Antimicrobial Activity from a Brazilian Propolis Oily Extract Compared with Other Propolis Extracts

Atividade Antimicrobiana de Extrato Oleoso de Própolis Brasileira e Comparações com Outros Extratos de Própolis

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Recebido em 02/09/2010 - Aceito em 19/03/2011.

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Abstract: The extraction method influences the composition and the most used solvent for propolis preparation is ethanol. Recently, our group used canola oil to prepare a propolis oily extract, which showed promising biological activities. Therefore, the study aims to evaluate the *in vitro* antimicrobial activity of the canola oily extract of propolis and its methanolic fraction and compare with the activities of ethanolic and aqueous extracts. Propolis samples were extracted with water, ethanol or edible vegetable oil, followed by filtration and drying steps. The oily extract was further submitted to a solvent partition and dried. The obtained dry mass was re-suspended and used for the antimicrobial assays using agar diffusion method. The oily extract of propolis showed potent antifungal activity compared to the ethanol extract against *Aspergillus fumigates*, and antibacterial activity similar to the aqueous extract. The oily fraction soluble in methanol also showed similar action against *Staphylococcus aureus* and *Listeria monocytogenes* in comparison to ethanol extracts 95 and 70%. Our results demonstrated that the propolis extract obtained with veg-

etable canola oil and its methanolic fraction showed *in vitro* antimicrobial activity similar to the ethanol extract.

Key words: antibacterial activity; antifungal activity; propolis oily extract.

Resumo: O método de extração influencia na composição do extrato de própolis, sendo que o solvente mais utilizado no preparo é o etanol. Recentemente, nosso grupo utilizou o óleo vegetal de canola para preparar um extrato oleoso de própolis, que demonstrou atividades biológicas promissoras. Dessa forma, este estudo teve como objetivos avaliar a atividade antimicrobiana *in vitro* do extrato oleoso de canola de própolis, de sua fração metanólica e comparar com as atividades dos extratos etanólicos e aquosos. As amostras de própolis foram extraídas com água, etanol ou óleo vegetal comestível de canola e, posteriormente, submetidas a etapas de filtração e secagem. Em seguida o extrato oleoso foi submetido a uma partição utilizando o metanol como solvente e, em seguida, desidratado. A massa seca foi ressuspensa e utilizada para os testes antimicrobianos pelo método de difusão em ágar. O extrato oleoso demonstrou uma potente atividade antifúngica contra *Aspergillus fumigates* se comparado ao extrato etanólico e atividade similar ao extrato aquoso. A fração solúvel em metanol também demonstrou atividade similar contra *Staphylococcus aureus* e *Listeria monocytogenes* em comparação aos extratos etanólicos 95 e 70%. Nossos resultados demonstraram que o extrato de própolis obtido com óleo vegetal de canola comestível e suas frações metanólicas possuem atividade antimicrobiana *in vitro* semelhante à dos extratos etanólicos.

Palavras-chave: atividade antibacteriana; atividade antifúngica; extrato oleoso de própolis.

1 Introduction

Propolis, also known as bee glue, has attracted the attention of researchers due to its various biological activities and therapeutic properties. The pharmacological properties of propolis includes anti-inflammatory, immunomodulatory, healing,

anesthetic, anticarcinogenic; antimicrobial, antiprotozoan, antiviral, anti-oxidant, antineoplastic and anti-ulcer [1, 2]. In addition, propolis extracts have shown to enhance the antibiotics action, and to prevent or reduce any gradual build-up in tolerance of Staphylococci to antibiotics [3]. Consequently, this aspect has increased the interest of pharmaceutical industry to search viable commercial formulations of propolis.

The extraction solvent influences the composition and consequently the biological activities. The most used solvent for propolis preparation is aqueous ethanol, followed by others such as ethyl ether, water, methanol and chloroform [4]. Recently, we used canola oil to prepare a propolis oily extract with promising preliminary antibacterial and cytotoxic activities. The oily extract presents some advantages against the usually used ethanolic extract [5]. Therefore, the aim of the present study was to evaluate the *in vitro* antimicrobial activity of the propolis oily extract and its methanolic fraction and to compare it with the ethanolic and aqueous extracts activities against pathogenic microorganisms *Aspergillus fumigatus*, *Listeria monocytogenes* and *Staphylococcus aureus*.

2 Material and methods

2.1 Propolis origin and extraction procedures

The propolis samples were collected in 2005 and gently supplied by Campolin & Schmidt Company from Prudentópolis city (Paraná State, Brazil). Propolis was stored at -18ºC until extraction. Propolis samples were ground and the hidroalcoholic extracts were obtained as water, 70% or 95% v/v aqueous ethanol during 10 days at room temperature and occasional shaking. After that period, the extractive solutions were filtered and extracts of propolis. The oily propolis extract was obtained as described by Buriol et al [5], however the extraction time was 90 days, and it was used in this form for the antimicrobial assays. In order to obtain the methanol soluble fraction from the oil extract half of it was submitted to partition into 80% v/v aqueous methanol and this methanolic phase was further dried in ro-

tatory evaporator. All obtained dried extracts were dissolved in aqueous ethanol yielding a concentration of 10% w/v to perform the antimicrobial assays.

2.2 Antifungal assay

The extracts were evaluated against *Aspergillus fumigatus* supplied by the Mycology Laboratory of Medicine Faculty of Ribeirão Preto/USP using the agar diffusion method following the National Committee of Clinical Laboratory Standard Guidelines [6]. The fungi was grown on plates with Sabouraud Dextrose Agar (30°C/15 days), it was added 1mL of sterile saline solution (0.85%) to prepare the spore suspension. The plates with Sabouraud agar (20 mL) were seeded by pour plate with 100 μL of the spore suspension. Volume 40, 80 and 100 μL of the ethanolic and oily extracts were added in wells (7mm), with final volume of 100 μL per well completed with the respectively solvent, then the plates were incubated at 37°C for 48h and the inhibition zone was measured with paquimeter. The assays were made in triplicate and Ethanol and Itraconazole (0.030 μg/mL) were used as negative and positive control, respectively.

2.3 Antibacterial assay

The extracts were evaluated against two gram positive bacteria: *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC 19111 by the agar diffusion method [7] and microdilution protocol (M7-A5) [8]. Bacterial inoculum was prepared according to the recommendations of the Clinical and Laboratory Standards Institute [8]. Briefly, 3 to 4 colonies of microorganisms, each 1mm or more in diameter, from 24h at 25°C on BHI agar (Himedia M063) subcultures were suspended in 2mL of Müller Hinton broth (MERCK, Germany). The resulting suspension was mechanically mixed and the cell turbidity adjusted to correspond to a 0.5 McFarland standard. This procedure yielded a stock suspension containing 1×10^8 CFU/mL. For the agar diffusion method, plates with trypticase soy broth were seeded by pour plate, and wells (7mm) were inoculated with 50 or 80 μL of the extracts (10% w/v),

incubated (37ºC/24 h) and the inhibition zone was measured with paquimeter, the assays were made in triplicate. An inhibitory zone with a diameter less than 10mm corresponded to the lack of activity, as reported by Packer and Luz [9]. Ethanol and gentamicine (50 μg/mL) were used as negative and positive control, respectively. The minimum inhibitory concentration (MIC) was evaluated for each extract according to the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards, NCCLS) microdilution protocol (M7-A5) [8]. Of this, 90μL of stock suspension was added in 96-wells plates (InLab, USA) with 10μL of the different propolis extracts. The number of colonies growing in the presence of the extracts at each concentration was determined by counting of Unit of Colony-Forming (UCF) that found the MIC that prevented in 30, >90 or 100% the growth of different species of bacteria.

2.4 Statistical analisys

The data are reported as mean # SEM and are representative of three independent experiments. The means from different groups were compared by analysis of variance (ANOVA) followed by Bonferroni's t-test for unpaired values. $p<0.05$ was considered to be statistically significant.

3 Results and discussion

All propolis extracts tested significantly inhibited the growth of the *Aspergillus fumigatus* fungi in the agar-well diffusion test, which was concentration-dependent. However, the oily extract was more potent in inhibiting the fungi growth, mainly, volumes of 40 and 80 μL promoted higher inhibition zone than that observed with the ethanolic extracts 70, 95% and Itracolazole (Figure 1A). In the antibacterial test, our data also showed that aqueous and oily extracts inhibited the growth of *S. aureus* in the agar-well diffusion test (Figure 1B). Moreover, the oily methanolic fraction resulted in greater inhibition zone against *S. aureus* when compared to aqueous or oily extracts (Figure 1B), demonstrating that the partition step is a good methodology to obtain a better oily extract.

In addition, we also compared the antimicrobial activity of the oily methanolic fraction against *L. monocytogenes* and *S. aureus*, (Table 1) and it promoted 100% of inhibition against *L. monocytogenes* and 90% against S. *aureus* at the concentration of 5μg/mL. The ethanolic extracts exerted similar effects against *L. monocytogenes*, but was less effective against *S. aureus* (Table 1).

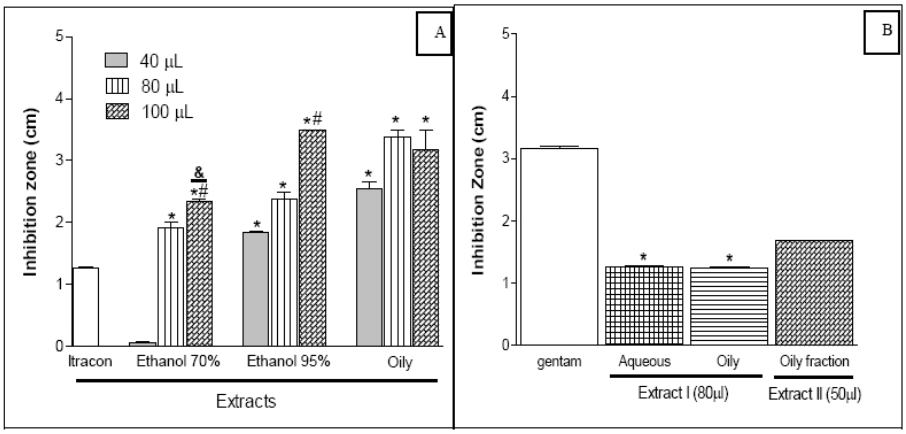

Figure 1. Antimicrobial activity of propolis extracts obtained with different solvents. (A) Effect of propolis extracts against Aspergillus fumigatus and (B) against Sthaphylococcus aureus. The fungi and bacterial growth inhibition were evaluated by agar diffusion method (cm). (A) $*P \leq 0.001$ compared to Itraconazole (Itracon); $\#P \leq 0.001$ compared to 80μL; $\&P \leq 0.001$ compared to Oily Extract 80μL; (B) $*P \leq 0.001$ compared to Oily fraction.

Table 1. Antibacterial activity of propolis extracts by agar diffusion method expressed as inhibition zone (cm) and by dilution method expressed as MIC or percentage of growth inhibition.

	Propolis Extracts		
Species/methods	Oily Methalonic fraction	Ethanolic 95%	Ethanolic 70%
Staphylococus aureus			
Diffusion method (cm)	1.51 ± 0.57	1.18 ± 0.53	1.57 ± 0.45
*MIC ($\mu g/mL$)	5.00	2.50	5.00
Inhibition%	> 90.00	30.00	> 90.00
Listeria monocytogenes			
Diffusion method (cm)	1.76 ± 1.00	1.38 ± 0.28	1.79 ± 0.50
MIC ($\mu g/mL$)	5.00	2.50	5.00
Inhibition%	> 100.00	100.00	> 100.00

*MIC: minimal inhibitory concentration; percentage of growth inhibition has as a reference the starting inoculum of $1 - 2 \times 10^8$ CFU/ml.

The antimicrobial properties of propolis have been known for many years. Several published reports have described the effect of propolis on a variety of microorganisms [10, 11]. However, there are few studies about antifungic activity of propolis against *Aspergillus fumigatus*, one of the principal microorganisms of aspergillosis, which is now the most common mold infection worldwide in immunosuppressed patients [12]. The current antifungal therapy has limited effectiveness and despite increased awareness and earlier management of invasive aspergillosis, there remains a critical need for a more effective and well-tolerated antifungal agent [13].

All evaluated extracts were more effective in inhibiting the growth of *L. monocytogenes* than the growth of *S. aureus*. Inhibition effect of propolis extract in the growth of *L. monocytogenes* was also demonstrated recently by our group and others [5, 14, 15]. Listeriosis is caused by *L. monocytogenes*, an emergent pathogenic microorganism, and results in an invasive disease that affects immunocompromised patients and has the highest case-fatality rate of food borne illnesses [16]. In addition, the oily methanolic fraction presented the same inhibitory activity against *S. aureus* as the 70% ethanolic extracts, demonstrating that this fraction can be used as antimicrobial agent without the inconvenient from ethanolic solutions. The antimicrobial effect of 70 and 95% ethanolic propolis extracts was also verified by Kujumgiev et al. [17], which evaluated extracts from many countries and Brazilian states, including one sample from the Prudentópolis region, which presented a zone inhibition of 1.0 and 1.3 cm, lower than the data observed in our work.

Moreover, the aqueous extract resulted in the same antibacterial activity as the oily extract. Data different were observed by Garedev et al. [18] that compared several types of propolis extracts and concluded that the water-extracted propolis solution had the weakest antibacterial and antifungal action. Maybe the difference to our results can be related to the intrinsic chemical composition of the propolis which is different depending on their geographical origin [19]. Water extracts presented other biologically activities such as *in vivo* anti-tuberculosis, anti-inflammatory and anti-oxidative effects [20–22]. The oily extract enables the production of gelatinous capsules which could be filled directly with the oily extractive solution requiring just one step of centrifugation and/or filtration and avoiding the need for removing the

hidroalcoholic solvent when ethanolic extractions are performed. Therefore, these results here presented are promising, and evidenced that the bioactive molecules were extracted and are present in the oily extract.

The flavonoids and phenolics content of the oily methanolic fraction and the ethanolics extracts were reported in Buriol et al. [5], where it can be seen that flavonoids content was similar between the extracts, however the phenolics percentage was smaller in the oily methanolic fraction. These led us to speculate that flavonoids exert important role in antimicrobial activity; maybe more crucial than the total phenolics content and that probably other molecules are present in the canola oily extract which co-exert this activity.

4 Conclusion

Taken together, our results suggest that the oily extract and its methanol soluble fraction exerted excellent antifugical and antibacterial activities *in vitro*. Therefore, the oily propolis extract, which is a less common propolis formulation, might be used for gelatinous capsules production, helpful to people who are unable to use propolis ethanolic solutions, as food preservative and as an extract with therapeutic potential against *A. fumigatus*. This putative medical application will have to be carefully investigated and researches are currently being done in our group to identify the bioactive chemicals in the oily propolis extract.

5 Acknowledgment

The authors thank to Fundação Araucária and CNPq for the financial support (10908/ Programa Primeiros Projetos/2006 e 7102/ Programa Paraná Inovação Fase I-2004 e Fase II-2006).

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