Atividade Antibacteriana do Extrato Aquoso da Própolis de *Appis mellifera* da Cidade de Ibaiti, PR

Antibacterial Activity of Aqueous Extract of *Appis mellifera* Propolis from the City of Ibaiti, PR

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Resumo

Foi preparada uma solução aquosa de compostos solúveis da própolis utilizando uma extração alcalina. No estudo da atividade antibacteriana pela metodologia de diluição em ágar, foram utilizadas bactérias Gram-positivas de interesse médico como *Streptococcus mutans, Staphylococcus aureus* e *Streptococcus pyogenes*, e Gram-negativas como *Enterobacter aerogenes, Salmonela typhimurium* e *Pseudomonas aeruginosa*. O extrato aquoso de compostos solúveis da própolis foi capaz de inibir o crescimento das bactérias testadas. A concentração mínima inibitória determinada para *Streptococcus mutans, Streptococcus pyogenes, Enterobacter aerogenes, Salmonela typhimurium* e *Pseudomonas aeruginosa* foi de 36 μg/ml e 9 μg/ml para *Staphylococcus aureus*.

Palavras-chave: Própolis. Extrato aquoso. Bactérias.

Abstract

An aqueous solution of soluble compounds extracted from propolis was prepared through alkaline treatment. In the study of antibacterial activity, using agar dilution method, Gram-positive bacteria of medical importance such as Streptococcus mutans, Staphylococcus aureus and Streptococcus pyogenes, and Gram-negative ones such as Enterobacter aerogenes, Salmonela typhimurium, and Pseudomonas aeruginosa were used. The aqueous extract from propolis soluble compound was able to inhibit the growth of all bacteria tested. The minimal inhibitory concentration of 36 g/ml was determined for Streptococcus mutans, Streptococcus pyogenes, Enterobacter aerogenes, Salmonela typhimurium, Pseudomonas aeruginosa and of 9 g/ml for Staphylococcus aureus.

Keywords: *Propolis. Aqueous extract. Bacteria.*

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1 Introduction

Propolis is a resinous substance with varieties of color and consistency that is secreted by bees, such as *Apis mellifera.* They collect from various buds and barks of trees and shrubs, and it is mixed with beeswax and enzymes which they secrete during the propolis collection. Propolis color and scent change depending on its botanical origin and chemical properties[1-3]. Propolis is used by bees to seal openings in the hives and to protect themselves against predators such as ants, hive moths, beetles and mices[1,4].

Several biological properties have been reported for the ethanolic extract of propolis such as antibacterial, anti-inflammatory, antiviral, hypotensive, antifungal, anesthetic, immunostimulatory, antitumoral and cytotoxicity activities[4,5]. Out of these activities, the antibacterial effect is the most extensively investigated. The differences between the ethanolic extracts exist due to some factors such as bees' species, plants, season, extract preparation and bacteria tested[2]. In spite of these factors, some researchers display evidence that *Baccharis dracunculifolia* is the main vegetable source of Brazilian propolis[6,7].

Those pharmacological activities probably exist due to a mixture of several compounds such as flavonoid aglycones, cinnamic acid derivatives and terpenoids groups[8]. On the other hand, several propolis samples other than the European ones which have therapeutic properties do not contain flavonoids compounds or contain relatively small amounts from these compounds[9].

More recently, several researchers are interested in obtaining propolis preparations with defined chemical composition, because of the wide possibilities for medical applications. Apart from some rare extracts[10,11] the major propolis preparations reported are ethanolic, methanolic or chloroform extracts[2,4,8,10-12,21]. It is important to point out that most of propolis alcoholic extracts contain allergic compounds such as 1,1 dimethyl-caffeic acid which means that these products have restricted therapeutic use[13]. The aim of this work was to establish an aqueous extract preparation method for displaying greater antibacterial activity.

2 Material and Method

2.1 Propolis sample

Propolis of *Apis mellifera* was obtained from bees' hives of an apiary association (APICAMPI) from Ibaiti, a city in the state of Paraná, Brazil.

2.2 Microorganisms strains

All microorganisms' strains *Streptococcus mutans* (ATCC35668), *Staphylococcus aureus* (ATCC6538), *Streptococcus pyogenes* (ATCC19615), *Enterobacter aerogenes* (ATCC13048), *Salmonela typhimurium* (ATCC14028) and *Pseudomonas aeruginosa* (ATCC35032) were obtained from the New ProvLaboratory**,** Pinhais, PR.

2.3 Aqueous extract of propolis

The aqueous extract of propolis was prepared as described by Funayama et al.14, with some modifications. In the extraction of water soluble compounds, 1000 g of propolis were used. The soft resin was fragmented in small pieces and extracted with three liters of 75% ethanol under intermittent agitation for 12 hours. After this time, the mixture was filtered through paper filter with a yield of 1200 mL of hydro alcoholic solution.

The filtrate was incubated in an aerated incubator at 40oC for 72 hours in order to the ethanol to evaporate. After the evaporation of the ethanol, two fractions were obtained, one of them was 350 mL of clear brownish aqueous fraction, whose pH was adjusted to 7.4 with 3 moles/L sodium hydroxide solution and tagged as aqueous extract of propolis (AEP). The other extract was obtained from 350 g of a yellowish water-insoluble resinous material which was washed three times with 80 mL of distilled water each time or until the rinse water did not look darkish. The washed resinous material was discarded. The total rinse water collected looked darkish. The pH solution was adjusted to 8.5, boiled for 5 minutes, cooled and added to the former AEP. The pH mixture was adjusted to 5.8 with 85% phosphoric acid, all insoluble material was eliminated by paper filtration and the pH was readjusted to 7.3. The filtrate obtained by this method was tagged as aqueous solution of soluble compounds of propolis (ASSCP).

2.4 Determination of total flavonoids concentrations

The total content of flavonoids was determined by spectrophotometric method using aluminium chloride as shift reagent[15]. The standard solution of quercetin (2mg/mL) was prepared in 95% ethanol. The total flavonoids content of samples were determined in triplicates and expressed as mg/mL.

2.5 ASSCP dry weight

A glass vessel of 10 mL capacity was incubated in an incubator at 60o C for 72 hours, cooled and its weight was determined. One milliliter of ASSCP was added in the vessel and treated in the same way for water evaporation. So, the dry weight of ASSCP was determined.

2.6 Mueller & Hinton agar plate containing ASSCP

The final volume of Mueller & Hinton agar plates containing different amounts of ASSCP was 25 mL. The concentrations of ASSCP in different plates were 144 mg/ml, 72 mg/ml, 36 mg/ml, 18 mg/ml, 9 mg/ml, 6 mg/ml and 3 mg/ml.

The plates were prepared by mixing agar Mueller & Hinton media (MH) melted with an amount of ASSCP to confirm the concentrations of ASSCP dry weight. The minimal inhibitory concentration (MIC) range was followed according to the Clinical and Laboratory Standards Institute guidelines16.

2.7 Strains of bacteria growth and cultures dilutions

In order to standardize the inocula density, a BaSO4 turbidity standard equivalent to a 0.5 McFarland scale was used. The 0.5 McFarland standard was prepared as described in CLSI (Clinical and Laboratory Standards Institute) 16. The bacteria strains were grown overnight in brain heart infusion medium (BHI at 35oC for (24 hours). The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity comparable to that of the 0.5 McFarland standards.

2.8 Minimal inhibitory concentration (MIC)

Agar surface of the plates containing different concentration of ASSCP and the control plate without antimicrobial agent received 5 μL of the inoculum suspension using a micropipette. Inoculation was done from the plate containing the lowest concentration of antimicrobial agent and the control plate was inoculated separately. Inoculated agar plates were allowed to stand until the inocula were completely absorbed. After that, the plates were incubated overnight at 35oC.

2.9 Interpretation of MIC results.

The MIC was defined as the smallest concentration of ASSCP (microgram dry weight) per milliliter of culture media, in which there was complete inhibition of bacteria growth. A visible haze of growth or a single colony was disregarded. The MICs were results obtained from triplicates experiments and interpreted according to the recommendation of CLSI16.

3 Results and Discussion

The propolis samples from Ibaiti utilized in this paper are probably the green propolis type, because of similar characteristics from that reported by specialized literature17. The propolis shows green color, has balsamic smell and hot taste. Furthermore, "alecrim do campo" (Baccharis dracunculifolia) is a native plant from the Ibaiti area. The hydro-alcoholic extracts of propolis contain several compounds such as flavonoids, cinnamic acid, fat acid,

terpenoids, resin[4,5,11]and depending on propolis origin, may also contain 1,1 dimethyl-caffeic acid[13]. Therefore, it is reasonable to suppose that extracts prepared with others organic solvents[2,5,12,21] have similar compositions. Based on this hypothesis, a new method was created for aqueous extract without 1,1 dimethyl-caffeic acid but with high flavonoids concentrations. The ethanol evaporation from hydro-alcoholic extracts obtained and described in this paper caused abundant hydrophobic compounds precipitation, but we were not able to obtain evidences for 1,1 dimethyl-caffeic acid in both AEP. By other hand the total flavonoids concentration in the former AEP and ASSCP were respectively 1.2 mg/mL and 3.65 mg/mL, which could be related with good bactericide activity. Probably, this is a characteristic of the typical propolis from Ibaití, since aqueous extracts of propolis from other sources were dismissed from this antibacterial capacity[14].

The MICs of ASSCP are shown in the table 1, where the amounts of ASSCP dry weight vary from 3 μg/ml to 144 μg/ml. Most of bacteria strains showed MIC of 36 μg/ml while *Staphylococcus aureus* showed an MIC of 9 μg/mL.

Table 1: The minimal inhibitory concentration (MIC) of ASSCP on Gram-positive and Gram-negative bacteria

Microorganism	ASSCP concentration (g/mL) in Mueller & Hinton agar plate						
	144	72	36	18	9	6	3
Staphylococcus aureus	-	-	-	-	-	+	+
Streptococcus mutans	-	-	-	+	+	+	+
Streptococcus pyogenes	-	-	-	+	+	+	+
Enterobacter aerogenes	-	-	-	+	+	+	+
Salmonela typhimurium	-	-	-	+	+	+	+
Pseudomonas aeruginosa	-	-	-	+	+	+	+

"+" indicates bacteria growth and "-"indicates bacteria growth inhibition.

The minimal inhibitory concentration (MIC) for *Staphylococcus aureus* was 9 μg/mL and for *Streptococcus mutans*, *Streptococcus pyogenes, Enterobacter aerogenes, Pseudomonas aeruginosa* and *Salmonela typhimurium* was necessary four times more, that is, 36μg/mL. These results show the ASSCP bactericidal efficiency and suggest its use in infection therapy against these and other microorganisms.

The MIC for *Pseudomonas aeruginosa* and *Staphylococcus aureus* reported by Rhajaoui and collaborators[19] were respectively 62 μg/mL and 31 μg/mL. On the other hand, the MIC reported by Hegazi & Abd El Hady[20] for *Staphylococcus aureus* was 5400 μg/mL. The ASSCP minimal inhibitory concentrations determined in the present work were three times less than those reported by Rhajaoui and collaborators[19] and 675 times less than those reported by Hegazi & Abd El Hady[20].

Nevertheless, the comparisons of these results are not conclusive, since they depend on the type of propolis, extraction method and bacteria tested. The fact that ASSCP holds good growth inhibition capacity for Gram-positive bacteria as *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus mutans* and Gram-negative bacteria as *Enterobacter aerogenes, Salmonella typhimurium* and *Pseudomonas aeruginosa,* indicates that these antibacterial activities do not exist due to the presence of one particular compound, but due to the actions of an active mixture of them.

Therefore, that ASSCP contains some active compounds which possess different mechanisms of antibacterial action. Finally, the present investigation suggests possible use of ASSCP as a bactericidal agent in human and veterinary medicine besides the necessity of additional investigations on this matter.

4 Conclusion

The alkaline treatment of darkish solution obtained from washing the resinous material releases active antibacterial water-soluble compounds that provide a significant increase of former AEP activity.

The ASSCP prepared through this procedure showed evidence of good growth inhibition activity against *Staphylococcus aureus, Streptococcus mutans, Streptococcus pyogenes*, *Enterobacter aerogenes, Salmonela typhimurium* and *Pseudomonas aeruginosa* cultures.

The fact that ASSCP has antibacterial activity against Gram-positive and Gram-negative bacteria, suggests that this substance is an active bactericidal compounds mixture.

References

1. Ghisalberti EL. Propolis: a review. Bee World 1979;60:59-84.

2. Nieva Moreno MI, Isla MI, Cudmani NG, Sampietro AR. Antioxidant activity of Argentine propolis extracts. J Ethnopharmacol 1999;68:239-46.

3. Marcucci MC, Ferreres F, Garcia-Viguera C, Bankova V, Castro SL, Dantas AP, et al. Phenolics compounds from Brazilian propolis with pharmacological activities. J Ethnopharmacol 2001;74:105-12.

4. Burdock GA. Review of the biological properties and toxicity of propolis**.** Food Chem Toxicol 1998;36:341-63.

5. Kujumgiev A, Tsvetkova I, Serkedjieva V, Bankova V, Christov R, Popov S. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J Ethnopharmacol 1999;64:235-40.

6. Bankova V, Krasteva GB, Sforcin JM, Frete X, Kujumgiev A, Rodella RM, et al. Phytochemical evidence for plant origin of Brazilian propolis from São Paulo State. Z Naturforsch 1999;54:401-5.

7. Kumazawa S, Yoneda M, Shibata I, Kanaeda J, Hamasaka T, Nakayama T. Direct evidence for the plant origin of Brazilian propolis by the observation of honeybee behavior and phytochemical analysis. Chem Pharm Bull 2003;51:740-2.

8. Maciejewicz W. Isolation of flavonoid aglycones from propolis by a column chromatography method and their identification by GC-MS and TLC methods. J Liquid Chromatog Related Technol 2001;24:1171-9.

9. Longhini R, Raksa SM, Oliveira ACP, Svidzinski TIE, Franco SL. Obtenção de extratos de própolis sob diferentes condições e avaliação de sua atividade antifúngica. Rev Bras Farmacogn 2007;17:388-95.

10. Matsuno T, Chen C. Basnet, P. A tumouricidal and antioxidant compound isolated from an aqueous extract of propolis. Med Sci Res 1997;25:583-4.

11. Park YK, Ikegaki M. Preparation of water and ethanolic extracts of propolis and evaluation of the preparations. Biosci Biotechnol Biochem 1998;62:2230-2.

12. Aga H, Shibuya T, Sugimoto K, Nakajima S. Isolation and identification of antimicrobial compounds in Brazilian propolis. Biosci Biotechnol Biochem 1993;58:945-6.

13. Hausen BM, Wollenweber E, Senf H, Post B. Contact Dermat 1987;17: 63-170.

14. Funayama S, Werner PR, Andrade UVC. Action of inhibitor compounds on growth cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from propolis of *Apis mellifera*. 10º Seminário de Pesquisa e 4º Seminário de Iniciação Científica. 2006. Disponível em: www.utp.br/proppe/x%20seminario_pesquisa/index.htm

15. Woisky RGR, Salatino, A. Analysis of propolis: some parameters and procedures for chemical quality control. J Api Res 1998;37:99-105.

16. CLSI 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved Standard-Eighth Edition. CLSI document M07-A8. Clinical and Laboratory Standards Institute; 2009.

17. Teixeira EW, Negri G, Meira RMSA, Message D, Salatino A. Plant origin of green propolis: bee behavior, plant anatomy and chemistry. Evid Based Complement Alternat Med 2005;2:85-92.

18. Grunberger D, Banerjee R, Eisinger K, Oltz EM, Efros L, Caldweel M et al. Preferential cytotoxicity on tumor cells by caffeic phenetyl ester isolated from propolis. Experientia 1988;44:2320-30.

19. Rhajaoui M, Ourmazil H, Faid M, Lyagoubi M, Elyachioui M, Benjouad A. Antibacterial activity of a Moroccan propolis extracts. Science Letters 2001;3:3.

20. Hegazi AG, Abd El Hady FK. Egyptian propolis: 3. antioxidant, antimicrobial activities and chemical compositions of propolis from reclaimed lands. Z Naturforsch 2001;57:395-402.

21. Bankova V, Castro SL, Marcucci MC. Propolis: recent advances in chemistry and plant origin. Apidologie 2000;31:3-15.