Industrial Crops and Products xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Seasonal variation in the chemical composition, antimicrobial and mutagenic potential of essential oils from *Piper cernuum*

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ARTICLE INFO

Article history:
Received 18 June 2016
Received in revised form 12 October 2016
Accepted 21 October 2016
Available online xxx

Keywords:
Piper cernuum
Essential oils
Antimicrobial activities
Dihydroagarofuran
Dermatophyte fungi

ABSTRACT

Environmental factors such as seasonality can influence the chemical composition, the pharmacological activity and potential toxicity of essential oils. The aim of this study was to evaluate the influence of seasonality on the chemical composition, the antimicrobial potential cytotoxic and mutagenic of essential oils and fractions from the hydrolate of *Piper cernuum*. The essential oils and hydrolate fractions obtained from the dried leaves, were analyzed by means of Gas Chromatography, Gas Chromatography–Mass Spectrometry and Carbon-13 Nuclear Magnetic Resonance. Twenty-seven constituents were identified, representing 92.9 to 98.4% of the oils in different seasons of the year. *trans*-Dihydroagarofuran, 4-epi-*cis*-dihidroagarofuran, γ-eudesmol, β-caryophyllene, elemol, α-pinene and camphene were found as the major compounds. Furthermore, was found that the essential oil did not exhibit mutagenic potential. The oils exhibited a very interesting antimicrobial profile against *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus pyogenes* and dermatofites including *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Epidermophyton flocosum* and opportunistic yeast *Criptococcus neoformans*. The dichloromethane and ethyl acetate fractions were more active against dermatophyte fungi. The seasonality influenced the antimicrobial activity and quantitative composition of substances in essential oils, but could not establish a direct correlation between the concentrations of compounds with improved antimicrobial activity.

1. Introduction

The *Piper* genus (Piperaceae) consists more than 2000 species distributed worldwide noting Brazil has the largest number, with 300 species in Amazon and 150 in the Atlantic forest, making this genus an important source of raw material for the research of bioactive compounds (Jaramillo and Manos, 2001; Parmar et al., 1997; Sengupta and Ray, 1980; Wanke et al., 2007). Members of this genus are widely used in the production of condiments or spices due to the strong aromatic smell (Di Stasi et al., 1989; Guimarães et al., 1978; Ma et al., 2004; Pio-Correa, 1974; Santos et al., 2012). Some studies with essential oil of different species of *Piper* reported antimicrobial activity on various microorganisms (Costantin et al., 2001; Dorman and Deans, 2000; Lago et al., 2005; Navickiene et al., 2006; Tirillini et al., 1996).

The use of essential oils needs detailed chemical characterization and evaluation of possible changes in its chemical composition due to population genetic variations, climatic influence and edaphic and toxicological profile (Anaya, 1974; Oliveira et al., 2007; Painter, 1951; Yunes and Cechinel-Filho, 2007).

Among the *Piper* genus, *P. cernuum* Vell. it is commonly found in the Amazon, northeast, southeast and south of Brazil. The infusion of leaves is used as analgesic, problems in digestive tract, liver and kidney, as well as for circulation (Di Stasi et al., 2002).

In the scientific literature was not found research about the seasonal influence on chemical composition, pharmacological activity and potential toxicity of the essential oils of this species. In this context the aim of this study was to evaluate the influence of seasonality on the chemical composition, the antimicrobial potential cytotoxic and mutagenic of essential oils and fractions from the hydrolate of *P. cernuum*. These aspects are susceptible to factors such as seasonality, since the amount and sometimes even the

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http://dx.doi.org/10.1016/j.indcrop.2016.10.030
0926-6690/

nature of the active substance is not constant throughout the year (Globbo-Neto and Lopes, 2007).

2. Materials and methods

2.1. Plant material

The leaves of *P. cernuum* were collected in Blumenau, a city in the state of Santa Catarina, Brazil (latitude: 26°97′69.66″S, longitude: 49/06′24.31″W, and altitude: 200 m) at the end of every season of the year: winter (September 15, 2012), spring (December 15, 2012), summer (March 15, 2013), and autumn (June 15, 2013). The material was classified by Prof. Andre Luis de Gasper, Curator Herbarium Dr. Roberto Miguel Klein Universidade Regional de Blumenau. Samples of the specimen were deposited in this Herbarium with the number 41606.

2.2. Extraction of the essential oils

Leaves of *P. cernuum* collected in the end of each seasons were dried at room temperature during ten days. After it were pulverized and submitted to hydrodistillation for four hours in a modified Clevenger-type apparatus. Each essential oil was dried over anhydrous sodium sulfate and stored at −4 °C protected from light for further analysis.

2.3. Hydrolate fractions

After the extraction of the essential oil, the water was filtered to yield the hydrolate. Approximately 1L of each hydrolate were subjected to liquid-liquid partition with immiscible solvents in increasing polarity order (dichloromethane and ethyl acetate). For each solvent, two extractions were performed using first 400 mL and then 200 mL of solvent. After separation of the phases, the organic phases were combined and dried over anhydrous sodium sulfate, filtered and taken to dryness in a rotary evaporator at a maximum temperature of 50 °C.

2.4. Gas chromatography–mass spectrometry (GC–MS)

The chemical analysis of the essential oils was performed by gas chromatography coupled to mass spectrometry (GC/MS Shimadzu, QP2010 S) and gas chromatography coupled to flame ionization detection (GC/FID Shimadzu, QP2010 S), using the methodology proposed by Costantin et al. (2001) with adaptations. The essential oils were diluted in dichloromethane at 1% concentration. Analyses were performed on a fused silica capillary column Rtx-1 (100% methylpolysiloxane, 30 m X 0.25 mm X 0.10 μm), helium was used as carrier gas at a flow rate of 0.8 mL/minute, the injector temperature for GC/MS was 300 °C and for GC/FID was 310 °C. Moreover, splitless injection was used for GC/MS and split injection for GC/FID, wherein the volume injected was 1 μm with split ratio of 40:1 for GC/MS and 1.4 μm with split ratio of 1:50 for GC/FID, with programed temperature from 80 to 310 °C and mass spectrometer operating at 70 eV. Essential oil constituents were identified by comparing the mass spectra with those in the data system library (NIST version 8.0) and by comparing the mass spectra and calculated linear retention indices (RI) with values in the literature (Adams, 2007; Alves and Victor, 2010; Mühlen, 2009). Retention indices (RI) were calculated based on retention times from a series of linear hydrocarbons (C_7-C_{40}–SUPELCO), analyzed in the same conditions described, and by the equation of Van Den Dool and Kratz (1963).

The ^{13}C NMR spectra of essential oils (concentration 40 mg/mL) were obtained on a Bruker AC–300 MHz 300F. Spectra were obtained in deuterated chloroform (purity 99.8% + 0.05% TMS) of Cambridge Isotope Laboratories Inc., with tetramethylsilane as internal reference (TMS). Chemical shifts were recorded in dimensionless values δ (ppm) indicating the sign as singlet (s), doublet (d), triplet (t), etc.

Analyses by infrared spectroscopy of essential oils was performed by DRIFTS (Diffuse Reflectance Infrared Fourier Transform Spectroscopy) in cells Zinc selenide, using the spectrometer by Fourier Transform IRPrestige-21 SHIMADZU (Tokyo, Japan).

Absolute density 0.989, 0.995, 0.988 and 0.983 g/ml from oils collected in winter, spring, summer and autumn, respectively.

2.5. Antimicrobial strains and media

The antibacterial activity of the essential oils were evaluated against three Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 19615), *Bacillus subtilis* (ATCC 14579), one Gram-negative *Escherichia coli* (ATCC 11775). The antifungal activities against the pathogenic fungi *Microsporum canis* (C112), *Microsporum gypseum* (C 115), *Trichophyton mentagrophytes* (ATCC 9972), *Trichophyton rubrum* (C 137), *Epidermophyton flocosum* (C 114), *Aspergillus fumigatus* (ATCC 26934), *Rhizopus* sp. (C 135), *Candida albicans* (ATCC 10231), and *Criptococcus neoformans* (ATCC 32264) were determined, using the dilution technique. The culture medium used for bacteria was Mueller-Hinton agar, whereas Sabouraud Dextrose 4% agar was used for growing the fungi. The incubation conditions used were 18 to 24 h at 35 °C for the bacteria, 24 to 48 h at 30 °C to yeasts and 5 to 15 days at 25 °C to filamentous fungi. These particular strains were standard reference of the American Type Culture Collection (ATCC) (Rockville, MD, USA) and Ceremic (C) – Mycological Reference Center (Facultad de Ciencias y Bioquímicas Farmacêuticas, Rosario, Argentina).

2.6. Antimicrobial assay

The minimum inhibitory concentration (MIC) of essential oils was determined by agar dilution assay according to the CLSI (2009), with some modifications. The assay was carried out on slants (1 mL) against bacteria, yeasts, and filamentous fungi. A series of dilutions was prepared in concentrations ranging from 25,000 ppm to 48 ppm. Afterwards, a volume of 1 μL of inoculum suspension was added to each slant, with the exception of the sterile control. A drug-free solution was also used as blank control. Each assay was repeated three times. The strains were incubated at 35 °C for 18 to 24 h for bacteria, 30 °C for 24 to 48 h for yeasts, and room temperature (25 °C) for 5 to 15 days for filamentous fungi. The MICs were visually recorded after 24 h for bacteria, 48 h for yeasts, and in accordance with control fungus growth for the remaining fungi (Zacchino and Gupta, 2007).

2.7. Mutagenicity and cytotoxicity on the Saccharomyces cerevisiae strain

Mutagenicity of the essential oils was determined using techniques described in Medina et al. (2008). The haploid wild-type strain of *S. cerevisiae* XV185-14c (MATa, *ade*2-2, *arg*4017, *his*1-7, *lys*1-1, *trp*5048, *hom*3-10) was provided by Dr. R. C. Von Borstel (Genetics Department, Alberta University, Canada). A suspension of 2×10^8 cells/mL of the yeast grew until the exponential phase was incubated for 3 h in several different *P. cernuum* essential oil concentrations (25000, 12500, and 6250 ppm). Survival was determined based on SC (5 days at 28 °C) and mutation induction (*Lys*+, *His*+ or *Met*+ revertants) was performed in appropriate deficient media. Assays were repeated at least three times, with plating done in triplicate for each dose. 4-nitroquinolein-1-oxide (4NQO, 5 μM) was used as mutagenic positive control.

Please cite this article in press as: Gasparetto, A., et al., Seasonal variation in the chemical composition, antimicrobial and mutagenic potential of essential oils from Piper cernuum. Ind. Crops Prod. (2016), http://dx.doi.org/10.1016/j.indcrop.2016.10.030