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Tese de Doutorado

INVESTIGAÇÃO DA ATIVIDADE ANTIOXIDANTE *IN VITRO* E DA COMPOSIÇÃO QUÍMICA DE DIFERENTES EXTRATOS DE PLANTAS MEDICINAIS

Romaiana Picada Pereira

PPGBT

Santa Maria, RS, Brasil

2011

INVESTIGAÇÃO DA ATIVIDADE ANTIOXIDANTE *IN VITRO* E DA COMPOSIÇÃO QUÍMICA DE DIFERENTES EXTRATOS DE PLANTAS MEDICINAIS

por

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

Doutora em Bioquímica Toxicológica.

Orientador: João Batista Teixeira da Rocha

Co-orientadora: Roselei Fachinetto

Santa Maria, RS, Brasil

2011

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

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elaborada por

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como requisito parcial para a obtenção de grau de
Doutora em Bioquímica Toxicológica.

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"O ESFORÇO É A PONTE QUE LIGA A REALIDADE AO SONHO. QUEM SE ESFORÇA FAZ EMERGIR A ESPERANÇA, A ESPERANÇA NASCE DO ESFORÇO"

(Daisaku Ikeda)

AGRADECIMENTOS

Agradeço de todo coração aos meus pais, Alberto e Lizane, pelos incansáveis incentivos, pelo total apoio de sempre, por todo enorme carinho, compreensão e amor incondicional.

Ao meu irmão, Rômulo, por todo apoio e exemplo de determinação e persistência no alcance de seus objetivos.

Ao meu orientador, Prof. João Batista Teixeira da Rocha, por todas as oportunidades proporcionadas, pelos incontáveis ensinamentos, pelos inúmeros desafios que sempre contribuíram para o meu crescimento profissional e pessoal e pela confiança depositada em mim.

À minha co-orientadora e grande amiga, Roselei Fachinetto, que sempre esteve presente durante a minha formação, por seus valiosos ensinamentos, pela amizade, confiança, paciência e preocupação.

Ao meu namorado André, um agradecimento muito especial pela sua presença ao meu lado em todos os momentos, pelo apoio, compreensão, incentivos, por todo amor, por tudo!

À Profª Margareth Linde Athayde, pela total receptividade de sempre, pelo apoio, pelo seu exemplo, pelos ensinamentos, pela amizade. E, também, um agradecimento muito especial à Aline, pela amizade e disposição de ajudar sempre.

A todos os meus queridos amigos e colegas do Lab. do Prof. João pela parceria, companheirismo, amizade, pelo harmonioso convívio diário.

Ao Prof. José Eduardo Tanus dos Santos e todos os alunos do seu laboratório pela total receptividade, disposição de ajudar, pela amizade e pelos ensinamentos.

Ao Prof. Félix e à Profª Maria Rosa, por seu exemplo, sua amizade, disposição para ajudar sempre, e também ao pessoal de seus laboratórios pela amizade.

Aos demais professores e funcionários do Programa de Pós-Graduação em Ciências Biológicas (Bioquímica Toxicológica) que contribuíram de alguma forma para minha formação. Um agredecimento especial à Mária pela disposição de ajudar sempre e ao Rinaldo pela amizade.

Ao CNPq e à CAPES pela bolsa de estudos e pelos recursos financeiros concedidos.

Enfim, agradeço à UFSM e ao Programa de Pós-Graduação em Ciências Biológicas (Bioquímica Toxicológica) por possibilitar a realização deste curso.

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LISTA DE ABREVIATURAS

AChE – Acetilcolinesterase

ACh – Acetilcolina

Aβ – peptídeo β-amilóide

3-ANP – Ácido 3 -Nitropropiônico

C. citratus – Cymbopogon citratus

DA – Doença de Alzheimer

EO – Estresse oxidativo

EROs – Espécies Reativas de Oxigênio

ERNs – Espécies Reativas de Nitrogênio

H_2O_2 – Peróxido de Hidrogênio

M. recutita – Matricaria recutita

M. officinalis – Melissa officinalis

MDA – Malondialdeído

MMP-2 – Metaloproteinase-2 da Matriz

$OH^{\cdot -}$ – Radical Hidroxil

TBA – Ácido Tiobarbitúrico

TBARS – Espécies Reativas ao Ácido Tiobarbitúrico

RLs – Radicais Livres

SNC – Sistema Nervoso Central

NPS – Nitroprussiato de Sódio

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APRESENTAÇÃO

Nos itens **INTRODUÇÃO e REVISÃO BIBLIOGRÁFICA**, consta uma revisão sucinta da literatura sobre os temas trabalhados nesta tese.

A metodologia realizada e os resultados obtidos que compõem esta tese estão apresentados sob a forma de artigos, os quais se encontram no item **RESULTADOS**. Nestes artigos constam as seções: Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas.

Os itens **DISCUSSÃO E CONCLUSÕES,** encontradas no final desta tese, apresentam descrições, interpretações e comentários gerais sobre os artigos científicos incluídos neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO**, **REVISÃO BIBLIOGRÁFICA, DISCUSSÃO** e **CONCLUSÕES** desta tese.

RESUMO

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

INVESTIGAÇÃO DA ATIVIDADE ANTIOXIDANTE *IN VITRO* E DA COMPOSIÇÃO QUÍMICA DE DIFERENTES EXTRATOS DE PLANTAS MEDICINAIS

AUTORA: Romaiana Picada Pereira
ORIENTADOR: João Batista Teixeira da Rocha
CO-ORIENTADORA: Roselei Fachinetto
LOCAL E DATA DA DEFESA: Santa Maria, 18 de março de 2011.

O estresse oxidativo (EO) está envolvido no desenvolvimento de várias doenças relacionadas ao sistema nervoso central, tal como a Doença de Alzheimer (DA). Considerando as limitações da terapia existente para a DA, existe ainda a necessidade de novas alternativas médicas. Na primeira parte deste estudo, foi investigada a capacidade antioxidante e a composição química de três plantas usadas popularmente para tratar desordens neurológicas: *Melissa officinalis*, *Matricaria recutita* e *Cymbopogon citratus*. O efeito antioxidante de alguns compostos fenólicos também foi examinado para fins comparativos. Todos os extratos de plantas foram efetivos no combate ao EO induzido por diferentes agentes, mas o extrato aquoso da *Melissa officinalis* apresentou o maior efeito antioxidante. Dentre os compostos purificados, a quercetina teve a maior atividade antioxidante seguida pelo ácido gálico, quercitrina e rutina. Neste trabalho, foi mostrado que os extratos de plantas protegeram contra o EO induzido por vários agentes pró-oxidantes que induzem peroxidação lipídica por diferentes processos. Baseado nestes

resultados, que indicam que a *Melissa officinalis* poderia ser um agente efetivo na prevenção de várias doenças neurológicas associadas ao EO, na segunda parte deste estudo, foram investigadas a composição química e a atividade antioxidante de diferentes frações do extrato de *Melissa officinalis*. Além disso, investigou-se o efeito da fração com maior potencial antioxidante na atividade da acetilcolinesterase (AChE) e o efeito do ácido gálico na atividade da metaloproteinase-2 da matriz (MMP-2). A fração acetato de etila apresentou maior conteúdo de flavonóides associada a uma maior atividade antioxidante quando comparado com as outras frações testadas e causou inibição da AChE. Além disso, o ácido gálico inibiu a atividade da MMP-2. Desta forma, sugere-se que a fração acetato de etila da *M. officinalis* poderia ser melhor investigada para o seu possível uso no tratamento da DA, devido às suas atividades antioxidante e anticolinesterase e a capacidade do ácido gálico, composto fenólico presente em sua composição, inibir a MMP-2.

Palavras-chave: *Melissa officinalis*; *Matricaria recutita; Cymbopogon citratus;* Estresse Oxidativo; Quercetina; Ácido Gálico; Doença de Alzheimer.

ABSTRACT

PhD Thesis
Graduate Course in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

INVESTIGATION ABOUT *IN VITRO* ANTIOXIDANT ACTIVITY AND CHEMICAL COMPOSITION OF DIFFERENT EXTRACTS FROM MEDICINAL PLANTS

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CO-ADVISOR: Roselei Fachinetto
DATE AND PLACE OF THE DEFENSE: Santa Maria, March, 18th, 2011.

Oxidative stress (OS) is involved in the development of several disorders involving the central nervous system, such as Alzheimer's disease (AD). Considering the limitations of current therapeutics for AD, there is still a great demand for discovery of new medical alternatives. In the first part of the present study it was investigated the antioxidant capacity and the chemical composition of three plants popularly used to treat neurological disorders: *Melissa officinalis*, *Matricaria recutita* and *Cymbopogon citratus*. The antioxidant effect of some phenolic compounds was also examined for comparative purposes. All plant extracts were effective in to combat OS, but *Melissa officinalis* presented the highest antioxidant effect. Among the purified compounds, quercetin had the highest antioxidant activity followed by gallic acid, quercitrin and rutin. In this work, we have demonstrated that the plant extracts could protect against OS induced by various pro-oxidant agents that induce lipid peroxidation by different process. Based on this study, which indicates that *Melissa officinalis* could be an effective agent in the prevention of various neurological diseases

associated with OS. In the second part of this study, it was investigated the chemical composition and antioxidant activity of different fractions from *Melissa officinalis* extract. Furthermore, it was verified the effect of the most antioxidant fraction on acethylcholinesterase (AChE) activity and the effect of gallic acid on the matrix metalloproteinase-2 (MMP-2) activity. Ethyl acetate fraction presented the highest flavonoids content as well as antioxidant activity when compared with other tested fractions and also it caused AChE inhibition. Moreover, gallic acid inhibited MMP-2 activity. In conclusion, *M. officinalis* ethyl acetate fraction is suggested to be further investigated for its possible use in the treatment of AD, due its antioxidant, anticholinesterase activities and the MMP-2 inhibitory capacity of their phenolic compound, gallic acid.

Keywords: *Melissa officinalis*; *Matricaria recutita; Cymbopogon citratus;* Oxidative stress; TBARS; DPPH; HPLC; Quercetin; Gallic Acid.

1. INTRODUÇÃO

Espécies reativas de oxigênio (EROs) e/ou espécies reativas de nitrogênio (ERNs) são geradas por processos metabólicos normais em todos os organismos aeróbicos (Frei, 1994; Finkel e Holbrook 2000; Silva e cols., 2005). Porém, a excessiva produção destas espécies pode exceder as defesas antioxidantes celulares e levar a uma condição denominada estresse oxidativo (EO). De particular importância, o EO tem sido implicado na patogênese de várias doenças através de diferentes mecanismos, os quais podem envolver mutação no DNA, oxidação de proteínas e peroxidação lipídica (Finkel e Holbrook 2000; Valko e cols., 2004; 2006; 2007). O dano oxidativo pode ocorrer em todas as células aeróbicas, porém o cérebro é um órgão particularmente susceptível devido ao seu alto consumo de oxigênio (aproximadamente 20% do consumo de todo organismo) (Halliwell, 2006), nível relativamente baixo de enzimas antioxidantes, alta concentração de metais de transição e, ainda, pela alta quantidade de ácidos graxos poliinsaturados, propiciando a ocorrência da peroxidação lipídica (Reiter, 1995; Lohr e cols., 2003). Desta forma, existem muitas desordens neurodegenerativas cuja patogênese tem sido relacionada ao EO (Halliwell, 2006) e vários trabalhos têm dedicado atenção especial ao seu papel em doenças como Parkinson e Alzheimer (Simonian e Coyle, 1996; Gilgun-Sherki e cols., 2001).

A doença de Alzheimer (DA) consiste num exemplo de desordem neurodegenerativa caracterizada pelo declínio cognitivo progressivo e perda generalizada de neurônios e suas sinapses no córtex cerebral e hipocampo. Os neurônios do hipocampo são especialmente vulneráveis às injúrias induzidas pela DA (Qu e cols., 2009). As causas e a cura para esta doença não são conhecidas, sendo um dos principais problemas médico e

de saúde pública nos países industrializados. A etiologia da DA permanece ainda não elucidada, porém, dados da literatura mostram que o EO está envolvido na patogênese do dano neuronal característico da DA (Johnson e cols., 2008; Huang e cols., 2009).

Um fator importante e bastante estudado na DA se baseia na deposição cerebral do peptídeo β-amilóide (Aβ) formando placas. Porém, os fatores que causam essa deposição de Aβ nos vasos, bem como as rotas ativadas nesse processo são ainda pouco compreendidas (Zhang-Nunes e cols., 2006). Dados da literatura mostram que Aβ induz a ativação de metaloproteinases da matriz (MMPs), endopeptidases dependentes de zinco que atuam no remodelamento da matriz extracelular, e, além disso, induz a ocorrência de EO (Jung e cols. 2003; Garcia-Alloza e cols., 2009). Outros estudos mostram que EROs também ativam MMPs (Cao e cols., 1995; Haorah e cols. 2007) e antioxidantes naturais reduzem a atividade de MMPs associadas com a deposição de Aβ (Demeule e cols. 2000).

Outra hipótese para as alterações observadas na DA consiste na hipótese colinérgica que relaciona a DA à deficiência de acetilcolina (ACh) cerebral. Assim, a maioria das estratégias de tratamento mais aceitas atualmente baseia-se em inibidores da acetilcolinesterase (AChE) (Perry, 1986). Porém, estas drogas produzem vários efeitos colaterais e relativamente poucos benefícios (Van Marum, 2008). Desta forma, pelo fato de não haver cura para a DA e de acordo com as limitações da terapia atual, há ainda uma grande demanda para a descoberta de novas alternativas para o tratamento da DA. Alguns produtos naturais têm recebido considerável atenção como candidatos para a terapia da DA, devido as suas propriedades anti-amiloidogênica, antioxidante e antiinflamatória (Singh e cols., 2008; Sun e cols., 2010). Plantas têm sido usadas no tratamento de desordens

cognitivas e podem levar a descoberta de novas drogas para o tratamento da DA (Dastmalchi e cols., 2007).

Dentre os compostos presentes na composição de extratos de plantas, possivelmente responsáveis por suas propriedades farmacológicas, estão os compostos fenólicos, como por exemplo, o ácido gálico. Dados da literatura mostram que tais compostos apresentam atividades antioxidante, antiinflamatória, antimutagênica e anticarcinogênica (Pereira e cols., 1996; Yang e cols., 1999; Thompson, 2000; Atoui e cols., 2005; Geetha e cols., 2005). Os flavonóides, tais como a quercetina e suas formas glicosídicas, quercitrina e rutina, fazem parte deste grupo de constituintes e com alta atividade antioxidante, sendo mais eficazes que as vitaminas C e E em proteger as células contra o dano causado por RLs (Vinson e cols., 1995; Wiseman e cols., 1997). Vale ressaltar, porém, que dados da literatura indicam que as propriedades farmacológicas de extratos brutos de plantas podem ser perdidas depois do isolamento de compostos específicos, indicando que parte de suas propriedades farmacológicas pode estar relacionada aos efeitos sinérgicos de diferentes classes de compostos presentes nestes extratos (Carlini, 2003).

Neste contexto, considerando a importância do EO na patogênese de várias doenças, incluindo desordens neurodegenerativas, e a presença de compostos com propriedades antioxidantes na composição de produtos naturais, torna-se interessante investigar as propriedades antioxidantes e neuroprotetoras de extratos brutos de plantas, visando sua aplicação na saúde humana.

2. OBJETIVOS

Objetivo geral

O objetivo do presente estudo consiste em investigar a composição química e a capacidade antioxidante *in vitro* das plantas *Melissa officinalis, Matricaria recutita* e *Cymbopogon citratus*, bem como avaliar o efeito *in vitro* da planta considerada mais promissora em parâmetros relacionados com a doença de Alzheimer.

Objetivos específicos

→ Avaliar o efeito dos extratos etanólico, metanólico e aquoso das plantas bem como dos compostos quercetina, quercitrina, ácido gálico e rutina frente à peroxidação lipídica cerebral de ratos induzida por diferentes conpostos pro-oxidantes *in vitro*;

→ Determinar o extrato com maior atividade antioxidante;

→ Avaliar o efeito de diferentes frações do extrato da planta considerada mais promissora frente à peroxidação lipídica cerebral de ratos induzida por sulfato ferroso *in vitro*;

→ Avaliar a atividade antioxidante dos extratos acima citados bem como as frações da *M. officinalis* através da reação com 1,1-difenil, 2-picrilidrazila (DPPH);

→ Determinar a concentração de compostos fenólicos nos extratos de *M. recutita*, *M. officinalis* e *C. citratus*, bem como das frações da *M. officinalis*;

→ Investigar a composição química dos extratos;

→ Avaliar o efeito do extrato da planta considerada mais promissora, sua fração mais antioxidante e compostos presentes em sua composição sobre a autoxidação da adrenalina;

→ Avaliar o efeito do ácido gálico e do ácido ascórbico na atividade da MMP-2;

→ Avaliar o efeito do extrato da planta considerada mais promissora, sua fração mais antioxidante e compostos presentes em sua composição sobre a atividade da AChE *in vitro* em cérebro de ratos.

3. REVISÃO BIBLIOGRÁFICA

3.1) ESTRESSE OXIDATIVO E DESORDENS NEURODEGENERATIVAS:

Sabe-se que todos os organismos aeróbicos necessitam de oxigênio para sua sobrevivência, porém a hiperóxia (excesso de O_2) causa toxicidade aos mesmos, incluindo neurotoxicidade (Chavko e cols., 2003). Estes organismos produzem espécies reativas de oxigênio (EROs) ou espécies reativas de nitrogênio (ERNs) constantemente em condições fisiológicas. Dentre estas espécies estão os radicais livres (RLs), que são definidos como moléculas ou fragmentos moleculares com um ou mais elétrons desemparelhados. Em condições normais, tais espécies não causam nenhuma toxicidade aos organismos devido à presença de mecanismos antioxidantes nas células.

A capacidade antioxidante endógena é composta por várias vitaminas e enzimas como, por exemplo, as enzimas catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx) e as vitaminas A, C e E. Desta forma, o EO só se origina na ocorrência de um desequilíbrio entre a capacidade antioxidante do organismo e EROs ou ERNs, em favor das últimas. Este desequilíbrio, definido como EO, está entre os fatores que predispõe a ocorrência de desordens neurológicas (Hayashi, 2009).

O dano oxidativo pode ocorrer em todas as células aeróbicas, porém o cérebro é um órgão particularmente susceptível. Uma das razões para isso é o seu alto consumo de O_2 (aproximadamente 20% do consumo de O_2 de todo corpo) (Halliwell, 2006), além disso, o cérebro apresenta um nível relativamente baixo de enzimas antioxidantes, níveis elevados

de metais de transição e, ainda, é rico em ácidos graxos poliinsaturados, propiciando a ocorrência da peroxidação lipídica (Reiter, 1995; Lohr e cols., 2003). Existem muitas desordens neurodegenerativas nas quais diferentes regiões cerebrais estão envolvidas, apresentando os mais variados sintomas e tendo diferentes causas, cuja patogênese se correlaciona com o EO (Halliwell, 2006). Dentre elas, podemos citar como exemplo, as doenças de Parkinson, Alzheimer e Huntington (Lohr, 1991; Halliwell, 1992; Liu e cols., 1996; Simonian e Coyle, 1996; Cohen, 2000; Gilgun-Sherki e cols., 2001; Lohr e cols., 2003; Onodera e cols., 2003; Honda e cols., 2004; Todorich e Connor, 2004).

Neste contexto, vários trabalhos têm sido feitos atualmente na busca de novos compostos com atividade antioxidante que tenham potencial para reverter o EO e assim atuar na cura e prevenção de uma série de doenças (Bastianetto e Quirion, 2002; Williams e cols., 2004; Wagner e cols., 2006; Patel e cols., 2007; Ávila e cols., 2008; Sudatti e cols. 2009). Dentre os compostos amplamente estudados, destacam-se os produtos naturais, tais como as plantas medicinais, devido à presença de uma série de compostos com propriedades antioxidantes que fazem parte de sua constituição química. Neste grupo de constituintes, destacam-se os compostos fenólicos, como por exemplo, o ácido gálico, por possuírem um papel chave na eliminação de RLs (Madsen e cols., 1996; Moller e cols., 1999; Ban e cols., 2008).

3.1.1) AGENTES PRÓ-OXIDANTES

Devido à importância do EO na patogênese de inúmeras doenças e a conseqüente demanda por novas substâncias com propriedades antioxidantes, vários métodos vêm sendo

desenvolvidos para este fim. Um importante exemplo de ensaio bastante utilizado há vários anos para detectar a peroxidação lipídica em diferentes amostras biológicas é o método de TBARS (Espécies Reativas ao Ácido Tiobarbitúrico) descrito originalmente por Okawa e colaboladores (1979). Sabe-se que em condições de EO, os RLs em excesso podem atacar uma diversidade de biomoléculas, sendo os ácidos graxos insaturados, encontrados nas membranas celulares, um importante alvo. Desta forma, este ataque dos RLs à membrana, denominado peroxidação lipídica, pode causar lise e morte celular. Neste contexto, o método de TBARS consiste numa medida dos níveis de peroxidação lipídica tecidual. Na reação em que se baseia este método, produtos da peroxidação lipídica, como o malondialdeído (MDA) reage com o ácido tiobarbitúrico (TBA) para formar um complexo colorido (TBA-MDA), o qual pode ser quantificado espectrofotometricamente em 532 nm. Este método vem sendo extensamente utilizado em várias pesquisas, onde substâncias são testadas frente a diferentes pró-oxidantes, que induzem peroxidação lipídica por mecanismos diversos (Puntel e cols., 2005; 2007). Um pró-oxidante bastante utilizado é o ferro, apesar de desempenhar importantes funções biológicas, íons ferro em excesso estimulam a produção de RLs (Braughler e cols., 1986; Minotti e Aust, 1987; Minotti e Aust, 1992). Dados da literatura mostram que o ferro livre induz neurotoxicidade (Bostanci e Bagirici, 2008) via estimulação da reação de Fenton, que gera radicais hidroxil ($OH^{\cdot}$) (Fraga e Oteiza, 2002). Além disso, sabe-se que os níveis de ferro encontram-se aumentados em algumas doenças neurodegenerativas (Qiane cols., 1997; Swaiman, 1991; Aisen e cols., 1999). O ferro também pode formar complexos com oxigênio, sendo estes responsáveis por iniciar reações de peroxidação lipídica (Oubidar e cols., 1996).

Outra substância que tem sido utilizada em diferentes estudos *in vitro* e *in vivo*

(Posser e cols., 2006; Prigol e cols., 2009) como um pró-oxidante é o nitroprussiato de sódio (NPS). O NPS pode causar EO e citotoxicidade através da liberação de cianeto e/ou óxido nítrico (NO), o qual pode gerar peroxinitrito (Arnold e cols., 1984; Bates e cols., 1990; Huie e Padmja, 1993; Pryor e Squadrito, 1995). Vários estudos enfatizam o papel do NO na patofisiologia de várias doenças, como Parkinson's e DA.

O ácido 3-nitropropiônico (3-ANP) induz EO via inibição irreversível da enzima succinato desidrogenase, causando disfunção mitocondrial e exercendo assim seus efeitos tóxicos (Alston e cols., 1977). O 3-ANP também tem sido utilizado em vários estudos, tanto *in vitro* quanto *in vivo* como um indutor de EO e neurotoxicidade, por exemplo, o tratamento com esta substância tem sido utilizado como um modelo experimental para o estudo da doença de Huntington's (Binienda e cols., 1998; Silva e cols., 2007; Sandhir e cols., 2010).

3.3) DOENÇA DE ALZHEIMER:

A DA consiste num exemplo de desordem neurodegenerativa progressiva que, segundo dados da literatura, afeta atualmente cerca de 36 milhões de pessoas em todo o mundo (Williams e cols., 2010). A doença foi originalmente descrita em 1906 por Alois Alzheimer baseado na observação de placas amilóides, emaranhados neuro-fibrilares e anormalidades vasculares durante a autópsia de Auguste Deter, um paciente que morreu com severos defeitos cognitivos (Alzheimer, 1907). A patogênese da DA é complexa, envolvendo tanto fatores genéticos como ambientais (Lahiri e cols., 2007), que contribuem para graus variados da doença, com ocorrência de morte aproximadamente 9 anos após o diagnóstico (Jakob-Roetne e Jacobsen, 2009).

Embora a patogênese da DA não seja ainda totalmente esclarecida, sabe-se que resulta da ocorrência de morte celular desencadeada por diferentes fatores. O cérebro com DA apresenta baixos níveis de acetilcolina (ACh), que pode se originar do acúmulo de peptídeos beta amilóide (βA) formando placas que, por sua vez, podem interferir com a habilidade da ACh em exercer seu efeito na transmissão sináptica e iniciar processos inflamatórios que produzem EROs (Wollen, 2010). Desta forma, a principal estratégia de tratamento atualmente aceita é o uso de inibidores da enzima AChE, cujo principal papel é a rápida hidrólise do neurotransmissor ACh à colina, levando consequentemente á interrupção da transmissão do impulso nervoso nas sinapses colinérgicas (Perry, 1986). Porém, a maioria destes fármacos produz uma série de efeitos colaterais, incluindo distúrbios gastrointestinais e problemas relacionados à biodisponibilidade (Melzer, 1998; Schulz, 2003; Van Marum, 2008).

Desta forma, considerando que ainda não há cura para a DA, mas apenas tratamentos sintomáticos, há uma grande necessidade de novas alternativas para o tratamento desta doença. Neste contexto, vários produtos naturais têm recebido uma atenção especial como possíveis candidatos para a terapia da DA, devido às suas propriedades anti-amiloidogenica, antioxidante, antiinflamatoria e anticolinesterase (Mukherjee e cols., 2007; Singh e cols., 2008; Sun e cols., 2010).

3.3.1) HIPÓTESE COLINÉRGICA:

O sistema colinérgico central é considerado um importante sistema neurotransmissor envolvido na regulação das funções cognitivas. Estudos em pacientes

com DA revelaram que a captação de colina e liberação de ACh estão reduzidas no cérebro destes pacientes. Estas reduções estão associadas com déficits colinérgicos pré-sinapticos, tidos como a principal característica da DA (Francis e cols., 1999). Estas observações levaram a "hipótese do déficit colinérgico" ou "hipótese colinérgica" para explicar a DA. Esta hipótese postula que a escassez de ACh causa muitos sintomas da DA, especialmente aqueles relacionados à dificuldade de aprendizado. Desta forma, para diminuir a progressão da DA e causar uma melhora cognitiva, pesquisadores tentam restaurar o equilíbrio colinérgico através da inibição da degradação de ACh mediada pela AChE (Francis e cols., 1999; van Marum, 2008).

Apesar de muitos fármacos serem atualmente aprovados para uso, seus benefícios são moderados. Por exemplo, os inibidores da AChE melhoram os déficitis de memória em geral apenas temporariamente e seus efeitos em testes cognitivos e medidas comportamentais são poucos. Além disso, estas drogas comumente causam efeitos colaterais gastrointestinais, como náusea e diarréia (Lleo e cols. 2006; van Marum 2008).

Atualmente, baseado no acúmulo de evidências que levam à rejeição da "hipótese colinérgica", autores sugerem que os poucos efeitos benéficos de inibidores da AChE poderiam ser atribuídos simplesmente a uma restauração do equilíbrio de ACh no cérebro (van Marum 2008). Desta forma, novas terapias que visam diretamente a causa da DA estão agora sob investigação. Dois inibidores da AChE atualmente utilizados na clínica para aliviar os sintomas cognitivos da DA são derivados de produtos naturais (galantamina e rivastigmina). Em consequência disso, várias pesquisas são direcionadas para a identificação de outros inibidores da AChE de fontes naturais (Mukherjee e cols., 2007).

3.3.2) HIPÓTESE AMILÓIDE:

A hipótese amilóide sucedeu a hipótese colinérgica, sendo introduzida em 1992 para explicar a patofisiologia da DA (Hardy e Higgins, 1992). De acordo com esta hipótese, clivagens proteolíticas anormais da proteína precursora amilóide levam ao acúmulo extracelular excessivo de Aβ no cérebro do paciente com DA (Selkoe, 1996). Essa produção aumentada de Aβ resulta em um conjunto de eventos patogênicos. O peptídeo Aβ patologicamente acumulado interage diretamente com membranas neuronais ou estimula indiretamente diversas rotas de sinalização intracelular prejudicando sinapses neuronais e causando ainda reações de EO e respostas inflamatórias (Roberson e Mucke, 2006; Heneka e O'Banion, 2007; van Marum, 2008). Através destes mecanismos multifatoriais, o acúmulo de Aβ leva à progressiva perda de neurônios, desintegração de circuitos neurais e ao declínio neurológico característico da DA (Roberson e Mucke 2006). Em suma, a hipótese amilóide sugere que o peptídeo Aβ é a chave para a profunda degeneração neuronal e sináptica em regiões do cérebro relacionadas ao aprendizado e memória (Hardy Higgins, 1992; Mattson, 2004). Desta forma, novas abordagens para a prevenção e tratamento da DA têm enfatizado estratégias para reduzir a patogênese do peptídeo Aβ (Roberson e Mucke 2006).

Apesar de todos os fatores acima expostos, já esclarecidos na literatura, os mecanismos envolvidos no acúmulo e deposição do peptídeo Aβ não são ainda completamente elucidados. Evidências recentes sugerem que o peptídeo Aβ induz ativação de MMPs (Jung e cols. 2003; Garcia-Alloza e cols., 2009). Estas enzimas são endopeptidases dependentes de zinco que atuam no remodelamento da matriz extracelular

(Cao e cols., 1995; Fu e cols., 2008). Além disso, outros estudos mostram que o peptídeo Aβ induz a ocorrência de EO, que também leva à ativação de MMPs (Cao e cols., 1995; Haorah e cols. 2007). Desta forma, antioxidantes poderiam bloquear a ativação de MMPs e o EO associados com o acúmulo de Aβ (Demeule e cols. 2000).

Por outro lado, um estudo recente mostra uma diminuição nos níveis de MMP-2 no fluído cérebro espinhal de pacientes com DA e níveis inalterados no plasma dos mesmos pacientes quando comparado com pacientes normais (Horstmann e cols., 2010). Desta forma, embora alguns estudos mostrem evidências de que as MMPs desempenham um importante papel na patogênese da DA, não está bem esclarecido se sua função é protetora ou destrutiva. Neste contexto, mais estudos são necessários para esclarecer estas questões e melhor investigar estratégias eficazes de tratamento para a DA.

3.4) PRODUTOS NATURAIS:

Há um crescente número de trabalhos mostrando os benefícios dos produtos naturais à saúde humana. Considerando estes produtos, destacam-se as plantas medicinais, que têm sido tradicionalmente usadas no tratamento de várias doenças. Suas propriedades terapêuticas são atribuídas a diferentes constituintes químicos isolados de seus extratos. Dentre tais constituintes, destacam-se aqueles com atividade antioxidante, que têm mostrado efeitos preventivos em várias doenças degenerativas, incluindo câncer, doenças cardiovasculares e desordens neurológicas, como, por exemplo, a doença de Alzheimer (Leite e cols., 1986; Hertog e cols., 1993; Au Kono e cols., 1996; Dreostic e cols., 1997;

Jankun e cols., 1997; Tijburg e cols., 1997; Wiseman e cols., 1997; Velioglu e cols., 1998; Carlini, 2003; Cui e cols., 2004; Evans e cols., 2006; Mentreddy, 2007).

Dados da literatura mostram que as propriedades farmacológicas de extratos brutos de plantas podem ser perdidas quando se isolam componentes específicos. Isto indica que parte destas propriedades pode estar relacionada com o efeito sinérgico de diversos compostos. Assim, extratos brutos, como por exemplo, extratos aquosos (chás), podem oferecer maiores vantagens do que compostos isolados, uma vez que oferecem um menor custo, apresentam atividade farmacológica, baixa toxicidade, além de ser a forma mais utilizada tradicionalmente pela população (Carlini, 2003; Pietrovski e cols., 2006; Pereira e cols., 2009).

Dentre os compostos que fazem parte da constituição dos extratos de plantas, estão os compostos fenólicos, tais como os flavonóides, que são substâncias químicas presentes na composição de plantas, sendo mais de 4000 diferentes estruturas já identificadas e descritas atualmente (Macheix e cols., 1990). Apesar de existirem pesquisas com estes compostos há várias décadas, atualmente estes estudos têm se intensificado devido a crescente compreensão do potencial benéfico que os efeitos destes compostos oferencem à saúde humana (Hollman e cols., 1996; Eastwood, 1999; Hollman e Katan, 1999). Estudos revelam que tais compostos apresentam atividades antioxidante, antiinflamatória, antimutagênica e anticarcinogênica (Pereira e cols., 1996; Yang e cols., 1999; Thompson, 2000; Atoui e cols., 2005; Geetha e cols., 2005). Além disso, estudos também mostram que estes compostos são mais efetivos que as vitaminas C e E em proteger as células contra o dano causado por RLs (Vinson e cols., 1995; Wiseman e cols., 1997).

Produtos naturais têm sido amplamente estudados como alternativas para o tratamento da DA, devido às propriedades anti-amiloidogênica, antioxidante e anti-inflamatória (Singh e cols., 2008; Sun e cols., 2010). Além disso, de particular importância, dados da literatura mostram a interação de compotos polifenólicos com MMPs em modelo de doença relacionada ao SNC (Park e cols., 2010).

Desta forma, considerando as informações acima expostas acerca dos benefécios proporcionados pelos produtos naturais, percebemos a necessidade de maiores investigações acerca dos mecanismos farmacológicos das plantas medicinais devido ao seu grande potencial de aplicação no tratamento de diversas doenças, podendo assim contribuir para a melhora da saúde humana.

3.4.1) *Melissa officinalis, Matricaria recutita* e *Cymbopogon citratus*:

A planta *Melissa officinalis* (*M. officinalis*), da família Lamiacea, é amplamente utilizada na forma de chá para tratar ou aliviar distúrbios do sono e desordens gastrointestinais. Alguns estudos têm mostrado efeitos antitumorais e neuroprotetores desta planta (Perry e cols., 1999; de Sousa e cols., 2004; Marongiu e cols., 2004; dos Santos e cols., 2006). De particular importância, dados da literatura mostram que a *M. officinalis* melhora funções cognitivas e reduz a agitação de pacientes com DA moderada (Akhondzadeh e cols., 2003). Além disso, um estudo mostrou que esta planta modula o humor e a cognição quando administrada a jovens adultos (Kennedy e cols., 2002). Tendo em vista estas evidências, atualmente vários estudos desta planta vêm sendo feitos, com a perspectiva de encontrar novas estratégias de tratamento para a DA (Orhan e Aslan, 2009; Dastmalchi e cols., 2009). Neste contexto, em alguns estudos, a *M. officinalis* inibiu a enzima AChE *in vitro*, o que é bastante relevante no contexto da DA, visto que os anticolinesterásicos utilizados atualmente apresentam uma série de efeitos colaterais e relativamente poucos benefícios (Melzer, 1998; Schulz, 2003;Van Marum, 2008), levando à necessidade de novos tratamentos que sejam eficazes e potencialmente menos tóxicos para o tratamento desta doença.

Outro fator importante e bastante explorado atualmente em relação *M. officinalis* está relacionado à sua atividade antioxidante (Lopez, 2007; Mencherini, 2007; López e cols., 2009). Isto é bastante interessante, visto que já está bem estabelecido que o EO está presente no desenvolvimento de desordens relacionadas ao SNC (Finkel e Holbrook, 2000). Sabe-se que, atualmente, existe um grande número de pessoas sofrendo de desordens neurodegenerativas no mundo, especialmente em países desenvolvidos, havendo um

crescente número de casos de doenças como Parkinson e DA e doenças psiquiátricas, como depressão e ansiedade (Khandhar e Marks, 2007; Sanders e Morano, 2008; Timonen e Liukkonen, 2008). Neste contexto, considerando a atividade antioxidante da *M. officinalis*, bem como de seus constituintes, torna-se bastante interessante explorar diferentes extratos brutos, frações e constituintes isolados desta planta nos mais diversos modelos experimentais de doenças neurológicas relacionadas ao EO, tanto *in vitro* quanto *in vivo*.

A planta *Matricaria recutita* (*M. recutita*), da família Asteraceae, popularmente conhecida como camomila, tem sido amplamente utilizada na medicina tradicional devido às suas propriedades antiinflamatória, sedativa, antibacteriana, antifúngica, entre outras (Avallone e cols., 2000; Zanoli e cols., 2000). Esta planta é popularmente conhecida como Camomila e é bastante comum o uso popular do chá de suas flores secas. Dados da literatura mostram que a camomila apresenta efeito antioxidante moderado *in vitro* (Dragland e cols. 2003). Estudos em modelos animais mostram atividade antimutagênica (Hernandez-Ceruelos e cols., 2002), ação hipocolesterolemiante (Al-Jubouri e cols., 1990) e também efeitos ansiolíticos (Avallone e cols., 2000; Nakamura e cols., 2002). Estudos em humanos e testes clínicos com esta planta são ainda escassos.

A planta *Cymbopogon citratus* (*C. citratus*), da família Gramineae, popularmente conhecida como “capim cidreira”, é utilizada popularmente como analgésico, antiinflamatório, antitérmico, diurético e sedativo (Carlini e cols., 1986). Esta planta é utilizada na indústria como componente de vários perfumes e cosméticos devido ao seu aroma semelhante ao do limão (Rauber e cols., 2005). Dados da literatura mostram atividade antioxidante do extrato desta planta (Cheel e cols., 2005; Tiware e cols., 2010). A infusão ou decocção das partes aéreas desta planta são as formas mais utilizadas na

medicina popular, sendo recomendada também para tratar desordens digestivas, diabetes e distúrbios nervosos (Masuda e cols., 2008).

Embora várias informações estejam disponíveis na literatura sobre as plantas aqui descritas, mostrando que elas são agentes promissores no que diz respeito à saúde humana, os mecanismos exatos envolvidos em suas propriedades terapêuticas ainda não estão completamente elucidados. Sendo assim, torna-se bastante interessante a realização de mais estudos explorando os extratos destas plantas assim como seus constituintes químicos.

4- RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se nos próprios artigos. O artigo está disposto na forma que foi publicado na revista Neurochemical Research. O manuscrito em preparação está disposto na forma em que será submetido para publicação.

4.1. Efeito antioxidante de diferentes extratos de *Melissa officinalis, Matricaria recutita* e *Cymbopogon citratus*

Artigo

ANTIOXIDANT EFFECTS OF DIFFERENT EXTRACTS FROM *MELISSA OFFICINALIS, MATRICARIA RECUTITA* AND *CYMBOPOGON CITRATUS*

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Neurochemical Research 34 (2009) 973–983

Neurochem Res (2009) 34:973–983
DOI 10.1007/s11064-008-9861-z

ORIGINAL PAPER

Antioxidant Effects of Different Extracts from *Melissa officinalis, Matricaria recutita* and *Cymbopogon citratus*

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Accepted: 24 September 2008 / Published online: 14 October 2008

Abstract Considering the important role of oxidative stress in the pathogenesis of several neurological diseases, and the growing evidence of the presence of compounds with antioxidant properties in the plant extracts, the aim of the present study was to investigate the antioxidant capacity of three plants used in Brazil to treat neurological disorders: *Melissa officinalis, Matricaria recutita* and *Cymbopogon citratus*. The antioxidant effect of phenolic compounds commonly found in plant extracts, namely, quercetin, gallic acid, quercitrin and rutin was also examined for comparative purposes. Cerebral lipid peroxidation (assessed by TBARS) was induced by iron sulfate (10 μM), sodium nitroprusside (5 μM) or 3-nitropropionic acid (2 mM). Free radical scavenger properties and the chemical composition of plant extracts were assessed by 1′-1′ Diphenyl-2′ picrylhydrazyl (DPPH) method and by Thin Layer Chromatography (TLC), respectively. *M. officinalis* aqueous extract caused the highest decrease in TBARS production induced by all tested pro-oxidants. In the DPPH assay, *M. officinalis* presented also the best antioxidant effect, but, in this case, the antioxidant potencies were similar for the aqueous, methanolic and ethanolic extracts. Among the purified compounds, quercetin had the highest antioxidant activity followed by gallic acid, quercitrin and rutin. In this work, we have demonstrated that the plant extracts could protect against oxidative damage induced by various pro-oxidant agents that induce lipid peroxidation by different process. Thus, plant extracts could inhibit the generation of early chemical reactive species that subsequently initiate lipid peroxidation or, alternatively, they could block a common final pathway in the process of polyunsaturated fatty acids peroxidation. Our study indicates that *M. officinalis* could be considered an effective agent in the prevention of various neurological diseases associated with oxidative stress.

Keywords Oxidative stress · Iron sulfate · 3-Nitropropionic acid · Sodium nitroprusside · Medicinal plants

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Introduction

Reactive oxygen species (ROS) are generated by normal metabolic processes in all organisms utilizing oxygen [1–3]. However, excessive ROS production can overcome cellular antioxidant defenses and can lead to a condition termed oxidative stress. Of particular importance, oxidative stress has been implicated in the installation and progression of several degenerative diseases, via either DNA mutation, protein oxidation and/or lipid peroxidation [3–6].

Literature data have given a special attention to the role of ROS and oxidative stress in chronic neurodegenerative

disorders such as Parkinson's and Alzheimer's diseases [7, 8]. In this context, several studies have focused in the potential use of natural and synthetic antioxidant compounds in a variety of in vitro and in vivo models of human pathologies, including neurotoxicity models [9–13].

Medicinal plants have been traditionally used in the treatment of several human diseases and their pharmacological and therapeutic properties have been attributed to different chemical constituents isolated from their crude extracts. Of particular importance, chemical constituents with antioxidant activity can be found at high concentrations in plants and can be responsible for their preventive effects in various degenerative diseases, including cancer, neurological and cardiovascular diseases [14–27]. Thus, the antioxidant properties of plants have a full range of perspective applications in human healthcare [2]. Interestingly, literature data have indicated that the pharmacological properties of crude extracts of plants can be lost after isolation of specific compounds, indicating that part of their pharmacological properties can be related to a combination of different classes of compounds [28, 29].

Lemon balm, *Melissa officinalis* L. (Lamiaceae) *(M. officinalis)* is widely used as herbal tea to treat or to relieve nervous disturbance of sleep and functional gastrointestinal disorders. Of particular importance, some studies have demonstrated antitumoral and neuroprotective effects of *M. officinalis* [30–33]. *Cymbopogon citratus* (DC) Stapf (Gramineae) *(C. citratus)* is an herb worldwide known as lemongrass. The tea made from its leaves is popularly used as antispasmodic, analgesic, anti-inflammatory, antipyretic, diuretic and sedative [34]. However, the mechanisms involved in its pharmacological properties are not well understood. *Matricaria recutita* L. (Asteraceae) *(M. recutita)*, particularly the dried flower heads of the plant, is widely used in traditional and herbal medicine because of its anti-inflammatory, spasmolytic, antipeptic, sedative, antibacterial and antifungal properties [35–37]. Nonetheless, the mechanisms involved in the therapeutic properties of these plants are still not elucidated.

In this context, considering the importance of the oxidative stress in the pathogenesis of various diseases, including those related to the central nervous system and the presence of a number of compounds with antioxidant properties in the plant extracts, the aim of the present study was to investigate, in a comparative way, the antioxidant capacity of the three popularly worldwide used plants on the oxidative stress induced by different agents in brain of rats. We have also investigated the effect of purified compounds, namely, quercetin, quercitrin, gallic acid and rutin. They are commonly found in plant extracts and could be involved in the antioxidant activity of plant extracts against in vitro iron sulfate-, sodium nitroprusside- and nitropropionic acid- induced cerebral lipid peroxidation.

Experimental Procedure

Chemicals

Tris–HCl, thiobarbituric acid (TBA), 3- nitropropionic acid (3-NPA), 1′-1′ diphenyl-2′ picrylhydrazyl (DPPH), rutin, quercetin, gallic acid and malonaldehyde bis- (dimethyl acetal) (MDA) were obtained from Sigma (St. Louis, MO, USA). Sodium nitroprusside (SNP) was obtained from Merck (Darmstadt, Germany). Iron sulfate (Fe_2SO_4), ascorbic acid, chloridric and acetic acid were obtained from Merck (Rio de Janeiro, RJ, Brazil). Quercitrin was isolated from *Solidago microglossa* D.C. and the purity of the isolated compound was 99.3% [38].

Extract Preparation

The plants were obtained from commercial sources. Ethanolic and methanolic extracts were obtained from 5 g of dried plant material (leaves of *C. citratus*, aerial parts of *M. officinalis* and flowers of *M. recutita*). These parts of the plants were macerated in the dark for 7 days with 50 ml of methanol or ethanol. After this, the extracts were evaporated to dryness under reduced pressure. The dry extracts were suspended in the same solvent. The aqueous extracts were obtained by infusion in hot water and they were prepared just before use.

Animals

Male Wistar rats (3.0–3.5 months of age and weighing 270–320 g) were maintained groups of 3–4 rats per cage. They had continuous access to food and water in a room with controlled temperature (22 ± 3°C) and on a 12-h light/dark cycle with lights on at 7:00 a.m. The animals were maintained and used in accordance to the guidelines of the Brazilian Association for Laboratory Animal Science (COBEA).

Tissue Preparation

Rats were killed and the encephalic tissue was rapidly dissected and placed on ice. Tissues were immediately homogenized in cold 10 mM Tris–HCl, pH 7.5 (1/10, w/v). The homogenate was centrifuged for 10 min at 4,000 × g to yield a pellet that was discarded and a low-speed supernatant (S1) that was used for the TBARS assay [39].

TBARS

An aliquot of 100 µl of S1 was incubated for 1 h at 37°C with freshly prepared Fe_2SO_4 (10 µM), SNP (5 µM) or 3-NPA (2 mM) in the presence or absence of plant extracts

Table 1 Tested concentrations in the TBARS assay for each plant extract and isolated compounds

Plant extracts or isolated compounds	Tested concentrations (μg/ml)
M. officinalis aqueous extract	83.3–1666.7
M. officinalis methanolic extract	97.143–914.2
M. officinalis ethanolic extract	194.3–1828.6
M. recutita aqueous extract	83.3–1666.7
M. recutita methanolic extract	133.3–1255
M. recutita ethanolic extract	183.8–1729.7
C. citratus aqueous extract	83.3–1666.7
C. citratus methanolic extract	133.3–1255
C. citratus ethanolic extract	183.8–1729.7
Quercetin	0.015–2
Quercitrin	0.5–25
Gallic Acid	0.5–25
Rutin	5–25

or purified quercetin, gallic acid, quercitrin and rutin. Then, TBARS production was determined as described by Ohkawa et al. [40] and Puntel et al. [39]. The extracts and purified compounds were tested in the range indicated in Table 1. Ethanol and methanol had no effect in TBARS production. Indeed the levels of TBARS production in the presence of water, ethanol and methanol were in the range indicated in Table 2.

Radical-Scavenging Activity-DPPH Assay

The antioxidant activity of the extracts was evaluated by monitoring their ability in quenching the stable free radical DPPH, according Choi et al. with minor modifications [41]. Free radical scavenging capacity (FRSC) of plant extracts was calculated as their IC_{50} values (the concentration necessary to inhibit 50% radical formation), using the method of Dixon and Web [19]. Six different ethanol dilutions of each extract (7.8, 15.6, 31.2, 62.5, 125 and 250 μg/ml) were mixed with 1.0 ml of a 0.3 mM DPPH ethanol solution. Ethanol (1.0 ml) plus plant extract solution was used as a blank. The absorbance was measured at 518 nm after 30 min of reaction at room temperature. DPPH was prepared daily and protected from light. Relative activities were calculated from the calibration curve of L-ascorbic acid standard solutions working in the same experimental conditions. Scavenging or inhibitory capacity in percent (IC%) was calculated using the equation:

$$IC\% = 100 - \left[\left(Abs_{sample} - Abs_{blank}\right) \times 100/Abs_{control}\right]$$

where Abs_{sample} is the absorbance obtained in the presence of different extract concentrations and $Abs_{control}$ is that obtained in the absence of extracts. Tests were carried out in triplicate.

Total Phenolic Compounds Determination

The total phenol content was determined by mixing the extracts with 1.25 ml 10% Folin-Ciocalteu's reagent (v/v) which was followed by the addition of 1.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated at 45°C for 15 min, and the absorbance was measured at 765 nm. Gallic acid (GA) was used as standard for phenolic compounds [42].

TLC Analysis

Concentrated extracts were chromatographed on silica gel TLC plates. Mixtures of hexane: acetone (9:1), dichloromethane: ethanol (9:1), hexane: ethyl ether (7:3), ethyl acetate: ethanol: water (77:15:8), ethyl acetate: formic acid: water (65:15:20) and *n*-butanol: acetic acid: water (40:10:50) were used as eluents. Sitosterol, sinapic acid, quercetin and rutin were used as standard compounds. After elution, the TLCs were observed under UV light at

Table 2 TBARS levels in the presence of different pro-oxidants and distinct solvents (water, methanol or ethanol)

Plants	Extractor solvent	Pro-oxidants		
		Iron	SNP	3-NPA
M. officinalis	Water	673.4 ± 26.5	449.3 ± 31.9	298.4 ± 29.6
	Ethanol	740.6 ± 13.1	410.1 ± 52.5	269.2 ± 20.1
	Methanol	741.6 ± 38.8	447.9 ± 40.4	268 ± 17.1
M. recutita	Water	750 ± 24.7	569.7 ± 47.7	316.7 ± 21.1
	Ethanol	826 ± 116.9	569.9 ± 62.2	317.3 ± 66.9
	Methanol	819 ± 31.7	516.6 ± 127.9	289 ± 30.1
C. citratus	Water	711.7 ± 29.9	416.1 ± 34.7	375.8 ± 33.5
	Ethanol	776.5 ± 40.9	418.9 ± 47.1	341.5 ± 30.6
	Methanol	778.5 ± 21.6	461.7 ± 92.5	375.6 ± 98.6

254 and 366 nm. Afterwards, the compounds were detected by anisaldehyde sulphuric acid, oxaloboric solution and phosphomolibdic acid. It was also carried out a bidimensional TLC to confirm the presence of rutin in small amounts in the aqueous extract from *C. citratus*. In this case, the eluting solvent used were ethyl acetate: formic acid: water (80:8:12) two runs, in both directions [43, 44].

Statistical Analysis

Data from TBARS and DPPH were statistically analyzed by one-way ANOVA, followed by Duncan's multiple range tests when appropriated. Data from IC_{50} and Phenolic compounds were analyzed by *t*-test. When these data did not present variance homogeneity, they were log transformed. The results were considered statistically significant for $P < 0.05$.

Results

Effects of *Melissa officinalis, Matricaria recutita* and *Cymbopogon citratus* on TBARS Production Induced by 10 μM of Iron Sulfate

Aqueous, methanolic and ethanolic extracts obtained from *M. officinalis* (Fig. 1a), *M. recutita* (Fig. 1b) and *C. citratus* (Fig. 1c) significantly inhibited iron-induced TBARS production in brain preparations (for all plants and extracts *P* values were between 0.001 and 0.01). However, the inhibitory potency of the different types of extracts varied from plant to plant. For *M. officinalis* the potency order was aqueous > methanolic > ethanolic extracts (Fig. 1a; Table 3, $P < 0.01$). For *M. recutita,* the order was methanolic > aqueous and ethanolic (Fig. 1b; Table 3, $P < 0.01$), whereas for *C. citratus* the potency order was methanolic > ethanolic > aqueous (Fig. 1c; Table 3, $P < 0.01$).

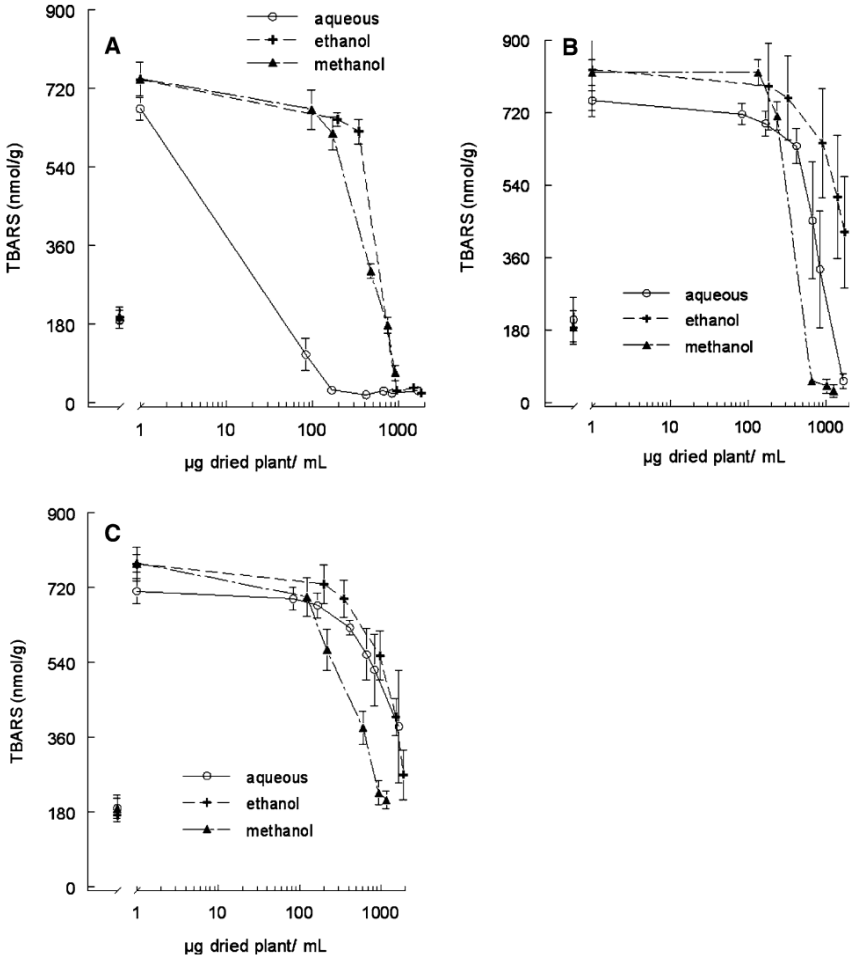

Fig. 1 Effects of different concentrations of aqueous, ethanolic and methanolic extracts from **a** *M. officinalis*, **b** *M. recutita* and **c** *C. citratus* on Iron (10 μM)-induced TBARS production in brain homogenates. The homogenates were incubated for 1 h with Iron and the plant extracts or without (basal). Data show means ± SEM values average from 3 to 4 independent experiments performed in duplicate

Table 3 IC_{50} (μg/ml) values for inhibition by plant extracts of TBARS production induced by different pro-oxidants in brain preparations

Plants	Extractor solvent	Pro-oxidants		
		Iron	SNP	3-NPA
M. officinalis	Water	15.67 ± 2.03[a]	11 ± 0.6[a]	77.4 ± 13.1[a]
	Ethanol	568.5 ± 10.4[b]	186.5 ± 51.3[c]	512.4 ± 103.9[c]
	Methanol	483 ± 25.5[c]	22.3 ± 1.9[b]	210.9 ± 24[b]
M. recutita	Water	848.9 ± 169.8[a]	58.4 ± 4.7[a]	202 ± 31.5[a]
	Ethanol	1874.3 ± 691[a]	826.3 ± 70.3[c]	1107.4 ± 49.4[c]
	Methanol	415 ± 14.2[b]	299.2 ± 8.1[b]	590.9 ± 25.5[b]
C. citratus	Water	2518.5 ± 913.8[c]	476.5 ± 200.2[a]	813.4 ± 236.9[b]
	Ethanol	1549.9 ± 124.9[b]	208.8 ± 28.2[a]	1270.3 ± 101.9[b]
	Methanol	535.8 ± 49.2[a]	313.5 ± 8.8[a]	355 ± 39.2[a]

Different alphabets indicate statistical significance among different extracts of the same plant against the same pro-oxidant

Effects of *Melissa officinalis, Matricaria recutita* and *Cymbopogon citratus* on TBARS Production Induced by 5 μM of Sodium Nitroprusside (SNP)

Aqueous, methanolic and ethanolic extracts obtained from *M. officinalis* (Fig. 2a), *M. recutita* (Fig. 2b) and *C. citratus* (Fig. 2c) inhibited significantly SNP-induced TBARS production in brain preparations (for all plants and extracts P values were between 0.001 and 0.006). However, for *M. officinalis* and *M. recutita* the inhibitory potency of the different types of extracts varied in the following order: aqueous > methanolic > ethanolic extracts (Fig. 2a and b; Table 3, $P < 0.01$).

Effects of *Melissa officinalis, Matricaria recutita* and *Cymbopogon citratus* on TBARS Production Induced by 2 mM of 3-Nitropropionic Acid (3-NPA)

Aqueous, methanolic and ethanolic extracts obtained from *M. officinalis* (Fig. 3a), *M. recutita* (Fig. 3b) and *C. citratus* (Fig. 3c) inhibited 3-NPA-induced TBARS production in brain (for all plants and extracts P values were between 0.001 and 0.003). However, for *M. officinalis* and *M. recutita,* the inhibitory potency of the different types of extracts varied in the following order: aqueous > methanolic > ethanolic extracts (Fig. 3a and b; Table 3), whereas for *C. citratus* the potency order was methanolic > aqueous and ethanolic extracts (Fig. 3c; Table 3, $P < 0.01$).

DPPH Radical-Scavenging Activity of *Melissa officinalis, Matricaria recutita* and *Cymbopogon citratus*

M. officinalis aqueous, methanolic and ethanolic extracts promoted an inhibition of DPPH radical with similar potency (Fig. 4a; Table 4, $P < 0.01$). The inhibitory potency of DPPH radical by different extracts of *M.recutita* was in the following order: methanol > ethanol > water (Fig. 4b; Table 4, $P < 0.01$). *C. citratus* methanolic and ethanolic extracts promoted an inhibition of DPPH radical with similar potency, which was higher than that obtained with aqueous extract (Fig. 4c; Table 4, $P < 0.01$).

Total Phenolic Compounds Determination

The amount of phenolic compounds for *M. officinalis* and *M. recutita* was in the following order: aqueous > methanolic > ethanolic extracts (P values were between 0.001 and 0.01). However, for *C. citratus*, the order was ethanolic > aqueous > methanolic extracts (Table 5, P values were between 0.001 and 0.05).

Effects of Quercetin, Gallic Acid, Quercitrin and Rutin on TBARS Production Induced by 10 μM of Iron Sulfate, 5 μM of Sodium Nitroprusside (SNP) or 2 mM of 3-Nitropropionic Acid (3-NPA)

Iron, SNP and 3-NPA-induced TBARS production in brain preparations was significantly decreased by Quercetin ($P < 0.001$), Gallic Acid ($P < 0.001$), Quercitrin ($P < 0.001$) and Rutin ($P < 0.01$) (Fig. 5). Quercetin exhibited the highest antioxidant activity as indicated by the IC_{50} values (Table 6).

TLC Analysis

The TLC analysis indicated the presence of terpenoids in the ethanolic extract of *M. officinalis*. Furthermore, greater amounts of flavonoids were found in the aqueous extract of *M. officinalis*. In line with this, the aqueous extracts from

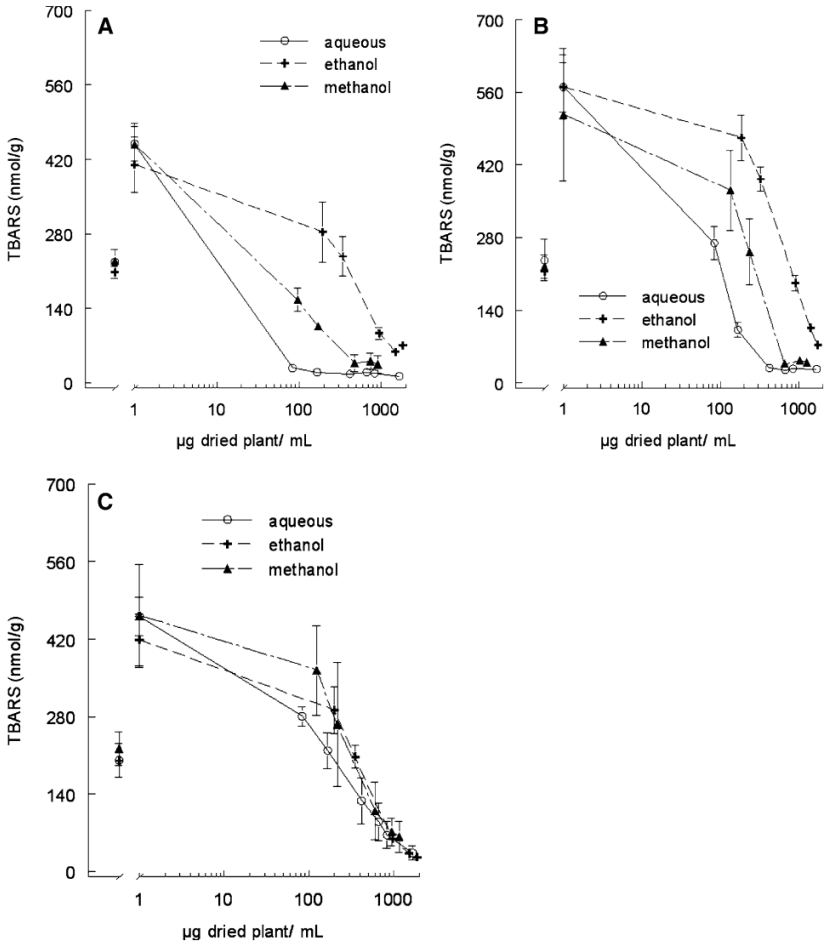

Fig. 2 Effects of different concentrations of aqueous, ethanolic and methanolic extracts from **a** *M. officinalis*, **b** *M. recutita* and **c** *C. citratus* on SNP (5 μM)-induced TBARS production in brain homogenates. The homogenates were incubated for 1 h with SNP and the plant extracts or without (basal). Data show means ± SEM values average from 3 to 4 independent experiments performed in duplicate

all these three plants presented more flavonoids than their respective ethanolic and methanolic extracts. For the ethanolic and methanolic extracts of *M. recutita*, simple phenolic compounds and flavonoids were detected in great amounts (data not shown).

Reducing agents were detected in all extracts. However they were more abundant in the aqueous extract of *M. officinalis*. This fact can explain the higher antioxidant activity of this extract. It was also possible to identify the presence of rutin in *C. citratus* aqueous extract by the bidimensional TLC (data not shown).

Discussion

In this study, we have tested the effect of three different plant species, *M. officinalis, M. recutita* and *C. citratus*, against well-known pro-oxidants, to investigate new potential antioxidants from the natural sources for the possible use in the diseases prevention.

The brain is particularly susceptible to free radical damage because of its high consumption of oxygen and its relatively low concentration of antioxidants enzymes and free radicals scavengers. Then, in this study, we used encephalic tissue for the TBARS assay and determine the quantity of phenolic compounds in the plant extracts to verify a possible relation with the antioxidant activity. These compounds are one of the largest and most ubiquitous groups of plant metabolites and there are current interest in their antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic activity [45–49].

In this work, the aqueous extract of *M. officinalis* had the highest activity against TBARS production induced by all tested agents, when compared with ethanolic and methanolic extracts. Interestingly, the inhibition of lipid peroxidation by *M. officinalis* extracts showed a relation

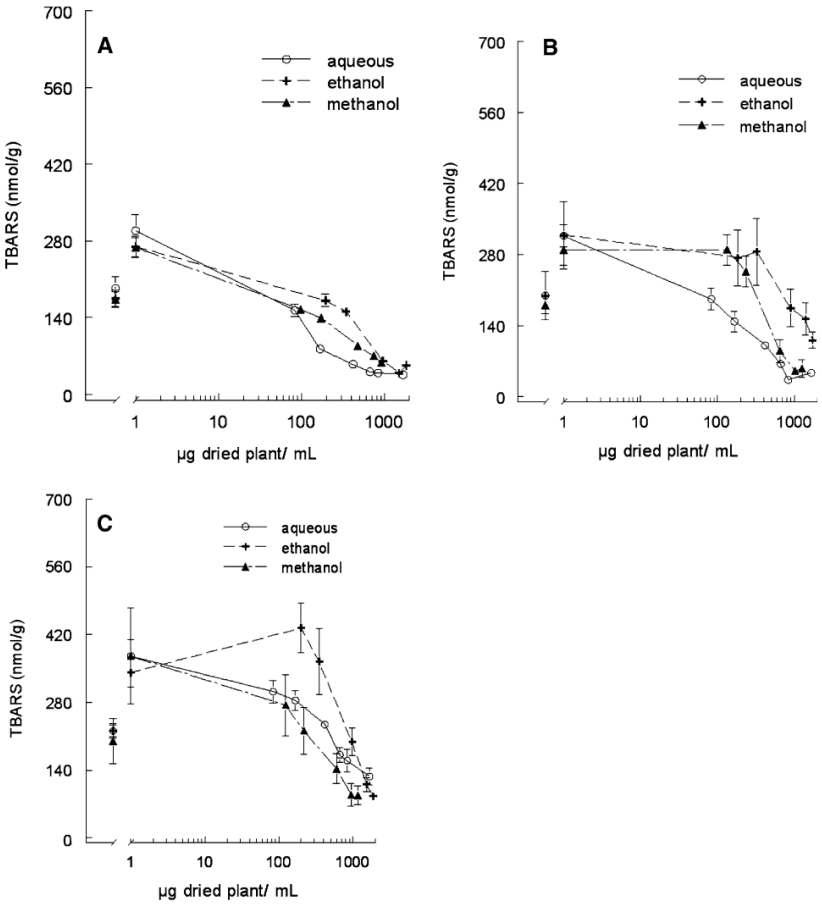

Fig. 3 Effects of different concentrations of aqueous, ethanolic and methanolic extracts from **a** *M. officinalis*, **b** *M. recutita* and **c** *C. citratus* on 3-NPA (2 mM)-induced TBARS production in brain homogenates. The homogenates were incubated for 1 h with 3-NPA and the plant extracts or without (basal). Data show means ± SEM values average from 3 to 4 independent experiments performed in duplicate

with its phenol content. However, in the DPPH assay, the three different extracts obtained from this plant (aqueous, ethanolic and methanolic) presented similar effect.

For *M. recutita* and *C. citratus*, TBARS inhibitory potency varied depending on the pro-oxidant used in a rather complex way. In contrast to *M. officinalis*, there was no clear relation between the antioxidant activity and phenolic contents. In the DPPH test, *M. recutita* methanolic extract presented lower IC_{50} than the ethanolic and aqueous extracts. Furthermore the free radical scavenger potency was not related to phenol concentrations. For *C. citratus*, the IC_{50} values for methanolic and ethanolic extracts were lower than aqueous extract. As in *M. recutita,* the free radical scavenger potency was not related with phenol concentrations.

Here we have used pro-oxidant agents that induce lipid peroxidation by different mechanisms. Free iron can induce neurotoxicity [50] via stimulation of Fenton reaction [51] and its levels are increased in some degenerative diseases [52–54]. SNP can cause oxidative stress and citotoxicity either by releasing cyanide and/or nitric oxide (NO) which can generate peroxynitrite [55–58]. Nitropropionic acid is thought to induce oxidative stress via inhibition of succinate dehydrogenase [59]. Although at first glance, the distinct antioxidant properties of plant extracts could indicate that they were acting via distinct mechanism. Although this can be the case, plant extracts could be inhibiting a common final (or downstream) pathway in polyunsaturated fatty acids peroxidation. Thus, we cannot exclude that a single mechanism is involved in the antioxidant of the tested extract.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical has been widely used to test the free radical scavenging ability of various natural products [60] and has been accepted as a model compound for free radicals originating in lipids [61, 62]. In the present study, the extracts obtained from *M. officinalis* exhibit lowest IC_{50} values, indicating the highest potential as free radical scavengers.

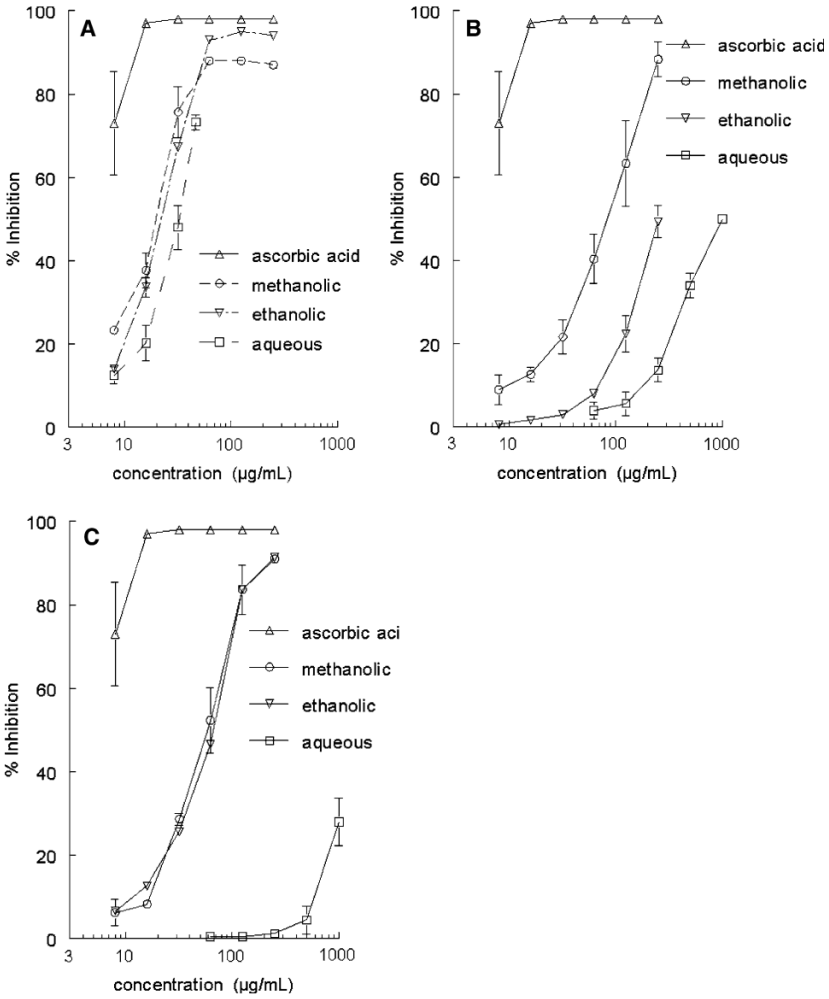

Fig. 4 Effects of different concentrations of aqueous, ethanolic and methanolic extracts from **a** *M. officinalis*, **b** *M. recutita* and **c** *C. citratus* on DPPH test. The results are expressed as percentage of inhibition and Ascorbic Acid was used as a positive control. Data show means ± SEM values average from 3 to 4 independent experiments performed in triplicate

Table 4 IC_{50} (µg/ml) values of tested plant extracts obtained by the reaction with DPPH free radical

Plants	Extractor solvent	IC_{50} (µg/ml)
M. officinalis	Water	32.9 ± 1.2^{b}
	Ethanol	28.2 ± 0.4^{a}
	Methanol	24.3 ± 2.1^{a}
M. recutita	Water	947.2 ± 22.5^{c}
	Ethanol	258.9 ± 13.3^{b}
	Methanol	115.9 ± 16.3^{a}
C. citratus	Water	1615.7 ± 302.2^{b}
	Ethanol	97.7 ± 0.2^{a}
	Methanol	85.7 ± 12.2^{a}

Different alphabets indicate statistical significance among different extracts of the same plant

Flavonoids are plant secondary metabolites widely distributed in the plant kingdom, and can be subdivided into six classes: flavones, flavanones, isoflavones, flavonols, flavanols, and anthocyanins based on their structure and conformation of the heterocyclic oxygen ring (C ring) of the basic molecule [63]. It has been demonstrated that flavonoid compounds in several aqueous extracts have very strong antioxidant and free radical scavenging activities, and are much more effective than vitamins C and E in protecting cells from free radical damage [24, 64]. Our study demonstrate the presence of flavonoid compounds in the extracts by TLC analysis, mainly in the aqueous extracts, which also presented important antioxidant activity, suggesting that these extracts could offer various health benefits, since flavonoids have been linked to

Table 5 Phenolic compounds determination in aqueous, ethanolic and methanolic extracts from *Melissa officinalis*, *Matricaria recutita* and *Cymbopogon citratus*

Plants	Extractor solvent	Phenol (nmol GA/g plant) mean ± SEM
Melissa officinalis	Water	389.65 ± 99.15[a]
	Ethanol	26.41 ± 0.09[c]
	Methanol	166.32 ± 18.92[b]
Matricaria recutita	Water	74.65 ± 12.23[a]
	Ethanol	18.71 ± 0.07[c]
	Methanol	30.01 ± 1.15[b]
Cymbopogon citratus	Water	64.24 ± 8.56[b]
	Ethanol	103.72 ± 6.43[a]
	Methanol	28.28 ± 1.60[c]

The results are expressed as nmol Gallic Acid (GA)/g dried plant. Data show means ± SEM values average from 3 to 4 independent experiments performed in triplicate. Different alphabets indicate statistical significance among different extracts of the same plant

Table 6 IC_{50} (μg/ml) values of compounds against different pro-oxidant agents- induced TBARS production in brain preparations

Compounds	Pro-oxidants		
	Iron	SNP	3-NPA
Quercetin	1.4 ± 0.03[a]	0.17 ± 0.03[a]	0.10 ± 0[a]
Gallic Acid	16.3 ± 4.5[b]	1.6 ± 0.2[b]	1.2 ± 0.2[b]
Quercitrin	12.2 ± 2.8[b]	1.4 ± 0.46[b]	1.3 ± 0.2[b]
Rutin	25.8 ± 5.8[b]	10.57 ± 2.1[c]	14.4 ± 1.3[c]

Different alphabets indicate statistical significance among different compounds against the same pro-oxidant

benefits in reducing the risk of certain cancers [22–25] and cardiovascular diseases [26–28]. Our data demonstrated also that the tested isolated compounds (flavonoids and phenolic compounds), that are present at a high quantity in plant extracts, showed an excellent activity against TBARS production induced by different agents, which promote

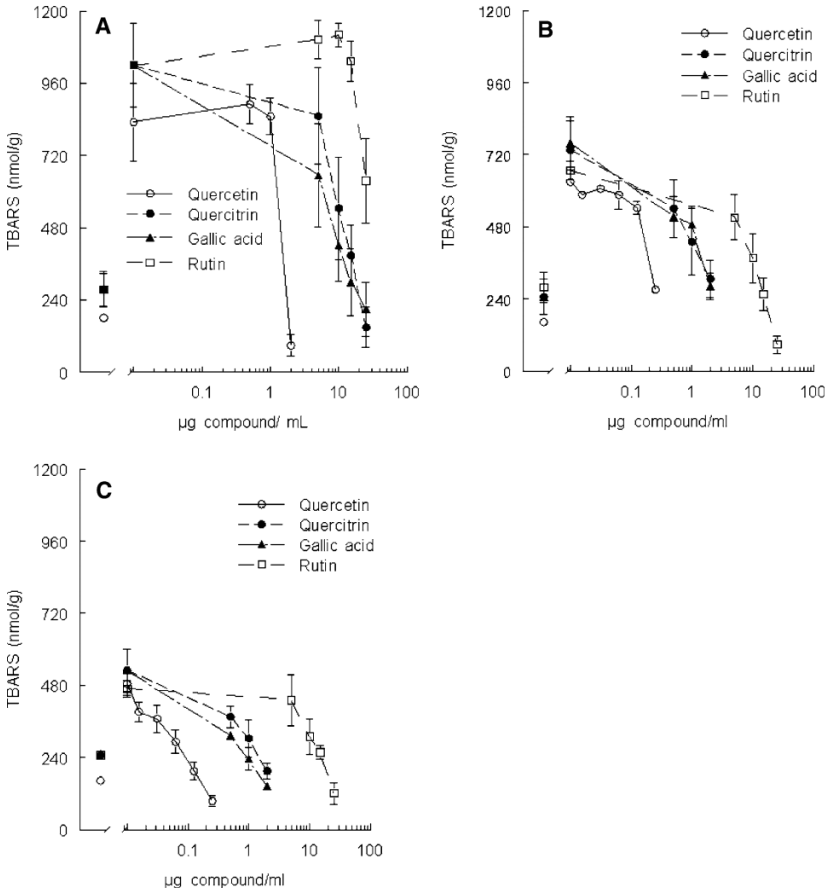

Fig. 5 Effects of different concentrations of Quercetin, Gallic Acid, Quercitrin and Rutin on **a** Iron (10 μM), **b** SNP (5 μM) or **c** 3-NPA (2 mM)-induced TBARS production in brain homogenates. The homogenates were incubated for 1 h with Iron, SNP or 3-NPA and compounds or without (basal). Data show means ± SEM values average from 3 to 4 independent experiments performed in duplicate

lipid peroxidation by different process. Quercetin was the most effective among the purified tested compounds, followed by gallic acid, quercitrin and rutin. This could be explained by the highest lipophilic characteristics of quercetin, which could increase its potency as a blocker of lipid peroxidation. In contrast, the lower antioxidant activity of rutin can be related to the presence of the glycoside hydrophilic group in its structure [65]. Interestingly, plant extracts are sources of a variety of potentially beneficial compounds, including the purified phenolic compounds tested here. The superior activity of the purified compounds in comparison with plant extracts can be explained in the basis of the lower concentration of the antioxidant compounds in the extracts. In spite of these, the use of crude plant extracts can be considered of pharmacological importance both in view of its easy availability and to the presence of different compounds that can have synergic effects in vivo.

In conclusion, all extracts tested here are effective inhibitors of TBARS production and also presented DPPH scavenger activity. In part, these effects can be related to their phenolic content, including the presence of flavonoids. Interestingly, *M. officinalis* aqueous extract presented the best antioxidant activities and the highest content of reducing agents, when compared to *M. recutita* and *C. citratus*. Consequently, this plant could be used as a potential agent for the prevention of various neurological diseases associated with oxidative damage. In line with this, recent data from literature have supported a protective role for *M. officinalis* intake against Alzheimer disease [29]. It is important emphasize that the aqueous extracts from plants tended to present highest antioxidant activities, which is the preparation used by the general population.

Acknowledgements The financial support by CAPES/SAUX/PROAP, VITAE Fundation, CNPq, FAPERGS, ICTP and FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" # 01.06.0842-00 is gratefully acknowledged.

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4.2. Composição química, atividade antioxidante e anticolinesterase da *Melissa officinalis*

Manuscrito em preparação

CHEMICAL COMPOSITION, ANTIOXIDANT AND ANTICHOLINESTERASE ACTIVITY OF *MELISSA OFFICINALIS*

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Chemical composition, antioxidant and anticholinesterase activity of *Melissa officinalis*

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Abstract

Oxidative stress is involved in the development of various important diseases, especially related with the central nervous system, such as Alzheimer's disease. Based on the innumerous benefits described for *M. officinalis* in previous studies, we investigated the chemical composition and antioxidant activity of different fractions from *M. officinalis* extract. Furthermore, we verified the effect of the most antioxidant fraction on acethylcholinesterase (AChE) activity and also investigated the effect of gallic acid, a phenolic compound present in *M. officinalis* composition, on the matrix metalloproteinase-2 (MMP-2) activity. High performance liquid chromatography (HPLC), gas chromatography coupled with mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) were used to analyze the chemical composition of the plant. TBARS, DPPH, epinephrine autoxidation were used to verify antioxidant properties. Ethyl acetate fraction presented the highest flavonoids content as well as antioxidant activity when compared with other tested fractions and it caused AChE inhibition. Moreover, our study demonstrates that gallic acid inhibited MMP-2 activity. In conclusion, *M. officinalis* ethyl acetate fraction is suggested to be further investigated for its possible use in the treatment of oxidative stress related diseases, such as Alzheimer's disease, due its antioxidant, anticholinesterase activities and the MMP-2 inhibitory capacity of their phenolic compound, gallic acid.

Keywords: Oxidative Stress; *Melissa officinalis*; Gallic Acid; Quercetin; Alzheimer's Disease.

Abbreviations: reactive oxygen species (ROS); reactive nitrogen species (RNS); oxidative stress (OS); Alzheimer's Disease (AD); matrix metalloproteinase (MMPs); thiobarbituric acid reactive substances (TBARS); acethylcholinesterase (AChE).

1. INTRODUCTION

Reactive oxygen and nitrogen species (ROS and RNS) are a class of highly reactive molecules generated by metabolic processes and by some external factors. An excessive production of ROS and RNS can lead to oxidative stress (OS), which is defined as an imbalance between generation of these species and the activity of the physiologic antioxidant defenses (Aruoma, 1998; Halliwel and Gutteridge, 1999). In OS conditions, the excessive presence of reactive species can cause DNA mutation and protein and lipid oxidation causing cellular failure and neuronal death (Finkel et al., 2000).

Of particular importance, the brain is an organ extremely susceptible to free radical damage because of its high consumption of oxygen and its relatively low concentration of antioxidant enzymes and free radicals scavengers. Consequently, OS has been directly implicated in the pathogenesis of a number of important chroni neurodegenerative diseases (Coyle and Puttfarcken,1993; Aliev et al., 2008; 2009). For instance, installation and progression of Alzheimer's disease (AD) has been linked to OS. AD is an age-related neurodegenerative disease recognized as one of the most important medical problems affecting the elderly. Brain aging is known to be related to excessive neuronal loss, decrease in ACh level, increase in inflammation and OS (Nie et al., 2009).

The "amyloid formation hypothesis" postulates that the 40 – 42 amino acid peptide amyloid-β (Aβ) fragment from β-amyloide precursor protein triggers the deposition of the senile plaques in the brain that are associated with AD development (Zhang-Nunes et al., 2006).

Recent evidence suggests that cerebrovascular abnormalities associated with AD may have been underestimated (Stopa et al. 2008). However, how AD pathology can influence or can be affected by these changes is unclear. Furthermore, the factors that cause Aβ deposits in vessels forming plaques, as well as the molecular pathways activated by vascular Aβ causing breakdown of the vessel wall are poorly understood. Among potential candidates are Aβ-induced activation of the extracellular matrix metalloproteinases (MMPs) and Aβ-induced OS (Garcia-Alloza et al., 2009).

MMPs are zinc-dependent endopeptidases with a major role in the remodeling of the extracellular matrix (Cao et al., 1995; Fu et al., 2008). Previous studies have shown that Aβ induces the expression and activity of MMP-2 in human cerebrovascular smooth muscle cells (Jung et al. 2003). Furthermore, other studies have shown that ROS can also activate MMPs (Cao et al., 1995; Haorah et al. 2007) and natural antioxidants can reduce MMP activity (Demeule et al. 2000). Consequently, antioxidant compounds could block Aβ plaques deposits formation, which can secondary to inhibition of Aβ plaques and/or OS-activated MMP. On the other hand, a recent study demonstrates decreased levels of MMP-2 in cerebral spinal fluid (CSF) of AD patients while MMP-2 in plasma of AD patients did not show any difference from controls (Horstmann et al., 2010). Thus, although some studies demonstrates evidences that MMPs play an important role in the pathogenesis of AD, and, in particular, may be involved in the processing pathway of Aβ, it is not known whether their functions are protective or destructive.

Based on “cholinergic hypothesis”, the most used treatment strategy in AD has been accepted as “cholinesterase inhibitors” that can inhibit AChE enzyme in order to increase acetylcholine level in the brain, which are deficient in AD (Perry, 1986). However,

a number of these drugs used to treat AD have been shown to produce several side effects and yield relatively modest benefits (Van Marum, 2008). To reverse these limitations of current therapeutics for AD, extensive research are in progress to identify drugs that are effective and free of undesirable side effects (Francis et al. 1999; Van Marum 2008; Dastmalchi et al. 2009).

Nevertheless, no cure is available for AD except for the symptomatic treatment. Consequently, there is still a great demand for discovery of new medical alternatives for AD treatment. Certain naturally occurring dietary phytochemicals have received considerable attention as alternative candidates for AD therapy, because of their anti-amyloidogenic, antioxidant and anti-inflammatory properties (Singh et al., 2008; Sun et al., 2010). Furthermore, literature data demonstrated MMP inhibition by a polyphenol in a brain disease model (Park et al., 2010). Accordingly, plants have been used in the treatment of cognitive dysfunction (Kennedy et al., 2002; Akhondzadeh et al., 2003). It has been shown that ethnopharmacological screening of plants may be useful in the discovery of new drugs for AD therapy (Dastmalchi et al., 2007). For instance, *Melissa officinalis* L. (Lamiaceae), has been assessed for its potential therapeutic efficacy in AD (Perry et al., 1999; Akhondzadeh et al., 2003; dos Santos-Neto et al., 2006). This plant is used in traditional medicine to prepare tea for its nerve calming effect and to treat nervous disturbance of sleep (Kennedy et al., 2004; Wheatley, 2005; 2006). A recent study of our group demonstrates that different extracts from *M. officinalis* presented a very pronounced antioxidant property against different pro-oxidants in brain homogenates (Pereira et al., 2009).

Considering the important role of OS in several neurological diseases, and the innumerous benefits described for *M. officinalis* in previous studies, it becomes interesting to further investigate its composition and to study the pharmacological properties of different *M. officinalis* extracts. So, in this study, we determine the chemical composition and antioxidant activity of different fractions from *M. officinalis* crude extract. Furthermore, we tested the effect of gallic acid, a phenolic compound found in this plant extract, on the MMP-2 activity. The potential inhibitory effect of *M. officinalis* crude extract and its fraction with higher antioxidant activity on the AChE activity was also determined.

2. METHODS

2.1. Chemicals, apparatus and general procedures

All chemicals were of analytical grade. Silica Gel 60, Silica Gel 60 F254 coated plates, solvents for the extractions and analytical procedures, dichloromethane, ethyl acetate, ethanol, methanol, n-butanol, acetonitrile, chlorogenic and caffeic acids were purchased from Merck (Darmstadt, Germany). Iron sulfate ($FeSO_4$), ascorbic acid, chloridric and acetic acid were obtained from Merck (Rio de Janeiro, RJ, Brazil). Rutin, quercetin, gallic acid, Tris–HCl, thiobarbituric acid (TBA), 1'-1' diphenyl-2' picrylhydrazyl (DPPH), malonaldehyde bis- (dimethyl acetal) (MDA) and all other reagents were obtained from Sigma (St. Louis, MO, USA).

NMR spectra were carried out on a Bruker AMX 400 spectrometer equipped with a broadband 5-mm probe, using a spectral width of 10 ppm (parts per million). Chemical shifts were expressed as ppm relative to the TMS, deuterated methanol and chloroform were used as solvent for the samples. High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto-Sampler (SIL-20A), equipped with Shimadzu LC-20 AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV–VIS detector DAD SPD-M20A and Software LC solution 1.22 SP1. Analyses in the Gas Chromatography coupled with Mass Spectrometry (GC-MS) were performed on gas chromatograph Hewlett-Packard 6890 Series Plus + equipped with an automatic split-splitless model HP 6890 Series GC Auto Sampler Controller and mass selective detector model HP 5973 MSD, using capillary chromatographic fused silica HP-5 MS (30 mx 0.32 mm internal diameter and thickness of

the film 0.25 mM) with 5% of phenyl and 95% of methylsiloxane. The carrier gas was helium (flow rate of 2 mL/min). Injector temperature was 250°C programming with a heating rate of 12ºC min-1 up to 280ºC. Ionization potential was 70 eV.

2.2. Plant collection and extractions

The plant was obtained from commercial sources. This material was macerated in the dark at room temperature with ethanol 70% for a week with daily shake-up. After filtration, the extract was evaporated under reduced pressure to remove the ethanol. The extract was suspended in water and partitioned successively with dichloromethane, ethyl acetate and n-butanol. The yield of each fraction was: 2.36% for dichloromethane fraction, 7.42% for ethyl acetate fraction and 7.95% for butanolic fraction. The aqueous extracts were obtained by infusion in hot water and they were prepared just before use.

2.3. Animals

Male Wistar rats weighing 270-320 g and with age from 3 to 3.5 months, from our own breeding colony were kept in cages of 3 or 4 animals each, with continuous access to foods and water in a room with controlled temperature (22±3 °C) and on a 12-h light/dark cycle with lights on at 7:00 am. The animals were maintained and used in accordance to the guidelines of the Brazilian Society of Association for Laboratory Animal Science (SBCAL) following the law 11.794/08.

2.4. Analysis of *M. officinalis* fractions composition by HPLC

Reversed phase chromatographic analyses were carried out under isocratic and gradient conditions using a C_{18}-column (250 mm x 4.6 mm), packed with 5-mm diameter particles; the mobile phase for rutin and quercetin was methanol/ acetonitrile/water (40:15:45 v/v/v/) containing 1% of acetic acid in isocratic conditions at flow rate of 0.9 mL/min. The phenolics acids analysis was carried out using a gradient system using Solvent A (water/ acetic acid [98:2 v/v] and solvent B [methanol]. The gradient program was started with 95% of A and 5% of B until 2 min and changed to obtain 25%, 40%, 50% and 100% B at 10, 20, 30 and 40 min, respectively, and flow rate was 0.8 mL/min. The mobile phases were filtered through a 0.45 mm membrane filter and then degassed by an ultrasonic bath prior to use. Quantifications were carried out by integration of the peaks using the external standard method, at 257 nm for gallic acid, 325 nm for caffeic and chlorogenic acids and 365 for quercetin and rutin. Identification of phenolics and flavonoids was achieved by comparing retention times and UV spectra with those of standards. Stock solutions of quercetin, rutin and gallic, caffeic and chlorogenic acids standards references were prepared in the HPLC mobile phase at a concentration range of 0.180 - 0.280 mg/mL for quercetin, 0.0125 - 0.200 mg/mL for rutin, and 0.0625 - 0.250 mg/mL for phenolics acids.

2.5. Isolation of dichloromethane fraction compounds

The chromatographic profile of the dichloromethane fraction was first investigated by TLC. A quantity of 2 µL of the fraction was spot in plate and eluted with dichloromethane: ethyl acetate: methanol (5: 3: 2) and then observed under UV light (254 and 366 nm) and sprayed with anisaldehyde-H_2SO_4/100ºC. Substances with triterpenoids

and flavonoids characteristics were visualized. This preliminary TCL screening prompts us to identify and quantify the major compounds in this fraction.

Therefore, 2.5 g of dried fraction was submitted to column chromatography on silica gel 60 using initially dichloromethane: hexane (50:50 v/v) as mobile phase and increasing the polarity by rising in 10% of dichloromethane until 100%. A new solvent system was used, starting with dichloromethane: ethyl acetate (90:10 v/v) and increasing in 10% of ethyl acetate until 100%. Finally the system started with ethyl acetate: methanol (90:10 v/v), increasing in 10% of methanol until 100%.

The procedure described above furnished ninety-eight (98) fractions of ± 50 mL each, which were analyzed by TLC and pooled together on the basis of similarities in their chromatographic profile. These fractions were combined to give two major fractions (I and II). Fraction I (sub-fractions 1–30, 0.8 g), was further purified by repeated chromatography column on silica gel 60 and eluted with dichloromethane: hexane (80: 20, v/v), to yield compounds 1 (35 mg). Fraction II (fractions 35–87, 1.3 g), was further purified by repeated column chromatography and eluted with CH_2Cl_2/EtOH (30:70 v/v), to yield compounds 2 (53 mg) and 3 (29 mg). The samples were submitted to NMR 1H and 13C analysis.

2.6. Analysis of *M. officinalis* fractions composition by GC-MS

The dichloromethane and ethyl acetate fractions were analyzed for CG-MS, all compounds were identified using the Adams MS database (Adams, 2001) and the NIST library of spectra, and additional literature data were also consulted.

2.7. TBARS

Rats were killed and the cerebral tissue was rapidly dissected and placed on ice. Tissues were immediately homogenized in cold 10 mM Tris–HCl, pH 7.5 (1/10, w/v). The homogenate was centrifuged for 10 min at 4,000 X g to yield a pellet that was discarded and a low-speed supernatant (S1) that was used for the TBARS assay. An aliquot of 100 µl of S1 was incubated for 1 h at 37ºC with freshly prepared $FeSO_4$ (10 µM) in the presence or absence of different fractions from crude extract of *M. officinalis*. Then, TBARS production was determined as described by Ohkawa et al. (1979) and Puntel et al. (2007). The fractions were tested at 5; 10; 25; 50 and 100 µg/mL.

2.6. Radical-Scavenging Activity-DPPH Assay

The antioxidant activity of the fractions from crude extract of *M. officinalis* was evaluated by monitoring their ability in quenching the stable free radical DPPH, according Choi et al. (2002) with minor modifications. Free radical scavenging capacity (FRSC) of plant extracts was calculated as their IC50 values (the concentration necessary to inhibit 50% radical formation), using the method of Dixon and Web (Oboh and Rocha, 2007). Five different ethanol dilutions of each fraction (5; 10; 50; 100 and 500 µg/ml) were mixed with 100 µL of a 0.3 mM DPPH ethanol solution. Ethanol (80 µL) plus plant extract solution was used as a blank. The absorbance was measured at 518 nm after 30 min of reaction at room temperature. DPPH was prepared daily and protected from light. Relative activities were calculated from the calibration curve of L-ascorbic acid standard solutions working in the same experimental conditions. Scavenging or inhibitory capacity in percent (IC%) was calculated using the equation: $IC\% = 100 - [(Abs_{sample} - Abs_{blank}) \times 100/Abs_{control})$ where

Abs_{sample} is the absorbance obtained in the presence of different extract concentrations and $Abs_{control}$ is that obtained in the absence of extracts. Tests were carried out in triplicate.

2.7. Epinephrine autoxidation

The potential superoxide anion scavenging activity of the *M. officinalis* aqueous extract, ethyl acetate fraction, gallic acid and quercetin were determined using the epinephrine autoxidation in an alkaline pH. Briefly, 100 µL of vehicle, ascorbic acid (100 µM) or tested samples were mixed with 2.8 mL of 50 mM of sodium carbonate buffer, pH 10.2 containing 0.1 mM of EDTA. The reaction was then initiated by adding 100 µL of epinephrine (epinephrine 10 mM prepared in HCl 10 mM) and the kinetics readings were performed at 480 nm, as described before (Misra and Fridovich, 1972).

2.8. *In vitro* effect of gallic acid or ascorbic acid on MMP-2 activity

To examine whether gallic acid directly inhibits MMP-2 activity *in vitro*, we measured human recombinant MMP-2 activity (R&D Systems, Minneapolis, MN, USA) in the absence or presence of gallic acid using the Gelatinolytic Activity Kit (E12055; Molecular Probes). Briefly, human recombinant MMP-2 (2.5 ng/µl) activity was measured using DQ gelatin (5 µg/ml; Molecular Probes) in Tris–$CaCl_2$ buffer. The activity was evaluated in a microplate spectrofluorimeter (excitation at 495 nm and emission at 515 nm; Gemini EM; Molecular Devices) after 120 min of incubation at 37°C in the absence or presence of gallic acid or ascorbic acid (0, 0.1, 1.0, 10, 50 and 100 µM), as previously described (Castro et al., 2009; Ceron et al., 2010). A standard curve of gelatinolytic activity was prepared as recommended by the manufacturer of the Gelatinolytic Activity Kit

(E12055; Molecular Probes). Phenanthroline (0.1 mmol/L) was used as positive control for MMP-2 activity inhibition.

2.9. *In vitro* AChE activity

Rats were killed and the cerebral tissue was rapidly dissected and placed on ice. Tissues were immediately homogenized in cold 50 mM Tris–HCl, pH 7.5 (1/10, w/v). The homogenate was centrifuged for 10 min at 4,000 X g to yield a pellet that was discarded and a low-speed supernatant (S1) that was used for the AChE assay. AChE was determined according to Ellman et al. (1961) modified by Rocha et al. (1993). The reaction mixture (2 ml final volume) was composed of 100 mM phosphate buffer pH 7.5, 1 mM 5,5' -dithio-bis-2-nitrobenzoic acid (DTNB). The method is based on the formation of yellow anion, 4,4'-dithio-bis-2-nitrobenzoicmeasured by absorbance at 412 nm during 5 min at 25ºC. An aliquot of 100 µl of S1 was pre-incubated in the presence or absence of plant extracts (0, 1, 10, 100 and 1000 µg/mL) or compounds (quercetin or gallic acid 0, 0.1, 1 and 10 µg/mL) for 20 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide. The enzyme activity was estimated in terms of percentage change in absorbance compared to the control. Physostigmine (Eserine) 1 µM was used as the reference standard.

2.10. Statistical Analysis

Data were statistically analyzed by one-way ANOVA, followed by Duncan's multiple range tests when appropriated (for TBARS and DPPH) or Bonferroni's multiple comparison tests (for epinephrine autoxidation, MMP-2 and AChE activities). The results were considered statistically significant for $P<0.05$.

3. RESULTS and DISCUSSION

3.1 HPLC analysis

The HPLC profile of dichloromethane, ethyl acetate and butanolic fractions from the leaves of *M. officinalis* is depicted in a representative chromatogram of each extract in Figure 1. The quantification of the major components from the extracts is depicted in Table 1. The samples of *M. officinalis* contains other minor compounds in addition to gallic acid (retention time (Rt) = 12.13 min, peak 1), chlorogenic acid (Rt = 21.71 min, peak 2), caffeic acid (Rt = 27.53 min, peak 3), rutin (Rt = 34.51 min, peak 4) and quercetin (Rt = 46.11 min, peak 5). Quercetin was the major component of dichloromethane and ethyl acetate fractions; and rutin for butanolic fraction (Table 1). Caffeic, chlorogenic and gallic acids were also detected in the extracts and their amount contents were in the order: ethyl acetate > dichloromethane > butanolic fractions.

3.2. GC-MS analysis

The dichloromethane fraction was submitted to GC-MS analysis (Figure 3) and containing propanoic acid (4.79 min, 1.93%, peak 1); propanoic acid–2-hydroxy butyl ester (7.95 min, 1.98%, peak 2); 2,5 pyrrolidinedione (11.57 min, 1.04%, peak 3); ethyl hydrogen succinate (12.71 min, 3.88%, peak 4); conhydrin (19.64min, 1.62%, peak 5); ethyl iso-allocholate (24.41 min, 5.06%, peak 6); heptatriacotonol (25.03 min, 1.33%, peak 7); acetic acid 2(2,2,6 trimethyl) 7-oxa-bicyclo hepteno (27.47 min, 2.05%, peak 8); gallic acid (31.40 min, 9.54%, peak 9), hexadecanoic acid (32.05 min, 1.97%, peak 10);

hexadecanoic acid methyl ester (33.55 min, 1. 09%, peak 11); β-sitosterol (34.64 min, 45.59%, peak 12); 9,12,15 octadecatrienoic acid methyl ester (34.78 min, 2.11%, peak 13); chlorogenic acid (34.85 min, 1.81%, peak 14); linoleic acid ethyl ester (35.10 min, 0.99%, peak 15); hexadecanoic acid, butyl ester (38.42 min, 2.97%, peak 16); lupeol (42.35 min, 0.98%, peak 17); caffeic acid (45.66min, 3.07%, peak 18); caffeine (52.95 mim. 1.26%, peak 19) and octanal (53.15 min, 2.10%, peak 20). Numerous aliphatic long-chain fatty acids such as hexadecanoic acid, hexadecanoic acid methyl ester, octadecatrienoic acid methyl ester, linoleic acid ethyl ester, and some compounds related to essential oils (heptatriacotonol and ethyl iso-allocholate) were identified. Besides caffeine, the sterols β-sitosterol and lupeol were identified in this fraction, sterols have received much attention because of their cholesterol-lowering properties, and several studies have shown a protective effect against cardiovascular disease as well as colon and breast cancer development (Ferretti et al., 2010).

The ethyl acetate fraction was submitted to GC-MS analysis as well (Figure 5), presented butanoic acid, ethyl ester (3.37 min, 4.32%, peak 1); acetic acid butyl ester (3.57 min, 3.78%, peak 2); 2,5 pyrrolidinedione (9.77 min, 0.84%, peak 3); ethyl hydrogen succinate (13.64 min, 1.45%, peak 4); butylated hydroxytoluene (20.4 min, 1.97%, peak 5); 1,6 anhydro-β-D-glucopyranose (21.11 min, 1.53%, peak 6); phenol,2 amino-6 (1-1-dimethyl ethyl) (21.54 min, 8.96%, peak 7); imidazole, 2 amino 5 (2 carboxy)- vinil (26.01 min, 8.15%, peak 8); gallic acid (31.42min, 4.86%, peak 9); hexadecanoic acid (32.07 min, 2.14%, peak 10); pterin 6 carboxylic acid (32.25 min, 2.44%, peak 11); β-sitosterol (34.72 min, 1.09%, peak 12); chlorogenic acid (34.87 min, 2.61%, peak 13); lupeol (41.27min, 0.91%, peak 14); caffeic acid (42.07 min, 5.32%, peak 15).

3.3. Isolation of compounds

After the detection and identification of specific compounds in the fractions, dichloromethane fraction was submited to further successive column chromatographic procedures, which led to the isolation of two compounds, whose structures were identified based on ^{1}H NMR and ^{13}C NMR spectra (Figure 4A-D), and by comparison with literature data (Boligon et al., 2009 a; Jayaprakasha et al., 2007). The ^{13}C NMR spectra of subfraction 1-30 showed 29 carbon atoms, two sp2 signals at δ 121.67 and 140.74 ppm, which indicates the presence of one double bonds, this signals are characteristic of steroids with a double bond at C5 and C6 (Forgo and Kövér, 2004; De-Eknankul and Potduang, 2003). The large singlet signal at δ 5.28 ppm in the ^{1}H NMR is characteristic of H-6 in the compound. Therefore, the substance 1 was characterized as β-sitosterol and this structure is shown in Figure 5. Some signs of compound 1: ^{1}NMR (400 MHz, $CDCl_3$): δH 3,46 (1H, m, H-3); 5,30 (1H, m, H-6); 1,02 (3H, s, H-19); 0,87 (3H, t, H-29). 13C-NMR (CDCl3, 100 MHz): δC 37,25 (C-1); 31,65 (C-2); 71,77 (C-3); 49,44 (C-4); 140,74 (C-5); 121,67 (C-6); 50,14 (C-9); 33,94 (C-10); 21,22 (C-11); 29,68 (C-12); 45,84 (C-13); 56,05 (C-14); 25,40 (C-15); 28,22 (C-16); 51,23 (C-17); 36,14 (C- 20); 23,08 (C-21); 33,94 (C-22); 28,22 (C-23); 19,16 (C-25); 19,80 (C-26); 19,05 (C-27).

The isolated compound 2 showed characteristics of flavonoids. Briefly, the ^{1}H NMR spectrum of compound showed two peaks at 6.12 (1H, d, J = 2.0 Hz) and 6.39 ppm (^{1}H, d, J=2.0 Hz) consistent with the meta protons H-6 and H-8 on A-ring and an ABX system at: δH 7.72 (1H, d, J = 2.1 Hz, H-20), 7.62 (1H, dd, J = 8.4, 2.1 Hz, H-60) and 6.87 (1H, d, J = 8.4 Hz, H-50) corresponding to the catechol protons on B-ring. The 13C NMR spectrum

indicated the presence of 15 carbon atoms, the signal at: δ 177.3 was attributed to a carbonyl carbon placed at C-4, the other signals were: 165.92 (C-7), 162.47 (C-5), 158.24 (C-9), 121.63 (C-6'), 116.32 (C-5'), 116.00 (C-2'), 99.79 (C-6), 94.67 (C-8), the spectral data were compatible with those of quercetin (Boligon et al., 2009 b; Fossen et al., 1998; Liu et al., 2008; Slimestad, 2003), the structure is shown in Figure 5.

3.4. *In vitro* effects of *Melissa officinalis* crude extract and fractions on iron-induced cerebral TBARS production

Considering that the brain is particularly susceptible to free radical damage, we used cerebral tissue for the TBARS assay. Hydroalcoholic extract and different fractions from *M. officinalis* significantly inhibited iron-induced TBARS production in brain preparations ($P < 0.001$). However, the inhibitory potency of the different fractions and crude extract varied. Ethyl Acetate fraction presented the highest inhibition relative to the other tested fractions, since it caused a TBARS decrease to the basal levels starting at 10 μg/mL (Figure 6). This result is in accordance with the phenolic and flavonoids composition of the fractions measured by HPLC (Table 1), because ethyl acetate fraction presented the highest content of majority of detected compounds, which have a very high antioxidant activity as demonstrated in the literature (Vinson et al., 1995; Wiseman et al., 1997; Wagner et al., 2006; Pereira et al., 2009;).

3.5. DPPH radical-scavenging activity of *Melissa officinalis* extract and fractions

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical has been widely used to test the free radical scavenging ability of various natural products (Brand et al., 1995) and has been accepted as a model compound for free radicals originating in lipids (Hatano et al., 1889; Yasuda et al., 2000).

Hydroalcoholic extract and different fractions from *M. officinalis* presented a significant DPPH radical-scavenging activity ($P < 0.001$). Ethyl acetate and dichloromethane fractions promoted a DPPH radical inhibition with a similar potency to that of positive control (ascorbic acid) (Figure 7 and Table 2). In accordance with data obtained in TBARS assay, ethyl acetate fraction presented the lowest IC50 value, probably due its high flavonoids content demonstrated in HPLC analysis (Table 1).

3.6. Effect of *M. officinalis* aqueous extract, ethyl acetate fraction, gallic acid and quercetin on epinephrine autoxidation

Here we tested *M. officinalis* aqueous extract (which presented the highest antioxidant activity in our previous study; Pereira et al., 2009), ethyl acetate fraction (which present the highest antioxidant activity determined in the present study), and two phenolic compounds, gallic acid and quercetin, present in *M. officinalis* extract as potential inhibitors of epinephrine autoxidation. *M. officinalis* aqueous extract and ethyl acetate fraction caused a significant inhibition of epinephrine autoxidation only at 1mg/mL. Gallic acid caused a significant inhibition of epinephrine autoxidation at 100 μg/mL. However, quercetin did not change epinephrine autoxidation (Figure 8).

3.7. Effect of gallic acid or ascorbic acid on human recombinant MMP-2 activity

Increased concentrations of ROS have been implicated as a cause of MMPs activation, because ROS react with thiol groups, which preserve MMP latency (Van Wart and Birkedal-Hansen, 1990). Recent studies indicated that MMPs plays an important role in AD (Garcia-Alloza et al., 2009). Furthermore, literature data demonstrate a polyphenol (compound with antioxidant activity) causing MMP inhibition. So, we evaluated the effect of gallic acid, a phenolic compound with recognized antioxidant activity (Pereira et al. 2009) on human recombinant MMP-2 activity. We have also determined the effect of ascorbic acid, which is frequently used as a standard antioxidant compound in several models, on MMP-2 activity.

As show in Figure 9A, ascorbic acid had no significant effect on human recombinant MMP-2 activity. However, gallic acid inhibited MMP-2 activity ($P<0.01$), at 50 and 100 μM (Figure 9B), similarly to positive control (phenanthroline). This result is very interesting and could be further investigated to determine the effect of gallic acid, as a possible potential drug in the treatment of AD. These results also suggest that the MMP-2 inhibitory effect of gallic acid seems not to be directly related with its antioxidant activity because ascorbic acid, a strong antioxidant compound, did not alter MMP-2 activity. However, more studies are needed to elucidate the exact mechanism involved in this inhibition.

3.8. Effect of *M. officinalis* on AChE activity

Based on the cholinergic hypothesis of AD, acetylcholinesterase inhibitors are used for alleviating the symptoms of patients with AD, because it can increase the cholinergic transmission by blocking the degradation of ACh, which levels are known to be decreased in AD. (Perry, 1986). So, considering that OS is involved in pathogenesis of AD and also occurs a decrease in ACh levels, we tested the effect of *M. officinalis* aqueous extract and ethyl acetate fraction on AChE activity.

M. officinalis aqueous extract did not change AChE activity (Figure 10A),. Conversely, ethyl acetate fraction significantly inhibited this enzyme when compared with control in a concentration dependent manner (figure 10B). So, we evaluated the effect of quercetin (Figure 10D) or gallic acid (figure 10C) on AChE activity and we did not found any inhibition. Although the inhibitory effect of a minority compound can not be ruled out, the effect observed for ethyl acetate fraction could be attributed to the synergistic effect or interaction between different constituents of the fraction. This data indicate that *M. officinalis* can be considered as potential alternative medicine for the AD treatment. Moreover, this result is in accordance with literature data that also demonstrate AChE inhibition by crude extracts from *M. officinalis* (Ferreira et al., 2006; Dastmalchi et al., 2009).

4. CONCLUSION

Our previous study demonstrates that different crude extracts of *M. officinalis* present a highest antioxidant activity when compared with other popularly used plants (Pereira et al. 2009). So, in the present study, we better investigate the properties of *M. officinalis*, analyzing antioxidant activity and chemical composition of different fractions from its crude extract. Furthermore, we demonstrated the *in vitro* inhibitory effect of an important phenolic compound constituent of this plant, gallic acid, against the MMP-2 activity. We have also demonstrated that the ethyl acetate fraction from *M. officinalis* presented the highest flavonoids content and antioxidant properties. Furthermore, the ethyl acetate fraction also exhibited a moderate inhibition of cerebral AChE. In conclusion, *M. officinalis* could be further investigated for its possible use in the treatment of AD, due its very pronounced antioxidant activity and anticholinesterase activity. In addition, gallic acid, a phenolic compound present in *M. officinalis* extract could be also a promising drug in the treatment of AD due its antioxidant and MMP-2 inhibitory activities showed here.

Acknowledgements

The financial support by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (014/2006) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) is gratefully acknowledged. The authors would like to thank Maria Angélica da Silveira Lima (Chemistry Department of Federal University of Santa Maria) for providing the NMR Spectra.

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LEGENDS FOR FIGURES

Figure 1) High performance liquid chromatography flavonoids profile of dichloromethane (a), ethyl acetate (b) and butanolic (c) fractions of the *Melissa officinalis*. Rutin (peak A) and quercetin (peak B).

Figure 2) High performance liquid chromatography phenolics profile of dichloromethane (a), ethyl acetate (b) and butanolic (c) fractions of the *Melissa officinalis*. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3) and not identified (peak 4).

Figure 3A) GC-MS dichloromethane fraction of *Melissa officinalis*. B) GC-MS ethyl acetate fraction of *Melissa officinalis*.

Figure 4A) ^{13}C –NMR spectra (400 MHz, $CDCl_3$): β-sitosterol. B) ^{1}H-NMR spectra (400 MHz, $CDCl_3$): β-sitosterol. 10C) ^{13}C –NMR spectra (100 MHz, CD_3OD): Quercetin.10D) ^{1}H-NMR spectra (400 MHz, CD_3OD): Quercetin.

Figure 5) Chemical structures of isolated compounds from dichloromethane fraction. β-sitosterol (**1**) and quercetin (**2**).

Figure 6) Effects of different concentrations of hydroalcoholic extract, ethyl acetate, dichlorometane and butanolic fractions from *M. officinalis* on Fe(II) (10 μM) - induced TBARS production in brain homogenates. The homogenates were incubated for 1 h with Iron and the

extracts or without (basal). Data show means ± SEM values average from 3 to 6 independent experiments performed in duplicate.

Figure 7) Effects of different concentrations of hydroalcoholic extract, ethyl acetate, dichlorometane and butanolic fractions from *M. officinalis* on DPPH test. The results are expressed as percentage of control and Ascorbic Acid was used as a positive control. Data show means ± SEM values average from 3 independent experiments performed in triplicate.

Figure 8A) *In vitro* effects of *M. officinalis* ethyl acetate fraction (1 to 1000 μg /mL), aqueous extract (1 to 1000 μg/mL) (B), quercetin (1 to 100 μM) (C) and gallic acid (1 to 100 μM) (C) on autoxidation of epinephrine. Data are shown as means ± SEM of three experiments (# $p < 0.05$ versus control). Ascorbic Acid was used as a positive control.

Figure 9A) *In vitro* effects of Ascorbic Acid (0.0, 0.1, 1.0, 10, 50 and 100 μM) and Gallic Acid (0.0, 0.1, 1.0, 10, 50 and 100 μM) (9B) on human recombinant MMP-2 activity. Human recombinant MMP-2 activity was measured using a Gelatinolytic Activity Kit, in the absence or presence of Ascorbic Acid or Gallic Acid. Phenanthroline (Phe) was used as positive control for MMP-2 activity inhibition (# $p < 0.01$ versus control). Data are shown as means ± SEM of three experiments done in duplicate.

Figure 10A) *In vitro* effects of *Melissa officinalis* aqueous extract (0.0, 1, 10, 100 and 1000 μg/mL), *Melissa officinalis* ethyl acetate fraction (0.0, 1, 10, 100 and 1000 μg/mL) (10B), gallic acid (0.0, 0.1, 1 and 10 μg/mL) (10C) and quercetin (0.0, 0.1, 1 and 10 μg/mL) (10E) on AchE activity. Eserine was used as positive control for AChE activity inhibition (# $p < 0.05$ versus control). Data are shown as means ± SEM of three independent experiments.

Figure 1

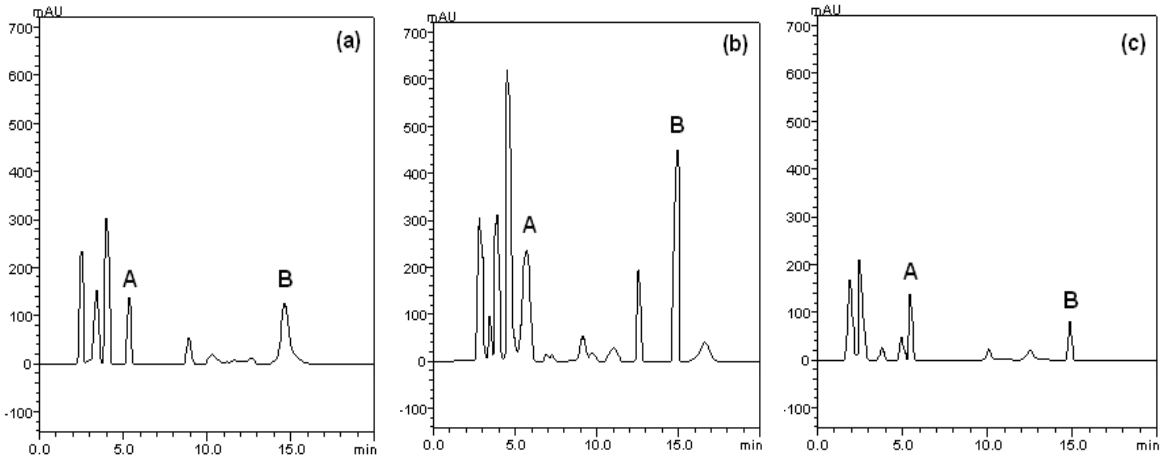

Figure 2

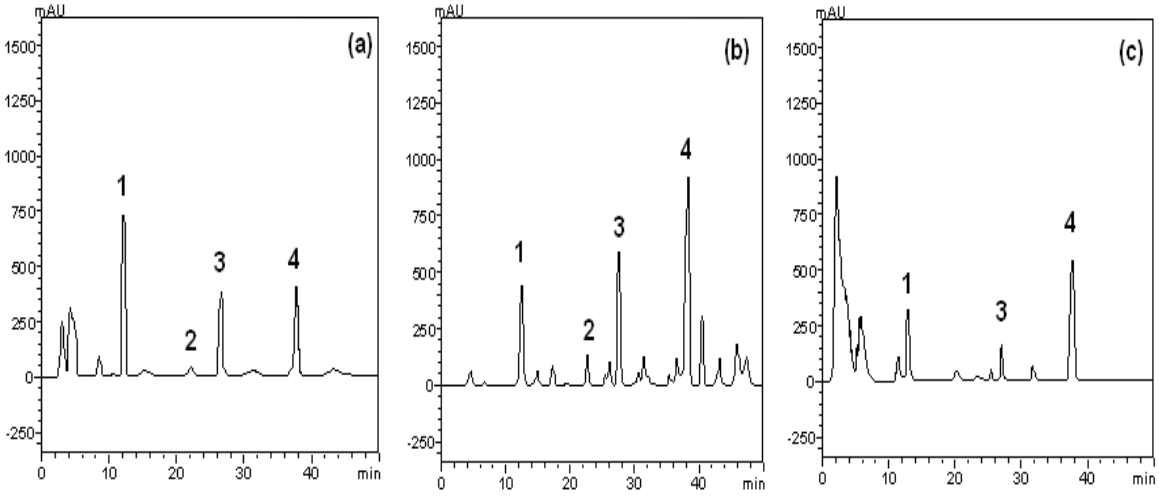

Table 1 - Phenolics and flavonoids composition of *Melissa officinalis*:

Compounds	Dichloromethane Fraction	Ethyl Acetate Fraction	Butanolic Fraction
Gallic Acid (%)	9.66 ± 0.05	4.91 ± 0.04	3.59 ± 0.01
Chlorogenic Acid (%)	1.83 ± 0.09	2.63 ± 0.10	-
Caffeic Acid (%)	3.10 ± 0.12	5.32 ± 0.09	1.36 ± 0.02
Rutin (%)	9.52 ± 0.03	16.81 ± 0.03	6.97 ± 0.07
Quercetin (%)	11.19 ± 0.01	19.22 ± 0.01	4.99 ± 0.05

Results are expressed as mean ± SEM of three determinations.

Figure 3A

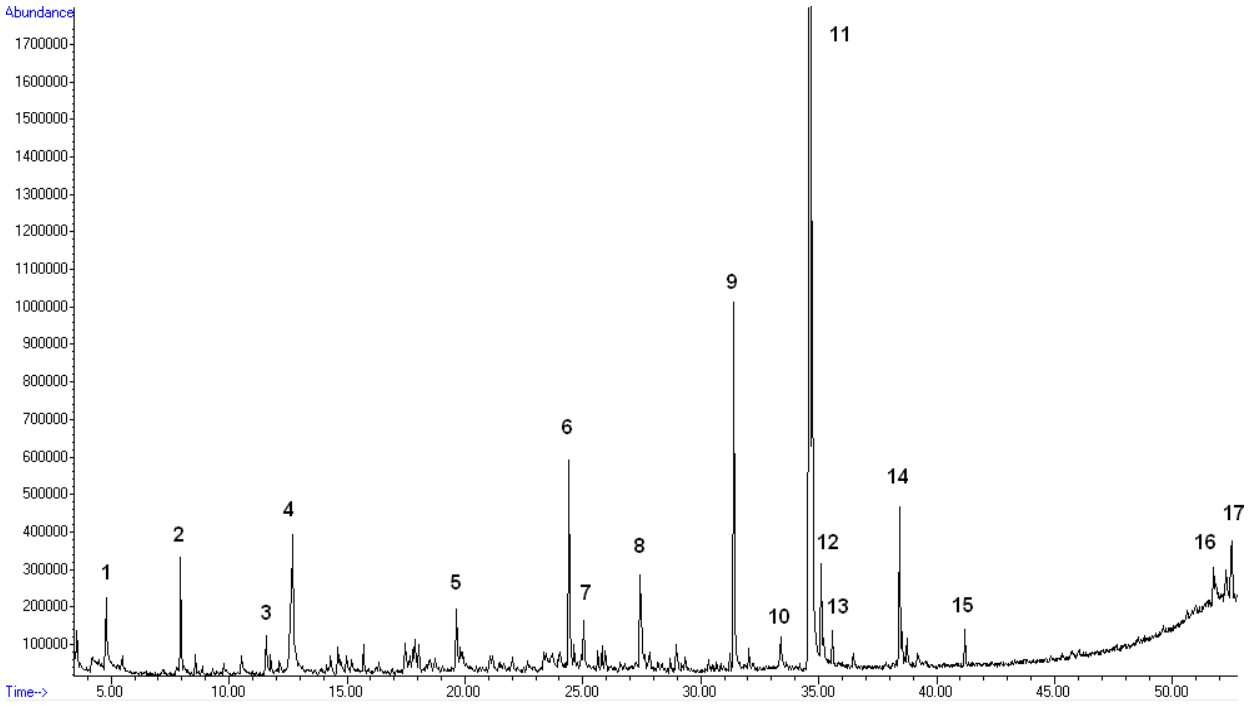

Figure 3B

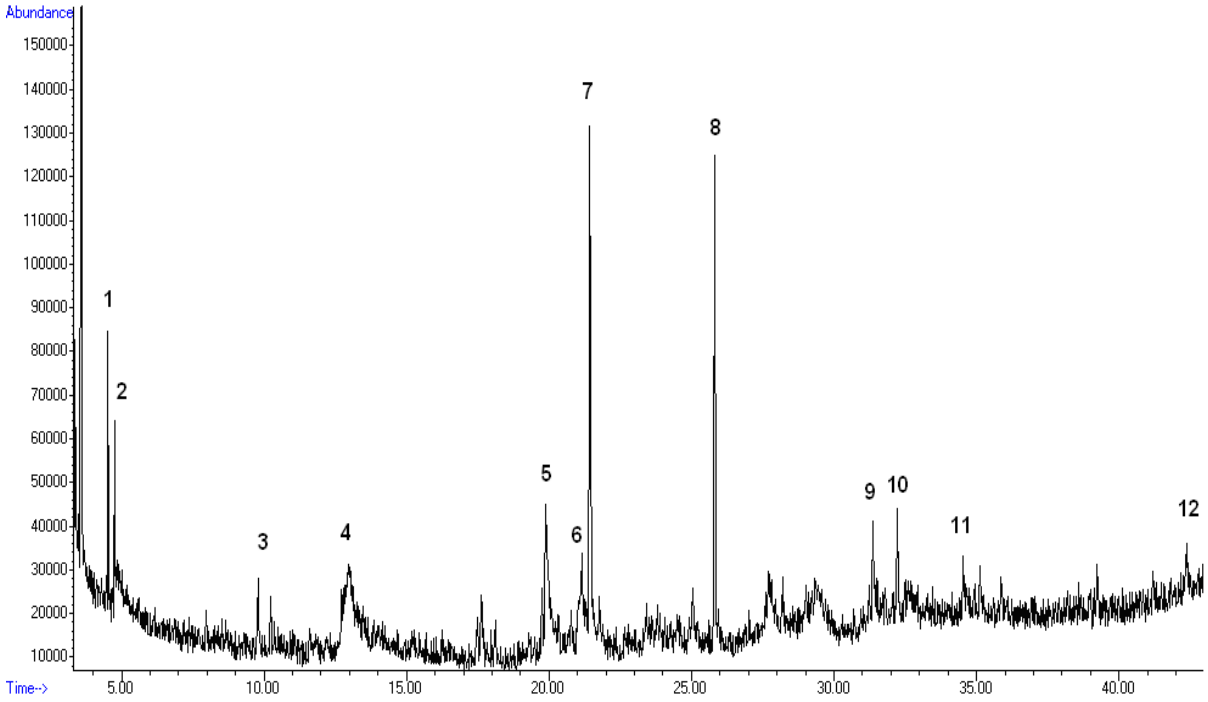
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Figure 4A

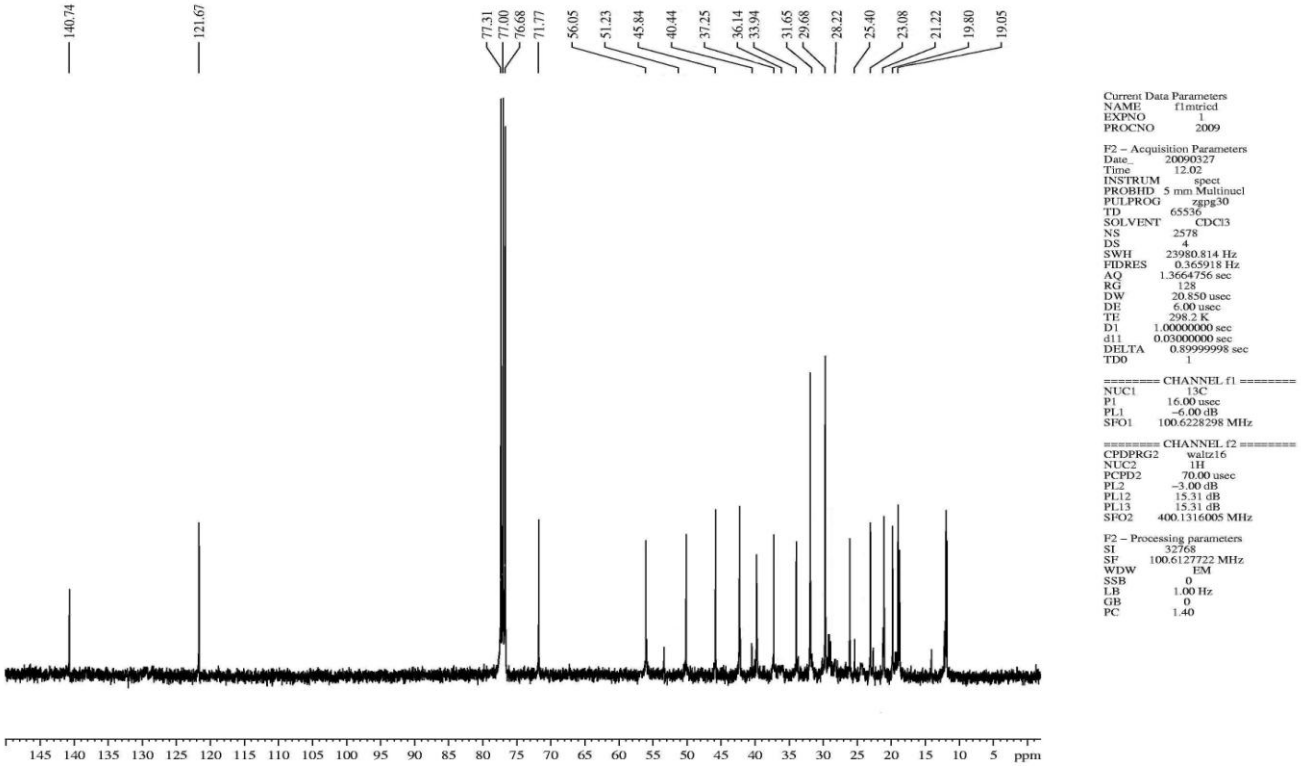

Figure 4B

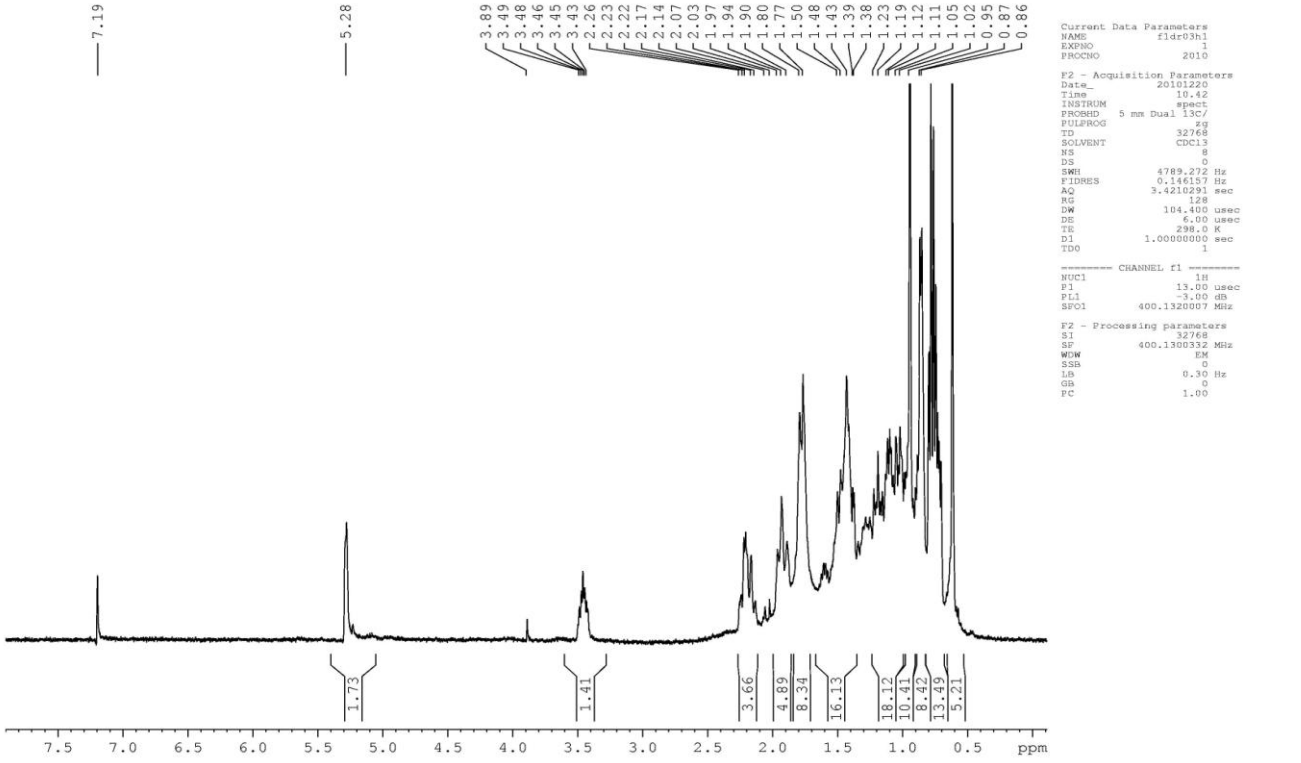

Figure 4C

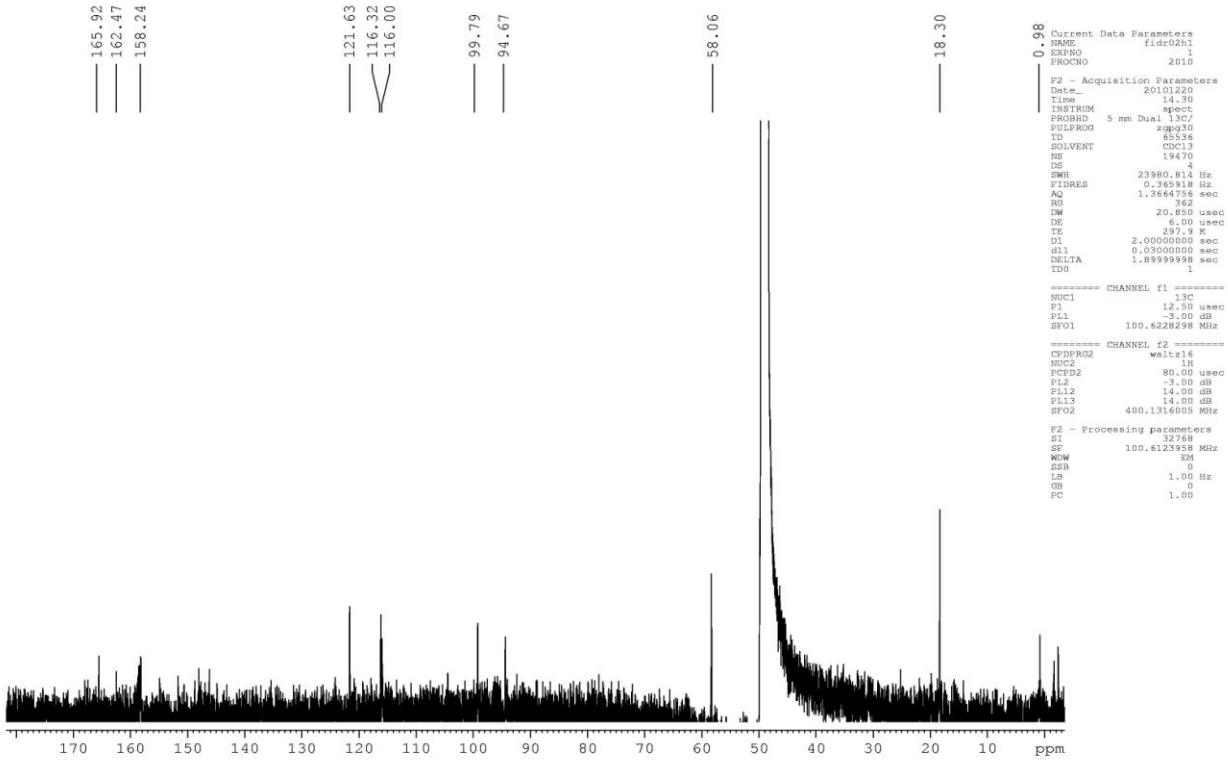

Figure 4D

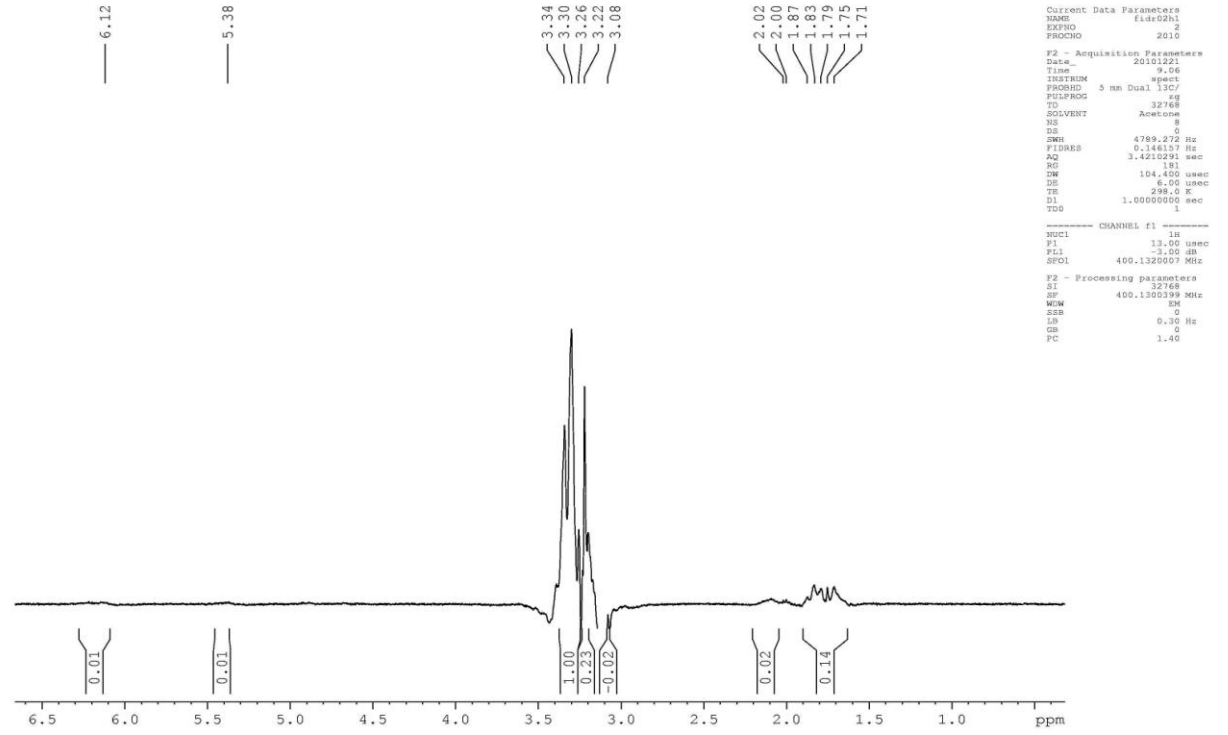

Figure 5

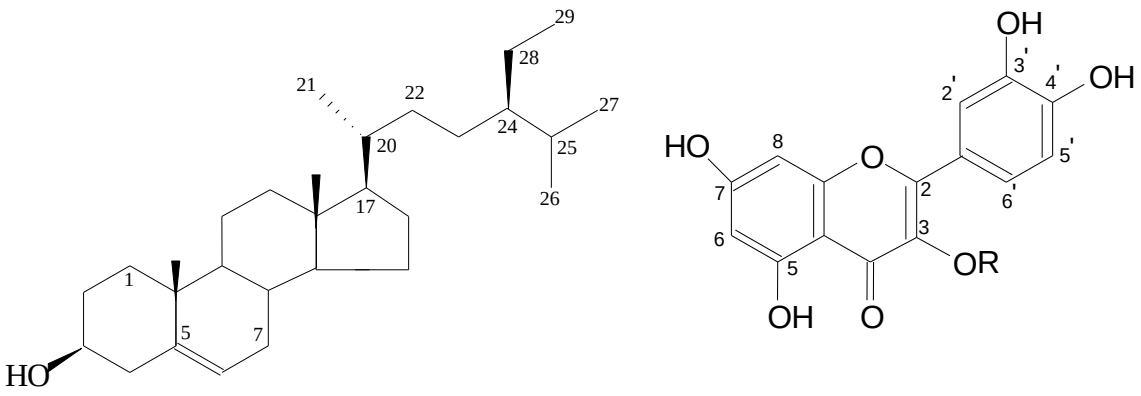

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Figure 6

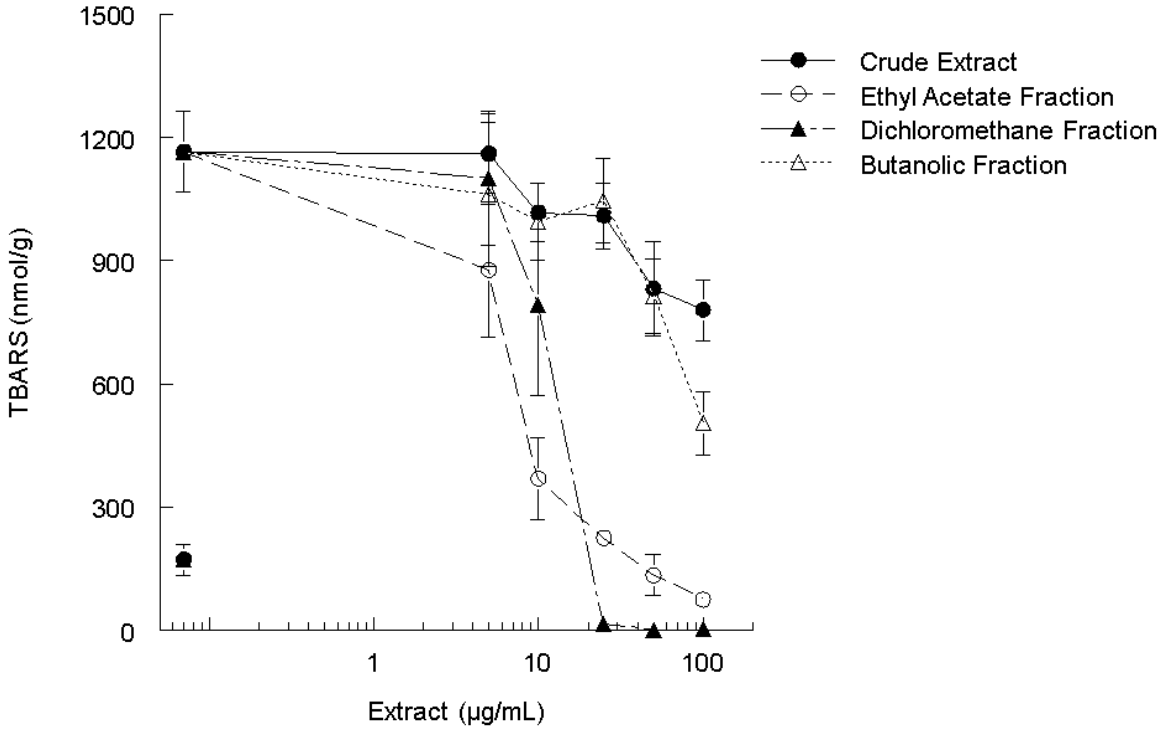


Figure 7

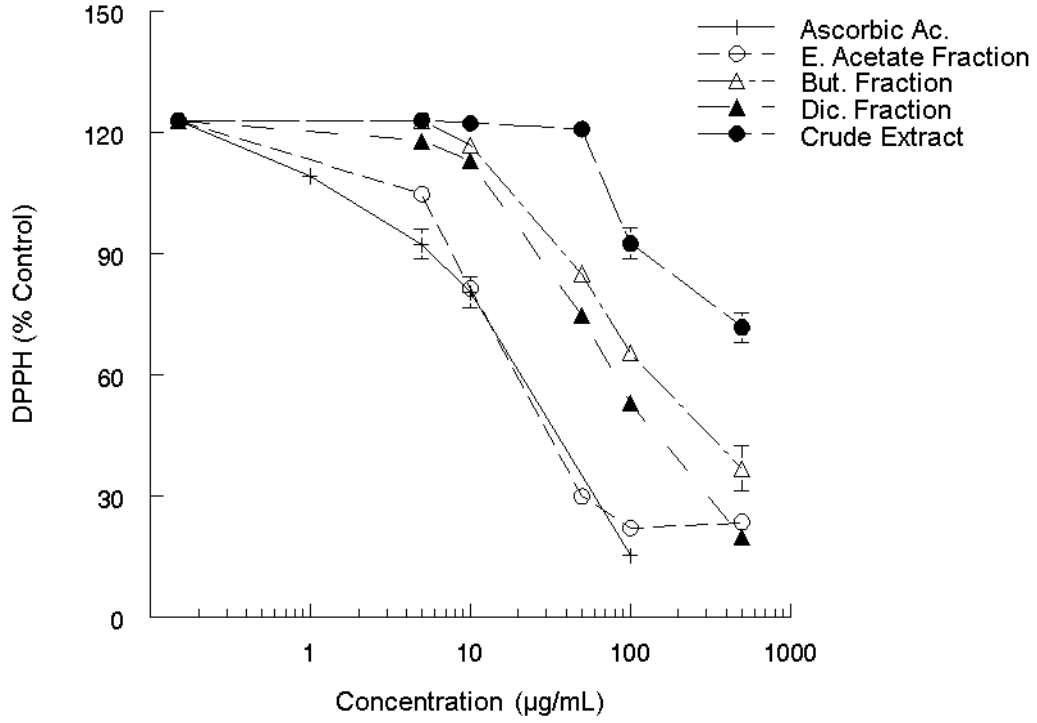


Table 2 - IC_{50} (µg/ml) values for inhibition by *M. officinalis* crude extract and fraction of DPPH. Data show means ± SEM values average from 3 independent experiments:

Ethyl Acetate Fraction	Dichloromethane Fraction	Butanolic Fraction	Crude Extract	Ascorbic Acid
30.413 ± 0.568	125.684 ± 12.5	384.7745 ± 62.3745	379.985 ± 50.445	56.105 ± 1.925
a	a	b	B	a

Different alphabets indicate statistical significance among different fractions, extract and ascorbic acid.

Figure 8A

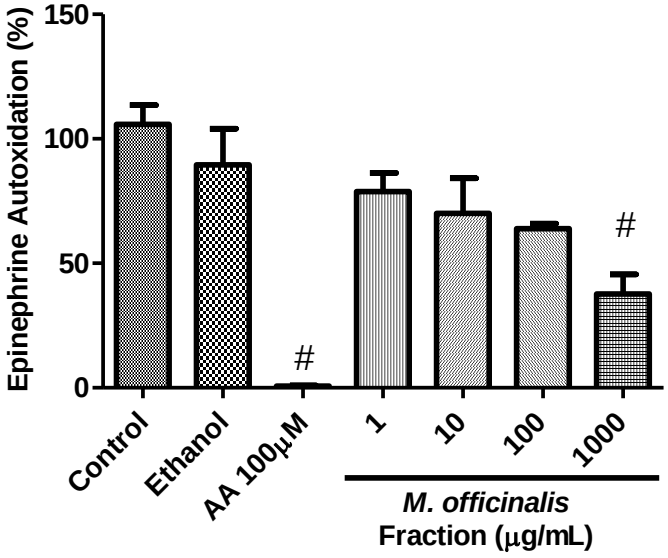


Figure 8B

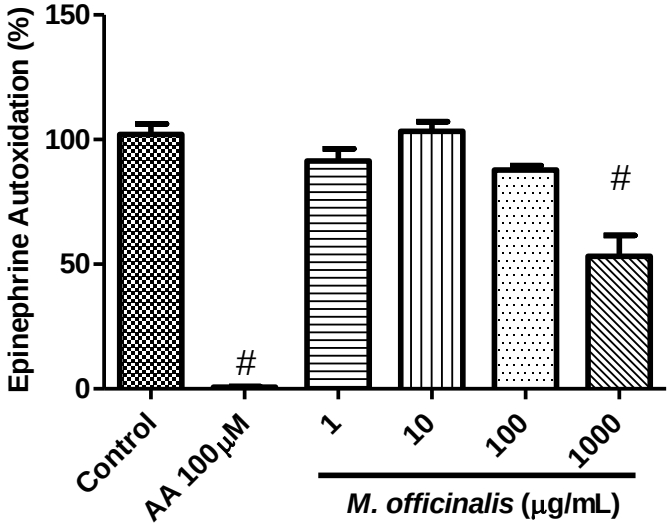


Figure 8C

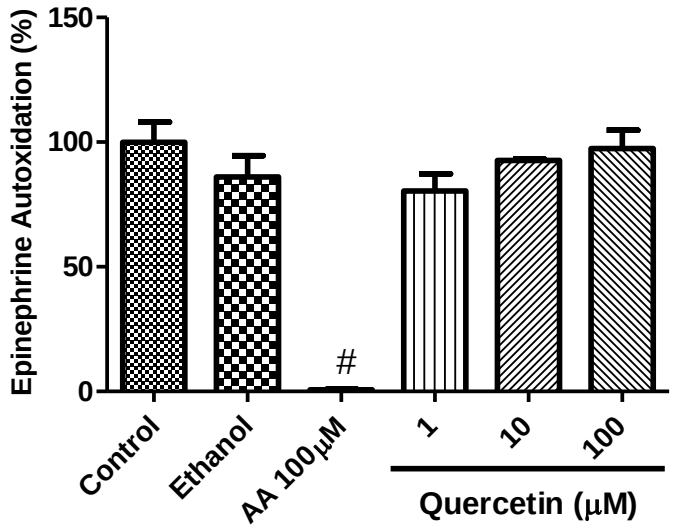


Figure 8D

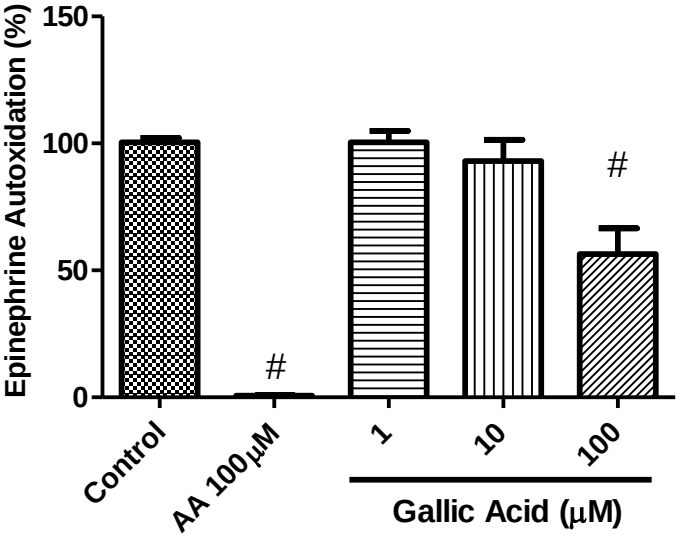


Figure 9A

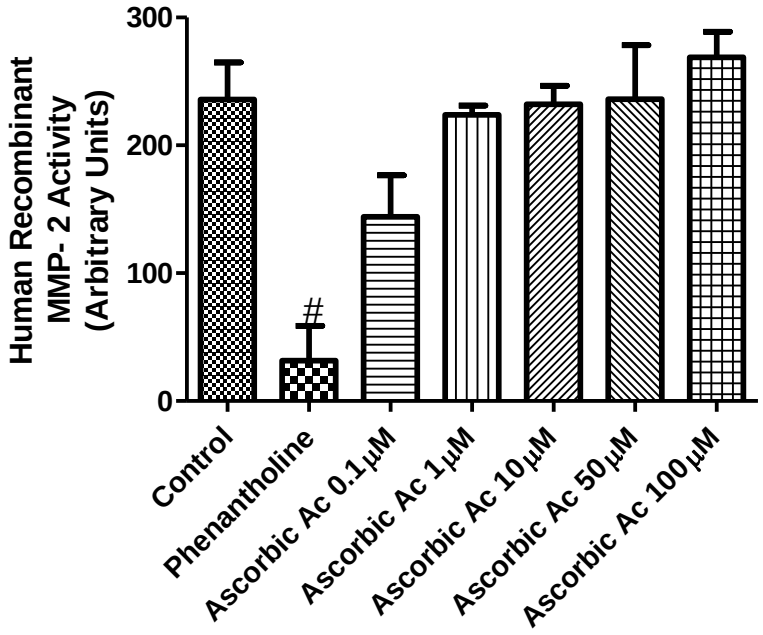


Figure 9B

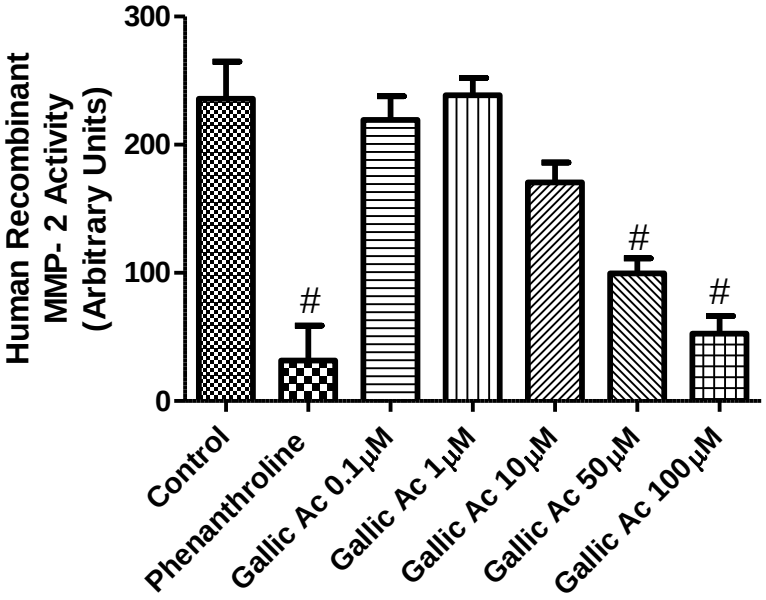


Figure 10A

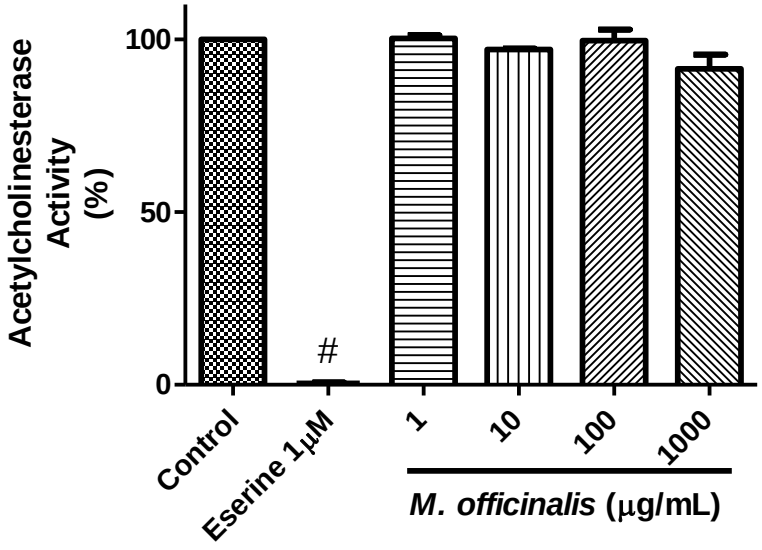


Figure 10B

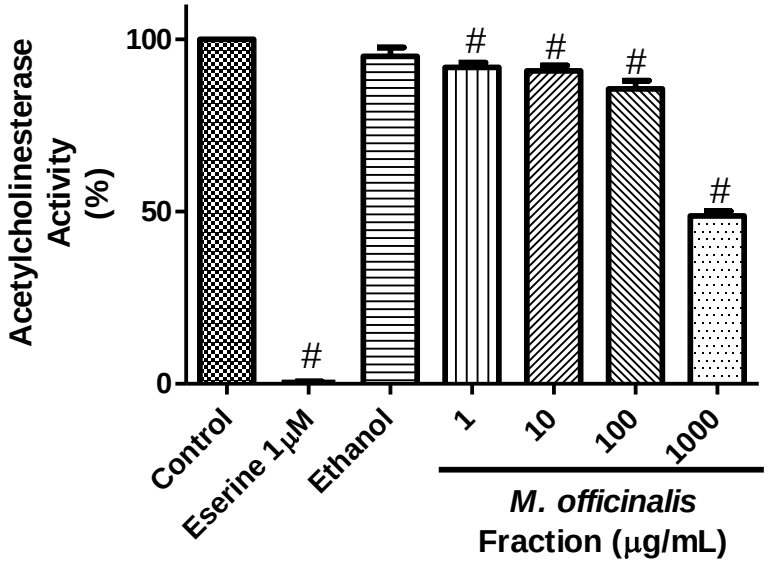


Figure 10C

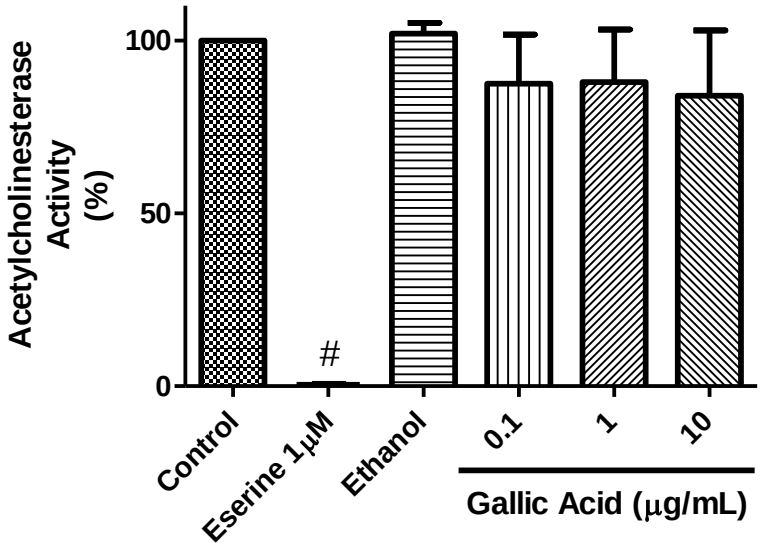
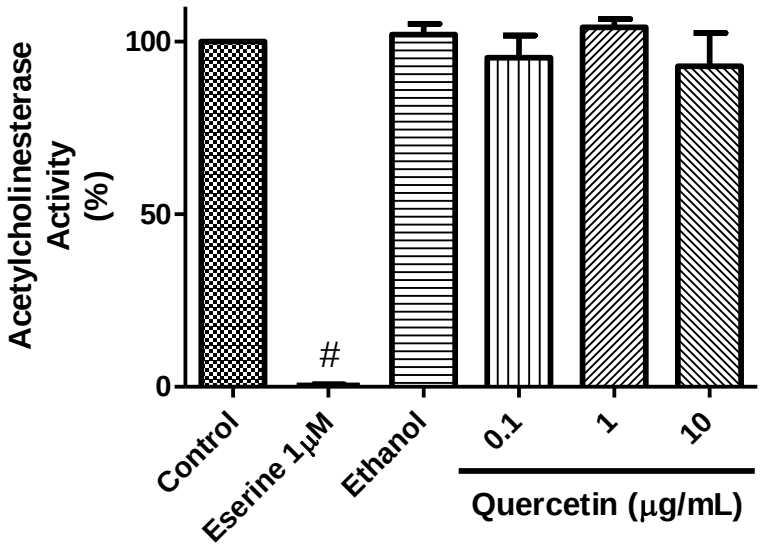


Figure 10D



5. DISCUSSÃO

Na primeira etapa do presente estudo, foi avaliado o efeito antioxidante e a composição química de diferentes extratos de três espécies de plantas utilizadas popularmente, *M. officinalis, M. recutita* e *C. citratus* contra agentes pró-oxidantes bem conhecidos, a fim de encontrar novas substâncias com potencial antioxidante para o possível uso na prevenção e/ou tratamento de doenças. Considerando a alta suscetibilidade do cérebro ao dano causado por RLs e a relação do EO com o desenvolvimento de doenças neurodegenerativas (Halliwell, 2006), foi escolhido o tecido cerebral para avaliar o potencial antioxidante das plantas frente a diferentes agentes pró-oxidantes através do ensaio de TBARS. Além disso, determinou-se a quantidade de compostos fenólicos nos extratos de plantas para avaliar uma possível relação destas substâncias com a atividade antioxidante. Estes compostos são metabólitos de plantas que têm despertado bastante interesse em várias pesquisas devido às suas atividades antioxidante, antiinflamatória e antimutagênica (Pereira e cols., 1996; Yang e cols., 1999; Thompson, 2000; Atoui e cols., 2005; Geetha e cols., 2005).

Neste trabalho, o extrato aquoso da planta *M. officinalis* apresentou a maior atividade contra a produção de TBARS induzida por todos os agentes pró-oxidantes testados, quando comparado com os extratos etanólico e metanólico. Além disso, a inibição da peroxidação lipídica pela *M. officinalis* mostrou uma relação com o seu conteúdo de compostos fenólicos. Porém, no ensaio do DPPH, os três diferentes extratos obtidos desta planta (aquoso, metanólico e etanólico) apresentaram um efeito semelhante entre si. No

caso das plantas *M. recutita* e *C. citratus*, a potência inibitória no ensaio de TBARS variou dependendo do pró-oxidante utilizado. Neste caso, a relação entre a atividade antioxidante e o conteúdo de compostos fenólicos não ficou clara. No DPPH, o extrato metanólico da *M. recutita* apresentou IC50 menor do que os extratos etanólico e aquoso. Além disso, o potencial para eliminar RLs não teve relação com a concentração de compostos fenólicos. Os extratos metanólico e etanólico da planta *C. citratus* apresentaram valores de IC50 menores que o extrato aquoso. Neste caso, o potencial para eliminar RLs também não apresentou relação com a concentração de compostos fenólicos.

Os agentes pró-oxidantes aqui utilizados induzem peroxidação lipídica por diferentes mecanismos. O ferro pode induzir neurotoxicidade (Bostanci e Bagirici, 2008) através da reação de Fenton (Fraga e Oteiza, 2002) e seus níveis encontram-se aumentados em algumas doenças neurodegenerativas (Aisen e cols., 1999; Qian e Wang, 1997; Swaiman, 1991). O NPS pode induzir EO e citotoxicidade pela liberação de cianeto e/ou ON, que pode gerar peroxinitrito (Arnold e col., 1984; Bates e cols., 1990; Huie e Padmaja, 1993; Pryor e Squadrito, 1995). O ácido nitropropiônico induz EO via inibição da enzima succinato desidrogenase (Alston e cols., 1977). Neste contexto, as variações nas propriedades antioxidantes dos extratos de plantas poderiam indicar que eles atuam por mecanismos distintos. Por outro lado, não se pode excluir a possibilidade de um mecanismo comum entre os extratos, como a inibição de uma rota final da peroxidação de ácidos graxos poli-insaturados.

O radical DPPH tem sido amplamente utilizado para testar a habilidade de vários produtos naturais em eliminar RLs (Brand e cols., 1995). Neste estudo, o extrato obtido da

M. officinalis exibiu os menores valores de IC50, indicando maior capacidade de eliminar RLs.

Os flavonóides são metabólitos secundários amplamente distribuídos nas plantas. Dados da literatura mostram que flavonóides de vários extratos aquosos apresentam uma atividade antioxidante bem pronunciada e atividade de eliminar RLs, sendo mais efetivos que as vitaminas C e E na proteção das células contra os danos causados por RLs (Wiseman e cols., 1997; Vinson e cols., 1995). O presente estudo mostra a presença de flavonóides nos extratos por análises de cromatografia em camada delgada (CCD), principalmente nos extratos aquosos, os quais também apresentaram considerável atividade antioxidante, sugerindo que estes extratos poderiam oferecer vários benefícios à saúde, visto que os flavonóides têm mostrado benefícios relacionados à redução no risco de certos cânceres (Dreostic e cols., 1997; Jankun e cols., 1997; Wiseman e cols., 1997; Hertog e cols., 1993) e doenças cardiovasculares (Au Kono e cols., 1996; Tijburg e cols., 1997; Pietrovski e cols., 2006). Os dados deste estudo mostram também que os compostos purificados exerceram uma excelente atividade contra a produção de TBARS por diferentes agentes. A quercetina foi a mais efetiva entre os compostos testados, seguida pelo ácido gálico, quercetina e rutina. Isto poderia ser explicado pelas características lipofílicas da quercetina, que melhoram seu acesso aos lipídios da membrana, aumentando assim sua potência como bloqueador da peroxidação lipídica. Neste contexto, a menor atividade antioxidante da rutina, pode estar relacionada com a presença de um glicosídeo em sua estrutura, tornando-a mais hidrofílica (Saija e cols., 1995). De maneira interessante, extratos de plantas são fontes de uma variedade de compostos potencialmente benéficos, incluído os compostos fenólicos aqui testados. A atividade antioxidante superior dos compostos purificados em

comparação com os extratos de plantas pode ser explicada com base na menor concentração dos compostos antioxidantes nos extratos. Apesar disso, o uso de extratos de plantas pode ter considerável importância farmacológica devido ao seu fácil acesso e à presença de diferentes compostos que podem ter efeitos sinérgicos *in vivo*. Em conjunto, estes dados mostram que apesar de todos os extratos testados terem sido inibidores efetivos da produção de TBARS e causarem eliminação do radical DPPH, o extrato aquoso da planta *M. officinalis* apresentou a maior atividade antioxidante e o maior conteúdo de agentes redutores. Este dado é bastante interessante, uma vez que o extrato aquoso ou chá de plantas é a forma de preparação mais utilizada pela população em geral. Além disso, a planta *M. officinalis* parece ser um agente promissor na prevenção de várias doenças neurodegenerativas associadas com o EO.

Baseado nas análises acima expostas, que mostram que o extrato aquoso da planta *M. officinalis* apresentou a melhor atividade antioxidante, foi realizada a segunda etapa deste estudo, onde as propriedades da *M. officinalis* foram investigadas em maior detalhe, analisando a atividade antioxidante e a composição química de diferentes frações e do extrato bruto desta planta. Considerando que o EO está envolvido na patogênese da DA e que o ácido gálico tem uma atividade antioxidante bem estabelecida, foi testado o efeito inibitório *in vitro* deste composto fenólico contra a atividade da enzima MMP-2. Vários trabalhos apontam para o fato de que esta enzima está alterada em pacientes com DA (Garcia-Alloza e cols., 2009; Horstmann e cols., 2010). Além disso, investigou-se o efeito do extrato e da fração mais antioxidante da *M. officinalis* na atividade da AChE. Fármacos capazes de inibir a enzima AChE são a principal estratégia de tratamento utilizada

atualmente na DA, devido à deficiência do neurotransmissor ACh característica desta doença (Francis e cols., 1999; Perry, 1986).

A fração acetato de etila apresentou maior conteúdo de flavonóides e atividade antioxidante quando comparado com as outras frações testadas. Além disso, esta fração causou uma inibição moderada da AChE cerebral. O composto fenólico ácido gálico inibiu a atividade da enzima MMP-2.

O efeito anticolinesterásico e o efeito antioxidante bem pronunciado da fração acetato de etila são resultados bastante interessantes, uma vez que o EO e a deficiência de Ach fazem parte do desenvolvimento da DA. Além disso, as drogas anticolinesterásicas disponíveis atualmente provocam sérios efeitos colaterais e poucos benefícios (Van Marum, 2008; Schulz, 2003; Melzer, 1998), tornando necessária a descoberta de novas drogas com melhores resultados para este propósito.

A partir dos resultados deste estudo, sugere-se que a fração acetato de etila da planta *M. officinalis* poderia ser submetida a maiores investigações considerando o seu possível uso no tratamento da DA, devido às suas atividades antioxidante e anticolinesterase e à capacidade do ácido gálico, composto fenólico presente em sua composição, inibir a MMP-2.

6. CONCLUSÕES

Baseando-se nos resultados apresentados nesta tese, pode-se concluir o seguinte:

→ Todos os extratos (etanólico, metanólico e aquoso) das três diferentes plantas estudadas (*Melissa officinalis, Matricaria recutita* e *Cymbopogon citratus*), foram capazes de reduzir a peroxidação lipídica induzida por diferentes agentes pró-oxidantes *in vitro*, porém a potência antioxidante variou entre os diferentes extratos da mesma planta e entre as diferentes plantas. Da mesma forma, os compostos fenólicos testados (quercetina, quercitrina, ácido gálico e rutina) apresentaram efeito antioxidante, porém a quercetina mostrou-se mais potente que os demais;

→ A planta *M. officinalis* apresentou um maior poder de eliminar RLs quando comparada com as demais plantas testadas (*Matricaria recutita* e *Cymbopogon citratus*), sendo o extrato aquoso mais potente que o etanólico e o metanólico.

→ Todas as frações bem como o extrato bruto da planta *M. officinalis* reduziram de forma significativa a peroxidação lipídica induzida por ferro *in vitro*, porém a fração acetato de etila parece ser mais potente em relação às demais frações analisadas;

→ Todas as frações bem como o extrato bruto da planta *M. officinalis* foram capazes de eliminar o radical DPPH, porém a fração acetato de etila foi mais potente em relação às demais frações analisadas;

→ O extrato aquoso da planta *M. officinalis* apresentou maior conteúdo de compostos fenólicos totais, justificando a maior atividade antioxidante detectada neste extrato em relação aos demais;

→ O extrato aquoso da planta *M. officinalis* apresentou grande quantidade de flavonóides e compostos redutores detectados por CCD, o que também está de acordo com sua maior atividade antioxidante em relação aos demais; Além disso, a fração acetato de etila apresentou maior conteúdo da maioria dos flavonóides analisados por HPLC.

→ O extrato aquoso da planta *M. officinalis* bem como a fração acetato de etila inibiram a autoxidação da adrenalina. O ácido gálico também apresentou efeito antioxidante, porém a quercetina não;

→ O ácido gálico inibiu a atividade da enzima MMP-2 *in vitro*, porém o ácido ascórbico não exerceu nenhum efeito sobre a atividade desta enzima;

→ A fração acetato de etila da planta *M. officinalis* causou inibição da enzima AChE *in vitro*, porém o extrato aquoso desta planta, bem como os compostos purificados (quercetina e ácido gálico) não apresentaram nenhum efeito sobre a atividade desta enzima.

7. OUTROS TRABALHADOS REALIZADOS DURANTE O DOUTORADO

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