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Artigo

Antioxidant capacity and biological activity of essential oil and methanol extract of *Hyptis crenata* Pohl ex Benth

Monaliza M. Rebelo,[1] Joyce Kelly R. da Silva,[2] Eloísa Helena A. Andrade,[3] José Guilherme S. Maia*,[3]

[1]Programa de Pós-Graduação em Ciências e Tecnologia de Alimentos, Universidade Federal do Pará, 66075-900 Belém-PA, Brazil,
[2]Programa de Pós-Graduação em Química, Universidade Federal do Pará, 66075-900 Belém-PA, Brazil,
[3]Faculdade de Engenharia Química, Universidade Federal do Pará, 66075-900 Belém-PA, Brazil

RESUMO: "Capacidade antioxidante e atividade biológica do óleo essencial e extrato metanólico de *Hyptis crenata* Pohl ex Benth". O óleo essencial das folhas e ramos finos frescos e secos de *Hyptis crenata* forneceu os seguintes rendimentos, 1,4% e 0,9%. Os constituintes voláteis principais foram α-pineno (22,0%; 19,5%), 1,8-cineol (17,6%; 23,2%), β-pineno (17,0%: 13,8%), cânfora (4,7%; 11,6%), limoneno (5,4%; 4,4%) e γ-terpineno (3,5%; 2,4%), totalizando mais de 70% nos óleos. A atividade de seqüestro do radical DPPH para o extrato metanólico (CE_{50}, 16,7 ± 0,4 μg/mL) foi comparável ao do BHT (19,8 ± 0,5 μg/mL) mostrando uma significante atividade antioxidante. Os óleos apresentaram baixa atividade. O teor de fenólicos totais (TP, 373,0 ± 15,9 mg GAE/g) e equivalente trolox (TEAC, 226,8 ± 0,5 mg TE/g) confirmaram a atividade antioxidante do extrato metanólico, que pode ser atribuída à presença de compostos fenólicos polares. No teste com larvas de camarão as concentrações letais para o óleo e extrato metanólico foram 6,7 ± 0,2 μg/mL e 13,0 ± 3,7 μg/mL**,** respectivamente, fornecendo importante evidência de suas atividade biológicas.

Unitermos: *Hyptis crenata*, Lamiaceae, "salva-do-marajó", óleo essencial, atividade antioxidante, teor de fenólicos totais, citotoxidade.

ABSTRACT: The essential oils of fresh and dried leaves and fine stems of *Hyptis crenata* furnished the following yields: 1.4% and 0.9%. The main volatile constituents were α-pinene (22.0%; 19.5%), 1,8-cineole (17.6%; 23.2%), β-pinene (17.0%; 13.8%), camphor (4.7%; 11.6%), limonene (5.4%; 4.4%) and γ-terpinene (3.5%; 2.4%), totalizing more than 70% in the oils. The DPPH radical scavenging activity (EC_{50}, 16.7 ± 0.4 μg/mL) of the methanol extract was comparable to BHT (19.8 ± 0.5 μg/mL) showing a significant antioxidant activity. The oils showed low activities. The amount of total phenolics (TP, 373.0 ± 15.9 mg GAE/g) and trolox equivalent (TEAC, 226.8 ± 0.5 mg TE/g) confirmed the antioxidant activity of the methanol extract that can be attributed to the presence of polar phenolic compounds. In the brine shrimp bioassay the lethal concentrations (LC_{50}) for the oil and methanol extract were 6.7 ± 0.2 μg/mL and 13.0 ± 3.7 μg/mL**,** respectively, providing important evidence of their biological activities.

Keywords: *Hyptis crenata*, Lamiaceae, salva-do-marajó, essential oil, antioxidant activity, total phenolic content, cytotoxicity.

INTRODUCTION

The genus *Hyptis* comprises nearly 400 species belonging to Lamiaceae and occurring in tropical America. The species *Hyptis crenata* Pohl ex Bentham is an annual herbaceous plant reaching 60-80 cm in height, growing spontaneously on sandy soil near streams in the Marajó Island, State of Pará, Brazil. The plant is known as "salva-do-marajó", "salsa-do-campo" or "hortelã-do-campo" and is used by the riverine communities as spices for food aromatization and anti-inflammatory medicine (Maia et al., 2001; Andrade et al., 2002).

Concerning the *Hyptis* species occurring in the Brazilian Amazon we previously analyzed the essential oil composition of *H. suaveolens*, *H. mutabilis* and *H. goyazensis* (Gottlieb et al., 1981; Luz et al., 1984 and 1989; Andrade et al., 2003). The essential oil of another specimen of *H. crenata* collected in the Brazilian Pantanal was first reported (Scramin et al., 2000).

Essential oils and plant extracts have been studied for their potential use as alternative dietary supplement to prevent infectious diseases and in the preservation of foods from the toxic effects of oxidants. The antioxidant properties of aromatic plants and spices

* E-mail: gmaia@ufpa.br, Fax +55-91-3201-7295
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have been indicated as effective in delaying the lipid peroxidation of foods (Bracco et al., 1981; Kramer, 1985; Lagouri et al., 1993; Souza et al., 2007). Tsimidou and Boskou (1994) observed that between the herbs and spices extensively studied, the plants belonging to the Lamiaceae family have significant antioxidant activity. In spite of the intensive investigation of the antioxidant activities of the European and Asian herbs and spices, little is known about the properties of these plants in South America (Stashenko et al., 2004; Sachettii et al., 2004; Costa et al., 2008; Sousa et al., 2008).

As part of an ongoing inventory of the Amazon odoriferous flora, including its herbs and spices, this study report the essential oil composition of a specimen of *Hyptis crenata* collected on the Marajó Island, PA, Brazil, as well as its antioxidant capacity, the total phenolic content and the cytotoxicity of the oils and methanol extract using the DPPH radical scavenging, the Folin-Ciocalteu reagent and the brine shrimp bioassay.

MATERIAL AND METHODS

Plant material

Samples of *Hyptis crenata* were collected in the locality of "Deus-me-ajude" (God, help me), Municipality of Salvaterra, Marajó Island, State of Pará, Brazil, in March 2005. The plant was identified by comparison with an authentic voucher of *H. crenata* (#MG174699) that is deposited in the herbarium of Emílio Goeldi Museum, city of Belém, State of Pará, Brazil.

Plant processing

The plant material was separated in fresh and air-dried portions (150 g each) and submitted to hydrodistillation using a Clevenger-type apparatus (3 h). The hydrodistilled oils were dried over anhydrous sodium sulfate and the percentage content calculated on basis of the plant dry weight. The moisture content of samples was calculated after the aqueous phase separation using a Dean-Stark trap (5 g, 30 min) and toluene. Similarly, the powdered air-dried sample was submitted to solvent extraction (75 g, 4 h) using a Sohxlet extractor and methanol. The solvent was eliminated by vacuum evaporation and the yield was calculated. The oils were codified as HcOf and HcOd, and the methanol extract was codified as HcEd.

Oil composition analysis

The analysis of the volatile compounds was performed on a Finnigan Mat INCOS XL GC-MS instrument, with the following conditions: WCOT DB-5ms (30 m x 0.25 mm; 0.25 μm film thickness) fused silica capillary column; temperature programmed: 60-240 °C (3 °C/min); injector temperature: 220 °C; carrier gas: helium, adjusted to a linear velocity of 32 cm/s (measured at 100 °C); injection type: splitless (2 μL, of a 1:1000 hexane sol.); split flow was adjusted to give a 20:1 ratio; septum sweep was a constant 10 mL/min; EIMS: electron energy, 70 eV; ion source temperature and connection parts: 180 °C. The quantitative data of the oils were obtained by peak area normalization using a HP 5890 GC/FID operated under the same GC-MS condition, except for the carrier gas that was hydrogen produced by a Packard hydrogen generator and a WCOT CP-Sil CB (25 m x 0.25 mm; 0.25 μm film thickness) fused silica capillary column. The individual components of oils were identified by comparison of both mass spectrum and their GC retention data with those of authentic compounds previously analyzed and stored in the data system. Additional identifications were made by comparison of mass spectra with those existing in the data system libraries and cited in the literature (Adams, 2007; NIST, 2005). The retention index was calculated for all volatiles constituents using an n-alkanes homologous series.

Antioxidant capacity evaluation

A stock solution of DPPH radical (0.5 mM) in methanol was prepared. The solution was diluted in methanol (60 μM approx.) measuring an initial absorbance of 0.62 ± 0.02 in 517 nm at room temperature. The reaction mixture was composed by 1950 μL of DPPH solution and 50 μL of the samples diluted in different methanol portions. For each sample a methanol blank was also measured. The absorbance was measured in the reaction starting (time zero), every 5 min during the first 20 min and then at continuous intervals of 10 min up to constant absorbance. All experiments were triplicate. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and BHT (butylated hydroxytoluene) were used as standard antioxidants. The radical scavenging activity of each sample was calculated by the DPPH inhibition percentage according the Eq. (1):

$$IP_{DPPH} = 100(A - B)/A, \quad (1)$$

where A and B are the blank and sample absorbance values in the end reaction. The radical scavenging activity, expressed as milligrams of trolox equivalent per gram of each sample, was also calculated by means of the Eq. (2):

$$TE = (A - B)/(A - C) \times 25/1000 \times 250.29/1000 \times 1000/10 \times D, \quad (2)$$

where A, B and C are the blank, sample and trolox absorbance values in the reaction end, and D is the dilution factor (Choi et al., 2000; Hu et al., 2004; Yamaguchi et al., 1998). The concentration of antioxidant required

for 50% scavenging of DPPH radicals (EC_{50}) were determined by linear regression using Windows/Excel.

Total phenolics evaluation

The amount of total phenolics (TP) of methanol extract was determined according to the Folin-Ciocalteu procedure (Singleton and Rossi, 1965; Kähkönen et al., 1999). The experimental calibration curve was prepared using 500 μL of aqueous solution of gallic acid mixed with 250 μL of Folin-Ciocalteu reagent (1.0 N) and 1250 μL of sodium carbonate (75 g/L) resulting in final gallic acid concentrations of 0.57, 1.14, 2.28, 3.42, 4.56, 5.70 and 6.84 mg/L. The absorbance was measured after 30 min at 760 nm and 25 ºC (UV-Vis spectrophotometer, ULTROSPEC 2000). The methanol extract was dissolved in methanol (2 mg/mL), diluted in water (1:99) and submitted to the same procedure. The total phenolics content was expressed as gallic acid equivalents (GAE) in milligrams per gram of extract, using the Eq. (3):

$$GAE\ (mg/L) = A \times D \times 7.93 \times d, \quad (3)$$

where A is the sample absorbance, D is the sample dilution, 7.93 is the angular coefficient, and d is the reaction dilution.

Table 1. Composition of volatiles identified in the oils of *Hyptis crenata*.

No.	Components	RI[a]	HcOf (%)[b]	CsOd (%)[b]
1	α-thujene	930	0.6	0.5
2	α-pinene	939	22.0	19.5
3	camphene	952	2.7	2.7
4	sabinene	975	0.6	0.1
5	β-pinene	979	17.0	13.8
6	myrcene	990	1.6	1.3
7	α-phellandrene	1003	0.2	0.2
8	α-terpinene	1117	1.1	0.8
9	*p*-cymene	1025	2.0	1.8
10	limonene	1028	5.4	4.4
11	1,8-cineole	1033	17.6	23.2
12	γ-terpinene	1060	3.5	2.4
13	terpinolene	1089	1.1	0.8
14	camphor	1142	4.7	11.6
15	borneol	1164	2.1	5.3
16	4-terpineol	1178	1.0	2.7
17	α-terpineol	1190	-	0.1
18	α-longipinene	1353	2.0	0.1
19	β-caryophyllene	1419	1.0	0.6
20	aromadendrene	1441	-	0.1
21	β-chamigrene	1475	2.3	0.2
22	β-himachalene	1499	1.3	0.2
23	himachalol	1647	1.3	0.1
	Total		91.1	92.5

[a]Retention indices calculated for all volatile constituents using a homologous series of *n*-alkanes.
[b]Percentages are the mean of three runs and were obtained from FID electronic integration.

Table 2. Data of antioxidant capacity for the plant oils and methanol extract of *Hyptis crenata*.

Samples	Concentration (μg/mL)	DPPH Inhibition[a] (%)	DPPH EC_{50} [a] (μg/mL)	TEAC[a] (mg TE/g)	TP[a] (mg GAE/g)
HcOf	15417.0	79.9 ± 1.5	1951.5 ± 54.5	1.1 ± 0.1	
	5781.0	64.5 ± 1.3			
	3558.0	52.3 ± 2.2			
	1658.7	50.1 ± 2.3			
	773.3	42.6 ± 1.9			
HcOd	15417.0	71.4 ± 0.5	6882.7 ± 522.9	1.0 ± 0.1	
	5781.0	57.6 ± 1.2			
	3558.0	42.6 ± 1.5			
	1658.7	32.5 ± 1.5			
	773.3	24.5 ± 1.5			
HcEd	40.0	94.0 ± 0.1	16.7 ± 0.4	226.8 ± 0.5	373.0 ± 15.9
	20.0	73.6 ± 0.5			
	10.0	38.7 ± 0.5			
	7.0	25.9 ± 1.1			
	5.0	14.2 ± 2.3			
Trolox	10	96.7 ± 0.5	4.7 ± 0.4	-	-
	4	53.2 ± 1.6			
	2	21.5 ± 2.3			
BHT[b]	-	-	19.8 ± 0.5	-	-

[a]Mean ± standard deviation, [b]Literature data.

Table 3. Lethal concentration of oil and methanol extract of *Hyptis crenata* for the brine shrimp bioassay.

Samples	Concentration (μg/mL)	Mortality (%)	LC_{50} (μg/mL)
HcOf	1	0,0	6.7 ± 0.2
	5	40.0	
	10	60.0	
	25	100.0	
HcEd	1.0	10.0	13.0 ± 3.7
	10.0	46.7	
	25.0	56.7	
	50.0	80.0	
	100.0	86.7	
	500.0	95.0	

Brine shrimp bioassay

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity (biological activity) of the oils and extract (Meyer et al., 1982; Lewan et al., 1992). Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a glass rectangular vessel (5 L), filled with sterile artificial seawater, prepared using water (2 L), NaCl (46 g), $MgCl_2 \cdot 6H_2O$ (22 g), Na_2SO_4 (8 g), $CaCl_2 \cdot 2H_2O$ (2.6 g) and KCl (1.4 g), with pH 9.0 adjusted with Na_2CO_3 under constant aeration for 48 h. After hatching active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in vials containing 5 mL of brine solution. In each experiment the oil (1%) and extract (5%) solutions were prepared using the brine solution and DMSO. From these oil and extract solutions four other solutions at different concentrations were prepared and then added to the 5 mL brine solution. The vials were maintained at room temperature for 24 h under the light and the surviving larvae were counted. Experiments were conducted along with control and different concentrations (1, 10, 100 and 1000 μg/mL) in a set of three tubes per dose. The percentage lethality was determined by comparing the mean value of surviving larvae of the test and the control tubes. Lethal concentration (LC_{50}) values were obtained from the best-fit line plotting concentration versus percentage lethality (Finney, 1971).

RESULTS AND DISCUSSION

The fresh (HcOf) and dried (HcOd) leaves and fine stems of *H. crenata* provided oil yields of 1.4% and 0.9%, respectively. The oil yield of the dried plant (HcOd, 0.9%) was equal to that before reported by us (Zoghbi et al., 2002). The oil yield of the fresh plant (HcOf, 1.4%) was greater and it was determined by the first time. At the same time, the chemical composition of the oil was similar to that of our previous analysis using plants collected on Marajó Island, Brazil (Zoghbi et al., 2002). For the present analyses the main constituents of fresh and dried plants were α-pinene (22.0% and 19.5%), 1,8-cineole (17.6% and 23.2%), β-pinene (17.0% and 13.8%), camphor (4.7% and 11.6%), limonene (5.4% and 4.4%) and γ-terpinene (3.5% and 2.4%), totalizing over 70% in the oils. The volatiles constituents identified in the oils of *H. crenata* are listed in Table 1.

The oils of *H. crenata* (HcOf and HcOd) were tested at high concentration, between 773.5 and 15417.0 μg/mL, to determine the DPPH scavenging activities. The kinetic reaction was slow with an average of 180 min. The resulting DPPH inhibition percent varied from 42.6% to 79.9% in the fresh oil (HcOf) and from 24.5% to 71.4% in the dried oil (HcOd) showing low antioxidant activities. On the other hand the methanol extract of dried plant (HcEd) was assayed at low concentrations, between 5 and 40 μg/mL, to determine the DPPH scavenging activities. The kinetic reaction was slow in all tested concentration with an average of 120 min. The resulting DPPH inhibition percent ranged from 14.2% to 94.0% in the dried plant extract (HcEd) indicating a significant antioxidant property.

The EC_{50} values for the plant oils (HcOf: 1951.5 ± 54.5 μg/ml; HcOd: 6882.7. ± 522.9 μg/mL), defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals, were very high, confirming that the oils do not have important antioxidant activity. Choi et al. (2000) has reported that the monoterpenes γ-terpinene and terpinolene showed a DPPH radical-scavenging effect three times stronger than the trolox (4.7 ± 0.4 μg/mL), while others, such as α-pinene, β-pinene and 1,8-cineole did not show significant antioxidant capacity. The percentage of γ-terpinene and terpinolene in the analyzed oils is very small and thus with low contribution to their antioxidant activity. On the other hand, the EC_{50} value obtained for the dried methanol extract (HcEd: 16.7 ± 0.4 μg/mL) is comparable with that of butylated hydroxytoluene (BHT, 19.8 ± 0.5 μg/mL) (Sökmen et al., 2004), a commercial synthetic antioxidant, showing that it has a very strong antioxidant activity.

The amount of total phenolics (TP, 373.0 ± 15.9 mg GAE/g) and trolox equivalent (TEAC, 226.8 ± 0.5 mg TE/g) for the dried methanol extract (HcEd) confirmed the significant antioxidant activity of *H. crenata* that could be attributed to the presence of polar phenolic compounds.

The data of DPPH inhibition, DPPH radical activity (EC_{50}), trolox equivalent antioxidant capacity (TEAC) and total phenolics (TP) for the plant oils and methanol extract of *H. crenata* are shown in Table 2.

For the brine shrimp bioassay performed with the plant oil (HcOf) and methanol extract (HcEd) the lethal concentration (LC_{50}) was 6.7 ± 0.2 μg/ml and 13.0 ± 3.7 μg/mL, respectively, showing a significant cytotoxicity where the oil was twice higher than the methanol extract. Oils and extracts from plants presenting lethal concentration values below 1000 μg/mL are considered as bioactive (Meyer et al., 1982). The data for brine shrimp bioassay are shown in Table 3.

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