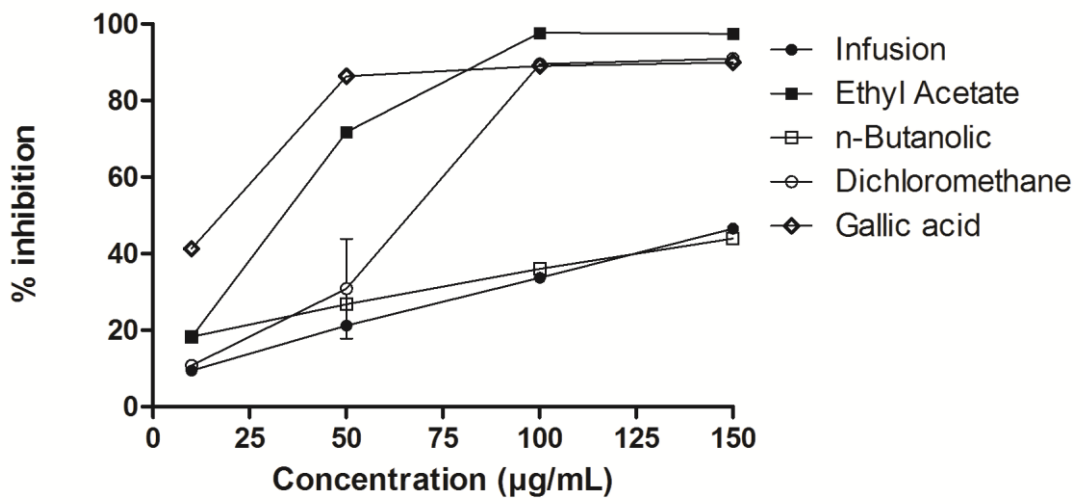
Results are expressed as mean ± SEM from three to four independent experiments performed in duplicate. Means followed by different letters, on each column test and were analyzed by ANOVA, followed by Newman–Keuls test when appropriated  $p < 0.05$ .

**Table 2**

<b>Phenolic Compounds</b> ( $\mu\text{g GAE/mg}$ of extract) mean $\pm$ S.E.M.	
Infusion	1.82 $\pm$ 0.26 <sup>a</sup>
Ethyl acetate	21.95 $\pm$ 0.44 <sup>b</sup>
<i>n</i> -Butanol	2.54 $\pm$ 0.01 <sup>a</sup>
Dichloromethane	11.71 $\pm$ 0.49 <sup>c</sup>

Results are expressed as mean  $\pm$  SEM from three to four independent experiments performed in duplicate. Means followed by different letters, on each column, differ statistically by Bonferroni's test at  $p < 0.05$ .

Figure 2





**Table 3**

<b>Extracts</b>	<b>IC<sub>50</sub> µg/mL</b>
Gallic acid (control)	13.33 ± 1.13 <sup>a</sup>
Infusion	164.3 ± 3.34 <sup>b</sup>
Ethyl acetate	38.86 ± 0.26 <sup>c</sup>
<i>n</i> -butanolic	175.4 ± 3.72 <sup>b</sup>
Dichloromethane	58.98 ± 2.34 <sup>c</sup>

Results are expressed as mean ± SEM from three independent experiments performed in duplicate. Means followed by different letters differ by Bonferroni's test at  $p < 0.05$ .

Figure 3

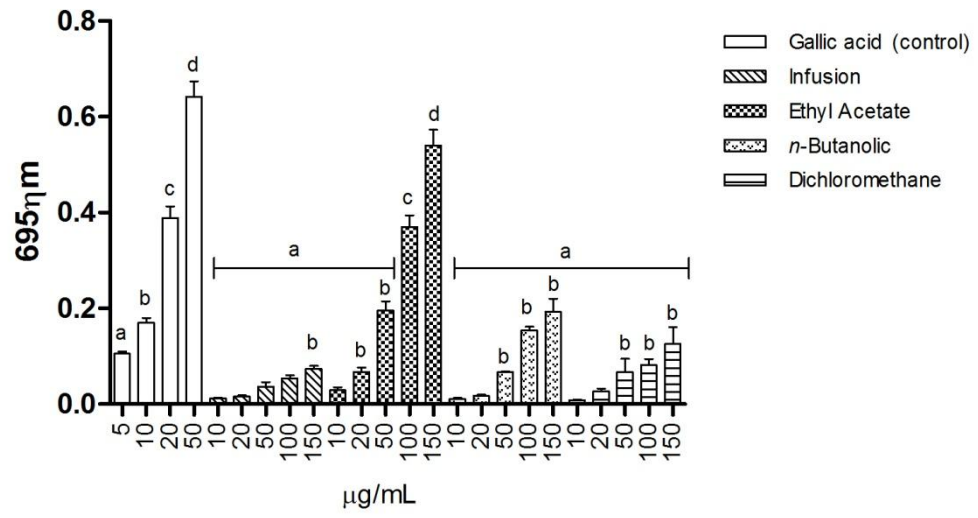


Figure 4

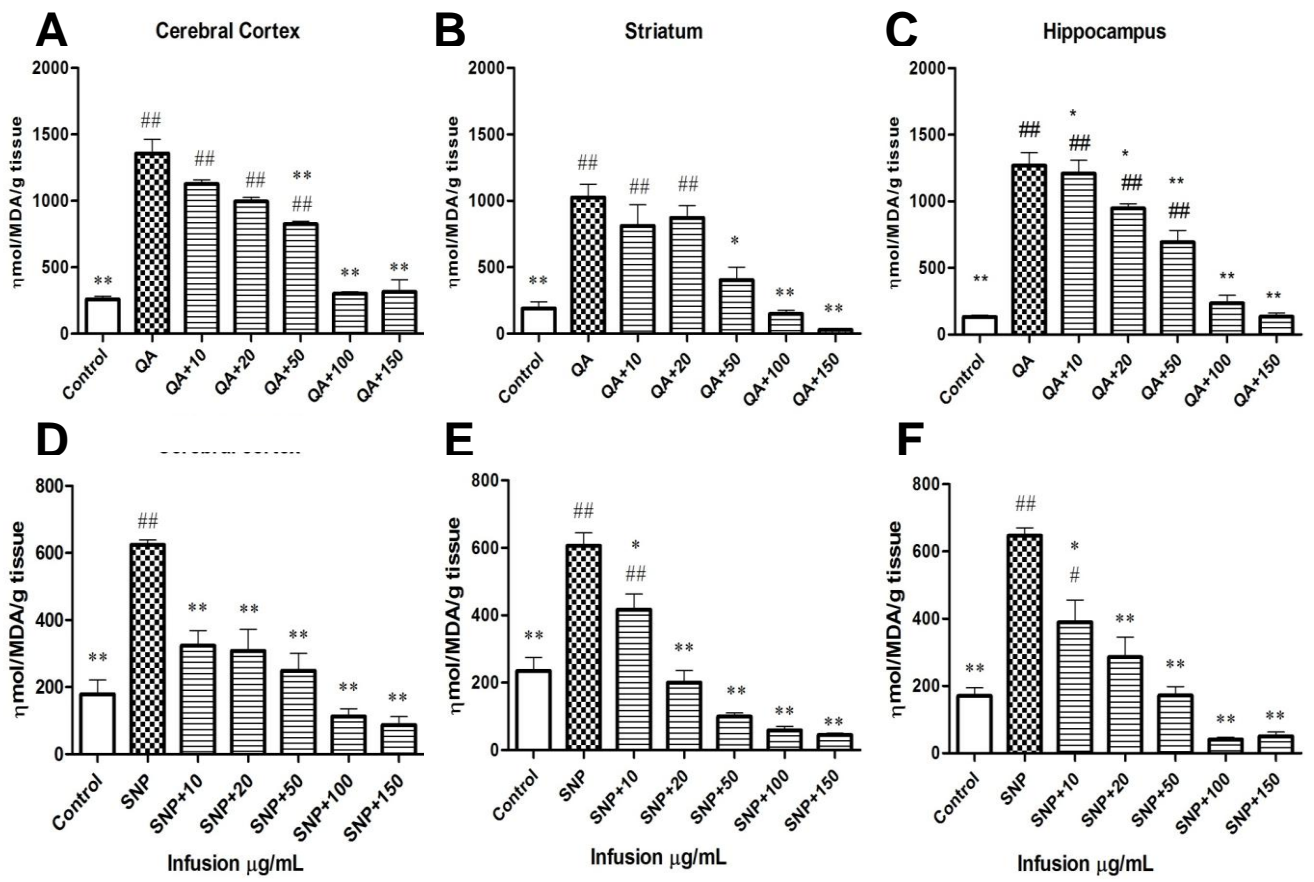


Figure 5

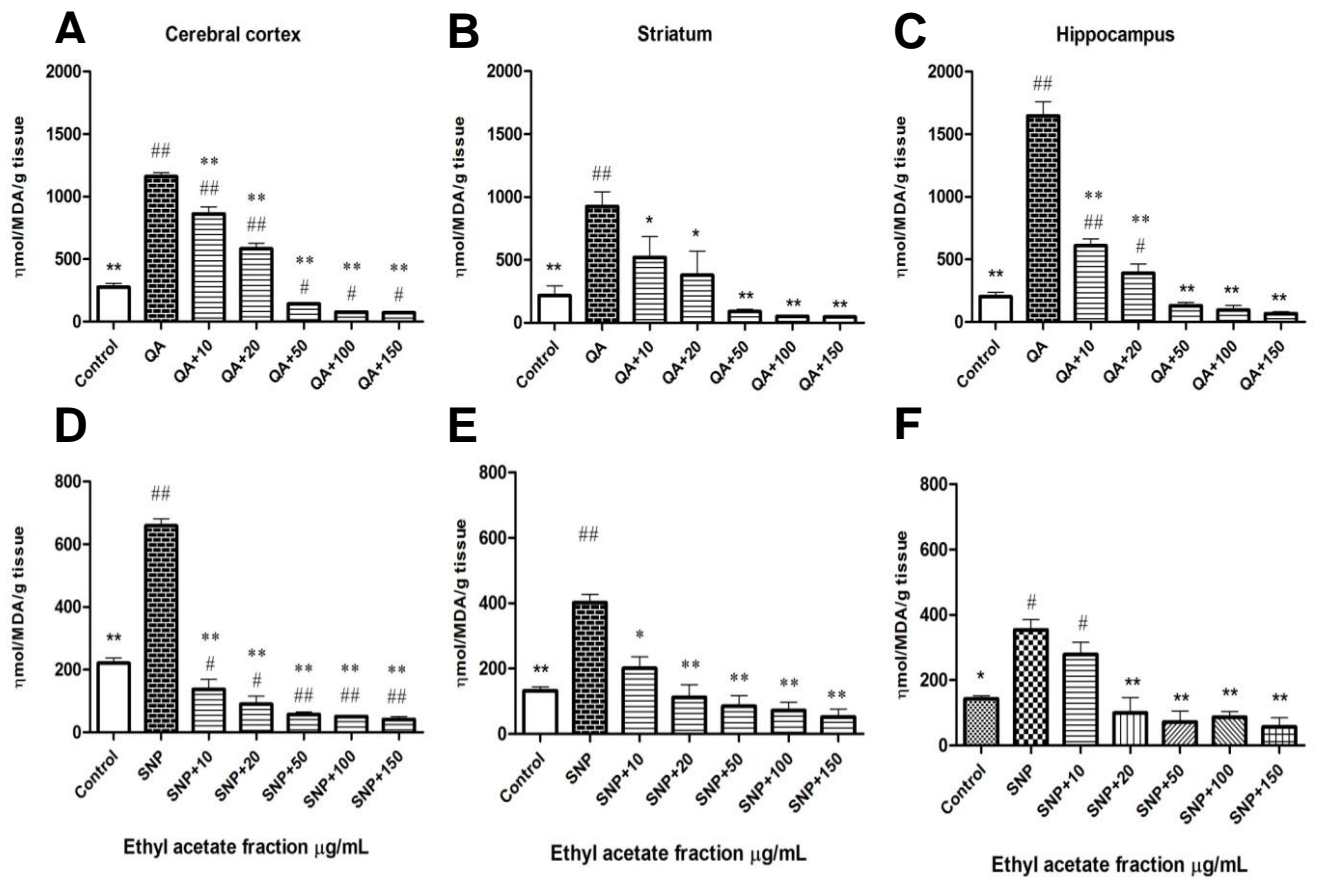


Figure 6

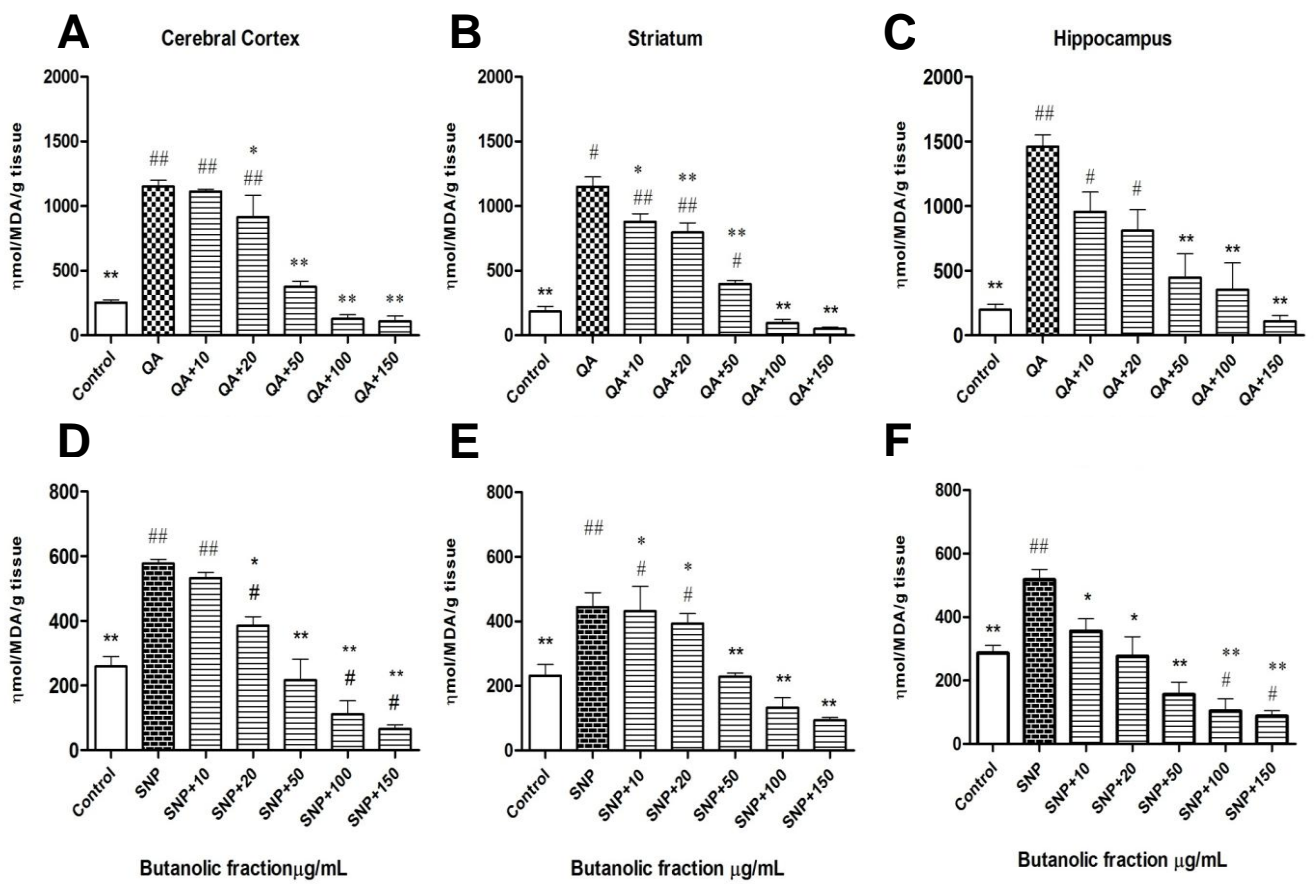
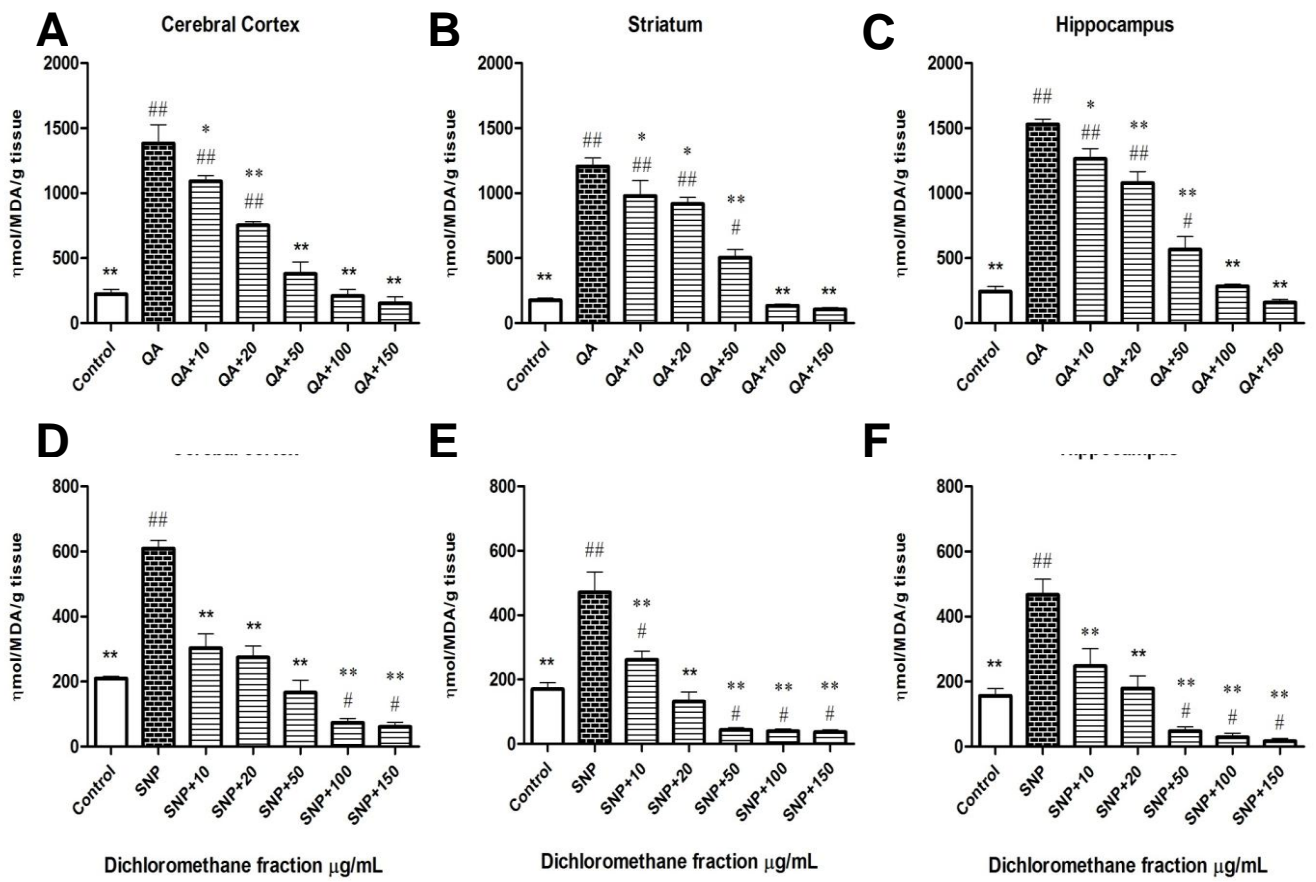


Figure 7



**Table 4**

Extracts	Pro-oxidants					
	QA			SNP		
	Cortex	Striatum	Hippocampus	Cortex	Striatum	Hippocampus
Infusion	72.85 ± 7.47 <sup>a,*</sup>	46.90 ± 10.4 <sup>a,*</sup>	52.64 ± 7.42 <sup>a</sup>	21.67 ± 2.20 <sup>a</sup>	16.12 ± 4.64 <sup>a,b</sup>	17.15 ± 2.03 <sup>a</sup>
Ethyl acetate	11.82 ± 2.54 <sup>b</sup>	13.91 ± 2.90 <sup>b</sup>	13.55 ± 6.33 <sup>b</sup>	10.82 ± 0.74 <sup>a</sup>	13.57 ± 4.32 <sup>b,e</sup>	21.83 ± 7.91 <sup>a</sup>
<i>n</i> -butanolic	25.36 ± 5.95 <sup>b</sup>	35.89 ± 1.42 <sup>ab</sup>	29.96 ± 2.45 <sup>ab</sup>	31.98 ± 0.57 <sup>a</sup>	47.94 ± 2.69 <sup>b,c</sup>	22.97 ± 5.53 <sup>a</sup>
Dichloromethane	28.19 ± 0.60 <sup>b</sup>	42.84 ± 4.88 <sup>ab</sup>	37.45 ± 5.76 <sup>ab</sup>	14.62 ± 0.10 <sup>a</sup>	11.05 ± 3.72 <sup>b,d,e</sup>	19.22 ± 2.59 <sup>a</sup>

Results are expressed as mean ± SEM from three to four independent experiments performed in duplicate. Means followed by different letters, on each column, differ statistically by Bonferroni's test at  $p < 0.05$ . While the lines, the values marked with (\*) differ.

## 4 CONCLUSÕES PARCIAIS

Com a análise dos resultados obtidos chegamos as conclusões parciais do trabalho. Todos os extratos (infusão, acetato de etila, *n*-butanol e diclorometano) mostraram capacidade antioxidante *in vitro*.

A análise por HPLC dos extratos mostrou a presença dos flavonoides quercetina, quercetrina, kampferol e rutina. Glicosídeos triterpenos, no caso o madecassosídeo, os taninos catequina e epicatequina e por fim os ácidos fenólicos ácido gálico, ácido clorogênico, ácido rosmarínico e ácido cafeico. Sendo que a fração acetato de etila apresentou uma alta quantidade da maioria dos compostos testados. A fração *n*-butanol e a infusão apresentaram alta quantidade de rutina e quercitina respectivamente. O maior conteúdo de madecassosídeo foi identificado na fração diclorometano e não foi identificado na fração *n*-butanol.

A quantificação de compostos fenólicos mostrou que a fração acetato de etila possui um maior conteúdo de GAE/mg, seguida da fração diclorometano > *n*-butanol > infusão.

A capacidade redutora dos extratos foi testada através do teste de redução do Mo<sup>6+</sup> a Mo<sup>5+</sup>. Todas as concentrações de *n*-butanol e diclorometano mostraram atividade similar à concentração de 5µg/mL de ácido gálico (AG). A infusão também demonstrou atividade semelhante a 5µg/mL de AG nas concentrações de 10, 20, 50 e 100µg/mL. Já a concentração de 150µg/mL da infusão, 20-50µg/mL da fração acetato de etila, 50-150 de *n*-butanol e diclorometano foram semelhantes ao AG a 10µg/mL. E por fim a única fração equivalente ao AG nas maiores concentrações de 20 e 50µg/mL foi a fração acetato de etila em 100 e 150µg/mL respectivamente.

Ácido quinolínico e nitroprussiato induziram a peroxidação lipídica *in vitro* em regiões do cérebro de rato e os extratos de *C.asiática* foram capazes de prevenir essa produção de espécies reativas na ordem de maior proteção descritas a seguir. Para o AQ: acetate de etila > *n*-butanol > diclorometano > infusão em córtex, estriado e hipocampo. E para NPS: acetate de etila > diclorometano > infusão > *n*-butanol no córtex; diclorometano > acetate de etila > infusão > *n*-butanol no estriado e para hipocampo foi infusão > diclorometano > acetato de etila > *n*-butanol.



## 5 CONCLUSÃO FINAL

Em conclusão, todos os extratos de *C.asiática* testados neste trabalho foram capazes de prevenir a peroxidação lipídica induzida por dois agentes pró-oxidantes diferentes, AQ e NPS em regiões do cérebro de rato. Também, a *C.asiática* apresentou atividade como scavenger de radicais DPPH· e redução de  $\text{Mo}^{6+}$  a  $\text{Mo}^{5+}$ . Em parte, esses efeitos podem estar relacionados com o conteúdo fenólico presente nos extratos, incluindo a presença de flavonoides, taninos e ácidos fenólicos na fração acetato de etila, que apresentou a melhor atividade antioxidante em comparação com as outras frações. Esses resultados são interessantes porque mostram que a *C.asiática* pode ser usada como um agente em potencial na prevenção de doenças neurológicas associadas com o estresse oxidativo. Porém, estudos adicionais são necessários para investigar o exato mecanismo responsável pelo efeito protetor da *C.asiática* na peroxidação lipídica, bem como seus efeitos em modelos *in vivo* de estresse oxidativo.

## 6 PERSPECTIVAS

Tendo em vista as conclusões deste trabalho as perspectivas para trabalhos posteriores são:

- Analisar a modulação do sistema glutamatérgico pelos efeitos do ácido quinolínico com extratos de *Centella asiática*;
- Testar a planta em modelos *in vivo* de estresse oxidativo;
- Esclarecer os mecanismos envolvidos na atividade protetora da planta sobre a peroxidação lipídica.

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