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RESEARCH NOTE

Pathogen control using a natural Chilean bee pollen extract of known botanical origin

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Abstract

C. Cabrera, and G. Montenegro. 2013. Pathogen control using a natural Chilean bee pollen extract of known botanical origin. **Cien. Inv. Agr. 40(1):223-230.** Bee pollen is a product from beehives that is contained in corbiculae, generally monospecific and is currently considered as a functional food due to its nutritional and medicinal properties. This study investigates the antimicrobial activity of a Chilean bee pollen extract of known botanical origin. The botanical origin of the pollen was determined by palynological analysis, and then an aqueous extract was prepared. The antibacterial properties of the extract were evaluated on human infectious agents (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pyogenes*) using a qualitative method (agar diffusion) and a quantitative method (minimum inhibitory and bactericide concentration) and on agricultural pathogens (*Alternaria alternata, Botrytis cinerea* and *Fusarium oxysporum*) using the poisoned food technique. The bee pollen (60% of *Azara petiolaris*) is considered native, endemic and monofloral. The *E. coli* and *P. aeruginosa* became resistant to the extract, while *S. aureus* and *S. pyogenes* were sensitive. Additionally, the minimum inhibitory concentrations (MICs) of these bacteria were 82.4 mg mL^{-1} for *E. coli*, 41.2 mg mL^{-1} for *P. aeruginosa*, and 20.6 mg mL^{-1} for *S. aureus* and *S. pyogenes*. Additionally, the extract did not have a complete inhibitory effect on the fungi, but it caused delayed growth in comparison to the control. The development of an aqueous extract from native and endemic bee pollen with antimicrobial properties creates the potential for future research and development of new Chilean natural products, favoring the development of national apiculture.

Key words: Antimicrobial activity, bee pollen, aqueous extract.

Introduction

Floral pollen is one of the main sources of protein for bees (Almeida-Muradian *et al*., 2005) and is used as food for their offspring. However, before transporting it to the beehive, bees mix pollen with nectar and digestive enzymes and store it within a structure on the hind legs, the corbicula, thus forming bee pollen (Nagai *et al*., 2004). The pollen contained in corbiculae is generally monospecific (Montenegro *et al*., 1992) because while travelling from the beehive, bees forage many flowers of the same species to fill their corbiculae (Montenegro *et al*., 1992; Sá-Otero *et al*., 2002).

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Generally, bee pollen is a directly consumed product due to its nutritional value in the human diet, and it is currently regarded as a functional food because vegetal proteins are important due to their scarcity in nature (Carpes *et al*., 2007). Pollen is primarily composed of polysaccharides, simple sugars, lipids, amino acids, proteins and other secondary compounds, such as flavonoids, vitamins, minerals and pigments such as carotenoids (Montenegro *et al*., 1997; Vit *et al*., 2008). The use of pollen in traditional medicine is strongly linked to its chemical composition, which depends on the floral origin of bee pollen (Sá-Otero *et al*., 2002). Additionally, phenolic compounds (principally flavonoids) present in pollen have recently aroused interest due to their antioxidant and antimicrobial activity (Leja *et al*., 2007; Vit *et al*., 2008) and positive effects on several pathologies such as diabetes, hypertension and cardiovascular disease (Nagai *et al*., 2004; Abouda *et al*., 2011).

Although there are few studies on the antimicrobial activity of bee pollen, some authors show that its antimicrobial activity depends on the botanical origin of the pollen, unlike propolis. Özcan *et al*. (2004) evaluated the antifungal activity of bee pollen methanolic extracts from different regions in Turkey and showed that they have an inhibitory effect on the growth of some phytopathogens. Additionally, Basim *et al*. (2006) evaluated the antibacterial activity of methanolic extracts against agricultural bacteria, which were also inhibited by pollen extracts from Turkey. Carpes *et al*. (2007) studied the effect of methanolic extracts from Brazilian pollen on the growth of pathogenic bacteria and also obtained significant results. However, Carpes *et al*. (2009) did not obtain positive results using ethanolic extracts from bee pollen from Southern Brazil on human and agricultural bacteria, demonstrating that the antimicrobial activity of bee pollen is dependent on its botanical origin. Finally, two studies from 2011 on the antimicrobial activity on bee pollen extracts from human pathogens (Abouda *et al*., 2011; Morais *et al*., 2011) and yeasts (Morais *et al*., 2011) both showed inhibitory effects.

The objective of this study was to evaluate the *in vitro* antimicrobial activity of aqueous bee pollen extracts derived solely from native Chilean flora on human and agricultural pathogens in relation to the growing interest in natural products, the importance of functional foods, and the effort to the increase the use of less environmentally harmful agricultural products.

Materials and methods

Sampling bee pollen

Bee pollen was collected in the Calera de Tango sector of the Metropolitan Region of Chile (33°36' S and 70°47' W) in January of 2011 and stored at -6 °C before use. The sample contains 250 grams of bee pollen. The pollen was palynologically analyzed according to the methodology of the Chilean Standard NCh3255-2011 (Montenegro *et al*., 2011) to determine its botanical and geographical origin.

Pathogen culture

The pathogenic bacteria used in this study, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pyogenes,* were acquired from the Instituto de Salud Pública (Santiago, Chile). The phytopathogenic fungi *Alternaria alternata*, *Botrytis cinerea* and *Fusarium oxysporum* were provided by Laboratorio AgroLab. The bacteria were grown overnight on trypticase soy agar at 37 °C for use the following day. The fungi were grown on potato dextrose agar (PDA) at 25 °C for seven days before use.

Aqueous extract from bee pollen

Aliquots containing 100 grams of bee pollen were suspended in 100 mL of distilled water. The mixture was submerged in a sonicating water bath (Branson 2200 Ultrasonic Cleaner,

Shelton, CT, USA; working frequency of 47 kHz at 30 °C) for 1 h and then centrifuged at 6,030 x g for 15 min to harvest the supernatant. The process was repeated four times with the resulting pellet. The five supernatants were combined and filtered using a vacuum pump and grade 4 filter paper. The filtered solution was desiccated in a rotavapor at 45 °C (Buchi, Flawil, Switzerland). The residue was brought to a final volume of 100 mL with distilled water. The solution was filtered again using sterile cellulose filters with 0.45 µm pores and 0.45 µm-syringe filters to remove the typical microbiological load from the bee pollen. Additionally, 1 mL of distilled water and 1 mL of the extract were weighed, and the extract concentration was calculated to be 82.4 mg of bee pollen mL^{-1} of extract by the difference in weight.

Determining the bacterial sensitivity

The bacteria were cultured on 90 x 15 mm-Petri plates containing 25 mL of soy agar. Three agar plugs were removed from each plate using a sterile 6 mm-diameter-punch, and 100 µL of the extract was placed inside each hole. Four concentrations of the extract were used (25, 50, 75 and 100%), and distilled water was used as control. This assay was run in triplicate. The plates were incubated at 37 °C for 24 h. The results were expressed as the diameter of the zone of inhibition (mm).

Determination of the antibacterial activity by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods

The dilution method was used to determine the minimum inhibitory concentration (MIC) (Hogg, 2005). Ten 12 x 75 mm-assay tubes containing 0.5 mL of soy broth were prepared for each bacterial species to be tested. Then, 0.5 mL of extract was added to the first tube and was subsequently diluted 1:2 by placing 0.5 mL from the first tube into the second and so on. Once the dilutions were made, 50 µL of bacterial culture was added to each tube (10^5 – 10^6 CFU mL^{-1}, 0.5 McFarland) and incubated for 24 h at 37 °C. The tube without bacterial growth was identified to determine the MIC (according to the turbidity compared with the control treatment). This assay was run in triplicate.

The minimum bactericidal concentration (MBC) was determined using the cultures from the MIC test as follows: a 10 µL aliquot was taken from each tube and plated within one of 10 pre-marked sections on a Petri dish containing trypticase soy agar. The plate was incubated for 24 h at 37 °C. The MBC was determined by identifying the section that lacked bacterial growth. This assay was run in triplicate.

Antifungal activity

The poisoned food technique was used to determine the fungistatic activity as previously described (Grover and Moore, 1962), but with slight modifications: 35 x 15 mm-Petri plates were filled with 3 mL of PDA and poisoned with three different concentrations of the extract (25, 50 and 75%). Agar plugs were removed from the fungal culture after seven days using a 3 mm-diameter punch and inserted into the center of each plate with poisoned medium. The plates were incubated at 25 °C, and the diameter of growth was measured daily until the fungus occupied the entire plate in the control treatment (PDA with distilled water). This assay was run in triplicate. The fungicidal activity of extract was determined based on the results of the fungistatic activity test. The agar plugs from plates on which no partial inhibition of growth was observed were placed in 35 x 15 mm-Petri plates with PDA medium. The plates were incubated at 25 °C, and the diameter was measured daily.

Experimental design and data analysis

The experiments were conducted under a completely randomized design. To assess the variation of the data obtained in the evaluation of antimicrobial activity, we performed an analysis of variance and means comparison test (LSD Fisher), using InfoStat software, version 2012.

Results and discussion

Determination of the pollen botanical origin

The analyzed bee pollen was composed of pollen from seven floral species (Figure 1), of which one was identified only at the genus level (*Brassica* sp.). Five of the seven species are introduced, and the other two are native and endemic from Chile (Table 1). *Azara petiolaris* is the predominant species in the sample (60%) followed by *Brassica* sp. (30%), *Raphanus sativus* and *Adesmia confusa* (3.33% each), *Datura stramonium*, *Rubus ulmifolius* and *Lotus corniculatus* (1.67% each) (Figure 1).

The bee pollen sample is categorized as monofloral, endemic and native according to the Chilean Standard NCh3255.c2011 (Montenegro *et al*., 2011) due to its 60% *A. petiolaris* pollen content, which may indicate that the antimicrobial properties of the bee pollen extract are derived primarily from the *A. petiolaris* pollen.

Antibacterial activity

The antibacterial activity results obtained by the agar diffusion method indicate that of the four evaluated bacterial species, only *S. aureus* and *S. pyogenes* were inhibited, whereas the extract did not have an effect on *E. coli* and *P. aeruginosa* growth. However, the inhibition of the effected bacteria occurred only at the 50, 75 and 100% concentrations, and the extract did not show activity at lower concentrations (25%) when compared to the control treatment. Additionally, *S. pyogenes* was more sensitive to the extract than *S. aureus*, reaching an average inhibition halo of 14.78 and 17.15 mm at 75 and 100%, respectively, whereas the halo formed in *S. aureus* reached an approximate diameter of 12.37 and 14.04 mm at the same concentrations (Figure 2). It was also observed that in *S. aureus,* the results obtained at the 50% concentration are statistically equivalent to those obtained with the 75% concentration, while the latter are comparable to those obtained with the

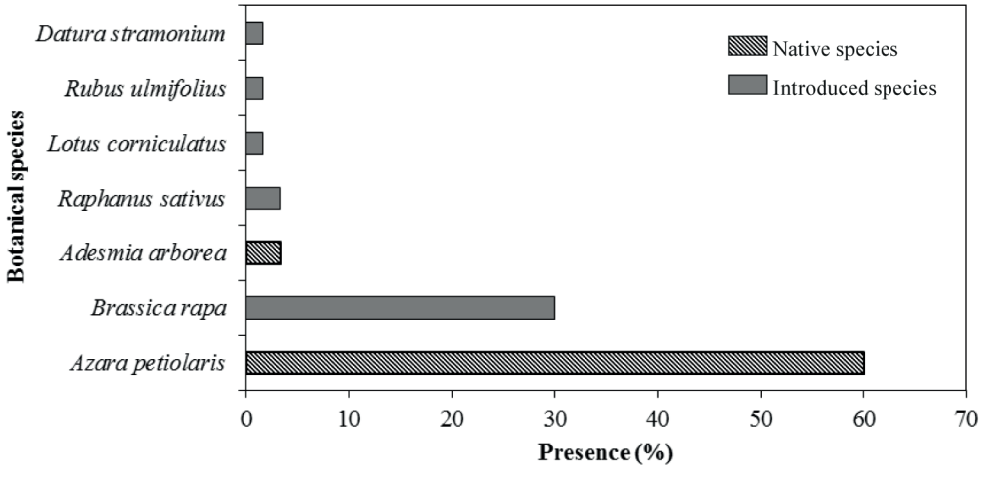

Figure 1. Botanical species, their respective presence (%), and typology (native or introduced) according to the botanical origin results for the bee pollen sample.

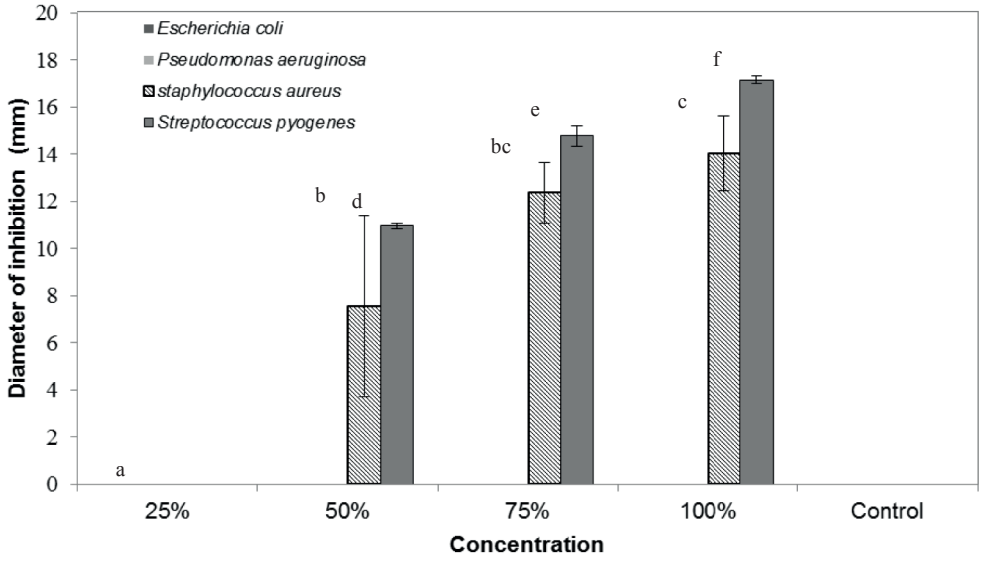

Figure 2. Determination of the bacterial sensitivity by the agar diffusion method. The diameters of the zones of inhibition are shown in mm at different concentrations of bee pollen extract for each bacterium, ± the standard deviation. Means with a common letter are not significantly different (P≤0.05).

100% concentration. However, for *S. pyogenes,* the results obtained for the three concentrations were significantly different.

With regard to the MIC results, the four bacterial species were inhibited by the extract, but at different concentrations. The MIC for *S. aureus* and *S. pyogenes* was 20.6 mg of pollen mL^{-1} of extract, for *P. aeruginosa,* the MIC was 41.2 mg of pollen mL^{-1} of extract and for *E. coli,* it was 82.4 mg of pollen mL^{-1} of extract. These results indicate that *E. coli* and *P. aeruginosa* are inhibited at a higher concentration of extract than *S. aureus* and *S. pyogenes*. On the other hand, the results from the MBC showed that the *A. petiolaris* extract only had a bactericidal effect on *S. pyogenes* at a concentration of 82.4 mg of pollen mL^{-1} of extract. The other three bacteria continued to grow when they were transferred to new culture medium; therefore, the extract only had an inhibitory effect on them.

The fact that Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were not affected by the extract in the agar diffusion method and were affected at higher extract concentrations (MIC) could be because the outer membrane present in this type of bacteria is absent in the Gram-positive bacteria (*S. aureus* and *S. pyogenes*). Thus, they are more resistant to hydrolytic enzymes, surfactants, biliary salts and hydrophobic antibiotics than the Gram-positive bacteria (Kim and Gadd, 2008). Additionally, the MBC results confirm the higher resistance of the Gram-negative bacteria to some types of treatment.

Fungistatic activity

With regard to the fungistatic activity of the extract, only the 25% and 50% concentrations caused a decline in the growth of three fungal pathogens compared to the control treatment, while the 75% concentration inhibited the growth of *A. alternate* until the fourth day of evaluation and completely inhibited the growth of *B. cinerea* and *F. oxysporum*. In addition, significant differences were found in mycelial growth when different evaluation days were compared (Table 1).

Moreover, the fungicidal activity results of the extract showed that mycelial growth resumed when the inocula were transferred to a new culture

medium, comparable to the treatment control; therefore, the extract showed no fungicidal activity on all three pathogens studied (Figure 3).

Although the extract did not show a complete inhibitory effect on the fungi, similar results have been observed for *A. alternata* and *F. oxysporum* (Özcan *et al*., 2004), where the effect of pollen extracts is thought to occur due to various phenolic compounds, which depend directly on the botanical origin of the bee pollen.

Table 1. Average growth per day (mm) of *Alternaria alternata*, *Botrytis cinerea* and *Fusarium oxysporum* with respect to three concentrations of bee pollen extract of *Azara petiolaris* and its respective control treatment.

		Average growth per day (mm)							
Pathogen	Concentration	1	2	3	4	5	6	7	LSD Concentration
Alternaria alternata	25%	3.0 a	7.0 b	10.8 c	14.2 d	16.6 e	21.3 f	21.8 f	c
	50%	1.8 a	3.3 b	6.3 c	9.0 d	11.0 e	15.3 f	15.7 f	b
	75%	0.0 a	0.0 a	0.0 a	0.0 a	0.3 ab	0.7 b	0.7 b	a
	Control	6.0 a	14.3 b	22.4 c	27.0 d	28.0 e	28.0 f	28.0 g	d
Botrytis cinerea	25%	6.9 a	17.0 b	22.3 c	23.8 cd	25.1 de	27.0 e	27.0 e	c
	50%	1.3 a	4.0 a	6.8 b	8.9 bc	11.0 cd	13.4 de	12.2 e	b
	75%	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	a
	Control	6.4 a	18.3 b	28.0 c	28.0 c	28.0 c	28.0 c	28.0 c	d
Fusarium oxysporum	25%	3.0 a	7.8 b	14.3 c	18.0 d	22.3 e	23.3 f	23.7 f	c
	50%	1.0 a	5.0 b	8.1 c	11.0 d	14.2 e	16.2 f	16.2 f	b
	75%	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	a
	Control	5.1 a	6.3 a	16.1 b	22.7 c	25.0 d	26.7 e	26.7 e	d

Means with a common letter are not significantly different (P≤0.05).

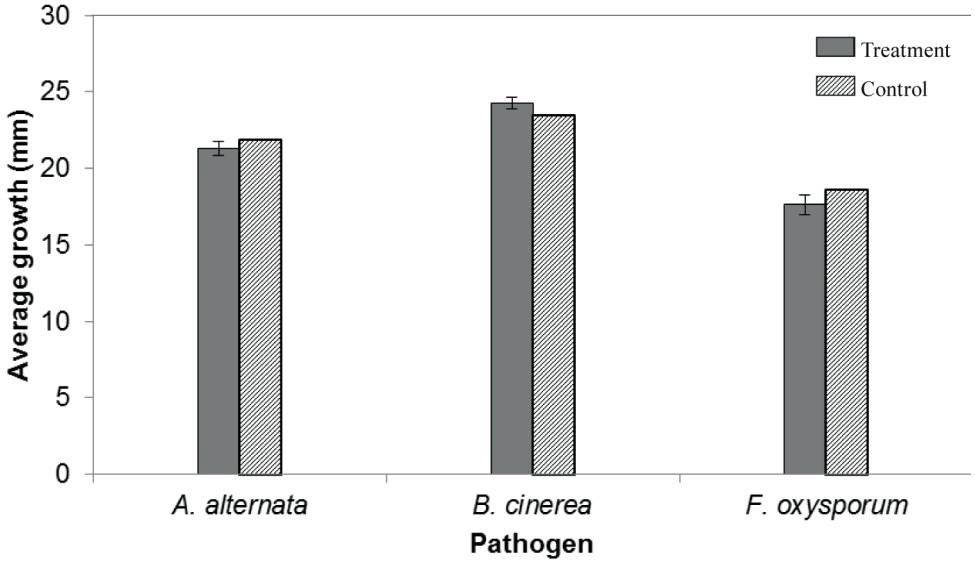

Figure 3. Evaluation of the fungicidal activity of bee pollen extract from *Azara petiolaris*, measured as average growth (mm) for each pathogen (*Alternaria alternata*, *Botrytis cinerea* and *Fusarium oxysporum*) with respect to their respective control treatment, ± the standard deviation. Means with a common letter are not significantly different (P≤0.05).

The main conclusions of the aforementioned research are as follows. The bee pollen extract from the native and endemic *Azara petiolaris* species showed antibacterial and antifungal activity against the pathogens analyzed. This is crucial, as other authors (Özcan *et al*., 2004; Basim *et al*, 2006; Carpes *et al*., 2007; Carpes *et al*., 2009; Abouda *et al*., 2011; Morais *et al*., 2011) agree that the antimicrobial properties of bee pollen are due mainly to their phenolic components, which are directly related to its botanical origin.

The antimicrobial activity of pollen has become important through the years, mainly due to the development of antibiotic resistant microorganisms; therefore, the development of native bee pollen extracts that are harmless to the environment and human beings is essential as a natural product with specific biological activity. Additionally, the *A. petiolaris* bee pollen extract is unique in the world due to the endemic nature of the species, giving an added value to the products of the hive.

It is noteworthy that the present research is preliminary with regard to the antimicrobial activity of bee pollen from *A. petiolaris*. Thus, additional experiments must be carried out to determine which factors positively influencing this activity.

Acknowledgements

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Resumen

C. Cabrera y G. Montenegro. 2013. Control de patógenos mediante el uso de un extracto natural de polen apícola chileno de origen botánico conocido. Cien. Inv. Agr. 40(1):223-230. El polen apícola es un producto de la colmena compuesto por corbículas, generalmente monoespecíficas y, debido a sus propiedades nutricionales y medicinales, hoy en día es considerado un alimento funcional. En la presente investigación se estudió la actividad antimicrobiana de un extracto de polen apícola chileno, de origen botánico conocido. Se determinó mediante análisis palinológico el origen botánico del polen y luego se preparó un extracto acuoso. La efectividad del extracto se evaluó en el control de bacterias humanas (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* y *Streptococcus pyogenes*), mediante un método cualitativo (difusión en agar) y otro cuantitativo (mínima concentración inhibitoria y bactericida) y sobre patógenos del agro (*Alternaria alternata, Botrytis cinerea y Fusarium oxysporum*), mediante el método del alimento envenenado. El polen apícola resultó ser monofloral (60% de *Azara petiolaris*), nativo y endémico. Las bacterias *E. coli* y *P. aeruginosa* resultaron ser resistentes al extracto, mientras que *S. aureus* y *S. pyogenes* fueron sensibles. Además, la concentración mínima inhibitoria (MCI), para *E. coli* fue de 82,4 mg mL^{-1}, 41,2 mg mL^{-1} para *P. aeruginosa* y 20,6 mg mL^{-1} para *S. aureus* y *S. pyogenes*. Además, se determinó que el extracto no tuvo un efecto inhibitorio completo sobre los hongos, pero sí retardó su crecimiento, con respecto al control. El desarrollo de un extracto acuoso de polen apícola nativo y endémico, con propiedades antimicrobianas, abre una puerta a futuras investigaciones y desarrollo de nuevos productos, favoreciendo así la apicultura nacional.

Palabras clave: Actividad antimicrobiana, extracto acuoso, polen apícola.

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