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Antioxidant activity of natural compounds of *Stachytarpheta cayennensis* by scavenger of mitochondrial reactive oxygen species

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Abstract: The etiology of a range of diseases is associated with an excessive generation of reactive oxygen species (ROS). Exacerbated ROS production leads to mitochondrial dysfunction, cellular damage and apoptosis. Nowadays, many strategies are being developed for the targeted delivery of antioxidants compounds to mitochondria, trying to minimize the damages. Production of ROS was investigated by the molecular probes CM-H_2DCFDA and Amplex Red. The purpose of this work is to evaluate the antioxidant capacity of verbascoside, martinoside, betulinic acid from the *Stachytarpheta cayennensis* and quercetin by an *in vitro* assay with isolated mitochondria from mice's brain The results showed that all compounds tested exhibited a scavenger effect on the ROS generated by the isolated mitochondria, which displayed a dependent dose increase.

Introduction

Antioxidants react differently with the diverse free radicals and oxidant sources (Huang et al., 2005). During the last decade, natural antioxidant compounds from medicinal plants have aroused much attention, what resulted on increasing efforts to search for plant-derived antioxidants against free radicals like reactive oxygen (ROS) and nitrogen species (RNS) (Huang et al., 2005; Pulido et al., 2005; Roginsky et al., 1996). Mitochondrial electron transport system (ETS) is considered to be one of the major sources of cellular ROS. The formation of ROS is inevitable during normal oxidative metabolism, and it is well established that some of the oxygen (O_2) consumed by the ETS generate ROS, mostly at complexes I and III (Cadenas et al., 1977, Cadenas & Davies, 2000; Chen et al., 2003; Boveris et al., 1976; Boveris & Chance, 1973; Turrens & Boveris, 1980). The partial reduction of molecular oxygen, during oxidative phosphorylation, leads to a constant flux of superoxide anion ($O_2^{\cdot-}$), the precursor of hydrogen peroxide (H_2O_2) radicals (Liu, 2002; Oliveira & Kowaltowski, 2004). It has been described in the literature that in pathological conditions ROS production can be exacerbated, resulting in an imbalance between ROS generation and the intracellular levels of antioxidant defenses (Brand et al., 2004; Turrens 2003). It is involved in the pathology of many neurodegenerative diseases like Parkinson's disease, Alzheimer's disease, Huntington's disease, epilepsy and atherosclerosis (Freitas et al., 2005; Schapira, 2006). Others studies showed important role of antioxidant drugs such as ascorbic acid and α-tocopherol can be used as scavengers of free radicals, reducing mice's brain injury (Tomé et al., 2010a; 2010b; Xavier et al., 2007).

Stachytarpheta cayennensis (Rich.) Vahl, Verbenaceae, is a Brazilian medicinal plant, commonly known as "gervao-roxo", "verbena" and "gerbao". The roots of this plant are used in Brazilian folk medicine for purulent ulcers and to treat sore skin wounds by topical application (De Souza., 2004;

Penido et al., 2006; Schapoval et al., 1998). The most frequent ethnomedical usages of roots and leaves of *S. cayennensis* are as inflammations, ulcers, fever and diuretics (Penido et al., 2006; Schapoval et al., 1998). The anti-inflammatory activity of *S. cayennensis* has been demonstrated in rats by and Penido et al. (2006).

The aim of this study was to demonstrate the antioxidant properties of verbascoside, martinoside and betulinic acid compounds isolated from the roots of *S. cayennensis*. The antioxidant capacity was evaluated by an *in vitro* assay using mitochondria isolated from mice's brains like ROS source. For that, we used two fluorescent probes, Amplex Red and CM-H_2DCFDA, that are highly sensitive and stables markers for mitochondrial generated ROS with selectivity to H_2O_2 and free radicals, respectively (Valeur, 2001; Votyakova & Reynolds, 2004; Zhou et al., 1997).

Materials and Methods

Collection and extraction of plant material

Roots of *Stachytarpheta cayennesis* (Rich.) Vahl, Verbenaceae, were collected at Conceição lake, Florianópolis, Santa Catarina, Brazil. Botanical identification was done by Inês M. Oliveira da Silva from the Department of Botany at Universidade Federal Rural do Rio de Janeiro, Brazil, where a voucher specimen is deposited (RBR 5979). The air-dried and powdered roots (367.8 g) were placed in a Soxhlet apparatus and exhaustively extracted with *n*-hexane followed by ethanol, yielding 5.0 g and 48.0 g of crude hexane and ethanol extracts, respectively. The dried ethanol extract was suspended in MeOH/H_2O 3:2 and extracted with hexane, chloroform, ethyl acetate and butanol, in this order. High-speed counter-current chromatography (HSCCC) was applied to the preparative separation of the compounds verbascoside (**2**) (300.0 mg) and martinoside (**3**) (54.0 mg) from the ethyl acetate fraction and is described elsewhere (Leitao et al., 2005). Betulinic acid (**4**) (45.0 mg) was obtained from the chloroform fraction from the ethanol extract by HSCCC.

Selection of two-phase solvent system by the tube partitioning test

A small quantity of the chloroform fraction from the ethanol extract of the roots of *S. cayennensis* was dissolved in a series of test tubes containing the solvent systems CH_2Cl_2:EtOH:H_2O (1:1:1, 1.5:1:1, 2:1:1 v/v) and hexane:CH_2Cl_2:EtOH:H_2O (1:0.5:1:1, 1:1:1:1, 1:2:1:1 v/v). The test tubes were shaken and the compounds allowed to partition between the two phases. Equal aliquots of each phase were spotted beside each other separately on silica gel TLC plates and developed with the solvent system $CHCl_3$:ethyl acetate 3:2. The results were visualized under UV light and by spraying with vanillin-sulfuric acid (1%) solution followed by heating. The solvent system was defined as hexane: CH_2Cl_2:EtOH:H_2O (1:2:1:1 v/v).

HSCCC separation procedure

The solvents of the selected solvent system were thoroughly equilibrated in a separation funnel at room temperature. The upper and lower phases were separated and degassed in an ultrasonic bath for 20 min before use. The sample was prepared by dissolving 400 mg of the chloroform fraction from *S. cayennesis* in 2.5 mL of each phase of the solvent system and loaded into an injection valve (Rheodyne model 5010 A) equipped with a 5 mL loop. A P.C Inc counter-current chromatograph equipped with a multi-layer coil equilibrated by a counterweight was used. The 80 mL coil was initially filled with the stationary upper phase of the solvent system. Then, rotation started (850 rpm) and the lower mobile phase was pumped into the coil in the head to tail (H→T) direction at a flow rate of 2.0 mL/min until hydrodynamic equilibrium was achieved. Under these conditions, the retention of the stationary phase (S_F) in the coil was 82.5 % (*Vm*=14.0 mL). CCC rotation was interrupted in tube 60 and the coil content (organic and aqueous phases) was collected ("wash-off"), giving a total of seventy fractions, of 5.0 mL each. The whole separation experiment was conducted at 27 °C. All fractions obtained by CCC were analyzed by TLC using dichloromethane: acetone: methanol (2:1:0.5) as eluent. The major compound, betulinic acid, was obtained in fractions (FC 38-40) and identified by ^{1}H and ^{13}C NMR spectroscopy and the data compared with those from the literature (Mahato 1994).

Animals and reagents

Male swiss mice, two month old, were maintained on a 12 h light/dark cycle (lights on at 7 a.m.), with free access to tap water and standard laboratory chow. The experimental protocols using animals were approved by the Committee for Ethics in Animal Research of the Universidade College for Animal Experimentation. The reagents were purchased from Sigma (USA), Amersham Biosciences (USA), Invitrogen (USA) and Merck (Germany) and Molecular Probes (Eugene, OR).

N-acetyl-3,7-dihydroxyphenoxazine (Amplex-Red) and 5-(-6)-chloromethyl-2`,7`-dichlorohydrofluorescein diacetate (CM-H_2DCFDA) probes were obtained from Sigma-Aldrich, USA. The Mili-Q water was used for preparing the solutions. Fresh rat brain homogenate was

used as mitochondrial source for oxidative stress assay. The experiments using Amplex Red and CM-H2DCFDA probes with isolated mitochondria were carried out at 37 °C. Quercetin was used as antioxidant standard, obtained from Sigma Chemicals and used in all experiments, due to its well-established antioxidant activity. All organic solvents used for High-Speed Countercurrent Chromatography (HSCCC) were of analytical grade and purchased from Tedia Brazil.

Mitochondrial isolation

Mitochondria from forebrains were isolated as described by Rosenshtraukh et al. (1978) with minor modifications, from two months old swiss mice. Their brains were rapidly removed (within 1 min) and put into 10 mL of ice-cold isolation buffer containing 300 mM mannitol, 1 mM K+-EGTA, 0.1% bovine serum albumin (BSA; free fatty acid), 10 mM K^+-HEPES pH 7.2. The tissues were cut into small pieces using surgical scissors and extensively washed. Then, they were ressuspend in a proportion of 1/10 (w/v) with the cold isolation buffer, and manually homogenized during two cycles of 10 s in a Teflon glass potter. The homogenates were centrifuged at 2.000 x *g* for 3 min in a Hitachi Himac SCR20B RPR 20-2 rotor. After centrifugation, the supernatant was centrifuged for 8 min at 12.000 x *g*. The pellet was suspended in 5 mL of isolation buffer containing 20 µL of 10% digitonin and centrifuged again for 8 min at 12.000 x g. The supernatant was discarded and the final pellet gently washed and suspended in cold isolation buffer at an approximate protein concentration of 10 mg/mL. The protein concentration in the samples was determined by the Folin-Lowry method using bovine serum albumin as standard (Lowry et al., 1951). All of the experiments with isolated mitochondria were carried out at 37 °C with continuous stirring in a respiration buffer containing 10 mM Tris-HCl, pH 7.4, 0.32 M mannitol, 8 mM inorganic phosphate, 4 mM $MgCl_2$, 0.08 mM EDTA, 1 mM EGTA and 0.2 mg/mL fatty acid-free bovine serum albumin.

Spectrofluorometric measurements of mitochondrial ROS generation

Mitochondrial release of reactive oxygen species (ROS) was assessed by two methods using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) to evaluate H_2O_2 or 5-(-6)-chloromethyl-2`,7`-dichlorohydrofluorescein diacetate (CM-H_2DCFDA) to access whole ROS release from mitochondria (Cadenas & Davies 2000; Cadenas et al., 1977; Chen et al., 2003; Turrens 2003; Turrens & Boveris 1980; Valeur 2001; Votyakova & Reynolds 2004). Briefly, for evaluation of H_2O_2 release from mitochondria, 0.5 mg protein/mL of isolated mitochondria were incubated in the standard respiration buffer supplemented with 1.5 µM Amplex Red and 10 units/mL horseradish peroxidase. (HRP). Amplex Red, is a nonfluorescent molecule that when oxidized by hydrogen peroxide in the presence of HRP originates resorufin, a highly fluorescent product. Fluorescence was monitored at excitation and emission wavelengths of 563 nm and 587 nm, respectively, using a multi-mode microplate reader spectrofluoremeter Spectra Max® M5 (Molecular Devices). For evaluation of whole mitochondrial ROS release, CM-H_2DCFDA fluorescence, 0.5 mg protein/mL of isolated mitochondria were incubated in the standard respiration buffer supplemented with 2 µM of CM-H_2DCFDA. Fluorescence was monitored at excitation and emission wavelengths of 485 nm and 535 nm, respectively, using a multi-mode microplate reader spectrofluoremeter Spectra Max® M5 (Molecular Devices). The acetate group of CM-H_2DCFDA is hydrolysed by esterases when it enters the cell and the molecule is trapped inside as the non-fluorescent 5-(-6)-chloromethyl-2`,7`-dichlorodihydrofluorescein (CM-H_2DCFH). Oxidation of CM-H_2DCFH by ROS yields the fluorescent product carboxymethyldichlorofluorescein (CM-DCF), and indirectly measures mitochondrially produced O_2- that has dismutated to H_2O_2 through the action of superoxide dismutase. Calibrations were performed by the addition of known quantities of H_2O_2. Each experiment was repeated at least three times with different mitochondrial preparations with increasing concentrations (1-45µM) of quercetin (**1**), verbascoside (**2**), martinoside (**3**) and betunilic acid (**4**) compounds. In all experiments, we detected small changes in the rate of H_2O_2 formation among different preparations, but the overall pattern of response to different modulators was not affected. The maximal rate (100%) of mitochondrial H_2O_2 or ROS formation was assumed to be the difference between the rate of H_2O_2 or ROS formation in the absence or in the presence of succinate.

Statistical analysis

Statistical analyses were performed using a Tukey's test and ANOVA. Differences were considered statistically significant for $p<0.05$. All results are expressed as means±S.E.M. for *n* independent experiments.

Results and Discussion

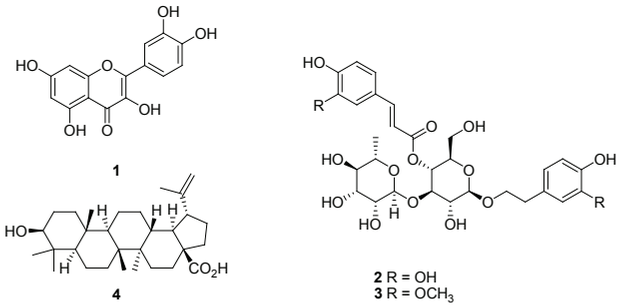
ROS generation rate decrease with increasing concentrations of the natural compounds in mice's brain mitochondria

The excess of ROS induce lipid peroxidation, especially in brain, since it is inadequately equipped with scavenger antioxidant defense mechanisms and which has easily peroxidizable fatty acid, high content of iron and ascorbate. These features turn brain tissue very vulnerable to free radical and ROS injury (Chen et al., 2003; Halliwell & Gutteridge, 2002; Halliwell, 2006; Liu, 2002; Sheu et al., 2006). Mitochondria is the most important cellular source of the free radical superoxide anion ($O_2^{\bullet -}$), which was established as the stoichiometric precursor of mitochondrial H_2O_2 (Chen et al., 2003; Sheu et al., 2006; Halliwell & Gutteridge, 2002).

Little information is available on the levels of natural antioxidant compounds in brain (Halliwell & Gutteridge, 2002). On the other hand, there is an evidence, based on animal studies, that some phenolics antioxidants compounds (feluric acid and flavonoids such as naringenin and hesperitin) can cross the blood-brain barrier (Halliwell & Gutteridge, 2002; Sheu et al., 2006, Spencer et al., 2004). Some of the pharmacological properties of medicinal plant like anti-inflammatory property may be linked to their antioxidant potential, antioxidant compounds, which can be due to their ability to scavenge free radicals and ROS, such as H_2O_2 and other very reactive non-radical species (Amaral et al., 2009).

In this context we tested the ROS production using two fluorescent probes, Amplex Red (AR) and CMH_2DCDFA, in the experiments using isolated mitochondria from mice's brain, in order to investigate the antioxidant potential of the natural compounds from *S. cayennensis*. The chemiluminescence of CM-H_2DCFDA and Amplex Red (AR) catalyzed by horseradish peroxidase (HRP) enzymatic coupled assays have been employed in several *in vitro* studies for the quantification of low concentrations of ROS and H_2O_2 (Chen et al., 2003; Sheu et al., 2006; Valeur, 2001). The addition of H_2O_2 to a sample consisting of AR and HRP. HRP in pH 7.4, buffer immediately generated a strong absorption band with maximum at 587 nm, indicating conversion of AR to resorufin. Increasing H_2O_2 produced more resorufin, the concentration of which was proportional to the concentrated H_2O_2 used (Chen et al., 2003; Sheu et al., 2006). The acetate group of CM-H_2DCFDA is hydrolysed by esterases when it enters the cell and the molecule is trapped inside as the non-fluorescent CM-H_2DCFH. CM-H_2DCFH was chosen because, unlike underivatized dichlorodihydrofluorescein, it is well retained in cells and, in this case, in the mitochondrial matrix (Valeur, 2001; Votyakova & Reynolds, 2004).

The verbascoside (**2**), martinoside (**3**) and betulinic acid (**4**) resulting from the roots of ethanol extract of *S.cayennensis* has been previously assayed by our group for antioxidant activities (DPPH free-radical assay and *Saccharomyces cerevisiae* cells, an eukaryotic cell model) (De Souza et al., 2010). All compounds afforded the best results in the two assays and were therefore, chosen for this study. The remarkable capacity of these natural compounds to function *in vitro* as antioxidants has been demonstrated in this study.



We induced the isolated mitochondria to produce ROS using the mitochondrial substrate succinate and the mitochondrial complex III inhibitor antimycin A. Each experiment was repeated at least three times with different mitochondrial preparations. The compounds verbascoside and martinoside presented the best antioxidant effects, according to their IC50 for ROS scavenger (Table 1). Table 2 and Table 3 show representative experiments. The differences between the antioxidant efficacy of verbascoside, martinoside and betunillic acid comparing to quercetin became clear in the concentration range of 1 to 20 μM (Table 2) and of 1 to 10 μM (Table 3).

Table 1. Effect of different compounds with antioxidant activities.

IC50 (uM) values for ROS scavenger		
Compounds	H_2O_2 (Amplex red) IC50	ROS (CMH_2DCFDA) IC50
Betunilic acid	28.11	2.07
Martinoside	19.34	0.54
Quercetin	-	7.27
Verbascoside	5.25	0.54

Table 1 shows for the Amplex red probe, after the titulation curve with H_2O_2, with the IC_{50} (inhibitory concentration, 50%) of 19,000 pmol of H_2O_2 consumed by each compound. For the CMH_2DCFDA probe, the IC50 was 29,325 pmol of H_2O_2 consumed by each compound.

On the other hand, the current study demonstrates that AR (Table 3) is more sensitive probe than CMH_2DCFDA (Table 2) when the assay is conducted at pH 7.4. Perhaps, the hydrolysis of CM-H_2DCFDA in buffer at physiological temperature and pH is not rapid, it must be taken into consideration in experiments lasting longer than

several minutes, and where buffer capacity is low and pH is variable. In addition, the oxidized product of Amplex Red has excitation and emission maxima at 563 and 587 nm, in comparison with those of CMH_2DCFDA at 485 and 535 nm. These superior spectral properties maybe are important for avoiding interference from autofluorescence in assays involving biological samples. Finally, there are a few factors affecting the sensitivity of resorufin for detecting H_2O_2 (Valeur, 2001).

The number of phenolic hydroxyl groups may play an important role in their scavenging activity (Justino et al., 2004; Van Acker et al., 1993). The literature reports that compounds with hydroxyl in the ortho-aromatic position, like quercetin, have more antioxidant activity against ROS (Justino et al., 2004; Soobratte et al., 2005). In the presence of H_2O_2, for example, phenolic compounds such as ortho-quinol suffer a reduction reaction generating ortho-quinones and water as products (Dewick, 2002; Justino et al., 2004; Soobratte et al., 2005; Van Acker et al., 1993). The verbascoside (**2**) and the flavonoid quercetin (**1**) have four hydroxyls in their structures capable of suffering oxidation reactions in the presence of peroxides, such as H_2O_2 (Dewick, 2002; Zhou & Sadik, 2008).

Table 3 shows the antioxidant activity of the tested compounds in H_2O_2. Again, the verbascoside presented the better antioxidant capacity among the compounds tested with about 10 µM of concentration. Both betulinic acid (**4**) and martinoside (**3**) showed similar antioxidant effect around the concentration of 10 µM (Table 3). A possible explanation for this would be that the carboxylic acid group presents a greater oxidation potencial comparing to the phenolic compound (Dewick, 2002; Justino et al., 2004). This property could give to betulinic acid a similar antioxidant capacity when compared with martinoside, which has only a phenolic hydroxyl group on each of its two aromatic rings. Surprisingly, both verbascoside and martinoside showed more antioxidant activity than quercetin that was used as standard, due to its well-established antioxidant activity (Table 1 and 3) (Belinha et al., 2007; Ho et al., 2004; Santos et al., 2008; Oken et al., 1998; Soobrattee et al., 2005; Spencer et al., 2004; Zhou & Sadik, 2008).

Nevertheless, only verbascoside has four hydroxyls on ortho position, in its two aromatic rings. This structural information could justify its high antioxidant activity when compared to the other compounds tested (Zhou & Sadik, 2008). Perhaps, the weak antioxidant activity of quercetin reported here may be related to the solubility and ability of quercetin to be distributed to a less or greater extent of its diffusion speed, between the lipid-water interface, or the distribution in the hydrophobic core of the mitochondrial membrane (Justino et al., 2004; Oliveira & Kowaltowski, 2004; Pulido et al., 2005; Spencer et al., 2004).

In summary, present results show that verbascoside, martinoside and betulinic acid are useful antioxidants for preventing oxidative stress-related mitochondrial dysfunction, and as such may find use in the management of a range of acute and chronic disease processes. However, very little is known about the

Table 2. ROS generation rate using the fluorescent probe CM-H_2DCFDA, during state 2 respiration induced by 10 mM succinate (suc), with increasing compounds concentrations (1-45 µM) of verbascoside, quercetin, betunilic acid and martinoside.

	Relative rate of mitochondrial ROS generation (Vo/Vi)					
Compounds	Control	1 (µM)	10 (µM)	20 (µM)	30 (µM)	45 (µM)
Verbascoside	0,0500±0,00666	0,0290±0,00120[a]	0,0270±0,00145[a]	0,0220±0,00361[a]	0,0150±0,00267[a]	0,0140±0,00451[a]
Martinoside	0,0440±0,01049	0,0130±0,00033[a]	0,0120±0,00115[a]	0,0070± 0,00033[a]	0,0050± 0,00088[a]	0,0050±0,00120[a]
Quercetin	0,0540±0,01357	0,0130±0,00033[a]	0,0100±0,00033[a]	0,0070±0,00066[a]	0,0050± 0,00120[a]	0,0040±0,00088[a]
Betulinic acid	0,0600±0,00549	0,0300±0,00524[a]	0,0240±0,00260[a]	0,0210±0,00291[a]	0,0160±0,00265[a]	0,0120±0,00176[a]

[a]$p<0.05$ relative to 1, 10,20,30 and 45 µM. The values are mean±SE from at least three independent mitochondrial preparations for each assay conditiona: control vs 1,10,20,30,45.

Table 3. ROS (H_2O_2) generation rate using the fluorescent probe Amplex Red, during state 2 respiration induced by 10 mM succinate (suc), with increasing compounds concentrations (1-45 µM) of verbascoside, quercetin, betunilic acid, and martinoside.

	Relative rate of mitochondrial ROS generation (Vo/Vi)					
Compounds	Control	1 (µM)	10 (µM)	20 (µM)	30 (µM)	45 (µM)
Verbascoside	6,911±0,08170	1,363±0,06059[a]	0,4367±0,01618[a,b]	0,2557±0,04156[a,b]	0,2800±0,05029[a,b]	0,2833±0,06164[a,b]
Martinoside	6,651±0,09650	1,381±0,06351 [a]	1,015±0,06784[a,b]	0,6773±0,07518 [a,b]	0,6100±0,08685[a,b,c]	0,5267±0,08225[a,b,c]
Quercetin	6,943±0,10513	1,362±0,6529 [a]	1,477±0,3324 [a]	1,107±0,2052 [a]	0,7627±0,1621	0,8267±0,1470[a]
Betulinic acid	6,873±0,06754	1,428±0,1159 [a]	1,053±0,1195[a]	0,9297±0,09556[a,b]	0,6543±0,07911[a,b]	0,5670±0,07365[a,b,c]

[a]$p<0.05$ relative to control, [b]$p<0.05$ relative to 1 µM, [c]$p<0.05$ relative to 10 µM. The values are mean±SE from at least 3 independent mitochondrial preparations for each assay condition. a: control vs 1,10,20,30,45; b: 1 vs 10,20,30,45; c: 10 vs 20,30,45.

effects of natural compounds isolated from plants in the cellular metabolism of humans, especially after cross the blood-brain barrier and interaction with brain cells (Justino et al., 2004; Spencer et al., 2004). Preclinical studies *in vivo* models are needed to evaluate the effectiveness and toxicity of mitochondrially targeted antioxidants before recommending specific doses for human treatment.

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