ANTIOXIDANT POTENTIAL OF *Casearia sylvestris* EXTRACTS

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Abstract. *Casearia sylvestris* is a plant widely used in popular medicine in Brazil and other American countries. In their extracts there are some substances of great interest in pharmaceutical and/or cosmetic industries, such as coumarins, flavonoids and diterpenes, especially casearins. Studies have been demonstrated citotoxic activity of casearins against microorganisms and tumor cells, suggesting antimicrobial and antitumor activities. *Casearia* extracts also presents sesquiterpenes as β- caryophyllene and α-humulene, which have proved citotoxic and anti-inflammatory activities. The aim of the present work was to compare the antioxidant capacity of *C. sylvestris* extracts obtained by supercritical fluid extraction (SFE) and by low pressure extractions Sohxlet (SOX) and maceration (MAC). The SFEs were conducted with CO_2 (100-300bar/40°C-60°C) and CO_2 added with co-solvents (2%-8% of ethanol and ethyl acetate), while SOX and MAC were applied using hexane (HX), ethanol (ETOH), dichloromethane (DCM), ethyl acetate (ETOAC) and chloroform (CLF) as solvents. The antioxidant potential was evaluated indirectly by the total phenolic content (TPC), through the Folin-Ciocalteau method, and directly by radical DPPH (2,2-diphenyl-1-picrylhydrazil) and β-carotene bleaching methods. The antioxidant potential by DDPH method resulted in effective concentration at 50% (EC_{50}) values of 231±6μg/mL (MAC-ETOH) and 245±4μg/mL (300bar/50°C + 8%ETOH). The TPC presented maximum values of 64.35±0.04mgGAE/g and 169.4±0.6mgGAE/g in the 300bar/50°C + 8%ETOH and SOX-ETOH samples, respectively. The results of β-carotene bleaching method ranged from 18±5%, in the SFE sample obtained at 100bar/60°C, to 111±3% in the SOX-ETOAC extract, after 120 minutes-reaction.

Keywords: supercritical fluid extraction, co-solvent, low pressure extractions, phenolic content, DPPH.

1. Introduction

The demand for natural antioxidants has been increased due to consumer concerns about the safety of synthetic antioxidants [1]. Phenolic compounds, particularly flavonoids, have been shown to possess an important antioxidant activity towards these radicals, which is principally based on their structural characteristics (number and position of phenolic hydroxyls, other groups, conjugation) [2].

The natural antioxidant compounds have been isolated from different kind of natural products, including flavonoids, phenolic acids, terpenes, tocopherols, phospholipids and polyfunctional organic acids [2].

Casearia sylvestris is a native medicinal plant in Brazil, Peru, Argentina, Uruguay and Bolivia. The leaves of the plant are popularly used in folk medicine as antiseptic, topical anaesthetic, antitumor and antiulcer agents, and also to heal skin wound diseases [3,4]. In the *Casearia* extracts there are some substances of great interest, such as coumarins, flavonoids and diterpenes, especially clerodane diterpenes as casearins [5-8]. Also, the casearvestrins, another clerodane diterpene, have been identified in the *C. sylvestris* leaves [9, 10].

The bioactive clerodanes (casearins and casearvestrins) are oxygenated tricyclic diterpenes with excellent cytotoxic and antitumor potential [7;11-13]. Despite their well-known antiproliferative actions, there are no studies showing how these diterpenes cause cell death, although Huang et al. [14] have suggested that

clerodane diterpenes isolated from *Casearia membranacea* induce apoptosis in PC-3 cells (human prostate tumor line). *Casearia* extracts also presents sesquiterpenes as β-cariofilene and δ-humulene, which have proved citotoxic and anti-inflammatory activities [15-17].

Supercritical fluid extraction (SFE) is an alternative process to conventional extractions in various applications due to the possibility in the obtention of solvent-free extracts and the use of low extraction temperatures. Other advantages include lower consumption of hazardous solvents, higher sample throughput and a complete separation between products and solvent [18-19].

Together with the importance of the extraction technique on product quality, the efficiency and reproducibility of extracting operations must be guaranteed by evaluating the process selectivity to bioactive compounds.

According to Silva et al. [20] the major compounds observed in the essential oil of *C. sylvestris* were: bicyclogermacrene (43.6 %), β-caryophyllene (18.1 %), spathulenol (15.9 %), germacrene B (5.2 %), α-humulene (4.7 %), α-humulene (4.7 %), α-pinene (4.0 %), germacrene D (3.9 %), globulol (3.0 %), α-muurolol (2.7 %). Among these compounds, β-caryophyllene and α-humulene are sesquiterpenes with high cytotoxic activity.

The aim of the present work was to compare the extracts of *C. sylvestris* obtained by supercritical fluid extraction (SFE) and by low pressure extractions Sohxlet (SOX) and maceration (MAC), in terms of antioxidant capacity, evaluated by total phenolic content, DPPH assay and β-carotene bleaching method.

2. Material and Methods

2.1. Sample preparation

Leaves of *C. sylvestris*, provided by Brazervas Laboratório Fitoterápico Ltda., Osório/RS, Brazil, were air-dried at room temperature up to 13.72 ± 0.05 % (w/w) of moisture. The dried material was ground in a knife mill (De Leo, Porto Alegre/RS, Brazil) and characterized by size classification in a vertical vibratory sieve shaker (Bertel Metalurgic Ind. Ltda., Caieiras/SP, Brazil). The mean particle diameter was calculated based on mean size distribution as described by Gomide [21], resulting in 372 ± 37 μm. The sample was stored at -18 ^{o}C until the extractions were performed.

2.2. Supercritical fluid extraction (SFE)

The SFE of *C. sylvestris* was performed in a dynamic extraction unit previously described by Zetzl et al [22], as well as the extraction procedure is completely explained by Michielin et al. [23]. Briefly, the extraction procedure consisted of placing 15 g of dried and milled material inside the column to form the particles fixed bed, followed by the control of the process variables (temperature, pressure and solvent flow rate). The extraction was performed and the solute collected in amber flasks and weighted in an analytical balance (OHAUS, Model AS200S, NJ, USA). The SFE assays were performed with CO_2 and CO_2 added with co-solvents. The SFE assays with CO_2 were conducted at 40 ^{o}C, 50 ^{o}C and 60 ^{o}C and 100, 200 and 300 bar, using CO_2 flow rate of 8.3 ± 2 g/min, during 3.5 h extraction. The extraction procedures with CO_2 added with co-solvents were done at 50 ^{o}C, 200 bar and CO_2 flow rate of 8.3 ± 2 g/min (3.5 h extraction), using 2 %, 5 % and 8 % (w/w) of ethanol and ethyl acetate, separately. The SFE assays were performed with 99.9 % pure carbon dioxide, delivered at pressure up to 60 bar (White Martins, Brazil).

2.3. Low pressure extractions (LPE)

The Soxhlet (SOX) and maceration (MAC) extraction methods were performed evaluating the following solvents: hexane (HX), dichloromethane (DCM), chloroform (CLF), ethyl acetate (EtOAc) and ethanol (EtOH), with ascending polarity values of 0, 3.1, 4.1, 4.4 and 5.2, respectively [24]. The SOX method consists of 150 mL of solvent recycling over 5 g of dried sample in a Soxhlet apparatus for 6 h extraction at the boiling temperature of different solvents. The MAC procedure was performed with 10 g of dried sample material placed in 100 mL of each solvent mentioned, with occasionally agitation, during seven days.

2.4. Solvent-solute separation

The resulting mixtures from the SOX and MAC techniques with different solvents and SFE performed with CO_2 added with co-solvents were concentrated by using reduced pressure to evaporate the solvents in a rotary evaporator (Fisatom, 802, Brazil), obtaining the extracts.

2.5. Antioxidant activity

The antioxidant activity was performed for the extracts obtained by SFE and by LPE. Also, the results were compared with a synthetic compound with antioxidant activity, BHT (butylated hydroxytoluene).

Total phenolic content (TPC). The TPC was determined for each extract sample according to the Folin–Ciocalteau method [25, 26]. Briefly, the reaction mixture was composed by 0.1 mL of extract (1667 mg/L), 7.9 mL of distilled water, 0.5 mL of Folin–Ciocalteau reagent and 1.5 mL of 20% sodium carbonate. The flasks were agitated, held for 2 h, and the absorbance was measured at 765 nm. The TPC was calculated according to a standard curve, prepared previously with gallic acid as standard. The results (mean value of the triplicate assays) were expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract (mg GAE/g).

Free radical scavenging activity. The free radical scavenging activity of the *C. sylvestris* extracts at 500 µg/mL was evaluated using 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenger method measured at 517 nm and converted in percentage of antioxidant activity (% AA). DPPH is a free radical, stable at room temperature, which produces a violet solution in ethanol. In presence of antioxidant compounds the DPPH is reduced producing a non-colored solution. This activity was also expressed as the effective concentration at 50 % (EC_{50}), i.e., the concentration of the solution required to give a 50 % decrease in the absorbance of the test solution compared [27,28].

Antioxidant activity with the β-carotene bleaching method. The β-carotene bleaching rate was determined by the difference in absorbance (470 nm) values of each extract at 0 min and at 120 min. The antioxidant activity from the β-carotene/linoleic acid system was carried out according to the method described by Matthäus [29] and Kang et al. [30]. Briefly, 40 mg of linoleic acid and 400 mg of Tween 20 were transferred into a flask, and 1 mL of a β-carotene-chloroform solution (3.34 mg/mL) was added. Chloroform was removed by rotary evaporation at 40 °C. Then 100 mL of distilled water was slowly added and vigorously agitated to form a stable emulsion. An aliquot of 5 mL of this emulsion was added with 0.2 mL of ethanolic *C. sylvestris* extract solution (1667 mg/mL) and the absorbance was immediately measured at 470 nm against a blank consisting of the emulsion without β-carotene. The tubes were placed in a water bath at 50 °C and the absorbance was measured every 15 min up to 120 min. The absorbance values (mean of the triplicate experiments) were converted into percentage of antioxidant activity (% AA).

2.6. Statistical analysis

The antioxidant activity results obtained by the methods described in section 2.5 were statistically evaluated by a one-way analysis of variance (ANOVA), using the Software Statistica for Windows 7.0 (Statsoft Inc., USA) in order to detect significant differences between samples. The significant differences ($p < 0.05$) were analyzed by Tukey test.

3. Results and Discussion

The results for antioxidant activity are shown in Table 1, calculated according to procedures described in section 2.5 for the analyses of TPC, DPPH and β-carotene, obtained for samples of *C. sylvestris* extracts for different extraction methods (SFE and LPE) and for BHT as standard sample.

The literature shows 50 ^{o}C as the optimum temperature for phenolic compounds extraction from vegetable matrixes due to the thermal sensivity of these kinds of compounds [32]. As it can be noticed in Table 1, most of the SFE conditions obtained at 50 ºC presented the best antioxidant results, confirming the behavior described in literature. Among the results from Table 1 some SFE conditions presented a good antioxidant potential.

According Table 1, the best TPC results were achieved by SOX-ETOH and MAC-ETOH (169,4 ± 0,6 mg GAE/g and 135 ± 2 mg GAE/g, respectively). For the SFE extractions, the best conditions were 100 bar/50 ^{o}C

(45 ± 1 mg GAE/g), 200 bar/50 °C + 2 % ETOAC (35,4^{defgh} ± 0,9 mg GAE/g) and 300 bar/50 °C (35,4 ± 0,6 mg GAE/g).

The highest %AA values (Table 1) determined by the DPPH method for LPE extractions were obtained by MAC-ETOH, SOX-ETOH and MAC-ETOAC. The highest %AA showed by SFE extractions were at 200 bar/50 °C ± 2% ETOAC, 200 bar/50 °C ± 8 % ETOH and 200 bar/50 °C ± 5 % ETOH. The best EC_{50} results were achieved by the same extracts.

Table 1. Antioxidant activity for *C. sylvestris* extracts from different methods and BHT.

Extraction[(1)]	Solvent[(2)]	TPC (mg GAE/g)[(3)]	EC_{50} (µg/mL)[(4)]	% AA (500 µg/mL)[(5)]	% AA (120 min)[(6)]
SOX	HX	48,9^{d} ± 0,9	699^{def} ± 16	35^{ij} ± 1	86^{bcdefg} ± 5
SOX	ETOH	169,4^{b} ± 0,6	244^{a} ± 2	90^{a} ± 1	99^{abcdef} ± 9
SOX	DCM	34,0^{defghi} ± 0,9	316^{abc} ± 8	79^{c} ± 2	98^{abcdef} ± 1
SOX	ETOAC	46de ± 1	297^{ab} ± 14	84^{b} ± 2	111^{ab} ± 3
SOX	CLF	44^{defg} ± 2	282^{ab} ± 2	83,2^{bc} ± 0,2	100^{abcde} ± 6
MAC	HX	41^{defg} ± 2	642^{bcde} ± 13	38,72^{hi} ± 0,09	75^{defg} ± 5
MAC	ETOH	135^{c} ± 2	231^{a} ± 6	92,2^{a} ± 0,9	35^{i} ± 2
MAC	DCM	47de ± 1	419^{abcd} ± 13	59^{f} ± 3	71^{fgh} ± 5
MAC	ETOAC	46,88^{de} ± 0,04	239,7^{a} ± 0,3	90,3^{a} ± 0,8	44^{hi} ± 4
MAC	CLF	44,1^{def} ± 0,4	421^{abcd} ± 2	58,4^{ef} ± 0,9	66^{gh} ± 4
SFE 40 ºC/100 bar	CO_2	33,4^{defghi} ± 0,6	4568^{j} ± 362	5,7^{m} ± 0,3	71,7^{efgh} ± 0,3
SFE 50 ºC/100 bar	CO_2	45^{def} ± 1	2674^{i} ± 335	8^{m} ± 2	66^{gh} ± 3
SFE 60 ºC/100 bar	CO_2	8,4^{j} ± 0,9	6251^{k} ± 600	4,4^{m} ± 0,1	18^{i} ± 5
SFE 40 ºC/200 bar	CO_2	24,5^{efghij} ± 0,4	1055^{fgh} ± 56	24^{k} ± 1	74^{efg} ± 3
SFE 50 ºC/200 bar	CO_2	20,4^{ghij} ± 0,4	980^{efg} ± 10	24,7^{k} ± 0,3	98^{abcdef} ± 6
SFE 60 ºC/200 bar	CO_2	25^{fghij} ± 1	1370^{h} ± 110	19^{l} ± 1	84^{cdefg} ± 3
SFE 40 ºC/300 bar	CO_2	19,5^{hij} ± 0,6	744^{def} ± 27	34^{ij} ± 1	82^{cdefg} ± 17
SFE 50 ºC/300 bar	CO_2	35,4^{defgh} ± 0,6	596^{abcd} ± 7	42,6^{h} ± 0,5	106^{abc} ± 4
SFE 60 ºC/300 bar	CO_2	13,7^{ij} ± 0,3	1224^{gh} ± 45	22,1^{kl} ± 1	19^{i} ± 5
SFE 50 ºC/200 bar	CO_2 ± 2% ETOH	22^{ghij} ± 1	680^{cdef} ± 20	38^{hi} ± 1	73^{efg} ± 16
SFE 50 ºC/200 bar	CO_2 ± 5% ETOH	29,4^{defghi} ± 0,3	409^{abcd} ± 3	62,1^{def} ± 0,2	84^{cdefg} ± 3
SFE 50 ºC/200 bar	CO_2 ± 8% ETOH	25^{fghij} ± 2	398^{abcd} ± 10	64de ± 2	80^{cdefg} ± 5
SFE 50 ºC/200 bar	CO_2 ± 2% ETOAC	35,4^{defgh} ± 0,9	398^{abcd} ± 14	65^{d} ± 3	110^{ab} ± 1
SFE 50 ºC/200 bar	CO_2 ± 5% ETOAC	25,7^{defghij} ± 0,4	770^{def} ± 30	33^{j} ± 2	103^{abcd} ± 18
SFE 50 ºC/200 bar	CO_2 ± 8% ETOAC	28^{defghij}±1	519^{abcd} ± 6	48^{g} ± 2	90^{abcdefg} ± 7
BHT[(7)]	-	268^{a} ± 13	261^{a} ± 12	89.7^{a} ± 0.5	113^{a} ± 7

Same letters in the same column indicate no significant difference at level of 5 % ($p < 0.05$).
[(1)]SFE: supercritical fluid extraction; SOX :Soxhlet extraction; MAC: Maceration extraction; [(2)]HX:hexane; DCM: dichloromethane; ETOAC:ethyl acetate; ETOH: ethanol; CLF: chloroform; CO_2: carbon dioxide; BHT: butylated hydroxytoluene; [(3)]TPC: Total phenolic content**;** [(4)]EC_{50}: Effective concentration at 50 %; [(5)]% AA (500 µg/mL): Antioxidant activity evaluated by free radical scavenging activity (DPPH); [(6)]% AA (120 min): Antioxidant activity evaluated by the β-carotene bleaching method; [(7)] Benelli et al. [31].

The values for the antioxidant activity by β-carotene bleaching method (Table 1) show that SFE obtained at 200 bar/ 50°C + 2 % ETOAC (110 ± 1 %), 200 bar/ 50°C + 5 % ETOAC (103 ± 18) and 200 bar/ 50°C + 8 % ETOAC (90 ± 7 %), 300 bar/ 50 °C (106 ± 4 %) and 200 bar/50 °C (98 ± 6 %) were statistically equal to BHT antioxidant potential (113 ± 7 %). In the same way, the LPE extractions equal to BHT potential were SOX-ETOAC (111 ± 3 %), SOX-CLF (100 ± 6 %) and SOX-DCM (98 ± 1 %).

The SFE results evidenced a tendency of all antioxidants potential increase and EC_{50} decrease with temperature enhancement from 40 °C to 50 °C, for the tested pressures. From 50 °C to 60 °C the behavior seems to be reverse. This effect may possibly be related to the crossover isotherms, which occurs between pressures of 140 bar and 160 bar (determined in a previous work).

According to Silva et al [20] β-caryophyllene and α-humulene are present in the chemical composition of *C. sylvestris* extracts. These compounds are important sesquiterpenes and have been extensively studied in the literature due to their anticarcinogenic and anti-inflammatory activities [33], while spathulenol has antibacterial properties [34-35]. Possibly, the good results of antioxidant activity may also be related to these compounds.

4. Conclusions

The results of the present work showed that SFE and LPE extractions presented good antioxidant potential extracts, similarly to BHT. The use of *C. sylvestris* as extraction raw material for antioxidants attainment is promising due to the presence of high quality compounds as β-caryophyllene and α-humulene, which have proved citotoxic and anti-inflammatory activities, according to literature data. These components may also be related to important antioxidant activity found by TPC, DPPH and β-carotene methods.

The next step of this work is the achievement of the extracts chemical profile by gas chromatography coupled to mass spectrometry analysis (GC-MS), in order to prove the presence of important compounds with biological activity in the SFE and LPE extracts.

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