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ORIGINAL ARTICLE

Phytochemical profile, antioxidant and antimicrobial activity of extracts obtained from erva-mate (*Ilex paraguariensis*) fruit using compressed propane and supercritical CO_2

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Abstract Traditionally, *Ilex paraguariensis* leaves are consumed in tea form or as typical drinks like mate and terere, while the fruits are discarded processing and has no commercial value. The aim of this work to evaluate phytochemical properties, total phenolic compounds, antioxidant and antimicrobial activity of extracts of *Ilex paraguariensis* fruits obtained from supercritical CO_2 and compressed propane extraction. The extraction with compressed propane yielded 2.72 wt%, whereas with supercritical CO_2 1.51 wt% was obtained. The compound extracted in larger amount by the two extraction solvents was caffeine, 163.28 and 54.17 mg/g by supercritical CO_2 and pressurized propane, respectively. The antioxidant activity was more pronounced for the supercritical CO_2 extract, with no difference found in terms of minimum inhibitory concentration for *Staphylococcus aureus* for the two extracts and better results observed for *Escherichia coli* when using supercritical CO_2.

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Introduction

Yerba (or erva) mate (*Ilex paraguariensis*) is a tree shrub belonging to Aquifoliaceae family, with natural growing in the states of Mato Grosso do Sul, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul, reaching also part of Paraguay and Argentina (Groppo 2015). The *Ilex paraguariensis* has been extensively studied due to a variety of pharmacological antioxidant properties (Bravo et al. 2007; Boaventura et al. 2013a), bactericidal ability (Burris et al. 2015), stimulant (Athayde et al. 2000), diuretic, antidiabetic (Boaventura et al. 2013b), usually attributed to the presence of phenolic compounds and alkaloids.

To get a sense of the *Ilex paraguariensis* market, only in South of Brazil it can be found more than 40 mate processing industries. To supply these industries, about 180 thousand medium and small rural properties dedicated almost exclusively to cultivate this raw material (Jacques et al. 2007a). Considering the fact that all of those industries direct their efforts to produce the same product (mate leaves for teas) the processing is still done in a very rudimentary form. Due to recent availability of this raw material from other countries, the strong competition established has required company investments towards improved agronomic techniques and also in the development of new processes to produce higher-value products (Jacques et al. 2007b).

The *Ilex paraguariensis* produces ripe fruits between January and March, presenting a purplish red color, being

eaten by native birds, which spread out the plant in the region (Peixoto et al. 2000). The bitter taste of the fruit and its strong color indicates the presence of phenolic compounds (Miró et al. 1998). According to Canhoto (2010) phenolic compounds are secondary metabolites produced by plants, which can generate competitive advantages as antimicrobial action. It is well known that the chemical structure of phenolic compounds is directly connected to their antioxidant activity, which stabilizes free radicals, and can inhibit for example LDL (low-density lipoprotein) cholesterol oxidation, named the bad cholesterol (Vaccari et al. 2009). It should also be noted that while an adult mate plant produces around 150 kg of leaves, up to 20 kg of fruits could be obtained, which may comprise a non-negligible amount of an interesting, non-waste, raw material.

Because of the economic and safety concerns related to the toxicity of conventional (organic solvent) extraction methods, the use of pressurized and supercritical fluids has emerged as a plausible alternative. Extraction of vegetable matrices with supercritical fluids is probably the most classical and well established application of supercritical fluid technology. Carbon dioxide is the most commonly used supercritical solvent in the extraction of flavor and fragrance compounds due to its well-known properties (Reverchon 1997; Reverchon and de Marco 2006; Melo et al. 2014). Nevertheless, propane and n-butane can also be a good choice because their critical pressures are relatively low compared to that of carbon dioxide and present a dielectric constant of around 1.7, which is quite similar to carbon dioxide, 1.6 (Weast et al. 1988). Of course, for safety reasons, the flammability of propane will require adoption of additional security policies.

As liquid propane exhibits low compressibility and very low solubility in water, it behaves like a piston fluid that increases the system pressure changing favorably the extraction of non-polar components. Furthermore, propane is plenty available, cheaper, can be used in much lower pressures compared to carbon dioxide and can also be easily separated from the final product by system depressurization. Besides the mild temperature and pressure operating conditions, the use of short-chain hydrocarbons, like propane and n-butane, allows reduction of extraction time. However, literature is somewhat scarce on the vegetable matrices extraction using pressurized propane (Illés et al. 1997, 2000; Hamdan et al. 2008; Daood et al. 2002; Corso et al. 2010; Freitas et al. 2008; Silva et al. 2015a, b; Santos et al. 2015; Zanqui et al. 2015a, b; Jesus et al. 2013; Pederssetti et al. 2011; Nimet et al. 2001; Sparks et al. 2006).

Carelli et al. (2011) extracted *Ilex paraguariensis* leaves using supercritical CO_2. The extract obtained demonstrated potential for natural antibiotics from this plant due to the antimicrobial activity exhibited against microorganisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Though literature is somewhat vast concerning supercritical-CO_2 extraction of mate leaves (Jacques et al. 2007a, b), to the best of our knowledge no report was found regarding compressed fluid extraction of mate plant fruits, which means that possible application of such waste biomass has been completely neglected.

In this context, the aim of this study was to perform the phytochemical characterization, to determine the amount of total phenolic compounds, and the antioxidant and antimicrobial activity (*Escherichia coli* (gram negative) and *Staphylococcus aureus* (gram positive) microorganisms) of extracts of *Ilex paraguariensis* fruits obtained from supercritical CO_2 and compressed propane extraction.

Materials and methods

Plant material

Ilex paraguariensis ripened fruits were collected in January, −26.87961 latitude, longitude −52.9538 in the Pinhalzinho city, western state of Santa Catarina-Brazil, and *Ilex paraguariensis* A. St. Hil. was identified as belonging to the Aquifoliaceae family for Marciela Batistela in Biology Laboratory of Unochapecó where one voucher specimen was deposited under the number 3169. Fruit samples were washed and then dried in oven with forced air circulation at 40 °C overnight followed by grinding, packing and stored under nitrogen atmosphere prior to extraction experiments.

Pressurized fluid extraction

The experimental apparatus consists basically of a solvent reservoir, two thermostatic baths, a syringe pump (ISCO 260D), a 130 cm^3 jacketed extraction vessel, an absolute pressure transducer (Smar, LD301) equipped with a portable programmer (Smar, HT 201) with a precision of 0.12 bar, a collector vessel with a glass tube, and a cold trap. Amounts of around 30 g of dried of finely comminuted fruits (average particle size of 0.14 mm) were charged into the extraction vessel. The solvent was pumped at a constant flow rate of 2 mL/min into the bed, which was supported by two 300 mesh wire disks at both ends and was kept in contact with the herbaceous matrix for at least 30 min to allow system stabilization. The extract was then collected by opening the micrometering valve and extraction continued until no significant mass was extracted, as measured on a precision scale balance (Shimadzu, Model AY220 with 0.0001 g accuracy).

For propane (White Martins, 99.5% purity, Florianópolis, Brazil) an experimental 2^2 full design was executed based on a previous work (Freitas et al. 2008) in the temperature range of 35–55 °C and from 40 to 60 bar. Solvent density was estimated using the HBT (P–V-T) correlation for compressed liquids (Reid et al. 1987). It should be noted that in the temperature and pressure ranges investigated in this work, propane behaves like a compressed liquid and thus exhibits small density variations, around 0.46 g cm^3.

For carbon dioxide (White Martins, 99.9% purity, Florianópolis, Brazil), an experimental 2^2 design was also followed in the temperature range of 35–55 °C and from 150 to 250 bar, such extraction conditions based on previous works (Jacques et al. 2007a, b). Carbon dioxide densities were estimated using Angus correlation (Angus et al. 1985). In both cases, experiments were accomplished isothermally at constant pressure. A whole experimental run lasted in general 6 h, including all steps involved: sample weighing, temperature stabilization (baths, extractor), extraction, and depressurization. Based on triplicate experiments carried out for all the experimental conditions of both experimental designs, the overall average standard deviation of the yields noticed was about 0.15 wt%.

The output of compressed fluids was computed based on the volume decay of the syringe pump reservoir, with a resulting accuracy of ±0.01 g in pressurized solvents delivery. With known values of pressure and temperature in the syringe pump reservoir, solvent densities were calculated and accordingly the mass of solvents charged into the extraction vessel. For more information on the methods used in the extraction process, the reader is addressed to the works of Jacques et al. (2007a, b) and Rodrigues et al. (2004).

The extracts obtained from the use of compressed solvents extraction, CO_2 and propane, were collected at the end of the extraction vessel through a glass flask immersed in a cold trap. The solvent was easily evaporated to constant weight, which was a simple task due to the high volatility of the solvents employed. Then, the extracts were stored in freezer (∼−18 °C) until the chemical and biological analyses. Extracts were then ready to follow the specific analyses mentioned.

Determination of total phenolic content

Total phenolics content was determined by the Folin–Ciocalteu method based on the technique described by Shahidi and Naczk (2002) with modifications. Triplicate solutions using both extracts were prepared at a concentration of 0.5 mg/mL in methanol analytical grade and protected from light. Afterwards, water was added and subsequently the Folin–Ciocalteu reagent (Vetec, Rio de Janeiro, Brazil) and after 3 min saturated Na_2CO_3 was added. After 1 h absorbance was read at 765 nm in a spectrophotometer. Quantification was performed based on a standard curve of gallic acid and the total phenolic was expressed in mg of gallic acid equivalents (GAE)/100 g of dry extract.

Gas chromatography/mass spectrometry

The extracts (from CO_2 and propane) were analyzed through GC/MS (Shimadzu QP5050A), using a capillary column DB5 (30 m × 0.25 mm, 25 µm). Column temperature was programmed 70 °C/3 min, 4 °C/min to 260 °C, 2.5 °C/min to 300 °C/25 min. Helium was the carrier gas and the injection port and detector temperatures were 290 and 300 °C, respectively. The components were identified by matching their mass spectra with those of Wiley library database and by comparison of retention times with standards. In all samples it was added an internal standard (biphenyl 100 ppm) and the content of each compound was calculated by the ratio between the peak area of the compound by the peak area of internal standard (Boligon et al. 2013). Caffeine, theobromine, phytol, palmitic acid, squalene, vitamin E, β-sitosterol and stigmasterol were quantified through the injection of authentic standards (Sigma-Aldrich Co). Three replicate were performed for each sample.

Antioxidant activity by DPPH (2,2-difenil-1-pricril-hidrazil) method

The antiradical powers of the different concentrations of the *Ilex paraguariensis* extracts (0.01, 0.02, 0.04, 0.08, 0.16 and 0.32 mg/mL) and 50 ml of each dilution was mixed with 1.95 ml of methanolic DPPH solution standard (0.24 mg/mL—Sigma-Aldrich, Steinheim, Germany) were determined by measuring the decrease in the DPPH absorbance at 517 nm after 24 h in the dark compared to a blank (Brand-Williams et al. 1995). This analysis was carried out in triplicate (n = 3), and the results are expressed as the means of % inhibition of the DPPH radical. The concentration of *Ilex paraguariensis* extracts that could scavenge 50% of the DPPH radical (IC50) was calculated via regression analysis using the GraphPad Prism Program version 6.0.

Microbiological analysis

Microbiological analyses were performed into sterile 96 well microplates-bottomed "U" in triplicate. A volume of 200 µL of the sample was prepared in a concentration of 2000 µg/mL, using 10% DMSO as a diluent and it was filtered (Millipore 0.45 µm) and inoculated with two fold

serial dilutions (1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8 μg/mL). The microbial inoculum at a concentration of 0.5 McFarland standard, which corresponds to 10^8 CFU/mL (Colony Forming Units), was subsequently diluted 10 times in sterile 0.9% saline, and from this solution a volume of 5 μL was added to all wells. The wells of the column 7 have been reserved for the negative control of the inhibitory activity of DMSO diluent. The wells in column 8 received only Mueller–Hinton (Merck®) broth and microbial inoculum, providing positive control of bacterial viability and column 9 wells received only Mueller–Hinton broth for verification plate sterility (Ostrosky et al. 2008). The microplates were incubated in bacteriological incubator at 35 °C for 18 h. Then, it was added to each well 20 μL of an aqueous solution of TTC (triphenyltetrazolium chloride, 0.5% w/v) and the microplates were again incubated for a further 3 h. The presence of a red color in the wells was interpreted as negative test of the inhibitory effect/anti-microbial activity, while the absence of red color as positive proof of inhibitory action. The minimum inhibitory concentration (MIC) was determined according to the methods described by Liu and Yang (2012) and was defined as the lowest concentration, in mg/mL able to inhibit microbial growth, tested in this work against *Escherichia coli* (gram negative) and *Staphylococcus aureus* (gram positive) microorganisms, acquired from NEWAPROV.

Results and discussion

The first step was to evaluate the effect of temperature and pressure on the extraction yield of mate fruits and results are shown in Table 1. As can be seen from this table, extraction with pressurized propane provided higher yields compared to supercritical CO_2 for almost all conditions, with the best results from the two solvents being 2.72 and 1.51 wt%, for propane and CO_2, respectively. The extractions with propane required the application of lower pressure and in all cases shorter extraction times, typically 90–120 min for propane compared to 3–4 h for CO_2.

Thus, in the temperature range investigated, compressed propane presented a hydrostatic behavior, working as mechanical press or piston fluid that increases the system pressure, changing favorably the extraction of non-polar components. Such characteristic may be advantageous due to the possibility of saving energy and reducing operational pressure expenditures with consequent lower capital investments. Thus, it seems that working at relatively lower pressures and mild temperatures may be advantageous over supercritical carbon dioxide use, since lower investment (capital expenditure—Capex) and operational costs (operational expenditure—Opex) are involved when using compressed propane.

Table 1 Experimental conditions and yields obtained from supercritical CO_2 and pressurized propane extraction

Experiment	T (°C)	*P* (bar)	Extraction yield (wt%)
Supercritical CO_2			
1	35	150	1.26 ± 0.12^{bc}
2	45	200	1.33 ± 0.15^{b}
3	55	250	0.91 ± 0.12^{e}
4	45	200	1.18 ± 0.13^{cd}
5	35	250	1.51 ± 0.15^{a}
6	45	200	1.13 ± 0.18^{d}
7	55	150	0.71 ± 0.17^{f}
Pressurized propane			
1	35	40	1.99 ± 0.11^{ab}
2	45	50	2.51 ± 0.13^{a}
3	55	60	2.56 ± 0.17^{a}
4	45	50	2.72 ± 0.16^{a}
5	35	60	1.88 ± 0.13^{b}
6	45	50	2.47 ± 0.14^{ab}
7	55	40	1.11 ± 0.18^{c}

Results are expressed as mean ± standard deviation

Means followed by the same letter do not differ by Tukey test ($p < 0.05$), statistical analysis was performed for each separately solvent, i.e., inside the same solvent group

According to Lanza et al. (2005) the oils are constituted by mostly triglycerides, which have high solubility in propane. Pederssetti et al. (2011) performed canola seed extraction with CO_2 from 40 to 60 °C and pressures from 20 to 25 MPa, while propane was employed from 30 to 60 °C and pressures of 8 to 12 MPa. They concluded that compressed propane required shorter times and lower pressures relative to carbon dioxide. Nimet et al. (2001) studied sunflower seed extraction with CO_2 at temperatures of 40 to 60 °C and pressures from 19 to 25 MPa and with propane at temperatures from 30 to 60 °C and pressures of 8 to 12 MPa and observed that for all conditions investigated propane allowed higher extraction yields of sunflower oil.

The extracts of *Ilex paraguariensis* fruits showed different concentrations of phenolic compounds for the solvents investigated, but with no significant difference, as can be seen in Table 2.

The antioxidant activity of different *Ilex paraguariensis* fruit extracts was measured by the DPPH method and Fig. 1 shows the antioxidant activity in DPPH radical capture percentage concentration of 0.32 mg/mL extract. Table 2 also shows the IC_{50} of the extracts, which is the concentration capable of inhibiting 50% of DPPH,

Table 2 Total phenolic compounds (TPC) and IC_{50} DPPH for *Ilex paraguariensis* fruit extracts obtained at the central point of the experimental designs

Solvent	TPC (mg GAE/100 g sample)	IC_{50} DPPH (mg/mL)
Supercritical CO_2	$9.25^{a} \pm 0.01$	$13.46^{a} \pm 4.92$ ($R^2 = 0.945$)
Pressurized propane	$9.01^{a} \pm 0.005$	$20.14^{a} \pm 7.81$ ($R^2 = 0.868$)

Results are expressed as mean ± standard deviation of three determinations

Means followed by the same letter do not differ by *T* test ($p < 0.05$) in the same column

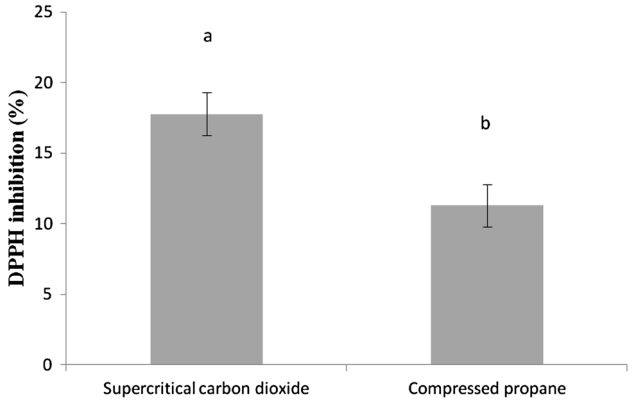

Fig. 1 Percentage of antioxidant activity by DPPH radical capture for the *Ilex paraguariensis* fruit extracts from supercritical CO_2 and pressurized propane obtained at the central point of the experimental designs. Columns with the same letter do not differ by *T* test ($p < 0.05$). Values represent the mean ±standard deviation

therefore the lower the better is the antioxidant activity IC_{50}. Compared to propane, the extract obtained with supercritical CO_2 showed a slightly higher amount of total phenolic compounds (9.25 mg GAE/100 g sample), but with no significant difference by Tukey test ($p < 0.05$). Ghafoor et al. (2012) obtained 2.52 mg GAE/mL of extract obtained from grape seeds with supercritical CO_2 at 46 °C and 167 bar. Pessoa et al. (2015) found total phenolic compounds between 14.6 and 17.0 mg GAE/g for the extract obtained from pequi with pressurized propane at 60 °C and 15 MPa. Mesomo et al. (2012) obtained better results in terms of phenolic compounds extraction from ginger using propane at 50 °C and 10 MPa than with CO_2 under 40 °C and 16.5 MPa.

The antioxidant activity conducted by the capture of DPPH radical was more efficient for the extract obtained with carbon dioxide, 17.77%, while that coming from propane showed 11.27% radical inhibition. The IC 50 was lower for the extracts obtained with CO_2, 13.46 mg/mL, while the extracts obtained by propane required 20.14 mg/mL. Such result might be related to the higher amount of phenolic compounds found in the CO_2 extracts and also due to the presence of vitamin E in this solvent, not found in the pressurized propane extracts.

Table 3 Quantitative chemical composition (mg/g) of *Ilex paraguariensis* fruit extracts obtained from pressurized propane supercritical CO_2 obtained at the central point of the experimental designs

Compound	Supercritical CO_2	Compressed propane
Caffeine	163.28 ± 0.81^{a}	54.17 ± 0.32^{f}
Theobromine	2.45 ± 0.19^{d}	0.93 ± 0.08^{g}
Phytol	1.87 ± 0.26^{de}	3.57 ± 0.45^{h}
Palmitic acid	11.34 ± 0.07^{b}	6.04 ± 0.21^{i}
Squalene	8.13 ± 0.15^{c}	27.15 ± 0.03^{j}
Vitamin E	0.51 ± 0.02^{e}	nd
β-Sitosterol	1.67 ± 0.09^{de}	4.03 ± 0.08^{h}
Stigmasterol	9.81 ± 0.10^{bc}	30.63 ± 0.12^{l}

Results are expressed as mean ± standard deviation of three determinations

Averages followed by different letters differ by Tukey test at $p < 0.05$

nd not-detected

Caffeine was the compound found in greater amount for the solvents, with 163.28 mg/g sample for CO_2 and 54.17 mg/g for pressurized propane. Peres et al. (2013) found caffeine concentrations up to 268 mg/mL in mate samples, but the compounds found in higher concentrations were caffeoylquinic acids with concentrations up to 289 mg/mL. Table 3 shows the concentration of the eight compounds quantitatively identified by GC/MS, with caffeine as the major compound. The well-known much more pronounced hydrophilic characteristic of supercritical carbon dioxide compared with propane, and consequently its extracting solvent power for polar compounds compared with propane is clearly demonstrated by the chemical profile of the extracts obtained, shown in Table 3. From this table it can be seen that supercritical carbon dioxide extracts much more caffeine and theobromine than propane, compounds for which the antioxidant activity is closely related. This is the reason why supercritical carbon dioxide extract show a better antioxidant activity compared with propane, which in turns provides greater extraction yield but loaded with (apolar) compounds (perhaps waxes, not characterized in this work) that do not contribute effectively to the antioxidant activity.

Table 4 Minimum inhibitory concentration for *Ilex paraguariensis* fruit extracts obtained from supercritical CO_2 and pressurized propane obtained at the central point of the experimental designs

Microorganism	Pressurized propane	Supercritical CO_2
Escherichia coli (ATCC 25922)	2000 μg/mL	1000 μg/mL
Staphylococcus aureus (ATCC 29213)	2000 μg/mL	2000 μg/mL

The minimum inhibitory concentration for the two extracts was tested on two microorganisms, as presented in Table 4. Duarte et al. (2007) and Wang et al. (2008) classified the extracts as strong inhibitors for MIC values below 500 μg/mL, moderate inhibitors for MIC between 600 and 1500 μg/mL and weak inhibitors for MIC above 1600 μg/mL. Thus, the two extracts obtained in this work may be considered weak inhibitors for *Staphylococcus aureus* with the minimum inhibitory concentration found of 2000 μg/mL.

For *Escherichia coli* culture, the extract obtained with CO_2 required a concentration of 1000 μg/mL and was considered a moderate inhibitor, while 2000 μg/mL was necessary for the extract obtained with propane pressurized, and thus was a weak inhibitor.

Oliveira et al. (2013) using grape extract obtained with supercritical CO_2, noted a moderate inhibitory effect against *Staphylococcus aureus* and *Escherichia coli*. Czaikoski et al. (2015) using Eupatorium *intermedium* flower extracts obtained with supercritical carbon dioxide and pressurized propane, noticed the efficacy against *Staphylococcus aureus*, whereas *Escherichia coli* was completely resistant to the extracts.

Conclusion

Extraction yields of mate fruits in the temperature and pressure range investigated were, in general, higher for compressed propane compared to supercritical carbon dioxide. Antioxidant and antimicrobial activity for both compressed extraction solvents tested were detected with slight advantage for the CO_2 extract. Microbiological test of both extracts showed the same weak minimum inhibitory concentration for *Staphylococcus aureus*, while for *Escherichia coli* the extract obtained from the application of supercritical CO_2 was classified as moderate inhibitor, and that obtained with propane was considered weak inhibitor. Regarding chemical profile, the supercritical CO_2 extract presented a higher concentration of caffeine, theobromine, palmitic acid and vitamin E, whereas for propane higher concentrations of phytol, squalene, β-sitosterol and Stigmasterol were found. Most importantly, this work demonstrates the potential use of an agro-industrial waste as a raw material, completely neglected, for producing value-added compounds.

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