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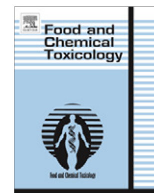

Baccharis dracunculifolia, the main source of green propolis, exhibits potent antioxidant activity and prevents oxidative mitochondrial damage

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ABSTRACT

Baccharis dracunculifolia DC (Asteraceae) is the main botanical source used by honeybees to produce Brazilian green propolis whose hepatoprotective properties have been already described. In this work we investigated the protective effects of the glycolic extract of *B. dracunculifolia* (GEBd) against oxidative stress in isolated rat liver mitochondria (RLM). The GEBd was prepared by fractionated percolation using propylene glycol as solvent. The total phenols and flavonoids, which are substances with recognized antioxidant action, were quantified in GEBd and the phytochemical analysis was carried out by HPLC. GEBd exhibited significant scavenger activity towards DPPH radicals and superoxide anions in a concentration-dependent manner, and also a Fe^{2+} chelating activity. GEBd decreased the basal H_2O_2 generation and the Fe^{2+}- or *t*-BuOOH-induced ROS production in isolated mitochondria. Lipid oxidation of mitochondrial membranes, protein thiol groups and GSH oxidation were also prevented by GEBd. This shows that *B. dracunculifolia* exhibit potent antioxidant activity protecting liver mitochondria against oxidative damage and such action probably contribute to the antioxidant and hepatoprotective effects of green propolis.

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1. Introduction

Oxidative stress is a condition characterized by the oxidative damage of biomolecules by free radicals when they are overproduced and/or the ability of the cellular antioxidant systems is decreased. These types of damage were thought to be involved in the ethyology of many human diseases (Brookes et al., 2004), including liver damage, aging, carcinogenesis, atherogenesis, immune disorders, and others (Ames et al., 1993). Mitochondria are directly implicated with oxidative stress conditions, due to the constant generation of superoxide anions ($O_2^{\cdot-}$) by the respiratory chain, a process which is normally eliminated by the antioxidant defense system composed of superoxide dismutase, glutathione peroxidase and reductase, GSH and NAD(P)H. However, in mitochondrial dysfunctions, the excessive formation of $O_2^{\cdot-}$, and consequently hydrogen peroxide (H_2O_2), generates the extremely reactive hydroxyl radical ($^{\cdot}OH$) by means of the Fenton–Haber–Weiss reaction (Sies, 1997). This process is able to oxidize mitochondrial lipids, thiols, proteins and nucleic acids that may result in necrotic or apoptotic cell death (Kowaltowski et al., 2009).

The vegetal secondary metabolism produces chemical substances responsible for the antioxidant properties *in vitro* and *in vivo* of many plant extracts. Such substances are mainly phenolic compounds, able to decrease the production and/or to eliminate produced free radicals (Dorta et al., 2006; Saija et al., 1995; Chen et al., 1990). Among these substances are flavonoids and other phenolic compounds, especially as synthesized in leaves, flowering tissues, and pollens that are intensively studied due to their presence in the human diet (Chen et al., 1990).

The large genus *Baccharis* presents approximately 350 species distributed specifically in America and several studies have been performed to identify secondary metabolites with biological activities and pharmacological potential (Brown, 1994; Labbe et al., 1986; Zdero et al., 1986). Several properties of *Baccharis* species have been described, including the inhibition of plant growth (Céspedes et al., 2002), anti-rheumatic, antifungal (Rahalison et al., 1995), and insecticide properties (Juan et al., 2008), among others. *Baccharis dracunculifolia* DC (Asteraceae), popularly known as "alecrim-do-campo", is largely distributed in South America

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from southeastern Brazil to Argentina and Uruguay and it is shown to exhibit a protective action against gastric ulcers, reducing the volume of gastric juice and increasing gastric pH (Lemos et al., 2007). In Brazil in particular, honeybees (*Apis mellifera*) utilize flower nectars of *B. dracunculifolia* to produce green propolis, whose healthy benefits, including the hepatoprotective effect, are well described in the literature (Seo et al., 2003). Interestingly, it was recently demonstrated that many chemical substances present in *B. dracunculifolia* are also present in green propolis, such as flavonoids and coumaric acid derivatives (Park et al., 2004; Kumazawa et al., 2003). Although several studies have demonstrated the antioxidant properties, *in vitro* and *in vivo,* of green propolis (reviewed in Burdock, 1998) there is no evidence regarding the antioxidant properties of *B. dracunculifolia*.

Since the secondary metabolites from *B. dracunculifolia* may be present in the human diet directly or indirectly via green propolis intake, in this work we investigated the antioxidant properties of the glycolic extract of *B. dracunculifolia* against oxidative stress in isolated rat liver mitochondria, and the mechanisms of this protection are discussed.

2. Materials and methods

2.1. Chemicals

All reagents were of the highest purity grade available commercially, and aqueous solutions were prepared with deionized Milli-Q water (mixed bed of ion exchanger, Millipore). The quercetin, gallic acid, caffeic, *p*-coumaric and *trans*-cinnamic acids were acquired from the Sigma Chem. Co. (St. Louis, USA); artepillin C (Wako Pure Chemical Industries Co., Osaka, Japan); isosakuranetin (ChromaDex, Irvine, Canada) and aromadendrin-4′-*O*-methyl ether, previously isolated and identified as described by Sousa et al. (2007) and kindly donated by the authors.

2.2. Plant source and extract preparation

Leaves of *B. dracunculifolia* DC were collected in August (winter in the southern hemisphere) in a farm located at Mogi das Cruzes, São Paulo, Brazil (GPS localization: 760 m, 23°29840′S). Voucher specimens of the plant were deposited at the Herbarium Mogiense, located at the University of Mogi das Cruzes, Mogi das Cruzes, São Paulo, Brazil. After being dried at 40 °C in a greenhouse by means of air circulation, plant leaves (100 g) were submitted to fractionated percolation with propylene glycol:H_2O (70:30) as an extractor solvent; 100 mL of the glycolic extract of *B. dracunculifolia* (GEBd) were obtained, and considered as 100% (crude extract).

2.3. Electronic absorption spectroscopy

Electronic absorption measurements of GEBd were conducted in a photodiode spectrophotometer MultiSpec-1501, equipped with a thermo bath TB-85 (Shimadzu Scientific Instruments Inc., Columbia, MD), using 10 mm optical quartz cuvettes, model 104-QS (Hellma, Germany) and a slit of 0.5 nm.

2.4. Quantification of total phenols and flavonoids

Soluble phenol derivatives were determined according to the Folin–Ciocalteau method (Singleton and Rossi, 1965) and expressed as μM of gallic acid equivalents based on a standard curve with gallic acid. To flavonoid quantification, an aliquot was incubated in a medium containing 60 μl of glacial acetic acid, 1.0 ml of pyridine:H_2O:$AlCl_3$ 12% solution (17:80:3), and 1.24 ml of dimethyl sulfoxide:H_2O (1:1) for 5 min at 25 °C. The reaction product was determined spectrophotometrically at 420 nm; the flavonoid content was determined based on a standard curve and expressed as μM of quercetin equivalents.

2.5. Phytochemical analysis of B. dracunculifolia glycolic extract by HPLC

GEBd was analyzed by HPLC using a Shimadzu apparatus equipped with a CBM-20A controller, a LC-20AT quaternary pump, a SPD-M 20A diode-array detector, and Shimadzu LC solution software, version 1.21 SP1. A Shimadzu Shim-Pack CLC-ODS column (4.6 × 250 mm, particle diameter of 5 μm, pore diameter of 100 Å) was used. The mobile phase consisted of methanol (B), and of a solution of water–formic acid (0.1% v/v), pH 2.7 (A). It was used a linear gradient of 20–95% of B over a period of 77 min at a flow rate of 0.8 ml/min with detection at 275 nm. GEBd was diluted with 5 ml of methanol (HPLC grade) in 10 ml volumetric flasks, sonicated for 10 min, and filled to volume with Milli-Q water. The sample was filtered through a 45 μm filter before analysis.

2.6. DPPH reduction assay

The reduction of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH, 100 μM) by GEBd was determined at 517 nm after 5 min incubation in 40 mM sodium acetate pH 5.5 (1.5 mL) plus ethanol (1.0 mL) at 30 °C (Blois, 1958).

2.7. Superoxide scavenger activity

Superoxide anions were generated by the xanthine/xanthine oxidase system and the scavenger activity of GEBd was evaluated by the inhibition of the reduction of nitroblue tetrazolium (NBT). Briefly, after the addition of 0.08 U/mL xanthine oxidase in phosphate buffer pH 7.5 containing 0.05 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM hypoxanthine and 0.1 mM NBT, the absorbance at 540 nm was recorded for 20 min at 37 °C (Hitachi U-2000 Spectrophotometer, Tokyo, Japan) (Parejo et al., 2002).

2.8. Competitive iron chelating assay

The amount of Fe^{2+} in the medium was determined spectrophotometrically in the presence and absence of GEBd by using the specific indicator batophenantroline disulfonic acid (0.2 mM BPS) according to Cowart et al. (1993).

2.9. Isolation of rat liver mitochondria

All experiments involving animals were previously approved by the Ethical Committee on Animal Experimentation and Manipulation of University of Mogi das Cruzes (CEUA/UMC) according to the Brazilian College of Animal Experiments. Rat liver mitochondria were isolated by conventional differential centrifugation in isosmotic medium from adult male Wistar rats as previously described (Santana et al., 2009).

2.10. Mitochondrial reactive oxygen species (ROS) and H_2O_2 measurements

General ROS and H_2O_2 production in mitochondria were estimated by using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Lebel et al. (1992) and Amplex® Red (Tahara et al., 2009), respectively. Briefly, mitochondria (1 mg/mL) were incubated in a medium 125 mM sucrose, 65 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)-KOH, pH 7.4, plus 1 μM H_2DCFDA or 25 μM Amplex® Red plus 1 U/ml horseradish peroxidase at 30 °C with continuous stirring in the presence of 5 mM potassium succinate (+2 μM rotenone) and the fluorescence was measured in a Hitachi F-2500 Spectrophotometer (Tokyo, Japan). The excitation/emission wavelength pair was 503/529 nm and 563/587 nm to H_2DCFDA and Amplex® Red, respectively.

2.11. Lipid oxidation assays

The lipid oxidation of mitochondrial membranes was estimated by measuring the thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) formation as described in details by Borges et al. (2004). The percentages of inhibition by GEBd were calculated in relation to positive control (*t*-BuOOH or Fe^{2+}/citrate).

2.12. GSH levels

After 30 min of incubation at 37 °C in a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES–KOH, pH 7.4, plus 5 mM potassium succinate (+2 M rotenone), the mitochondria suspension was treated with 0.5 ml of 13% trichloroacetic acid and centrifuged at 900*g* for 3 min. Aliquots (100 μl) of the supernatant were mixed with 2 ml of 100 mM sodium phosphate buffer pH 8.0, containing 5 mM ethylene glycol tetraacetic acid (EGTA). One hundred microliters of *o*-phthalaldehyde (1 mg/mL) were added and the fluorescence was measured 15 min later, using the 350/420 nm excitation/emission wavelength pair and an excitation/emission slit of 5 nm in a fluorescence spectrophotometer Hitachi F-2500 (Tokyo, Japan) (Hissin and Hilf, 1976).

2.13. Data analyzes

The graphical, mathematical and statistical analyzes were performed by using Microcal (TM) Origin® version 6.0, Microcal Software Inc., Northampton, MA, USA. Data are presented as mean ± s.d.; the statistical differences were calculated by Student's t-test, and the values of $p < 0.05$ were considered significant.

3. Results

3.1. Characterization and standardization of the glycolic extract of B. dracunculifolia (GEBd)

As a preliminary characterization, the electronic absorption UV–vis spectra of the glycolic extract of *B. dracunculifolia* (GEBd) was acquired at different concentrations. It was observed that GEBd contains substances that exhibit strong absorption in the UV region (200–400 nm) and the mixture presents a band of maximum absorption at around 290 nm (Fig. 1). Such electronic absorption might be attributed to unrelated substances presenting benzene rings or conjugated bonds. However, flavonoids and phenolic compounds, substances with recognized antioxidant action, were thought to absorb light at this spectral region (Jurd and Geissman, 1956). In the inset of Fig. 1, the maximal absorption at 290 nm was plotted in the function of GEBd concentration, and the linear correlation observed may be used to accompany the stability of the obtained extract or for comparison with other extractions. However, the term 'absorptivity' may not be applied here because the extract contains a mixture of chemical substances. Considering the large occurrence of flavonoids and other phenolic substances as secondary metabolites in plants associated to the well-described antioxidant properties, the quantification of these substances in GEBd was performed in order to provide a standardization of this and other glycolic extracts of *B. dracunculifolia* obtained by the same method. To quantify the total phenols and flavonoids in the extract, standard curves from 0 to 60 μM were performed with gallic acid and quercetin, respectively. GEBd at a 0.05% concentration presented 21.18 ± 0.156 μM of total phenols (as gallic acid equivalents) and 13.64 ± 0.01 μM of flavonoids (as quercetin equivalents). Phytochemical analysis of the GEBd was carried out by HPLC and it allowed the identification of caffeic, *p*-coumaric and *trans*-cinnamic acids, aromadendrin-4-*O*-methyl ether, isossakuranetin, and also artepillin C, a characteristic compound commonly found in Brazilian green propolis and *B. dracunculifolia* (Supplementary material). The quantification of these substances is presented in Table 1.

3.2. Scavenger and iron chelating activities in non-biological systems

The free radical scavenger activity of a plant extract may be screened by using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), which exhibits a strong absorption band

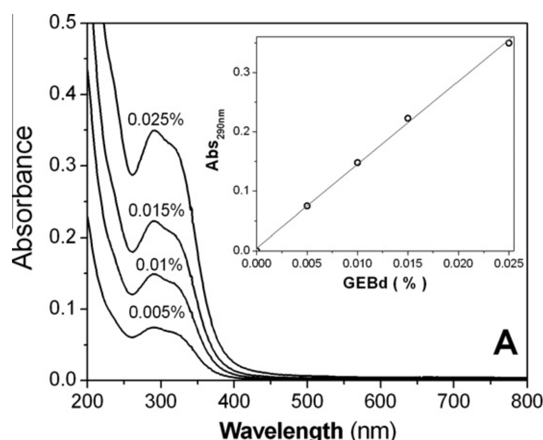

Fig. 1. UV–vis spectra of the glycolic extract of *B. dracunculifolia* (GEBd). (A) UV–vis electronic absorption spectra of different concentrations of GEBd in pure water recorded from 200 to 800 nm. The inset shows the linear correlation between the absorbance at 290 nm and the extract concentration.

Table 1
Chemical profile presentation of the glycolic extract of *Baccharis dracunculifolia.*

Compounds	GEBd* (mg/g)
Caffeic acid	0.692 ± 0.0076
p-Coumaric acid	0.533 ± 0.0058
Cinnamic acid	0.044 ± 0.0006
Aromadendrin	0.117 ± 0.0015
Isosakuranetin	0.586 ± 0.0078
Artepelin C	0.650 ± 0.0159

* Data are presented as mean ± s.e.m. (n = 3).

at 517 nm, bleached with its reduction to a non-radical species. As observed in Table 2, GEBd was able to reduce the DPPH radical in a concentration-dependent manner. The EC_{50}, defined as the GEBd concentration necessary to reach 50% of the observed effect calculated by using a hyperbolic fit, was 0.005%. The flavonoid quercetin was used as a reference and 0.05% GEBd presented a similar effect of 10 μM quercetin to reduce DPPH (not shown). Since DPPH is not a biological free radical, we investigated the ability of GEBd to scavenge superoxide anions generated by the xanthine/xanthine oxidase system, with NBT as an indicator (Table 2). GEBd was able to decrease the NBT reduction comparatively to control indicative of $O_2^{\cdot-}$ scavenger activity ($EC_{50} \sim 0.0732\%$). GEBd was able to chelate 77.57 ± 0.30% of the Fe^{2+} available in the medium (not shown). The presence of flavonoids in *B. dracunculifolia* probably provides this ability to the extract, since iron chelating activity has already been demonstrated for these compounds (Dorta et al., 2008).

3.3. GEBd decreases basal H_2O_2 production in succinate-energized rat liver mitochondria

The antioxidant activity of GEBd was evaluated on isolated rat liver mitochondria and the mechanisms of antioxidant protection were investigated. First, the effects of GEBd on the H_2O_2 production by succinate-energized mitochondria were analyzed fluorimetrically by using Amplex Red®. RLM produced a basal amount of H_2O_2 as a product of spontaneous $O_2^{\cdot-}$ dismutation or by SOD (Fig. 2, dotted line). The pre-incubation of mitochondrial suspension with GEBd promoted a decrease in the fluorescence slope in a concentration-dependent manner, indicating the elimination of the H_2O_2 produced (peroxidase activity) or the decrease in its formation in state 4 respiring RLM, e.g., by scavenging superoxide anions.

3.4. Protection of RLM against t-BuOOH- or Fe^{2+}/citrate-induced oxidative stress by GEBd

The antioxidant action of GEBd towards oxidative stress promoted by *t*-BuOOH on isolated rat liver mitochondria was evaluated. The pre-incubation of the mitochondrial suspensions with GEBd at different concentrations resulted in the inhibition of ROS generation (Fig. 3A) and lipid oxidation (Fig. 3B) induced by

Table 2
Scavenger activity of GEBd against DPPH and superoxide anion ($O_2^{\cdot-}$) radicals.

GEBd (%)	DPPH (%)	$O_2^{\cdot-}$ (%)
0.005	45.43 ± 0.30	13.03 ± 0.24
0.01	78.45 ± 5.70	28.90 ± 1.15
0.025	90.92 ± 5.45	29.74 ± 0.28
0.05	–	36.96 ± 1.90
0.1	–	64.67 ± 6.21

Data are the percentage of radical scavenged in relation to control (absence of GEBd) presented as mean ± s.e.m.

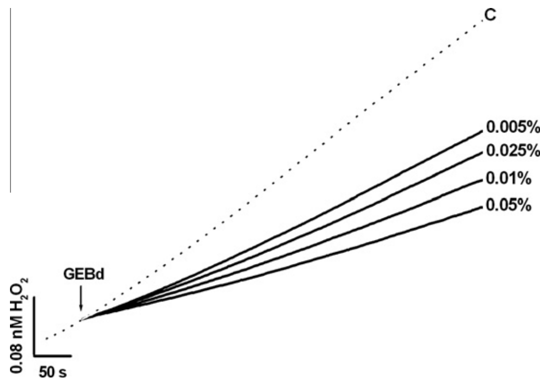

Fig. 2. Inhibition of H_2O_2 production by GEBd on isolated rat liver mitochondria. After addition of 5 mM succinate, 2 μM rotenone, and 10 μM $CaCl_2$ to the mitochondrial suspension (1.0 mg/mL), the fluorescence emissions of Amplex Red were recorded for 10 min. Traces are representative of three experiments with different mitochondrial preparations, and the GEBd concentrations were expressed in a percentage (v/v) in relation to the crude extract, considered as 100%. C: absence of GEBd (dotted line).

t-BuOOH. The EC_{50} for the inhibition of the lipid oxidation was 0.0065%. Additionally, in the same experimental conditions, GEBd prevented the oxidation of thiol groups of mitochondrial proteins (Fig. 3C) and GSH (Fig. 3D).

Hydroxyl and peroxyl radicals generated by Fe^{2+}-catalyzed Fenton type reactions and their oxidative effects on mitochondrial protein and lipids were estimated. Similarly to the effects observed with *t*-BuOOH, GEBd also inhibited the Fe^{2+}/citrate-mediated ROS generation (Fig. 4A) and lipid oxidation (Fig. 4B) in RLM. The EC_{50} value for inhibition of lipid oxidation induced by Fe^{2+} was 0.017%. The protection of mitochondrial membranes by GEBd against oxidation was further demonstrated by the decrease of lipid hydroperoxides (LOOH) production induced by Fe^{2+}/citrate (Fig. 4C, EC_{50} 0.012%). Thus, GEBd inhibited the formation of the intermediate and final products of lipid peroxidation during the oxidative chain reactions. Besides the lipid protective effect, GEBd also prevented the thiol oxidation of mitochondrial proteins induced by Fe^{2+} (Fig. 4D).

4. Discussion

Propolis is a honeybee product that has received worldwide attention as food and nutraceutical products due to its medicinal properties. Despite its economic importance, propolis presents a wide range of biological properties, including antibacterial, antiviral, anti-inflammatory, antifungal, antitumor, antioxidant, and hepatoprotective activities (Banskota et al., 2000, 2001). Such effects have been attributed to the presence of polyphenols such as flavonoids and phenolic acids in its composition (Bankova,

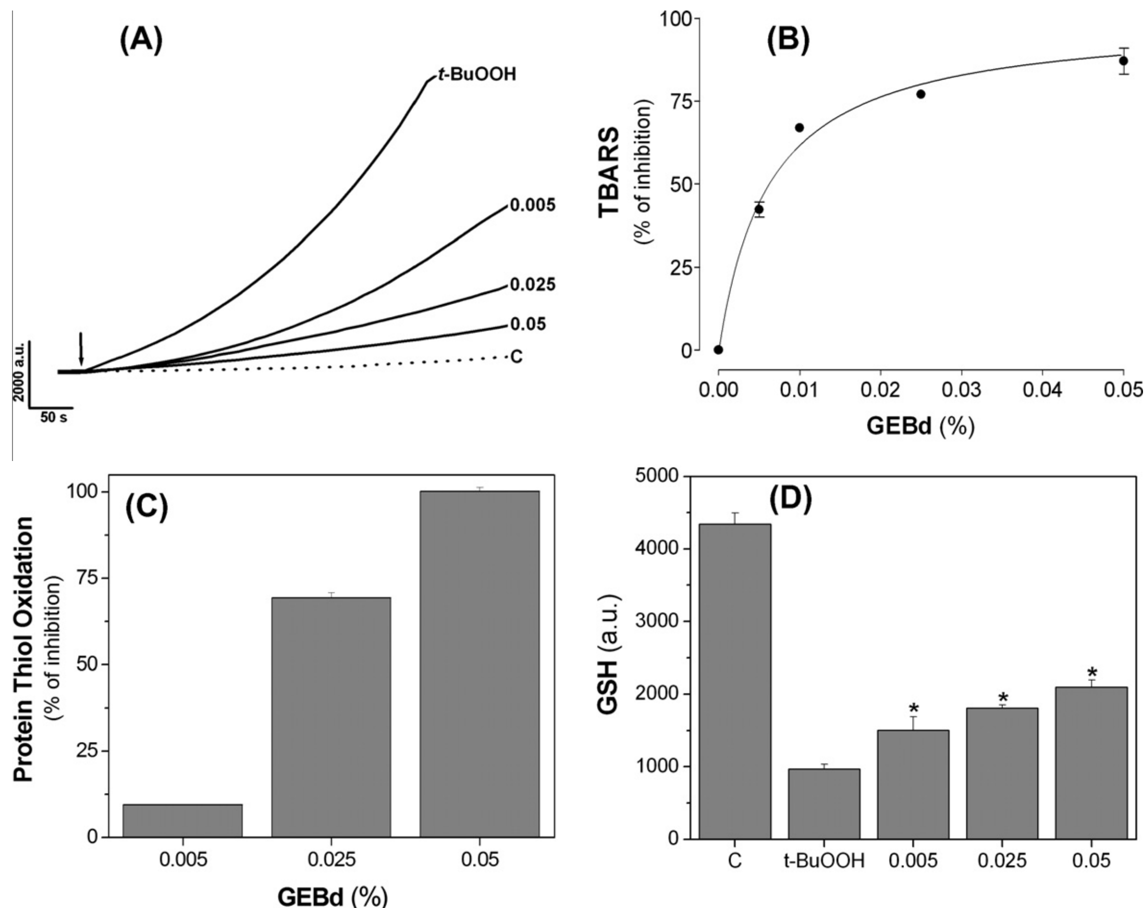

Fig. 3. Protective effects of GEBd against *t*-BuOOH-induced oxidative damage on isolated rat liver mitochondria. Mitochondria (1.0 mg/mL) were pre-incubated with GEBd followed by the addition of *t*-BuOOH. (A) ROS generation was assayed as DFC fluorescence. C: absence of GEBd and *t*-BuOOH (control, dotted line); *t*-BuOOH: 0.6 mM *t*-BuOOH (positive control); numbers are the GEBd concentration in the % pre-incubated followed by the addition of *t*-BuOOH (as indicated by the arrow). Traces are representative of three experiments with different mitochondrial preparations. (B) Inhibition of the *t*-BuOOH-induced lipid oxidation of mitochondrial membranes by GEBd estimated as TBARS. (C) Inhibition of the *t*-BuOOH-induced thiol oxidation of mitochondrial proteins by GEBd measured with DTNB. (D) Inhibition of the *t*-BuOOH-induced GSH oxidation by GEBd estimated fluorimetrically. In B–D, the data were presented as the mean ± s.d. of three experiments with different mitochondrial preparations and GEBd concentrations were expressed in percentage (v/v) in relation to the crude extract (100%). For each assay, see details in Section 2.

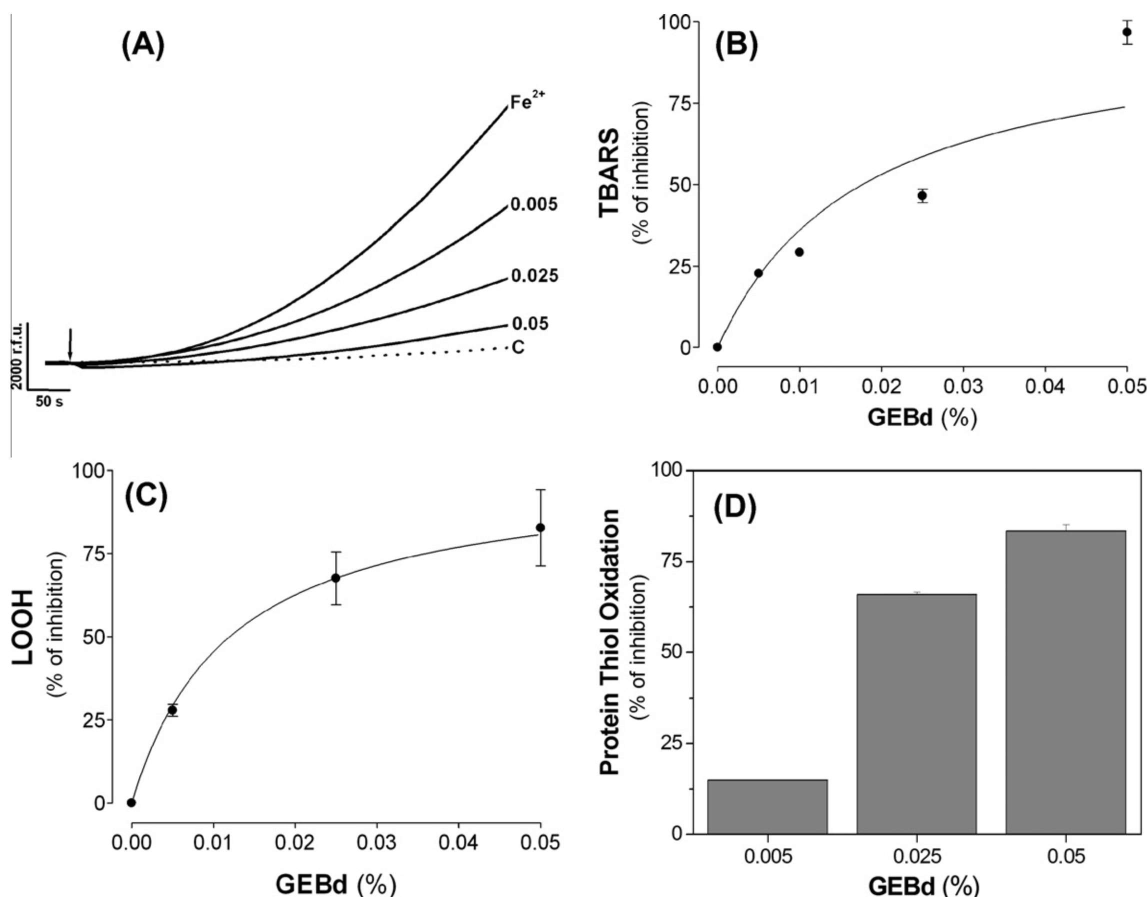

Fig. 4. Inhibition of Fe^{2+}/citrate-induced oxidative damage on isolated rat liver mitochondria by GEBd. Mitochondria (1.0 mg/mL) were pre-incubated with GEBd, followed by the addition of Fe^{2+}/citrate. (A) ROS generation was assayed as DFC fluorescence. C: absence of GEBd and Fe^{2+}(control, dotted line); 50 M (NH4)2Fe(SO4) 2 plus 2 mM citrate (positive control); numbers are the GEBd concentration in % pre-incubated, followed the addition of Fe^{2+}(as indicated by the arrow). Traces are representative of three experiments with different mitochondrial preparations. (B) Inhibition of the Fe^{2+}-induced lipid oxidation of mitochondrial membranes by GEBd, estimated as TBARS. (C) $Fe2^{2+}$-induced LOOH production was measured by the oxidation of Fe^{2+} in the presence of xylenol orange in the presence of different GEBd concentrations. (D) Inhibition of the Fe^{2+}-induced thiol oxidation of mitochondrial proteins by GEBd measured with DTNB. In B–D, data were presented as the mean ± s.d. of three experiments with different mitochondrial preparations and GEBd concentrations were expressed in percentage (v/v) in relation to crude extract (100%). In (C), hyperbolic fit was used to calculate the IC_{50} values. See details in Section 2. SM01 – Chromatographic profile of GEBd. (1) Caffeic acid; (2) *p*-Coumaric acid; (3) Cinnamic acid; (4) Aromadendrin; (5) Isosakuranetin; (6) Artepellin C. The chromatogram was acquired at 275 nm by using reverse phase high performance liquid chromatography (RP-HPLC) equipped with a C18 column (Shim-pack, CLC-ODS (M), 25 cm × 4.6) and gradient elution with methanol and acidic water (formic acid pH 2.7).

2005). Particularly in Brazil, *B. dracunculifolia* DC (Asteraceae) is the main botanical source used by honeybees to produce green propolis. In fact, ethanolic extracts of Brazilian Green Propolis and its botanical origin *B. dracunculifolia* showed a similar chromatographic profile (Park et al., 2004) and the major compound in both extracts was artepillin C.

Propolis chemical composition is complex, presenting predominantly 50% resin, which contains flavonoids and related phenolic acids, 30% wax, 10% essential oils, 5% pollen and 5% other organic substances, and this composition varies according to geographical origin (Gómez-Caravaca et al., 2006). Chemical profile presented in this study by GEBd corroborate the similarity between *B. dracunculifolia* and green propolis, since it was demonstrated the presence of the main compounds in the glycolic extract such as caffeic, *p*-coumaric and cinnamic acids, aromadentrin-4-methyl-ether, isosakuranetin and finally, artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) (Sousa et al., 2007, 2011). The potent antioxidant action presented by GEBd probably results from the sum or synergistic effect of each compound in the extract, mainly phenols and flavonoids – substances with well known scavenger properties (Dorta et al., 2006). Caffeic acid, quercetin, kaempferol, phenethyl caffeate and artepillin C was found to reduce DPPH (Kumazawa et al., 2004). It was also demonstrated that artepillin C inhibited the lipid peroxidation and the development of pulmonary cancers (Kimoto et al., 2001).

Oxidative mitochondrial damage is associated with cell death and dysfunctions of tissue and organ systems, including hepatotoxicity (Brookes et al., 2004). The antioxidant activity exhibited by GEBd in non-biological systems was confirmed also in mitochondria, preventing the oxidative damage of lipids and proteins. Since the effectiveness of GEBd against membrane lipids and thiol protein oxidation was more pronounced than the effectiveness against GSH oxidation probably the substances present in the extract exert their antioxidant action not directly eliminating peroxides, but through scavenging free radicals formed in the system able to oxidize these macromolecules. The protective effectiveness of GEBd against oxidative damage induced by different pro-oxidants demonstrates its potent antioxidant capacity in biological systems. Such efficiency was demonstrated here by different and complementary methods *in vitro*, providing evidences for the molecular mechanisms of the antioxidant action of GEBd as discussed below.

Despite the DPPH-reducing activity of GEBd, its scavenger activity of $O_2^{\cdot-}$ is particularly interesting because this radical is produced *in vivo* and may originate other highly reactive and oxidizing radicals such as hydroxyl (Halliwell, 2009). The mechanisms and

kinectics of the reactivity of $O_2^{\cdot-}$ with polyphenols are extensively studied. Phenolic compounds are weak acids acting as efficient hydrogen donors (Thavasi et al., 2009) able to react with $O_2^{\cdot-}$ through different mechanisms, depending on the nature and number of the substituents in the phenolic ring (René et al., 2010). Structural characteristics of flavonoids important for their free radical scavenging activities includes the 2,3 double bond conjugated with a 4-oxo function, the *o*-di-OH group in the B ring, and the presence of hydroxyl groups at positions 3 and 5 (Soobrattee et al., 2005; Dorta et al., 2008; René et al., 2010). Such structural features contribute also to the reactivity of phenols and flavonoids with hydroxyl ($^{\cdot}OH$) and lipid-derived radicals, such as lipid peroxyl (LOO•), inhibiting lipid peroxidation reactions (Ozyürek et al., 2008; Wilms et al., 2008). Flavonoids are particularly effective as inhibitors of the lipid oxidation chain reactions because (i) they are able to react with the radicalar species involved in this process, and (ii) the flavonoid-derived radical formed is relatively stable due to the possibility of resonance in the phenolic rings (René et al., 2010). Also, another important feature that confers antioxidant action is the metal-chelating activity that occurs when the oxidative damage is caused by metal-catalyzed Fenton-type reactions (Halliwell, 2009). The high content of phenolic compounds and flavonoids in GEBd probably confers to this extract the potent antioxidant activity demonstrated in this study that comes from both free radical scavenging and iron chelating activities.

5. Conclusions

GEBd exert its antioxidant activity by a sum of free radical scavenging and iron chelating activities able to protect membranes from oxidation and consequently from a loss of function. Moreover, the oxidation of thiol groups of mitochondrial proteins and GSH depletion was also prevented by GEBd. Considering the similarity in chemical composition, the antioxidant properties exhibited by *B. dracunculifolia* contribute at least partially to the hepatoprotection described for Brazilian green propolis, and show evidence for its potential as a hepatoprotective agent against oxidative-induced liver damage.

Conflict of Interest

The authors declare that there are no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fct.2011.11.014.

References

Ames, B.M., Shigenaga, M.K., Hagen, T.M., 1993. Oxidants, antioxidants and degenerative diseases of aging. PNAS 90, 7915–7922.
Bankova, V., 2005. Chemical diversity of propolis and the problem of standardization. J. Ethnopharmacol. 100, 114–117.
Banskota, A.H., Tezuka, Y., Adnyana, I.K., Midorikawa, K., Matsushige, K., Message, D., Huertas, A.A., Kadota, S., 2000. Cytotoxic, hepatoprotective and free radical scavenging effects of propolis from Brazil, Peru, the Netherlands and China. J. Ethnopharmacol. 72, 239–246.
Banskota, A.H., Tezuka, Y., Adnyana, I.K., Ishii, E., Midorikawa, K., Matsushige, K., Kadota, S., 2001. Hepatoprotective and anti-*Helicobacter pylori* activities of constituents from Brazilian propolis. Phytomedicine 8, 16–23.
Blois, M.S., 1958. Antioxidant determinations by the use of a stable free radical. Nature 181, 1199–1200.
Borges, M.B.D., Santos, C.G., Yokomizo, C.H., Sood, R., Vitovic, P., Kinnunen, P.K.J., Rodrigues, T., Nantes, I.L., 2004. Characterization of hydrophobic interaction and antioxidant properties of the phenothiazine nucleus in mitochondrial and model membranes. Free Rad. Res. 44, 1054–1063.
Brookes, P.S., Yoon, Y., Robotham, J.L., Anders, M.W., Sheu, S.S., 2004. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. AJP-Cell 287, C817–833.
Brown, G., 1994. Phenylpropanoids and other secondary metabolites from *Baccharis linnearis*. Phytochemistry 35, 1037–1042.
Burdock, G.A., 1998. Review of the biological properties and toxicity of bee propolis (Propolis). Food Chem. Toxicol. 36, 347–363.
Céspedes, C.L., Uchoa, A., Salazar, J.R., Perich, F., Pardo, F., 2002. Plant growth inhibitory activity of p-hydroxyacetophenones and tremenones from Chilean endemic *Baccharis* species and some analogous: a comparative study. J. Agric. Food Chem. 50, 2283–2292.
Chen, Y.T., Zheng, R.L., Jia, Z.J., Ju, Y., 1990. Flavonoids as superoxide scavengers and antioxidants. Free Rad. Biol. Med. 9, 19–21.
Cowart, R.E., Singleton, F.L., Hind, J.S., 1993. Comparison of bathophenanthrolinedisulfonic acid and ferrozine as chelators of iron (II) in reduction reactions. Anal. Biochem. 211, 151–155.
Dorta, D.J., Curti, C., Rodrigues, T., 2006. Effects of flavonoids on mitochondria: an overview on pharmacological and toxicological aspects, In: Moreno, A.J.M., Oliveira, P.J., Palmeira, C.M. (Eds), Mitochondrial Pharmacology and Toxicology, Transworld Research Network, pp. 147–161.
Dorta, D.J., Pigoso, A.A., Mingatto, F.E., Rodrigues, T., Pestana, C.R., Uyemura, S.A., Santos, A.C., Curti, C., 2008. Antioxidant activity of flavonoids in isolated mitochondria. Phytother. Res. 22, 1213–1218.
Gómez-Caravaca, A.M., Gómez-Romero, M., Arráez-Román, D., Segura-Carretero, A., Fernández-Gutiérrez, A., 2006. Advances in the analysis of phenolic compounds in products derived from bees. J. Pharm. Biomed. Anal. 41, 1220–1234.
Halliwell, B., 2009. The wanderings of a free radical. Free Rad. Biol. Med. 4, 531–542.
Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74, 214–226.
Juan, H.V.E., Saad, J.R., Giordano, O.S., García, C., Martín, T., Martín, V.S., Sosa, M.E., Tonn, C.E., 2008. Insect growth regulatory effects of linear diterpenoids and derivatives from *Baccharis thymifolia*. J. Nat. Prod. 71, 190–194.
Jurd, L., Geissman, T.A., 1956. Absorption spectra of metal complexes of flavonoid compound. J. Org. Chem. 21, 1395–1401.
Kimoto, T., Koya-Miyata, S., Hino, K., Micallef, M.J., Hanaya, T., Arai, S., Ikeda, M., Kurimoto, M., 2001. Pulmonary carcinogenesis induced by ferric nitrilotriacetate in mice and protection from it by Brazilian propolis and artepillin C. Virch. Arch. 438, 259–270.
Kowaltowski, A.J., Souza-Pinto, N.C., Castilho, R.F., Vercesi, A.E., 2009. Mitochondria and reactive oxygen species. Free Rad. Biol. Med. 47, 333–343.
Kumazawa, S., Yoneda, M., Shibata, I., Kaneda, J., Hamasaka, T., Nakayama, T., 2003. Direct evidence for the plant origin of Brazilian propolis by the observation of honey bee behavior and phytochemical analysis. CPB 51, 740–742.
Kumazawa, S., Hamasaka, T., Nakayama, T., 2004. Antioxidant activity of propolis of various geographic origins. Food Chem. 84, 329–339.
Labbe, C., Rovirosa, J., Faini, F., Mahu, M., San-Martin, A., Castillo, M., 1986. Secondary metabolites from Chilean *Baccharis* species. J. Nat. Prod. 49, 517–518.
Lebel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231.
Lemos, M., Barros, M.P., Sousa, J.P., Silva Filho, A.A., Bastos, J.K., Andrade, S.F., 2007. *Baccharis dracunculifolia*, the main botanical source of Brazilian green propolis, displays antiulcer activity. J. Pharm. Pharmacol. 59, 603–608.
Ozyürek, M., Bektaşoğlu, B., Güçlü, K., Apak, R., 2008. Hydroxyl radical scavenging assay of phenolics and flavonoids with a modified cupric reducing antioxidant capacity (CUPRAC) method using catalase for hydrogen peroxide degradation. Anal. Chim. Acta. 616, 196–206.
Parejo, I., Viladomat, F., Bastida, J., Rosas-Romero, A., Flerlage, N., Burillo, J., Codina, C., 2002. Comparison between the radical scavenging activity and antioxidant activity of six distilled and nondistilled Mediterranean herbs and aromatic plants. J. Agric. Food Chem. 50, 6882–6890.
Park, Y.K., Paredes-Guzman, J.F., Aguiar, C.L., Alencar, S.M., Fujiwara, F.Y., 2004. Chemical constituents in *Baccharis dracunculifolia* as the main botanical origin of the southeastern Brazilian propolis. J. Agric. Food Chem. 52, 1100–1103.
Rahalison, L., Benathan, M., Monod, M., Frenk, E., Gupta, M.P., Solis, P.N., Fuzzati, N., Hostettmann, K., 1995. Antifungal principles of *Baccharis pedunculata*. Planta Med. 61, 360–362.
René, A., Abasq, M.L., Hauchard, D., Hapiot, P., 2010. How do phenolic compounds react toward superoxide ion? A simple electrochemical method for evaluating antioxidant capacity. Anal. Chem. 82, 8703–8710.
Saija, A., Scalese, M., Lanz, M., Marzullo, D., Bonina, F., Castelli, F., 1995. Flavonoids as antioxidant agents: importance of their interaction with biomembranes. Free Rad. Biol. Med. 19, 481–486.
Santana, D.P., Faria, P.A., Paredes-Gamero, E.J., Caires, A.C.F., Nantes, I.L., Rodrigues, T., 2009. Palladacycles catalyse the oxidation of critical thiols of the mitochondrial membrane proteins and lead to mitochondrial permeabilization and cytochrome c release associated with apoptosis. Biochem. J. 417, 247–256.

Seo, K.W., Park, M., Song, Y.J., Kim, S.J., Yoon, K.R., 2003. The protective effects of Propolis on hepatic injury and its mechanism. Phytother. Res. 17, 250–253.

Sies, H., 1997. Oxidative stress: oxidants and antioxidants. Exp. Phys. 82, 291–295.

Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144–148.

Soobrattee, M.A., Neergheen, V.S., Luximon-Ramma, A., Aruoma, O.I., Bahorun, T., 2005. Phenolics as potential antioxidant therapeutic agents: mechanism and actions. Mut. Res. 11, 200–213.

Sousa, J.P.B., Bueno, P.C.P., Gregório, L.E., Silva Filho, A.A., Furtado, N.A.J.C., Sousa, M.L., Bastos, J.K., 2007. A reliable quantitative method for the analysis of phenolic compounds in Brazilian propolis by reverse phase high performance liquid chromatography. J. Sep. Sci. 30, 2656–2665.

Sousa, J.P.B., Leite, M.F., Jorge, R.F., Resende, D.O., Silva-Filho, A.A., Furtado, N.A.J.C., Soares, A.E.E., Spadaro, A.C.C., Magalhães, P.M., Bastos, J.K., 2011. Seasonality Role on the Phenolics from Cultivated *Baccharis dracunculifolia*. Evid. Based Complement Alternat. Med. 2011, 1–8.

Tahara, E.B., Navarete, F.D.T., Kowaltowski, A.J., 2009. Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation. Free Rad. Biol. Med. 2009 (46), 1283–1297.

Thavasi, V., Bettens, R.P., Leong, L.P., 2009. Temperature and solvent effects on radical scavenging ability of phenols. J. Phys. Chem. A 113, 3068–3077.

Wilms, L.C., Kleinjans, J.C., Moonen, E.J., Briedé, J.J., 2008. Discriminative protection against hydroxyl and superoxide anion radicals by quercetin in human leucocytes in vitro. Toxicol. In Vitro 22, 301–307.

Zdero, C., Bohlmann, F., King, R.M., Robinson, H., 1986. Diterpene glycosides and other constituents from Argentinian *Baccharis* species. Phytochemistry 25, 2841–2855.