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EFFECTS OF PROPOLIS ON *STREPTOCOCCUS MUTANS, ACTINOMYCES NAESLUNDII* AND *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

It is known that formation of dental caries is caused by the colonization and accumulation of oral microorganisms and extracellular polysaccharides which are synthesized from sucrose by glucosyltransferases of *Streptococcus mutans*. *Actinomyces naeslundii* and *Staphylococcus aureus* are associated with human root caries and some oral mucosa infections, respectively. In this research *Streptococcus mutans* Ingbritt 1600 exhibiting glucosyltransferase activity was used to test whether different ethanolic extracts of propolis (EEP) inhibit or not the enzyme activity and growth of the bacteria. Antimicrobial activity of EEP against *A. naeslundii* and *S. aureus* was also examined. All EEP from various regions in Brazil inhibited both glucosyltransferase activity and growth of *S. mutans*, *A. naeslundii* and *S. aureus*, but one of propolis from Rio Grande do Sul (RS2) demonstrated highest inhibition of the enzyme activity and growth of the bacteria. It was also found that propolis (RS2) contained highest concentrations of pinocembrin and galangin.

Key words: Dental caries, glucosyltransferase, propolis, antimicrobial activity, *Streptococcus mutans*, extracellular polysaccharides.

INTRODUCTION

A number of investigations have shown a positive correlation between the number of *Streptococcus mutans* in dental plaque and the prevalence of dental caries (9, 17). Substantial numbers of *S. mutans* and lesser numbers of *S. sanguis*, *S. mitis* and species of genus of *Actinomyces* were isolated from cemental surface caries during an extensive survey of oral microbiology (30).

It was found that formation of dental plaque was caused by the colonization and accumulation of oral microorganisms and extracellular polysaccharides which are synthesized from sucrose by glucosyltransferase of *S.mutans* on hard surface of teeth (12). The extracellular polysaccharides consist of glucans containing predominantly α-1,6 bonds that are similar to classical dextrans and of polymers containing more than 50% α-1,3 linkages (12). The latter polysaccharides are highly insoluble and have been termed mutan. Both types of polymers have been detected in samples of human dental plaque (12).

Several studies have shown that species of genus *Actinomyces*, in particular *Actinomyces naeslundii*, are associated with human root caries (15, 26) and gingivitis (22, 27). Staphylococci and aerobic Gram-negative rods are not commonly involved with odontogenic infections, however, *Staphylococcus aureus* is associated with infections of the oral

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mucosa (8). In addition, strains of *S. aureus* have been isolated in periapical lesions and periodontal abscess (1).

It is now clear that microorganisms play an essential role in the pathogenesis of dental caries, gingivitis and some oral mucosa infections and consequently provide a prime target for the prevention of these diseases using antibiotics and vaccine (6, 7). On the other hand, it was reported that mutastein and ribocitrin, which were isolated from the culture supernatants of *Aspergillus terreus* and *Streptomyces sp.*, inhibited the glucosyltransferase of *S.mutans* (10, 24), but did not show antibacterial activity. In the case of the ethanolic extract of propolis, both the growth of *S. mutans* and the activity of glucosyltransferase were inhibited (19). It was also found that propolis has antibacterial activity against *S. aureus* (5, 13, 21). Propolis is a resinous hive product collected by bees from tree buds and mixed with beeswax, which they secrete. The propolis is used by the bees as a glue to seal the opening of the hives (14). It is known that the ethanolic extract of propolis exhibits various pharmacological activities such as antibacterial, antiviral, antifungal, anaesthetic, anti-inflammatory, hypotensive, immunostimulatory and cytostatic properties (3, 11). The objective of this work was to further investigate the use of propolis, collected from various regions of Brazil, for its antibacterial activity and inhibition of glucosyltransferase.

MATERIALS AND METHODS

Microorganisms. Bacterial strains used in this research were *Streptococcus mutans* Ingbritt 1600, *Actinomyces naeslundii* ATCC 12104 and a strains of coagulase positive *Staphylococcus aureus*. The strain of *S. mutans* Ingbritt 1600 was previously selected by its glucosyltransferase activity by both aerobic and anaerobic cultivation, as compared to three others oral microorganisms: *Streptococcus sanguis* ATCC 10556, *Streptococcus sp.* isolated from saliva in our laboratory and *Actinomyces naeslundii* ATCC 12104. *S. sanguis* ATCC 10556 and *Actinomyces naeslundii* ATCC 12104 were donated from Department of Dental Research, University of Rochester, NY, USA. *S. mutans* Ingbritt 1600 was donated from Eastman Dental Research Center, Rochester, NY, USA. A Gram negative bacteria, *Escherichia coli,* was also used for antimicrobial test.

Propolis samples. The propolis samples collected by *Apis mellifera* were obtained from the states of Minas Gerais, São Paulo, Goiás, Mato Grosso do Sul, Paraná and Rio Grande do Sul for this investigation. The specimens of propolis were further dehydrated using low vacuum pump and the extracts of the dried propolis prepared as described by Koo and Park (20). The dried propolis samples were ground into a fine powder and 2 g of the propolis powder mixed with 25 ml of 80% ethanol in a test tube and shaken at 70°C for 30 min. After extraction, the mixture was centrifuged to obtain the supernatants, which were denominated as an ethanolic extract of propolis (EEP).

Production of the enzyme. The strains of *Streptococcus mutans, Streptococcus sanguis, Streptococcus sp.* and *Actinomyces naeslundii* were inoculated into 100 ml conical flasks containing 20 ml of 3% tryptic soy broth and incubated at 37°C for 48 hr with shaking (150 rpm). In the case of anaerobic cultivation, the conical flasks were incubated statically in an anaerobic incubator. After cultivation for 48 hr, the culture media were centrifuged and the supernatans used as crude glucosyltransferase.

Assay of glucosyltransferase activity. Glucosyltransferase activity was determined as described by Smith *et al.* (28). A mixture of 0.9 ml of sucrose 0.25 M in 0.2 M phosphate buffer, pH 7.0 and 0.1 ml of the crude enzyme solution was incubated at 37°C for 2 hr. After incubation, reducing sugars were measured by the method of Somogyi-Nelson (29). The quantitative analysis of fructose was performed by HPLC using a chromatograph equipped with a differential refractometer detector and a Shodex Ionpak KS-802 column, the eluent being water with a flow rate of 1 ml/min. Authentic standards were fructose, glucose and sucrose. One unit of enzyme activity was defined as 1 μmol of fructose/ml of enzyme/hr.

Inhibition of glucosyltransferase activity. The incubation mixture containing 0.9 ml of 0.25 M sucrose in 0.2 M phosphate buffer pH 7.0, 0.1 ml of the crude enzyme solution, 0.005 ml of the ethanolic extract (EEP) (in case of control 0.005 ml of 80% ethanol) and 0.2 M phosphate buffer pH 7.0 to a total volume of 2 ml was incubated at 37°C for 2 hr (19). After incubation, concentration of fructose were determined as described above.

Antimicrobial activity of propolis. Actively growing cultures in tryptic soy broth of the test strains of microorganisms were inoculated onto tryptic soy agar plates with sterile swabs and then the EEP disks were applied on the inoculated plates which were

incubated overnight at 37°C under anaerobic condition. EEP disks were prepared by submerging sterile paper disks (Whatman filter paper nº 3, 5 x 0.5 mm) in EEP solutions, kept drying under low vacuum at room temperature overnight and then incubated at 60°C for 4 hr (4).

High Performance Thin-Layer Chromatography (HPTLC) of EEP. HPTLC precoated plates of silica gel RP-18 $F_{254}S$ were purchased from Merck Co. EEP 5 µl were applied to the lower edge of the plate, and ascending chromatography run using a mobile phase of ethanol:water (55:45, v/v). The detection of flavonoids was carried out by UV-radiation at 366 nm.

Reversed Phase High Performance Liquid Chromatography (HPLC) of EEP. A quantitative analysis of the flavonoids was performed by reversed phase HPLC using a chromatograph equipped with YMC PACK ODS-A column. The mobile phase was acetic acid:methanol:water (5:75:60, by vol.) and the flow rate was 1 ml/min and detection with a diode array detector. Chromatograms were recorded at 254 nm. The quantities of flavonoids in the EEP were calculated by using authentic standards of flavonoids purchased from Extrasynthese A .A . Co., France.

RESULTS AND DISCUSSION

Production of glucosyltransferase. *Streptococcus mutans* produced the highest activity of the enzyme by both anaerobic and aerobic cultivation (9.60 ± 0.12 and 9.00 ± 0.20 units, respectively) as compared to other three strains. *S. sanguis* and *A. naeslundii* produced the lowest activity of the enzyme (0.30 ± 0.06 and 0.48 ± 0.09 units, respectively, for anaerobic cultivation; and 0.30 ± 0.06 and 0.18 ± 0.08, respectively, for aerobic cultivation). *Streptococcus sp.* isolated from saliva also demonstrated lower activity of the enzyme (1.80 ± 0.15 and 2.10 ± 0.28 units for anaerobic and aerobic cultivation, respectively) than *S. mutans*. Therefore, the strain of *S. mutans* was used for further studies.

Effect of propolis on glucosyltransferase activity. The effect of propolis on glucosyltransferase activity from *S. mutans* was determined by incubating the enzymatic reaction mixture as described in the method which contained ethanolic extract of propolis (EEP) at 37°C for one hour. The results are shown in Table 1. All propolis samples inhibited the enzyme activity and propolis sample from Rio Grande do Sul (RS2) state demonstrated highest inhibition of the enzyme activity.

Antimicrobial activity of propolis. Susceptibility of *S. mutans, A. naeslundii* and *S. aureus* to EEP collected from different regions in Brazil were investigated and the results were shown in Table 2. All propolis samples exhibited an inhibitory action on the growth of the bacteria and one of the sample from Rio Grande do Sul state (RS2) demonstrated highest inhibition zone of the bacterial growth. Nevertheless, none of EEPs inhibited the growth of *E. coli*. Earlier studies reported that propolis is more active on Gram positive than on Gram negative bacteria, as *E. coli* (5, 13). It is interesting to note that the propolis from Rio Grande do Sul (RS2) demonstrated both higher antimicrobial activity and inhibition of glucosyltransferase activity. Therefore, analysis of flavonoids in propolis was carried out by using High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) because these two techniques are most often used (16, 31). The results were shown in Figs. 1 and 2. HPTLC and HPLC (Figs. 1 and 2) demonstrated that patterns of chromatograms of propolis from SP, MG1, MG2, GO

Table 1 - Effect of propolis on activity of glucosyltransferase from *S. mutans*.

Place of Collection of Propolis	Glucosyltransferase activity (Unit)*		Inhibition of the enzyme (%)
	whitout propolis	with propolis**	
Paraná State (PR)	2.9 ± 0.25	2.4 ± 0.38	17.2 ± 0.14
São Paulo State (SP)	2.5 ± 0.21	2.3 ± 0.27	8.0 ± 0.25
Rio Grande do Sul State (RS)	3.6 ± 0.30	2.5 ± 0.20	30.5 ± 0.18
Minas Gerais State (MG)	3.0 ± 0.15	2.6 ± 0.22	13.3 ± 0.16
Goiás State (GO)	3.2 ± 0.34	2.7 ± 0.45	15.6 ± 0.27
Mato Grosso do Sul State (MS)	3.6 ± 0.52	2.9 ± 0.31	19.4 ± 0.23

Each value represents the mean ± standard deviation of a triplicate analysis ($p<0.001$)
* One unit was defined as 1 µmol of fructose/hr/ml of enzyme
** Ethanolic extract of propolis (2 g of propolis / 25 ml of 80% ethanol)

Table 2 - Antimicrobial activity of ethanolic extracts of propolis from different regions to *Streptococcus mutans*, *Actinomyces naeslundii* and *Staphylococcus aureus*. (Overnight incubation at 37°C under anaerobic condition)

Regions of propolis collected	Zone of inhibition of microbial growth (mm)		
	S. mutans	*A. naeslundii*	*S. aureus*
Paraná State (PR)	1.0	1.5	1.0
São Paulo State (SP)	1.5	2.0	1.0
Goiás State (GO)	0.5	1.0	0.5
Mato Grosso do Sul State (MS)	1.5	2.5	1.0
Minas Gerais State (MG1)	1.5	2.5	1.0
(MG2)	0.5	2.0	0.5
Rio Grande do Sul State (RS1)	1.5	2.0	1.0
(RS2)	3.0	3.5	2.0
80% ethanol as control	0	0	0

Propolis samples were collected from two (2) different places in Minas Gerais and Rio Grande do Sul States
Ethanolic extract of propolis (2g of propolis / 25 ml of 80% ethanol)

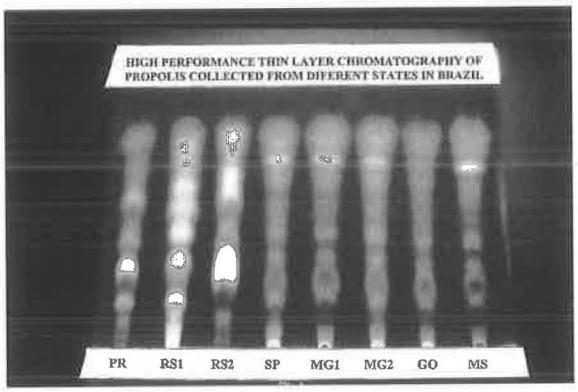

Figure 1. High performance thin layer chromatography of propolis collected from diferent states in Brazil.

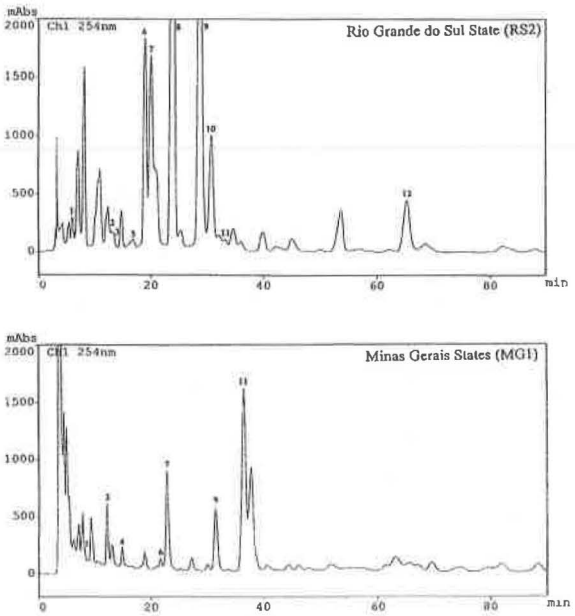

Figure 2. RP-HPLC of ethanolic extract of propolis. (1) quercetin, (2) kaempferol, (3) apigenin, (4) isorhamntin, (5) rhamnetin, (6) pinocembrin, (7) sakuranetin, (8) chrysin, (9) acacetin, (10) galangin, (11) kaempferide and (12) tectocharysin.

and MS (Southeastern and Central Brazil) are entirely different as compared to PR, RS1 and RS2 (Southern Brazil). These different patterns of chromatogram are due to different flavonoids in propolis as reported previously (25). The results of quantitative analysis of flavonoids in propolis were shown in Table 3. It is apparent that propolis RS2 contained highest concentrations of pinocembrin as compared to others. Both RS1 and RS2 contained higher concentrations of galangin than other states. It was already reported that ethanolic extract of propolis showed strong inhibitory activity against various microorganisms *in vitro* test (21). Some flavonoids in propolis are considered to be antimicrobial agents and pinocembrin (32) and galangin (33) were identified as antimicrobial flavonoids. Later, Metzner *et al* (23) also reported that antimicrobial activity of propolis was due to presence of pinocembrin, galangin, pinobanksin, pinobanksin-3-acetate which are flavonoids, and caffeic acid ester. The propolis RS2 also contained higher concentrations of quercetin and mainly chrysin than others. Iio *et al.* (18) showed that quercetin and chrysin inhibited *in vitro* the glucosyltransferase activity and glucan formation.

In conclusion, all the samples of propolis tested showed antimicrobial activity and inhibition of glucosyltransferase activity, however propolis from Rio Grande do. Sul-RS2 (Southern Brazil) demonstrated better results than others, propably due its highest concentrations of pinocembrin, galangin

Table 3. Quantitative analysis of propolis from different regions in Brazil.

	Propolis							
Flavonoids	RS1	RS2	PR	SP	MG1	MG2	GO	MS
Quercetin	1.4	2.0	1.3	1.0	0.9	1.2	0.4	0.8
Kaempferol	2.9	0.6	1.1	1.9	2.4	1.2	1.1	1.8
Apigenin	1.8	0.5	1.5	0.5	-	0.5	1.0	2.4
Isorhamnetin	-	-	2.3	-	1.1	-	-	0.2
Rhamnetin	-	0.8	0.5	-	-	-	-	-
Pinocembrin	7.3	16.8	12.6	0.4	1.8	1.3	1.7	9.6
Sakuranetin	12.3	25.0	7.5	15.3	19.6	9.2	1.6	12.6
Isosakuranetin	-	-	-	-	-	0.2	-	5.9
Chrysin	-	21.9	9.7	1.8	-	2.1	1.1	10.7
Acacetin	-	28.8	4.9	13.0	4.3	8.3	3.9	4.9
Galangin	13.5	9.4	7.0	0.6	-	0.5	0.6	1.5
Kaempferide	3.2	1.1	3.2	-	22.2	0.4	0.5	1.1
Tectochrysin	-	3.3	1.0	-	-	-	-	-

All results represent mg/g of propolis

(antimicrobial activity), quercetin and chrysin (inhibition of glucosyltransferase activity and glucan formation).

RESUMO

Efeito da própolis sobre *Streptococcus mutans*, *Actinomyces naeslundii* e *Staphylococcus aureus*

Sabe-se que a cárie dental está relacionada com a colonização e acúmulo de microrganismos e polissacarídeos extracelulares sintetizados a partir da sacarose pelas enzimas glicosiltransferases produzidas pelos *Streptococcus mutans*. *Actinomyces naeslundii* e *Staphylococcus aureus* estão associados, respectivamente, com cárie de raiz e algumas infecções da mucosa oral em seres humanos. Neste experimento, *Streptococcus mutans* Ingbritt 1600 exibindo alta atividade glicosiltransferásica foi usado para analisar extratos etanólicos de própolis (EEP) quanto à possível inibição da atividade enzimática e do crescimento bacteriano. Também foi analisado a atividade antimicrobiana do EEP sobre *A. naeslundii* e *S. aureus*. Todos os EEPs testados, obtidos de diversas regiões do Brasil, inibiram tanto a atividade de glicosiltransferase como o crescimento de *S.mutans, A. naeslundii* e *S. aureus;* entretanto, uma amostra de própolis proveniente do Rio Grande do Sul (RS2) demonstrou maior inibição da atividade enzimática, bem como do crescimento bacteriano. Também foi observado que a própolis RS2 apresentou maiores concentrações de pinocembrina e galangina.

Palavras-chave: Cárie dental, glicosiltransferase, própolis, atividade antimicrobiana, *Streptococcus mutans*, polissacarídeos extracelulares.

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