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CROP PROTECTION

RESEARCH NOTE

The *in vitro* antifungal evaluation of a commercial extract of Chilean propolis against six fungi of agricultural importance

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Abstract

M. Curifuta, J. Vidal, J. Sánchez, A. Contreras, L.A. Salazar, and M. Alvear. 2012. The *in vitro* antifungal evaluation of a commercial extract of Chilean propolis against six fungi of agricultural importance. Cien. Inv. Agr. 39(2): 347-359. Propolis has been used for millennia for its antimicrobial and pharmaceutical properties, whereas its use as an agricultural antifungal agent has only recently been assessed. The chemical characteristics of six ethanolic extracts of Chilean propolis (EEP-1 to EEP-6) were evaluated. The total polyphenols ranged between 7.8 and 42.3 mg mL^{-1} equivalents of a 2:1 pinocembrin: galangin standard, and the total flavonoid contents ranged between 4.0 and 19.7 mg mL^{-1} equivalents of quercetin. A high-performance liquid chromatographic (HPLC) analysis allowed the identification of caffeic acid, myricetin, quercetin, kaempferol, apigenin, pinocembrin, galangin, caffeic acid phenethyl ester (CAPE) and rutin. The EEP-3 extract was selected for additional studies based on the higher total polyphenols and flavonoids contents (42.3 and 19.7 mg mL^{-1}, respectively). The antifungal effects of four different concentrations (0.5, 1.0, 2.5 and 5.0 %) of EEP-3 on *Alternaria alternata*, *Fusarium* sp., *Ulocladium* sp., *Botrytis cinerea*, *Penicillium expansum* and *Trichoderma reesei* were evaluated *in vitro* using the agar dilution method. Although significant differences occurred among the extract concentrations, the results indicated that EEP-3 inhibited the mycelial growth of the six fungi evaluated.

Key words: *Alternaria alternata*, antifungal activity, *Botrytis cinerea*, flavonoids, *Fusarium* sp., *Penicillium expansum*, polyphenols, propolis.

Introduction

In Chile and other countries around the world, phytopathogenic fungi cause serious economic losses in horticultural production systems and post-harvest diseases in fruits and vegetables. Although fungicide use is controversial, it is an important component of pest and disease management programs in horticultural production systems. To ensure the sustainability of these systems, a balance needs to be found between controlling the risks of fungal diseases in crops and protecting terrestrial and aquatic ecosystems (Wightwick *et al*., 2010). During the last few years, considerable efforts have been developed to identify natural products for controlling the diseases of crops, and the use of

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natural compounds, such as the ethanolic extracts of propolis, was suggested as an approach for reducing certain phytopathogenic fungi (Giovanelli, 2008). The first report of the antimicrobial action of propolis against the fungal pathogens of plants was that of Ghaly *et al*. (1998) who determined the efficacy of an ethanolic extract of propolis against the ascomycete mold fungus *Aspergillus flavus* with the goal of reducing aflatoxin production.

Currently, fungal diseases cause crops losses approaching 12% of the world's production (ElShafei *et al*., 2010), causing a threat to our food supply (Strange and Scott, 2005). Due to the high level of genetic variation, the loss of efficacy of chemical fungicides on fungal pathogens (Latorre *et al*., 2001) results in a rapid adaptability to these fungicides (Zhao *et al*., 2009). Therefore, the use of such natural products as propolis for the control of fungal diseases in plants is considered a promising alternative to synthetic fungicides because of their lower negative impact on the environment (Ordóñez *et al*., 2011).

Propolis (or bee glue) is a natural substance produced by *Apis mellifera* that is composed of botanical sources, including buds and exudates; it is chewed by the bees, enriching it with salivary secretions, for storage and transportation to the hive where it is then mixed with wax and pollen. The composition of propolis is variable: approximately 50% resin, 30% wax, 10% essential oils, 5% pollen and 5% other organic compounds (Russo *et al*., 2004; Gómez-Caravaca *et al*., 2006; Falcão *et al*., 2010). Propolis is thought to be used to seal the beehive (Lotti *et al*., 2010) to exclude draught and protect it against external invaders. However, its main function is to prevent the decomposition of organic matter (*i.e*., organisms that have been killed by the bees after invasion) within the hive by inhibiting microbial growth. Therefore, the presence of propolis may provide an environment that is unsuitable for the growth of fungi and other microorganisms, thus maintaining an aseptic hive (Falcão *et al*., 2010) and creating a protective barrier against enemies (Melliou *et al*., 2007). Propolis, therefore, is considered to be the most important "chemical weapon" of bees against pathogenic microorganisms (Bankova, 2005a,b). Over 300 compounds have been detected in propolis, and 160 of these have been identified of which 50% are phenolic compounds, mainly flavonoids (flavones, isoflavones, and flavones), aromatic acids and esters (caffeic acid, cinnamic and others), aromatic aldehydes (vanillin and isovanillin), coumarins and phenolic triglycerides. In addition, other groups of compounds and minerals are of fundamental importance for the biological activity of propolis, including provitamin A, certain B vitamins, lactones, polysaccharides, amino acids and other substances not yet identified (Quiroga *et al*., 2006; Hroboňová *et al*., 2008; Peña, 2008; Kalogeropoulos *et al*., 2009; Falcão *et al*., 2010). The propolis produced by bees is generally purified by extraction with different solvents to remove the wax and organic waste and to preserve the polyphenol fraction, which contains most of the bioactive components of propolis (Gómez-Caravaca *et al*., 2006; Kalogeropoulos *et al*., 2009).

Numerous studies have demonstrated the versatile pharmacological activities of propolis, including antibacterial, antifungal, antiviral, antiinflammatory, hepatoprotective, antioxidant, and antitumor properties (Hegazi and Abd El Hady, 2002; Tolosa and Cañizares, 2002; Castagna *et al*., 2004; Garedew *et al*., 2004; Popova *et al*., 2005; Quintero-Mora *et al*., 2008; Popova *et al*., 2009; Herrera *et al*., 2010; Saavedra *et al*., 2011). The control of phytopathogenic fungi by propolis has been proposed by Hegazi and Abd El Hady (2002) who evaluated the use of Egyptian propolis to control nine postharvest fungi and reported successful inhibition of the fungi, with minimum inhibitory concentration values ranging between 1.2-3.6 mg mL^{-1}. In other countries, samples of the local propolis have been evaluated by Özcan *et al*. (2004), Quiroga *et al*. (2006), Giovanelli (2008), Meneses *et al*. (2009), and Özdemir *et al*. (2010) who obtained interesting results against phytopathogenic fungi. However, the development of a propolis extract for use as an agricultural fungicide has not been given much

attention (Hegazy and Abd El Hady, 2002; Quiroga *et al*., 2006) in Chile, and data regarding its action against important economical agricultural fungi are limited.

The main goals of this study were a) to determine the chemical characteristics of the six commercial ethanolic extracts of Chilean propolis and b) to evaluate the *in vitro* antifungal activity of one of these extracts (EEP-3, with higher contents of total polyphenols and flavonoids than the others) in the control of pathogenic fungi in plants (*A. alternata*, *Fusarium* sp., and *Ulocladium* sp.), the control of postharvest pathogenic fungi (*B. cinerea* and *P. expansum*). The fungus *T. reesei* was used as a biological control.

Materials and methods

Commercial propolis extracts

Six commercial ethanolic extracts of propolis (EEP-1, EEP-2, EEP-3, EEP-4, EEP-5 and EEP-6) were purchased from stores of natural products in Temuco, Chile.

Microorganisms

The fungal strains used in this study belong to the collection of the Laboratorio de Bioquímica y Biología Molecular de Suelo, Universidad de La Frontera, Temuco, Chile. The fungi tested are of agricultural interest and included *Alternaria alternata*, *Fusarium* sp., *Ulocladium* sp., *Botrytis cinerea*, *Penicillium expansum* and *Trichoderma reesei*. The fungi were morphologically and molecularly characterized.

Identification of fungi by PCR

The genomic DNA of the fungal isolates was obtained from fresh mycelial cultures. The samples were frozen in liquid nitrogen, ground to a powder using a mortar and pestle and resuspended in TE buffer (10 mMTris/Cl pH 8.0, 1 mM EDTA), with an equal volume of lysis buffer was added (2% SDS, 10 mM Tris/Cl, and 1 mM EDTA, pH 7.0). After incubation on ice for 15 min, the mixture was centrifuged at 3,000 x g for 10 min, and the supernatant was subjected to phenol/chloroform extraction, followed by ethanol precipitation (Sambrook and Russel, 2001). The resulting pellet was resuspended in TE buffer containing 100 µg mL^{-1} RNaseA (Fermentas®) and incubated at 37 ºC for 30 min. The suspension was then subjected to phenol/chloroform extraction and ethanol precipitation; the genomic DNA was dissolved in deionized and nuclease-free water. The DNA samples were subjected to PCR using the technique developed by Saiki *et al*. (1988). The primers used were forward, ITS1 5'-TCCGTAGGTGAACCTGCGG-3', and reverse, ITS4 5'-TCCTCCGCTTATTGATATGC-3', as described by White *et al*. (1990) and synthesized by Invitrogen, USA. The primers were used to amplify a fragment of rDNA that includes the ribosomal RNA gene (partial sequence), internal transcribed spacer (ITS) 1, the 5.8S ribosomal RNA gene, ITS 2 (complete sequence), and the 28S ribosomal RNA gene (partial sequence). The PCR amplifications were performed in a total volume of 50 µL, with 15 ng template DNA, 20 µM each primer, 25 µM each dNTP, 2.0 U Taq DNA polymerase (recombinant) and 10 × Taq Buffer (100 mM Tris/Cl [pH 8.8 at 25 ºC], 500 mM KCl, and 0.8% v/v Nonidet-P40 [Fermentas®]). These reactions were subjected to an initial denaturation of 5 min at 95 ºC, followed by 30 cycles of 1 min at 94 ºC, 1 min at 55 ºC, and 2 min at 72 ºC, with a final extension of 5 min at 72 ºC using a Labnet MultiGene™ 96-well Gradient Thermal Cycler. Aliquots (2 µL) were analyzed by electrophoresis through a 1.2% (w/v) agarose gel in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.0), staining with ethidium bromide and imaging using a transilluminator. The molecular size marker was the GeneRuler™ 100 bp Plus DNA Ladder (Fermentas®).

The fragments of the ribosomal DNA region, including the ITS1 and ITS2 spacers and 5.8S (complete sequence), SSU and LSU (partial sequence) genes were sequenced in both directions using an automated DNA sequencing system (ABI, Model 3730XL Genetic Analyser, by Macrogen, Korea). The sequences were compared using the Basic Local Alignment Search Tool (BLAST) database.

Determination of total polyphenols

The Folin-Ciocalteu method was used to determine the total polyphenol content of the six extracts evaluated (Singleton *et al*., 1999). Briefly, each extract was diluted 1:10 in 70% ethanol and then 1:10 in distilled water. Subsequently, 40 µL of this dilution was mixed with 560 µL distilled water, 100 µL of the Folin-Ciocalteu reagent (Merck, Germany) and 300 µL of 7.5% sodium carbonate (w/v). The absorbance was measured at 760 nm after 2 h of incubation at room temperature. The concentrations were calculated from a calibration curve and were expressed in mg mL^{-1} equivalents of the mixture of pinocembrin:galangin standards in a 2:1 ratio (Popova *et al*., 2007). All of the measurements were performed in triplicate.

Total flavonoids

The total flavonoid content was determined according to the aluminum chloride colorimetric method (Salamanca *et al*., 2007). Briefly, each EEP was diluted 1:25 in 70% ethanol, and 0.5 mL of the sample was mixed with 0.1 mL 1 M potassium acetate, 0.1 mL 10% aluminum chloride and 0.3 mL distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm using a spectrophotometer (Jenway 6320D) with a 70% ethanol blank. Quercetin was chosen as the standard. The concentrations were calculated from a calibration curve and expressed in mg quercetin equivalents mL^{-1}.

Chromatographic analysis

High-performance liquid chromatography (HPLC) was performed using an HPLC system (Merck-Hitachi model L-4200) equipped with a pump (model L-6200), a UV-visible detector and a Sphere Column Heater (Phenomenex Terma model TS-130). The separation was performed using an RP-18 column (12.5 x 0.4 cm, particle size of 5 µm) (Merck, Germany) at 25 ºC with a mixture of 5% formic acid in water (A) and methanol (B) as the mobile phases. The separation of the compounds was performed using an isocratic 0-10 min run with the mixture 70% A and 30% B, followed by a gradient up to 100% B for 70 min. The compounds were detected at 290 nm, with a 0.001 sensitivity; the injection volume was 10 µL. The identification of the phenolic compounds was performed using the following standards: myricetin, kaempferol, quercetin, caffeic acid, galangin, pinocembrin, apigenin, caffeic acid phenethyl ester (CAPE) and rutin (Sigma, USA).

Antifungal activity of propolis

The antifungal potential of propolis was evaluated using the agar dilution method according to the procedure described by Quintero-Mora *et al*. (2008) and Garedew *et al*. (2004), with some modifications. The medium was potato-dextrose- agar (PDA) sterilized at a temperature of 55 ºC, and four culture media were prepared with the propolis selected extract, obtaining concentrations of 0.5, 1.0, 2.5 and 5% (v/v), according to the volume of medium used, and a control medium without propolis. The mixture was homogenized and poured into sterile Petri dishes (5.2 cm in diameter). As controls, two synthetic fungicides were used: benzimidazole for *B. cinerea*, *Fusarium* sp., *T. reesei*, *P. expansum* and *Ulocladium* sp. and dicarboximide for *A. alternata*. The control treatment contained only the PDA culture medium. Fungal plugs (5 mm in diameter) obtained from the actively growing

margin of five-day-old cultures of each species were obtained and placed at the center of Petri dishes with the PDA media containing the various concentrations of the extracts. The cultures were incubated at 28 ± 2 ºC, and the radial growth of the mycelia were measured daily for 6 days for *B. cinerea*, 13 days for *A. alternata* and 18 days for *Fusarium* sp., *T. reesei*, *P. expansum* and *Ulocladium* sp. *B. cinerea* presented a rapid radial growth of the mycelia and reached the edge of the plates after six days. The percentage of inhibition was calculated on the basis of the growth in the control plates, as follows:

$$\text{Percentage of mycelial growth inhibition} = \frac{\text{mycelial growth in control} - \text{mycelial growth in propolis}}{\text{mycelial growth in control}} \times 100$$

The antifungal effect was measured using a random design with three replications.

Determination of the median and ninety effective concentrations

Little and Hill (1976) have proposed a method for determining the median effective concentration (EC_{50}) and ninety effective concentration (EC_{90}), defined as the inhibition of 50 and 90%, respectively, of the mycelial growth. For this analysis, it was necessary to transform the concentration of propolis to the natural logarithm, and the percentage of inhibition of the fungi were transformed angularly.

Statistical analysis

The results were analyzed using an Analysis of Variance (One-Way ANOVA), and the comparisons between the means were performed using the multiple comparison test of Tukey, with a significance level of 5% (P≤0.05). The statistical program demo SPSS 11 was used.

Results

Determination of total polyphenols and flavonoids

There was a clear difference in the concentrations of the total polyphenols and flavonoids between the six extracts evaluated (Table 1).

Table 1. Total polyphenols and flavonoids present in six commercial ethanolic extracts of Chilean propolis.

Commercial ethanolic extract of propolis (EEP)	Total Polyphenols, mg mL^{-1}	Total Flavonoids, mg mL^{-1}
EEP-1	8.23 a[1]	4.83 a[1]
EEP-2	17.33 c	8.83 b
EEP-3	42.30 d	19.70 d
EEP-4	18.37 c	10.53 c
EEP-5	14.27 b	11.00 c
EEP-6	7.77 a	3.97 a

[1]Lowercase letters indicate significant differences (P≤0.05) between the total polyphenols and total flavonoids.

EEP-3 demonstrated the highest concentration: 42.3 mg mL^{-1} 2:1 pinocembrin: galangin equivalents for the total polyphenols and 19.7 mg mL^{-1} quercetin equivalents for the total flavonoids. The EEP-1 and EEP-6 extracts exhibited the lowest contents, with no significant differences (P>0.05) between them (Table 1).

Chromatographic analysis

The chromatographic analysis detected at least 35 compounds in the six EEPs studied, with the identification and quantification of caffeic acid, myricetin, quercetin, kaempferol, apigenin, pinocembrin, galangin, CAPE and rutin (Figure 1, Table 2). The EEP-3 extract has a higher content of kaempferol, galangin and pinocembrin compared to the other extracts (Table 2).

Table 2. Selected flavonoids of six commercial ethanolic extracts of Chilean propolis (mg mL^{-1}).

EEP	Caffeic acid	Myricetin	Quercetin	Kaempferol	Apigenin	Pinocembrin	Galangin	CAPE	Rutin
EEP-1	0.013±0.0006	0.00	0.00	0.15±0.004	0.01±0.0002	0.01±0.0001	0.07±0.003	0.00	0.00
EEP-2	0.00	0.00	0.07±0.003	0.16±0.003	0.02±0.0002	0.01±0.0001	0.07±0.002	0.05±0.0002	0.00
EEP-3	0.029±0.0008	0.04±0.0003	0.08±0.002	0.64±0.001	0.04±0.0004	0.06±0.0003	0.11±0.004	0.10±0.0006	0.02±0.0008
EEP-4	0.00	0.01±0.0001	0.07±0.002	0.15±0.002	0.01±0.0001	0.01±0.0001	0.07±0.002	0.05±0.0002	0.00
EEP-5	0.015±0.0005	0.00	0.07±0.003	0.00	0.01±0.0001	0.01±0.0001	0.07±0.003	0.05±0.0003	0.02±0.0009
EEP-6	0.00	0.01±0.0001	0.07±0.003	0.00	0.00	0.01±0.0001	0.07±0.003	0.00	0.00

CAPE: Caffeic acid phenethyl ester.

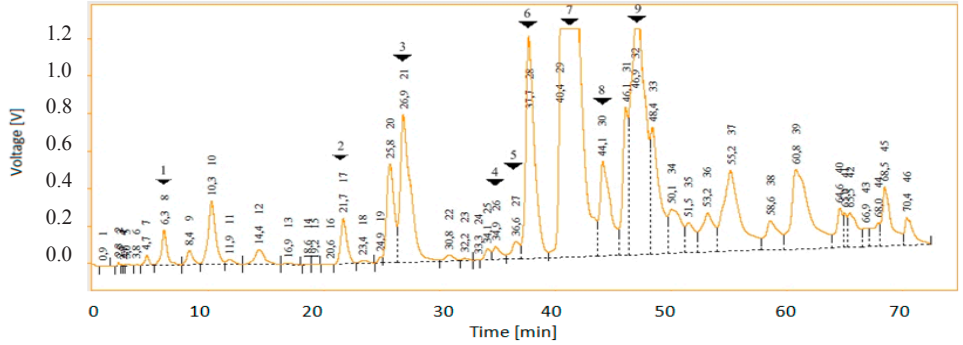

Figure 1. Chromatographic pattern of commercial extract EEP-3. Wavelength: 290 nm. Injection volume: 10 µL. Identified compounds: 1, caffeic acid; 2, myricetin; 3, quercetin 4, kaempferol; 5, apigenin; 6, rutin; 7, pinocembrin; 8, galangin; 9, caffeic acid phenethyl ester (CAPE).

Fungal identification

The morphological analysis of the fungal collections revealed that the genera correspond to *Trichoderma*, *Botrytis*, *Alternaria*, *Fusarium*, *Ulocladium* and *Penicillium*. The PCR identification of the six fungi is shown in Figure 2.

The nucleotide sequence of the ITS regions containing the 5.8S ribosomal gene were obtained from isolates of these six fungi, and the sequences were compared using BLAST. Test strains obtained from the culture collections were also molecularly characterized, and their identities as *B. cinerea*, *A. alternata*, *Fusarium* sp., *Ulocladium* sp. and *Trichoderma reesei* were confirmed.

The GenBank accession number of the reference sequence is FJ004274 *Penicillium expansum*.

Antifungal activity

Considering its high concentration of total polyphenols and flavonoids (Table 1 and Table 2), EEP-3 was selected for further experiments.

The six assessed fungi were sensitive to the high concentration of EEP-3 (Figures 3 and 4).

The efficacy of the treatment with EEP-3 decreased over time. In the first week, no statistically significant differences (P>0.05) between

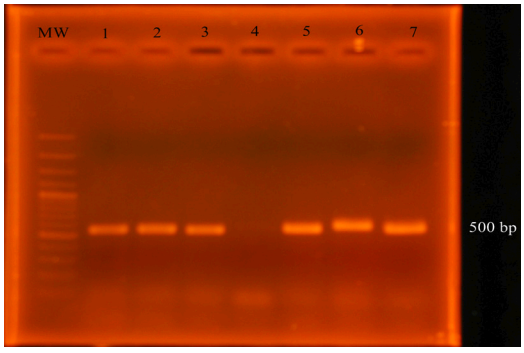

Figure 2. PCR products of the six fungi used for identification. Agarose gel (2%) stained with ethidium bromide. MW, molecular weight marker; lane 1, *Penicillium expansum*; lane 2, *Botrytis cinerea*; lane 3, *Alternaria alternata*; lane 4, negative control; lane 5, *Fusarium* sp.; lane 6, *Ulocladium* sp. and lane 7, *Trichoderma reesei*.

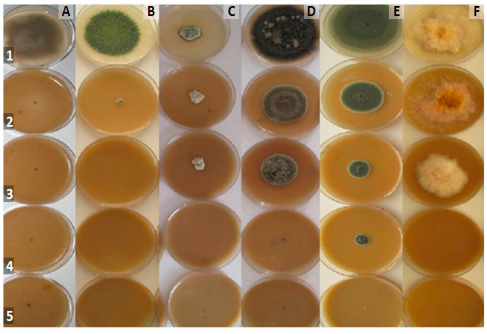

Figure 3. Evaluation of the antifungal activity when the control treatment reached its maximum growth. The letters indicate the fungus used in experiment, and the numbers correspond to the concentrations of the propolis extract added to the culture medium. Fungi: A, *Ulocladium* sp.; B, *Trichoderma reesei*; C, *Penicillium expansum*; D, *Alternaria alternata*; E, *Botrytis cinerea*; F, *Fusarium* sp. EEP-3 extract: 1, medium alone; 2, 0.5%; 3, 1.0%; 4, 2.5%; 5, 5%.

Table 3. *The effective concentration* (EC) for a 50 and 90 response for the fungi tested (%).

	Alternaria alternata	*Fusarium sp.*	*Ulocladium sp.*	*Botrytis cinerea*	*Penicillium expansum*	*Trichoderma reesei*	Average
EC_{50}	0.53	1.23	0.001	0.48	0.99	0.01	0.54
EC_{90}	1.73	2.35	0.001	2.35	2.70	0.31	1.57

the treatments of 0.5% and 1% EEP-3 versus the chemical treatment; this trend was also observed in the second week, with the 2.5% propolis treatment behaving the same way in the third week. The sensitivity of the six fungi to EEP-3 was demonstrated in this study (Figure 4), decreasing over 60% of the mycelial growth at the lowest concentration studied and nearly 100% growth inhibition at the highest concentration used (0.5 and 5%, respectively).

Determination of the EC_{50} and EC_{90}

The EC_{50} and EC_{90} values of the six fungi evaluated are shown in Table 3. The lowest concentrations of EEP-3 that inhibited approximately 50% of the mycelial growth of *Ulocladium* sp. and *T. reesei* were 0.001 and 0.01%, respectively. The lowest concentrations inhibiting approximately 90% of the mycelial growth (0.001%-0.31%) were found for the same fungi.

Discussion

The total polyphenol and flavonoid concentrations are similar to those reported by Vidal *et al*. (2009) and Herrera *et al*. (2010) who also evaluated Chilean commercial ethanol extracts of propolis and were similar to those reported in samples of Argentine (Bedascarrasbure *et al*., 2004, 2006; Chaillou and Nazareno, 2009) and Brazilian propolis (Castro *et al*., 2007).

The levels of total polyphenols and flavonoids account for the variability in the chemical composition of the commercial extracts of propolis and are attributable to both endogenous and exogenous factors (Table 1). The observed variability can be explained by the EEPs of a homemade product for which there is not requirement to declare the extraction process. In addition, the composition of propolis is influenced by the flora surrounding the apiary (Chaillou and Nazareno, 2009; Agüero *et al*., 2010), the

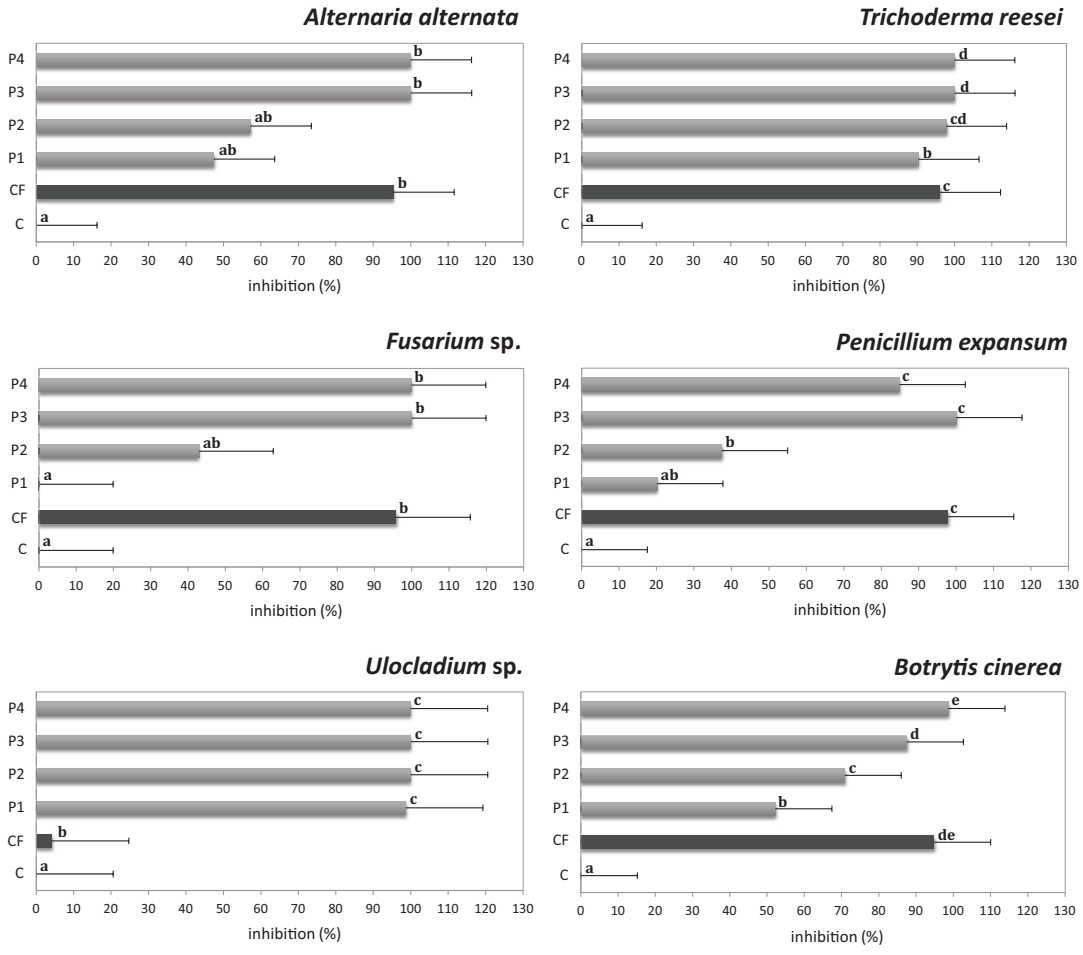

Figure 4. Percentages of inhibition by EEP-3. C: control without fungicide. CF: chemical fungicide. P1, 0.5% EPP-3; P2, 1% EPP-3; P3, 2.5% EPP-3 and P4, 5% EPP-3. Each bar represents the average of 3 independent plates, plus the standard deviation. The letters on the bars indicate significant differences (P≤0.05).

geographical features and local climatic (Seidel *et al*., 2008) and seasonal effects (Valencia *et al*., 2012). The exogenous factors are related to the extraction by the beekeeper and include the harvest season (Simões-Ambrosio *et al*., 2010), the method used (Sales *et al*., 2006), the type of extractant (Tosi *et al*., 1996; Longhini *et al*., 2007) and the extractant concentration (Dos Santos *et al*., 2003).

EEP-3 is produced by an association of beekeepers in the region La Araucanía that has a reproducible protocol for preparing ethanol extracts of propolis each year. Over the last four years (2007, 2008, 2009 and 2010), the EEP produced has had similar values of total polyphenols and total flavonoids (data not shown), indicating the importance of the association of beekeepers that have standardized methods (FIA, 2009).

The fungi used in this study are common causal agents of various agricultural diseases, spoilage, food contamination and mycotoxin production. New control agents are needed to manage the pathogenic fungi in plants (*A. alternata*, *Fusarium* sp., and *Ulocladium* sp.) and manage postharvest pathogenic fungi (*B. cinerea* and *P. expansum*). The results presented here showed that EEP-3 possesses *in vitro* antifungal activities against a broad spectrum

of plant pathogenic fungi and could be used as a potential antifungal agent for the control of fungal plant diseases. Furthermore, EEP-3 also presented an antifungal activity against the biological control fungus (*T. reesei)*. Garedew *et al*. (2004) indicate that the minimum concentration of propolis that caused the growth inhibition of filamentous fungi was between 0.5% and 2.5%, which is similar to that obtained in this study for the fungi tested. Basim *et al*. (2006) examined the antimicrobial activity of extracts of pollen and propolis and found that the antimicrobial activity of EEP was greater than the pollen extract, which is a result that was attributable to the presence of phenolic compounds. Quiroga *et al*. (2006) assayed the use of Argentinean propolis against several species of xylophagous and phytopathogenic fungi and, similar to Hegazi and Abd El Hady (2002), successfully demonstrated the inhibition of the fungal pathogens by the propolis samples.

Another important aspect to consider is that, as noted by Bankova (2005b), the function of propolis is to protect the bees against infections that occur in the hive. Therefore, independent of whether the protective chemical variability is maintained, the inhibitory activity of propolis is associated with the synergy of its components, consistent with Garedew *et al*. (2004), Meneses *et al*. (2009) and Petrova *et al*. (2010), and it has not been shown experimentally that only a single component has greater activity than the total extract (Agüero *et al*., 2010). However, Treutter (2006) discussed the significance of flavonoids in the protection and resistance of plants to phytopathogens, particularly fungi, and phenolics and flavonoids are proposed to be the main antimicrobial components of propolis. Some flavonoids with strong antimicrobial activities have been identified in propolis collected from various regions. Pinocembrin, pinobanksin, chrysin and galangin in Chinese propolis demonstrated strong antifungal activities against *P. italicum* (Yang *et al*., 2011), whereas the main antimicrobial compound was found to be galangin in Bulgarian propolis (Campana *et al*., 2009). In Sonoran propolis (Mexico) pinocembrin, pinobanksin 3-acetate, chrysin, CAPE, acacetin and galangin were present in propolis samples collected four seasons (Valencia *et al*., 2012). We identified caffeic acid, myricetin, quercetin, kaempferol, apigenin, pinocembrin, galangin, CAPE and rutin in EEP-3 (Table 2) and suggest that pinocembrin and galangin could be responsible for the antifungal activity.

In summary, our results indicate that the ethanolic extract of Chilean propolis has the capacity to inhibit certain important economical agricultural fungi *in vitro*. The results obtained in this study are promising and support the importance of further *in vivo* investigations into the antifungal capacity of propolis from the region of La Araucanía. The inclusion of such natural products as propolis extract in crop protection strategies will help to maintain the balance of agroecosystems and the safety of the harvested products.

Acknowledgments

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Resumen

M. Curifuta, J. Vidal, J. Sánchez, A. Contreras, L.A. Salazar y M. Alvear. 2012. Evaluación de la actividad antifúngica *in vitro* de un extracto comercial de propóleos Chileno contra seis hongos de interés agrícola. Cien. Inv. Agr. 39(2): 347-359. El propóleo ha sido utilizado por el hombre desde hace milenios por sus propiedades antimicrobianas y farmacéutica. Sin embargo, su uso como un agente antifúngico agrícola sólo recientemente ha sido evaluado. Con el objetivo de estudiar su capacidad antifúngica se caracterizaron químicamente seis extractos etanólicos (EEP1-EEP6), comercializados en la Región de La Araucanía. Se evaluó el contenido de polifenoles y flavonoides totales presentes en los EEP, estos presentaron concentraciones de polifenoles totales en un rango de 7,8 a 42,3 mg mL^{-1} equivalentes de la mezcla pinocembrina: galangina 2:1, y de flavonoides totales en un rango que varió entre 4,0 y 19,7 mg mL^{-1} equivalentes de quercetina. Análisis por cromatografía líquida de alta resolución (HPLC) permitió la identificación del ácido cafeico, miricetina, quercetina, kaempferol, apigenina, pinocembrina, galangina, éster del ácido cafeico (CAPE) y rutina. Se seleccionó el EEP-3, pues presentó las mayores concentraciones de polifenoles y flavonoides totales 42,3 y 19,7 mg mL^{-1}, respectivamente. El efecto antifúngico de cuatro diferentes concentraciones (0,5; 1,0; 2,5 y 5,0%) del EEP-3 sobre los hongos *Alternaria alternata*, *Fusarium* sp., *Ulocladium* sp., *Botrytis cinerea*, *Penicillium expansum* y *Trichoderma reesei*, fue evaluado *in vitro* mediante el método de dilución en agar. Se presentaron diferencias significativas entre las concentraciones de extracto ensayadas. Los resultados indican que el EEP-3 inhibió el crecimiento micelial en los seis hongos evaluados.

Palabras clave: *Alternaria alternata*, antifúngico, *Botrytis cinerea*, flavonoides, *Fusarium* sp., *Penicillium expansum*, polifenoles, propóleos.

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