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Antioxidant activity of twenty wild Spanish *Thymus mastichina* L. populations and its relation with their chemical composition

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ABSTRACT

The antioxidant activity and chemical composition of essential oils and methanolic extracts of twenty Spanish *Thymus mastichina* L. populations were studied. Both essential oils and methanolic extracts possessed antioxidant properties. However, the total phenol contents of the methanolic extracts varied between 2.90 and 9.15 mg GAE/g_{extract} and the EC₂₅ values of DPPH free radical scavenging activity between 0.90 and 3.45 mg/mL for the methanolic extracts and 78–241 mg/mL for essential oils, these showing low antioxidant potential. Actually, in essential oils the main compound determined was the 1,8-cineole (56.8–69.6%), whereas thymol, γ -terpinene, terpinolene and geraniol (species with considerable DPPH scavenging activity) were observed in low amounts. Concerning methanolic extracts, rosmarinic acid was the most abundant polyphenol (1.70–9.85 mg/g), followed by methoxysalicylic acid, apigenin, kaempferol and luteolin.

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1. Introduction

The species of *Thymus* genus are herbaceous perennial shrubs, commonly used as spices and/or medicinal herbs, with several pharmacological properties, such as antispasmodic, antiseptic, antitussive, expectorant and flatulence-reducing actions (Evans, 1998). Thyme oils are also used in dietary supplementation, as well as in the development of health products, particularly pharmaceuticals. Several studies over the antimicrobial activity of *Thymus* essential oils have shown their potential against important pathogenic microorganisms, such as *Staphylococcus aureus* (Bounatirou et al., 2007; Rasooli & Mirmostafa, 2002), *Helicobacter pylori* (Hazzit, Baaliouamer, Veríssimo, Faleiro, & Miguel, 2009) and *Candida albicans* (Faleiro et al., 2003; Hazzit et al., 2009), suggesting their ability in foodborne pathogens control.

Some species of *Thymus* are endemic in Iberian Peninsula, such as *Thymus mastichina*. The composition of essential oils of this specie had only been studied in Portuguese plants (Salgueiro et al.,

1997; Miguel, Duarte, Venâncio, & Tavares, 2004; Miguel et al., 2005; Miguel, Guerrero, et al., 2004), some oils showing a high 1,8-cineole content. Linalool is another major constituent in the essential oils of some populations of *T. mastichina* subsp. *mastichina* (Miguel, Duarte, et al., 2004; Miguel, Guerrero, et al., 2004; Salgueiro et al., 1997) and *Thymus albicans* (Salgueiro et al., 1997). Borneol is also found in significant amounts in essential oils of *T. mastichina* subsp. *donyanae* (Salgueiro et al., 1997).

Besides improving organoleptic properties of food products, spices and aromatic plants are also known to contribute to their preservation. In food industry, for example, antioxidants are very used with this end. These compounds prevent or delay oxidation reactions, in order to maintain food quality for longer periods and to extend shelf life. Some synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary-butylhydroquinone (TBHQ) have been used; however, the use of these synthetic antioxidants is now under discussion due to their questionable safety. Thus, it is highly desirable to find out natural antioxidants able to substitute them.

In Iberian Peninsula, several wild species of the genus *Thymus* have been found. Regarding *T. mastichina*, this is very frequent and popular in Iberian Peninsula, except in East region, Cataluña and Aragón. To our knowledge, until now no study over the antioxidant activity of essential oils and extracts of Spanish *T. mastichina* populations has been performed. In order to get insight on this, the

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antioxidant activity of *T. mastichina* populations collected in five Spanish provinces from Castilla y León region, was determined in the present work. In more detail, this study I) Evaluated the antioxidant activities of essential oils and methanolic extracts of twenty *T. mastichina* populations collected in five Spanish provinces, namely Salamanca, León, Burgos, Segovia and Soria; and II) Determined relationships between the antioxidant activities of the essential oils and methanolic extracts with their chemical composition.

2. Material and methods

2.1. Plant material

Samples of the aerial parts (leaves + flowers) of *T. mastichina* growing wild in Spain in five different provinces of Castilla y León, namely: Salamanca, León, Burgos, Segovia and Soria, were collected during the flowering phase (June–July 2008). At each province, four *Thymus* populations were collected in different localities at least 30 km apart. For each population 30 plants were collected in order to have a composed sample. The province, municipality, locality and altitude of the sampling sites are indicated in Table 1. At the laboratory, the plants were dried during one month in dark at room temperature (24–28 °C) and relative humidity between 60 and 70%.

2.2. Chemicals and reagents

Apigenin, caffeic acid, kaempferol, quercetin, rosmarinic acid, p-coumaric acid, abscisic acid, emodin, syringic acid, α -terpinyl acetate, isoborneol, camphene, α -phellandrene, α -pinene, methoxysalicylic acid, hesperetin and xanthone were purchased to Sigma–Aldrich (St. Louis, MO, USA). Eucalyptol, linalool, camphor, terpinen-4-ol, borneol, limonene, β -pinene, luteolin and chlorogenic acid were obtained from Fluka (Steinheim, Switzerland). Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and ferric chloride [FeCl₃·6H₂O] were of analytical grade and also supplied by Sigma–Aldrich (St. Louis, MO, USA). Folin and Ciocalteu's phenol

reagent, sodium carbonate and trichloroacetic acid (TCA) were obtained from Fluka (Steinheim, Switzerland), Panreac (Barcelona, Spain) and Merck (Darmstadt, Germany), respectively. Phosphate buffer (pH 6.6) was prepared from sodium dihydrogen phosphate (NaH₂PO₄·2H₂O) and disodium hydrogen phosphate (Na₂HPO₄·2H₂O), purchased from Merck (Darmstadt, Germany) and Panreac (Barcelona, Spain), respectively. Anhydrous sodium sulfate was also obtained from Panreac (Barcelona, Spain). Methanol was obtained from Sigma–Aldrich (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Essential oils isolation

The essential oils of the *T. mastichina* were isolated from 180 g of dried material by hydrodistillation in 2 L of water for 150 min, using a Clevenger-type apparatus (European Pharmacopoeia, 1996). The essential oils were dried over anhydrous sodium sulfate and stored under nitrogen in tightly closed dark vials between –20 °C and –30 °C until analysis.

2.4. Preparation of methanolic extracts

To obtain the methanolic extracts, 0.5 g of each dried powder plant material ($\leq 1200 \mu\text{m}$ mesh) was mixed with 15 mL of petroleum ether for 24 h to eliminate chlorophyll and fats. After that it was filtered (Whatman filter paper No.1) and dried in an oven at 40 °C during 24 h and then extracted with pure methanol for 2.40 h in a Soxhlet apparatus (around 70 °C). At the end, the extracts were concentrated under vacuum at 50 °C, using a rotary evaporator. All extracts were kept in the dark at –20 °C until further analysis. All subsequent determinations were made on triplicate.

2.5. Determination of total phenol contents

Total phenol contents of the extracts were estimated by a colorimetric assay based on the procedure described by Singleton and Rossi (1965) which has been frequently used in research studies (Oliveira et al., 2008; Safaei-Ghomi, Ebrahimabadi, Djafari-Bidgoli, & Batooli, 2009; Sahin et al., 2004), with some modifications. Prior to the determination of total phenol contents, the extracts were redissolved in methanol. Then, 1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the mixture and the volume adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Thermo Electron Corporation Genesis 10uv Spectrophotometer). A blank without any extract was used for background subtraction. The total phenol content of each extract was determined from standard curves (0.01–0.8 mmol/L; correlation coefficients (r) > 0.99) prepared daily, using gallic acid as standard. Results were expressed as milligrams of gallic acid equivalents (GAEs) per gram of extract.

2.6. Antioxidant activity

2.6.1. Free radical scavenging (DPPH) assay

The radical scavenging activities of the methanolic extracts and essential oils were determined by the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, according to the methodologies described by Oliveira et al. (2008) and Cao et al. (2009), respectively. The DPPH-scavenging effect was calculated as the percentage of DPPH discoloration, using the equation: % Scavenging effect = $[(A_{\text{DPPH}} - A_S)/A_{\text{DPPH}}] \times 100$, where A_S was the absorbance of the solution in which the plant extract or essential oil had been

Table 1

Provinces, localities and altitudes of the sampling sites where *Thymus mastichina* populations studied in the present work were collected, as well as the oil yields and reducing powers (EC₅₀ expressed as mg/mL) of the methanolic extracts.

Province	Locality	Sample	Altitude (m)	Oil yield (mL/100 g)	EC ₅₀ ^b (mg/mL)
Salamanca	Béjar	TM07	1210	3.13	5.35 ± 0.35
	Valdemierque	TM08	932	5.31	5.90 ± 0.80
	Mozarbez	TM09	913	5.39	3.83 ± 0.62
León	Golpejas	TM11	808	4.72	4.66 ± 0.26
	Carrocera	TM37	1029	4.06	4.25 ± 0.77
	Boñar	TM43	1017	2.27	3.20 ± 0.21
	Truchas	TM14	957	5.12	4.65 ± 0.43
	Peranzanes	TM33	507	5.22	5.16 ± 0.50
Burgos	Salas de los Infantes	TM40	947	2.60	4.69 ± 0.18
	Lerma	TM39	828	3.93	3.66 ± 0.19
	Oña	TM20	570	— ^a	6.23 ± 0.10
Segovia	Oña	TM32	550	4.27	4.26 ± 0.27
	Villacastin	TM38	1056	5.25	7.24 ± 1.15
	Riaza	TM23	814	4.89	3.69 ± 0.64
	Coca	TM26	790	4.84	5.00 ± 0.17
	Prádena	TM22	709	4.50	4.10 ± 0.55
Soria	Vinuesa	TM41	1090	3.43	5.81 ± 0.60
	Aldealpozo	TM18	1061	5.00	5.59 ± 0.76
	Almazán	TM17	933	6.48	4.73 ± 0.21
	Langa de Duero	TM25	434	4.64	5.37 ± 0.70

^a Insufficient sample to determine essential oil yield.

^b Mean ± SD ($n = 6$).

added at a particular level and A_{DPPH} was the absorbance of the DPPH solution. The extract or essential oil concentrations providing 25% inhibition (EC_{25}) were determined from the graphs of the scavenging effect percentages against the extract or essential oil concentrations.

2.6.2. Reducing-power assay

The reducing powers of the methanolic extracts and essential oils were determined by the procedure described by Berker, Güçlü, Tor, and Apak (2007) with some modifications. Briefly, various concentrations of the methanolic extracts or essential oils (1 mL) were mixed with 2.5 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) solution of $K_3Fe(CN)_6$. The mixtures were incubated at 50 °C in a water bath for 20 min. The incubated mixtures were left to cool to room temperature and 2.5 mL of 10% (w/v) TCA solution was added. The solutions were mixed thoroughly. Aliquots of 2.5 mL were withdrawn and to these, 0.5 mL of 0.1% (w/v) $FeCl_3 \cdot 6H_2O$ solution was added. After 2 min the absorbances of the resulting Prussian blue solutions were measured at 700 nm (A_{700}) against a reagent blank. The EC_{50} values that corresponded to the extract or essential oil concentrations which provide 0.5 of absorbance were determined from the graphs of A_{700} against the correspondent extract or essential oil concentrations.

2.7. Chromatographic analysis of the essential oils and methanolic extracts

The identification and quantification of the volatile components present in the essential oils were carried out using an Agilent Technologies gas chromatograph, model 7890A, equipped with a Flame Ionization Detector (FID). The analysis was performed with an HP 5 Column (Agilent Technologies) 50 m long \times 0.320 mm i.d., and 1.05 μ m film thickness. The temperature programme began at 50 °C and increased by 2 °C min^{-1} until 190 °C and then by 10 °C min^{-1} until reaching 280 °C (10 min). Helium X50 PRM (Carbueros metálicos) was used as carrier with a pressure of 12 psi. The injector and detector temperatures were 250 °C and 280 °C, respectively. The volume of the sample injected was 1 μ L and the components were identified by comparison of their GC retention indices calculated by linear interpolation with those of pure compounds and of a series of *n*-alkanes (C_7 – C_{25}).

Flavonoids and organic acid contents of the methanolic extracts, previously redissolved in methanol, were determined by an Agilent Technologies 1200 series High Performance Liquid Chromatograph (HPLC), equipped with a capillary column Zorbax Eclipse XDB-C18 (4.6 m long, 150 mm diameter and 5 μ m film thickness) at 25 °C, coupled to a Diode Array Detector (DAD). Acetic acid (2%) (A) and acetonitrile (B) were used as mobile phase, in the following ranges: 0 min – 90% A + 10% B; 10 min – 78% A + 22% B; 12 min – 62% A + 38% B; 17 min – 62% B + 38% A and 20 min – 100% B, at 1.2 mL/min. The volume of the sample injected was 20 μ L. Compound identification was done by comparing their retention times and UV–visible spectra with their respective pure standards at a wavelength of 254, 280 or 350 nm depending on the maximum absorption of each compound. The phenolic compounds were quantified using the external standard method and the respective calibration curve of each quantified phenolic compound.

2.8. Statistical analysis

The results were analyzed statistically using the SAS v.9.1.3 program. Depending on the existence of normality and homogeneity of the data, one-way analysis of variance (ANOVA), ANOVA-Welch or Kruskal–Wallis Tests were performed. Pearson correlation coefficients were also determined in order to compare the

antioxidant activities of the essential oils and methanolic extracts with their chemical compositions. Additionally, a Principal Component Analysis (PCA) was applied to the data sets of the methanolic extracts and essential oils of the thyme populations.

3. Results and discussion

3.1. Essential oil yields, total phenol contents and antioxidant activities of the methanolic extracts and essential oils of *T. mastichina* populations

The oil yields varied between 2.27 and 6.48 mL/100 g (Table 1). Similar ranges of essential oil yields were obtained for the five Spanish provinces: Salamanca – 3.13–5.39; León – 2.27–5.22; Burgos – 2.60–4.27; Segovia – 4.50–5.25; and Soria – 3.43–6.48 (mL/100 g). Even though in each Castilla y León provinces the thyme populations were collected in different altitudes, in this work no significant correlation ($p > 0.05$) was obtained between the essential oil yields and altitude, in line with Kizil (2010).

Total phenol contents of the methanolic extracts of the twenty *T. mastichina* samples, varied between 2.90 and 9.15 mg GAE/g_{extract} (0.29–0.915 g GAE/100g_{extract}), as shown in Fig. 1. The concentration ranges were the following: Salamanca – 2.90–8.17; León – 4.95–9.15; Burgos – 4.63–8.28; Segovia – 3.74–6.64; and Soria 5.48–6.05 mg GAE/g_{extract}. Some province intra-variability was detected. For example, in Salamanca the lowest total phenol content was obtained (TM07) (2.90 mg GAE/g_{extract}), as well as the highest third content (TM08) (8.17 mg GAE/g_{extract}). This last sample was not statistically different to those collected in other two Castilla y León provinces, such as, the TM37 collected in León and TM39 collected in Burgos. An exception to this intra variability was observed in Soria province. In fact, the four *T. mastichina* populations showed very similar total phenol contents, varying between 5.48 and 6.05 mg GAE/g_{extract} and not being statistically different ($p > 0.05$). However, our results were lower than those reported by Barros, Heleno, Carvalho, and Ferreira (2010) for *T. mastichina* extracted with methanol at 25 °C during 24 h (165.29 mg GAE/g_{extract}). This difference might be due to the application in our study of a preliminary extraction with petroleum ether, probably resulting in the extraction of compounds with reducing capacity and so decreasing total phenol contents.

In relation to the antioxidant activity, the methanolic extracts and essential oils isolated in the present work were screened for their possible antioxidant activities by using two *in vitro* methods:

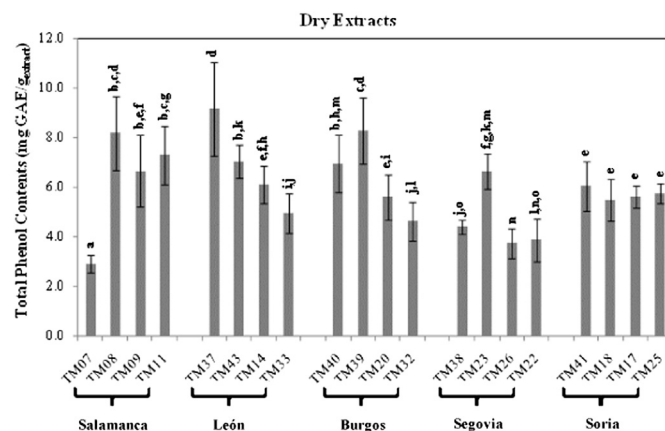


Fig. 1. Total phenol contents (mg GAE/g_{extract}) of methanolic extracts of twenty *T. mastichina* populations collected in five Spanish provinces, namely, Salamanca, León, Burgos, Segovia and Soria. Results are expressed as Mean \pm SD ($n = 6$).

the inhibition of DPPH free radical and the reductive potential assays.

3.1.1. DPPH Free radical scavenging activity

All methanolic extracts and essential oils isolated from *T. mastichina* collected in Castilla y León provinces followed a similar pattern in relation to the scavenging ability on DPPH radicals. Fig. 2A shows the results obtained for the samples collected in Soria province. All methanolic extracts and essential oils followed a similar concentration-dependent pattern, increasing the scavenging activity against DPPH radical when the concentrations also increased; however, much higher DPPH radical scavenging abilities were obtained with the methanolic extracts than with the essential oils. For all methanolic extracts, inhibitions of DPPH radicals between 86.6% (TM38 sample) and 93.9% (TM33 sample) were determined when the highest methanolic extract concentration was used. On contrary, the essential oils presented a much lower DPPH radical-scavenging activity, varying between 30.8% (TM33 sample) and 57.7% (TM41 sample) even when the highest concentrations were used. When analyzing *Thymus pallescens* and *Thymus algeriensis* essential oils, Hazzit et al. (2009) also stated some variability on DPPH radical scavenging abilities. Concentrations of 1.0 mg/mL of these *Thymus*, showed DPPH radical-scavenging activities between 59.9 and 93.4%, and 6.3–53.4%, respectively; however, these essential oils seemed to have a higher antioxidant potential than those analyzed in the present work, where much higher essential oil concentrations gave lower or similar DPPH radical-scavenging effect. Nevertheless, when analyzing *Thymus capitatus* essential oils, Bounatirou et al. (2007) obtained similar DPPH scavenging activities to those reported in the present work because scavenging effects of 5.7–17.1% and 7.6–46.6% were determined for essential oils concentrations of 100 and 250 mg/mL, respectively. On contrary, Kulisic, Radonic, and Milos (2005) obtained much higher percentages of inhibition of DPPH than those

reported in the present work when studying *Thymus vulgaris* and *Thymus serpyllum* essential oils, as 2 mg/mL concentrations gave scavenging activities of 91.3 and 82.0%, respectively. When comparing all of these percentages with ours and taking into account the tested concentrations, the present study suggests that the isolated *T. mastichina* oils had weaker DPPH radical-scavenging ability than other *Thymus* found worldwide, such as *T. vulgaris*, *T. pallescens*, *T. algeriensis* or *T. serpyllum*. In relation to Portuguese *T. mastichina* populations, only one reference involving the determination of DPPH free radical scavenging activity was available (Bentes et al., 2009). In that study just one sample of essential oil was analyzed, being also stated its ineffectiveness as antioxidant.

The scavenging effects of the methanolic extracts and essential oils were further expressed as EC₂₅ values, as represented in Fig. 2B. A higher EC₂₅ value meant a lower antioxidant activity.

Taking into account our results, some variability was again observed on the EC₂₅ values of each Castilla y León province. For the methanolic extracts, the values varied between 0.90 and 2.70, 1.43 to 1.93, 1.45 to 3.45, 1.65 to 2.57 and 2.11 and 2.49 mg/mL for Salamanca, León, Burgos, Segovia and Soria, respectively. In relation to the essential oils, higher values were obtained, varying from 104 to 124, 86 to 241, 147 to 152, 78 to 191, and 79 and 151 mg/mL, for Salamanca, León, Burgos, Segovia and Soria, respectively. Similar results were reported by Safaei-Ghomi et al. (2009), when analyzing essential oils and subfractions of methanol extracts of *Thymus caramanicus*. In that study the polar subfraction had a much higher radical-scavenging activity, almost six and nine times higher, than the essential oil and the nonpolar subfraction, respectively.

3.1.2. Reducing power

This assay measures the ability of a sample to act as an electron donor that is able to convert Fe³⁺-ferricyanide complex to the ferrous form. The reductive potentials were only determined for the

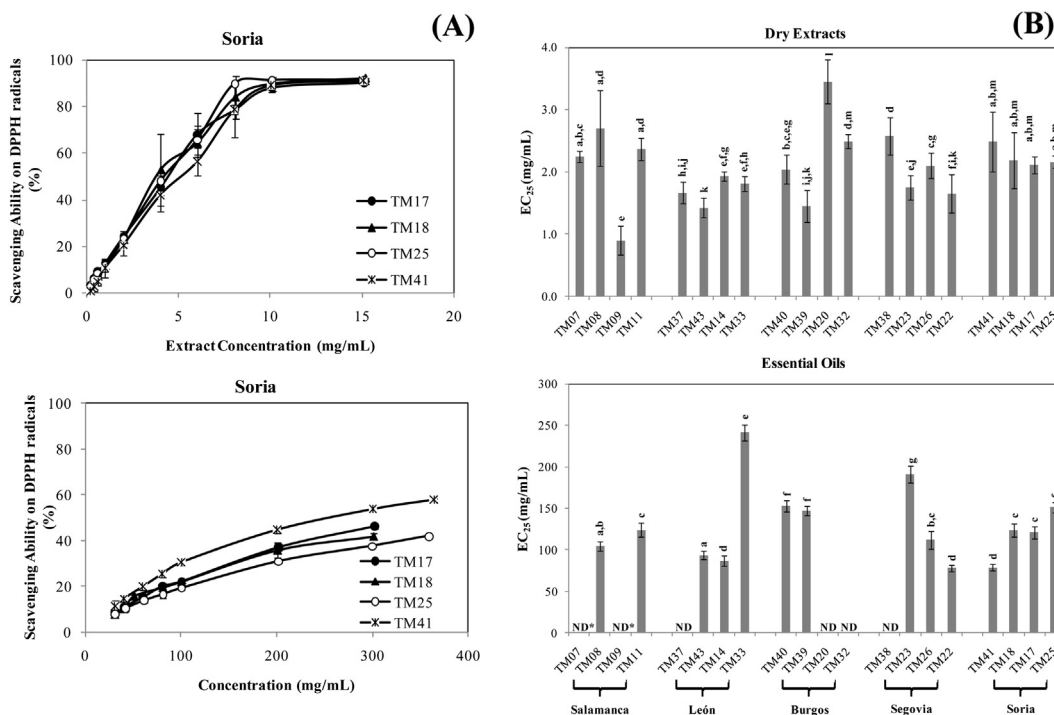


Fig. 2. (A) DPPH Free Radical Scavenging abilities (%) of the methanolic extracts and essential oils of the *Thymus mastichina* populations collected in the Soria province and (B) EC₂₅ (mg/mL) values of DPPH Free Radical Scavenging abilities of methanolic extracts and essential oils of twenty *T. mastichina* populations collected in five Spanish provinces, namely, Salamanca, León, Burgos, Segovia and Soria. Results are expressed as Mean ± SD (n = 6).

methanolic extracts because it was not possible to perform correctly the assay with the essential oils. This was due to the existence of two distinct phases, formed immediately after the addition of the essential oil to the phosphate buffer (pH 6.6), outcome of their different polarities. The reducing powers of the methanolic extracts of *T. mastichina* collected in Castilla y León provinces, increased with increasing concentrations (data not shown). When observing the results obtained for the EC₅₀ values of the reducing power of the methanolic extracts (Table 1), some intra-variability was again detected between the samples collected in distinct provinces. The ranges obtained for each Castilla y León provinces were the following: Salamanca – 3.83–5.90 mg/mL; León – 3.20–5.16 mg/mL; Burgos – 3.66–6.23 mg/mL; Segovia – 3.69–7.24 mg/mL; and Soria – 4.73–5.81 mg/mL.

When comparing our results with those reported for a Portuguese *T. mastichina* sample, the Spanish populations seemed to have lower reducing power. In Bentes et al. (2009) study an absorbance around 1.0 at 700 nm was obtained with a smaller concentration (0.1 mg/mL).

3.2. Antioxidant activities of essential oils and methanolic extracts versus their chemical composition

In order to better understand the antioxidant activities of the methanolic extracts and essential oils of *T. mastichina* populations studied in the present work, their chemical compositions were determined. The methanolic extracts were analyzed in terms of their contents in organic acids and flavonoids (Table 2), as some of these compounds possess antioxidant properties. Rosmarinic acid was the most abundant polyphenol carboxylic acid, varying between 1.70 and 9.85 mg/g_{extract}, corresponding these values to TM14 (León) and TM23 (Segovia) samples, respectively. Other compounds were detected in lower but significant quantities, such as methoxysalicylic acid (0.53–1.80 mg/g), apigenin (0.15–0.91 mg/g), kaempferol (0.20–0.65 mg/g) and luteolin (0.35–1.85 mg/g). Generally, it was observed that the maximums of these compounds were distributed along the *Thymus* populations sampled in the five provinces. The highest methoxysalicylic acid concentration was obtained for the TM43 sample, collected in León. Another sample of this province, TM14 sample, presented the highest kaempferol concentration. TM8 and TM17 samples of Salamanca and Soria provinces, respectively, were those with the highest contents in apigenin and luteolin. On contrary, other compounds were present in much lower concentrations, such as flavonol quercetin (<0.01–0.08 mg/g) and caffeic acid (<0.01–0.12 mg/g). Furthermore, other compounds were only detected in a small number of samples (data not shown), such as: I) Coumaric acid that was only detected in two samples, TM20 and TM32, both collected in Burgos; II) Abscisic and chlorogenic acids, both detected in four samples, namely TM33, TM39, TM23, TM22 and TM43, TM33, TM23, TM22, respectively; III) Emodin in two samples, TM23 and TM26; IV) Hesperetin in TM25

sample; V) Syringic acid in seven samples - TM8, TM9, TM11, TM43, TM23, TM26 and TM22; and VI) Xanthone in TM26 sample. These results are in line with those reported by Pereira and Cardoso (2013) who observed that species of *Mentha* and *Thymus* usually comprise derivatives of caffeic acid and distinct glycosidic forms of the flavonoids luteolin, apigenin, eriodictyol and naringenin, and Gordo et al. (2012) who identified nine compounds on dichloromethane and ethanol extracts of *T. mastichina* L., including rosmarinic acid, 6-hydroxyluteolin-7-O-β-glucopyranoside, and 6-hydroxyapigenin-7-O-β-glucopyranoside.

In more detail and regarding the chemical composition of the methanolic extracts of *T. mastichina* populations, some intra-variability inside each province was again detected. The ranges of rosmarinic acid contents for the five provinces were the following: Salamanca – 2.41–4.67; León – 1.70–6.86; Burgos – 3.62–7.37; Segovia – 2.32–9.85; and Soria – 2.08–4.78 mg/g. In relation to methoxysalicylic acid, the following values were determined: Salamanca – 0.53–1.29; León – 0.89–1.80; Burgos – 0.65–0.99; Segovia – 0.66–1.43; and Soria – 0.79–1.11 mg/g. The ranges of kaempferol were equal to: Salamanca – 0.20–0.54; León – 0.42–0.65; Burgos – 0.27–0.49; Segovia – 0.33–0.42; and Soria – 0.31–0.63 mg/g. In relation to luteolin, the following ranges were determined: Salamanca – 0.35–1.43; León – 0.80–1.67; Burgos – 0.47–1.52; Segovia – 0.43–0.83; and Soria – 0.50–1.85 mg/g.

Regarding possible relationships between the antioxidant activities of methanolic extracts and their chemical composition, significant negative correlations were only found between EC₂₅ values of the DPPH assay and methoxysalicylic acid ($p = 0.00126$) and between EC₅₀ values of the Reducing Power assay and methoxysalicylic and rosmarinic acids ($p = 0.00123$ and 0.0245, respectively). These results are in line with those reported by Chizzola, Michitsch, and Franz (2008) who demonstrated that rosmarinic acid influenced the DPPH activity, as well as the ferric reducing antioxidant power (FRAP), when analyzing ethanolic extracts of *T. vulgaris* leaves. Thus, our results suggest that the methanolic extracts of *T. mastichina* populations with high contents in methoxysalicylic and rosmarinic acids, seemed to be able to act as antioxidants and to suppress radical chain reactions by converting free radicals to more stable products.

In relation to the composition of essential oils, the major components are shown in Table 3. It is worth noting that eucalyptol, also known as 1,8-cineole, was the main compound in all *T. mastichina* populations studied, varying between 56.80 and 69.60%. This was expected because a high 1,8-cineole content is a discriminatory and common feature of the essential oils of the Section *Mastichina* (Salgueiro et al., 1997). Linalool was the following component present in almost all samples; however, its percentage (0.62–15.7%) was always much smaller than that of eucalyptol. In fact, only two samples, TM17 and TM25 (both of Soria), presented a linalool percentage higher than 10%, equal to 13.1 and 15.7%, respectively. Some monoterpene hydrocarbons,

Table 2
Major organic acids and flavonoids (mg/g of extract) found on the methanolic extracts of *Thymus mastichina* studied in the present work.

	Salamanca				León				Burgos				Segovia				Soria			
	TM 7	TM 8	TM 9	TM 11	TM 37	TM 43	TM 14	TM 33	TM 40	TM 39	TM 20	TM 32	TM 38	TM 23	TM 26	TM 22	TM 41	TM 18	TM 17	TM 25
Methoxysalicylic acid	0.53	0.73	1.29	1.01	0.89	1.80	1.13	1.33	0.95	0.99	0.65	0.79	0.66	1.23	0.90	1.43	1.11	0.79	0.99	0.87
Apigenin	0.15	0.91	0.68	0.31	0.53	0.57	0.59	0.46	0.39	0.64	0.29	0.28	0.36	0.29	0.46	0.36	0.76	0.34	0.74	0.38
Caffeic acid	0.04	0.12	0.05	0.05	0.04	0.05	0.04	0.03	0.03	0.05	tr.	0.02	0.03	0.05	0.03	0.05	0.04	0.03	0.03	0.04
Kaempferol	0.20	0.45	0.54	0.34	0.42	0.58	0.65	0.46	0.27	0.49	0.27	0.33	0.33	0.33	0.42	0.38	0.63	0.32	0.54	0.31
Luteolin	0.35	1.35	1.43	0.70	1.07	1.27	1.67	0.80	0.47	1.52	0.56	0.53	0.43	0.48	0.70	0.83	1.44	0.90	1.85	0.50
Quercetin	tr.	0.03	0.03	0.03	0.03	0.03	0.03	0.08	0.02	0.06	0.03	0.05	0.07	0.03	0.07	0.03	0.05	0.03	0.05	0.05
Rosmarinic acid	2.75	2.85	2.41	4.67	5.18	6.86	1.70	5.86	3.99	5.50	3.62	7.37	3.40	9.85	2.32	8.54	3.32	4.09	2.08	4.78

Note: tr.- Trace amount. All of the RSD were <10% ($n = 3$).

Table 3
Essential oils chemical composition (%) of the *Thymus mastichina* populations studied in the present work.

	Salamanca			León			Burgos			Segovia			Soria							
	TM 7	TM 8	TM 9	TM 11	TM 37	TM 43	TM 14	TM 33	TM 40	TM 39	TM 20	TM 32	TM 38	TM 26	TM 22	TM 41	TM 18	TM 17	TM 25	
α -Pinene	3.00	3.00	3.20	2.60	0.54	2.90	2.70	3.60	3.30	2.90	3.43	3.15	0.84	3.30	3.60	3.40	3.60	2.51	2.58	2.70
β -Pinene	3.50	4.40	4.50	3.70	1.72	4.50	4.40	5.63	5.00	4.40	5.18	4.87	2.14	4.90	4.80	4.70	4.80	3.66	3.85	4.00
α -Phellandrene	2.00	2.80	3.00	2.30	1.27	2.90	2.60	3.81	3.20	2.90	3.38	3.31	1.40	3.60	3.20	3.30	3.20	2.05	2.39	2.60
Camphene	2.10	0.20	0.70	0.50	0.14	0.20	n.d.	0.14	0.40	0.20	0.09	0.14	0.23	0.30	1.30	0.60	1.30	0.44	0.20	0.40
Limonene	5.10	3.60	2.20	3.00	1.74	1.70	2.40	1.71	1.40	2.20	4.31	1.14	1.54	1.90	1.10	4.60	1.30	2.62	1.07	1.40
Isoborneol	1.20	1.70	1.70	1.40	2.05	1.40	1.70	1.65	1.80	1.40	1.74	1.47	1.53	1.50	1.30	1.60	1.50	0.98	1.52	1.30
Borneol	2.90	0.20	0.80	0.80	1.28	0.40	n.d.	0.20	0.70	0.30	0.10	0.14	1.28	0.30	1.90	1.20	1.50	0.80	0.34	0.70
Terpinen-4-ol	1.00	0.90	0.80	0.90	1.02	0.80	0.90	0.69	0.90	0.80	0.41	0.57	1.11	0.60	0.80	0.70	0.60	0.64	0.58	0.80
Terpineol	2.70	4.80	4.90	3.50	5.99	3.90	4.50	5.28	5.30	4.10	5.22	4.29	4.29	4.70	3.80	4.70	4.80	2.07	4.05	4.10
Camphor	1.70	n.d.	0.60	0.30	0.36	0.10	n.d.	n.d.	0.20	0.10	n.d.	0.13	0.35	0.30	0.80	0.10	0.10	0.17	n.d.	0.10
α -Terpinyl acetate	1.10	0.50	n.d.	0.90	2.72	n.d.	0.40	1.12	0.40	1.80	0.46	1.63	4.48	1.20	2.00	0.90	1.80	2.89	0.32	1.10
Linalool	5.00	7.30	5.00	6.90	4.18	4.00	4.90	1.27	5.60	4.80	0.62	1.30	3.57	5.50	5.10	5.10	2.90	4.78	13.1	15.7
Eucalyptol	59.60	62.20	65.10	64.10	66.42	66.70	69.60	67.63	64.10	63.90	64.31	68.60	68.04	64.00	65.00	61.70	64.50	67.26	59.59	56.80
Terpene	18.50	17.70	17.90	15.30	9.04	15.80	15.00	19.30	17.20	16.20	22.69	16.50	9.49	19.40	18.00	20.80	18.80	14.57	13.92	14.30
hydrocarbons																				
Alcohols	8.10	7.70	8.70	7.10	11.14	6.80	7.40	8.11	9.00	7.20	7.90	6.84	8.75	7.50	8.20	8.50	8.70	5.17	7.22	8.30
Phenols	n.d.	n.d.	n.d.	n.d.	0.27	0.50	n.d.	n.d.	0.10	n.d.	n.d.	n.d.	0.68	n.d.	n.d.	n.d.	0.10	0.16	n.d.	n.d.
Ketones	1.70	n.d.	0.60	0.30	0.36	0.10	n.d.	n.d.	0.20	0.10	n.d.	0.13	0.35	0.30	0.80	0.10	0.10	0.17	n.d.	0.10
Esters	1.10	0.50	n.d.	0.90	2.72	n.d.	0.40	1.12	0.40	1.80	0.46	1.63	4.48	1.20	2.00	0.90	1.80	2.89	0.32	1.10
Linalool	5.00	7.30	5.00	6.90	4.29	4.10	4.90	1.27	5.70	4.90	0.62	1.30	3.57	5.50	5.10	5.10	2.90	4.78	13.26	16.10
Cineol	59.60	62.20	65.10	64.10	66.42	66.70	69.60	67.63	64.10	63.90	64.31	68.60	68.04	64.00	65.00	61.70	64.50	67.26	59.59	56.80

Note: n.d. — Not detected. All of the RSD were <10% (n = 3).

such as α -pinene, β -pinene and camphene were detected in almost all samples, varying in the following ranges: 0.54–3.60, 1.72–5.63, and not detected to 2.10%, respectively.

Taking into account these results, the main group of constituents present in *T. mastichina* populations studied in the present work was the cineol group, followed by the terpenic hydrocarbons. In general, all essential oils seemed to be rather like, even been extracted from *T. mastichina* populations collected in different provinces.

Our results were similar to those reported by Salgueiro et al. (1997) who analyzed 18 populations of *T. mastichina* ssp. *mastichina* collected in Portugal. The average of 1,8-cineole contents was equal to 53.3%, close to the range determined in the present work; however, these authors found a higher range for the 1,8-cineole content (15.0–64.2%), when compared to the present work. Our results were also identical to the findings reported by Fraternali, Giampieri, and Ricci (2003) who determined 55.5% of 1,8-cineole and 24.5% of linalool in an essential oil isolated from plantlets of *T. mastichina* L. ssp. *mastichina* cultured *in vitro*, as well as to those reported for *T. mastichina* samples collected in the North of Portugal (58%) (Miguel, Simões, et al., 2004) and in South of Portugal (49%) (Miguel et al., 2005). Nevertheless, *T. mastichina* collected in the five Castilla y León provinces were different to the population of *T. mastichina* ssp. *donyanae* collected in Portugal by Salgueiro et al. (1997). Beyond presenting a high content of 1,8-cineole (38.4%), this last *Thymus* population also had a high borneol concentration (15.3%); however, this situation was not detected in the present work because the borneol maximum obtained in our *T. mastichina* populations was only equal to 2.90%. The contents of 1,8-cineole determined for the *T. mastichina* populations analyzed in this work were also higher than those reported for *T. albicans*, collected in Portugal (29.0–42.9%) by Salgueiro et al. (1997). Nevertheless, these last samples presented a similar range of linalool, 3.2–22.0%, to the determined in this work. Our *T. mastichina* populations also presented higher contents of 1,8-cineole than the one reported for a Portuguese *T. mastichina* population (44%) analyzed by Bentes et al. (2009).

Concerning the antioxidant activity, no negative significant correlation was found between EC₂₅ values of DPPH assay and the individual compounds determined in the essential oils. Similar results were obtained when the main classes were considered. These results might be due to the low concentrations of thymol (less than 0.7%) (data presented as Supplemental material) determined in the essential oils. Thymol is an aromatic monoterpene, biosynthesized by the aromatization of γ -terpinene to *p*-cymene, followed by its hydroxylation (Poulose & Croteau, 1978), being only detected in six samples. Thymol is recognized to be a strong antioxidant, and so essential oils rich in this compound, generally present the highest activities in DPPH and FRAP tests (Chizzola et al., 2008). Recently, Hazzit et al. (2009) referred that the antioxidant activity of essential oils has been attributed to the presence of phenolic constituents, especially thymol and/or carvacrol. Thus, the low thymol concentrations determined in the essential oils extracted from *T. mastichina* populations studied in the present work, were possibly one of the reasons that explain their low antioxidant potential. Additionally, the low contents of γ -terpinene (0.18–0.40%), terpinolene (not detected to 0.20%) and geraniol (0.10–0.68%) (data presented as supplemental material) may also explain our results. In fact, some of these species have shown considerable DPPH reducing activity, as referred by Choi, Song, Ukeda, and Sawamura (2000), being some of them considered strong antioxidants (Emami, Javadi, & Hassanzadeh, 2007). On the other hand, all *T. mastichina* essential oils studied in the present work presented α -pinene (0.54–3.60%), β -pinene (1.72–5.63%) and limonene (1.07–5.10%); however, as stated by Emami et al. (2007),

these species even in the pure state, showed no or low DPPH free radical scavenging activities, reinforcing our results.

After applying a principal component analysis (data not shown), it was not possible to differentiate the methanolic extracts, as well as, the essential oils of the twenty *T. mastichina* populations by provinces, after considering the main classes of compounds or the individual ones, as well as, the values related with the antioxidant activity (EC₂₅ values of the DPPH assay and EC₅₀ values of the reductive potential assay).

4. Conclusions

In conclusion, our work contributed to solve a lack of information about one plant extensively used by local populations, as food spice or medicinal herb, in terms of its antioxidant potential and chemical composition.

In detail, the methanolic extracts and essential oils of the twenty *T. mastichina* L. populations studied in the present work showed antioxidant properties; however, the methanolic extracts showed higher antioxidant power than the essential oils. This might be due to their chemical composition. In fact, essential oils had low contents of thymol, γ -terpinene, terpinolene and geraniol, species with considerable antioxidant activity, whereas *T. mastichina* methanolic extracts were rich in rosmarinic acid that is a polyphenol carboxylic acid and known by possessing antioxidant activity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.lwt.2013.12.041>

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