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## RESEARCH ARTICLE

### Antibacterial Effect of Peruvian Propolis Collected During Different Seasons on the Growth of *Streptococcus Mutans*

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#### Abstract:

##### Introduction:

Propolis is a gummy, resinous substance made by bees from the buds and exudates of plants. The antibacterial activity of propolis has been widely studied and is known to vary according to its geographical origin, the type of surrounding flora, the collecting bee species, the mode of its collection and even the season in which it is collected. Unfortunately, these observations have not been corroborated experimentally.

##### Aim:

To compare the antibacterial activities of ethanolic extracts of propolis collected in the summer and autumn on the growth of *Streptococcus mutans* ATCC 25175.

##### Materials and Methods:

Propolis samples were collected in the summer and autumn and labeled “A” or “B” by an individual who was not directly involved in the study. Then, 5% ethanolic extracts of propolis were prepared for each sample. *S. mutans* was plated onto brain heart infusion agar plates into which wells were formed, and the plates were divided into four groups to test the antibacterial effectiveness of both the extracts and the positive (0.12% chlorhexidine digluconate) and negative (96% ethanol) controls.

##### Results:

Inhibition halos of 26.4±2.6 and 18.2±1.8 mm were observed for the autumn and summer propolis extracts, respectively, while those of the negative and positive controls were 0 and 13 mm, respectively. These differences were statistically analyzed using Student’s t-test.

##### Conclusion:

The significantly higher growth of *S. mutans* in the extracts made from propolis collected in autumn than that grown on extracts collected in summer indicates that the season in which propolis is collected does indeed influence its antibacterial activity.

**Keywords:** Peruvian propolis, Seasons, *S. mutans*, Antibacterial effectiveness, β-glucosidase, Polyethylene bags.

#### Article History

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## 1. INTRODUCTION

Propolis is a gummy, resinous substance made by bees from the buds and exudates of plants as well as β-glucosidase, which is secreted from the glands and hypopharynx of bees.

The word propolis is derived from the Greek word “pro”, meaning “defense”, and “polis”, meaning “community” or “city”, which altogether means “defending the city” (in reference to the hive), as bees use this product to seal cracks and holes in the hive walls and to embalm invading insects for self-protection [1 - 5].

In general, propolis is composed of resins, waxes, polyphenols, polysaccharides, volatile materials, and other

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substances. Since antiquity, this product has been known to possess various biological and pharmacological activities, such as antiviral, antifungal, antioxidant, anti-inflammatory, anti-tumor, immunomodulatory and, especially, antibacterial properties. These properties are directly related to the organic compounds present in propolis, primarily polyphenols, such as flavonoids, as well as terpenoids, steroids, naphthalene, stilbene derivatives, and fatty acids [6 - 12].

Research on propolis has primarily focused on its antibacterial activity [13 - 28] because it inhibits gram-positive bacteria. Interestingly, the antibacterial activity of propolis is known to vary according to the purification technique, the type and concentration of solvent used for its purification, its geographical origin, type of surrounding flora, the collecting bee species, the collection method used, and the season and even the site of its collection. However, these observations have not been corroborated experimentally by studies that have exclusively focused on evaluating these variables [4]. Thus, the objective of this study was to compare the antibacterial activity of ethanolic extracts of propolis collected in Santiago de Chuco in the summer and autumn on the growth of *Streptococcus mutans* ATCC 2517.

## 2. METHODOLOGY

### 2.1. Study Design

This study had an *in-vitro* experimental, prospective, cross-sectional and analytical study design.

### 2.2. Strains and Sample Size

#### 2.2.1. Population

The bacterium used in this study was *S. mutans* (ATCC 25175).

#### 2.2.2. Sample

The statistically determined sample size was a total of 6 repetitions divided in two for each Petri dish for each experimental group.

The technique used was microbiological observation, and microbiological methods were used.

### 2.3. Collection of Propolis

The protocol published by Quintero *et al.* [28], was followed with some modifications. Propolis was directly obtained from beekeepers in Santiago de Chuco with a plastic spatula during two different seasons (summer and autumn). The samples were placed directly in tapers made of nontoxic and transparent glass, after which they were covered with dark polyethylene bags, sealed, and transported to the Biochemistry laboratory of the School of Pharmacy of the Universidad Nacional de Trujillo for processing.

### 2.4. Obtaining the Extract

To obtain the propolis extract, the protocol published by Tolosa [29, 30] was followed with some modifications.

Crude propolis (10 g) was mixed with 100 ml of 96%

ethanol to yield a homogeneous mixture.

#### 2.4.1. Vacuum Filtration

Samples were prepared for the hot vacuum filtration process using the following materials: a funnel; filter paper; a distillation flask for each propolis sample that had been previously washed, disinfected, and labeled with the letter "A" or "B"; and a membrane pump connected to a glass device that joined the funnel and the flask to prevent the entry of air or the escape of substances.

#### 2.4.2. Determination of the Concentrations of Propolis in the Ethanolic Extracts

- All samples were removed from refrigerators and allowed to reach room temperature.
- Previously described solubility tests were performed using test tubes with different equivalent amounts of ethanolic extract of propolis and ethanol (100 ml) to determine the maximum solubility (%) of the ethanolic extract of propolis.
- The maximum solubility of propolis was the concentration at which it was subsequently used.

#### 2.4.3. Antibacterial Effect Against *S. mutans*

The protocol provided by the ATCC was followed with some modifications.

- *S. mutans* ATCC 250175 was obtained from the ATCC, stored at -80°C, and reactivated when cultured at 37°C
- *Streptococcus mutans* ATCC 25175 was incubated at 37°C for 24 hours in 2 tubes containing 6 ml of brain heart infusion (BHI) broth. The cultures were subsequently centrifuged at 2500 rpm for 8 minutes and decanted, and then the cell pellet was resuspended in BHI broth to an optical density of 0.270.
- Bovine Hydroxyapatite (BHA) was prepared, distributed in 12 Petri dishes, allowed to solidify and then seeded with the bacterial dilution. Subsequently, wells were punched in the plates, and 100  $\mu$ l of each propolis extract was added, with each assay performed in duplicate.
- The negative control was 96% ethanol, and the positive control was chlorhexidine digluconate.

The Petri dishes were incubated for 24 hours at 37°C in a microaerophilic environment, after which inhibition zone measurements were performed.

## 3. RESULTS

For the propolis extracts from different seasons, average inhibition halos of 18.15 and 26.4 mm were observed for extracts of propolis obtained in summer and autumn, respectively. Using Student's t-test to assess the difference between these values, a *p*-value of 6.427 was obtained with a significance of  $0.000 < (0.05)$ , showing that the inhibition halo diameters observed for the two extracts were significantly different.

The inhibition halos of the extract of propolis collected in the summer and autumn were  $18.2 \pm 1.8$  and  $26.4 \pm 2.6$  mm,

respectively, with no effect observed for the negative control (96% ethanol). Differences between the efficacy of the extracts were assessed by ANOVA ( $p=0.000<0.05$ ), and Dunnett's test results indicated a superior effect of both extracts compared to the negative control (96% ethanol).

**Table 1. *In vitro* comparison of the effect of the ethanolic extracts of propolis collected in Santiago de Chuco in the summer and autumn on the growth of *Streptococcus mutans* ATCC 25175.**

-	Inhibition Halo (mm)	
	Summer	Autumn
Mean	18.15	26.4
S.D.	1.80	2.58
Student's t-test	6.427	
Significance ( $p$ )	0.000	

Source: Data provided by the author.

**Table 2. *In vitro* comparison of the effect of ethanolic extracts of propolis collected in Santiago de Chuco in the summer and autumn versus 96% ethanol on the growth of *Streptococcus mutans* ATCC 25175.**

-	Inhibition Halo (mm)		
	Summer	Autumn	Negative Control (96% Ethanol)
Mean	18.2	26.4	0.0
SD	1.8	2.6	0.0
ANOVA: F	332.09		
$p$	0.000		
Dunnett's	a	a	b

Source: Data provided by the author.

**Table 3. *In vitro* comparison of the effect of the ethanolic extracts of propolis collected in Santiago de Chuco in the summer and autumn versus 0.12% chlorhexidine digluconate on the growth of *Streptococcus mutans* ATCC 25175.**

-	Inhibition Halo (mm)		
	Summer	Autumn	Positive Control (0.12% Chlorhexidine Digluconate)
Mean	18.2	26.4	13.0
SE	1.8	2.6	0.0
ANOVA: F	83.18		
$p$	0.000		
Dunnett's	a	a	b

Source: Data provided by the author.

The inhibition halos of the extracts of propolis collected in the summer and autumn were  $18.2\pm 1.8$  and  $26.4\pm 2.6$  mm, respectively, whereas that of the positive control (chlorhexidine gluconate) was 13 mm. Differences between the efficacy of the extracts were assessed by ANOVA ( $p=0.000<0.05$ ), and Dunnett's test and the results indicated that both extracts were more effective than the positive control (chlorhexidine gluconate).

#### 4. DISCUSSION

Giralt [10] suggested that the biological activity of propolis is highest during the autumn because the rainy season yields a variety of plant sources and the number of plant pests is still low, and these factors positively modify the chemical composition of propolis. Additionally, the lower temperatures favor the transportation of waxes to the hive. This hypothesis agrees with the results of this study, as the extract of propolis collected in autumn showed a greater antibacterial effect than that of propolis collected in the summer. This result is also in agreement with the results of a study by Samara *et al.* [6], who observed that propolis had antibacterial effects at low temperatures (23 and 14°C). However, in our study, the greatest effect was observed at a high temperature (23°C), which could possibly indicate that extremely low temperatures are not required to obtain an adequate antibacterial effect.

Giralt [10] also noted that there is a higher production of propolis in autumn, leading to a higher concentration of metabolites such as polyphenols and flavonoids that produce the antimicrobial effect. This hypothesis agrees with the results of a study by Veloz *et al.* [5], who investigated whether the collection year influences the antibacterial activity of propolis against *S. mutans*. In their study, propolis samples were collected during the same season in 2008, 2010 and 2011. Their results showed a higher concentration of total polyphenols, flavones and flavonols for the year 2010. Although no significant difference was observed in the inhibition of *S. mutans*, the propolis collected in 2010 showed a greater ability to inhibit biofilm formation, demonstrating that the one-year difference between the samples allowed a greater amount of sample to be collected for the year 2010, which consequently yielded a greater concentration of metabolites. The observations from this study are consistent with their results, as the largest amount of propolis was observed in autumn. However, this result contradicts the results of a study by Manrique and Egea [17], who observed that there is a higher production of propolis in summer than autumn because summer is when bees show a preference for collection, as the warm weather promotes the growth of plant species.

Additionally, in this study, favorable results were observed for both types of propolis obtained in the Andean region of Peru with respect to the growth inhibition of *S. mutans*, as the 5% extracts collected in summer and autumn produced inhibition halos of  $18.2\pm 1.8$  and  $26.4\pm 2.6$  mm, respectively. However, better results were obtained by Jara [7] using an extract of propolis collected in Oxapampa, which yielded an inhibition halo of 33.15 mm against *S. mutans*, demonstrating that the location from which the propolis is collected may have an effect as well as the concentration used. This phenomenon was also observed by Huayhua *et al.* [8], who compared the effects of propolis extracts and showed greater antimicrobial effects at greater concentrations, similar to the results of Ramirez *et al.* [9]. However, the opposite results were described by Eguizábal *et al.* [14], who showed that a lower concentration of propolis had a greater antibacterial effect. These findings may reflect the relationship between the effective concentration and the location of propolis collection because the effectiveness of the concentration may depend on the collection site.

In this study, the antibacterial effect of extracts of propolis collected in the summer and autumn was compared with that of 0.12% chlorhexidine digluconate. Both summer and autumn extracts showed a better result than the positive control, with 0.12% chlorhexidine digluconate producing an inhibition halo that was only 13±1.3 mm in diameter. This result agrees with the findings of Eguizábal *et al.* [15] and Veloz *et al.* [5], who also observed that propolis extracts exerted a better inhibitory effect than chlorhexidine digluconate, demonstrating that the components of propolis have a better antibacterial effect than the components of 0.12% chlorhexidine digluconate.

## CONCLUSION

The ethanolic extract of propolis from autumn showed a greater antibacterial effect toward *Streptococcus mutans* ATCC 25175 than the ethanolic extract of propolis from summer. The diversity of flora is dependent on climate change, temperature, humidity, soil type, and location, as demonstrated in this study.

## ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available in the [Los Angeles de Chimbote Catholic University] at [<http://repositorio.uladech.edu.pe/handle/123456789/5028>],

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None.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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