Antioxidant and Antifungal Effects of Six Plant Essential Oils Against *Penicillium digitatum* and *Penicillium italicum*

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Article

Antioxidant and Antifungal Effects of Six Plant Essential Oils Against *Penicillium digitatum* and *Penicillium italicum*

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Abstract

Six aromatic plants (*Lavandula pedunculata* subsp. *sampaioana*, *Lavandula stoechas* subsp. *luisieri*, *Mentha* × *piperita*, *Origanum vulgare* subsp. *virens*, *Thymus mastichina*, and *Thymus zygis* subsp. *sylvestris*) were analysed to evaluate their essential oil yield, chemical composition, antioxidant activity and antifungal capacity against two mold species, green mold (*Penicillium digitatum* (Pers.) Sacc.) and blue mold (*Penicillium italicum* Wehmer). The antioxidant activity was found to be at its lowest in *Lavandula pedunculata* subsp. *sampaioana* (3.84 ± 0.26) and at its highest in *Thymus zygis* subsp. *sylvestris* (161.70 ± 0.15). Similarly, the in vitro antifungal capacity assay produced different results depending on the essential oil used: the lowest value was produced by *Thymus mastichina* essential oil, and the highest by *Thymus zygis* subsp. *sylvestris*. All the data collected reveal a positive correlation between antioxidant activity, as measured by DPPH and ABTS assays, and the inhibition halo created by the essential oils used in this study.

Keywords: antifungal activity; antioxidant; essential oil; *Lavandula*; *Mentha*; *Origanum*; *Penicillium*; *Thymus*

1. Introduction

Fruit and vegetables postharvest diseases caused by fungal infections (*Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, …) due to wounds or insect bites produce elevated loss of food during storage, distribution and sale [1–7]. Citrus major sources of postharvest diseases are green mold (*Penicillium digitatum* (Pers.) Sacc.) and blue mold (*Penicillium italicum* Wehmer), which cause economic losses of 15–30% and affect 50–90% of production, particularly in developing countries [8–13]. Traditionally, several methods based on synthetic chemical fungicides have been developed to reduce post-harvest losses; however, intensive use of these methods generates resistance, reducing their effectiveness [9,14,15]. Moreover, consumer trends demand products that are free of chemical residues and more environmentally friendly. Together with legislative restrictions on the use of phytosanitary products, this creates the need for new, more effective and environmentally friendly postharvest management. These alternatives include biocontrol strategies involving the use of antagonist yeast or bacteria, immersion in aqueous extracts of medicinal plants or citrus fruits, vaporization of essential oils from medicinal plants, wax coatings containing essential oils or plant extracts, new biopolymers and heat treatments, among others [6,11,16–21].

Studies of the antifungal capacity of medicinal plants extracts or essential oils have reported the ability of fight various fungal infections caused by *Aspergillus* spp., *Candida* spp., *Cryptococcus* spp., *Epidermophyton* spp., *Fusarium* spp., *Microsporum* spp. *Penicillium* spp., and *Trichophyton* spp. [22–27].

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Several studies have shown that essential oils from species such thyme, oregano, clove, cinnamon or citrus have a high inhibitory capacity against the in vitro growth of fungal colonies from *Penicillium* species such as *P. digitatum* and *P. italicum*) [28–36]. The antifungal properties of these essential oils have contributed to the development of new research aimed at preventing post-harvest infections caused by *P. digitatum* and *P. italicum* [37–45].

The use of essential oils from medicinal plants whose chemical composition includes antifungal compounds (e.g., thymol, carvacrol, terpinen-4-ol, etc. [46]) makes it possible to search for local medicinal species commonly used in traditional medicine that are rich in these kinds of compounds, creating a new local employment opportunity that are more environmentally friendly. For that purpose, the main objective of this research is to evaluate the inhibitory and antioxidant activities of different aromatic plants, native to the SW from the Iberian Peninsula, against two mold species (*P. digitatum* and *P. italicum*), which cause postharvest disease in citrus fruits (e.g., oranges).

2. Materials and Methods

2.1. Plant Material, Essential Oil Extraction, and Chemical Characterization of Essential Oils

Aerial parts of six aromatic plants (*L. pedunculata* subsp. *sampaioana*, *L. stoechas* subsp. *luisieri*, *Mentha* × *piperita*, *O. vulgare* subsp. *virens*., *Th. mastichina*, and *Th. zygis* subsp. *sylvestris*) were collected from the experimental crops at Institute of Agrarian Research "La Orden-Valdesequera" (CICYTEX) (near of Guadajira, Spain). Representative samples were collected during the flowering stage, which took place between May and June 2024.

Fresh stems, leaves, and flowers from each specie were cut in small pieces and submitted to hydro-distillation in Clevenger-type apparatus for 2 h. The essential oils (EOs) were stored in amber vial at 4ºC.

The chemical analysis of the essential oils was carried out using a combination of two gas chromatography techniques (GC-FID + GC-MS), chemical compounds were identified by CG-MS and quantified by CG-FID. The analysis was performed on Agilent 8890 GC paired with the 5977B MSD (Mass Selective Detector). Polar column DB-WAX UI (60 m long, 0.25 mm diameter and 0.5 µm film thicknesses) was employed using Helium carrier gas at constant flow of 2 mL/min. Apolar column HP-5MS UI (60 m long, 0.25 mm diameter and 0.25 µm film thicknesses) was employed using Helium carrier gas at constant flow of 1 mL/min. The column temperature stared at 50ºC and increased to 240ºC (polar column) and 285ºC (apolar column).

2.2. Antioxidant Activity

The antioxidant activity of each essential oil samples was determined by ABTS and DPPH assay method. The absorbance was measured using a spectrophotometer (Beckman Coulter DU® 730).

The standard line from each assay was designed using Trolox (6-hidroxy-2,5,7,8-tetramethylchroman-carboxylic acid) (Sigma-Aldrich 238813) between 1mM and 2mM concentration and measured the absorbance at 734nm (ABTS) and 517nm (DPPH).

All the essential oil samples were analysed in triplicate. The sample volume used was 3 milliliters (2,95 ml from DPPH/ABTS + 50 µl from essential oil sample). The results, from both analyses (ABTS and DPPH) were presented as millimoles (mM) of Trolox equivalents and grams of Trolox equivalents per gram of essential oil, with the main objective of developing a data matrix comparable between each other.

2.2.1. ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] Assay

ABTS assay is based on the ability of molecules to scavenge the free radical of ABTS in comparison with Trolox [47]. Absolute ethanol was used to prepare the working solution of ABTS (Sigma-Aldrich A1888) at a concentration of 7 mM, which was then adjusted to obtain a final absorbance of 0.7 ± 0.02 (at 734 nm). To determine antioxidant activity, the essential oil samples

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remained in the dark at ambient temperature for 30 minutes and, thereafter, the absorbance was measured at 734nm.

2.2.2. DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

The DPPH protocol to measure antioxidant activity was based on the description in reference [48]. Methanol (100%) was used as the solvent to prepare a working solution 75 µmol/L of DPPH (Sigma-Aldrich D9132), which was then adjusted to a final absorbance of 0.7 ± 0.02 (at 517 nm). For antioxidant activity determination, the samples remained in the dark at ambient temperature for 120 minutes, after which the absorbance was measured at 517 nm.

2.3. In Vitro Antifungal Activity Assay

2.3.1. Fungal Isolation

The fungal species used are *P. digitatum* and *P. italicum*, obtained from infected *Citrus aurantium* L. fruit. The isolation was realized in Petri dishes containing Sabouraud Dextrose agar (6%) and incubated for 7 days at 27ºC ± 1ºC, in complete darkness. The differential isolations were transferred to new Petri dishes containing Sabouraud Dextrose agar and re-sown each week until a pure fungal culture of each species was obtained. Finally, the standardization of the fungal colonies was achieve using a 0.85% saline solution suspension to obtain the 0.5 McFarland standard (1,5 · 108 CFU/ml) [49]. Morphological characterization (macro and microscopic) was performed using dichotomous keys as a reference [50–52].

2.3.2. Antifungal Activity

The disk diffusion method was used to evaluate the antifungal activity of each of the essential oils [53,54]. The fungal suspension was sown in Petri dishes (87,8 mm diameter) containing 25 ml of Sabouraud Dextrose Agar. A sterile swab was used to spread the mold suspension evenly across the surface of the dish to ensure a homogeneous development of the mold. Essential oils were inoculate using a 10 mm diameter filter disk soaked with 25µl of each essential oil sample and placed in the center of the Petri dish.

The study included a control group and a study group with 3 repetitions of each for each species of essential oil. Thus, 12 Petri dishes were used for each essential oil species (3 control dishes + 3 study dishes for each one of the analysed molds, *P. digitatum* and *P. italicum*). The Petri dishes were incubated for 5 days (96 hours) in an incubator chamber at 27ºC ± 1ºC in complete darkness and in the normal position (not inverted) to avoid affecting the mold growth. Finally, the Petri dishes were checked, photographed and measured every 24 hours. Measurements were taken by evaluating the inhibitory halo of growth around the filter disk using a caliper.

2.4. Statistical Analysis

Descriptive and inferential statistical analysis were performed using R v 4.3.3 software) [55] to determine the relationship between the inhibitory halo of growth results and the antioxidant activity obtained from the samples. The 48-hour data from inhibitory halo of growth were used to developed the statistical analysis (to ensure a correct understanding of the data and avoid mixing up inhibition and natural absence of growth).

3. Results

3.1. Essential Oil Composition

Table 1 shows the essential oils yield obtained for each of the aromatic plant, expressed in grams of essential oil per kilogram of fresh plant and as a percentage (w/w). The highest yields were obtained in *Thymus mastichina* (L.) L., *Lavandula pedunculata* subsp. *sampaioana* (Rozeira) Franco, and

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Thymus zygis subsp. *sylvestris* (Hoffmanns. & Link) Cout. with values of 2.43%, 1.28%, and 0.88% respectively. The species with the lowest yields were *Mentha* × *piperita* L., *Lavandula stoechas* subsp. *luisieri* (Rozeira) Rozeira, and *Origanum vulgare* subsp. *virens* (Hoffmans. & Link) Bonnier & Layens (0.62%, 0.42% and 0.41%, respectively).

Table 1. Yield of the essential oil extraction by hydrodistillation.

Specie	Code	Yield (w/w)	% (w/w)
Origanum vulgare subsp. *virens*	OVV	4.09	0.41
Lavandula pedunculata subsp. *sampaioana*	LPS	12.80	1.28
Lavandula stoechas subsp. *luisieri*	LSL	4.22	0.42
Thymus zygis subsp. *sylvestris*	TZS	8.78	0.88
Thymus mastichina	TM	24.29	2.43
Mentha × *piperita*	MP	6.17	0.62

The essential oils have a rich monoterpene-based chemical composition (Table 2). The majority of the detected compounds are: thymol (68.83% in *Th. zygis* subsp. *sylvestris* and 36.72% in *O. vulgare* subsp. *virens*), 1.8-cineole (66.06% and 17.71% in *Th. mastichina* and L. *stoechas* subsp. *luisieri* respectively), camphor and fenchone (35.51% and 34.20% respectively in *L. pedunculata* subsp. *sampaioana*), gamma-terpinene (30.69% in *O. vulgare* subsp. *virens*), menthone and L-menthol (29.12 and 27.56% respectively in *M* × *piperita*), and trans-alpha-necrodyl acetate (20.46% in *L. stoechas* subsp. *luisieri*).

3.2. Antioxidant Activity

Obtained results (Table 3) show that the essential oils of the *L. stoechas* subsp. *luisieri*, *O. vulgare* subsp. *virens* and *Th. zygis* subsp. *sylvestris* species have higher antioxidant activity. These species have in common a high percentage of the chemical's thymol, gamma-terpinene and trans-alpha-necrodyl acetate in their essential oils.

Table 2. Composition of the essential oils.

RI-WAX	RI-HP5	Compound	OVV	LPS	LSL	TM	TZS	MP
1025	933	Alpha-Pinene	0.67	6.35	1.83	3.44	0.51	0.74
1029	918	Alpha-Thujene	1.63	0.01		0.21	1.32	0.06
1069	953	Camphene	0.29	2.29	0.10	0.10	0.14	0.02
1114	978	Beta-Pinene	0.18	0.05	0.30	5.11	0.13	1.17
1126	972	Sabinene	0.29	0.03	0.12	3.83	0.07	0.67
1133	940	Cymene Isomer			2.67			
1165	991	Beta-Myrcene	2.28	0.17	0.07	1.87	1.91	0.33
1186	1018	Alpha-Terpinene	3.76	0.03			1.33	0.26
1206	1021	Limonene	0.35	2.03	0.20	1.17	0.32	3.42
1222	1039	1,8-Cineole	0.02	0.93	17.71	66.06		6.72
1238	1035	Cis-Beta-Ocimene	2.15	0.15	0.45	0.02	0.01	0.27
1254	1058	Gamma-Terpinene	30.69	0.05	0.12	1.79	5.72	0.41
1278	1025	Para-Cymene	5.26	0.24	0.14	1.03	9.36	0.08
1418	1090	Fenchone		34.20	0.28			
1465	1074	Trans-Sabinene Hydrate	0.16			0.70	0.68	0.82
1484	1124	Menthone						29.12
1500	1164	Menthofuran						4.94
1510	1166	Isomenthone						4.31
1541	1149	Camphor		36.51	1.00			
1553	1100	Linalool	0.16	2.00	2.29	4.14	0.86	0.26
1574	1294	Menthyl Acetate						2.36
1592	1239	Thymol Methyl Ether	1.88				0.01	
1595	1119	Fenchol<endo->		0.86				
1599	1288	Bornyl Acetate		0.98	0.06	<0,01		

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1606	1280	Trans-Alpha-Necrodyl Acetate			20.46			
1607	1239	Carvacrol Methyl Ether	2.38				0.05	
1608	1165	Neo-Menthol						4.11
1617	1450	Trans-Beta Caryophyllene	1.61	0.04	0.22	0.12	1.41	1.18
1619	1284	Lavandulyl Acetate		0.20	4.01			
1636	1296	Arbozol			2.24			
1653	1169	L-Menthol						27.56
1662	1170	Delta-Terpineol				1.50		0.22
1665	1244	Pulegone						3.98
1668	1187	5-Methylene-2,3,4,4-tetrame-2-Cyclopentenone			2.37			
	1860	Unknown Sesquiterpenol			2.06			
1679	1172	Trans-Alpha-Necrodol			6.56			
1696	1195	Alpha-Terpineol	0.11	0.29	0.29	4.86	0.13	0.44
1713	1167	Borneol	0.65	0.78		0.12	0.34	0.02
2168	1293	Thymol	36.72				68.83	0.08
2192	1316	Carvacrol	0.28			0.17	2.54	

Table 3. Antioxidant Activity results (ABTS and DPPH methods).

Code	ABTS		DPPH	
	mM TROLOX eq.	g TROLOX eq. / g EO	mM TROLOX eq.	g TROLOX eq. / g EO
OVV	76.45 ± 3.02	433.01 ± 17.10	25.15 ± 1.69	142.45 ± 9.57
LPS	3.84 ± 0.26	20.79 ± 1.41	2.17 ± 0.16	11.73 ± 0.87
LSL	24.06 ± 0.64	131.33 ± 3.47	33.91 ± 1.21	184.99 ± 6.58
TM	9.76 ± 0.41	54.19 ± 2.30	0.96 ± 0.03	5.31 ± 0.16
TZS	161.70 ± 0.15	864.20 ± 0.81	25.34 ± 1.08	135.42 ± 5.78
MP	4.83 ± 0.09	26.94 ± 0.51	3.83 ± 0.13	21.34 ± 0.74

3.3. In Vitro Antifungal Activity Assay

3.3.1. Fungal Isolation

The *P. italicum* species was observed to grow more quickly and be less susceptible to contamination in in vitro conditions than *P. digitatum* (Figure 1).

(**a**) (**b**)

Figure 1. Fungal isolation: (**a**) *P. digitatum*; (**b**) *P. italicum*.

3.3.2. Antifungal Activity

The Petry dishes were photographed every 24 hours, and it could be observed that the filter disk infused with the essential oils is able to inhibit the fungal species development (inhibition halo) and delay maturation of both species (Figure 2).

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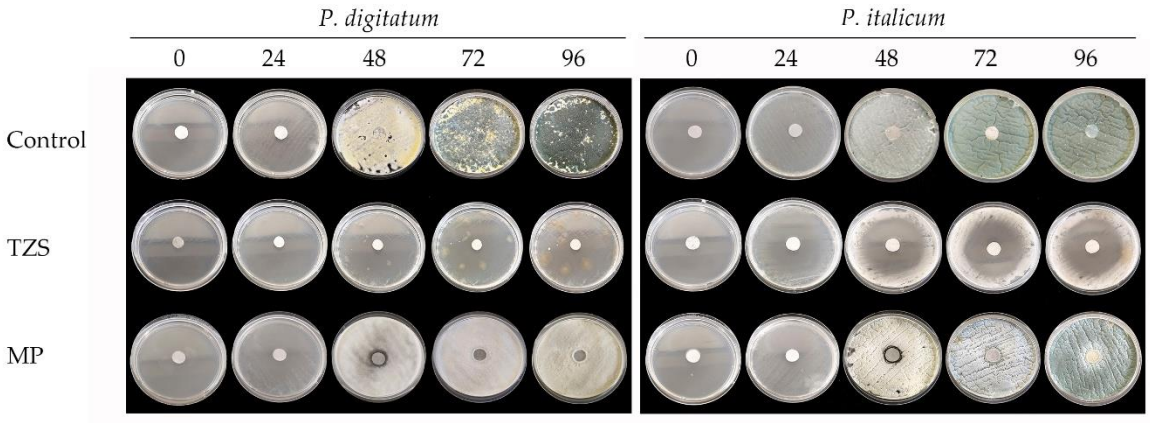

Figure 2. Photographic progression of *P. digitatum* and *P. italicum* (time in hours).

Table 4 displays statistical parameters obtained from the inhibition halo measurements obtained after 48 hours of growing. Measurement results show a higher inhibitory capacity from essential oil over *P. digitatum* specie than *P. italicum* (inhibition halo mean: 29.69 mm in *P. digitatum* and 27.81 mm in *P. italicum*). Besides, the observed skew shows a positive trend. On the other hand, the kurtosis of the antioxidant activity shows a platykurtic curve, indicating fewer extreme values than a normal distribution. However, the inhibition halo differs in the kurtosis depending on the fungal species (slightly leptokurtic in *P. digitatum* and platykurtic in *P. italicum*).

Table 4. Statistical parameters from inhibition halo measurement.

EO	$\bar{x}$	s	Me	Max	Min	SEM	g_1	g_2
				P. digitatum				
OVV	31.33	3.21	30.00	35.00	29.00	1.86	0.34	-2.33
LPS	20.17	3.25	20.00	23.50	17.00	1.88	0.05	-2.33
LSL	30.67	1.15	30.00	32.00	30.00	0.67	0.38	-2.33
TM	16.17	0.29	16.00	16.50	16.00	0.17	0.38	-2.33
TZS	60.50	5.77	60.00	66.50	55.00	3.33	0.09	-2.33
MP	19.33	3.21	18.00	23.00	17.00	1.86	0.34	-2.33
				P. italicum				
OVV	27.00	6.38	28.50	32.50	20.00	3.69	-0.22	-2.33
LPS	14.17	0.76	14.00	15.00	13.50	0.44	0.21	-2.33
LSL	37.33	2.52	37.00	40.00	35.00	1.45	0.13	-2.33
TM	13.67	1.26	13.50	15.00	12.50	0.73	0.13	-2.33
TZS	54.33	2.93	55.50	56.50	51.00	1.69	-0.34	-2.33
MP	20.33	3.06	21.00	23.00	17.00	1.76	-0.21	-2.33

Finally, the data distribution in relation to each essential oil species is presented in a boxplot for each fungal species (Figure 3). It is possible to observe that the *Th. zygis* subsp. *sylvestris* essential oil produced the most extensive inhibition halo for both fungal species, with a clear difference compared to the rest of the samples.

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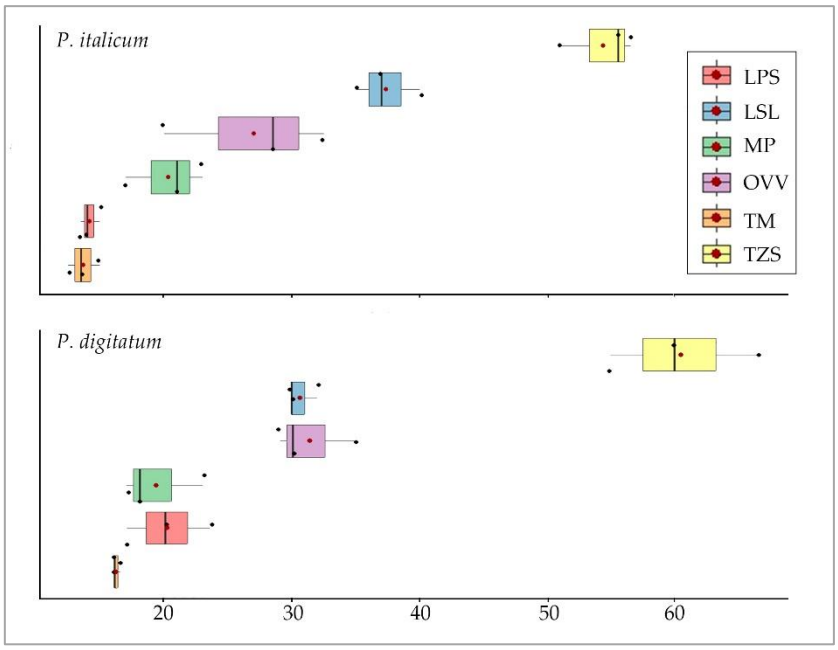

Figure 3. Inhibition halo boxplot produced by the different essential oil samples.

3.3.3. Statistical Analysis

The Shapiro-Wilk test applied to the data base obtained in the study indicate the absence of normal distribution (Table 5), for that reason the statistical analysis was based on non-parametric correlation test (Spearman's correlation).

Table 5. Shapiro-Wilk test results.

variables	W	p-value	W	p-value
	P. digitatum		*P. italicum*	
inhibition halo (mm)	0.80130	0.00159	0.86399	0.01418
ABTS (mM)	0.72045	0.00014	0.72045	0.00014
ABTS (g)	0.72651	0.00017	0.72709	0.00017
DPPH (mM)	0.79313	0.00122	0.79468	0.00122
DPPH (g)	0.79468	0.00128	0.79468	0.00128

The results of the Spearman's correlation test show a p-value of less than 0.05 (significance level), indicating a linear relationship between the pairs of variables studied at the ordinal level and showing that this relationship is not due to chance (Table 6.).

A significant statistical linear correlation was found between the different measurements of antioxidant activity (using the ABTS and DPPH methods) and the inhibition halo using the various essential oil samples on the two species of mesophilic mold (*P. digitatum* and *P. italicum*) (Figure 4).

Table 6. Spearman's correlation test results obtained in inhibition halo measurements.

variables	S	p-value	ϱ	S	p-value	ϱ
	P. digitatum			*P. italicum*		
ABTS (mM)	245.38	$3.70 \cdot 10^{-4}$	0.75	247.88	$3.98 \cdot 10^{-4}$	0.74
ABTS (g)	235.35	$2.75 \cdot 10^{-4}$	0.76	247.88	$3.98 \cdot 10^{-4}$	0.74
DPPH (Mm)	232.34	$2.51 \cdot 10^{-4}$	0.76	176.77	$3.42 \cdot 10^{-5}$	0.82
DPPH (g)	238.36	$3.01 \cdot 10^{-4}$	0.75	194.80	$6.98 \cdot 10^{-5}$	0.80

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4. Discussion

The obtained data shows the essential oils of *Th. zygis* subsp. *sylvestris*, *O. vulgare* subsp. *virens* and *L. stoechas* subsp. *luisieri* to have high antioxidant activity and elevated antifungal activity for both ABTS and DPPH. Conversely, the essential oils of *Th. mastichina*, *M.* × *piperita* and *L. pedunculata* subsp. *sampaioana* exhibited low antioxidant and anti-fungal activity against the two evaluated fungal species, *P. italicum* and *P. digitatum*.

High antifungal and antioxidant activity from *Th. zygis* has been widely recognized in several studies [56–61]. *Th. zygis* essential oil can have different chemotypes (thymol, carvacrol, carvacrol/thymol, linalool, geranyl acetate/geraniol, …) [58,60–63]. However, only the carvacrol, thymol and carvacrol/thymol chemotypes, have demonstrated elevated antifungal and antioxidant capacity [47,49–53]. The presence of thymol and carvacrol compounds in essential oil from other species of *Thymus* L. genus is well known [33,64–66]. Furthermore, research into the antifungal activity indicates that they have a higher inhibitory capacity for fungal growth than the pure compounds – thymol or carvacrol [57,64]. Regarding *P. digitatum* and *P. italicum* molds, *Th. zygis* subsp. *sylvestris* essential oil has an elevated inhibitory capacity against "in vitro" growth, as observed in other *Penicillium* species [33,36,56].

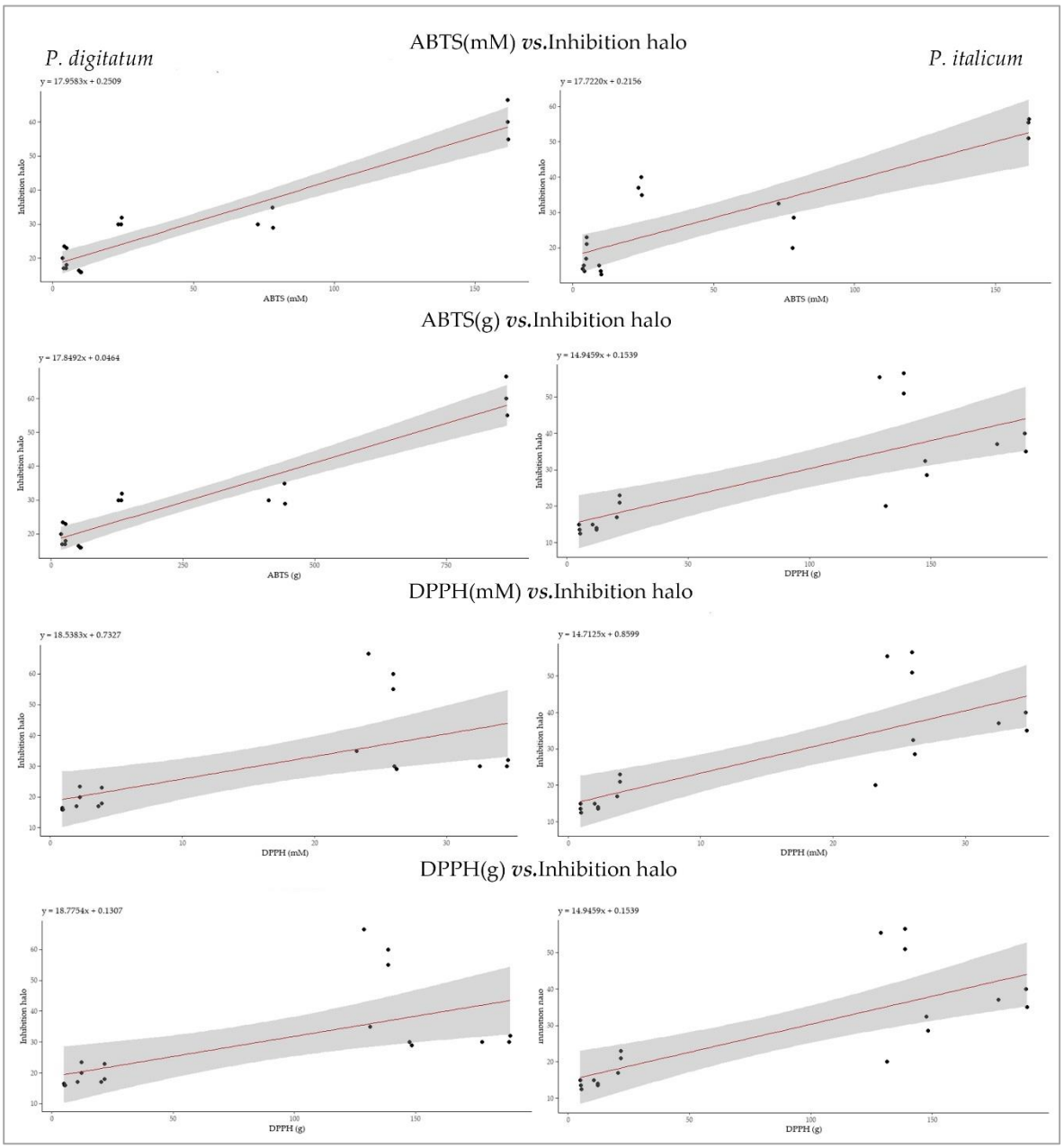

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Figure 4. Scatter plots.

On the other hand, the other thyme species included in this study, *Th. mastichina*, has an essential oil rich in 1,8-cineole [59,67–69], with a poor antioxidant and antifungal capacity against the *Penicillium* species studied. However, other studies have shown it to have good antifungal properties against other fungal species, such as *Sclerotinia* spp., *Fusarium* spp., *Alternaria* spp. or *Candida* spp. [59,70–72]. This makes it possible to use it to fight fungal infections in crops or on the skin.

O. vulgare subsp. *virens* essential oil has a thymol/gamma-terpinene chemotype, which is unusual for this species [73,74]. This coincides with what was observed in research involving the carvacrol chemotype of *O. vulgare*, which exhibits high antioxidant and antifungal activity against *P. digitatum* and *P. italicum* [36,74–78].

The two *Lavandula* L. subspecies studied exhibit different antifungal capacities, with *L. stoechas* subsp. *luisieri* demonstrating greater activity than *L. pedunculata* subsp. *sampaioana* [79], and notably the inhibitory effect on *P. digitatum* growth is higher than on *P. italicum*. The antioxidant activity of *L. stoechas* subsp. *luisieri* essential oil is very high, mainly due to the presence of necrodiol derivatives [80]. On the other hand, *L. pedunculata* subsp. *sampaioana* has an essential oil rich in fenchone, camphor and 1,8-cineole, which are compounds with low antioxidant capacity [59,68].

M. × *piperita* essential oil exhibits the lowest of all the essential oils studied in the present research. However, other studies indicate good inhibitory capacity against several species of the *Penicillium* genus, including *P. digitatum* [81–84]. This divergence in results could be due to variation in the essential oil's chemical composition, including different percentages of menthol, menthone, limonene, alpha-pinene, and betha-pinene, among others.

5. Conclusions

The *Th. zygis* subsp. *sylvestris*, *O. vulgare* subsp. *virens* and *L. stoechas* subsp. *luisieri* essential oils have a high antioxidant capacity and can effectively inhibit the "in vitro" growth of the molds that mainly cause postharvest damages in *Citrus* genus fruits. Furthermore, all the essential oils studied exhibited a higher inhibition response against green mold (*P. digitatum*) than blue mold (*P. italicum*).

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