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Antifungal activity of some botanical extracts on *Fusarium oxysporum*

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Abstract: The present paper describes the antifungal activity of some plant extracts on the development of *Fusarium oxysporum* f.sp. *lycopersici*. The best extracts were selected to be tested as a phytofungicide to control crop diseases, with the ultimate goal of developing a green alternative to synthetic fungicides. Using the conidia germination assay, of the 24 plant extracts tested, 15 reduced conidia germination and 6 completely inhibited germination. Extracts of *Rivina humulis*, *Brassica carinata*, *Brunfelsia calyicina, Salvia guaranitica* and *Punica granatum* showed good antifungal activity. The relationship between total phenolic content (TPC) in each plant extract tested and the percentage of mycelial growth inhibition showed a significant correlation (R^2 = 0.69), while no correlation was found between total flavonoid content (TFC) and percentage mycelial growth inhibition. Among all extracts tested, *Punica granatum* and *Salvia guaranitica* showed the best inhibitory effect against *Fusarium oxysporum* f.sp. *lycopersici*. Our results indicate that plant extracts with a good antifungal activity generally had a high level of total polyphenolic content and titratable acidity, and low values of pH.

Keywords: *Fusarium;* plant extract; antifungal activity; protectant fungicide

1 Introduction

Fungal diseases of crops are usually controlled using resistant cultivars, long rotations, and fumigants, but mainly by using fungicides. The use of synthetic fungicides is not an eco-friendly approach as many are reported to have serious health risks and have been linked to an increased occurrence of several types of cancer. Alternative methods to control fungal diseases have been studied by using compounds derived from plant sources in an attempt to reduce the use of synthetic fungicides. *Agapanthus africans* leaf extracts have shown good antifungal activity [1]. Plant products have proved toxic for a large number of fungal and bacterial pathogens. Soil pathogens such as *Pythium* sp. could be controlled by extracts of *Larrea tridentata* Cov. [2] while *Punica granatum* was effective against *Fusarium oxysporum* [3]. *Allium ursinum* [4] flower extract inhibited mycelial growth of *Aspergillus niger*, *Botrytis cinerea*, *Penicillium gladioli*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum*. The quality and quantity of biologically active compounds from *Allium* species greatly depended on the target species, the plant organ and harvest time. Four plant extracts (*Adhatoda vasica*, *Jatropha curcas*, *Sapindus emarginatus* and *Vitex negundo*) were able to control wilt disease of *Solanum melogena* [5]. *Piper betle* was more effective in controlling *Fusarium* populations in soil than "carbendazim", a commercial fungicide [6]. Extracts of *Allium sativum*, *Coriandrum sativum*, *Curcuma longo and Cuminum cyminum* possessed a strong antifungal activity [7]. Forty plants of different families were tested against *Fusarium oxysporum* f.sp. *cicero*, with *Chenopodium ambrosioides* having the highest inhibition [8]. More recently, the antifungal activity of more than 500 plant species has been assessed [9]. Of all plants tested, only 3% showed a high antifungal activity. Many authors have also studied the importance of secondary metabolites in fungal inhibition. The antifungal activity of *Quillaja saponaria* extract could be due to the presence of saponins and phenolic compounds [10]. The relationship between antifungal activity and total phenolic content

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has also been reported [11-14]. Contrasting results are reported in literature regarding the effect of flavonoids on antifungal activity. Some authors [11,15] found that flavonoids were not correlated with antifungal activity while others [16,17] reported that the inhibition of fungi was mainly due to flavonoids. The aim of the present work was to evaluate the antifungal activity of water extracts of various plant species using phytochemical screening. Total phenolic and flavonoid content, acidity and pH were also determined.

2 Experimental procedures

2.1 Plants for extraction and fungus used

Twenty four different plant species from various plant families were kindly provided by the botanical garden at "La Sapienza University", in Rome (Table 1). The fresh plant material was collected into plastic bags and stored in a freezer at -20°C. *Fusarium oxysporum* f. sp. *lycopersici* (strain CRA-PAV collection n. ER1372) was used as the target fungus. The fungus was maintained on potato dextrose agar (PDA, oxoid cm 0139) and stored at 4°C. When needed, the isolate was grown for 8 days on PDA in the dark at 25 ± 2°C. The conidial suspension obtained was filtered through a double layer of cheesecloth to remove leaf debris and centrifuged at 2500 r.p.m. for 3 min. Conidia were than counted and used at a concentration of 5×10^4 conidia ml^{-1}.

2.2 Preparation of powders and extracts

Fresh material of leaves, bulbs or peel were cut into small pieces and placed together in the solvent (water). The heterogeneous mixture was stirred overnight. The material was sonicated for 3 min (3 s on and 7 s off) and the extract obtained was then centrifuged at 15 000 (r.p.m.) for 10 minutes and the supernatant filtered through a 0.22 µm PTFE membrane. The solvent was vacuum evaporated in a rotatory evaporator, frozen at -80°C for 24 h and finally freeze dried (-40°C; 7×10^{-2} mbar) for 2 days. The powder of the extract obtained was stored in a freezer at -20°C for further use.

2.3 Antifungal screening

2.3.1 Conidia germination assay

A microtiter plate assay was used to rapidly detect the antifungal activity of plant extracts. 200 µL of a mixture containing: 80 µL of conidial suspension, 100 µL of Czapek Dox Broth and 20 µL of plant extract were pipetted into each well of the microtitration plate. One plate row was filled with untreated spore suspension in Czapek Dox Broth as a positive control. Changes in optical density following conidial germination were measured 48 h after inoculation using a microplate reader (Multiscan – Plus MK II, Labsystems OY, Helsinki, Finland) at a wavelength of 405 nm. Conidial germination 24 hours after inoculation was assessed by mounting 10 µl samples on a glass slide and counting the number of germinated spores on a gridded square hemocytometer at 4×10^{-2} mm^2. The percentage germination recorded for the eight wells was averaged. The test was repeated three times.

2.3.2 Mycelial growth inhibition assay

The inhibitory effect of extracts of plant species reported in Table 1 were also tested using cultures in Petri dishes. 200 mg of each powder plant extract were added to 9.8 mL of Potato Dextrose Agar (PDA) and subsequently put into sterile 50 mm diameter Petri plates. In addition, a plate containing a specific standard fungicide (Marisan 50 PB, Dicloran 60%, SIAPA s.r.l., Milano, Italy) was used at the recommended concentration to serve as a negative control to determine the effectiveness of the extracts by comparison. PDA with sterile water served as the control. Antifungal activity tests were performed by placing 5 mm mycelial agar discs cut from the actively growing margin of 8 days old *F. oxysporum* colony in the centre of each plate. Four replicates for each species extract were used and the whole experiment was repeated three times. Radial growth was measured each day, starting 4 days after incubation in the dark at 25°C, until the 6th day. The percentage growth inhibition of each extract was calculated by the formula: % inhibition = [growth in control - growth in sample/ growth in control] × 100.

2.4 Determination of total phenolic and flavonoids content

Total phenolic content of all plant extracts was determined by the Folin-Ciocalteu method [18]. 20 µL of each extract solution were transferred into separate tubes, which were then added with 1.58 mL of ultra-pure water. 100 µL of the Folin-Ciocalteu reagent was then added to the mixture, mixed well and left for 8 min. After that, 300 µL of 2% sodium carbonate was added, tubes were uncapped and shaken two seconds on a vortex and left in the dark for 1 h at room temperature. Measurement was conducted on

Table 1. Plant species used in the experiments.

Genus	Species	Family	Common name	Parts used
Allium	*sativum*	Alliaceae	Garlic	Bulb
Allium	*triquetrum*	Alliaceae	Angled onion	Leaves
Antholyza	*aethiopica*	Iridaceae	Cobra lily	Leaves
Arctium	*lappa*	Asteraceae	Greater burdock	Leaves
Boehmeria	*nivea*	Urticaceae	Ramie	Leaves
Brassica	*carinata*	Brassicaceae	Ethiopian mustard	Seeds
Brunfelsia	*calycina*	Solanaceae	Yesterday-today-tomorrow	Leaves
Campsis	*radicans*	Bignoniaceae	Trumpet vine	Leaves
Celtis	*glabrata*	Cannabaceae	Hackberry	Leaves
Citrus	*limon*	Rutaceae	Limon	Leaves
Coffea	*arabica*	Rubiaceae	Coffea arabica	Leaves
Conium	*maculatum*	Apiaceae	Poison Hemlock	Leaves
Cycas	*revoluta*	Cycadaceae	Sago cycad	Fruit
Lavandula	*multifida*	Lamiaceae	Fernleaf lavender	Leaves
Mallotus	*japonicus*	Euphorbiaceae	Japanese mallotus	Leaves
Petrea	*volubilis*	Verbenaceae	Sandpaper vine	Leaves
Philodendron	*crassinervium*	Araceae	Thick-nerved Philodendron	Leaves
Polygonatum	*odoratum*	Asparagaceae	Angular Solomon's seal	Leaves
Punica	*granatum*	Lythraceae	Pomegranate	Peel
Rivina	*humilis*	Phytolaccaceae	Pigeonberry	Leaves
Salvia	*guaranitica*	Lamiaceae	Anise-scented sage	Leaves
Strelitzia	*reginae*	Strelitziaceae	Bird of paradise	Leaves
Taraxacum	*officinale*	Asteraceae	Dandelion	Leaves
Yucca	*elephantipes*	Asparagaceae	Giant yucca	Leaves

a spectrophotometer (Varian Cary 100 Conc UV-Vis) at λ = 760 nm against a ultra-pure water as blank. Gallic acid was used as a standard phenolic compound to make the calibration curve that ranges between 0 to 500 mg/L (r^2 = 0.9913). The results are expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw) of lyophilized plant extract.

Flavonoid content was estimated using the $AlCl_3$ method [19]. 0.5 mL of each extract was taken, and 1.5 mL of methanol was added. 0.1 mL of 10% $AlCl_3$ and then 0.1 mL of 1M Potassium acetate was added to the reaction solution. The volume of the solution was made up to 5 ml with distilled water and the reaction mixture was incubated at room temperature for 30 minutes. Absorbance was read at 415 nm at the UV-Vis spectrophotometer. A calibration curve was generated, using Rutin as a standard flavonoid compound, from 5 to 100 mg/L (r^2 = 0.9969). Total flavonoid content was expressed as Rutin Equivalent (mg/L) of the extract. The experiment was repeated twice.

2.5 Acidity and pH analysis

Acidity was determined by titration with a 0.01 N alkaline sodium hydroxide solution. Phenolphthalein (1%) was used as the indicator (2 drops in 20 mL of each sample before starting the analysis). Sodium hydroxide was added dropwise with constant swirling until the solution turned pink throughout. The volume of base required to reach the equivalence point was used to calculate the acidity of the extracts expressed in meq NaOH/g. The pH value of each extract was determined with a Hamilton pH electrode sensor. All measurements were repeated twice within a period of 10 days.

2.6 Statistical analysis

A randomized experimental design was used. Statistical analysis ANOVA was carried out and mean values compared by Fisher's protected LSD test at $P \leq 0.05$. SigmaPlot version SPW10 and Sigma Stat version 3.5 were used to create graphics.

3 Results

3.1 Conidia germination assays

Analysis of optical density and conidia germination showed a high correlation between difference of absorbance (DA) at 48 h and the percentage of conidia germination observed after 24 h (Table 2). Plant extracts with a DA < 0.04 showed no or very low conidia germination. The extracts with a DA ≤ 0.3 showed a percentage higher than 70; when the DA was > 0.4, the percentage rose over 80. In the present assay, out of the 24 plant extracts tested, 15 were able to reduce conidia germination and 6 completely inhibited germination.

3.2 Mycelial growth inhibition assays

The extracts of *R. humulis*, *B. carinata*, *B. calyicina* and *S. guaranitica* showed a high antifungal activity: 4 days after inoculation, the mycelial growth was 33.6, 33.3, 28.2 and 26.0 mm respectively. In contrast, the nontreated control had mycelial growth of 42.9 mm (Table 3). The highest antifungal activity was recorded in *Punica granatum* extract, where the radial growth was 16.7 mm, even lower than with Marisan 50 PB, the synthetic fungicide (17.8 mm), though the data are not statistically significant. Fungal growth inhibition (Fig. 1) showed that from the 5th day the mycelium covered the whole control plates. The percentage of inhibition over control in *P. granatum* was 62.77 (4th day), 48.53 (5th day), 42.71 (6th day). These data were followed by *S. guaranitica* extract 39.35; 23.35, 12.74, *B. carinata* 33.14, 26, 14.7 and *B. calycina* 31.7, 27.62, 22.06.

3.3 Total phenolic and flavonoids content, acidity and PH analysis

Total phenolic content (TPC) and flavonoid content (TFC) varied widely among plant extracts (Table 3). TPC, expressed as mg GAE/g DW, ranged from 8.29 mg GAE/g DW in *C. radicans* to 542 mg GAE/g DW in *P. granatum*. TFC, expressed as mg RE/g DW, ranged from 2.71 in *P. crassinervium* to 102.76 in *P. granatum*.

There was a significant correlation (R^2 = 0.69) between TPC content in each plant extract tested and

Table 2. Difference of absorbance and percentage of conidia germination of 24 plant extracts. Values are the mean of four replications. Means in the same column followed by same letter are not statistically different at P = 0.05 according to the Fisher LSD Method.

Plant species	Difference of absorbance 48 h after inoculation	Percentage of conidia germination 24 h after inoculation
Control	0.41a	80a
Allium sativum	0.00d	0e
Allium triquetrum	0.04d	5e
Antholyza aethiopica	0.44a	≥ 80a
Arctium lappa	0.03d	15d
Boehmeria nivea	0.45a	≥ 80a
Brassica carinata	0.01d	0e
Brunfelsia calycina	0.01d	0e
Campsis radicans	0.41a	75a
Celtis glabrata	0.00d	5e
Citrus limon	0.00d	10de
Coffea arabica	0.04d	5e
Conium maculatum	0.53a	75a
Cycas revoluta	0.02d	10de
Lavandula multifida	0.29b	70ab
Mallotus japonicus	0.93a	≥ 80a
Petrea volubilis	0.00d	0e
Philodendron crassinervium	0.33b	70ab
Polygonatum odoratum	0.47a	≥ 80a
Punica granatum	0.03d	0e
Rivina humilis	0.00d	5e
Salvia guaranitica	0.04d	0e
Strelitzia reginae	0.50a	≥ 80a
Taraxacum officinale	0.26b	55cb
Yucca elephantipes	0.10c	65b

Table 3. Effect on mycelia growth of *F. oxysporum* (four days after inoculation) and TPC, TFC, total acid, and pH of some plant extracts tested at a concentration of 1%.

Plant species	Mycelia growth		TPC	TFC	Acidity	pH
	mm		*mgGAE/g DW*	*mgRE/g DW*	*meq NaOH/g*	
No treated (positive control)	42.9	a	-	-	-	-
Fungicide (negative control)	17.9	i	-	-	-	-
Allium sativum	34.7	ed	10.14	5.43	0.120	6.57
Allium triquetrum	42.2	a	29.85	15.12	0.248	6.27
Antholyza aethiopica	*		43.29	21.14	0.284	5.78
Arctium lappa	40.1	b	59.85	7.02	0.284	5.70
Boehmeria nivea	*		35.86	9.84	0.212	6.28
Brassica carinata	33.4	e	129.57	31.53	0.540	5.98
Brunfelsia calycina	29.2	g	136.71	10.94	0.344	5.28
Campsis radicans	*		8.29	1.53	0.300	6.24
Celtis glabrata	41.0	b	93.14	17.69	0.104	8.10
Citrus limon	40.0	b	133.57	36.71	0.280	6.08
Coffea arabica	38.5	c	106.57	33.02	0.220	5.82
Conium maculatum	*		33.28	9.69	0.192	6.23
Cycas revoluta	42.1	a	50.14	11.43	0.152	6.18
Lavandula multifida	40.7	b	95.10	14.30	0.916	4.75
Mallotus japonicus	38.1	c	193.70	54.87	0.700	5.15
Petrea volubilis	36.5	d	187.71	59.94	0.416	5.66
Philodendron crassinervium	*		13.42	2.71	0.220	6.5
Polygonatum odoratum	*		46.88	21.28	1.240	5.6
Punica granatum	16.7	i	542.50	102.76	1.376	4.08
Rivina humilis	33.6	e	87.28	10.51	0.292	5.79
Salvia guaranitica	26.0	h	210.28	44.20	0.480	5.94
Strelitzia reginae	*		27.86	8.35	0.200	6.57
Taraxacum officinale	41.1	b	84.85	15.79	0.148	6.93
Yucca elephantipes	*		52.70	16.61	0.588	5.66

*=Mycelial growth is greater than control. Values are the mean of four replications. Means in the same column followed by same letter are not statistically different at P = 0.05 according to the Fisher LSD Method.

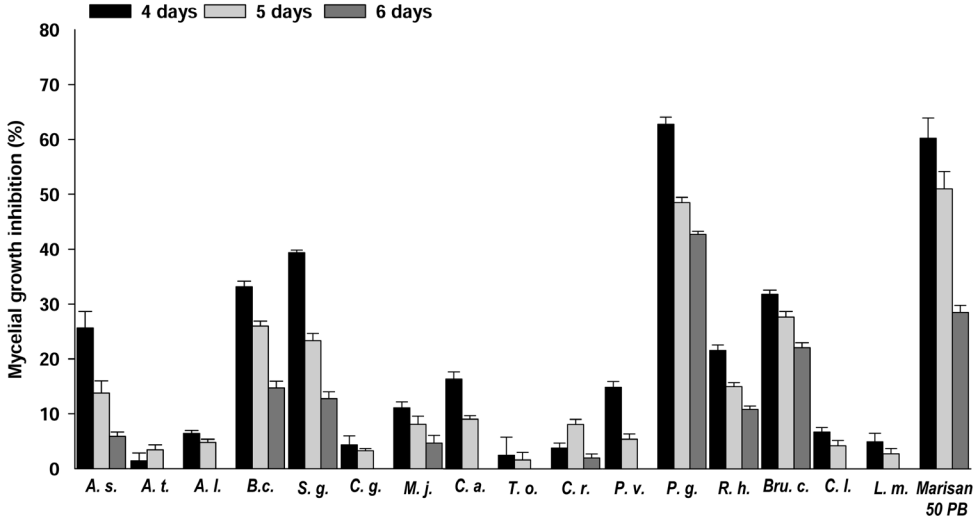

Figure 1. Percentage of mycelial inhibition of *F. oxysporum* f.sp *lycopersici* observed with water extracts of some plant species tested at concentration of 1%. From the 5th day the mycelium covered the whole plate in the non-treated plates. Abbreviations: *Allium sativum* (A.s.), *Allium triquetrum* (A.t.), *Arctium lappa* (A.l.), *Brassica carinata* (B.c.), *Salvia guaranitica* (S.g.), *Celtis glabrata* (C.g.), *Mallotus japonicas* (M.j.), *Coffea arabica* (C.a.), *Taraxacum officinale* (T.o.), *Cycas revoluta* (C.r.), *Petrea volubilis* (P.v.), *Punica granatum* (P.g.), *Rivina humulis* (R.h.), *Brunfelsia calycina* (Bru.c.), *Citrus limon* (C.l.), *Lavandula multifida* (L.m.)

the percentage of mycelial growth inhibition (Fig. 2a). In most of the extracts tested, TPC is positively correlated with antifungal activity, except for *A. sativum* and *M. japonicus* (Table 3). The extracts of *A. sativum* showed a significant antifungal activity (31.9 mm) but low values of phenolic content (10.14 mg GAE/g DW), while *M. japonicus* showed a low antifungal activity (38.1 mm) but high value of phenolic content (193.7 mg GAE/g DW). No significant correlation ($R^2 = 0.45$) was found between flavonoid compounds and the percentage of mycelial growth inhibition (Fig. 2b). We did not find any direct relationship with the inhibitory effect for titratable acidity and pH values in our experiment. However, the extracts with the highest inhibitory effects (*Punica granatum, Salvia guanaritica, Brassica carinata* and *Brunfelsia calycina*) have high values of TPC and acidity, and low values of pH (Table 3).

4 Discussion

Conidia germination assays of plant extracts that show low values of DA, have a very low percentage of conidia germination. In extracts with DA values close to zero, there is no germination.

Fourteen plant extracts were able to reduce the radial growth of *F. oxysporum* f.sp. *lycopersici*, compared to the non-treated control. The efficiency of *Rivina humulis* could be due to the presence of alkaloids, flavonoids and resin, that are known to be bioactive compounds against bacterial and fungi [20]. *Brassica carinata* has a high content of glucosinolates that, after enzymatic-catalysed hydrolysis, produce cytotoxic compounds with antifungal activity. The mechanisms of action are still not clear, but the S-containing compounds, such as carbon disulfide, dimethyl disulfide, dimethyl sulfide and methanethiol produced during degradation of glucosinolates, could have an important role in suppression of fungi. *Brunfelsia calycina* belongs to the *Solanaceae*, a family with a very good source of alkaloids, flavonoids, saponins, tannins, and glycosides. *Salvia guaranitica* extract has a high sesquiterpene content [21] which occurs as hydrocarbons or in oxygenated forms. Sesquiterpenes are considered by some authors [22] to significantly inhibit mycelial growth and spore germination of *F. oxysporum*, and this antifungal activity is based on the permeability of the cellular walls of fungi. It is also known that bioactivity of sesquiterpenes is mainly due to their reactions with –SH group of amino acids, proteins and enzymes. Moreover, in the Lythraceae some phenolic compounds, like punicalagin and ellagic acid, may be responsible for inhibiting fungal mycelial growth. There is no significant difference between the inhibitory effects of *Punica granatum* and the standard fungicide (Marisan 50PB) at the 4th and 5th day, while at the 6th day the percentage of inhibition in *P. granatum* is significantly higher than with the fungicide. This may be because the antifungal compounds of the pomegranate extract, although they are natural, are more persistent than those contained in the chemical fungicide.

The clear positive correlation found between TPC and antifungal activity could be due to the water used in the extraction, a polar solvent able to extract many polyphenol compounds from the plants. From chemical analysis, the antifungal activity could be due, at least partly, to the presence of polyphenol compounds that are usually the major antifungal compounds of most plant

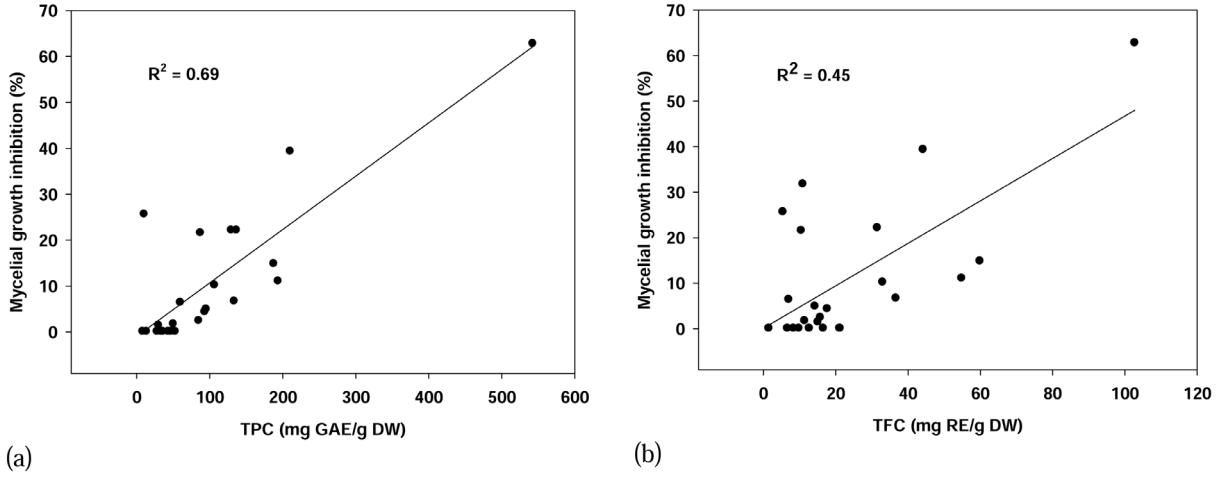

Figure 2. Linear correlation between the total phenol content (TPC) and mycelial growth inhibition (a); and between total flavonoid content (TFC) and mycelial growth inhibition (b).

extracts. The high antifungal activity of *Zizyphus spina-christ* extract is due to phenolic compounds [12,14,23]. These bioactive polyphenol compounds, singly or in combination, interfere with the life process of fungi by binding their protein molecules, acting as chelating agents, altering structural component synthesis, weakening or destroying the permeability barrier of the cell membrane and changing the physiological status of the cells.

In our study we found no correlation between flavonoids and percentage of mycelial growth inhibition. These findings are in general agreement with some previous studies. However, the effects of flavonoids on phytopatogenic fungi are not well documented. A few studies indicate that flavonoids are not correlated with antifungal activity [11] or can stimulate spore germination [15], while other authors found that extracts of some plant species are able to inhibit fungi and bacteria and that their ability is mainly due to flavonoids [16,17]. Only 2 flavonoids, pisatin and medicarpin, have been shown to be active against *F. oxysporum*. The lack of relationship that we find between TFC and antifungal activity could be due to the absence of pisatin and medicarpin in the extracts tested, or to the predominance of flavonoids that can stimulate fungal growth. These results are in agreement with [24] and [25] who found that inhibition effects were higher when polyphenols were in combination with organic acids. Similarly, pH may have, in general, a great impact on the antimicrobial activity of various phenolic compounds [26].

We have identified extracts from six plant species belonging to six different families (*Alliaceae*, *Brassicaceae*, *Lythraceae*, *Lamiaceae*, *Solanaceae* and *Verbenaceae*), showing a good level of antifungal activity against *F. oxysporum* f.sp. *lycopersici*, completely inhibiting conidial germination. *Punica granatum* and *Salvia guaranitica* seem to have the best inhibitory effect. Plant extracts with a good antifungal activity generally have high concentrations of total polyphenolic content and high levels of titratable acidity. Further studies are needed with the aim of purifying and characterizing the polyphenolic compounds of the plant species tested and of promoting their use in agriculture to reduce fungicide applications. This work allowed us to select the best extract which may be used as a phytofungicide to control crop diseases, with the ultimate goal of developing a green alternative to synthetic fungicides.

Conflict of interest: Authors declare nothing to disclose.

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