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Seasonality modifies rosemary's composition and biological activity

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ABSTRACT

The rosemary (*Rosmarinus officinalis* L.) essential oil is widely used as an ingredient in food formulations and fragrance industry. However, there are few studies on the effects of seasonal variation on the composition and biological activities of this oil. Thus, the aim of present study was to evaluate the effects of seasonal variation (2012–2013) on composition and antioxidant and antimicrobial activities of rosemary essential oils and ethanolic extract. Composition of essential oil was determined by GC-MS and that of the extract by LC-MS/MS. Antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and beta-carotene/linoleic acid methods. Total phenolic compounds were determined by Folin–Ciocalteu method, and antimicrobial activity was evaluated by the determination of the minimum inhibitory concentration. Camphor was found as the major compound in essential oil (24.3–35.9%, mainly in January/2013), while carnosic acid was found in largest amount in January/2013 (summer) in the ethanolic extract, which presented the largest antioxidant activity, similar to butylated hydroxytoluene (BHT) and alfa-tocopherol. The results showed that essential oil and extracts harvested in summer exhibited strong activity against *Staphylococcus aureus*, even higher than sulfanilamide, due to the high amount of carnosic acid and camphor. Altogether, the essential oil and the extracts collected in summer showed better antioxidant and antimicrobial activities, which can be explained by the high levels of camphor and carnosic acid.

1. Introduction

Rosmarinus officinalis L., commonly known as rosemary, belongs to the *Lamiaceae* family and is a plant commercialized worldwide. Its essential oil (Olmedo et al., 2013) and extracts (Gao et al., 2014) are used for food preservation and for the treatment of several illnesses. Recent studies using rosemary essential oil reported several activities, such as antibacterial (Ojeda-Sana et al., 2013), antifungal (Soylu et al., 2010), insecticide (Zoubiri and Baaliouamer, 2011), antioxidant (Ojeda-Sana et al., 2013), and hepatoprotective (Amin and Hamza, 2005).

Pharmacological properties of this plant, derived from its essential oils or extracts, have been exhaustively studied and presented to many industrial uses (principally as food additive). However, taking into consideration the great supply of the above-mentioned applications, industries should observe the seasonality effects on essential oils or extracts, these effects can cause abrupt shifts in temperature and soil humidity, consequently, alterations in chemical composition and activity of the essential oils. To ratify this, studies with *Sonoran propolis* (Valencia et al., 2012), *Mentha spicata* L. (Kofids et al., 2006), and *Origanum majorana* L. (Trivino and Johnson, 2000) showed that seasonality indeed influenced composition and activity of the plants.

Variations on composition and thus on biological activity caused by seasonality must be monitored so that the essential oil presents the same characteristics throughout the year, in order to keep a quality standard. Considering there are no data for this influence to Brazilian crop samples, the objective of this study was to evaluate the effects of seasonal variation on composition and antioxidant and antimicrobial activities of *R. officinalis* essential oils and ethanolic extract.

2. Materials and methods

2.1. Chemicals

2,2-Diphenyl-1-pycrilhydrazyl (DPPH) radical, beta-carotene, tween 40, and standards of tert-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT),

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gallic acid, rosmarinic acid, quercetin, rutin, apigenin, and alpha-tocopherol, as well as triphenyl tetrazolium chloride (TTC), Folin–Ciocalteu's reagent, sodium carbonate and the antibiotics sulfanilamide, penicillin G, and tetracycline were purchased from Sigma–Aldrich (St. Louis, USA). Agar and the Mueller–Hinton broth were obtained from Himedia Laboratories PVT (Mumbai, India), while linoleic acid was bought from Neon (São Paulo, Brazil).

2.2. Vegetal samples

Rosemary samples (*R. officinalis* L., variety Tuscan Blue) were cultivated at a farm in Marechal Floriano–ES, Brazil (latitude: −20.40331794° and longitude: −41.03258371°). A total of 125 samples was harvested during winter (July/2012), spring (October/2012), summer (January/2013), autumn (april/2013), and winter (July/2013). The plant was identified by botanist Ms. Solange Zanotti Schneider, and a voucher was stored at the Vila Velha University (Vila Velha, ES, Brazil) herbarium under registration number UVVES2311.

2.3. Essential oil extraction

The essential oil of the rosemary leaves was extracted by hydrodistillation with a Clevenger apparatus. Fresh leaves were manually separated from the branches and ground in a blender with Milli-Q water. The extraction process was done for two hours. Essential oils were stored in amber bottles wrapped in aluminum foils and placed under refrigeration prior to use. All procedures were performed in triplicates.

2.4. Extracts

Extraction was done by maceration with dried leaves for seven days using ethanol as solvent (40 g–200 mL of ethanol). The extracts were then filtered in filter paper and the vegetal material was submitted to remaceration with another 200 mL of the solvent for 24 h. After filtering, the organic fractions were blended and concentrated in a rotatory evaporator until dryness. The extract was later kept in an oven at 50 °C until completely dried and then stored under refrigeration prior to use. The extracts prepared in July/2012 were accidentally lost.

2.5. Total phenolic compounds

Determination of total phenolic compounds (TPC) was done on the extracts by the classic Folin–Ciocalteu method by Scherer and Godoy (2014). A 0.5 mL aliquot of the extracts at a 0.5 mg/mL concentration in methanol and 2.5 mL of the Folin reagent diluted in distilled water (1/10) were injected into a test tube. After 5 min, 2 mL of a 7.5% aqueous solution of sodium carbonate were added to the system. The reaction occurred for 2 h in the dark and absorbance was monitored at 740 nm. All analyses were done in triplicate.

2.6. Chromatographic analyses

2.6.1. Determination of the essential oil composition by GC–MS

Identification of the chemical constituents of the essential oils was done in a gas chromatographer (TraceUltra–Thermo) tandem mass spectrometer (DSQII–Thermo). Volatile substances were separated in a DB-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 μm, J&W Scientific, Folson, California, USA). Temperature was programed as follows: 60 °C at first, increasing up to 240 °C in a linear 3 °C/min slope, maintaining this final level for 7 min. The carrier gas was helium at constant flow rate of 1 mL/min. Temperatures of the injector and of the detector were kept at 230 and 250 °C, respectively. Samples were diluted in hexane (1 mg/mL) and the injected volume was 1.0 μL with the injector operating in splitless mode. Compound identification was performed at first by comparison of the similarity of the resulting mass spectra with those of the literature (NIST 98; Adams, 1995) and by further comparison of the Kovats index (KI) with that of the literature (Adams, 1995).

2.6.2. Determination of rosmarinic acid and carnosic acid by LC-ESI-MS/MS0

An Agilent 1200 series high performance liquid chromatographer (HPLC) tandem triple quadrupole API 3200 mass spectrometer (MS) (Applied Biosystems) was used. Ionization was done by electrospray source in negative mode (−4500 V). All separations were performed in an Agilent Eclipse Plus C18 column (150 mm × 4.6 mm i.d., 5 μm) at 35 °C. The mobile phase consisted of (A), formic acid aqueous solution (0.1%, v/v), and (B), formic acid methanolic solution (0.1%, v/v). Flow rate was kept at 0.8 mL/min in an elution gradient of 30–90% of B between 0–3 min, staying at this level until 5 min, and the reequilibration time was set until 9 min. The monitored transitions for the precursor ions and for the product ions were, respectively, 359/160.9 and 359/132.9 for rosmarinic acid and 331.3/287.3 for carnosic acid. Quantification was done by external standard with a five point curve with concentrations ranging from 0.98 to 62.5 μg/mL, using the first product ion. The following validation parameters were evaluated: selectivity, linearity, precision, and limits of detection and quantification.

Selectivity was verified by the clearness of the spectra obtained from the mass spectrometer. Linearity, obtained from external standard, was evaluated by the coefficient of determination of a calibration curve prepared in 6 concentration levels. Linear range was determined by the statistic method of the least squares, in which only points with average residuals lower than 15% were approved. Precision was analyzed by repeatability and intermediate precision parameters in three concentration levels (low, medium, and high). For repeatability, seven consecutive repetitions of each concentration were done and, for the intermediate precision, 21 assays were performed, with seven repetitions in three different days. Limits of detection and quantification were calculated by the signal-to-noise ratio method, with ratios of 3:1 and 10:1, respectively, obtained after seven injections of the blank. Practical quantification limit (PQL) was defined as the lowest concentration of the linear working range.

2.7. Antioxidant activity

2.7.1. DPPH free radical scavenging assay

Antioxidant activity of the samples was determined by the DPPH method, according to Scherer and Godoy (2009). Antioxidant activity of the essential oils and extracts was compared to the action of synthetic antioxidants BHA, BHT, and TBHQ, to the phenolic compounds rutin, quercetin, rosmarinic acid, and to alpha-tocopherol.

2.7.2. Beta-carotene/linoleic acid assay

Antioxidant activity of the rosemary samples was also determined by the beta-carotene/linoleic acid method, according to Jayaprakasha et al. (2001) Antioxidant activity of the essential oils and extracts was compared to the action of the synthetic antioxidants BHA and TBHQ, to the phenolic compound quercetin, and to alpha-tocopherol. The results were expressed as percentage of inhibition after a 2 h period.

2.8. Antimicrobial activity

Determination of the minimum inhibitory concentration (MIC) was done in a 96 well culture plate, following the regulations of CLSI (Clinical, 2003). The tested microorganisms were: *Staphylococus aureus* (ATCC 2592), *Escherichia coli* (ATCC 10,231), and *Salmonella*