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Received November 14, 2013
Revised February 25, 2014
Accepted March 19, 2014

Research Article

Phenolic characterization and antimicrobial activity of folk medicinal plant extracts for their applications in olive production

Phytophthora spp is important in plant pathology due to the importance of the diseases it causes. In olive trees, severe damages are caused by the disease known as "dry branch" occasioned by *Phytophthora nicotianae*, *P. citrophthora* and *P. palmivora*. Much effort has been made to find efficient methods of control, with a low negative impact on environment. In this regard, treatment with plant extracts is a valid strategy. The aims of the present study are (i) to determine the polyphenol composition of extracts of *Thymus vulgaris*, *Origanum vulgare*, *Matricaria recutita*, and *Larrea divaricata* by CZE, (ii) correlate the analytical composition of these extracts with the inhibition on the mycelial growth, and (iii) determine the individual antimicrobial activity of the most active ingredients. A simple methodology was developed for the determination of catechin, naringenin, cinnamic acid, syringic acid, chlorogenic acid, apigenin, vanillic acid, luteolin, quercetin, and caffeic acid in plant extracts by CZE. The extraction of phenolic compounds in extract was performed by a miniaturized solid phase extraction using a home-made minicolumn packed with suitable filtering material (C_{18}, 50 mg). The optimized analyses conditions were: 30 mM boric acid buffer, pH 9.50; capillary, 57 cm full length, 50 cm effective length, 75 μm id, hydrodynamic injection 30 mbar, 2 s; 25 kV; 25°C, detection by UV absorbance at 290 nm. Sample results suggest that phenolic composition seems to have a great influence on inhibition of pathogens. The highest inhibitions of mycelial growth were observed for cinnamic acid and naringenin.

Keywords:
Capillary Electrophoresis / Medicinal Plant Extracts / Olive / Phenolic Compounds / *Phytophthora*
DOI 10.1002/elps.201300562

1 Introduction

Pathogens belonging to the genus *Phytophthora* are worldwide responsible for the most destructive and economically important agricultural problems [1–6].The species of *Phytophthora* are some of the most abundant soil pathogens responsible for significant losses in numerous crops affecting roots, foliage, and fruits [7]. These pathogens can infect both via the root and the canopy, causing roots and the basal part of the stem to rot, especially in woody species such as olive [8, 9].

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Abbreviations: API, apigenin; **CAF**, caffeic acid; **CAT**, catechin; **CIN**, cinnamic acid; **CLH**, chlorogenic acid; **%IPc**, percentage of inhibition of *Phytophthora citrophthora*; **%IPn**, percentage of inhibition of *Phytophthora nicotianae*; **%IPp**, percentage of inhibition of *Phytophthora palmivora*; **LUT**, luteolin; **NAR**, naringenin; **PCA**, principal component analysis; **QUE**, quercetin; **SYR**, syringic acid; **TPC**, total phenolic compounds; **VAN**, vanillic acid

In olive trees severe damages are caused by the disease known as "dry branch" caused by *Phytophthora nicotianae*, *P. citrophthora* and *P. palmivora* [10]. The name "Dry Branch" has been used to distinguish it from other diseases, where the tree canopy looks like *Verticillium* wilt or insect damages [10, 11]. Diseased plants show rootlets death, leaves yellowing, or branch defoliation [12]. Moreover, this disease is responsible of plant death and significant losses in olives and oil production [13, 14].

Until now chemical control is the most effective strategy used to protect crops against these pathogens. The main products used to handle the diseases are metalaxyl, fosetyl-Al and copper-based fungicides [2, 15, 16]. However, this method increases production costs, generates environmental damage, the selection of resistant pathogen strains, and the presence of toxic residues in the foods [16–22]. Furthermore, the increasing public demand for organically grown produce, and the intended phasing out by the EU (European Union) of the use of copper-containing formulations, has precipitated an urgent need for alternative control methods [5]. These facts motivate the search for new control alternatives such as the use of essential oils, plant extracts, or biological control agents [5, 23–27].

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Nowadays a great attention has been paid to plant extracts, because they constitute a rich source of bioactive chemicals such as polyphenols, flavonoids, quinons, tannins, alkaloids, saponins, and sterols [27–31]. The antioxidant and antimicrobial activity of many plant extracts have been associated with the presence of polyphenols; especially phenolic acids and flavonoids [27, 30, 32–34].

Phenolic compounds are one of the most important groups of compounds occurring in fruits, vegetables, nuts, seeds, leaves, and flowers, where they are widely distributed, comprising of at least 8000 different known structures. They are synthesized by plants during normal development and as a response to different biotic or abiotic stress situations [35].

Sample preparation represents an especially important step in the analytical process for the analysis of phenols in plant extracts. Effective extraction and preconcentration of phenolic constituents from the aqueous extracts matrix is essential for their accurate analysis. Many recent studies have been reported as an alternative to the traditional extraction. Therefore, sample preparation methods have been significantly improved towards: (i) use of smaller initial sample sizes, small volumes or no organic solvents; (ii) greater selectivity in extraction; (iii) minimum sample preparation steps; (iv) better reproducibility and recovery; (v) good accuracy; (vi) higher throughput sample; and, (vii) minimum cost per analysis [36–38]. Traditional sample preparation techniques such as liquid–liquid extraction (LLE) suffer from limitations including low sample throughput, large volume of organic solvent consumption, and potential loss of analytes during extraction. In contrast, solid-phase extraction (SPE) is still the most widely used technique due to its ease of use and wide-ranging applicability [39, 40].

The separation and quantification of phenolic compounds in plant extracts has been mainly determined routinely by reversed phase (RP)-HPLC with UV detection [39, 40]. Capillary electrophoresis (CE) has become a valuable alternative to HPLC due to its versatility and high separation efficiency [41–43]. CE has several advantages over HPLC: (i) very small sample size requirement, (ii) high efficiency (iii) shorter analysis time, and (iv) lower cost. [44, 45]. The use of capillary zone electrophoresis (CZE) for the analysis of the phenolic profile of plant extracts can have benefits in terms of robustness and ruggedness, versatility, cost, and time [42, 46, 47].

Plants traditionally used in folk medicine due to their antifungal and/or antiseptic properties were selected for the present study; *Thymus vulgaris* (common thyme), *Origanum vulgare* (oregano), *Matricaria recutita* (chamomile), and *Larrea divaricata* (creosote bush). The aims of the present study were (i) to determine the polyphenol composition of extracts obtained from leaves of *T. vulgaris, O. vulgare, M. recutita,* and *L. divaricata* by CZE, (ii) correlate the analytical composition of the extracts with the in vitro impact of studied extracts on the mycelial growth of *P. nicotianae*, *P. palmivora*, and *P. citrophthora*, and (iii) determine the individual antimicrobial activity of the most active ingredients.

2 Materials and methods

2.1 Chemicals and standards

Phenolic compounds (catechin, naringenin, cinnamic acid, syringic acid, chlorogenic acid, apigenin, vanillic acid, luteolin, quercetin and caffeic acid), constituents of BGE (30 mM boric acid buffer) and organic modifiers (ACN and methanol (MeOH)) were purchased from Sigma (St. Louis, MO).

Ultrapure water (resistivity 18.3 MΩ cm) obtained from Barnstead EASY pure RF water system (Iowa) was used to prepare solutions including the electrolyte solutions. Millex-HV Syringe Filters (0.22 μm pore size) were purchased from Millipore.

2.2 Solvents and reagents

Standard stock solutions of the analytes were prepared by dissolving an appropriate amount of each pure substance in 10 mL of a HPLC-grade methanol to obtain a final concentration of 1000 mg/L. The resulting solutions were stored at 4°C in amber glasses.

Working standard solutions at a 5 mg/L concentration were prepared on a daily basis by diluting appropriate aliquots of the previous standard stock solutions in methanol. Before use, all solutions were degassed by sonication for 5 min and filtered through 0.22 μm nylon filters.

2.3 Plant extraction and purification

The extracts were prepared from leaves (20 g) of chamomile, oregano, common thyme, and creosote bush using the methodology proposed by Widmer and Laurent [16] with modifications. Then, the leaves of each plant were placed into Erlenmeyer flasks containing 200 mL of distilled water. The flasks were closed with a cotton ball, covered with aluminum foil, and autoclaved for 45 min at 121°C at 1 atm. The liquid was filtered through a surgical drape into a sterile beaker and the volume was reduced by boiling in laminar-flow hood to an approximate volume of 20 mL. The extract was centrifuged to remove any solids. The supernatant was placed into sterile tubes and autoclaved for 20 min at 121°C at 1 atm. Then the extracts were stored at 4°C.

The extract samples (1g/L) were thoroughly mixed with five parts (1:5, w/v) of acidic water until complete homogenization. An aliquot of 250 μL of each extract was used for extraction by SPE. The extraction of phenolic compounds in the extracts was performed by a miniaturized SPE using a home-made column packed with suitable filtering material. C18 cartridges (50 mg) were made in 1 mL syringes using 25 mg of glass wool as frits. These cartridges were placed in a vacuum elution apparatus (Varian VacElut 20 manifold and a Vacuubrand vacuum pump ME 2C) and preconditioned by passing 5 mL of methanol and 5 mL of acid water (water pH 2 with HCl). The samples were loaded onto the

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preconditioned column, leaving the sample on the solid phase under vacuum. Then, the column was washed with 1 mL of acid water (water pH 2, with HCl). The phenolic fraction was eluted with methanol (500 μL). The eluent was directly injected and analyzed by CZE.

2.4 Phenolic compounds in plant extracts by CZE

Phenolic acids were separated in a BGE consisting of a 30 mM boric acid solution at pH 9.5 that was prepared by weighing the required amount of boric acid and adjusting its pH with a few drops of sodium hydroxide.

All solutions and buffers were degassed by sonication for 5 min before use in order to avoid changes during ionization and ensure acceptable reproducibility. pH measurements were made with a Altronix model TPX-I pH meter furnished with a combined glass electrode.

CE analysis of all standards and extract samples were performed on a Capel 105 system (Lumex, St Petersburg, Russia) equipped with an automatic autosampler and a UV–vis absorbance detector. The data were collected on a PC configured with Elforun software version 3.2.2. The capillary columns used for separation were bare fused-silica capillaries with a full length of 57 cm, 50 cm effective length, 75 μm id, 375 μm OD from MTC MicroSolv Technology (Eatontown, NJ).Samples were introduced to the capillary by hydrodynamic injection at 30 mbar for 2 s. Direct UV detection was performed at 290 nm. All operations were carried out at 25°C. Before first use, fused-silica capillaries were washed (1000 mbar) with water (5 min), 0.1M NaOH for 5 min, followed by water for another 2 min and finally with the buffer for 5 min. At the start of each working day, the capillary was rinsed with 0.1 M NaOH for 10 min then with water for 10 min. Between runs, the capillary was flushed with water (5 min), 0.1 M NaOH (2 min), water (2 min) and fresh buffer (2 min). At the end of the day the capillary was washed for 5 min with 0.1 M NaOH, followed by 5 min with water, and stored overnight in water.

2.5 Antimicrobial activities of plant extracts by solid agar bioassay

The isolates of *P. nicotianae, P. palmivora*, and *P. citrophthora* were obtained from the microorganism's collection of the Department of Plant Pathology at the Faculty of Agricultural Sciences in Luján de Cuyo, Mendoza. The isolates were recovered from necrotic roots of young olive trees in Mendoza and were maintained on clarified V8 agar (V8A; commercial V8 juice, 200 ml; $CaCO_3$, 2 g; agar, 17 g; and distilled water, 800 ml).

P. nicotianae, P. palmivora, and *P. citrophthora* were grown on V8 agar plates at 24°C for 5 days. Individual agar disks (4 mm in diameter) were removed from the edge of an actively growing culture of each pathogen and placed at the center of each Petri dish (5.5 cm in diameter) containing V8A amended with a test extracts at different concentrations (1.5, 10, 50, 100, 200, 300 and 500 mg/mL). The extracts were added to V8A after autoclaving when the agar had cooled to approximately 60°C. Control plates were run simultaneously, using the growth medium without extracts. Three replicate plates of each of extracts and for each concentration as well as control were prepared. The experiment was repeated once. The Petri plates were kept at 25 ± 2°C for 4 days. After the incubation period the test was considered concluded. In order to evaluate the mycelial growth, inhibition was determined as the mean colony area. These mean growth values were converted into the inhibition percentage of mycelial growth related to the control treatment by using the formula of Mine Soylu and Kurt [24]:

$$\text{Inhibition Percentage} = \left(c - {}^{t}\!/_{t}\right) \times 100\,, \quad (1)$$

where c is control mean colony area, and t is treated mean colony area.

2.6 Individual Effects of selected phenolic compounds on mycelial growth

The effect of the pure phenolic compounds showing positive correlation with the antimicrobial activity against *Phytophthora* spp was evaluated. For this purpose, the solid agar bioassay described above was performed. In this case, a 4 mm disk of culture medium, containing mycelium of *P. nicotianae, P. palmivora*, or *P. citrophthora* was placed in V8A amended with different concentrations of phenolic compounds. Control plates were run simultaneously, using the growth medium without phenolic compounds. Three replicate plates of each of phenolic compound and for control were prepared. The Petri plates were kept at 25 ± 2°C for 4 days. After the incubation period the test was considered concluded. Mycelial growth inhibition was determined as described above.

2.7 Statistical analysis

Statistical analysis was performed by ANOVA, and means were compared using Tukey test. All the analyses were done in triplicate. The results were significant at $P < 0.05$ unless specified otherwise. Statistical analyses were carried out using InfoStat-Statistical Software. The principal component analysis (PCA) was used to find the relationships between phenolic compounds and percentage of inhibition of *Phytophthora* spp. Statistical analyses were carried out using InfoStat-Statistical Software and Statgraphics Centurion XVI.II.

3 Results and discussion

3.1 CE methodology development

With a view to establishing the best possible compromise between sensitivity, resolution, and analysis time in the

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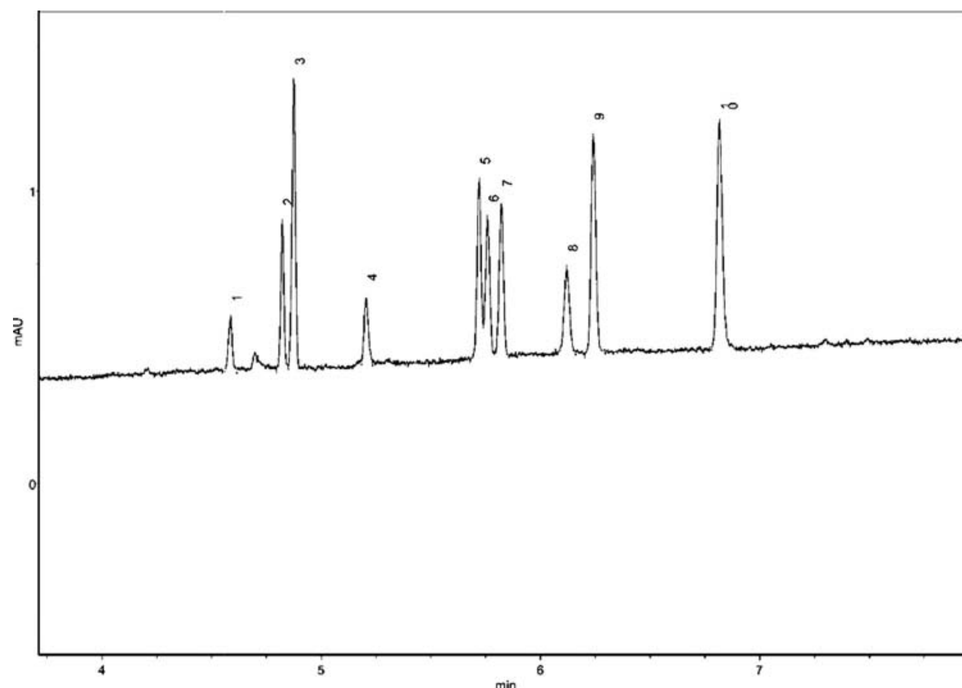

Figure 1. CZE-UV electropherograms of standard mix at 290 nm; catechin (1), naringenin (2), cinnamic acid (3), chlorogenic acid (4), syringic acid (5), luteolin (6), apigenin (7), quercetin (8), vanillic acid (9), caffeic acid (10).

separation of all analytes, the following parameters were consecutively optimized: BGE composition and concentration, injection volume and mode, and other electrophoretic parameters such as electrophoretic separation voltage, and capillary temperature and conditioning.

Taking into consideration the acidic nature of the analytes under study, the effect of the buffer pH was investigated within the range of 8.00/10.00 at a fixed buffer concentration, adjusted by 0.10 mol/L NaOH and 0.01 mol/L HCl. At pH 9.50 baseline separation was achieved.

Sodium tetraborate and boric acid were tested as BGEs, but the one producing the best results considering selectivity, reproducibility, baseline, and current performance, was boric acid. Keeping other parameters constant (pH: 9.50, 25 kV, 25°C) the buffer concentration was varied from 10 to 75 mM. Increases in migration times as well as current were observed when the concentration of buffer increased. Resolution also increased for higher buffer concentrations, but no appreciable improvements were observed for buffer concentrations above 30 mM. So, optimal separation was obtained with 30 mmol/L boric acid buffer, pH 9.5.

The effect of the applied voltage was studied over the range 5–35 kV. A potential of 25 kV was found to provide the best results in terms of run-time and resolution between peaks.

The effect of temperature on electrophoretic separation was examined over the range 15–30°C. A temperature of 25°C was selected as optimal because it provided the best compromise between migration time and peak resolution. In fact, raising the capillary temperature reduced migration time through a decreased electrolyte viscosity, but also led to lower resolution.

The injection mode giving the best response concerning reproducibility and linear range was the hydrodynamic mode. Injection parameters were optimized by varying the lengths of sample (2–8 s) and pressure injection until optimal conditions were reached. The best results were obtained for the following experimental parameters: hydrodynamic injection mode 30 mbar, 3 s.

Figure 1 shows a typical electropherogram for the standard mixture solution under the optimum conditions. The electropherograms were monitored at 290 nm. The peaks were baseline separated within about 7 min.

In order to determine the repeatability of the methodology, replicate injections ($n = 6$) of a standard mixture solution (2.00 μg m/L for each analyte) under the selected optimum conditions were carried out. The intraday %RSDs of the migration time, corrected area (peak area/tr), were between 0.90 and 2.20, 1.90, and 4.80 respectively. The interday values for the same performance criteria were 0.90–2.20 and 1.60–4.90 respectively.

Calibration curves for the determination of the ten compounds were constructed under the optimum conditions. Six concentrations were used for each calibration curve. As shown in Table 1, most of the correlation coefficients of area ratio equations were >0.96. The LODs and LOQs were evaluated on the basis of S/N of 3 and 10, respectively.

3.2 Phenolic composition of plant extracts

The optimized CZE method was then applied to determine the phenolic compounds in plant extracts of *T. vulgaris* (common thyme), *O. vulgare* (oregano), *M. recutita* (chamomile),

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Table 1. Results of regression analysis on calibration and the detection limits

Compound	Regression equation $y = a + bx$	Correlation coefficient R	LOD (mg/mL)	LOQ (mg/mL)	tm ± SE*
Catechin	$y = 0.173x - 0.080$	0.926	2.355	7.849	4.618 ± 0.004
Naringenin	$y = 1.244x + 0.391$	0.989	0.106	0.354	4.882 ± 0.005
Cinnamic acid	$y = 5.258x + 0.561$	0.987	0.002	0.007	4.907 ± 0.003
Chlorogenic acid	$y = 2.366x - 0.915$	0.988	0.012	0.040	5.222 ± 0.004
Quercetin	$y = 2.609x + 0.930$	0.986	0.019	0.065	5.754 ± 0.002
Vanillic acid	$y = 1.350x - 0.183$	0.983	0.063	0.208	5.826 ± 0.010
Luteolin	$y = 3.639x - 0.396$	0.983	0.006	0.021	5.854 ± 0.010
Syringic acid	$y = 2.479x - 0.622$	0.987	0.014	0.046	6.150 ± 0.020
Apigenin	$y = 2.128x - 0.923$	0.989	0.029	0.096	6.327 ± 0.002
Caffeic acid	$y = 3.375x - 1.136$	0.968	0.010	0.035	6.838 ± 0.010

*tm ± standard error (SE)

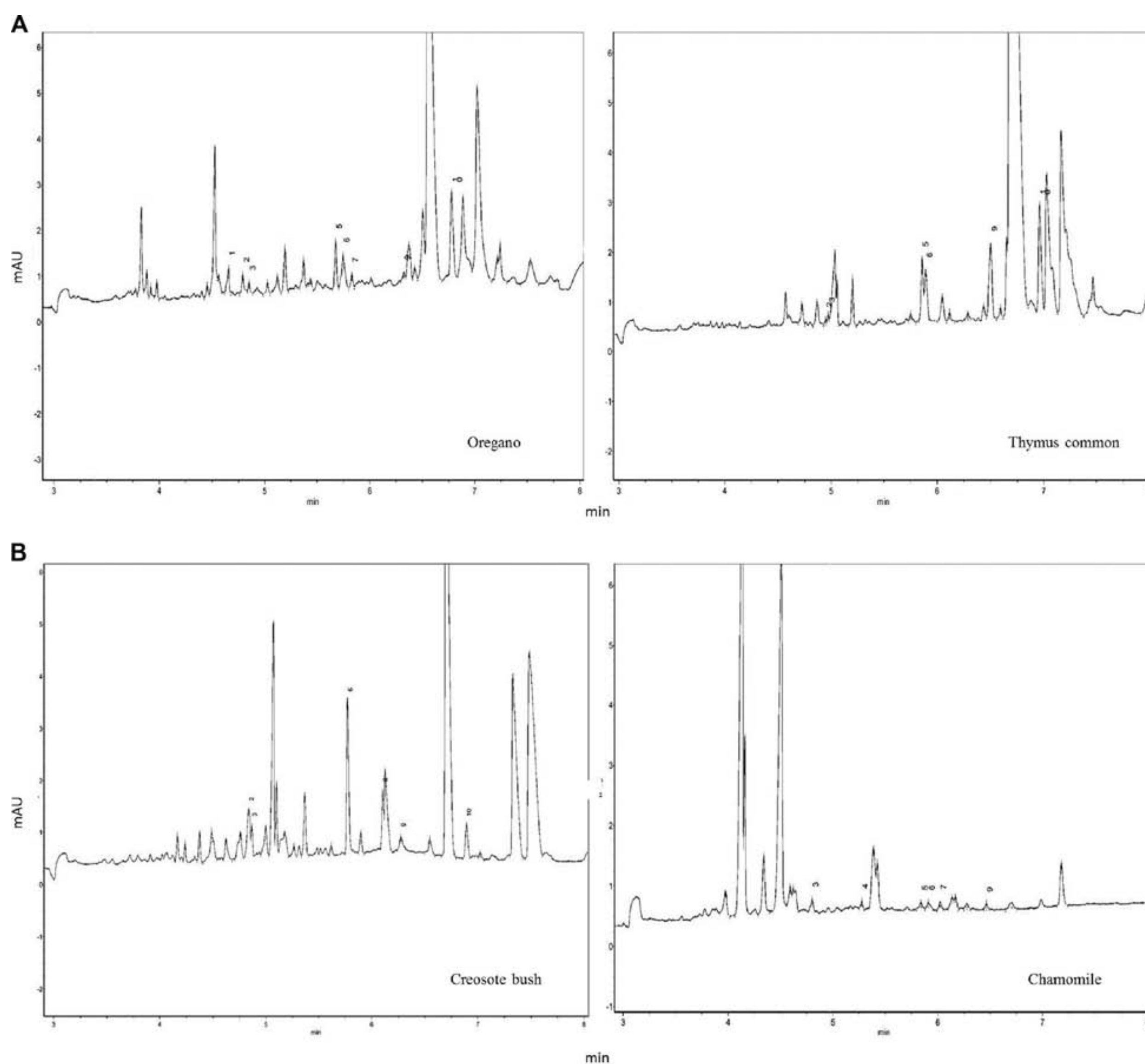

Figure 2. CZE-UV electropherograms of (A) oregano and thymus common extracts and (B) creosote bush and chamomile extracts, at 290 nm; catechin (1), naringenin (2), cinnamic acid (3), chlorogenic acid (4), syringic acid (5), luteolin (6), apigenin (7), quercetin (8), vanillic acid (9), caffeic acid (10).

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and *L. divaricata* (creosote bush). Representative electropherograms of the sample extracts are shown in Fig. 2. Significant qualitative and quantitative differences in the phenolic profile of plant extracts were observed.

The results of the quantitative determination of phenolic compounds in the extracts from different plants are presented in Table 2. The total amount of phenolic compounds ranged between 4.46 and 161.50 mg/L. Oregano was the extract with higher phenolic content, while chamomile showed the lowest phenolic content. Quercetin was found only in creosote bush extract while catechin was exclusively found in oregano. This extract showed a higher amount of naringenin, cinnamic, and luteolin than the other extracts. Chlorogenic acid was only detected in chamomile. Oregano showed the highest levels of apigenin, caffeic, and syringic acids. Common thymus was the extract showing the highest contents of vanillic acid (Table 2).

3.3 Antimicrobial activity of plant extracts

The development of effective control methods of *Phytophthora* species has been fraught with difficulties because of their ability to produce several forms of inoculum (zoospores, sporangia, chlamydospores, oospores and mycelium) rapidly and repeatedly, their ability to penetrate and infect a host plant within a few hours, their propensity to exist in soil to depths that allow them to escape most antagonists, and some cases their wide host range [1].

The biological control based in the use of plant extracts has gained much attention during the past several years, because they are promising natural antifungal agents with potential applications in agro industries to control phytopathogenic fungi causing severe destruction to crops [27–30, 48, 49].

It is often quite difficult to compare the results obtained from different studies, because the compositions of the extracts can vary greatly depending upon the geographical region, the variety, age of the plant, and the method of extraction of the extract [50]. Different approaches were tested for plant extract preparation [16, 51]. The best conditions including yield, reproducibility, and contamination risks were obtained as described in Section 2.3. It has to be pointed out that two autoclave sterilization steps were necessary for complete sterilization.

The effects of different concentrations of plants extracts on the mycelial growth of *P. nicotianae*, *P. palmivora*, and *P. citrophthora* are shown in Fig. 3, 4 and 5. These data were recorded on the fourth day after inoculation of the agar medium. All extracts were found to inhibit the growth of *P. nicotianae*, *P. palmivora*, and *P. citrophthora* in a dose-dependent manner.

The mycelial growing inhibition of *P. nicotianae*, *P. palmivora*, and *P. citrophthora* observed in this study was highly variable among the different plant extracts. Creosote bush extract was the most effective extract.

Table 2. Determination of phenolic compounds in plant extract (95% confidence interval; $n = 3$)

Plan textracts	Phenoliccompounds (mg/L)[a]										
	Catechin	Naringenin	Cinnamic acid	Chlorogenic acid	Syringic acid	Luteolin	Apigenin	Quercetin	Vanillic acid	Caffeic acid	Total phenolic
Creosote bush	n.d. a	64.20 ± 0.05 d	21.52 ± 0.02 b	n.d. a	n.d. a	42.44 ± 1.18 b	n.d. a	43.32 ± 0.05 b	17.20 ± 0.02 b	40.20 ± 0.85 b	229.08 ± 0.90 c
Chamomile	n.d. a	n.d. a	3.01 ± 0.01 a	1.92 ± 0.01 b	3.16 ± 0.01 b	5.92 ± 0.02 a	26.76 ± 0.01 b	n.d. a	2.04 ± 0.01 a	2.60 ± 0.01 a	20.88 ± 0.01 a
Oregano	10.1 ± 0.07 b	39.80 ± 0.07 b	23.60 ± 0.07 b	n.d. a	30.32 ± 0.39 d	35.36 ± 0.01 b	6.69 ± 0.10 c	n.d. a	31.12 ± 0.16 c	79.24 ± 0.34 d	315.76 ± 0.95 d
Common thyme	n.d. a	1.84 ±0.01 c	3.84 ± 0.01 a	n.d. a	28.20 ± 0.01 c	9.32 ± 0.01 a	n.d. a	n.d. a	41.76 ± 0.01 d	61.32 ± 0.03 c	153.24 ± 0.02 b

a) Mean values ± standard error. n.d. – not detected
Columns with diverse letters differ significantly according to Tukey's test, $p < 0.05$

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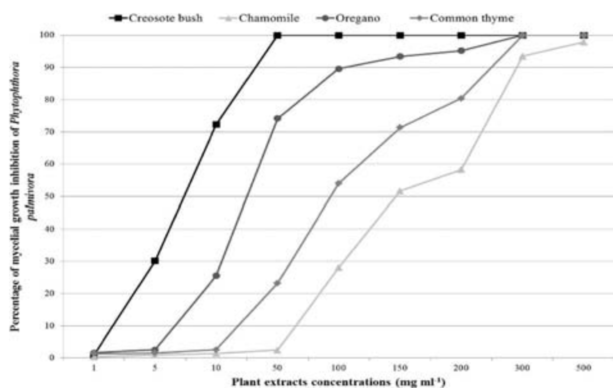

Figure 3. Antimicrobial activity of plant extracts on mycelial growth of *P. palmivora*.

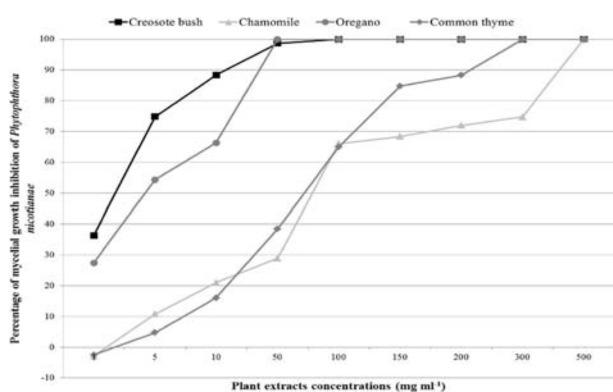

Figure 4. Antimicrobial activity of plant extracts on mycelial growth of *P. nicotianae*.

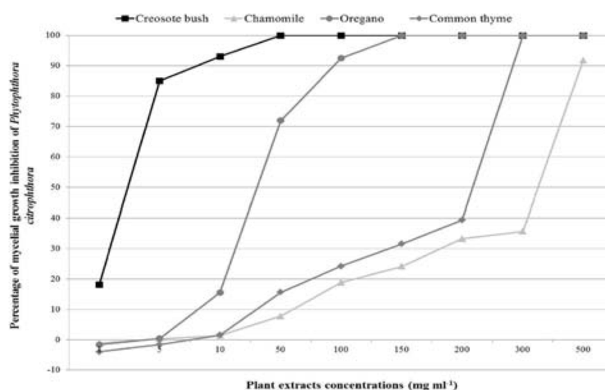

Figure 5. Antimicrobial activity of plant extracts on mycelial growth of *P. citrophthora*.

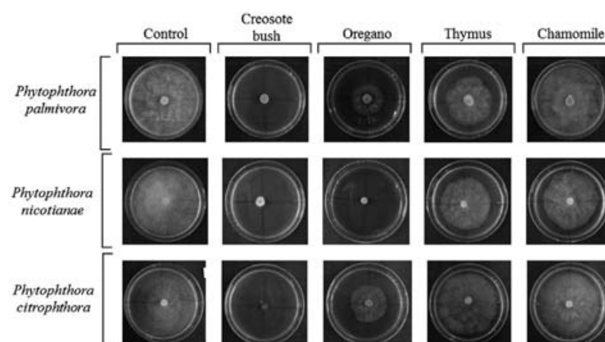

Figure 6. Influence of plant extracts (50 mg/ml) on mycelial growth of *P. palmivora*, *P. nicotianae* and *P. citrophthora*.

A 100% inhibition was observed at 50 mg/mL of creosote bush extract for all *Phytophthora* spp. evaluated.

Mycelial growth of *P. nicotianae* was totally inhibited by oregano extract at a concentration of 50 mg/mL, while for the same concentration of thyme and chamomile extracts the inhibition percentage was 39 and 28%, respectively (Fig. 6). *P. palmivora* was totally inhibited by oregano, thyme and chamomile extracts at concentrations of 300, 300 and 500 mg/mL respectively. The extracts of creosote bush at 50 mg/mL, oregano at 150 mg/mL and thyme at 300 mg/mL mycelial growth inhibited *P. citrophthora* completely, whereas for the chamomile extract at 500 mg/mL the percentage of inhibition was 92%.

3.4 Correlation between the analytical composition of the extracts and the in vitro antimicrobial activity

PCA was used to highlight the data structure and to find the relationships between phenolic compounds and percentage of inhibition of *P. palmivora*, *P. nicotianae,* and *P. citrophthora*. When performing a PCA of the different phenolic compounds, three principal components having eigen values greater than 1 were obtained. The first principal compound (PC) accounted for 53.81% of variance; the second PC contributed 35.07%, while the third PC contributed 10.71%.The most important PCs contributed to 99.59% of the total variance. The interpretation of the results of PCA is usually carried out by the visualization of the loadings plot (Fig. 7). High concentration of naringenin, quercetin, luteolin, and cinnamic acid characterized the creosote bush extract, this could contribute to higher percentage of inhibition of *Phytophthora* spp. Catechin, caffeic acid and total phenols compounds were typical indicators for Oregano extract. Chamomile was characterized by high concentrations of chlorogenic acid and low content of the other phenolic compounds; this would be associated to a lower percentage of inhibition the pathogens tested. Our results are in agreement with by Avula et al., where the chamomile flower extract showed a high content of chlorogenic acid [52]. Sisti et al. demonstrated that chlorogenic acid has no antimicrobial activity on mycelial growth of *Acremonium* sp., *Beauveria* sp., *Fusarium solani*, *Microsporumcanis* sp., *Scopulariopsis brevicaulis,* and *Trichoderma* sp. [53].

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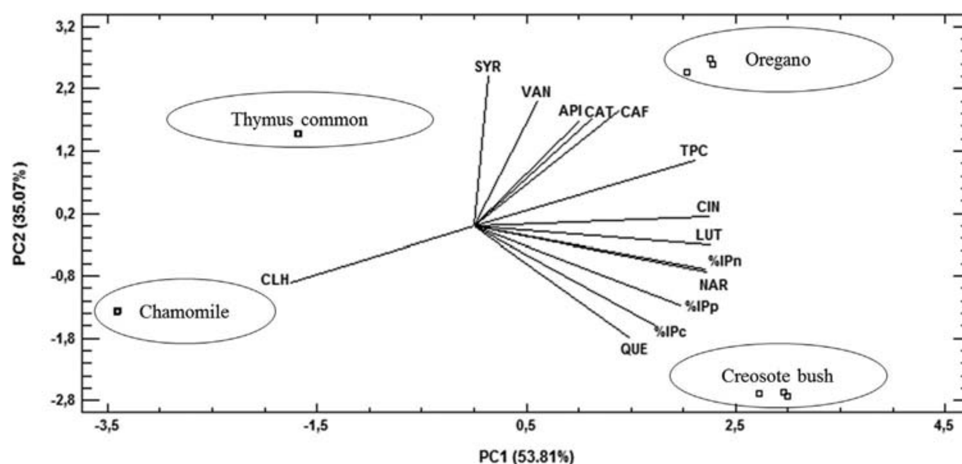

Figure 7. PCA plot of four plant extracts calculated on the basis of their phenolic compounds.

Table 3. Antimicrobial activity of naringenin, luteolin and cinnamic acid on mycelial growth of *Phytophthora* spp

Phenolic compound	Concentration (μg/ L)	Inhibition (%) of *Phytophthora* ± SE[a]		
		P. nicotianae	*P. palmivora*	*P. citrophthora*
Naringenin	0.06	20.60 ± 0.36	−4.38 ± 0.80	10.67 ± 0.23
Naringenin	3.22	35.79 ± 0.11	12.41 ± 0.57	23.57 ± 0.83
Naringenin	32.20	64.04 ± 0.29	55.25 ± 0.58	59.84 ± 0.59
Luteolin	0.04	−36.32 ± 0.23	−28.45 ± 0.80	1.58 ± 0.03
Luteolin	2.12	29.29 ± 0.36	−13.21 ± 0.37	9.58 ± 0.12
Luteolin	21.22	42.09 ± 0.79	5.09 ± 0.49	24.87 ± 0.47
Cinnamic acid	0.02	6.40 ± 0.34	−14.05 ± 0.25	14.38 ± 0.10
Cinnamic acid	1.18	9.26 ± 0.24	1.67 ± 0.03	42.91 ± 0.21
Cinnamic acid	11.80	35.32 ± 0.27	72.29 ± 0.89	74.28 ± 0.60

a) The mean inhibition percentage of mycelial growth of *P. nicotianae*, *P. palmivora* and *P. citrophthora* ± standard error.

3.5 Effect of naringenin, luteolin and cinnamic acid on mycelial growth of *P. nicotianae, P. palmivora,* and *P. citrophthora*

The antifungal activity of different plant extracts have been ascribed to the fungicidal action of some phenolic compounds [27, 30, 32–34]. These compounds not only attack fungi cell membranes, affecting the permeability and release of intracellular constituents but also they interfere with membrane function [50]. Diverse biological properties have been attributed to these phenolic compounds [54–61]. Pharmacological properties such as antioxidant, antifibration, anticancer, antiatherogenic, and antiproliferative activities have been demonstrated for naringenin [62]. Gatto et al. reported antioxidant, anticancer, antithrombotic, antimicrobial, and antiviral activities for quercetin [63]. Luteolin are known to possess numerous biological activities. In vivo and in vitro studies have acknowledged that luteolin and its glycosides exert antioxidative antibacterial, and anti-inflammatory activities [64]. Cinnamic acid and its derivatives occur in plants and fruits, providing a natural protection against infections by pathogenic microorganisms [65]. Many important biological activities have been attributed to cinnamic acid and its derivatives, including anti-inflammatory, antioxidative and antimicrobial properties [66]. Also this phenolic compound has shown a variety of pharmacologic properties including hepatoprotective and antidiabetic activities [62].

In order to further elucidate the antimicrobial role of the plant extracts under study, the individual effect of the most significant phenolic compounds was studied. Considering the principal component analysis (Fig. 7), naringenin, luteolin, and cinnamic acid were selected. These compounds showed the highest concentration in the plant extracts with higher antimicrobial activity (creosote bush and oregano). The following concentrations were tested: naringenin (0.06; 3.22 and 32.22 μg/L), luteolin (0.04; 2.12 and 21.22 μg/L) and cinnamic acid (0.02; 1.18 and 11.80 mg/L).

The effects of naringenin, luteolin, and cinnamic acid on the mycelial growth of *P. nicotianae*, *P. palmivora,* and *P. citrophthora* are shown in Table 3. These data were recorded on the 40th day after inoculation of the agar medium. The highest inhibitions of mycelial growth of the pathogens were observed for cinnamic acid and naringenin compared with luteolin.

P. nicotianae was more susceptible to naringenin, while that *P. citrophthora* and *P. palmivora* were more sensitive to the cinnamic acid. All phenolic compounds added at highest tested concentrations inhibited mycelial growth of the

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pathogens. Interestingly, in some cases, the phenols added at the lowest concentration induced the mycelial growth of the pathogens. From the principal component analysis between the analytical results and the microbial activity of the plant extracts and the effect of individual significant ingredients, other effects than the individual action of the phenolic compounds influence the control of pathogens by plant extracts, such as synergistic, antagonist and/or matrix effects.

4 Concluding remarks

In the present work, a SPE-CZE sustainable analytical strategy for the determination of ten phenolic compounds in the complex matrix of plant extracts was developed. LC-MS/MS analysis are being carried out in our laboratory in order to confirm the chemical identity of the most active components of each extract. The markedly qualitative and quantitative analytical differences in the phenolic profile of plant extracts found in this study contribute to the understanding of the phenolic compounds associated with inhibition of growth of *P. nicotianae*, *P. palmivora*, and *P. citrophthora*. Higher antimicrobial activities were shown by creosote bush and oregano extracts.

The results of this work suggest that the plant extracts tested could be considered as potential alternatives to the use of fungicides in food production or in olive production. Those claims are further supported by our findings; indicating that high contents of naringenin, cinnamic acid, and luteolin contribute to antifungal activity of the plant extracts against soil-born pathogens involving some type of synergism or antagonism with other active components. Differences between antimicrobial activity of plant extracts and individual phenolic compounds can be caused by a synergistic effect between these phenolic or other compounds as such as terpenes and alkaloids.

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2189) and Secretaría de Ciencia Tecnología y Posgrado (SECTyP) of Universidad Nacional de Cuyo.

The authors have declared no conflict of interest.

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