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Comparative chemistry and biological properties of the solid residues from hydrodistillation of Spanish populations of *Rosmarinus officinalis* L.

R. Sánchez-Vioque[a,b,✉], M.E. Izquierdo-Melero[a], M. Polissiou[c], K. Astraka[c], Petros A. Tarantilis[c], D. Herraiz-Peñalver[a], M. Martín-Bejerano[a] and O. Santana-Méridas[a,b]

[a]Centro Agrario de Albaladejito, Junta de Comunidades de Castilla-La Mancha, Ctra. Toledo-Cuenca Km 174, 16194 Cuenca, Spain
[b]Instituto de Recursos Humanos para la Ciencia y la Tecnología (INCRECYT), Fundación Parque Científico y Tecnológico de Albacete, Paseo de la Innovación 1, 02006 Albacete, Spain
[c]Agricultural University of Athens, Department of Food Science & Human Nutrition, Laboratory of Chemistry, Iera Odos Street 75, 11855 Athens, Greece

✉Corresponding author: rsanchezv@jccm.es

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SUMMARY: Solid residues from the hydrodistillation of selected Spanish populations of rosemary (*Rosmarinus officinalis* L.) have been analyzed for their polyphenol composition, and antioxidant and bioplaguicide activities. The objective was to evaluate and select the most suitable plant materials as sources of natural antioxidants and crop protectants. Total polyphenol content and polyphenol composition of rosemary populations were very dependent on the growth location: populations from Aranjuez showed a higher content of total polyphenols and were richer in rosmarinic acid as compared with their equivalent populations from Cuenca, whereas these latter were characterized by an overall higher content in genkwanin and carnosol. Most of the antioxidant activities were highly correlated with the total content of polyphenols although some polyphenols like carnosic acid and carnosol seemed to favor such activities. The extracts from *R. officinalis* were strong antifeedants against *Leptinotarsa decemlineata* Say and moderate against *Spodoptera littoralis* Boisd and *Myzus persicae* Sulzer, according to their feeding ecologies. The biological effects of the active samples cannot be accounted by their chemical composition, suggesting additive or synergistic effects. Both the phytotoxic and stimulating effects on *Lactuca sativa* L., and *Lolium perenne* L. leaf and/or root growth were observed.

KEYWORDS: *Antifeedants; Antioxidants; Health-promoting compounds; Natural crop protectants; Phytotoxic activity; Residue valorization*

RESUMEN: ***Composición química y propiedades biológicas de los residuos sólidos de la hidrodestilación de poblaciones españolas de*** **Rosmarinus officinalis *L***. Se ha analizado la composición en polifenoles y las actividades antioxidante y bioplaguicida de los residuos sólidos procedentes de la hidrodestilación de poblaciones seleccionadas de romero (*Rosmarinus officinalis* L.). El objetivo fue evaluar y seleccionar los materiales vegetales más adecuados como fuente de antioxidantes y bioplaguicidas naturales. El contenido total y la composición en polifenoles de las poblaciones de romero dependieron mucho de la localidad de cultivo: las poblaciones de Aranjuez mostraron un mayor contenido en polifenoles y fueron más ricas en ácido rosmarínico en comparación con sus equivalente poblaciones de Cuenca, mientras que éstas últimas en general se caracterizaron por un mayor contenido de genkwanina y carnosol. La mayoría de las actividades antioxidantes estuvieron altamente correlacionadas con el contenido total en polifenoles aunque algunos polifenoles como el ácido carnósico y el carnosol parecen favorecer dichas actividades. Los extractos de *R. officinalis* mostraron una fuerte actividad fagorepelente frente a *Leptinotarsa decemlineata* Say y moderada sobre *Spodoptera littoralis* Boisd.

y *Myzus persicae* Sulzer, de acuerdo a sus adaptaciones tróficas. Los efectos sobre el comportamiento alimentario observados no se justifican por la composición química del extracto, lo que sugiere la existencia de efectos aditivos y/o sinérgicos. Se observaron efectos tanto fitotóxicos como estimulantes sobre la elongación de la raíz y del tallo de *Lactuca sativa* L. y *Lolium perenne* L.

PALABRAS CLAVE: *Actividad fitotóxica; Antioxidantes; Bioplaguicidas naturales; Compuestos funcionales; Fagorepelentes; Valorización de residuos*

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1. INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is an aromatic perennial shrub (Lamiaceae) which is native to the Mediterranean area and very appreciated in toiletries, cosmetics and medicine, as a flavoring in foods, or as an ornamental plant. Rosemary is one of the most important aromatic plants in terms of the commercialization of essential oil, which in addition to its olfactory characteristics has numerous biological activities including antioxidant and antimicrobial properties of especial significance in human and animal health and in food preservation (Burt, 2004; Miguel, 2010).

Essential oil is obtained by distillation of the aerial part of the plant with water and/or steam. Nevertheless, the yield of distillation is only between 0.8 and 2.5 grams of essential oil per 100 grams of dry plant, which results in a world production of solid residue (10–20×10^3 Tm/year) that may result in environmental concerns if it is not properly managed (Angioni *et al*., 2004). Moreover, solid residue from distillation is rich in non-volatile bioactive compounds like polyphenols that can be exploited as natural antioxidants in food and feed or anti-aging ingredients in cosmetics, as health-promoting compounds, or as natural crop protectants in organic agriculture, and thus provide an additional profit to the crop in a sustainable way (Navarrete *et al*., 2011; Santana-Méridas *et al*., 2012; Sánchez-Vioque *et al*., 2013; Santana-Méridas *et al*., 2014).

The polyphenols in rosemary include rosmarinic acid, carnosic acid, carnosol, rosmanol, epirosmanol, rosmadial, genkwanin, cirsimaritin, hinokione and scutellarein, among others, which are endowed of antioxidant and bioplaguicide activities (Borrás-Linares *et al*., 2011; Navarrete *et al*., 2011; Santana-Méridas *et al*., 2014). However, the relative content of each polyphenol in aromatic plants can greatly differ as a result of genetic and environmental factors affecting the plant, and these differences in turn affect the biological activity of the residue (Cuvelier *et al*., 1996). The use of cultivated plants minimizes in part the variations in the chemical composition due to more controlled growing conditions in comparison with wild ones. This guarantees a more consistent quality and supply of the plant material, thus increasing interest in the industry and in farmers toward the use of cultivated aromatic species (Lubbe and Verpoorte, 2011).

In this work, we have tentatively identified the major phenolic compounds present in the solid residue after the hydrodistillation of cultivated populations of rosemary and evaluated its antioxidant and bioplaguicide activities. These populations have been previously selected from a survey throughout the natural distribution area of the species in Spain according to the yield and the variability in the chemical composition of their essential oils. Subsequently, the populations were cultivated in two locations. The objective was to evaluate the differences among populations and locations and select the plant materials most suitable as sources of natural antioxidants and crop protectants.

2. MATERIALS AND METHODS

2.1. Reagents and standards

Gallic acid, catechin, 2,6-Di-tert-butyl-4-methylphenol (BHT), Folin-Ciocalteu's phenol reagent, linoleic acid, potassium ferricyanide (III), iron (III) chloride, iron (II) chloride tetrahydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (ferrozine), pyrocatechol violet (PV) and sodium nitrite were purchased from Fluka-Sigma-Aldrich® (St. Louis, MO, USA). Aluminium chloride anhydrous and 2,4-dinitrophenylhydracine were obtained from Panreac (Barcelona, Spain). The phenolic compound standards caffeic acid, genkwanin and apigenin were from Extrasynthese® (Genay, France), and rosmarinic acid, carnosol and carnosic acid from Fluka-Sigma-Aldrich® (St. Louis, MO, USA). The Juglone standard was from

Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Plant material and solid residue extraction

An extensive prospection of *Rosmarinus officinalis* L. wild populations was carried out in Spain in the years 2007, 2008 and 2009. The plant material was collected at the full-flowering time, hydrodistilled and chemically analyzed. Ten of these populations were selected on the basis of the yield and chemical variability of their essential oil. Table 1 provides a description of the collecting places based on geographical parameters. Individual plants from each selected population were vegetatively propagated and rooted under greenhouse conditions in 2010. The cultivation plots were finally settled during March and April 2011 in two different locations: Centro Agrario de Albaladejito (Cuenca, Spain) and Finca Casa Concha (Aranjuez, Spain). Seventy-five individual plants from selected populations were planted in three furrows (25 plants each) with 1 m between plants and amongst rows.

The aerial parts (inflorescences, leaves and stems) of *R. officinalis* were randomly collected from the experimental fields at the flowering period and dried at room temperature for 5 days prior to the hydrodistillation. Around 100 g of dry sample were hydrodistilled for 4 hours using a Clevenger type apparatus according to Boland *et al.* (1991). The solid residue from hydrodistillation was recovered and dried at room temperature.

Approximately 50 g of dried solid residue were thoroughly extracted with ethanol in a *Soxhlet* apparatus for 48 h. The crude extracts were subsequently filtered (Whatman grade 1) under vacuum and evaporated in a rotary evaporator to obtain the dried extracts that were kept in sealed tubes at 4 °C until analysis.

2.3. Total polyphenol and total flavonoid contents

The content of total polyphenols was determined by the Folin-Ciocalteu method as described in Slinkard and Singleton (1977). Gallic acid was used as standard and the total polyphenol content was expressed as gram equivalents of gallic acid per 100 grams of dry extract. The total flavonoid content was determined using catechin as standard according to Zhishen *et al.* (1999) and expressed as gram equivalents of catechin per 100 grams of dry extract.

2.4. LC-MS analysis of the ethanol extract of *R. officinalis*

Solid residue extracts were analyzed using the LC-MS (ESI) technique in a Shimadzu LC/MS-2010A equipped with an LC-10ADvp binary pump, a DGU-14A degasser, a SIL-10ADvp auto sampler, and a SPD-M10Avp Photo Diode Array Detector. Dry extracts solubilized in methanol (10 mg/mL) were injected into a 250 mm ×4.6 mm, 5 µm particle size Discovery HS-C18 column (Supelco, Bellefonte, PA, USA) at a flow rate of 0.4 mL·min^{-1}. The general experimental procedures and conditions as described in Santana-Méridas *et al.* (2014) were followed.

2.5. DPPH radical-scavenging activity

The scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined according to Braca *et al.* (2001). The method is based on the reduction of the stable free radical DPPH in the presence of a hydrogen-donating antioxidant, and the formation of the non-radical form DPPH-H as result of the reaction. This reduction can be monitored at 517 nm by measuring the bleaching of DPPH (violet) to DPPH-H (yellow). Distilled water and BHT were used as negative

TABLE 1. Original collecting locations of *R. officinalis* populations in the Iberian Peninsula

PN	Locality	Province	Latitude	Longitude	Altitude (m)[a]
P1	Almorox	Toledo	40°17′29″N	004°21′54″W	715
P2	Alcaudete de la Jara	Toledo	39°49′57″N	004°52′09″W	477
P3	Lorca	Murcia	37°52′22″N	001°53′20″W	833
P4	Moratalla	Murcia	38°08′41″N	002°13′43″W	1161
P5	Lliria	Valencia	39°45′21″N	000°41′04″W	502
P6	Alcocer	Guadalajara	40°28′09″N	002°32′52″W	710
P7	Huete	Cuenca	40°08′29″N	002°41′58″W	847
P8	Pina de Ebro	Zaragoza	41°35′14″N	000°20′29″W	407
P9	Pontils	Tarragona	41°28′59″N	001°27′05″E	704
P10	Flix	Tarragona	41°16′15″N	000°35′14″E	149

PN, Population number.
[a]Meters above sea level.

control and as standard antioxidant of the assay, respectively. The inhibition activity (%) of DPPH radicals was calculated as $[(A_0–A_1)/A_0] \times 100$, where A_0 was the absorbance of the negative control, and A_1 was the absorbance of the extracts. The concentration of sample required to reduce 50% of DPPH radicals (IC_{50}) was calculated from a linear regression analysis.

2.6. Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power was determined according to the method of Oyaizu (1986) with modifications. This method determines the ability of a sample to reduce Fe^{3+} to Fe^{2+}, which is recorded by measuring the formation of Perl's Prussian blue at 700 nm. BHT was used as the standard antioxidant of the assay. The concentration of sample required to obtain 0.5 absorbance units (A.U.) was calculated from linear regression analysis.

2.7. Metal chelating activity

The Fe^{2+}-chelating activity was determined according to Carter (1971) with modifications. The method is based on the chelation of this metal ion with ferrozine to yield a red colored (562 nm) complex. The Cu^{2+}-chelating activity was determined according to Saiga *et al*. (2003) based on the formation of the blue colored (632 nm) complex between Cu^{2+} and pyrocatechol violet (PV). In the presence of other chelating agents, the complex formation is disrupted and the color of the complexes decreases. The measurement of the rate of color reduction therefore allows for the estimation of the chelating activity. Distilled water and gallic acid were used as the negative control and as the standard metal chelator of the assay, respectively. The percentage of inhibition of complex formation was calculated as $[(A_0–A_1)/A_0] \times 100$, where A_0 was the absorbance of the negative control, and A_1 was the absorbance of the extracts.

2.8. Oxidation of linoleic acid

Extracts of *R. officinalis* were incubated with linoleic acid in order to evaluate their capacity to inhibit the oxidation of this unsaturated fatty acid. One milligram of dry extract was dissolved in 6 mL of distilled water (167 µg dry extract·mL^{-1}) in screwed tubes, thoroughly mixed with five microliters of linoleic acid, and incubated at 60 °C. Oxidation of linoleic acid was monitored at 24, 48 and 72 h of incubation by measuring the formation of oxidation products in the medium, including conjugated dienes, lipid hydroperoxides, and aldehydes and ketones (carbonyl groups). Distilled water and BHT were used as the negative control and as the antioxidant standard of the assay, respectively.

The formation of conjugated dienes was determined by measuring their absorbance at 233 nm and expressed as the increase in the initial absorbance. The determination of lipid hydroperoxides was based on their capacity of oxidation of Fe^{2+} to Fe^{3+}, and the subsequent analysis of Fe^{2+} by ferrozine (Carter, 1971) as described in section 2.7. The results were expressed as percentage of initial absorbance at 562 nm, and calculated as $(A_t/A_i) \times 100$, where A_i is the absorbance before incubation and A_t is the absorbance at 24, 48 or 72 h of incubation. Aldehydes and ketones were determined by the reaction of their carbonyl groups with 2,4-dinitrophenylhydracine (460 nm), and the results were expressed as the increase in the initial absorbance (Henick *et al*., 1954).

2.9. Insect bioassays

Spodoptera littoralis Boisd. (Lepidoptera: Noctuidae), *Leptinotarsa decemlineata* Say (Coleptera:Chrysomelidae) and the aphid *Myzus persicae* Sulzer (Hemiptera:Aphididae) were reared on an artificial diet (Poitut and Bues, 1970), of potato foliage (*Solanum tuberosum*) and bell pepper (*Capsicum annum*) plants, respectively; and maintained at 22±1 °C, >70% relative humidity, with a photoperiod of 16:8 h (L:D) in a growth chamber. The bioassays were conducted with newly emerged *S. littoralis* L6 larvae or *L. decemlineata*/*M. persicae* adults. Feeding or settling inhibition (%FI or %SI) were calculated as $FI = [1-(T/C)] \times 100$, where T and C are the consumption of treated and control leaf disks, respectively, or as $\%SI = [1–10\ (\%T/\%C)] \times 100$ where %C and %T are percent aphids settled on the control and treated leaf disks, respectively, as described in Burgueño-Tapia *et al.* (2008).

2.10. Phytotoxic activity

The experiments were conducted with *Lactuca sativa* cv Teresa (Fito, España) and *Lolium perenne* (Batlle) seeds, as described (Moitero *et al*., 2006). Briefly, a 2.5 cm diameter filter paper with 20 µL of the test compound (10 µg·µL^{-1} for extracts and 5 µg·µL^{-1} for pure compounds) were placed on 12-well plates (Falcon). Five hundred µL H_2O/well and 10/5 seeds (*L. sativa*/*L. perenne* presoaked in distilled water for 12 h) were added and the covered plates were placed in a plant growth chamber (25 °C, 70% RH, 16:8 L: D). Germination was monitored for 6 days and the root/leaf length was measured at the end of the experiment (25 plantlets randomly selected for each experiment and digitalized with the application ImageJ 1.43, http://imagej.nih.gov/ij/). Juglone (JU) (5 µg·µL^{-1}) was included as a positive control.

2.11. Statistical analysis

The antifeedant effects (FI/SI) were analyzed for significance by the non-parametric Wilcoxon signed-rank test. Extracts (100 μg·cm^{-2}) and pure compounds (50 μg·cm^{-2}) with an FI or SI >70% were tested in a dose–response experiment to calculate their relative potency (EC_{50}, the effective dose to give a 50% feeding reduction) which was determined from the linear regression analysis (%FI/SI on log dose). A Kruskall–Wallis test and an analysis of variance (ANOVA) were performed on germination and radicle/leaf length data, respectively. In all cases, the significance level was set at $p<0.05$. The relative concentration data (% values) was subjected to a principal component analysis. Relative concentration data, antioxidant and antifeedant effects were subjected to the Pearson correlation. All statistical analyses were carried out using the IBM® SPSS® Statistics ver. 22 (IBM corp.©, 2013) package.

3. RESULTS AND DISCUSSION

3.1. Solid residue extraction and chemical composition

The extraction of the solid residues of *R. officinalis* by a *Soxhlet* system yielded a high recovery of ethanol soluble compounds that ranged from 20.4 to 26.2 g extract·100 g^{-1} dry matter.

Tables 2 and 3 show the chemical profile and the composition data (area percent) of the residues extracted from ten *R. officinalis* populations cultivated in Cuenca (Castilla-La Mancha, Spain) and Aranjuez (Madrid, Spain). The chemical composition of the rosemary solid residue was similar to that described for other *R. officinalis*

TABLE 2. Major polyphenols detected and tentatively identified by LC-DAD-MS in solid residues from different *R. officinalis* populations

Peak	t_R (min)[a]	UV λ_{max} (nm)	[M-H]$^-$ (*m/z*)	Tentative identification	Reference
1	29.67–29.74	231, 274, 342	477	n.i.	–
2	30.76–30.86	330	359	Rosmarinic acid[b]	Standards
3	32.08–32.33	239, 270, 333	461	n.i.	–
4	34.08–34.13	270, 340	461, 299	n.i.	–
5	37.50–37.66	254, 350	285	Luteolin[b]	Standards
6	39.24–39.44	230, 284	345, 301	Rosmanol/Rosmanol isomer	Kontogianni *et al*. (2013), Zhang *et al.* (2012)
7	40.47–40.65	267, 337	269	Apigenin[b]	Standards
8	40.68–40.86	230, 288	345	Rosmanol/Epirosmanol	Zhang *et al*. (2012)
9	42.71–42.88	274, 334	313	Cirsimaritin	Herrero *et al*. (2010), Zhang *et al*. (2012)
10	43.22–43.39	270	375	n.i.	–
11	43.46–43.70	284	375	n.i.	–
12	45.56–45.74	239, 289	345	Rosmanol/Epirosmanol	Kontogianni *et al*. (2013), Zhang *et al.* (2012)
13	47.81–48.01	267, 339	283	Genkwanin[b]	Standards
14	48.37–48.45	235, 289	359, 329	Methoxycarnosol	Kontogianni *et al*. (2013)
15	48.51–48.70	243, 304	343	n.i.	–
16	49.84–50.18	233, 285	329, 285	Carnosol[b]	Standards
17	50.41–50.66	231, 289	373, 329	Epirosmanol ethyl ether/ Rosmarinic acid methyl ether	Herrero *et al.* (2010), Zhang *et al.* (2012)
18	51.37–51.55	238, 286	343, 315	Rosmadial	Kontogianni *et al*. (2013), Zhang *et al*. (2012)
19	52.58–53.00	268, 328	359	n.i.	–
20	53.46–53.69	278	315	Rosmaridiphenol	Kontogianni *et al*. (2013), Borras-Linares *et al.* (2011)
21	57.66–58.38	234, 285	331, 685	Carnosic acid[b]	Standards
22	60.51–61.28	234, 282	345	Methyl carnosate	Herrero *et al*. (2010)

[a]Range of retention time (t^R) detected during the injection of samples from the different populations.
[b]Identification confirmed using commercial standards.
n.i., Not identified.

TABLE 3. Comparison of relative percentages (% area) of main compounds detected in solid residues from the *R. officinalis* populations cultivated in Cuenca (C) and Aranjuez (A). Peaks numbers (1–22) as in Table 2

		Peak area (%) of the major compounds detected																					
NP	**L**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
P1	C	3.20	1.01	0.66	0.00	2.79	3.56	1.50	4.62	1.28	0.87	1.65	1.70	6.53	0.97	1.07	17.86	9.93	5.81	2.77	2.13	0.59	1.90
	A	4.04	4.65	1.42	1.42	2.05	1.13	1.22	5.94	2.12	1.28	3.53	1.91	6.39	0.00	4.45	6.26	10.78	3.29	2.58	2.91	0.41	2.92
P2	C	4.40	3.09	1.43	0.00	1.19	2.25	1.05	3.72	1.89	1.33	2.31	1.07	7.71	0.00	3.46	19.51	7.03	3.84	4.16	1.48	0.54	1.74
	A	5.99	4.52	1.58	3.29	3.34	1.26	2.05	4.73	2.09	0.62	3.16	2.13	4.57	0.00	3.76	12.82	5.96	3.34	4.49	1.66	0.48	1.54
P3	C	1.39	2.67	0.33	0.00	1.47	1.67	1.41	2.35	3.81	0.40	2.35	0.90	6.79	0.00	4.92	24.45	3.65	4.68	1.77	1.79	10.35	0.00
	A	1.98	4.56	0.75	2.99	1.21	1.25	1.59	4.44	2.33	4.89	1.45	1.45	10.77	0.00	6.71	14.34	6.83	2.60	2.48	2.07	0.38	0.87
P4	C	1.95	1.85	0.69	0.00	0.91	1.33	0.97	3.63	5.18	4.02	0.64	0.84	14.51	0.00	5.52	16.67	9.07	2.71	1.37	1.37	0.55	2.07
	A	3.42	11.12	1.71	3.35	1.81	1.80	1.85	5.38	2.11	0.75	1.34	2.72	4.69	0.00	4.74	0.25	8.78	6.86	2.43	1.72	0.48	1.62
P5	C	2.09	3.55	0.69	0.00	1.34	1.29	1.02	3.94	2.90	3.12	0.95	1.39	9.57	0.00	8.33	16.26	5.63	3.45	2.07	3.22	0.06	2.06
	A	4.21	7.81	2.20	5.46	2.32	1.01	1.96	2.85	3.62	0.33	1.54	1.21	4.37	0.00	5.13	11.79	3.69	2.99	2.04	1.08	2.67	0.79
P6	C	2.87	0.60	0.19	0.00	0.50	0.71	0.00	4.41	2.10	14.28	0.96	0.66	27.52	0.00	6.10	0.36	8.87	3.32	0.73	3.48	0.22	2.89
	A	3.81	7.43	1.21	2.91	2.19	0.86	1.96	5.69	2.45	0.55	1.48	2.05	4.91	0.00	3.50	8.63	6.63	2.83	4.60	2.00	0.32	1.97
P7	C	2.07	0.72	0.42	0.00	1.17	1.24	0.72	6.68	2.40	9.41	1.12	1.39	19.31	0.00	8.04	0.62	12.31	3.52	1.97	3.61	0.40	2.56
	A	5.05	19.24	1.99	7.58	1.99	1.07	1.43	2.95	1.91	0.12	0.96	1.55	2.98	0.00	1.00	6.82	2.11	1.66	2.57	0.85	0.27	0.61
P8	C	1.16	0.88	0.36	0.00	0.84	1.17	0.00	8.04	4.72	8.66	1.51	1.71	22.94	0.00	6.07	2.81	11.18	4.65	1.24	2.82	0.27	1.51
	A	4.99	8.01	0.61	2.17	0.96	0.81	2.39	0.00	3.56	0.00	2.43	0.26	6.70	0.00	0.02	10.73	0.26	1.56	2.97	0.05	17.00	0.70
P9	C	2.76	2.05	0.67	0.00	0.67	0.55	0.00	6.28	3.37	13.16	0.89	0.75	18.44	0.00	12.75	2.81	6.91	2.00	2.03	2.25	0.41	1.47
	A	5.66	15.21	2.34	3.10	0.71	0.42	2.19	0.00	1.88	0.00	1.69	0.24	4.51	0.00	0.00	8.24	0.21	2.09	4.14	0.32	8.35	0.33
P10	C	2.97	2.09	0.86	0.00	2.29	3.39	0.00	7.35	3.35	0.41	1.34	1.62	8.49	2.18	1.35	18.35	9.54	4.69	2.56	1.29	0.54	2.74
	A	5.05	12.9	2.23	3.66	0.98	0.82	2.21	2.52	2.45	0.19	1.24	1.90	3.53	0.00	0.13	4.46	6.52	2.49	2.66	0.53	0.34	0.86

PN, population number (as in Table 1).
L, place of cultivation (C, Cuenca; A, Aranjuez).

extracts (Herrero *et al*., 2010; Borras-Linares *et al*., 2011; Navarrete *et al*., 2011; Zhang *et al*., 2012; Kontogianni *et al*., 2013; Santana-Méridas *et al*., 2014). Rosmarinic acid (2), genkwanin (13), carnosol (16), epirosmanol ethyl ether/rosmarinic acid methyl ether (17) and carnosic acid (21) were the most abundant compounds tentatively identified and represented between 28% and 48% of the total area of peaks in all populations (Table 3). Significant differences in the area percent of compounds were found among populations and notably between cultivation places (Table 3, Figure 1).

The composition data (area percent) of the 20 extracts of *R. officinalis* populations from Table 3 were submitted to principal component analysis (Figure 2). As a result of the analysis, three main components explaining 80.11% of the original variability of the data pool were obtained. The first component explained 41.44% of the variability and was highly correlated both negatively with compounds 1, 2, 3, 7; and positively with compounds 8, 10, 13, 15, 17 (numbers according to Table 2). The second component was negatively correlated with carnosol (16) and carnosic acid (21); and explained 23.41% of the variability. The third component explained 15.56% of the variability and was correlated with the rest of compounds.

Plots using principal components from first to second as axes (64.85%) did not show groupings of populations according to their original collecting places, although clear trends were observed according the growing area (Figure 2). The first principal component showed the highest score values for P6-P9 cultivated in Cuenca which shared low relative contents of rosmarinic acid and high contents of genkwanin. The values for the second component were higher for populations P4, P7 and P10 from Aranjuez with a relatively high content in rosmarinic acid and low-medium content in carnosol. P3 from Cuenca and P8 and P9 cultivated in Aranjuez ranked the highest in carnosic acid contents and appeared separated from the rest of populations in the plot.

Our results support the hypothesis that the relative amounts of polyphenols depend on the specific environmental conditions in which the plant is developed (Josuttis *et al*., 2013), and could be considered a common adaptation of plants to adverse environmental conditions (Lattanzio *et al*., 2012). This information can be used to establish classifications according to the geographical conditions of the growing regions (Carrillo *et al*., 2014).

3.2. Total polyphenol and flavonoid contents in the extracts

A great variability among populations and locations was observed in the total polyphenol content in the residues of *R. officinalis*, which ranged from 4.4 g·100 g^{-1} of dry extract in P6 from Cuenca to 12.6 g·100 g^{-1} in P8 from Aranjuez (Table 4). These data were consistent with the recovery of polyphenols from other aromatic plants (Sánchez-Vioque *et al*., 2013) although an overall minor content in total polyphenols was observed in the residues from the populations cultivated in Cuenca in comparison with those from Aranjuez. The total content in polyphenols and flavonoids were well correlated (r=0.78, p<0.05) (Table 5) but flavonoids showed a variability even higher than that observed for the content in polyphenols, from 0.6 (P6, Cuenca) to 6.2 g·100 g^{-1} of dry extract (P7, Aranjuez) (Table 4). The content of total flavonoids was superior in populations cultivated in Aranjuez in comparison with those from Cuenca, up to more than 7.5 times higher in the case of P7 and P10.

3.3. DPPH radical-scavenging activity

The large differences in the contents in polyphenols and flavonoids resulted in very different DPPH radical-scavenging activities among populations, whose IC_{50} values ranged from of 21.6 (P8, Aranjuez) to 80.5 μg·mL^{-1} (P6, Cuenca) (Table 4). The IC_{50} of BHT was 4.9 μg·mL^{-1}. The correlation between total polyphenols and DPPH activities was very high (r=−0.85, p<0.05) (Table 5). The agreement between the results of DPPH radical-scavenging activity and total polyphenols was expected, and it is consistent with the fact that both determinations are based on a chemical reduction: either Folin-Ciocalteu's reagent in the case of determination of the total polyphenols or DPPH radicals. Unlike total polyphenols, the method used for the determination of total flavonoids is not based on a reduction but on a complexation of Al (III) by flavonoids. Consequently, DPPH activities were less correlated with flavonoids (r=−0.53, p<0.05) than with total polyphenols (Table 5). Obviously, the DPPH activities depend on the total content of flavonoids but also on the flavonoid composition of the extract since each individual flavonoid has a specific antioxidant activity against DPPH radicals. For example, the extracts of P3 from Cuenca and of P6 from Aranjuez had similar flavonoid contents, 1.5 and 1.7 g·100 g^{-1} dry extract, respectively, but P3 (IC_{50}=24.5 μg·mL^{-1}) was a much better reducer of DPPH radicals than P6 (IC_{50}=56.3 μg·mL^{-1}) (Table 4). Similarly, the extracts of P1 and P7 from Cuenca (both with 0.8 g flavonoids/100 g dry extract) showed DPPH scavenging activities (IC_{50}) of 37.0 and 56.6 μg·mL^{-1}, respectively. The polyphenol compositions of these populations showed large differences: P1 and P3 from Cuenca were mainly characterized by a high content of carnosol, 17.9 and 24.4% of the total peak area,

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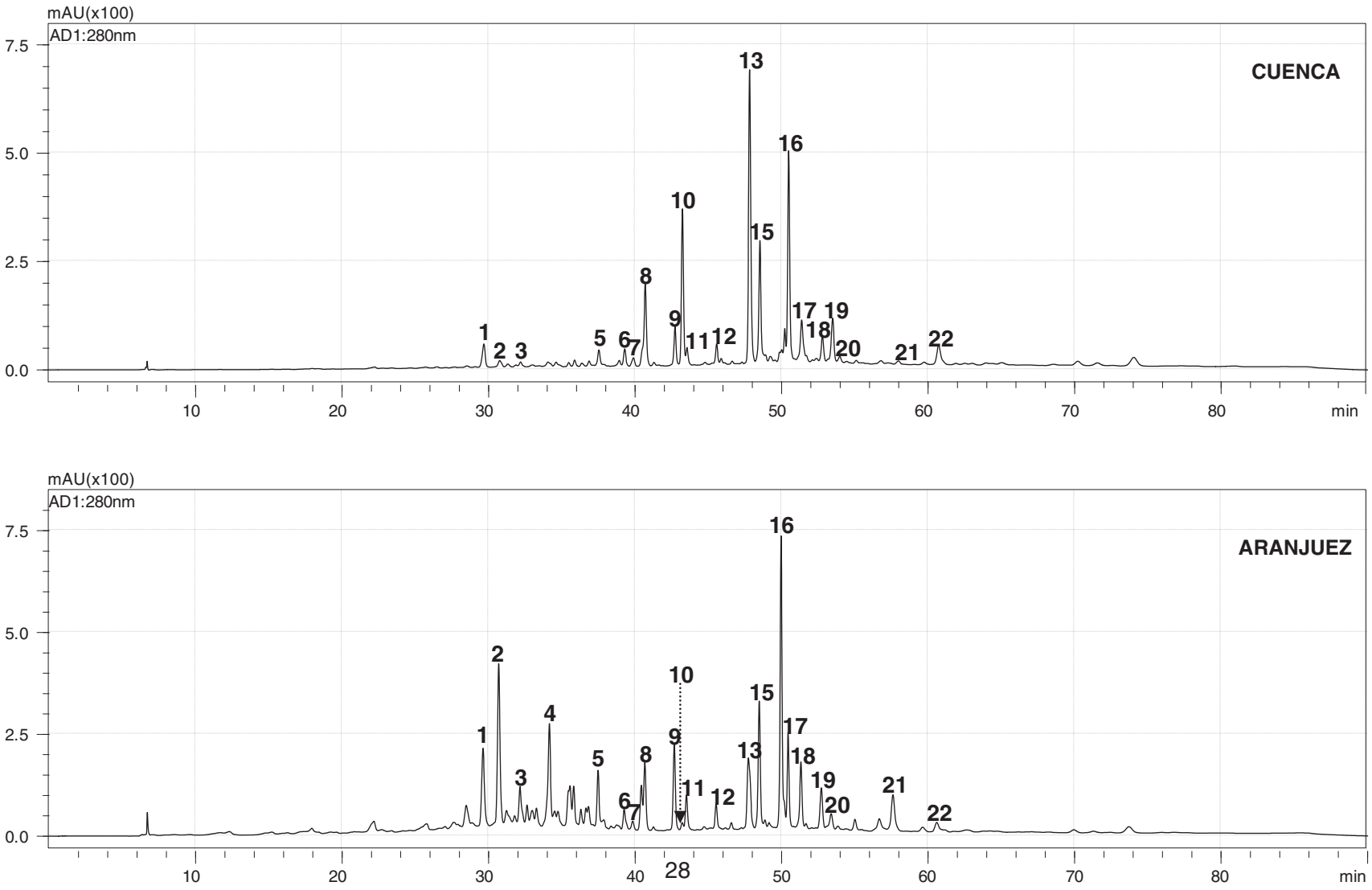

FIGURE 1. Comparative LC-DAD-MS chromatograms from solid residue of *Rosmarinus officinalis* from Zaragoza (P8) cultivated in two different locations (Cuenca and Aranjuez). Peak numbers as in Table 2.

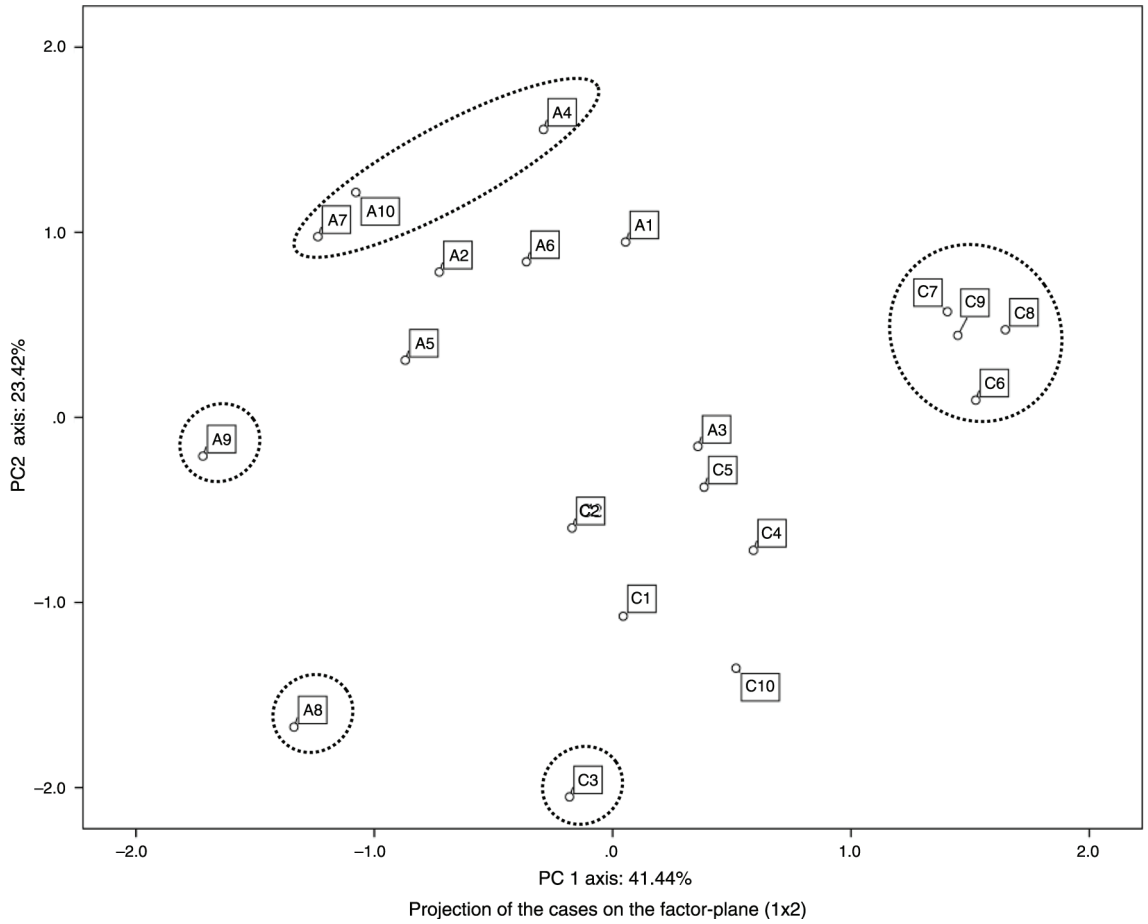

FIGURE 2. Principal component plot for *R. officinalis* populations grown in Cuenca (C) and Aranjuez (A).

respectively, whereas the content was much lower in the P6 from Aranjuez (8.6%) and marginal in the P7 from Cuenca (0.6%) (Table 3). Moreover, the compounds other than flavonoids endowed with reducing capacity may be present in the extracts and increase the DPPH activity.

3.4. Ferric reducing antioxidant power (FRAP)

The data of the reduction in Fe (III) by *R. officinalis* extracts were comparable to those of DPPH activities, showing a very high correlation with total polyphenols (r=−0.86, p<0.05), and a moderate correlation with total flavonoids (r=−0.70, p<0.05) (Table 5). The concentration of extract to yield 0.5 A.U. ranged from 21.0 (P8, Aranjuez) to 81.3 μg·mL^{-1} (P6, Cuenca) (Table 4). The standard BHT attained such absorbance at 8.0 μg·mL^{-1} of concentration. As in the previous antioxidant test, the FRAP of extracts was very dependent on the location and on the population; those cultivated in Aranjuez were more antioxidant than their equivalent populations from Cuenca.

3.5. Metal chelating activity

The Fe^{2+}-chelating activities (IC_{50}) of *R. officinalis* extracts also depended on the population and location, and ranged from 238 (P7, Aranjuez) to 410 μg·mL^{-1} (P9, Aranjuez) (Table 4). In terms of comparison, the IC_{50} of the standard gallic acid was 52.6 μg·mL^{-1}. Nevertheless, the Fe^{2+}-chelating activities of *R. officinalis* extracts were not significantly correlated with the total contents of either polyphenols or flavonoids (Table 5). A similar low correlation has been observed in several aromatic plant residues and attributed to the fact that only polyphenols having neighboring hydroxyl and/or keto groups in their chemical structures are able to chelate divalent metals, as well as to the possible presence of other compounds in the extracts that could act as chelators (Sánchez-Vioque *et al*., 2013; Santana-Méridas *et al*., 2014). None of the polyphenols showed a significant and negative correlation with Fe^{2+}-chelating activities (IC_{50}), and only carnosol showed a certain significant but positive correlation (r=0.46, p<0.05). Nevertheless, some observations with respect to the polyphenol composition and the chelation of Fe^{2+}

Table 4. Total polyphenols (TP), total flavonoids (TF), DPPH radical-scavenging activity (DPPH), ferric reducing antioxidant power (FRAP), iron chelating activity (FeCA) and copper chelating activity (CuCA) of *R. officinalis* populations cultivated in Cuenca (C) and Aranjuez (A). Values are means (± SD) of three determinations

		Population								
Assay	**Location**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
TP[a]	C	6.4±0.2	9.1±0.1	10.3±0.4	7.0±0.2	8.0±0.0	4.4±0.0	5.3±0.2	8.1±0.1	6.7±0.2	6.1±0.1
	A	8.4±0.1	8.7±0.1	7.9±0.1	8.9±0.0	9.9±0.1	6.0±0.4	11.4±0.2	12.6±0.1	7.4±0.3	12.3±0.4
TF[a]	C	0.8±0.0	1.5±0.0	1.5±0.0	0.8±0.0	1.2±0.2	0.6±0.0	0.8±0.0	1.3±0.1	1.3±0.0	0.8±0.0
	A	2.2±0.0	2.3±0.1	2.0±0.1	3.4±0.0	3.4±0.0	1.7±0.1	6.2±0.0	4.1±0.1	3.8±0.1	6.1±0.1
DPPH[b]	C	37.0±1.3	29.0±0.3	24.5±0.2	37.9±0.7	32.3±0.5	80.5±0.8	56.6±0.1	31.8±1.7	45.4±1.3	47.9±0.3
	A	28.9±0.2	29.4±0.5	31.5±0.3	31.5±0.6	27.8±0.2	56.3±1.6	25.7±0.7	21.6±0.6	37.6±0.3	24.6±0.6
FRAP[c]	C	43.0±1.6	38.4±0.1	32.5±0.3	47.0±0.1	44.8±0.6	81.3±0.1	58.2±0.7	43.3±0.0	45.1±0.2	49.6±1.1
	A	32.8±0.4	32.1±0.5	35.3±1.0	31.0±0.8	29.3±0.7	52.0±0.5	23.9±0.6	21.0±1.4	32.7±0.7	22.4±0.9
FeCA[b]	C	338±7	292±3	375±4	407±20	320±11	313±17	291±20	298±20	281±1	395±6
	A	251±3	249±1	278±1	266±2	293±7	313±1	238±2	256±3	410±7	288±2
CuCA[b]	C	587±19	529±9	371±5	633±5	481±6	583±11	542±5	452±4	501±2	695±1
	A	443±1	439±7	483±18	535±8	409±8	656±54	383±2	271±2	478±9	288±2

[a] g·100 g^{-1} of dry extract.
[b] IC_{50}, μg·mL^{-1}.
[c] Concentration of extract (μg/ml) required to obtain 0.5 A.U.

can be made. Thus, the Fe^{2+}-chelating activities (IC_{50}) of the extracts of P6 from Cuenca and of P6 from Aranjuez were identical (313 μg·mL^{-1}) despite the fact that the P6 from Cuenca had a flavonoid content of around 3 times lower than that of P6 from Aranjuez (Table 4). Both populations showed a very different polyphenol composition: the P6 from Cuenca was characterized by a high content in genkwanin (27.5% of total peak area) while the major polyphenols in the P6 from Aranjuez were carnosol (8.6%), rosmarinic acid (7.4%), epirosmanol ethyl ether/rosmarinic acid methyl ether (6.6%) and rosmanol/epirosmanol (5.7%) (Table 3). Likewise, the extract of P9 from Cuenca (1.3 g flavonoids·100 g^{-1} dry extract) was a better Fe^{2+}-chelator (IC_{50}=281 μg·mL^{-1}) than P9 from Aranjuez (3.8 g flavonoids·100 g^{-1} dry extract; IC_{50}=410 μg·mL^{-1}). The polyphenol profile of P9 from Cuenca showed a predominance of genkwanin (18.4%), whereas rosmarinic acid (15.2%), carnosic acid (8.3%) and carnosol (8.2%) were the major polyphenols tentatively identified in P9 from Aranjuez. Extracts from P7 (19.3% genkwanin) and P9 (22.9% genkwanin) from Cuenca also showed good chelating activities in relation to their modest contents in total flavonoids. This suggests that a high content of genkwanin improves the Fe^{2+}-chelating activity, which is consistent with the chelating activity of the 4-keto and 5-hydroxyl groups present in the structure of this flavonoid (Van Acker *et al*., 1996).

The Cu^{2+}-chelating activities (IC_{50}) of *R. officinalis* extracts ranged from 271 (P8, Aranjuez) to 695 μg/mL (P10, Cuenca), whereas the IC_{50} of gallic acid was 24.7 μg·mL^{-1}. In contrast to the chelation of iron, the Cu^{2+}-chelating activities were highly correlated with the total content in polyphenols (r=−0.86, p<0.05), and moderately correlated with the total content in flavonoids (r=−0.70, p<0.05) (Table 5). In addition to these good correlations, some differences among populations in the Cu^{2+}-chelating activities could be in part attributed to their polyphenol composition. Carnosic acid (r=−0.52, p<0.05) showed the best correlation with Cu^{2+}-chelating activity, and its predominance in some extracts seemed to promote this activity. Thus, the extracts from populations P2 and P3 from Cuenca had the same flavonoid content, 1.5 g·100 g^{-1} dry extract, but P3 (IC_{50}=371 μg·mL^{-1}) was a better copper chelator than P2 (IC_{50}=529 μg·m^{-1}) probably due to the higher content in carnosic acid in P3 (10.3%) in comparison with P2 (0.5%) (Tables 3 and 4). P8 and P9 from Aranjuez also showed similar flavonoid contents but P8 (IC_{50}=271 μg·mL^{-1}) was a much better chelator than P9 (IC_{50}=478 μg·mL^{-1}). The polyphenol composition of P8 was characterized by a higher content in carnosic acid as compared with P9 (17.0% versus 8.3%) These comparisons suggest that for equal contents of flavonoids, carnosic acid could enhance the Cu^{2+}-chelating activity in comparison with other flavonoids of *R. officinalis* extracts.

TABLE 5. Pearson's linear correlation coefficients between antioxidant *in vitro* tests: Total polyphenols (TP), total flavonoids (TF), DPPH radical-scavenging activity (DPPH), ferric reducing antioxidant power (FRAP), iron chelating activity (FeCA), copper chelating activity (CuCA), formation of lipid hydroperoxides (LOOH) and formation of conjugated dienes (CD)

***In vitro* test**	**TP**	**TF**	**DPPH**	**FRAP**	**FeCA**	**CuCA**	**LOOH**	**CD**
TP	1							
TF	0.78*	1						
DPPH	−0.85*	−0.53*	1					
FRAP	−0.86*	−0.70*	0.90*	1				
FeCA	n.s.	n.s.	n.s.	n.s.	1			
CuCA	−0.86*	−0.70*	0.67*	0.65*	0.47*	1		
LOOH	0.52*	n.s.	n.s.	n.s.	n.s.	−0.54*	1	
CD	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	−0.70*	1

*$p<0.05$, bilateral.
n.s., not significant.

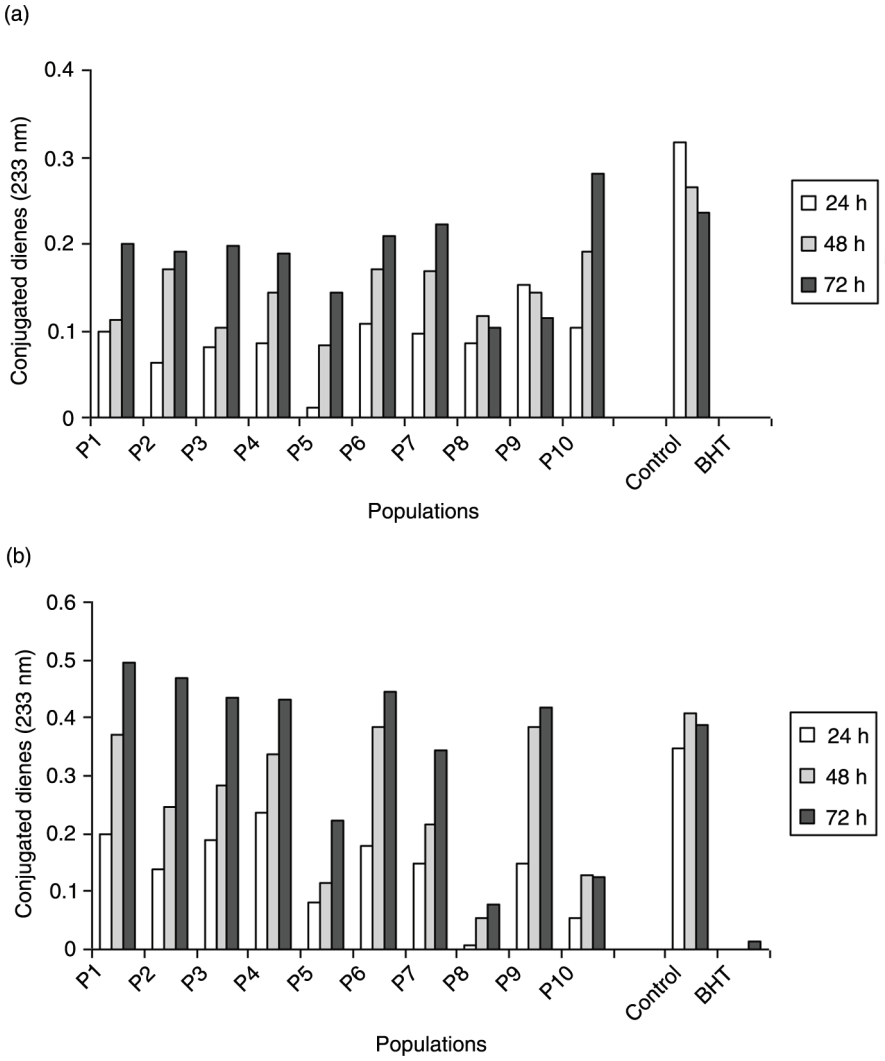

FIGURE 3. Formation of conjugated dienes during the incubation of linoleic acid with extracts (167 $\mu g \cdot mL^{-1}$) of *R. officinalis* populations cultivated in Cuenca (a) and Aranjuez (b).

3.6. Oxidation of linoleic acid

The oxidation of unsaturated fatty acids like linoleic acid can be monitored by the determination of the numerous oxidation compounds generated. The presence of antioxidant compounds in the medium delays the generation of oxidation compounds whose determination therefore allows for an indirect evaluation of their antioxidant capacity. Conjugated dienes are compounds formed at the early stages of the oxidation (primary oxidation products) and their determination constitutes an usual method of detecting the initiation of lipid oxidation. After 24 h of incubation, all the *R. officinalis* extracts reduced the generation of conjugated dienes with respect to the control, and especially effective were the extracts from populations P2 and P5 from Cuenca, and P5, P8 and P10 from Aranjuez. (Figure 3). The formation of conjugated dienes logically increased with the incubation time, but even after 72 h of incubation, the extracts from populations P5, P8 and P9 from Cuenca, and P5, P8 and P10 from Aranjuez still showed a remarkable reduction in the content of conjugated dienes as compared with the control.

Lipid hydroperoxides are also primary oxidation products whose generation was clearly inhibited by *R. officinalis* extracts (Figure 4). After 24 h of incubation, most of the rosemary extracts reduced the generation of hydroperoxides with respect to the control. The extracts from populations P2, P3, and P5 cultivated in Cuenca, and P8 and P10 from Aranjuez were the most effective in delaying the formation of lipid hydroperoxides throughout incubation. All these populations, except for P10 from Aranjuez, were characterized by a high content in carnosic acid and carnosol (16–35% total peak area). In fact, carnosic acid was the only polyphenol significantly correlated (r=0.61, p<0.05) with the inhibition of lipid hydroperoxide generation. As both lipid hydroperoxides and conjugated dienes are primary oxidation products, their generation was well correlated (r=−0.70, p<0.05) (Table 5).

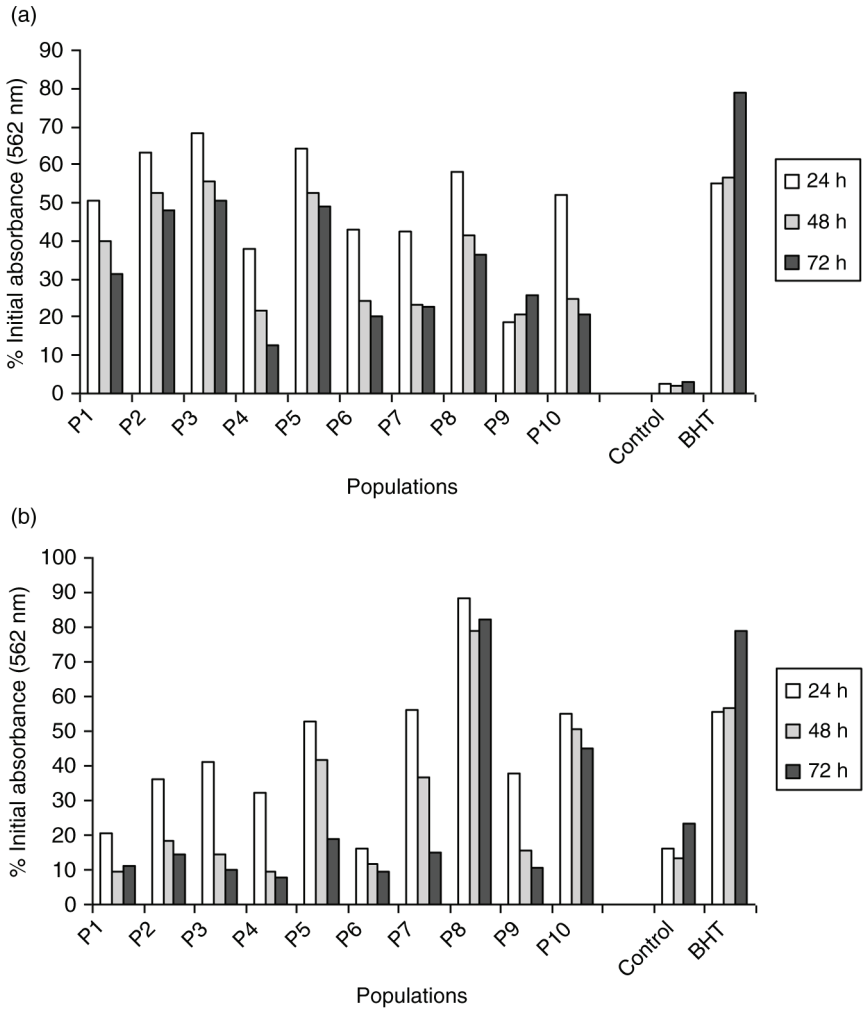

FIGURE 4. Formation of lipid hydroperoxides during the incubation of linoleic acid with extracts (167 μg·mL^{-1}) of *R. officinalis populations* cultivated in Cuenca (a) and Aranjuez (b).

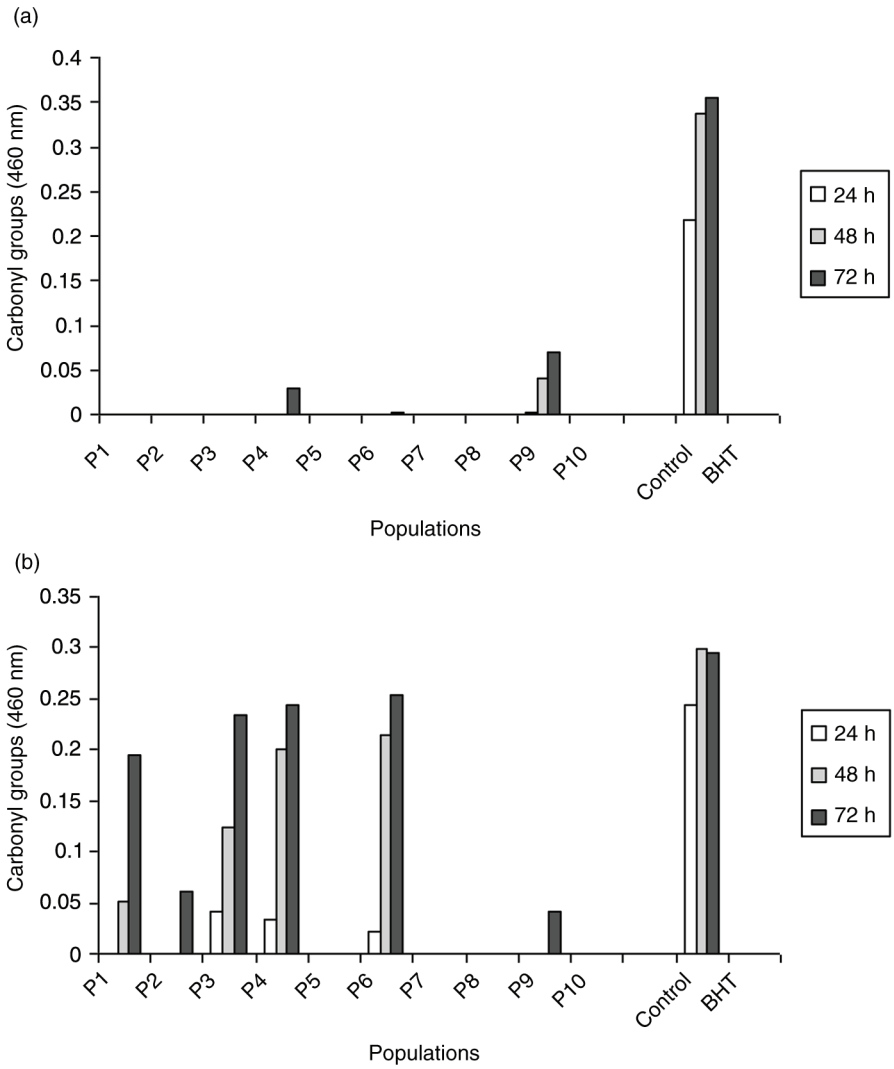

FIGURE 5. Formation of aldehydes and ketones (carbonyl groups) during the incubation of linoleic acid with extracts (167 μg·mL^{-1}) of *R. officinalis* populations cultivated in Cuenca (a) and Aranjuez (b).

Aldehydes and ketones are compounds generated when oxidation has reached an advanced stage (secondary oxidation products) and the development of rancidity is clearly perceptible. Their formation was stopped or drastically reduced by all the *R. officinalis* extracts after 24 h of incubation, and after 72 h the detection of carbonyl groups was only significant in incubations with extracts from populations P1, P3, P4 and P6 from Aranjuez (Figure 5).

The effectiveness of *R. officinalis* extracts to delay the oxidation of linoleic acid cannot be exclusively related with their total content of polyphenols and other compounds presumably present in the extracts like tocopherols, which could help to prevent such oxidation as deduced from the data on the formation of oxidation products. Thus, whereas some extracts with a high contents in polyphenols, like P2 and P3 from Cuenca, or P5, P8 and P10 from Aranjuez, extensively reduced the generation of oxidation products, others with similar contents in polyphenols, like P4 and P7 from Aranjuez, did not. Conversely, the extract from P5 from Cuenca was very effective as an antioxidant despite the fact that its content in polyphenols was lower.

3.7. Antifeedant effects

Table 6 shows the antifeedant effect of the extracts from 10 populations of *R. officinalis* cultivated in Cuenca and Aranjuez. According to their feeding ecologies, *L. decemlineata* (olyphagous) was more sensitive to the extracts of *R. officinalis* populations when compared with *S. littoralis* and *M. persicae* (polyphagous). Based on the EC_{50} values, the extracts from most of the populations cultivated in Aranjuez (P2-P5 and P7-P10) were

TABLE 6. Comparative antifeedant effects (%FI, %SI; 100 μg·cm^{-2}) of solid residues from different *R. officinalis* populations cultivated in Cuenca and Aranjuez and pure compounds (50 μg·cm^{-2}) on *L. decemlineata* adults, *S. littoralis* larvae and *M. persicae* apterous adults in choice test.

	% FI[a]±SE EC_{50} (μg·cm^{-2})[c]				% SI[b]±SE EC_{50} (μg·cm^{-2})[c]	
	L. decemlineata		*S. littoralis*		*M. persicae*	
Populations/compounds	**Cuenca**	**Aranjuez**	**Cuenca**	**Aranjuez**	**Cuenca**	**Aranjuez**
P1	71.82±10.9*	74.32±9.1*	80.10±6.1*	58.37±7.1	42.93±4.6	66.08±5.4
	12.12 (3.22, 25.54)	12.33 (2.6, 26.8)	4.45 (0.87, 12.69)	n.c.	n.c.	n.c.
P2	84.55±7.7*	83.22±8.2*	55.98±7.0	39.20±6.7	74.29±4.5*	80.83±5.1*
	4.15 (0.75, 12.77)	3.45 (0.49, 9.81)	n.c.	n.c.	>50	>50
P3	77.87±8.6*	86.46±7.7*	42.14±10.3	42.01±9.8	52.70±6.9	48.95±5.9
	3.98 (0.27, 28,27)	3.18 (1.06, 9.02)	n.c.	n.c.	n.c.	n.c.
P4	71.74±7.9*	75.27±8.3*	45.88±8.3	50.53±8.5	90.56±4.2*	53.89±4.3
	>50	9.68 (2.72, 24.38)	n.c.	n.c.	>50	n.c.
P5	60.89±10.0	81.20±7.8*	26.71±8.8	43.86±7.6	36.17±6.6	45.66±3.5
	n.c.	6.28 (1.55, 25.49)	n.c.	n.c.	n.c.	n.c.
P6	70.87±8.2*	61.86±9.6	57.62±8.9	20.49±7.4	69.62±6.9*	63.49±4.7
	>50	n.c.	n.c.	n.c.	n.c.	n.c.
P7	86.47±5.8*	90.54±10.4*	49.09±6.2	51.28±9.7	82.10±5.5*	66.23±3.9*
	13.20 (5.19, 23.52)	5.89 (1.54, 22.51)	n.c.	n.c.	~50	n.c.
P8	75.26±10.9*	87.73±9.5*	33.44±6.6	34.76±7.5	54.97±6.4	60.47±5.3
	~50	4.89 (1.80, 13.25)	n.c.	n.c.	n.c.	n.c.
P9	82.24±9.5*	89.61±8.4*	80.95±11.1*	25.83±8.3	65.53±5.1	49.39±4.6
	3.78 (0.45, 11.4)	6.72 (2.66, 16.97)	2.51 (0.38, 16.22)	n.c.	n.c.	nc
P10	82.28±8.2*	86.34±10.2*	61.10±12.5	35.79±9.4	58.81±5.4	67.11±5.9*
	2.13 (0.19, 14.00)	5.91 (0.91, 18.34)	n.c.	n.c.	n.c.	n.c.
Carnosic acid	81.5±9.6		47.61±5.2		n.t.	
	0.63 (0.04, 4.12)		n.c.		–	
Genkwanin	39.36±8.9		22.33±6.1		n.t.	
	n.c.		n.c.		–	
Rosmarinic acid	95.83±2.5		24.15±16.1		40.42±7.81[d]	
	0.60 (0.07, 1.82)		n.c.		n.c.	

n.t., not tested; n.c., not calculated (% FI/SI <70); *p<0.05, Wilcoxon signed-rank test; [a]% FI = [1− (T/C)] ×100, where T and C are the consumption of treated and control leaf disks, respectively; [b]% SI = [1− (%T/%C)] ×100, where %T and %C are the % of aphids on treated and control surface, respectively; [c]EC^{50} 95% confidence limits (lower, upper), concentration needed to produce 50% feeding/setting inhibition; [d]From Julio *et al*. (2014).

strong antifeedants against *L. decemlineata*, whereas only the extracts from populations P2, P3 and P9, P10, grown in Cuenca, showed strong activity on this pest. The extracts from P1 and P9 cultivated in Cuenca showed antifeedant effects on *S. littoralis*, while the rest of the samples were inactive. When tested against *M. persicae*, only the extracts from P2 (Cuenca and Aranjuez), P4 (Cuenca) and P7 (Cuenca) had antifeedant effects at the maximum concentration tested (100 μg·cm^{-2}). Among the major pure compounds present in the *R. officinalis* residue, rosmarinic and carnosic acids acted as strong antifeedants against *L. decemlineata*, while genkwanin was inactive. None of these compounds had activity against *S. littoralis* (Table 6).

Previous reports showed the strong antifeedant effects afainst *L. decemlineata* and moderate against *S. littoralis* of residues from rosemary (Santana-Méridas *et al*., 2014) and other economically important aromatic plants (Santana-Méridas *et al*., 2012). However, to our knowledge, this is the first report on a population-based survey including the comparative chemistry and biological properties of the solid residues from the distillation of *R. officinalis*.

The antifeedant effects observed for the extracts of *R. officinalis* populations were not accounted for by their chemical composition. A Pearson correlation analysis was performed to ascertain the relationship between the antifeedant activity and the major individual compounds detected in the

extracts and no significant correlation was observed (data not shown). However, we found a significant positive correlation between the antifeedant activity against *L. decemlineata* (%FI) and the total content in flavonoids (r=0.55, p=0.01) or the total content in polyphenols (r=0.52, p=0.03), suggesting that the antifeedant activity of rosemary extracts could be explained by the additive or synergistic effects of their individual phenolic components. Similar results have been reported by Julio *et al*. (2014) when comparing the conventional and supercritical extracts from two *Lavandula luisieri* populations. In the same way, Rojht *et al*. (2012) reported the potential repellent effect of a non-toxic ethanol extract from the aerial part of *R. officinalis* against adults of *Acanthoscelides obtectus* and *L. decemlineata*, and propose a synergistic effect among the volatile compounds as responsible for the activity. Interestingly, copper chelating activity was correlated with the antifeedant activity of the extracts on *L. decemlineata* (r=0.49, p=0.03), suggesting a possible impairment of the metal-based enzymes of insects by metal chelators (Dittmer and Kanost, 2012).

The involvement of plant phenolics in the plant mechanism against insects and its role on the feeding behavior of herbivores is well known through the ages, and has been recently reviewed (Lattanzio *et al*., 2012). Specific polyphenols, either individually or in combination, have been associated with defense against insect herbivores in *Quercus oleoides* (Moctezuma *et al*., 2014). Fornoff and Gross (2014) reported the increase in polyphenols as one of the induced resistance and defensive traits of *Myriophyllum spicatum* to prevent herbivore damage. In the same way, the feeding activity of some chewing insects has been correlated with the concentration of polyphenols and flavonoids in plant tissues (Lattanzio *et al*., 2012).

3.8. Phytotoxic activity

None of the samples tested had a phytotoxic effect on the germination and radicle growth of *L. sativa*. On the contrary, most of the extracts and especially those from populations cultivated in Cuenca had a strong stimulating effect on *L. sativa* radicle length (Table 7). The effects observed in the case of *L. perenne* were more controversial. The rosemary residues from P1 cultivated in Cuenca and Aranjuez showed the strongest inhibition of *L. perenne* germination, effective up to 144 h. The root and leaf growth of *L. perenne* were strongly affected by P2 grown in Cuenca (60 and 43%, respectively) and Aranjuez (88 and 52%, respectively) while the rest of the samples had stimulating effects, these being more evident in populations cultivated in Aranjuez (Table 8).

The allelopathic potential of phenolic compounds is reviewed by Li *et al*. (2010), and some phenolic acids present in rosemary, such as rosmarinic and caffeic acids have shown allelopathic effects on *Arabidopsis* seeds (Jian *et al*., 2010). More recently, López-Iglesias *et al*. (2014) proposed that enhanced plant growth or, on the contrary, plant growth inhibition, could be the result of a positive or negative balance between nutrient and polyphenol concentration. In a previous work, Santana-Méridas *et al*. (2014) reported a stimulating effect of rosemary residues on *L. sativa* and a moderate phytotoxic effect of *L. perenne*. Rojht *et al*. (2012) did not find significant differences in germination between the control and treated beans with rosemary ethanol extract, whereas Guimarães-Araújo *et al*. (2013) reported heterogeneous effects on the growth of hypocotyls and radicle of the seeds of lettuce and onion after the application of ethanol extract and fractions obtained from *R. officinalis*.

Table 7. Phytotoxic activity of solid residues from different *R. officinalis* populations cultivated in Cuenca and Aranjuez against *L. sativa*. Values are means ± SE.

PN	L	Germination[a]			Radicle length[a]
		24h	48h	72h	
P1	C	100.0±0.0	100.0±0.0	100.0±0.0	260.50±0.1[b]
	A	100.0±0.0	100.0±0.0	100.0±0.0	152.50±0.1[b]
P2	C	100.0±0.0	100.0±0.0	100.0±0.0	257.01±0.1[b]
	A	95.0±2.9	100.0±0.0	100.0±0.0	169.88±0.1[b]
P3	C	100.0±0.0	100.0±0.0	100.0±0.0	220.31±0.1[b]
	A	95.0±2.9	100.0±0.0	100.0±0.0	212.07±0.2[b]
P4	C	100.0±0.0	100.0±0.0	100.0±0.0	166.06±0.2[b]
	A	97.5±2.5	100.0±0.0	100.0±0.0	225.21±0.2[b]
	A	100.0±0.0	100.0±0.0	100.0±0.0	155.76±0.2[b]
P5	C	100.0±0.0	100.0±0.0	100.0±0.0	164.73±0.1[b]
	A	97.5±2.5	100.0±0.0	100.0±0.0	157.75±0.1[b]
P6	C	100.0±0.0	100.0±0.0	100.0±0.0	166.99±0.1[b]
	A	97.5±2.5	100.0±0.0	100.0±0.0	118.73±0.1
P7	C	100.0±0.0	100.0±0.0	100.0±0.0	183.24±0.1[b]
	A	85.0±8.07	100.0±0.0	100.0±0.0	93.30±0.1
P8	C	100.0±0.0	100.0±0.0	100.0±0.0	180.21±0.1[b]
	A	92.5±4.8	97.5±2.5	100.0±0.0	195.74±0.1[b]
P9	C	100.0±0.0	100.0±0.0	100.0±0.0	137.77±0.1[b]
	A	100.0±0.0	100.0±0.0	100.0±0.0	118.43±0.1
P10	C	100.0±0.0	100.0±0.0	100.0±0.0	188.45±0.1[b]
	A	92.5±4.8	100.0±0.0	100.0±0.0	174.27±0.1[b]

PN, Population number (see Table 1).
L, Place of cultivation (C: Cuenca, A: Aranjuez).
[a]Expressed as % control.
[b]p<0.05, ANOVA-LSD test.

4. CONCLUSIONS

The total polyphenol and flavonoid contents as well as the polyphenol composition of rosemary populations were very dependent on the growth

Table 8. Phytotoxic activity of solid residues from different *R. officinalis* populations cultivated in Cuenca and Aranjuez against *L. perenne*. Values are means ± SE

		Germination[a]			Length[a]	
PN	**L**	**96h**	**120h**	**144h**	**Radicle**	**Leaf**
P1	C	55.6±6.5[b]	52.2±8.2[b]	60.7±7.5[b]	81.56±0.6	77.11±0.4
	A	25.0±0.0[b]	36.4±4.1[b]	48.0±7.1[b]	121.11±0.3[c]	103.71±0.2
P2	C	38.9±4.8[b]	39.1±6.3[b]	46.2±4.1[b]	40.05±0.4[c]	57.27±0.4[c]
	A	40.9±6.3[b]	59.1±4.8[b]	60.9±6.5[b]	12.06±0.02[c]	48.02±0.2[c]
P3	C	66.7±14.7	73.9±11.1	80.8±7.5	112.13±0.6	85.41±0.4
	A	62.5±6.5	86.4±4.8	92.0±4.8	123.83±0.5[c]	94.41±0.3
P4	C	88.5±6.3	95.7±5.0	100.0±0.0	106.22±0.5	82.96±0.3
	A	100.0±0.0	100.0±0.0	100.0±0.0	142.42±0.4[c]	109.39±0.2
P5	C	73.9±6.3	76.9±10.0	83.3±8.5	108.92±0.5	89.76±0.4
	A	100.0±0.0	100.0±0.0	100.0±0.0	144.27±0.4[c]	121.16±0.3[c]
P6	C	61.1±11.8[b]	73.1±11.1	80.8±13.1	95.97±0.5	77.86±0.3
	A	81.3±6.3	81.8±9.6	88.0±6.5	132.34±0.5[c]	123.78±0.3[c]
P7	C	66.7±7.1	73.9±10.3	76.9±9.1	149.74±0.8[c]	98.46±0.5
	A	100.0±0.0	100.0±0.0	100.0±0.0	168.32±0.4[c]	145.79±0.3[c]
P8	C	50.0±2.5[b]	56.5±2.5[b]	61.5±7.1[b]	116.75±0.65	76.50±0.4
	A	81.3±4.8	86.4±10.3	96.4±7.5	131.10±0.4[c]	108.43±0.3
P9	C	53.8±10.4[b]	60.9±10.4[b]	69.2±11.9	119.16±0.7	77.56±0.4
	A	87.5±2.9	100.0±0.0	100.0±0.0	142.81±0.5[c]	120.39±0.3[c]
P10	C	95.7±2.9	100.0±0.0	100.0±0.0	120.82±0.6[c]	93.83±0.4
	A	93.8±4.8	95.5±7.5	100.0±0.0	167.94±0.5[c]	132.34±0.3[c]

PN, Population number (see Table 1).
L, Place of cultivation (C: Cuenca, A: Aranjuez).
[a]Expressed as % control.
[b]$p<0.05$, Kruskall–Wallis test.
[c]$p<0.05$, ANOVA-LSD test.

location, which points to a significant influence of the environmental conditions on these traits. In general, the populations from Aranjuez showed higher contents in polyphenols and flavonoids in comparison with their equivalent populations from Cuenca. The populations from Cuenca were characterized by an overall higher content in genkwanin and carnosol, whereas those from Aranjuez were richer in rosmarinic acid. DPPH radical-scavenging activity, ferric reducing antioxidant power (FRAP) and Cu^{2+}-chelating activity were in general higher in populations from Aranjuez as compared with those from Cuenca due to the high correlation of these activities with the total content in polyphenols. Nevertheless, Fe^{2+}-chelating activity did not depend on the polyphenol content, which suggests the presence of others compounds participating as iron chelators in the extracts. The extracts from rosemary residues delayed the oxidation of linoleic acid, especially in the advanced stages when the secondary oxidation products responsible for the rancidity off-flavor are generated. Some polyphenols like carnosic acid and carnosol seemed to enhance the antioxidant and copper chelating activities of the extracts, whereas genkwanin could be related with the chelation of iron. The extracts from *R. officinalis* acted as strong antifeedants against *L. decemlineata*, this activity being more evident in the populations grown in Aranjuez. Low to moderate antifeedant effects on *S. littoralis* and *M. persicae* were observed. The antifeedant activity was correlated with the total content in flavonoids and polyphenols, suggesting additive or synergistic effects and supporting the possible defensive role of phenolic compounds in plant-insect interactions. Most of the extracts, and especially those from populations cultivated in Cuenca, had strong stimulating effects on the *L. sativa* radicle length. Controversial behavior including phytotoxic and stimulating effects was observed in the case of *L. perenne*, depending on population and growing area.

These results provide valuable data on the potential as high added-value compounds of processing-based residues from rosemary distillation.

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