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

Romaiana Picada Pereira[a,**], Aline Augusti Boligon[c], André Storti Appel[b], Roselei Fachinetto[f], Carla Speroni Ceron[d], José Eduardo Tanus-Santos[d], Margareth Linde Athayde[c], João Batista Teixeira Rocha[b,e,*]

[a] *Departamento de Química, Programa de Pós-Graduação em Química Aplicada, Universidade Estadual de Ponta Grossa, PR, Brazil*
[b] *Departamento de Química, Programa de Pós-Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria, RS, Brazil*
[c] *Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, RS, Brazil*
[d] *Departamento de Farmacologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, SP, Brazil*
[e] *Abdus Salam International Centre for Theoretical Physics, Italy*
[f] *Departamento de Fisiologia e Farmacologia, Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria, RS, Brazil*

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ABSTRACT

Oxidative stress is associated with various diseases, in particular those related with the central nervous system, such as Alzheimer's disease. Based on the various benefits of *Melissa officinalis*, we investigated the chemical composition and antioxidant activity of different fractions from *M. officinalis* extract. Furthermore, the fraction with the highest antioxidant activity was tested as a potential acetylcholinesterase (AChE) inhibitor. Gallic acid, an important water soluble constituent of *M. officinalis*, was tested 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 *M. officinalis*. TBARS, DPPH, epinephrine autoxidation were used to verify antioxidant properties of *M. officinalis* or its constituents. Ethyl acetate fraction presented the highest flavonoids content as well as the antioxidant activities when compared with other tested fractions. The ethyl acetate fraction was also a weak inhibitor of brain AChE. Moreover, gallic acid inhibited MMP-2 activity. In conclusion, *M. officinalis* ethyl acetate fraction should be further investigated for its possible use in the treatment of oxidative stress related diseases, such as Alzheimer's disease.

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; Halliwell and Gutteridge, 1999). In OS conditions, the excessive presence of reactive species can cause DNA, protein and lipid oxidation, which can cause cellular failure and neuronal death (Finkel and Holbrook, 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 chronic 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.,

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; OS, oxidative stress; AD, Alzheimer's disease; MMPs, matrix metalloproteinase; TBARS, thiobarbituric acid reactive substances; AChE, acetylcholinesterase.

* Corresponding author at: Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Bioquímica Toxicológica, 97105-900 Santa Maria, RS, Brazil. Tel.: +55 21 55 220 8140; fax: +55 21 55 220 8978.
** Corresponding author at: Centro de Ciências Exatas e Naturais, Programa de Pós-Graduação em Química Aplicada, 84030-900 Ponta Grossa, PR, Brazil. Tel.: +55 21 42 220 3062.
E-mail addresses: romaiana@yahoo.com.br (R.P. Pereira), jbtrocha@yahoo.com.br (J.B.T. Rocha).

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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 could blunt the subsequent OS activation of MMP.

Based on "cholinergic hypothesis" of AD, the most common treatment strategy in AD has involved the use of "cholinesterase inhibitors" aiming to re-establish acetylcholine level in the brain (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).

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 have demonstrated MMP inhibition by epigallocatechin gallate, a polyphenol found in greentea, 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, 2006; Wheatley, 2005). 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 neurological 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, gallic acid, chlorogenic acid, ellagic acid, caffeic acid, catechin and epicatechin 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 and quercetin, 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 m × 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 12 °C min^{-1} up to 280 °C. 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

Reverse phase chromatographic analyses were carried out under gradient conditions using C18 column (4.6 mm × 150 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40 and 50 min, respectively, following the method described by Sabir et al. (2012) with slight modifications. *M. officinalis* fractions were analyzed at a concentration of 10 mg/mL, and *M. officinalis* infusion was analyzed at a concentration of 20 mg/mL. The presence of eight antioxidants