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Contribution of the main polyphenols of *Thymus mastichina* subsp. *mastichina* to its antioxidant properties

I. Méndez-Tovar[a,*], S. Sponza[b], M.C. Asensio-S-Manzanera[a], J. Novak[b]

[a] Instituto Tecnológico Agrario de Castilla y Leon (ITACyL), Ctra. Burgos Km. 119, 47071 Finca Zamadueñas, Valladolid, Spain
[b] Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine Vienna, Veterinaerplatz 1, A-1210 Vienna, Austria

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ABSTRACT

The antioxidant activity and phenol content of 14 populations of *Thymus mastichina* grown in an experimental plot, was analyzed by DPPH, FRAP and Folin-Ciocalteu method to define their antioxidant activity. Polyphenols were analyzed by HPLC-DAD and the relationship between polyphenols and antioxidant capacity was established. Populations means for DPPH activity ranges were 44–98 mg TE/g DW while FRAP antioxidant capacity was 52–115 mg TE/g DW. Total phenol content ranged between 11 and 38 mg of CAE/g DW for the different populations. The polyphenols identified were: chlorogenic acid, caffeic acid, rosmarinic acid, luteolin glucoside and luteolin. The main polyphenols were rosmarinic acid, ranging from 1.7 to 43 mg/g DW, one unidentified polyphenol designated as Peak 3 (0.53–15 LE eq mg/g) and luteolin glucoside ranging from 0.96 to 19 LE eq mg/g. Rosmarinic acid contributed mainly to the FRAP antioxidant capacity and to the total phenols, while peak 3 contributed mainly to the DPPH assay. Luteolin, chlorogenic acid and caffeic acid had a range of 0–2.7, 0.07–2.2, 0–0.46 mg/g DW, respectively. The study showed high intra-populations variability and above all high inter-populations variability. The Carrocera population had the highest antioxidant activity and amount of phenols and it could be selected for its content.

1. Introduction

Thymus mastichina L. is an endemic species from the Iberian Peninsula belonging to the Lamiaceae family. It is divided into two subspecies: *T. mastichina* subsp. *mastichina* and *T. mastichina* subsp. *donyanae* (Morales, 2002). The first subspecies grows in most of Spain and Portugal, while *T. mastichina* subsp. *donyanae* is found only in the southwest of the Iberian Peninsula. Spanish marjoram is a species rich in essential oil and its main essential oil compounds are 1,8-cineole and linalool (Salgueiro et al., 1997).

Since ancient times, this plant species has been used as a food spice and currently has numerous applications (Barros et al., 2010). In the field of food, it is mainly used as an infusion or as a dressing for olives; in the field of medicine, for its antitussive and antiseptic properties, and in the perfume and cosmetic industry, its essential oil is used as an ingredient in perfumes, creams, soaps, etc. The biological activities of this species have also been investigated recently showing anti-inflammatory (Albano and Miguel, 2011), anti-carcinogenic activity (Gordo et al., 2012) and antifungal properties (Leal et al., 2013).

T. mastichina is also known for its antioxidant properties. The existence of polyphenols such as rosmarinic acid and luteolin was reported in this plant species (Gordo et al., 2012) as well as in other species from the genus *Thymus* (Costa et al., 2012). The analysis of the antioxidant activity of essential oils in *T. mastichina* with colorimetric methodologies has been widely studied by several authors (Bentes et al., 2009; Galego et al., 2008; Miguel et al., 2005, 2007; Salgueiro et al., 1997) however, the antioxidant activity in plant extracts of *T. mastichina*, has only been reported by Barros et al. (2010) when evaluating samples from Bragança (Portugal) and by Albano and Miguel (2011) on analysing samples from the Algarve (Portugal).

The antioxidant variability among populations coming from different origins has not yet been evaluated in *T. mastichina*. This might be because it is an endemic species adapted to the Iberian Peninsula however its study is very important for the growers. The high variability that may exist among different origins of the same species can be a problem for the commercialization of this plant species. Furthermore environmental and external factors play a secondary role for the qualitative and quantitative accumulation of secondary metabolites (Horwath et al., 2008). Antioxidant variability may

* Corresponding author. Tel.: +34 675668616; fax: +34 983414780.
E-mail address: mentovin@itacyl.es (I. Méndez-Tovar).

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occur by seasonal variation and is also influenced by environmental factors such as weather conditions or temperature (André et al., 2009; Skrzypczak-Pietraszek and Pietraszek, 2012). The evaluation of the variability in antioxidants under the same environmental factors, finding populations or individual plants with high antioxidant activity is an important issue to standardize plant raw material for production and to further optimize it by plant breeding.

This work aims to evaluate the variability in antioxidant activity and main polyphenols composition of *T. mastichina* from Spain. Fourteen populations were studied in order to find specimens with high antioxidant levels and to know the contribution of the phenolic profile to the antioxidant properties of the species. The samples were analyzed using colorimetric methodologies and HPLC-DAD analysis.

2. Materials and methods

2.1. Plant material

The samples of this study come from a field trial comprising fourteen populations of *T. mastichina* located in the Instituto Tecnológico Agrario de Castilla y León (ITACyL) in Valladolid (Spain). The populations came from several localities in Spain (Table 1); they were multiplied by vegetative reproduction establishing the assay in 2010 (Asensio et al., 2010). To avoid the influence of environmental and external factors, all the samples were placed in the same experimental field under the same environmental and weather conditions.

All the samples were collected at the beginning of the flowering phase during June and early July 2012. The flowers and leaves of 7–10 individual plants of each population were collected separately and air dried at room temperature in a dark room and conserved under these conditions until extraction.

2.2. Chemicals and solvents

Luteolin, caffeic acid, chlorogenic acid, rosmarinic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma–Aldrich (Steinheim, Germany). Folin-Ciocalteu reagent, methanol (p.a.), sodium acetate trihydrate, sodium nitrite, and ferric chloride hexahydrate were obtained from Merck (Darmstadt, Germany). Trolox was purchased from Fluka (Denmark, Germany). Sodium carbonate anhydrous, sodium hydroxide, hydrochloric acid, acetic acid, acetonitrile and HPLC grade methanol were obtained from Carl Roth (Karlsruhe, Germany).

2.3. Extraction

The extracts were prepared from 100 mg of dry flowers and leaves of *T. mastichina* that were previously ground. After an extraction in 16 mL methanol 50% for 1 h at room temperature in an ultrasonic bath, the samples were filtered using Pasteur pipettes covered with cotton on their tip and stored in a cooling room (+4 °C) until analysis.

2.4. Free radical scavenging activity method (DPPH)

The free radical scavenging activity was measured by DPPH radical, following the methodology described by Lamien-Meda et al. (2010). The extracts were diluted using 200 μL of the original extract and filled up to 500 μL with methanol 50%. 10 μL of this dilution was adjusted to 100 μL of methanol and mixed with 100 μL of DPPH solution (0.015%). The microplate was covered with parafilm, incubated in darkness for 30 min and was then read at 490 nm with a microplate reader (BIO-RAD Tokyo, Japan). The calibration curve was prepared with six different concentrations of trolox (0–2.48 μg/mL) using the highest concentration as a blank. Every result is a mean of a quadruplicate analysis and is expressed as mg of trolox equivalent per gram of dry weight (mg TE/g DW).

2.5. Ferric reduction antioxidant power assay (FRAP)

The FRAP assay was carried out to evaluate the capacity of the extracts to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) and produce ferrous-tripyridyltriazine. The colour of the samples changes depending on the reducing power of each extract. Ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) dissolved in methanol (6.26 mg in 10 mL of MeOH) and tripyridyltriazine (TPTZ) (31.2 mg of TPTZ in 10 mL of HCL 40 mM) were prepared before analysis. 25 mL of acetic acid buffer (pH = 3.6) were mixed with 2.5 mL of TPTZ solution and 2.5 mL of chloride hexahydrate to form the working reagent. 1 μL of the original extract was mixed with 180 μL of working reagent and 23 μL of H_2O. Trolox was used as a standard to prepare the calibration curve with a total of eight different concentrations (0–400 μg/mL). 6 μL of trolox was mixed with 180 μL of working reagent and 18 μL of H_2O. The blank was performed with 180 μL of working reagent and 24 μL of H_2O. The microplate was covered with parafilm and incubated in darkness for 5 min then read in the microplate reader (BIO-RAD Tokyo, Japan) at 595 nm. The results are expressed in mg TE/g DW and are presented as means of quadruplicate analyses.

2.6. Total phenol content

The Folin-Ciocalteu method to determine the total content of phenols was carried out according to the methodology of Chizzola et al. (2008). The original extract solution was diluted (100 μL of the original extract mixed with 300 μL methanol 50%) and 10 μL of this dilution were used for analysis mixing it with 225 μL of H_2O, 5 μL of Folin-Ciocalteu reagent and 10 μL of Na_2CO_3 solution (35% in H_2O). A calibration curve with eight different concentrations (0–23 μg/mL) of caffeic acid was performed using 10 μL of each concentration. A blank was performed using 10 μL of methanol 50%. The microplate was covered with parafilm and incubated in darkness for 30 min. Then the plate was measured at 750 nm with a microplate reader model 450 (BIO-RAD Tokyo, Japan). Every analysis was carried out in quadruplicate and the results are expressed as mg of caffeic acid equivalents per gram of dry weight (mg CAE/g DW).

2.7. High performance liquid chromatography analysis (HPLC-DAD)

The analyses of polyphenols of the *T. mastichina* extracts were carried out with a HPLC system from Water Corporation (Mildford, MA). The system was equipped with a quaternary pump, an auto-sampler (Waters 717) and a photo diode array detector (DAD Detector Waters 996). The column used was reverse phase symmetry C18 (4.6 mm × 150 mm, 5 μm pore size) Luna 5u (Phenomenex). The two mobile phases used were: 1% acetic acid/acetonitrile (85:15, v/v) as solvent A and methanol as solvent B. The gradient elution profile started with A and B (90:10). Solvent B was gradually increased to 100% with a flow rate of 1.5 mL/min at 30 min and kept constant for 10 min. The identification of polyphenols was achieved by comparison of their retention times and UV spectra with those of reference standards. Detection was performed at 330 nm for rosmarinic acid, chlorogenic acid, luteolin and caffeic acid. Calibration curves were made for each standard with the