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Changes in essential oil composition and phenolic fraction in *Rosmarinus officinalis* L. var. *typicus* Batt. organs during growth and incidence on the antioxidant activity

Zaouali Yosr*, Chograni Hnia, Trimech Rim, Boussaid Mohamed [1]

Department of Biology, Laboratory of Plant Biotechnology, National Institute of Applied Science and Technology, B.P. 676, 1080 Tunis Cedex, Tunisia

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ABSTRACT

Rosmarinus officinalis var. *typicus* Batt. essential oils and acetonic extracts were isolated from different organs at different growth stages of branches developed after decapitation of the main stem apices of clonal plants. Oils assessed by GC–MS were present in high concentration (1.23 and 1.43%) in leaves being collected during the vegetative and flowering stages. Significant variations of component contents were observed according rather to organs than to phenological stages of branches. 1.8-Cineole (35.8%) was the main constituent in leaves, while, β-caryophyllene (16.7%) was identified as the main constituent for stems. Flowers were characterized by high levels of caryophyllene oxide (11.9%). The discrepancy could be due, either to differential oil accumulation or physiological and biochemical interactions within and among organs during morphogenesis.

Total polyphenols (4.8–37.4%), flavonoids (3.0–28.5%) and condensed tannins (0.2–2.4%) were identified in all plant parts. The total polyphenols and condensed tannins were more accumulated in leaves (28.6 and 24.0 mg EAG/g DW) and their concentration was high at the vegetative and fruiting stages, suggesting that flowering contribute to the modification of their accumulation. The amounts of flavonoids in leaves and stems (14.7–17.5 mg ER/g DW) did not vary significantly in course of time. The level of antioxidant activity of acetonic extracts estimated by DPPH and FRAP test systems was high for leaf and stem extracts isolated at the flowering stage. Compared to the acetonic extracts, essential oils exhibited lower antioxidant activity.

1. Introduction

Rosemary, Rosmarinus officinalis L. (*Lamiaceae*), is an evergreen plant typical of the Mediterranean region. It has been used since ancient times for medicinal purposes and known for its antiseptic (Bult et al., 1985; Rampart et al., 1986), anti-rheumatic (Makino et al., 2000), anti-inflammatory (Juhás et al., 2009; Benincá et al., 2011) and antispasmodic properties.

R. officinalis extracts exhibit hepatoprotective (Sotelo-Felix et al., 2002; Amin and Hamza, 2005), anti-diabetic (Bakirel et al., 2008; Abu-Al-Basal, 2010), anti-ulcerogenic (Dias et al., 2000) and antidepressant (Machado et al., 2009) effects. The main identified antioxidant compounds are carnosic acid, carnosol, abietanes diterpenes, rosmarinic acid and a hydroxycinnamic acid ester (Frankel et al., 1996; Del Bano et al., 2003; Wellwood and Cole, 2004). Based on main compounds of essential oils, different chemotypes such as α-pinene and verbenone (Pintore et al., 2002) or 1.8-cineole, verbenone and camphor (Celiktas et al., 2007) were identified according to geographical and climatic regions. Essential oils are synthesized by both peltate and capitate trichomes located on the abaxial and adaxial leaf surfaces (Marin et al., 2006). The capitate trichomes were predominant on both leaf sides. They also are present, with branched or non-branched and non-glandular trichomes on young stems at the earlier vegetative stage.

Studies on the chemical composition and biological activity of rosemary extracts using aerial parts taken before, during and after flowering periods of plants have been performed. However, the variation of the chemical composition (individual compounds and/or compound classes) for the same organs in course of time, and its relationships with the global development of plants remains little reported (Del Bano et al., 2003).

In Tunisia, *R. officinalis* is represented per four botanical varieties: var. *typicus* Batt., var. *laxiflorus* De Noé, var. *troglodytorum* Maire and var. *lavandulaceum* Batt. (Pottier-Alapetite, 1981). They include 1.8-cineole chemotype represented by varieties *typicus*, *laxiflorus* and *lavandulaceum* and a camphor chemotype

* Corresponding author. Tel.: +216 71703829/929; fax: +216 71704329.
E-mail addresses: yosrzaouali@yahoo.fr (Z. Yosr), mohamed.boussaid@insat.rnu.tn (B. Mohamed).
[1] Tel.: +216 71703829/929; fax: +216 71704329.

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represented by *R. officinalis* var. *troglodytorum* (Zaouali et al., 2010). *R. officinalis* var. *typicus* Batt., widely represented in Tunisia (from the sub-humid to the low semi-arid bioclimates), is diploid ($2n = 2x = 24$), characterized by erected growth habit, short inflorescences, pale blue corolla pinked purple and a large puberulent calyx axillated by deciduous bracts (Pottier-Alapetite, 1981).

The aim of this study is (i) to assess the essential oil and polyphenol compound contents of Tunisian rosemary var. *typicus* leaves, stems, flowers and achenes collected on branches of clonal plants and compare their distribution during vegetative, flowering and fructification of branches, and (ii) to evaluate the antioxidant activity of oils and acetonic extracts isolated from these organs in course of time.

2. Materials and methods

2.1. Plant material

The starting material consists of mature plants growing wild in Ezzit Djebel Mountains, located in the northeast of Tunisia (latitude 35°49′N, longitude 10°59′E, located at 60 km from Tunis). The site belongs to the sub-humid bioclimatic zone with a rainfall ranging between 500 and 600 mm/year and situated at an altitude of 350 m. Genetical studies carried out using isozymic and RAPDs markers on individuals from this population, allowed low genetic intra-population diversity (Zaouali and Boussaid, 2008). Six adjacent plants connected by the same sprout to avoid genetic and ecological factors effects were sampled. From each plant 7–10 long branches (20–30 cm in high) after fructifying (July 2009), and without apparent secondary ramifications were cut 5–7 cm just below the inflorescential zone to permit the development of the axillary buds (Fig. 1). The earliest growth of these buds begins one week after the decapitation of branches with synchronous leaf development. Leaves and portions of stems from the same proximal zone of the five first new developed ramifications were collected at the vegetative (end of September 2009), flowering (December 2009) and after fruiting (March 2010) stages of branches. All leaves are 25–30 mm in length and the diameter of stems ranged from 2–5 mm (vegetative stage) to 5–7 mm (flowering and fructifying stages).

2.2. Chemicals

Folin-Ciocalteu reagent, DPPH (1,1-diphenyl-2-picrylhydrazyl), TPTZ (2,4,6 tripyridyl-s-triazine), gallic acid, quercetin, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), sodium carbonate, $AlCl_3$, $FeCl_3 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$ were purchased from Sigma–Aldrich (St. Louis, MO). All solvents and reagents used were of the highest purity.

2.3. Isolation of oils and phenols

Phenolic extracts from each of all organs at each development stage of branches were obtained by magnetic stirring for 12 h of 2.5 g of dry organ powder with 25 ml of aqueous acetone (80:20, v/v). Extracts were kept at 4 °C for 24 h, filtered through a Whatman No. 4 filter paper, and evaporated to dryness under vacuum. They were stored at 4 °C until analysis.

The essential oils have been extracted from (100 g) air-dried leaves, stems and flowers by hydrodistillation for 3 h, using a Clevenger-type apparatus. Oil yields were then estimated on the basis of the dry weight of plant material. Oils were recovered directly, from above the distillate without adding any solvent, and stored in dark vials at 4 °C.

2.4. Essential oils identification

The essential oils were analyzed by Gas chromatography–mass spectrometry (GC–MS) using a HP 5975 C mass spectrometer (Agilent technologies) with electron impact ionization (70 eV). A HP-5MS capillary column (30 m × 250 μm coated with 5% phenyl methyl silicone, 95% dimethylpolysiloxane, 0.25 μm film thickness) was used. Oven temperature was programmed to rise from 60 to 220 °C at a rate of 4 °C/min; transfer line temperature was 230 °C. The carrier gas was He with a flow rate of 0.8 ml/min and a split ratio of 50:1. Scan time and mass range were 1 s and 50–550 m/z, respectively.

The identification of oil components was assigned by comparison of their retention indices (RI) relative to (C8–C22) n-alkanes with those of literature or with those of authentic compounds available in the authors' laboratory. Further identification was made by matching their recorded mass spectra with those stored in the Wiley/NBS mass spectral library of the GC/MS data system and other published mass spectra (Adams, 2001). Determination of the percentage composition was based on peak area normalization without using correction factors. Analyses were performed in triplicate.

2.5. Phenolic compounds content and identification

2.5.1. Total phenolic content

The total phenolic content of organs at the different development stage of branches was assessed using the Folin–Ciocalteu reagent, following Singleton and Rosi's (1965) method, based on the reduction of a phosphowolframate–phosphomolybdate complex by phenolics to blue reaction products and slightly modified by Dewanto et al. (2002). An aliquot of each diluted sample extract (0.5 ml) was mixed with 2 ml Folin-Ciocalteu reagent. After 5 min, 2.5 ml of sodium carbonate solution (7.5%) was added. After incubation (90 min) in dark, the absorbance at 760 nm was read versus the prepared blank. The total phenolic content of the plant parts was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW) through the calibration curve with gallic acid. All samples were analyzed in three replications.

2.5.2. Total flavonoid content

Flavonoid contents were determined according to aluminum chloride colorimetric method (Djeridane et al., 2006). One milliliter of diluted acetonic extract was mixed with 1 ml of 2% $AlCl_3$ methanolic solution. After incubation at room temperature for 15 min, the absorbance was measured at 430 nm. Total flavonoids were expressed as mg rutin equivalent/g DW (mg CE/g DW), through the calibration curve of rutin (0–400 μg/ml range). All samples were analyzed in three replications.

2.5.3. Total condensed tannins

Total condensed tannins were measured using the modified vanillin assay described by Sun et al. (1998). Three milliliters of 4% methanol vanillin solution and 1.5 ml of concentrated H_2SO_4 were added to 50 μl of suitably diluted sample. The mixture was allowed to stand for 15 min, and the absorbance was measured at 500 nm against methanol as a blank. The amount of total condensed tannins was expressed as mg catechin equivalent/g DW. All samples were analyzed in three replications.

2.6. Antioxidant activity of oils and acetonic extracts

The antioxidant activity of oils and acetonic extracts from all organs was assessed using both free radical-scavenging activity (RSA) and ferric reducing antioxidant power assay (FRAP) systems.