Ciencia e Investigación AGRARIA

Cien. Inv. Agr. 37(1):75-84. 2010
www.rcia.uc.cl

RESEARCH PAPER

The antifungal effect of six commercial extracts of Chilean propolis on *Candida* spp.

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Abstract

C. L. Herrera, M. Alvear, L. Barrientos, G. Montenegro, and L. A. Salazar. 2010. The antifungal effect of six commercial extracts of Chilean propolis on *Candida* spp. Cien. Inv. Agr. 37(1): 75 - 84. Propolis has been used in traditional medicine for many centuries because of its beneficial health properties, including its antimicrobial capacity. Prosthesis stomatitis affects a significant percentage of users of removable dentures; *Candida albicans* is the most common fungal species associated with the development of this pathology. Thus, the objectives of this study were: a. To evaluate the antifungal activity of six commercial propolis extracts against *Candida* spp. that was isolated from the oral cavity of removable dentures users, and b. To determine chemical characteristics of the propolis extracts evaluated. Among the results, we note that these concentrations of polyphenols varied between 9 ± 0.3 and 85 ± 2.1 mg mL^{-1}. Chromatographic analysis was able to detect 35 compounds, among which were caffeic acid, myricetin, quercetin, kaempferol, apigenin, pinocembrin, galangin, and caffeic acid phenyl ester (CAPE). All strains tested were inhibited by the liquid extracts of propolis. The MID ranged between 1:40 and 1:1280, and the MIC for *C. albicans* ranged from 197 µg mL^{-1} to 441 µg mL^{-1}. From the results obtained in this investigation, we can conclude that all propolis extracts evaluated are capable of inhibiting the development of *Candida* spp. However, they show significant differences in the concentration of polyphenols present and in antifungal activity.

Key words: Antifungal activity, *Candida albicans*, propolis.

Introduction

Propolis has been used in traditional medicine for centuries; however, its beneficial properties for human health have been explained scientifically only in recent decades.

It is well known that propolis composition shows great variability, both qualitatively and quantitatively, and its characteristics depend on botanical and geographical origin (Hegazi *et al*., 2000; Marcucci *et al*., 2000; Majiene *et al*., 2004; Peña, 2008). Salomao *et al.* (2004) states that the main components of an ethanolic extract of Brazilian propolis are aromatic acids, unlike Bulgarian propolis, which showed a predominance of flavonoids.

Received 7 April 2009. Accepted 28 May 2009.
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This variable composition might be responsible for the differences observed in the biological activity detected in propolis with different origins (Cafarchia *et al*., 1999; Kujumgiev *et al*., 1999; Velikova *et al*., 2000). However, it is noteworthy to say that chemically different propolis, in some cases, shows similar biological activity (Salomao *et al*., 2004). Thus, beyond the activity of the individual components, the result is the consequence of a synergistic effect, which results in propolis having diverse pharmacological properties (Banskota *et al*., 2001; Kujumgiev *et al*., 1999). Currently, there is considerable scientific evidence showing not only their important role in the inflammatory response and cancer development but also their antioxidant activity and antimicrobial properties (Banskota *et al*., 2001; Burdock, 1998; Sforcin, 2007).

The antifungal activity of propolis has been specifically evaluated against different fungi. The genera *Aspergillus* (Aly and Elewa, 2007), *Candida* (Santos *et al*., 2005; Silici *et al*., 2005; Oliveira *et al*., 2006; Silici and Koc, 2006; Ghasem *et al*., 2007; Quintero-Mora *et al*., 2008;), *Trichopyton* (Koc *et al*., 2005; Siqueira *et al*., 2008), *Trichosporon* (Oliveira *et al*., 2006), *Rhodotorula* (Silici *et al*., 2005) and others have been analyzed regarding their susceptibility to propolis or to some of its components. These and other analyses have determined that pinocembrin, galangin and pinobanksin are the predominant compounds in the studied propolis (Hegazi *et al*., 2000; Uzel *et al*., 2005; Quiroga *et al*., 2006).

Yeasts of the genus *Candida* are part of the microbial flora of human beings; the skin and the mucosal membranes of airways, gastrointestinal and genito-urinary tract serve as their habitat. This genus may be found in 20% to 70% of the population. However, the incidence has increased due to the use of dentures, xerostomia, endocrine disorders, use of multiple antibiotics, use of immunodepressants, use of antineoplasics and other factors (Gonsalves *et al*., 2007).

Oral candidiasis is defined as an infection of the mucous membrane of the oral cavity caused by yeasts of the genus *Candida*. In users of removable dentures, *C albicans* is the most common species associated with the development of subprosthestic stomatitis, the inflammation of oral mucous in contact with prosthesis. Dar-Odeth *et al*. (2003) found that *C. albicans* is responsible for 72% of the cases of subprosthestic stomatitis; although a variety of other species of this genus may also be responsible for this pathology (Pereira *et al*., 2008).

In recent years, the use of propolis has spread due not only to its beneficial properties but also to the emergence of propolis-supplemented food and cosmetics (Banskota *et al*., 2001). Although the consumption of alcoholic and non-alcoholic extracts of propolis has become popular in Chile, knowledge of the characteristics of these products is limited, unlike in other countries. According to different data bases, the study of the characteristics, composition and properties of Chilean propolis is restricted to a mere 12 scientific articles in the last ten years, and some type of biological activity has been evaluated only in four of them (Valcic *et al*., 1998; Valcic *et al*., 1999; Astudillo *et al*., 2000; Montenegro *et al*., 2000; Montenegro *et al*., 2001a; Montenegro *et al*., 2001b; Muñoz *et al*., 2001a; Muñoz *et al*., 2001b; Kumazawa *et al*., 2004; Montenegro *et al*., 2004; Russo *et al*., 2004; Hernández *et al*., 2005). Consequently, in addition to evaluating its botanical origin and chemical characteristics, Valcic *et al*. (1999) assessed its antimicrobial activity against *Mycobacterium avium*, *M. tuberculosis* and *Staphylococcus aureus*. Astudillo *et al*. (2000) evaluated its antioxidant activity, and Kumasawa *et al*. and Russo *et al*. (2004) evaluated its antioxidant and antitumoral activity, respectively.

Finally, with regard to the aforementioned studies, and to address the lack of studies evaluating the activity of the Chilean propolis against *Candida* spp., the objectives of the present study were: a. To evaluate the antifungal activity of six commercial extracts of propolis on *Candida* spp. strains that were isolated from the oral cavity of removable dental prosthesis users and b. To determine some of the chemical characteristics of the propolis extracts.

Materials and methods

Propolis samples

Six liquid extracts of propolis (mother tincture) were purchased from drugstores and stores of natural products in Temuco, Chile.

Microorganisms

The strains of *Candida* spp. used in this study were isolated from the oral cavity of removable dental prosthesis users from Temuco, Chile. The study protocol was approved by the Ethics Committee of the Universidad de La Frontera, and all subjects gave written, informed consent according to the basic principles of biomedical investigation enumerated in the Helsinki Declaration.

The isolation and identification of the microbial species were conducted in the Laboratory of Molecular Biology and Pharmacogenetics of Universidad de La Frontera, Temuco, Chile. For *C. albicans*, identification was made using CHROMagar Candida (Laboratorios Linsan S.A., Chile), the germinal tube formation test and Polymerase Chain Reaction (PCR). The primers used and conditions of reaction were previously described by Baquero *et al*. (2002). Identification by PCR of *C. albicans* strains is shown in Figure 1. In addition, the identification of *C. dubliniensis*, which presents phenotypical characteristics similar to that of *C. albicans*, was evaluated by monitoring development at 45°C in Sabouraud dextrose agar (Sharlau, Spain) (Pinjon *et al*., 1998), and by PCR-restriction fragment length polymorphism (PCR-RFLP), using the restriction endonuclease *Bln*I (Fermentas, Lithuania) (Mirhendi *et al*., 2005). Other species of the genus *Candida* were identified through the use of CHROMagar Candida.

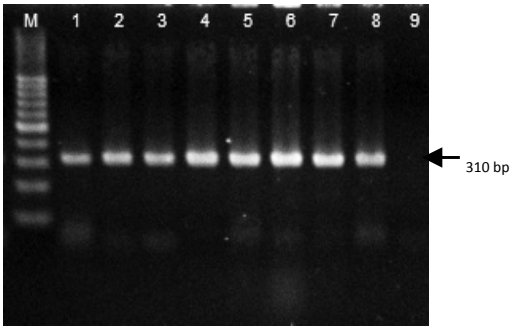

Figure 1. PCR products of *Candida albicans* identification. Agarose gel (2%), stained with ethidium-bromide. M: molecular weight marker, lanes 1-8: clinical samples, lane 9: negative control.

Total polyphenols

The Folin-Ciocalteu method was used to determine the total polyphenol content of the 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 of 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} equivalent to the mixture of the pinocembrin:galangin standards in a 2:1 ratio (Popova *et al*., 2007). All measurements were made three times.

Chromatographic analysis

High performance liquid chromatographic (HPLC) analysis was made on 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 made in an RP-18 column (12.5 x 0.4 cm, particle size

5 µm) (Merck, Germany), which separates at 25ºC using a mixture of formic acid 5% in water (A) and methanol (B) as mobile phase. The separation of the compounds was carried out by an isocratic-0 to 10 min-run, with the mixture 70% A and 30% B, followed by a gradient up to 100% B at 70 min. The compounds were detected at 290 nm, with 0.001 sensitivity; the injection volume was 10 µL. The identification of the phenolic compounds was made using the following standards: myricetin, kaempferol, quercetin, caffeic acid, galangin, pinocembrin, apigenin, caffeic acid phenyl ester (CAPE) and resveratrol (Sigma, USA).

Antifungal activity of propolis

The minimum inhibitory dilution (MID) and the minimum inhibitory concentration (MIC) were determined by the broth microdilution method following the standards of the National Committee for Clinical Laboratory Standards (NCCLS, 1997) with some modifications. The culture medium used for this test was RPMI-1640 broth with L-glutamine, which was supplemented with glucose at 2% and buffered with morpholine-propanosulphonic acid (MOPS) at pH 7.0 (Silva *et al*., 2003).

The yeast suspensions were prepared in sterile physiological serum at a concentration of 1.5 x 10^6 cfu mL^{-1}. The absorbance of this suspension was determined by spectrophotometry and was compared to the MacFarland standard. This suspension was diluted (1:50) in sterile physiological serum and then in the culture medium (1:20) to obtain a final concentration of 0.75 x 10^3 cfu mL^{-1} in the culture plate.

The commercial extracts of propolis were diluted serially in the culture plate to obtain dilutions varying from 1:10 to 1:2560 and to obtain ethanol proportions varying from 7 to 0.03%, respectively. Positive, negative and solvent controls were conducted for all conditions.
Plates were incubated for 48 h at 37°C, and the results were determined by the presence or absence of microorganism development in a specific propolis dilution. The MICs were determined as the lowest propolis concentration where a microbial development was not observed, and the MID was determined as the lowest propolis dilution where a microbial development was not observed.

Statistical analysis

Averages, standard deviations, and maximum and minimum values were calculated when necessary. Analysis of variance (ANOVA) was used for comparing the chosen level of statistical significance and was $p < 0.05$. The data analysis was made using the program GraphPad Prism, version 3.0 (Graphpad Software, San Diego, CA, USA).

Results

Microorganisms

For the evaluation of antifungal activity of the propolis extracts, a total of 21 isolates were used: *C. albicans* (18), *C. tropicalis* (2) and *C. glabrata* (1). The PCR identification of *C. albicans* is shown in Figure 1.

Total polyphenols

The propolis extracts evaluated showed considerable differences in concentration of total polyphenols, with almost a 10-fold difference between the highest and the lowest concentration obtained. In addition, no relation between the concentrations determined in this study and the propolis concentration declared by the manufacturers was found (Table 1).

Chromatographic analysis

The chromatographic analysis of the propolis samples detected 35 compounds in sample I (Figure 2), which was the largest number of

Table 1. Total polyphenol content in extracts of commercial Chilean propolis. Method: Folin-Ciocalteu.

Propolis extract	Propolis concentration[1] %	Total polyphenols[2] mg mL^{-1}
I	20	85 ± 2.1
II	10	9 ± 0.3
III	20	41 ± 0.4
IV	nd	16 ± 0.2
V	nd	19 ± 0.9
VI	20	16 ± 0.4

[1]Propolis concentration declared by manufacturers. nd: Not determined.
[2]Values expressed as mean, ± standard deviation.

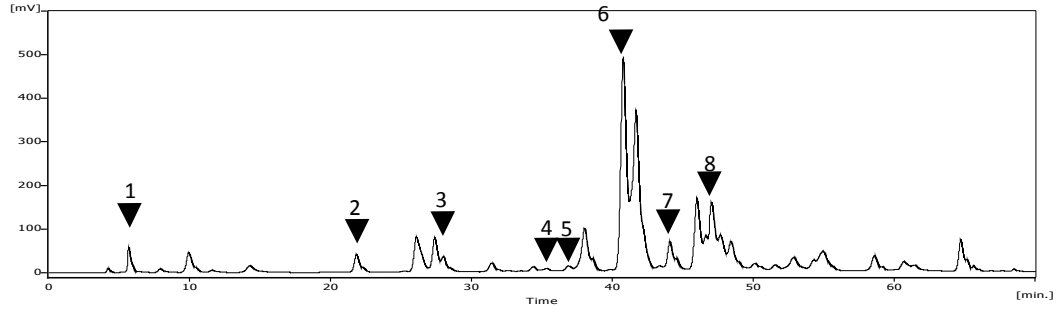

Figure 2. Chromatographic pattern of commercial extract I of Chilean propolis. Wavelength: 290 nm. Injection volume: 10 µL. Identified compounds: 1, caffeic acid; 2, myricetin; 3, quercetin; 4, kaempherol; 5, apigenin; 6, pinocembrin; 7, galangin; 8, caffeic acid phenyl ester (CAPE).

compounds detected. On the other hand, only seven compounds were detected in sample II. Regardless of these differences, all the samples had similar chromatographic patterns, with pinocembrin being one of the predominant compounds in these propolis extracts.

Antifungal activity

It was determined that all propolis samples inhibited the development of the tested strains of *Candida* spp. However, there were important differences in extract dilutions that showed inhibition of *Candida* spp. (Table 2). For *C. albicans*, a 1:320 dilution had the highest MID with propolis I, while a 1:40 dilution of propolis II and V produced the highest MID. A similar result was obtained with *C. tropicalis*. Also, in the case of *C. glabrata*, the propolis with the highest and lowest activity was the same, but the inhibition was observed at higher dilutions.

In the case of *C. albicans*, when comparing the respective concentrations for dilutions inhibiting development, significant differences were observed in propolis extracts IV and VI, which were active at lower concentrations, compared with propolis extracts I, III and V, which were active at higher concentrations.

Discussion

The evaluation of the biological activity of propolis, particularly its antifungal activity, has been studied previously (Sawaya *et al*., 2002; Santos *et al*., 2005; Silici and Kutluca, 2005; Silici *et al*., 2005; Oliveira *et al*., 2006; Ghasem *et al*., 2007; Quintero-Mora *et al*., 2008). However, the information obtained

Table 2. Minimum inhibitory dilution (MID)[1] and minimum inhibitory concentration (MIC) of commercial Chilean propolis on Candida spp.

	C. albicans		*C. tropicalis*		*C. glabrata*	
Propolis extracts	MID	MIC µg ml^{-1}	MID	MIC µg ml^{-1}	MID	MIC µg ml^{-1}
I	6	287±93	6	265	8	66
II	3	224±74	3	227	4	113
III	5	324±117	5	253	5	253
IV	4	197±59	4	197	5	99
V	3	441±103	3	476	5	119
VI	4	198±65	4	204	6	51

[1]MID 1=1:10; MID 2=1:20; MID 3=1:40; MID 4=1:80; MID 5=1:160; MID 6=1:320; MID 7=1:640; MID 8=1:1280. For *C. albicans,* MID values are expressed by mode.

in this research is needed in Chile because of unique propolis characteristics and the dependence of its chemical/biological characteristics on its botanical origin.

Bankova (2005) states that, as the concentration and activity of the components of different propolis extracts are unknown, it is necessary to consider each extract as an active principle in itself. On this basis, not only were the concentrations of total polyphenols in propolis extracts determined, but important differences in the concentrations of active compounds were also found; this is relevant because the concentration, in part, establishes the degree of the extracts's biological activity. This relevance was evident when the samples with significant differences expressed in MID (propolis I and II: 1/320 and 1/40, respectively) showed no difference when compared to the MIC (propolis I and II: 287 ± 93 and 224±74 µg mL^{-1}, respectively).

Chromatographic analysis detected at least 35 compounds in propolis I and identified caffeic acid, myricetin, quercetin, kaempferol, apigenin, pinocembrin, galangin and caffeic acid phenyl ester (CAPE). Regardless of the differences in the number of compounds detected among the samples analyzed, the chromatographic profiles exhibited great similarities. Differences were mainly associated with compound concentration differences.

The activity of propolis on *C. albicans* has been evaluated by different methodologies (e.g., agar dilution, agar diffusion, macro and microdilution broth). Due to variations both in the methodologies used and in the modes of determining the concentration of active substances, comparing the results from different studies becomes difficult. For example, throught broth microdilution, Oliveira *et al*. (2006) showed that a concentration of 50 µg mL^{-1} of total flavonoids was able to inhibit the development of 67 onychomycosis fungal species; specifically, the MIC_{90} on *C. albicans* was 25 µg mL^{-1}. These results are different from those informed by Sawaya *et al*. (2002), who determined that the MIC on *C. albicans* varied between 6 and 12 µg mL^{-1} using agar dilution. In addition, these researchers could not obtain satisfactory results by broth macrodilution and agar diffusion.

In our study, it was observed that all propolis samples evaluated were able to inhibit the development of *Candida* spp., although differences were observed regarding the MIC; ranging between 278 µg mL^{-1} for *C. albicans* and 117 µg mL^{-1} for *C. glabrata*.

Currently, subprosthesis stomatitis arises as a real problem for an important number of users of removable dentures. Infection by *C. albicans* is clearly identified as one of the main predisposing factors in its development (Baena-Monroy *et al*., 2005; de Resende *et al*., 2006; Bilhan

et al., 2008). The evaluated propolis demonstrated antifungal activity; but there is a new possibility for Chilean propolis to be used in the elaboration of products that prevent and/or treat subprosthesis stomatitis. An experience of this kind, conducted in Brazil showed that patients with subprosthesis stomatitis and treated with an ethanolic extract of propolis obtained a similar effect when compared with a group of patients treated with nystatin; however, the number of patients considered in that study was small (Santos *et al*., 2005).

Although many products in the Chilean market are supplemented with or based on propolis, the results obtained in this research show that the extracts of propolis studied were not equivalent in relation to the concentration of total polyphenols. These results show the need for regulation both in determining the geographical and botanical origin of propolis as well as in determining the chemical characterization of extracts. Therefore, a clear standardization of characteristics of this product and knowledge of its composition are needed to extrapolate from composition to particular biological properties. These practices would not only add value to the national propolis, but it would also allow the population to choose and use products with known characteristics, as appointed by Peña (2008).

In summary, all of the propolis samples evaluated in this study have the ability to inhibit the development of *Candida* spp. since their qualitative characteristics are similar; however, they show important differences both in the concentration of polyphenols present and in antifungal activity.

Acknowledgements

This study was supported by grants from Dirección de Investigación y Desarrollo (DIDUFRO INI 110605) Universidad de La Frontera, and CONICYT (FONDEF D05I-10021), Chile.

Resumen

C. L. Herrera, M. Alvear, L. Barrientos, G. Montenegro y L. A. Salazar. 2010. The antifungal effect of six commercial extracts of Chilean propolis on *Candida* spp. Cien. Inv. Agr. 37(1): 75- 84. El propóleo ha sido utilizado por la medicina tradicional desde hace muchos siglos debido a sus propiedades benéficas para la salud, entre las que destaca su capacidad antimicrobiana. La estomatitis subprotésica, afecta a un porcentaje importante de usuarios de prótesis dental removible, siendo *Candida albicans* la especie fúngica más común asociada al desarrollo de esta patología. Así, los objetivos de este estudio fueron: a) evaluar la actividad antifúngica de seis extractos de propóleos comerciales sobre cepas de *Candida* spp. aisladas de la cavidad oral de usuarios de prótesis dental removible, y b) determinar algunas características químicas de los extractos de propóleos utilizados. Entre los resultados obtenidos, podemos señalar que éstos mostraron concentraciones de polifenoles que variaron entre 9 ± 0,3 y 85 ± 2,1 mg/mL. El análisis cromatográfico permitió detectar 35 compuestos, entre los cuales se logró identificar la presencia de ácido cafeico, miricetina, quercetina, kaempferol, apigenina, pinocembrina, galangina y ácido cafeico fenil éster (CAPE). Todas las cepas de *Candida* spp. evaluadas fueron inhibidas por los seis extractos líquidos de propóleos, observándose que la DIM varió entre 1/40 y 1/1280, y la CIM para *C. albicans* varió entre 197 µg/mL y 441 µg/mL. A partir de los resultados obtenidos en esta investigación podemos concluir que todos los propóleos evaluados son capaces de inhibir el desarrollo de *Candida* spp., sin embargo, éstos muestran importantes diferencias en la concentración de los polifenoles presentes y en la actividad antifúngica.

Palabras claves: Actividad antifúngica, *Candida albicans*, propóleos.

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