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Short communication. Influence of phenological stage on the antioxidant activity of *Thymus zygis* s. l. essential oil

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Abstract

When a species has proven antioxidant capacity and possible commercial application it is important to know whether or not this activity varies with the phenological stage of the raw material commodity. The present work aims to assess this topic for the case of *Thymus zygis* s.l. We analyzed the yield and the chemical composition (gas chromatography-mass spectrometry) of its essential oil in five wild populations from the Southwest of Iberian Peninsula at flowering and fruiting stage. We measured their TEAC (Trolox equivalent antioxidant activity) values. The main components were *p*-cymene (flowering: 33.6-16.2%; fruiting: 43.2-30.3%), γ-terpinene (flowering: 13.5-5.78%; fruiting: 4.67-1.96%) and thymol (flowering: 46.45-22.2%; fruiting: 54.24-13.9%); the latest, especially high (flowering: 46.45%; fruiting: 54.24%) in one of the populations, identified as *Thymus zygis* ssp. *gracilis* (Boiss.) R. Morales, which showed the highest antioxidant activity as well. In the set of samples TEAC oscillated between 16.12-30.52 mmol Trolox L^{-1} (flowering) and 10.96-40.24 mmol Trolox L^{-1} (fruiting). No significant differences between antioxidant activity of both stages were found, but yields were significantly higher at flowering time. These levels of antioxidant activity are close to those achieved as highly antioxidant food products, as coffee or wine. This species can be a good raw material for agribusiness use, specially the population of *Thymus zygis* ssp. *gracilis*.

Additional key words: antioxidant capacity; chemical composition; Iberian Peninsula; Lamiaceae; phytogenetic resources; thyme.

Resumen

Comunicación corta. Influencia del estado fenológico en la actividad antioxidante del aceite esencial de *Thymus zygis*

Cuando se ha demostrado la capacidad antioxidante de una especie y es factible su aplicación comercial, es importante saber si esta actividad varía según el estado fenológico de la materia prima de los productos básicos. El presente trabajo tiene como objetivo evaluar esta cuestión para el caso de *Thymus zygis* s.l. Se analizó el rendimiento y la composición química (cromatografía de gases-espectrometría de masas) de su aceite esencial en cinco poblaciones silvestres del suroeste de la Península Ibérica, en los estados de floración y fructificación. También se midieron sus valores de TEAC (*Trolox equivalent antioxidant activity*). Los principales componentes fueron *p*-cimeno (floración: 33,6-16,2%; fructificación: 43,2-30,3%), γ-terpineno (floración: 13,5-5,78%; fructificación: 4,67-1,96%) y timol (floración: 46,45-22,2%; fructificación: 54,24-13,9%); el último de ellos, especialmente alto (floración: 46,45%; fructificación: 54,24%) en una de las poblaciones que fue identificada como *Thymus zygis* subsp. *gracilis* (Boiss.) R. Morales, y que también

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Abbreviations used: FL (flowers and leaves); TEAC (Trolox equivalent antioxidant activity); WP (whole plant).

mostró la mayor actividad antioxidante. Los valores de TEAC estuvieron entre 16,12 y 30,52 mmol Trolox L^{-1} en floración y 10,96 y 40,24 mmol Trolox L^{-1} en fructificación. No se observaron diferencias significativas entre la actividad antioxidante de ambos estados, si bien fue significativamente mayor el rendimiento en floración. Los valores de actividad antioxidante son similares a los alcanzados por los productos alimenticios como el café o el vino, que se consideran de alta actividad. Esta especie puede ser una buena materia prima por su utilidad agroindustrial, especialmente la población estudiada de *Thymus zygis* subsp. *gracilis*.

Palabras clave adicionales: capacidad antioxidante; composición química; Lamiaceae; Península Ibérica; recursos fitogenéticos; tomillo.

Introduction

Thymus zygis s. l., is a plant endemic to the Iberian Peninsula and northern Morocco, which is a pioneer scrub on all types of soils. Its traditional use is well known in its native range (Vázquez, 2008; Morales, 2010) and its essential oil has been chemically characterized using material from North Africa, Spain and Portugal (Blanco, 2005; Figueiredo *et al*., 2008). Some authors (Jiménez *et al*., 1993; Jordán *et al*., 2009) have pointed out its antioxidant power. This capacity makes it a plant of possible use in the food industry, important to inhibit oxidation, one of the major causes of food spoilage. It prevents rancidity and/or deterioration of the nutritional quality, colour, flavour and texture (Antolovich *et al*., 2002).

When a species has proven antioxidant capacity and possible commercial application it is important to know whether or not this activity varies with the phenological stage of the raw material commodity. Studies addressing this question have not been made and it is a highly interesting topic from the applied point of view. Thus, the present work aims to assess the influence of the phenological stage on the antioxidant activity of *Th. zygis*.

Plant material (30 individuals/population) of wild origin from five populations (Table 1) was collected in Badajoz (SW Spain), first in the flowering stage and then in the fruiting stage. It was dried in an airy room in darkness, and conserved for two months in paper bags. Two extractions of the essential oil were carried out for each sample (hydrodistillation; European Pharmacopoeia, Council of Europe, 1996); one with the whole plant (WP) and another with flowers and leaves (FL). Essential oil yield and its percentage composition were calculated.

Analyticals gas chromatography, gas chromatography-mass spectrometry and qualitative analyses were carried out following Blanco *et al.* (2010)'s method. Antioxidant activity was measured using the 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)/horseradish peroxidase decoloration method (Arnao *et al*., 2000; Cano *et al*., 2000). Results were statisticaly analyzed (IBM SPSS Statistics 19).

We found that the yields of the populations studied oscillated between 0.5 and 1.73% for the whole plant (WP) and between 0.96 and 3.42% for the flowers and leaves (FL) (Table 2). The high yields of this plant have made it one of the most comercial Spanish thymes (Jordán *et al*., 2009). Our results may suggest that it is much more advantageous the extraction in flowers and leaves than in the whole plant, because yields are significantly higher in the flowering (WP = 0.9-1.73%; FL = 1.43-3.42%) than fruiting (WP = 0.35-0.93%; FL = 0.96-1.92) [Wilcoxon test, (WP), n = 4, *, $p = 0.043$; (FL), n = 4, *, $p = 0.043$].

Table 1. Provenance of the study material, indicating locality (all in Badajoz province), altitude, UTM coordinates, collection date, voucher specimens in the HSS Herbarium (*Herbario del Suroeste de España*, Badajoz, Spain), and population taxon (subspecies)

Population	Locality	Altitude (m a.s.l.)	UTM coordinates	Collection date: Flowering /Fruiting	Herbarium	Subspecies
P1	Badajoz	219	29SPD70	June/August	HSS 10847	*gracilis*
P2	La Albuera	256	29SPC88	June/August	HSS 8939	*sylvestris*
P3	Guadajira	211	29SPD90	May/August	HSS 8871	*sylvestris*
P4	Los Santos de Maimona	636	29SQC25	June/August	HSS 9092	*sylvestris*
P5	Solana de los Barros	239	29SQC18	April/August	HSS 9209	*sylvestris*

Table 2. Percentage composition (whole plant, WP) and yield (WP and flowers and leaves, FL) of the essential oil of five *Thymus zygis* populations (P)

	RI[2]	RTM[3]	Flowering stage[4]					Fruiting stage[4]					Significance level[5] (n = 10)
			P1	P2	P3	P4	P5	P1	P2	P3	P4	P5	
Compounds[1]													
1	926	4.93	0.4	0.5	t	0.1	0.4	0.2	0.2	0.2	0.3	0.1	ns, 0.523
2	933	5.07	0.36	1.07	0.87	1.09	0.86	0.39	0.93	0.96	0.93	1.08	ns, 0.827
3	949	5.32	1.34	2.27	0.87	2.5	2.02	1.13	1.98	2.6	2.35	2.48	ns, 0.344
4	976	5.59	0.29	t	t	0.97	t	t	t	t	t	0.32	ns, 0.439
5	981	5.70	t	t	t	0.1	t		0.1	t	t		ns, 1.000
6	985	5.76	0.46	3.0	0.37	1.95	2.98	1.0	3.24	6.2	4.4	3.95	ns, 0.470
7	991	5.86	2.93	2.4	2.0	4.21	2.36	1.21	1.89	2.19	2.19	2.36	ns, 0.071
8	1,005	6.18	0.1	t	0.3	0.2	0.1	0.4	0.1	0.1	t	t	ns, 0.585
9	1,018	6.41	2.1	0.8	0.1	1.8	0.5	0.5	t	1.1	0.3	t	ns, 0.141
10	1,021	6.53	22.8	33.6	31.1	16.2	20.2	30.3	43.2	37.3	40.1	37.81	*, 0.028
11	1,025	6.62	2.09	1.36	0.59	1.06	1.58	0.78	t	t	t	0.54	*, 0.015
12	1,025	6.69	0.6	t	0.2	0.4	0.2	0.2	0.1	0.1	t	0.1	ns, 0.133
13	1,025	6.69	0.5	0.3	0.2	0.3	t	0.2	0.1	0.2	0.4	0.3	ns, 0.748
14	1,038	6.91	t	1.55	5.67	21.1	1.23	t	4.86	6.6	5.78	4.63	ns, 0.675
15	1,060	7.17	10.4	12.8	5.78	9.6	13.5	4.31	4.67	1.96	2.82	3.86	**, 0.009
16	1,066	7.35	0.2	t	0.4	0.1	t	0.1	0.1	0.2	0.3	0.1	ns, 0.588
17	1,091	7.77	0.2	0.5	t	t	0.2	0.4	0.1	0.3	0.1	0.1	ns, 0.750
18	1,102	7.93	1.83	4.7	9.62	10.1	7.09	1.44	5.34	11.18	12.2	4.14	ns, 0.917
19	1,148	9.01	t			0.1	t	0.1	t				ns, 1.000
20	1,172	9.44	0.3	1.34	1.77	1.02	1.56	1.1	2.35	4.27	5.4	1.74	ns, 0.760
21	1,216	9.68	1.02	2.95	5.3	2.25	2.3	0.7	4.26	7.54	t	5.48	ns, 0.753
22	1,221	10.03	0.2	0.71	t	0.76	t	0.1	0.78	0.78	0.76	0.79	ns, 0.094
23	1,228	10.61	0.1	t	1.12	0.6		0.2	t	1.0	1.34	0.56	ns, 0.396
24	1,255	11.12	0.1		t		0.1	t					ns, 0.134
25	1,290	12.01	46.45	28.4	32.1	22.2	40.04	54.24	23.6	13.9	18.1	27.23	ns, 0.251
26	1,298	12.23	3.8	1.71	1.6	1.19	1.92	0.97	1.86	1.21	1.91	2.28	ns, 0.832
27	1,416	15.00	0.92	t	t	0.1	0.83	t	t	t	t	t	ns, 0.054
28	1,505	16.68	0.1				t		t				ns, 0.317
29	1,578	18.32	0.4		t	t	t	t	0.2	0.1	0.3		ns, 0.410
Oil yield (v/w)													
WP			1.73	1.52	0.9	1.09	1.26	0.73	0.66	0.5	0.35	0.93	
FL			3.42	2.19	1.43	2.25	2.27	1.84	1.33	0.96	1.31	1.92	

[1] Compounds: 1) α-thujene; 2) α-pinene; 3) camphene; 4) sabinene; 5) 1-octen-3-ol; 6) β-pinene; 7) myrcene; 8) α-phellandrene; 9) α-terpinene; 10) *p*-cymene; 11) limonene; 12) β-phellandrene; 13) 1,8-cineole; 14) (*Z*)-β-ocimene; 15) γ-terpinene; 16) *cis*-sabinene hydrate; 17) terpinolene; 18) linalool; 19) camphor; 20) borneol; 21) terpinen-4-ol; 22) α-terpineol; 23) citronellol; 24) geraniol; 25) thymol; 26) carvacrol; 27) (*E*)- caryophyllene; 28) β-bisabolene; 29) caryophyllene oxide. [2] RI = retention index according to n-paraffins on DB-1 column. [3] RTM = Retention time in GLC-MS (min). [4] t = trace (< 0.1%); [5] Kruskal-Wallis test significance levels for the comparison of the essential oil compounds between flowering and fruiting stage. ns: not significant ($p > 0.05$). *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$.

Even though, for an industrial extraction process, it seems more logical to use the whole plant, because the separation of flowers and leaves consumes many hours of factory work.

Lipophilic antioxidant activity of essential oil (WP) of *Th. zygis*, showed the highest of the individual values in the range 16.12-30.52 mmol Trolox L^{-1} (TEAC, Trolox equivalent antioxidant activity) in the flowering stage and 10.96-40.24 mmol Trolox L^{-1} in the fruiting stage (Fig. 1). They can be considered in the range of oils which have a great antioxidant activity, such as those from black pepper (*Piper nigrum* L.: 25.36 mmol Trolox L^{-1}). They are lower than the ones from clove (*Syzygium aromaticum* L.: 69.05 mmol Trolox L^{-1}), oregano (*Origanum vulgare* subsp. *hirtum* (Link) Letsw.: 61.55 mmol Trolox L^{-1}) or Spanish thyme

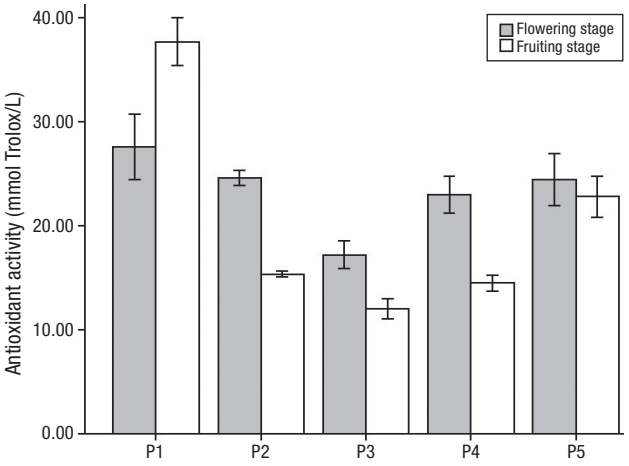

Figure 1. Antioxidant activity of the essential oil of the studied populations (whole plant) in flowering and fruiting stages. Average of four replicates per population. Flowering stage: P1-P5 (respectively) = 27.56, 24.6, 17.21, 22.97, and 24.4 mmol Trolox L^{-1}. Fruiting stage: 37.69, 15.35, 11.98, 14.49, and 22.81 mmol Trolox L^{-1}.

(*Thymbra capitata* (L.) Cav.: 45.59–52.06 mmol Trolox L^{-1}) (Dorman *et al*., 2000b; Sacchetti *et al*., 2005; Blanco *et al*., 2010). But food products commercially known as with high activity (Arnao *et al*., 2000; Pellegrini *et al*., 2003), for example coffee (26.96-36.54 mmol Trolox L^{-1}), wine (0.38-12.14 mmol Trolox L^{-1}), tea (black Tea 3.60 mmol Trolox L^{-1}; green tea 6.01 mmol Trolox L^{-1}), or different fruit juices (Orange juice 3.02 mmol Trolox L^{-1}; pear juice 2.56 mmol Trolox L^{-1}; apple juice 1.83 mmol Trolox L^{-1}; lemon juice 2.21 mmol Trolox L^{-1}), have similar or lower values than those obtained for *Th. zygis*. Other essential oils, also considered as chemicals with high antioxidant activity, have lower values as well: geranium (*Pelargonium graveolens* L'Herit: 5.84 mmol Trolox L^{-1}), nutmeg (*Myristica fragans* Houtt.: 5.00 mmol Trolox L^{-1}), ginger (*Zingiber officinale* Roscoe 0.94 mmol Trolox L^{-1}), pine (*Pinus radiata* D. Don 0.85 mmol Trolox L^{-1}), cypress (*Cupressus sempervirens* L. 0.79 mmol Trolox L^{-1}), eucalyptus (*Eucalyptus globulus* Labill. 0.50 mmol Trolox L^{-1}) or lemon balm (*Melissa officinalis* L.: 0.28 mmol Trolox L^{-1}) (Dorman *et al*., 2000b; Sacchetti *et al*., 2005).

No significant differences were found in antioxidant activity between the stages of flowering and fruiting (Wilcoxon test, n = 4. P1 = ns, 0.068; P2 = ns, 0.068; P3 = ns, 0.068; P4 = ns, 0.068; P5 = ns, 0.102). Then the quality of the product for industrial purposes, will not be influenced by phenology. However, as significant differences do exist between the yields of one and another collection season, it is advisable to collect in bloom when yields are higher.

P1 population [subspecies *gracilis* (Boiss.) R. Morales] obtained a higher antioxidant activity than P2-P5 populations [subspecies *sylvestris* (Hoffmanns. & Link) Brot. ex Coutinho] with differences statistically significant in both, flowering and fruting season (Kruskal-Wallis test: flowering stage, n = 20, *, $p = 0.023$; fruiting stage, n = 20, *, $p = 0.002$) (Fig. 1).

Table 2 summarizes chemical composition of essential oils studied. Chemical composition of thyme essential oil may vary throughout the growing season (Blanco *et al*., 2010). Our results offered only significant differences between the flowering and fruiting season in three of the components identified (Kruskal-Wallis test, *p*-cymene: n = 10, *, $p = 0.028$; limonene, n = 10, *, $p = 0.015$; γ-terpinene: n = 10, **, $p = 0.009$: see Table 2), without impact on the final antioxidant activity, although two of them did not represent residual amounts in the total mixture. It is evident that the total antioxidant activity is due to the sum of the activities of each component, many of them with demonstrated antioxidant capacity have been tested individually by Dorman *et al.* (2000a).

Finally, it is also noteworthy that this essential oil has a high proportion of thymol. This fact gives a remarkable quality of the product, given the particular organoleptic characteristics of this phenolic derivative, top rated by industry (Sotomayor, 1998), and much more competitive from a business perspective than others as carvacrol, present in related species but with unpleasant smelling, irritating properties, and a clearly lower value.

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