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Potential application of Northern Argentine propolis to control some phytopathogenic bacteria

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Summary

The antimicrobial activity of samples of Northern Argentine propolis (Tucumán, Santiago del Estero and Chaco) against phytopathogenic bacteria was assessed and the most active samples were identified.
Minimal inhibitory concentration (MIC) values were determined by agar macrodilution and broth microdilution assays. Strong antibacterial activity was detected against *Erwinia carotovora* spp carotovora CECT 225, *Pseudomonas syringae* pvar tomato CECT 126, *Pseudomonas corrugata* CECT 124 and *Xanthomonas campestris* pvar vesicatoria CECT 792. The most active propolis extract (Tucumán, T1) was selected to bioguide isolation and identified for antimicrobial compound (2′,4′-dihydroxychalcone). The antibacterial chalcone was more active than the propolis ethanolic extract (MIC values of 0.5—1 $\mu g\,ml^{-1}$ and 9.5—15 $\mu g\,ml^{-1}$, respectively). Phytotoxicity assays were realized and the propolis extracts did not retard germination of lettuce seeds or the growth of onion roots. Propolis solutions applied as sprays on tomato fruits infected with *P. syringae* reduced the severity of disease. Application of the Argentine propolis extracts diluted with water may be promising for the management of post harvest diseases of fruits.

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Introduction

At present, quick and effective management of plant diseases and microbial contamination in several agricultural commodities is generally achieved by the use of synthetic pesticides (Agrios 1997). Considering this situation, there is an urgent need of alternative agents for the control of the plant microbial diseases (Rice et al. 1998).

Propolis (bee glue) is a natural matter made up of resinous collected by bees from buds and trees to reinforce honeycombs and cover cracks in the hive. The propolis has different chemical compositions according to the botanical origin. Propolis was found to have many biological properties, including anti-inflammatory, antioxidant, antiviral, cytostatic, antiproliferative, cytotoxic, hepatoprotective and antimicrobial activities (Agüero et al. 2010; Chaillou and Nazareno 2009; Isla et al. 2001, 2005, 2009; Nieva Moreno et al. 1999, 2000, 2005; Sawaya et al. 2002). Some Argentine propolis shows antibacterial activity against antibiotic resistant human pathogenic bacteria (Isla et al. 2005; Nieva Moreno et al. 1999). Propolis extracts, due to their antibacterial action, were also employed in zootechnology, in order to sanitize close environments (Bevilacqua et al. 1997). The acaricidal and insecticide properties of propolis have been established (Garedew et al. 2002). Propolis has been found to inhibit postharvest pathogens, *Botrytis cinerea* and *Penicillum expansum* (Lima et al. 1998). At present, the natural product available to control phytopathogenic bacteria is limited. Hence, it is very important to detect natural products with activity on them.

Hence, propolis has been incorporated as a dietary supplement into the Argentine Food Code by means of Res. 357/08 of the Secretaría de Agricultura, Ganadería, Pesca y Alimentos and Res. 94/08 of the Ministerio de Salud. For these reason we chose six propolis samples used by local inhabitants of the Argentina for treating different disorders in humans and animals to assay its effect on phytopathogenic microorganisms with relevance in the food industry.

Selected microorganisms

Bacterial spot and bacterial speck of tomato are serious problems of processing and fresh market tomatoes (*Lycopersicon esculentum* L.). Bacterial soft rot (*Erwinia carotovora*) is probably the principal cause and the most serious in terms of crop losses. Foliar and fruit diseases of tomato such as bacterial spot (*Xanthomonas* sp.), bacterial speck (*Pseudomonas syringae* pv. tomato) and pith necrosis (*E. carotovora*) can severely curtail the production of this crop (Byrne et al. 2005).

Materials and methods

Chemicals

All reagent and chemicals used were of analytical or microbiological grade. 2′,4′-Dihydroxychalcone was commercially obtained from Indofine Chemicals Co.

Propolis

The samples were collected from beehives in 2008—2009 in different phytogeographical places of Argentina. Six samples were obtained from: Tucumán (T), Santiago del Estero (SE) and Chaco (CH) and named as T1, T2, SE1, SE2, CH1 and CH2, respectively. The samples were hand gathered by scraping from hives. Once frozen at −20 °C, propolis was ground and extracted with n-hexane first and with ethanol next (2 g of samples in 100 ml 80% ethanol, IRAM-INTA 15935-1; Norma IRAM-INTA 2008). The propolis ethanolic extracts (PE) were obtained.

Separation of propolis components

Fractionation procedures

The most active propolis extract (Tucuman1-EP) was fractionated by column chromatography (CC) on silica gel (200 g, 0.063—0.2 mesh, Merck 60) and eluted stepwise with petroleum ether (PE) and then with mixtures of PE/ethylacetate (EtOAc) to reach 100% EtOAc. Fractions of 100 ml were collected, evaporated to dryness in a rotary evaporator at 50 °C. The column fractions were analyzed by TLC (Merck, Kieselgel 60 F254, 0.2 mm); chloroform ($CHCl_3$):EtOAc 80:20 was used as the mobile phase; detection was under UV light followed by spraying with 1% methanolic solution of diphenylboric acid aminoethyl ester (Wagner et al. 1984). Fractions with similar TLC patterns were combined and evaluated for antibacterial activity by bioautography agar overlay method. The active fractions were purified by Sephadex LH-20 (column length, 40 cm; i.d., 1.5 cm; PE/MeOH/$CHCl_3$ 2:1:1). The more active compound was characterized by NMR and UV spectra. The profiles and retention time were compared with 2′,4′-dihydroxychalcone commercial standard (Indofine Chemicals Co.).

Analysis of flavonoids

Total phenol content was quantitatively determined by the method of Folin–Ciocalteau (Singleton et al. 1999). Results were expressed as micrograms of gallic acid ml^{-1} ($\mu g\,GAE\,ml^{-1}$).

Total flavonoids were quantitatively analyzed according to Woisky and Salatino (1998).

Microorganisms

Bacterial strains were obtained from the Spanish Type Culture Collection (Burjasot, Valencia, Spain). *E. carotovora* spp carotovora CECT 225, *P. syringae* pvar tomato CECT 126, *Pseudomonas corrugata* CECT 124, *Xanthomonas campestris* pvar vesicatoria CECT 792 were used.

Antimicrobial assay

Bioautography assay

Comparative bioautography of PE were realized, 6 μg GAE of each extract/plate were separated by TLC (Merck, Kieselgel 60 F254) using $CHCl_3$:EtOAc 80:20 as eluant. Two ml of soft medium (0.6% BHI agar) containing 1×10^5 CFU (colony forming units ml^{-1}) of *P. syringae* was added and incubated at 27 °C for 24 h. Then, the plates were revealed according to Nieva Moreno et al. (1999).

Agar diffusion method

Examination of propolis sensitivity to different phytopathogenic bacteria was determined according to the method described by Nieva Moreno et al. (1999). Sterile filter disks (Whatman No. 4, 5 mm diameter) were impregnated with 5 μl of different dilutions of PE (5–320 $\mu g\,GAE\,ml^{-1}$). Bacterial growth inhibition was determined as the diameter of the inhibition zones around the disks as recommended by the Clinical and Laboratory Standards Institute (CLSI 2006).

Serial agar macrodilution method

This test was performed in sterile Petri dishes. PE (4.75–305 $\mu g\,GAE\,ml^{-1}$) and 2′,4′-dihydroxychalcone (0.1–3 $\mu g\,GAE\,ml^{-1}$) dilutions (final volume 1 ml) were performed. Then, 9 ml of Müller–Hinton agar (MHA) was added (CLSI 2006). The dishes were inoculated with 2 μl of each bacterial cell suspension (1×10^4 CFU) and incubated aerobically for 18 h at 27 °C. A growth control of each tested strain was included.

MIC (minimal inhibitory concentration) was defined as the lowest concentration of sample at which no colony was observed after incubation.

Broth microdilution susceptibility assay

PE (4.75–305 $\mu g\,GAE\,ml^{-1}$) and 2′,4′-dihydroxychalcone (0.1–3 $\mu g\,ml^{-1}$) dilutions were prepared in 96-well microplates. After evaporating all samples to dryness, the residues were dissolved in dimethyl sulphoxide (DMSO). The concentration of DMSO (1%) added to the control did not influence the growth of any bacteria. The wells were filled with 100 μl cation-adjusted (Ca^{2+} 8 $mg\,l^{-1}$ and Mg^{2+} 5 $mg\,l^{-1}$) Müller–Hinton broth. The final inoculum was 1×10^5 CFU. The inoculated plates were incubated aerobically at 27 °C during 18 h.

MICs were determined as the first well where no pellets appeared on the well bottom.

MIC was defined as the lowest concentration of PE or isolated compound able to restrict growth to an absorbance <0.05 at 625 nm (no macroscopically visible growth). To confirm MIC and to establish MBC, 10 μl of culture medium from each microplate well was inoculated in agar plates and incubated aerobically 16–20 h at 27 °C. Then, the number of surviving organisms was determined.

MBC (minimal bactericidal concentration) was defined as the lowest extract concentration at which 99.9% of the bacteria have been killed.

All experiments were carried out in triplicate.

In vivo assay

The tomato fruits (*L. esculentum* L., $n = 5$) were provided by local market, washed with distilled water, $NaHClO_4$ 1% (v/v) for 5 min, and then three times with sterile distilled water. They were dried at room temperature in laminar flow and separated in 5 groups of 5 fruits each in cardboard boxes. The boxes were previously exposed to UV light (Casiba VL-1) for 10 min. The fruits were wounded on the surface (skin) with a sterile scalpel before inoculating with each bacterial suspension (*P. syringae* pv. tomato, 10^5 CFU ml^{-1}) or sterile distilled water for the control. The fruits were sprayed with aqueous solutions obtained by adding the standardized propolis ethanolic solution (1 mg of GAE) to 100 ml of water 24 h prior to inoculation with *P. syringae* or 2 h after inoculating with bacteria. They were kept at room temperature or 4 °C for 7 days. The evaluation of the decay development (external symptoms such as necrotic areas) was carried out for each 48 h up to 7 days. The test was performed by triplicate and the percentage of fruits with postharvest disease was calculated according to the formula:

$$\% = \frac{\text{Number of diseased tomato}}{\text{Total tomato number}} \times 100$$

The assay included a lot of fruit without treatment (fruit control), a group of tomato with wound (wound control), a lot with wound + extract + inoculum (extract effect prior to inoculation), a lot with wound + inoculum (inoculum control) and a lot with wound + inoculum + extract (extract effect after inoculation).

Phytotoxicity bioassay

Lactuca sativa test

Lettuce seeds (*L. sativa* L., var. Grand Rapids, Florensa) were chosen for bioassay as test plants. Seeds were surface sterilized for 5 min prior to treatment in a 1% commercial hypochlorite solution, rinsed with running tap water for 20 min and air dried. Twenty seeds were placed on two layers of Whatman No. 1 paper in 9 cm diameter Petri dishes and watered with 3 ml of propolis sample diluted as described above.

The covered Petri plates were placed in a germination chamber at $25 \pm 1\,^{\circ}C$ with a 12 h photoperiod for 3 days. Germinated seeds were counted, frozen at $-20\,^{\circ}C$ for 24 h to stop radicle growth, and the percent of radicle elongation inhibition was calculated by reference to that of control seeds.

Allium cepa test

Non-germinated onion bulb was maintained in distilled water for 48 h. Then, the roots were exposed to different propolis extract concentrations for 24 h. Root length was considered as a toxicity index (Fiskesjo 1985). The roots growing in water were used as negative control.

Statistical analysis

All measurements and the data were analyzed by analysis of variance.

Results

The chemical composition of propolis varies according to the geographical origin, local flora and gathering time. As a consequence, its biological activity would also vary. *Apis mellifera* propolis samples from three provinces of Argentina were collected: Two samples were from Santiago del Estero (SE), two samples from Tucumán (T) and two from Chaco (CH). All propolis samples contained higher concentrations of phenolic compounds (20—90 mg/g) and flavonoids (15—50 mg/g). The antibacterial activity was determined by bioautographic assays, agar diffusion and MIC/MBC. In

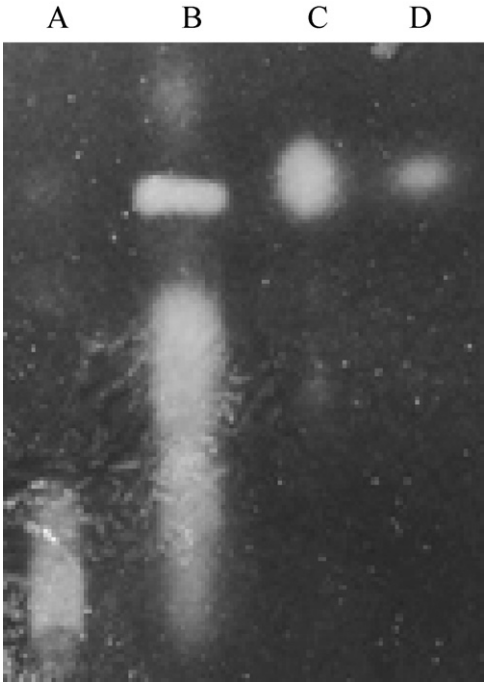

Figure 1. Comparative bioautography of propolis extracts from Argentina. Lane A: CH2-PE; Lane B: SE1-PE; Lane C: T1-PE (6 μg of phenolic compounds of each extract/plate); Lane D: 2′,4′-dihydroxychalcone (2 μg). The extract components were separated by TLC (Merck Kieselgel 60 F254) using $CHCl_3$:EtOAc 80:20 as eluant. 2 ml of soft medium (0.6% BHI agar) (containing 1×10^5 CFU ml^{-1}) of *P. syringae* was added and incubated at 27 °C for 24 h. Then, the plates were revealed using 2.5 mg ml^{-1} MTT solution (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) in PBS (10 mM sodium phosphate buffer, pH 7, with 0.15 M NaCl). Plates were incubated at 27 °C for 1 h in the dark for colour development.

bioautographic assays, T1-PE and T2-PE showed one major antibacterial component (R_f of 0.75) against phytopathogenic bacteria coincident with flavonoids (Fig. 1) and SE-PE showed two inhibition bands of growth inhibition (R_f of 0.5 and 0.75). Phytocomplexes (total extract) of CH1 propolis are necessary to obtain inhibition of phytopathogenic bacteria growth in bioautographic assays. All PEs were found to be effective against phytopathogenic bacteria by agar diffusion methods. Tucumán propolis samples had the highest bacterial growth inhibition (1—1.5 mm) as compared with other samples (0.5—1 mm). The MIC values of PE shown in Table 1 indicate that propolis extracts exhibited the following order of potency for all phytopathogenic bacteria growth inhibition: T1 > T2 > SE1, SE2 > CH1, CH2. *X. campestris* were the most resistant to all of them with MIC values of 38—152 μg GAE ml^{-1}. MBC values were similar to or two fold higher than MIC values. Stored (1 year at 4 °C) and fresh propolis

Table 1. Minimum inhibitory concentration (MIC) of propolis ethanolic extracts against phytopathogenic bacteria.

Propolis extracts	*E. carotovora*	*P. syringae*	*P. corrugata*	*X. campestris*
MIC (μg GAE ml^{-1})				
SE1	9.5 ± 0.5	9.5 ± 0.5	9.5 ± 0.5	76 ± 20
SE2	9.5 ± 0.2	9.5 ± 0.5	9.5 ± 0.8	76 ± 25
T1	4.7 ± 0.1	4.7 ± 1.0	4.7 ± 0.1	38 ± 10
T2	9.5 ± 0.3	9.5 ± 0.5	9.5 ± 0.5	38 ± 10
CH1	38.0 ± 5.0	38.0 ± 7.0	38.0 ± 5.0	152 ± 20
CH2	38.0 ± 3.0	38.0 ± 5.0	38.0 ± 4.0	152 ± 25

MIC values were determined by macrodilution (Müller–Hinton agar) and microdilution (Müller–Hinton broth) assays. The inoculated plates were incubated aerobically at 27 °C for 18 h. Each value represents the mean ± standard deviation of a triplicate analysis.

samples had similar antibacterial activity. No difference was obtained from samples collected in 2008 and 2009.

In consequence T1-PE was selected to realize a bioactivity-guided separation of bactericidal compounds. The isolated antimicrobial compound was identified as 2′,4′-dihydroxychalcone. The UV spectrum in MeOH showed three maximum at λ_{max} 264; 319 and 345 nm, in MeOH·AcONa at 267; 300 and 395 nm and two maximum in the presence of $AlCl_3$ at 358 and 396 nm that was not changed in the presence of HCl. Spectral features shown by (^{1}H NMR and ^{13}C NMR) were similar to those previously reported (Patra et al. 1982; Wollenweber and Seigler 1982) and agreed with those of an authentic compound. Additional support of the proposed structure was provided by comparison of TLC and HPLC behaviour with that of an authentic sample obtained commercially. An ethanolic solution of isolated chalcone was used for the determination of MIC and MBC on phytopathogenic bacteria. The results are presented in Table 2. The MIC and MBC values obtained with 2′,4′-dihydroxychalcone were significantly lower than those of the propolis extract.

Table 2. Antibacterial activity of 2′,4′-dihydroxychalcone assayed by agar macrodilution and broth microdilution methods.

Microorganisms	MICs (μg ml^{-1})	MBC(μg ml^{-1})
E. carotovora	0.30 ± 0.03	0.90 ± 0.03
P. syringae	0.50 ± 0.05	2.00 ± 0.05
X. campestris	1.00 ± 0.10	3.00 ± 0.05
P. corrugata	0.50 ± 0.05	1.00 ± 0.02

Each value represents the mean ± standard deviation of a triplicate analysis.

The T1-PE and their bioactive compound isolate did not have a phytotoxic effect on lettuce or onion in the range of concentrations that show antibacterial activity.

The preventive effect of T1-PE diluted with water (0.01 mg GAE ml^{-1} of water) against postharvest disease was evaluated on tomato fruits inoculate with a bacterial suspension of *P. syringae*

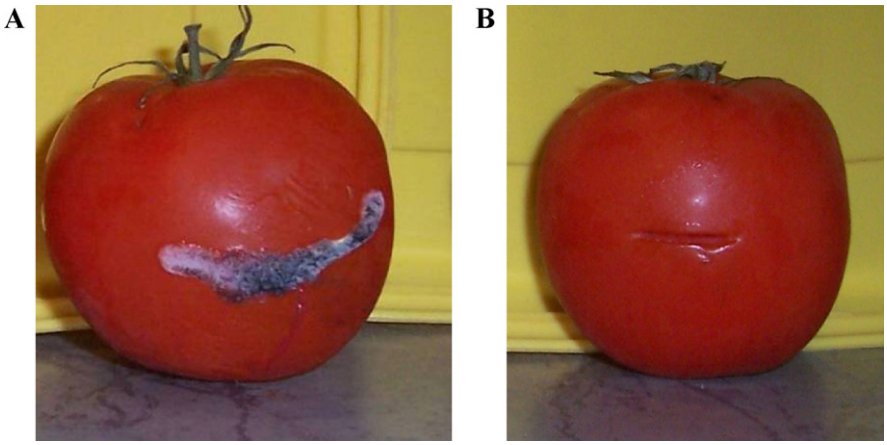

Figure 2. Symptoms caused by *Pseudomonas syringae* pv. tomato in harvested ripe fruits of *L. esculentum*: (A) without T1-PE treatment and (B) with T1-PE treatment. A propolis solution was sprayed on mature fruit 24 h prior to inoculation with bacterial suspension (1×10^5 CFU ml^{-1}). The fruits were maintained at 4 °C and the symptoms were determined 7 days after inoculations.

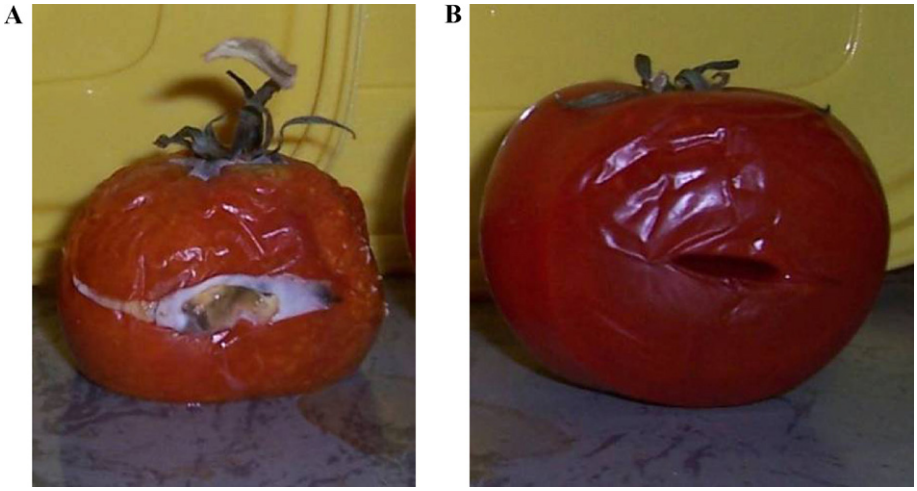

Figure 3. Symptoms caused by *Pseudomonas syringae* pv. tomato in harvested ripe fruits of *L. esculentum*: (A) without T1-PE treatment and (B) with T1-PE treatment. A propolis solution was sprayed on mature fruit 24 h prior to inoculation with bacterial suspension (1×10^5 CFU ml^{-1}). The fruits were maintained at 27 °C and the symptoms were determined 7 days after inoculations.

pv. tomato. According to our results there were no macroscopic changes in the untreated fruits (fruit control) and in tomato wounding (wound control) after 1 week of treatment. According to our results, an application of hydroalcoholic propolis solution was very effective in reducing lesions and necrotic areas developed by *P. syringae* pv. tomato on harvested mature tomatoes. The antibacterial activity was more effective when the fruits were maintained at 4 °C than at room temperature (Figs. 2 and 3). Similar results were obtained when the treatments with T1-PE were realized prior or after *P. syringae* inoculation.

Discussion

The use of natural products for the control of bacterial diseases in plants is considered as an interesting alternative to synthetic bactericides or fungicides due to their lower negative impact on the environment. Bacterial diseases are managed primarily with fixed copper compounds, although control is generally inadequate due to the prevalence of cooper-resistant strains and weather conditions that often favor bacterial diseases in the field. In this work, we compared six propolis samples from Northern Argentina and examined the antimicrobial activity of their extracts against phytopathogenic bacteria (*P. syringae*, *P. corrugata*, *X. campestris* and *E. carotovora*). According to our results, T1-PE was the most active and could be considered a promising antibacterial agent against phytopathogenic bacteria. The main antibacterial compound of T1-PE was identified as 2′,4′-dihydroxychalcone. In the previous work, it was demonstrated that this compound was isolated from *Zuccagnia punctata*, a medicinal plant that grow in different regions of Argentina, had antibacterial activity against Gram-negative multiresistant bacteria (Zampini et al. 2005) and on phytopathogenic fungi (Svetaz et al. 2007; Agüero et al. 2010), antioxidant activity (Morán Vieyra et al. 2009) and had not showed genotoxic or mutagenic potential (Zampini et al. 2008). T1-PE and their bioactive compound isolate did not show a phytotoxic effect on lettuce or onion. The low phytotoxicity of T1-PE makes it an ideal candidate for microbial control in harvested fruits. Propolis water solutions applied as sprays on tomato fruit, resulted in the reduction of the severity of bacterial disease. The efficacy of a propolis solution sprayed prior to the inoculation with *P. syringae* pvar tomato was comparable to that obtained after inoculations. Our results demonstrated the bactericidal effect of propolis *in vivo*. In accordance with our results, the application of T1-PE may be promising, economical and non contaminant treatment for the management of post harvest diseases of fruits and/or vegetables.

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