LWT - Food Science and Technology 57 (2014) 412–418

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

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

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ARTICLE INFO

Article history:
Received 2 July 2011
Received in revised form
27 December 2013
Accepted 28 December 2013

Keywords:
Thymus mastichina L.
Methanolic extracts
Essential oils
Antioxidant activity
Chemical composition

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 EC_{25} 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.

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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http://dx.doi.org/10.1016/j.lwt.2013.12.041

antioxidant activity of *T. mastichina* populations collected in five Spanish provinces from Castilla y Léon 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 [$FeCl_3 \cdot 6H_2O$] 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 ($NaH_2PO_4 \cdot 2H_2O$) and disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$), 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).

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 (EC_{50} 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
	Golpejas	TM11	808	4.72	4.66 ± 0.26
León	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
	Oña	TM32	550	4.27	4.26 ± 0.27
Segovia	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 ($n = 6$).

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 (≤1200 μm 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 = $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S was the absorbance of the solution in which the plant extract or essential oil had been