

ANTI-COAGULASE-NEGATIVE *Staphylococcus* ACTIVITY OF ETHANOLIC EXTRACTS OF PROPOLIS FROM TWO BRAZILIAN REGIONS AND SYNERGISM WITH ANTIMICROBIAL DRUGS BY THE E-TEST METHOD

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ABSTRACT: Propolis is a natural resinous substance collected by bees from vegetal sources and its therapeutic properties have been investigated. In this work, we evaluated the inhibitory activity of ethanolic extracts of propolis (EEP) from the Southeast and South of Brazil on coagulase-negative *Staphylococcus* (CNS) growth as well as the EEP *in vitro* synergism with antimicrobial drugs by using the diffusion method (E-test). The EEP chemical characteristics (dry weight, pH, flavonoid and phenolic compounds) were determined. Seven drugs were tested, and synergism was observed between three drugs and Southeast EEP, six drugs and South EEP, and one drug and ethanol control. Ethanolic extracts of propolis from the South of Brazil presented the greatest flavonoid content and synergism rate, while EEP from the Southeast presented the greatest anti-CNS activity and phenolic compound content. Results showed the correlation among anti-CNS activity, synergism rate and chemical characteristics of propolis.

KEY WORDS: Brazilian propolis, coagulase-negative *Staphylococcus*, E-test, antimicrobial drugs, synergism.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Propolis is a complex resinous material produced by honeybees from plant exudates, beeswax, and bee secretions (17) and has protective function on honeycombs, especially against microorganisms (3). The chemical composition of *Apis mellifera* propolis and its wide spectrum of biological activities (hepatoprotective, antitumour, antioxidative, antimicrobial, and anti-inflammatory properties) have attracted the attention of researchers (2). It is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% several substances, including organic debris; but this composition varies according to the vegetal source (4). In the last few years propolis from tropical regions, especially from Brazil, has become a subject of increasing interest, and investigations revealed that flavonoids are important components of these samples (1).

The antimicrobial mechanism of propolis is complex and could be attributed to the synergistic activity between phenolic and other compounds (16), mainly to the flavonoids pinocembrin, galangin, and pinobanksin (5). Also, several mechanisms of the activity of propolis on bacterial growth have been reported: (1) inhibition of cell division; (2) bacterial cytoplasm, cell membranes, and cell walls collapse; (3) bacteriolysis; and (4) protein synthesis inhibition (27). Galagin and caffeic acid from EEP are enzyme inhibitors of the bacterial metabolism (11, 12, 15).

A stronger activity on Gram-positive bacteria growth has been reported (4) and a correlation between this activity and flavonoids content were related (10). Antimicrobial activity was observed against *Staphylococcus aureus* (8, 16, 25), *Streptococcus pyogenes* and *S. mutans* (3, 15), Gram-positive and Gram-negative bacteria species and *Candida* (7, 25, 26), anaerobic bacteria of human oral cavity (23), *Salmonella* (21,22) and on miscellaneous microorganisms including *Mycobacterium* (2).

In vitro synergism between propolis and antimicrobial drugs has been investigated (6, 8, 13, 16, 21, 24, 26) and the protein synthesis inhibitors in bacterial metabolism showed the greatest synergism rate with ethanolic extract of propolis (8).

Antimicrobial susceptibility methods in clinical laboratories utilize the principle of diffusion, known as the Kirby and Bauer (disk diffusion) and E- test (strip diffusion) methods. The E-test method uses the combination of both principles – dilution and diffusion. Drugs are impregnated in a strip, an antimicrobial density gradient is

established in an agar plate, and the minimal inhibitory concentration (MIC), in mg/ml, is evaluated (18).

Staphylococci species are initially differentiated by the coagulase test. The following coagulase-producing *Staphylococcus* are *S. aureus*, *S. intermedius*, *S. delphini*, and some strains of *S. hyicus* and *S. schleiferi*. *Staphylococci* that do not produce coagulase are referred to as coagulase-negative *Staphylococcus* (CNS). The most clinically significant species in this group are *S. epidermidis* and *S. saprophyticus* which have been known to be responsible for a variety of hospital-acquired infections and to be associated with urinary tract infections (18).

The aim of the present work was to investigate *in vitro* synergism between two ethanolic extracts of Brazilian propolis (EEP) and seven drugs employed against CNS strains using the susceptibility test denominated E-test method.

MATERIALS AND METHODS

Ethanolic Extract of Propolis (EEP)

A crude sample of *Apis mellifera* propolis was collected from Botucatu, São Paulo State, Southeast of Brazil (latitude 22°53'09" south, longitude 48°26'42" west) and Uribici, Santa Catarina State, South of Brazil (latitude 28°00'48" south, longitude 49°35'22" west); EEP were obtained treating 25g crude propolis in 100ml of 70% ethanol, and extracted at room temperature. After three days, the extract was filtered (Whatman paper) and stored at 5°C. Some physicochemical characteristics of EEP (dry weight, pH, phenolic and flavonoid content) were measured according to the methodology of Gonsales *et al.* (10).

Coagulase-Negative *Staphylococcus* (CNS) Strains

Thirty-two CNS strains were isolated from clinical specimens of newborns admitted to the Neonatal Unit of the University Teaching Hospital, Botucatu, São Paulo State, Brazil. Strains were isolated in Sheep's Blood Agar and after biochemical identification (14) they were stored in brain heart infusion (BHI) plus agar. The strains tested were *S. haemolyticus* (n=6), *S. warneri* (n=4), *S. epidermidis* (n=13), *S. lugdunensis* (n=3), *S. xylois* (n=2), *S. hominis* (n=2) and *S. cohnii* (n=2).

Minimal Inhibitory Concentration (MIC) of EEP

MIC of EEPs were carried out for 32 CNS strains by diluting the extracts in Mueller-Hinton Agar (MHA) media (%v/v), as recommended by the CLSI/NCCLS (19,20). Petri plates containing EEP concentrations varying from 0.1 to 5.0%v/v and control plates with MHA and MHA plus 70% ethanol were inoculated with CNS strains (10^5 CFU) using a Steers replicator and were incubated at 37°C/24h. The MIC was determined as the lowest concentration of EEP that inhibited the growth of CNS strains, and the MIC 90% was calculated for two EEP and ethanol 70% control.

Synergism Assays between EEP and Antimicrobial Drugs

In vitro synergism was performed by E-test diffusion method on Mueller Hinton Agar (MHA) (20) on ten CNS strains - *S. epidermidis* (5) and *S. saprophyticus* (5). One-fourth of MIC 90% to CNS strains was considered as sub-inhibitory concentration of EEP and ethanol 70% in the synergism assays (18). Seven drugs were evaluated: oxacillin (OXA), vancomycin (VAN), cephalothin (CFL), chloramphenicol (CLO), netilmicin (NET), tetracycline (TET), and clindamycin (CLI). Three susceptibility tests, in duplicate, were performed for each CNS strain in control plates with plain MHA and in plates containing MHA plus one-fourth of MIC 90% of EEP and ethanol 70%. The MIC values in the E-test method ($\mu\text{g/ml}$) were recorded by observing the elliptical inhibitory areas for each strip after incubation at 37°C/18h.

Statistical Analysis

Results from synergism assays were subjected to the Mann-Whitney non-parametric test in order to compare the values ($\mu\text{g/ml}$) of the inhibitory zone in the strip diffusion method (Minitab Statistical Software version 13.32). Results were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

The results presented in Table 1 indicate that EEP from the South of Brazil (Urubici, Santa Catarina State) showed the greatest amount of flavonoid content, and EEP from the Southeast of Brazil (Botucatu, São Paulo State) showed the greatest phenolic compounds content. These results also show that EEP from the Southeast of Brazil had the greatest antimicrobial action ($p < 0.001$) on CNS growth (Table 2),

although presenting the lowest flavonoid content. The antimicrobial mechanism of propolis seems to be complex. It may vary according to the composition (2, 9) and can be explained by the synergism between the active substances found in propolis.

In vitro synergism between antimicrobial drugs and propolis, using E-test, has been reported (6, 8, 13, 16, 24, 26), however, no reports of the EEP chemical characteristics and synergism profile was related. Results from *in vitro* synergism assays between the two EEP and the seven antimicrobial drugs performed by the E-test method are presented in Table 3. The propolis samples showed the potential to enhance the antimicrobial drugs efficacy, but the greatest synergism profile was observed in the propolis from the South of Brazil. This propolis sample also showed the greatest flavonoid content although it was less active against CNS strains than Southeast EEP. The sub-inhibitory MICs determined in the synergism assays were 0.21%v/v for Southeast and 0.41%v/v for South EEP samples, and this protocol resulted in greater flavonoid content in Petri plates prepared with South EEP than in those with Southeast EEP. The results support the conclusion that EEP chemical characteristics, especially the flavonoid content, can make clear the synergism between EEP and antimicrobial drugs, but the establishment of the molecular basis of the synergistic effect between EEP and antimicrobial drugs along with further microbiological, pharmacological and clinical trials is needed.

Thus, the results were encouraging and confirmed the strong anti-CNS activity of propolis; determination of the chemical characteristics of EEP showed that the phenolic compounds were responsible for the greatest anti-CNS activity of Southeast EEP, and the flavonoid content was responsible for the greatest synergism rate of South EEP. Since bacteria may be resistant to several antimicrobial drugs, the synergism reported here is relevant, and propolis may constitute an alternative for treating these pathogens.

Table 1. Physicochemical characteristics of ethanolic extracts of propolis from two regions of Brazil.

Propolis samples	Dry weight (mg/ml)	pH	Flavonoids %	Phenolic compounds (%)
Botucatu (São Paulo State-Southeast)	80.3	5	0.597	5.9
Urubici (Santa Catarina State-South)	87.7	4	0.871	4.7

Table 2. MIC 90% (*) from ethanolic extracts of propolis (EEP) and ethanol (%v/v) control against 32 coagulase-negative *Staphylococcus* strains and one-fourth of MIC 90% values used in synergism assays.

	Brazilian Regions		
	Southeast sample (%v/v)	South sample (%v/v)	Ethanol Control (%v/v)
MIC 90 % (1/4 of MIC 90%)	0.83 (0.21)	1.63 (0.41)	12.72 (3.18)
Range	0.35–1.0	0.4–5.0	5.0–13.0

(*) Antimicrobial activity of Southeast EEP > South EEP > Ethanol control: $p < 0.05$

Table 3. Synergism (X)* between ethanolic extracts of propolis (South and Southwest samples); ethanol control and antimicrobial drugs by the E-test method against 10 coagulase-negative *Staphylococcus* strains: *S. epidermidis* (5) and *S. saprophyticus* (5).

Antimicrobial drugs	Brazilian regions		
	Southeast sample	South sample	Ethanol control
(Cephalotin) CFL	-	X	-
(Oxacillin) OXA	-	X	-
(Netilmicin) NET	X	X	-
(Clindamycin) CLI	X	X	X
(Vancomycin) VAN	-	X	-
(Tetracycline) TET	X	X	-
(Chloranphenicol) CLO	-	-	-

*Synergism when $p < 0.05$

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REFERENCES

- 1 BANKOVA VS., CASTRO SL., MARCUCCI MC. Propolis: recent advances in chemistry and plant origin. *Apidologie.*, 2000, 31, 3-15.
- 2 BANSKOTA AH., TEZUKA Y., KADOTA S. Recent progress in pharmacological research of propolis. *Phytother. Res.*, 2001, 15, 561-71.
- 3 BOSIO K., AVANZINI C., D'AVOLIO A., OZIMO O., SAVOIA D. *In vitro* activity of propolis against *Streptococcus pyogenes*. *Lett. Appl. Microbiol.*, 31, 2000, 174-7.
- 4 BURDOCK GA. Review of the biological properties and toxicity of bee propolis (Propolis). *Food Chem. Toxicol.*, 1998, 36, 347-63.
- 5 CASTALDO S., CAPASSO F. Propolis, an old remedy used in modern medicine. *Fitoterapia*, 2001, 73, S1-S6.
- 6 DETOMA P., OZINO OL. Azione della propoli su microrganismi dell'ambiente ospedalino. *Ann. Microbiol. Enzimol.*, 1991, 41, 231-6.
- 7 DRAGO L., MOMBELLI B., DE VECCHI E., FASSINA MC., TOCALLI L., GISMONDO MR. *In vitro* antimicrobial activity of propolis dry extract. *J. Chemother.*, 2002, 12, 390-5.
- 8 FERNANDES JUNIOR A., BALESTRIN EC., BETONI JEC., ORSI RO., CUNHA MLRS., MONTELLI AC. Propolis: anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. *Mem. Inst. Oswaldo Cruz*, 2005, 100, 563-6.
- 9 GEBARA ECE., LIMA LA., MAYER MPA. Propolis antimicrobial activity against periodontopathic bacteria. *Braz. J. Microbiol.*, 2002, 33, 365-9.
- 10 GONSALES GZ., ORSI RO., FERNANDES JUNIOR A., RODRIGUES P., FUNARI SRC. Antibacterial activity of propolis collected in different regions of Brazil. *J. Venom. Anim. Toxins incl. Trop. Dis.*, 2006, 12, 276-84.
- 11 HAVSTEEM B. Flavonoids, a class of natural products of high pharmacology potency. *Biochem. Pharmacol.*, 1983, 32, 1141-8.
- 12 IKENO K., IKENO T., MIYAZAWA C. Effects of propolis on dental caries in rats. *Caries Res.*, 1991, 25, 347-51.

- 13 KEDZIA B., HOLDERNA E. Investigations upon the combined action of antibiotics and propolis on *Staphylococcus aureus*. *Herba Polonica*, 1986, 32, 187-95.
- 14 KONEMAN EW., ALLEN SD., JANDA NM., SCHRECKEMBERGER PC., WINN JR WC. *Diagnóstico Microbiológico: Texto e Atlas Colorido*. 5.ed. Rio de Janeiro: Medis, 2005.
- 15 KOO H., ROSALEN PL., CURY JA., PARK YK., BOWEN WH. Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity. *Antimicrob. Agents Chemother.*, 2002, 46, 1302-9.
- 16 KROL W., SCHELLER S., SHANI J., PIETSZ G., CZUBA Z. Synergistic effect of ethanolic extract of propolis and antibiotics on the growth of *Staphylococcus aureus*. *Arzneimittel-Forsch.*, 1993, 43, 607-9.
- 17 KUSUMOTO T., MIYAMOTO RH., DOI S., HIROYUKI S., YAMADA H. Isolation and structures of two new compounds from the essential oil of Brazilian propolis. *Chem. Pharm. Bull.*, 2001, 49, 1207-9.
- 18 MAHON CR., MANUSELIS JR G. *Textbook of Diagnostic Microbiology*. Philadelphia: WB Saunders, 1995.
- 19 NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 6.ed. Wayne: NCCLS, 2003. [Approved Standard M7.A6]
- 20 NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing: fifteenth informational supplement*. Wayne: CLSI, 2005. M100-S15.
- 21 ORSI RO., SFORCIN JM., FUNARI SRC., FERNANDES JUNIOR A., BANKOVA V. Synergistic effect of propolis and antibiotics on the *Salmonella typhi*. *Braz. J. Microbiol.*, 2006, 37, 108-12.
- 22 ORSI RO., SFORCIN JM., RALL VLM., FUNARI SRC., BARBOSA L., FERNANDES JUNIOR A. Susceptibility profile of *Salmonella* against the antibacterial activity of propolis produced in two regions of Brazil. *J. Venom. Anim. Toxins incl. Trop. Dis.*, 2005, 11, 109-16.
- 23 SANTOS FA., BASTOS EMA., UZEDA B., CARVALHO MAR., FARIAS ESA., BRAGA FC. Antibacterial activity of Brazilian propolis and fractions against oral anaerobic bacteria. *J. Ethnopharmacol.*, 2002, 80, 1-7.

24 SCHELLER S., DWORNICZAK S., WALDEMAR KK., RAJCA M., TOMCZIK A., SHANI J. Synergism between ethanolic extract of propolis (EEP) and anti-tuberculosis drugs on growth of mycobacteria. *Z. Naturforsch.*, 1999, 54, 549-53.

25 SFORCIN JM., FERNANDES JUNIOR A., LOPES CAM., BANKOVA V., FUNARI SRC. Seasonal effect on Brazilian propolis antibacterial activity. *J. Ethnopharmacol.*, 2000, 73, 243-9.

26 STEPANOVIC S., ANTIC N., DAKIC I., SVABIC-VLAHOVIC M. *In vitro* antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs. *Microbiol. Res.*, 2003, 158, 353-7.

27 TAKAISI-KIKUNI NB., SCHILCHER H. Electron microscopy and microcalorimetric investigations of the possible mechanism of the antibacterial action of a defined propolis provenance. *Planta Med.*, 1994, 60, 222-7.