

In vitro* antibacterial effect of Argentine propolis extracts on *Streptococcus mutans* and *Actinomyces viscosus

Efecto antibacteriano *in vitro* de extractos de propóleos argentinos sobre *Streptococcus mutans* y *Actinomyces viscosus*

Efeito antibacteriano *in vitro* de extratos de própolis argentinos sobre *Streptococcus mutans* e *Actinomyces viscosus*

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ABSTRACT

Aim: In the present study, the antibacterial activity of the Ethanol Extract of Propolis (EEP), collected from various regions (Mendoza, Santiago del Estero, and Corrientes) in Argentina, against *Streptococcus mutans* ATCC® 35668™ and *Actinomyces viscosus* ATCC® 15987™ (MicroBioLogics Inc., USA) was investigated. **Methods:** Identification of geographic and botanical origin was based on a reconnaissance survey. Phytochemical screening of propolis was carried out on ethanolic extracts using standard methods to identify the constituents (aluminum chloride colorimetric method, Folin-Ciocalteu colorimetric method, thin layer chromatography). The agar diffusion method (discs and wells) and serial dilution method (plates and tubes) were used to evaluate the antibacterial activity of EEP. **Results:** EEP exerted various degrees of antibacterial activity against *S. mutans* and *A. viscosus*, depending on the geographic area of collection. Phytochemical screening showed that the bioactive compounds correspond to phenolic compounds and flavones. EEP from Tunuyán (Mendoza), where the most abundant vegetation belongs to *Populus* sp., showed the highest content of phenolic compounds (220.92±2.01 mg/g) and flavonoids (30.39±0.25 mg/g). This sample showed the most profound antibacterial activity among the EEP tested. By the agar-well diffusion method, we found a high susceptibility with an inhibitory halo of 11.25 ± 4.68 mm and 10.90 ± 4.21 mm against *S. mutans* and *A. viscosus*, respectively. It also presented low Minimum Inhibitory Concentration and Minimum Bactericidal Concentration values against *S. mutans* (MIC 0.05 mg/mL - MBC 0.46 mg/mL) followed by *A. viscosus* (MIC 0.11 mg/mL - MBC 0.93 mg/mL). **Conclusions:** The combined results from all methods indicated that *S. mutans* is more susceptible to the effect of the Tunuyán EEP than *A. viscosus*.

Uniterms: Propolis. *Streptococcus mutans*. *Actinomyces viscosus*. Microbial sensitivity tests.

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INTRODUCTION

Propolis is a semisolid mixture of plant-derived compounds, produced by honeybees (*Apis mellifera*). The constituents of propolis vary widely, depending on the climate, season, and geographic location where it was collected. In general, propolis contains approximately 50% resin and vegetable balm, 30% wax, 10% essential and aromatic oils, 5% pollens, and 5% other trace substances, including organic debris¹.

Its chemical composition is predominantly comprised of phenolic compounds, including flavonoids, phenolic acids, and their esters. Due to the presence of flavonoids and phenolic esters, propolis is responsible for its potential effects through a specific reagent².

Propolis has strong bacteriocidal, antiviral, antiparasitic, fungicidal, and antioxidative properties³. Studies have shown propolis antibacterial activity against Gram-positive and Gram-negative bacteria^{4,5}. The use of different solvents changes the activity of the main biologically active constituent in propolis, which are responsible for its many biological properties, which also changes according to dosage⁶.

Antibacterial action of EEP against *Streptococcus mutans* (gram-positive cocci, facultative anaerobic bacterium) has been widely investigated⁷⁻⁹. However, relatively few studies were aimed at the influence of EEP on the growth of *Actinomyces viscosus* (a gram-positive facultative anaerobic bacterium)^{10,11}.

Both *S. mutans* and *A. viscosus* have been associated, alone or in combination, with caries, gingivitis, alveolar bone loss, and the delayed healing of extraction sites. *S. mutans* also possesses the ability to combat harsh physiological conditions of the oral environment¹². Ethnopharmacological surveys show that several plant species are used empirically by the population to combat oral diseases. However, it is necessary to check the properties of these plant species¹³. In the process of developing new pharmacologically active compounds from natural products for use in dentistry, EEP remains an underestimated compound.

Therefore, considering the wide range of therapeutic properties of propolis, further investigations are needed to validate a dose required to eliminate pathogenic microorganisms of the oral cavity.

The purpose of this paper is to evaluate the antibacterial effect of Argentinian propolis extracts on *S. mutans* and *A. viscosus*.

MATERIAL AND METHODS

NATURAL PRODUCT

Six Argentinean samples of propolis from apiaries from the regions of Corrientes, Mendoza, and

Santiago del Estero were used. Organoleptic features and physicochemical properties were analyzed according to current Argentine standards¹⁴.

Propolis was ground to a fine powder and extracted with 80% ethanol by maceration and agitation in the dark and at room temperature. After three days, the propolis was frozen overnight to -20°C, and the mixture was then centrifuged to obtain the supernatant, which was filtered through filter paper. This supernatant was dried by evaporation under vacuum at 40°C, and the crude propolis EEP was stored in the dark at 4°C until use¹⁵.

The botanical origin of propolis, collected from hives of *Apis mellifera* bees, was a native and cultivated flora from Tunuyán: *Populus* sp, *Pinus* sp, *Larrea cuneifolia*, *Larrea divaricata*, *Salix humboldtiana*, *Prosopis* sp, *Schinus* sp, and *Geoffraeadeorticans*; Santiago del Estero: *Eucalyptus* sp, *Prosopis* sp, *Schinus molle*, and *Zizyphus mistol*; and Corrientes: *Mangifera indica*, *Nectandra angustifolia* (= *Nectandra falcifolia*), and *Pouteriagardneriana* (= *Pouteriasauvis*).

CHARACTERIZATION OF THE ETHANOL EXTRACTS

The extracts were standardized based on phenolic compounds and flavonoid content according to IRAM-INTA guidelines¹⁶. All experiments were carried out in triplicate. Total flavonoid content was determined using the aluminum chloride colorimetric method¹⁷, and the total phenolic content was analyzed using the Folin-Ciocalteu colorimetric method¹⁸. Antioxidant property screening was determined by the potassium permanganate assay¹⁹. The chemical composition and radical scavenging activity of EEP were determined using thin layer chromatography (TLC) and bioautographic analysis^{20,21}.

ANTIBACTERIAL ACTIVITY

The bacteria used in these experiments were: *Streptococcus mutans* ATCC 35668 and *Actinomyces viscosus* ATCC 15987 (2012 Microbiologics®, Inc. USA). Aliquots of frozen stocks in 20% glycerol of both strains were inoculated on agar plates. Brain Heart Infusion Agar (BHI), together with 5% defibrinated sheep blood (S-BHI-A), was used to recover bacteria. *S. mutans* and *A. viscosus* were cultured under microaerophilic conditions, at 37°C for 18-24h. The resulting colonies were suspended in phosphate buffer solution (PBS), to reach concentrations equivalent to the McFarland Scale No. 1.

The antimicrobial susceptibility test was performed according to the agar diffusion method, and disc and well techniques²². Suspensions were spread onto the plates with a sterile cotton swab.

Then a total of 20 µg of phenolic compounds were placed on each disk or well. After incubation, the average diameter of the three readings of the clear zone surrounding the disk or well was taken as the measure of the inhibitory level from EEP against the bacteria on test and recorded as mean ± standard deviation, in mm.

The serial dilution method was carried out using plate and tube techniques. Minimum Inhibitory Concentration (MIC) for Tunuyán propolis against the tested bacterial species was determined using the propolis extract in serial concentrations: 0.15, 0.30, 0.45, 0.60, and 0.75 mg/mL. Control plates with serial concentrations of ethanolic solution were also tested; control (BHI+inoculum). In serial dilutions in tubes, bacterial growth is not distinguished due to the haze that propolis caused in the medium. The isolated organism on the blood agar was incubated at 37°C for 18-24 hrs. After incubation, the plates were observed. The concentration that exhibited no bacterial growth was considered the Minimum Bactericidal Concentration (MBC) value. All tests were performed in triplicate.

STATISTICAL ANALYSIS

ANOVA followed by *post-hoc* test (LSD) were used to check for significant differences between groups (differences between total phenol and flavonoid concentrations in the extracts or diameter inhibition zones). Differences were considered significant at $p < 0.05$.

RESULTS

Quality control analysis of raw propolis and its ethanolic extract met physical, chemical, and sensory requirement standards according to Argentine standards. Mendoza EEP showed the highest content of phenolic compounds and flavones, as well as an oxidation index of two seconds on average. Corrientes EEP showed the lowest phenolic compound and flavone concentrations and the highest oxidation index. Santiago del Estero EEP also contained high concentrations of phenolic compounds and flavonoids and low oxidation index values close to those reported for EEP from Tunuyán (Table 1), but their content of mechanical impurities is greater, and raw propolis consistency is more rigid.

Table 1 - Oxidation index and means and Standard Deviation (M±SD) of phenolic compound and flavonoid contents of analyzed Argentine propolis samples

Propolis sample origins	Oxidation index(s)*	Phenolic compounds (mg/g)**	Flavonoids (mg/g)**
Caá Catí Corrientes	110	16.43±0.01	2.06±0.00
Saladas Corrientes	17	103.75±5.05	1.51±0.01
Bella Vista Corrientes	12	86.50±5.01	0.79±0.05
Loreto Corrientes	95	70.08±2.05	0.48±0.12
Santiago del Estero	2.5	208.58±2.81	28.51±0.05
Tunuyán Mendoza	2	220.92±2.01	30.39±0.25

Legend: *Seconds, **mg per g of propolis. Studies performed in triplicate.

Phytochemical analysis reveals that the analyzed samples did not contain alkaloids. Figure 1 shows the chromatographic profile of all propolis samples analyzed in this study. Phytochemical analysis reveals that the analyzed samples did not contain alkaloids. Samples from Santiago del Estero and Mendoza have various phenolic compounds; samples 1 and 3 from Corrientes have a very similar profile, containing 3 components with similar R_f values. More concentrated EPP should be used for samples 2 and 4 from Corrientes in order to make

the already existent components clear. Due to these conditions, stains were not observed with proper resolution in this study. Using vanillin-sulfuric acid as a reagent to observe components, spots in the blue-violet range proved to be evident in samples from Corrientes. In the case of samples from Santiago del Estero (sample 5) and Mendoza (sample 6), these components presented clear brown, yellow and orange tones in compounds with intermediate and high polarity, as compared to blue-violet tones in less polar compounds.

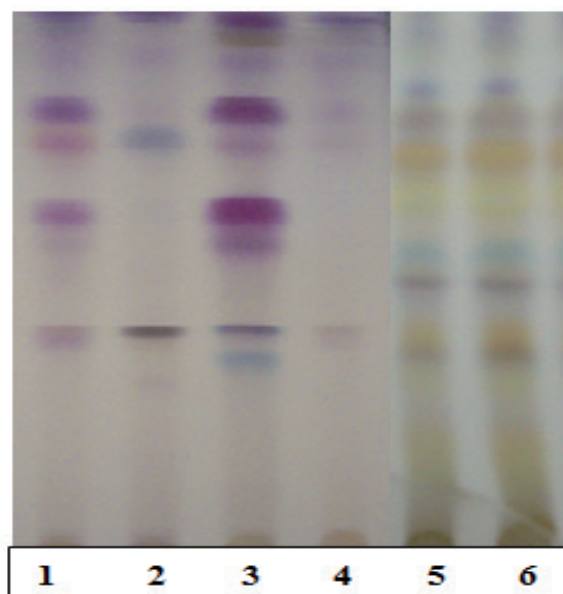


Figure 1 - Thin layer chromatography (TLC) of EEP. Solvent run: hexane: ethyl acetate: acetic acid (60:30:1), developer: vanillin-sulfuric acid. Observation: numbers indicate the origin of the propolis samples: Corrientes (1-4), Santiago del Estero (5), and Mendoza (6).

TLC plates compared the compositions of the propolis extracts. The number of components in the propolis extracts increased proportionally to the ethanolic concentration in the solvent used for extraction evidenced by the intensity of the bands (Figure 2, A). Therefore, the extracts obtained using 30% ethanol contained only the most polar compounds, followed by those obtained using 50% ethanol. The samples extracted with 80% ethanol were similar in composition with

those extracted with absolute ethanol. Bioautography of the TLC plate showed a large area containing substances with antioxidant activity, as evidenced by the discolored areas (Figure 2, B). The higher yield of the extraction, according to the increase of the alcoholic degree was also observed in the content of flavonoids and phenolic compounds determined spectrophotometrically (Figure 2, C). The coloration of the extracts varied from medium yellow to dark brown (Figure 2, D).

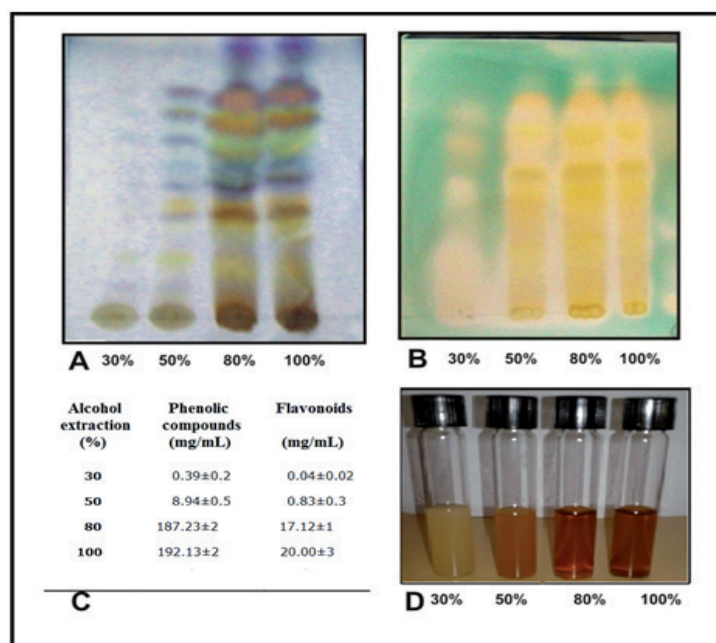


Figure 2 - **A.** Phytochemical profile of EEP obtained with different extractive hydroalcoholic mixtures (30, 50, 80, 100%). Mobile phase: toluene:chloroform:acetone. Developer: vanillin-sulfuric acid. **B.** Antioxidant activity by autographic method. **C.** Content of phenolic compounds and flavonoids in propolis from Mendoza obtained with different hydroalcoholic mixtures. The results represent the average of 3 determinations ± standard deviation. **D.** Appearance of the extracts mentioned above.

Propolis samples from the Central-West region of Argentina (Mendoza “Tunuyán”) were more effective than the samples obtained in the Central-North (Santiago del Estero) and Northeast region (Corrientes: “Caá Catí”, “Loreto”, “Bella Vista”, and “Saladas”) against *S. mutans* and *A. viscosus* strains. The propolis from Caá Catí and Bella Vista (Corrientes) was the most effective, while that from Loreto and Saladas (Corrientes) proved to be the least effective of the Corrientes region.

When the inhibition zones of agar disk diffusion method were compared, a significant

difference was observed among them ($p=0.0001$), in which some showed no inhibition zones or they were diminished in contrast to the well method. The susceptibility of *S. mutans* and *A. viscosus* to Tunuyán propolis samples showed that the inhibition zones were smaller for the disk method and larger for the well method (> 15 mm). Susceptibility of both bacteria to Santiago del Estero propolis samples showed no inhibition zones for the disk method and greater than 10 mm for the well method (Table 2). All of the propolis samples had higher antibacterial activity values against *S. mutans* than *A. viscosus*.

Table 2 - Antibacterial activity of Argentine Ethanol Extract Propolis. Mean and Standard Deviation (M \pm SD) (mm) of inhibition zones in agar diffusion test

EEP Samples	Propolis Antimicrobial Activity (20ug/mL)			
	Inhibition zones (mm)			
	Streptococcus mutans		Actinomyces viscosus	
	W	D	W	D
Caá Catí Corrientes	8.76 \pm 1.04	7.83 \pm 0.76	8.16 \pm 0.68	7.83 \pm 0.28
Loreto Corrientes	7.93 \pm 0.81	0	7.70 \pm 0.65	0
Bella vista Corrientes	8.60 \pm 0.36	8.16 \pm 0.90	7.76 \pm 0.92	0
Saladas Corrientes	8.80 \pm 0.26	0	8.50 \pm 0.50	0
Santiago del Estero	14.66 \pm 2.51	0	14.66 \pm 1.52	0
Tunuyán Mendoza	19.33 \pm 3.51	9.00 \pm 0.50	18.00 \pm 2.00	8.16 \pm 0.28
Total	11.25 \pm 4.68	4.16 \pm 4.32	10.90 \pm 4.21	2.66 \pm 3.88

Legend: W: agar well diffusion method, D: agar disk diffusion method, 0: no inhibition halos. Studies performed in triplicate.

The MIC and MBC from Tunuyán propolis against *S. mutans* and *A. viscosus*, determined by the agar dilution in

the tube method, are presented in Table 3. In both cases, MIC and MBC values showed differences in dilutions.

Table 3 - Antibacterial activity of EEP Tunuyan Argentine Propolis Samples. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against *S. mutans* and *A. viscosus*

N° Tubes	Concentration (mg/mL)	<i>S. mutans</i>		<i>A. viscosus</i>	
1	15	-	-	-	-
2	7.5	-	-	-	-
3	3.75	-	-	-	-
4	1.87	-	-	-	-
5	0.93	-	-	-	MBC
6	0.46	-	MBC	-	+

Legend: (-) inhibition; (+) growth.

DISCUSSION

In this research, propolis from *A. mellifera* has shown antibacterial activity against *S. mutans* and *A. viscosus* in disc and well diffusion methods, MIC, and MBC.

This result is supported by Rezende et al. (2006)⁶ and Liberio et al. (2011)²³, who found a diameter of the zone of inhibition against *S. mutans* to be greater than 10 mm.

This study is in accordance with Dziedzic et al. (2013)⁸, who verified that the growth of Gram-positive bacteria is inhibited by low propolis concentrations. Drago et al. (2000)²⁴ also observed that in low concentrations propolis shows bacteriostatic rather than bactericidal activity.

In the present study, the MIC and MBC values were at a relatively low level (Table IV). The obtained result is similar to findings from Moreno et al. (2007)²⁵, showing that EEP from Mendoza exhibits strong antibacterial activity against *S. mutans* with an MBC value of 0.46 mg/mL. Kim et al. (2011)²⁶ stated that MIC values of Korean propolis for *S. mutans* were 0.03 mg/mL, which were close to this study's values. However, it should be taken into account that the determination of MIC values depends on technical details that may vary between laboratories and the bacteria's inherent virulence and susceptibility.

The verification of the antibacterial action of the propolis extract is not surprising. The primary function of propolis in the hive is to act as a biocide, being active against invasive bacteria, fungi and even invading larvae. A number of studies have documented the biocidal functions of propolis, its extracts, and its constituents²⁷.

Although there are many bee species that can produce propolis, especially stingless bees, such as *Melipona fasciculata* and *Tetragonula carbonaria*, *A. mellifera* was chosen, since it is commonly cultured for honey. It is an easy to manage species in apiaries, and thus makes access to propolis on a commercial, as well as an environmentally sustainable, scale feasible. In addition, the bioactivities of propolis are reported to depend on geographic regions, seasons, and other external factors²⁸.

Results of this research showed that propolis had a distinct antibacterial activity according to the raw material's collection areas. Propolis composition differed between these samples and is responsible for their different antibacterial activity. Propolis chemical composition is variable depending on the region and season of collection. Consequently, the active compounds may not be present in sufficient quantities or quality²⁹.

Santiago del Estero and Mendoza EEP showed a high content of phenolic compounds, similar to those reported for propolis from Brazil and other propolis from Argentina^{21,30}. Moreover, samples from

Santiago del Estero and Mendoza showed the highest values of flavonoids. These findings show similarity with results from Lozina et al. (2010)³⁰ and Salas et al. (2014)²¹. These results revealed that the quantitative chemical composition of propolis depends on the phytogeographic region where the hives are located.

According to a wide range of reports, within the presence and concentration of phenolic compounds and flavonoids lies the reason why the molecular structure of propolis contributes to its biological properties, which act as scavengers of free radicals and inhibitors of nitric oxide and inflammatory cytokine production by macrophages and neutrophils³¹.

The antimicrobial activities observed in the present study may be a product of high phenolic compound concentration or, as reported for propolis produced by other bee species, a result of a synergistic action between flavonoids and other compounds present in these extracts.

Phenolic compounds and flavonoids have been reported to be the most abundant and most effective antioxidants in propolis^{32,33}. In this study, Santiago del Estero and Tunuyán EEP contents of phenolic compounds and flavonoids, as well as the oxidation index, were high, indicating a correlation between phenolic compound and flavonoid contents and antioxidant activity.

It is also possible to report that the extract preparation may also influence these results, although all of them were ethanolic extracts. Extraction methodology may result in ingredient preparations with different safety and efficacy profiles². In this paper, differences were observed in compositions of the EEP according to solvent concentrations.

The higher antioxidant and antibacterial activity of Tunuyán EEP, as demonstrated in the present study, was most likely due to the higher phenolic compound and flavonoid contents, as well as to the better solubility of phenol constituents in 80% ethanol. The screening of antimicrobial activity by contact bioautography, which was used for qualitative antibacterial activity detection, demonstrated that the highest number of bands with antibacterial activity was observed in the extracts obtained using 80% ethanol.

CONCLUSIONS

According to the results, it may be concluded that *S. mutans* bacteria were more susceptible to Argentine EEP antibacterial action than *A. viscosus* bacteria. The 80% EEP was more effective than 30% EEP and 50% EEP. Propolis showed a different antibacterial activity due to the geographic origin. The findings suggest that Tunuyán propolis is a very effective antibacterial agent, which may be due to high levels of phenolic and flavonoid compounds.

Therefore, the composition of propolis depends upon the vegetation of the area from which it is collected and is influenced by the solvent preparation of EEP.

RESUMEN

Objetivo: En el presente estudio, fue investigada la actividad antibacteriana de los Extractos Etanólicos de Propóleos (EEP), coleccionados de diversas regiones (Mendoza, Santiago del Estero, Corrientes) de Argentina, contra *Streptococcus mutans* ATCC® 35668™ y *Actinomyces viscosus* ATCC® 15987™ (MicroBioLogics Inc., USA.). **Métodos:** La identificación del origen geográfico y botánico se basó en el estudio de reconocimiento. El tamizaje fitoquímico de propóleos se llevó a cabo en extractos etanólicos utilizando métodos estándar para identificar los componentes (método colorimétrico de cloruro de aluminio, método colorimétrico de Folin-Ciocalteu, cromatografía en capa fina). El método de difusión en agar (discos y pocillos) y métodos de diluciones en serie (placas y tubos) se llevaron a cabo para evaluar la actividad antibacteriana de los EEP. **Resultados:** EEP ejercieron diversos grados de actividad antibacteriana contra *S. mutans* y *A. viscosus*, dependiendo de la zona geográfica de recolección de propóleos. El tamizaje fitoquímico mostró que los compuestos bioactivos corresponden a compuestos fenólicos y flavonoides. El EEP de Tunuyán (Mendoza), donde la vegetación más abundante pertenece a *Populus* sp., mostró el mayor contenido de compuestos fenólicos (220.92±2.01 mg/g) y flavonoides (30.39±0.25 mg/g) y la más importante actividad antibacteriana entre los EEP estudiados. Por el método de difusión en agar en pocillos, se apreció una alta susceptibilidad con un halo inhibitorio de 11,25 ± 4,68 mm y 10,90 ± 4,21 mm frente a *S. mutans* y *A. viscosus*, respectivamente. Se observaron valores bajos de Concentración Inhibitoria Mínima y valores mínimos de concentración bactericida contra *S. mutans* (CIM 0,05 mg/ml - CBM 0,46 mg/ml) seguido de *A. viscosus* (CIM 0,11 mg/ml - CBM 0,93 mg/ml). **Conclusiones:** Los resultados combinados de todos los métodos indicaron que *S. mutans* es más susceptible a los efectos de EEP que *A. viscosus*.

Palabras clave: Propolis. *Streptococcus mutans*. *Actinomyces viscosus*. Pruebas de sensibilidad microbiana.

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