PROPOLIS OBTAINED BY MEANS OF ALKALINE HYDROLYSIS AND ACTION ON *Staphylococcus aureus*

PRÓPOLIS OBTIDA POR HIDROLISE ALCALINA E AÇÃO SOBRE STAPHYLOCOCCUS AUREUS

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SUMMARY

The Brazilian propolis is investigated for its antibacterial properties. The aim of this work was to establish the extraction and purification method to display the antibacterial activity. Propolis of *Apis mellifera* was obtained from bee hives cultivated in Parana State, Brazil. In this study, we used ATCC 25923 *Staphylococcus aureus* strains and 158 *Staphylococcus aureus* strains isolated from cows with clinical mastitis. Extraction and partial purification: Several fractions were obtained during the partial purification: a crude ethanolic extract of propolis, a resinous material in ethanolic solution and an alkaline hydrolysis water-soluble compounds solution. The results show that the alkaline hydrolysis water-soluble compounds solution had a minimal inhibitory concentration of approximately 155.46 μg/mL for *Staphylococcus aureus*. In this work, we obtained an aqueous solution of organic compounds extracted from *Apis mellifera* propolis that bears physical-chemical and biological characteristics capable to inhibit the proliferation of Gram-positive bacteria.

KEY-WORDS: Propolis; *Apis mellifera*; Antibacterial activity; Alkaline hydrolysis.

RESUMO

A própolis brasileira é investigada por suas propriedades antibacterianas. O objetivo deste trabalho foi estabelecer o método de extração e purificação para demonstrar a atividade antibacteriana. A própolis de *Apis mellifera* foi obtida de colméias de abelhas oriundas do Estado do Paraná, Brasil. Neste estudo, foram usados cepas de *Staphylococcus aureus* ATCC 25923 e 158 cepas de *Staphylococcus aureus* isolados de vacas com mastite clínica. Várias frações foram obtidas durante a purificação parcial: um extrato etanólico bruto de própolis, um material resinoso em solução alcoólica e uma solução de compostos solúveis por hidrólise alcalina em água. Os resultados mostram que a solução de compostos solúveis por hidrólise alcalina em água apresentou uma concentração inibitória mínima de cerca de 155,46 mg / mL para *Staphylococcus aureus*. Neste trabalho foi obtida uma solução aquosa de compostos orgânicos extraídos da própolis de *Apis mellifera* que possui características físico-químicas e biológicas capazes de inibir a proliferação de bactérias Gram positivas.

PALAVRAS-CHAVE: Própolis; *Apis mellifera*; atividade antibacteriana; hidrólise alcalina.

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INTRODUCTION

Propolis is a resinous substance secreted by the *Apis mellifera* bees from various buds and barks of trees and shrubs, mixed with beeswax and β-glucosidase secreted during the propolis collection (KÖNIG, 1985, BURDOCK, 1998).

Several biological activities have been reported for the ethanolic extract of propolis such as antibacterial, anti-inflammatory, antiviral, antifungal, anaesthetic, immunostimulatory, antitumoral and cytotoxicity activities (AGA et al., 1993, FERNANDES et al., 1997, MATSUNO et al., 1997, MIRZOEVA et al., 1997, BANKOTA et al., 1998, BURDOCK, 1998, PARK et al., 1998, PARK and IKEGAKI, 1998, KUJUMGIEV et al., 1999). Among these activities, the antibacterial action is the most extensively investigated and the differences between the ethanolic extracts are due to some factors such as bee species, propolis origin, extract preparation and bacteria tested (FERNANDES et al., 1997, MATSUNO et al., 1997, MIRZOEVA et al., 1997).

These pharmacological activities are probably due to a mixture of several compounds as flavonoid aglycones, cinnamic acid derivatives and terpenoids groups (SIMUTH et al., 1986, TAKAISI and SCHILCHER 1994, KOO and PARK 1997, PARK et al., 1998, MACIEJEWICZ, 2001). Hence, the interest shown by several researchers to obtain propolis preparations with defined chemical composition has increased because of the wide possibilities for medical applications. Besides some rare exactor extracts (MATSUNO et al., 1997, PARK and IKEGAKI, 1998), the major propolis preparations reported are ethanolic, methanolic or chloroform extracts (KÖNIG, 1985; AGA et al., 1993, FERNANDES et al., 1997, KOO and PARK, 1997, MATSUNO et al., 1997, MIRZOEVA et al., 1997, BANKOTA et al., 1998, BURDOCK, 1998, PARK et al., 1998, PARK and IKEGAKI, 1998, KUJUMGIEV et al., 1999, MACIEJEWICZ, 2001). Since all properties have shown discrete antibacterial activity, such as those against *Staphylococcus aureus* (KÖNIG, 1985, AGA et al., 1993, FERNANDES et al., 1997, KOO and PARK,1997, MATSUNO et al., 1997, MIRZOEVA et al., 1997, BANKOTA et al., 1998, BURDOCK, 1998, PARK et al. 1998, PARK and IKEGAKI, 1998, KUJUMGIEV et al., 1999, MACIEJEWICZ, 2001), the aim of this work was to establish the extraction and purification method to display the antibacterial activity.

MATERIAL AND METHODS

Propolis

Apis mellifera propolis was obtained from bee hives cultivated in Parana State, Brazil.

Microorganisms

ATCC 25923 Staphylococcus aureus strains (deve aparecer "strains" aqui?) and 158 strains of Staphylococcus aureus isolated from cows with clinical mastitis.

Extraction and partial purification

The extraction process was adapted according to FUNAYAMA et al (2006). An amount of 250 g of *Apis mellifera* propolis was used. The samples of propolis were stored at 0.5 -1^{o} C to harden and then ground to powder by means of an electric blender. A total of 600 mL of 75% ethanol was added and the apparatus was turned on five times during a period of 3 minutes each. These operations were repeated after 3 hours and the suspension allowed to stand for 16 hours in maceration and then filtered through paper filter. The yield was approximately 490 mL of crude ethanolic extract of propolis (CEEP), which was concentrated through aeration during 6 hours. After evaporation of the ethanol, two fractions were obtained: an aqueous solution and a yellowish water-insoluble resinous material. About 110 g of the latter was washed with distilled water until the rinse water did not seem cloudy. The washed resinous material was dissolved in 150 mL of 99.8% ethanol and tagged as resinous material ethanolic solution. The pH of this solution was adjusted to 7.5 with 15 mL NaOH 2.5 N and stored at 20^{o} C for at least 20 hours.

After this period, the volume of this hydrolyzed mixture was reduced to half through aeration and both liquid and resinous fractions were collected. The water-soluble compounds released through alkaline hydrolysis were collected from the resinous material by carefully washing it 3 times with 50 mL of distilled water or until the rinse water did not seem cloudy. All fractions of rinsed water were added to the former one. After storage at 0.5^{o}–2^{o} C for 48 hours, the aqueous solution became cloudy. Keeping it under the same temperature, the preparation was spindown at 10,000 x g for 10 minutes, the supernatant was collected, tagged as alkaline hydrolysis water-soluble compounds solution (AHWSCS) and the pellet was discarded.

Preparation of filter paper disks containing propolis extracts

Filter paper disks (8 x 1 mm) were incubated at 50^{o} C for 48 hours and their weights were determined. The extracts of propolis disks were prepared by applying 10 μl of extract for 5 times on each filter paper disk; they were then incubated at 30^{o} C during 20 minutes between subsequent applications and kept at the same temperature during 24 hours for drying, after which their weights were again determined.

Determination of antibacterial specific activities of propolis extracts

Estimation of bacteria susceptibility to propolis extracts was performed by inoculating 0.1 mL of a Brain Heart Infusion culture for microorganisms in Mueller-Hinton agar plates. The culture was spread using Drigalski's handle in circular movements until the agar plates had absorbed the broth culture. The filter paper disks containing propolis extracts were placed on the inoculated Mueller-Hinton agar plates and incubated at 37^{o} C for 24 hours. The process was adapted according to FUNAYAMA et al. (2006).

Minimal inhibitory concentration of AHWSCS

Serial AHWSCS concentrations (77 - 2328 μg/mL) were performed in Petri dishes containing 20 mL of Mueller-Hinton agar to test bacterial growth. Simultaneously, plates containing medium without AHWSCS were used as control. The agar was allowed to solidify and 0.1 mL of a culture containing approximately 10^3 cells/mL was inoculated on each plate and incubated at 37° C for 24 hours. The minimal inhibitory concentration (MIC) is defined as the lowest concentration that totally inhibits bacterial growth after incubation at 37° C for 24 hours (FUNAYAMA et al, 2006).

RESULTS AND DISCUSSION

Table 1 summarizes the extraction, partial purification and determination of specific activities of propolis extracts fractions on *Staphylococcus aureus* cultures; table 2 summarizes the determinations of minimal inhibitory concentrations of AHWSCS. The MIC determined for *S. aureus* was 155.46 μg/mL.

Table 1 - Isolation, partial purification and antibacterial specific activities of propolis fractions

Fractions	Total volume (mL)	Means and standard deviations by dry weight (mg/50 μl)	Means and standard deviations by diameter of inhibition zone on *S .aureus* culture (mm)	Specific activity (Inhibition zone/dry weight)	Purification
CEEP	490	11.1 ± 0.43	12 ± 0.87	1.08	----
AHWSCS	260	2.3 ± 0.12	16 ± 0.44	6.4	6.4

CEEP = Crude ethanolic extract of propolis ; AHWSCS = Alkaline hydrolysis water-soluble compounds solution

Although the chemical analysis of AHWSCS from this propolis preparation was not performed, their antibacterial activity is certainly due to a complex mixture of propolis compounds. This statement is based on differences found in the Brazilian propolis chemical composition. The data on growth results of Table 1 show the specific activities of each purification step of propolis compounds and their effects on *Staphylococcus aureus*. An evidently interesting point is that extraction of propolis antibacterial compounds was increased 7 times with alkaline hydrolysis when compared to former ethanolic extraction.

Table 2- Determination of minimal inhibitory concentrations of AHWSCS

Mueller & Hinton Agar plate ([b]PSa)	AHWSCS (mL)	AHWSCS (μg/mL)	*S. aureus* culture containing 10^3cells/mL (mL)	*S. aureus* (Number of [a]CFU)
PSa_0	----	----	0.10	210
PSa_1	0.05	77.73	0.10	64
PSa_2	0.10	155.46	0.10	0
PSa_3	0.20	310.92	0.10	0
PSa_4	0.30	466.38	0.10	0
PSa_5	0.40	621.84	0.10	0

([a]CFU) Colony formation unit; ([b]PSa) Mueller & Hinton agar plate inoculated with *S. aureus.*

The AHWSCS has antibacterial activity (MIC of approximately 155.46 μg/mL) for *Staphylococcus aureus* (Gram-positive bacteria). funayama et al. (2006) report having found an alkaline hydrolysis water-soluble compounds solution with a minimal inhibitory concentration of approximately 291 μg/mL for *Staphylococcus aureus.* Therefore, AHWSCS contains some active compounds that possess different mechanisms of antibacterial action. These results indicate that these antibacterial activities were not due to the presence of one particular compound, but to the actions of an active compounds mixture. Similar results were described by AGA et al.(1993) and Fernandes et al.(1997).

CONCLUSIONS

The alkaline treatment of resinous material ethanolic solution hydrolyzes esters releasing active water-soluble compounds that provide a significant increase in antibacterial activity of AHWSCS. Since AHWSCS has antibacterial activity against *Staphylococcus aureus* (Gram-positive bacteria), it is the strongest evidence that these antibacterial activities were due to an active compounds mixture. Finally, the present investigation suggests a possible use of AHWSCS as a bactericidal agent in human and veterinary medicine, and the need of additional investigation on this matter.

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