Article published online: 2021-11-01

Original Article

Antibacterial Activity of Ethanol Extract of the Leaves of *Rosmarinus officinalis* L. at Different Concentrations versus *Streptococcus mutans*: An *In vitro* Comparative Study

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Abstract

Aim: The study aimed to compare the *in vitro* antibacterial activity of an ethanol extract of *Rosmarinus officinalis* "romero" (EERO) at different concentrations with *Streptococcus mutans* ATCC 25175. **Materials and Methods:** We worked with three concentrations of 25%, 50%, and 75% of the EERO and compared these with the positive control chlorhexidine 0.12% and distilled water as a negative control. Seeding was performed in Müller–Hinton agar medium. The inhibitory effectiveness of EERO was determined using the disc diffusion method with the experimental solutions. The seeded and inoculated plates were incubated at 37°C, for 24 and 48 h. The reading was carried out according to the Kirby–Bauer method by measuring the diameter (mm) of the inhibition halo formed by the bacteria using a Vernier caliper and recorded on a data collection sheet. **Results:** In the 24-h group, chlorhexidine 0.12% showed the highest inhibition halos with a mean of 12.8 ± 0.8 mm, followed by EERO 75% with a similar mean of 12.1 ± 0.6 mm. Similarly, at 48 h, chlorhexidine 0.12% had the highest antibacterial activity of 13.0 ± 0.7 mm followed by EERO 75% with 12.2 ± 0.5 mm. **Conclusions:** The EERO showed inhibitory effectiveness against *S. mutans*, with the concentration of 75 mg/ml showing similar results, albeit not statistically significantly different, to those of chlorhexidine.

Keywords: Antibacterial activity, *in vitro*, *Romero*, *Rosmarinus officinalis*

Introduction

Diverse microorganisms inhabit the oral cavity, of which many are involved in the beginning and progression of dental caries and other pathologies at an oral level. Among these, the family of the genus *Streptococcus* is the most relevant in the process of dental caries. On the other hand, *Enterococcus faecalis* is especially frequent in infections of dental origin, being considered one of the main pathogens in dentistry.[1] These microorganisms are usually complex microorganisms and have not yet been fully investigated. The oral cavity is characterized by containing different structures in which microorganisms can replicate. Each part in the mouth contains a specific characteristic biofilm, involving many different bacterial species which interact dynamically leading to changes in the flora of the oral cavity and in the life of the host.[1-3]

The prolonged use of antimicrobials and antiseptics can lead to adverse reactions at the oral level, such as pigmentation of the teeth and alterations of taste, and, therefore, new treatment alternatives without adverse effects and that are adjuvants to tooth brushing are being investigated for the treatment of bacterial plaque removal.[3-6]

In the area of phytotherapy, plants such as rosemary are rich in active principles and have multiple actions on the human organism. Rosemary is grown in our country and in the last decade, studies on the properties of this plant have been developed in order to provide information on its therapeutic potential,[7-9] *Rosmarinus officinalis* has some applications in

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Quick Response Code:

Website: www.ejgd.org

DOI: 10.4103/ejgd.ejgd_148_20

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How to cite this article: Janampa E, Perez H, Mauricio F, Alvítez-Temoche D, Mauricio-Vilchez C, Gallo W, Mayta-Tovalino F. Antibacterial activity of ethanol extract of the leaves of *Rosmarinus officinalis* L. at different concentrations versus *Streptococcus mutans*: An *In vitro* comparative study. Eur J Gen Dent 2020;9:152-6.

Submitted: 04-May-2020 **Revised:** 30-Jun-2020
Accepted: 06-Jul-2020 **Published:** 15-Sep-2020

dentistry especially in relation to disease prevention, is being used in dental hygiene, and is used as an anti-inflammatory medication. However, to date, there has been little research into the antibacterial action of this herb.[10-13]

Therefore, the objective of this study was to compare the *in vitro* antibacterial activity of an ethanol extract of *R. officinalis* (EERO) at different concentrations with that of *Streptococcus mutans* ATCC 25175. The hypothesis of this research was to demonstrate that this ethanol extract has effectiveness similar to that of chlorhexidine.

Materials and Methods

Study design

This research was an experimental, longitudinal, and comparative study. It was carried out in the Faculty of Dentistry of the Universidad Nacional Federico Villarreal and in the Faculty of Pharmacy and Biochemistry of the Universidad Nacional Mayor de San Marcos (UNMSM), Lima, Peru.

Sampling method

The sample size was calculated using the mean comparison formula with Stata® 15 software (Texas, USA). A sample of 60 wells inoculated with the experimental substances divided into four groups of 15 discs each was used.

Group distribution

- Group 1: *S. mutans* versus EERO 25%
- Group 2: *S. mutans* versus EERO 50%
- Group 3: *S. mutans* versus EERO 75%
- Group 4: *S. mutans* versus chlorhexidine 0.12%.

Sample preparation

Two kilograms of *R. officinalis* was collected and taken to the Analytical Control Center (CCA) of the Faculty of Pharmacy and Biochemistry of the UNMSM [Figure 1], where the ethanolic extract was processed and three concentrations at 25%, 50%, and 75% were obtained [Figure 2]. Then, the extract was stored in a sterile amber bottle and kept refrigerated. To evaluate the antibacterial activity, biological material and the strain of *S. mutans* ATCC 25175 were used. Seeding was carried out in Müller–Hinton agar culture medium in Petri dishes, and four wells were prepared: three for the concentrations of the extract and one for 0.12% chlorhexidine which was the positive control. The base of each plate was marked in order to differentiate the type of solution and position of each well, and each Petri dish was labeled. Then, 50 µl of each experimental solution was placed on the surface of the medium in each well and allowed to stand for 30 min before incubation. The Petri dishes were then placed inside the incubator at 37°C for 24 and 48 h, and the inhibition halo measurements were read with the help of a King Foot or caliper [Figure 3].

Statistical analysis

For descriptive analysis, we used means, standard deviations, and maximum and minimum values of the variable antibacterial activity. Next, normality was calculated using the Shapiro–Wilk test. For inferential analysis, the Student's *t*-test and the Bonferroni's *post hoc* test were used. A level of significance of $P < 0.05$ was established, and the analyses were performed using the Stata® 15 Software.

Figure 1: Obtaining the leaves of *Rosmarinus officinalis*

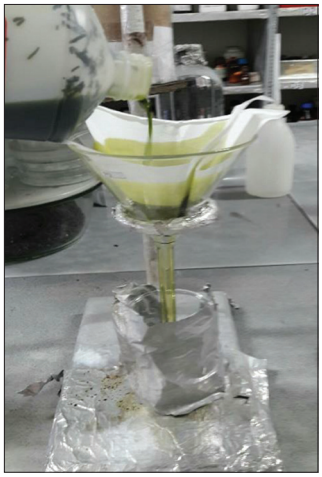


Figure 2: Preparation of ethanol extract of *Rosmarinus officinalis*

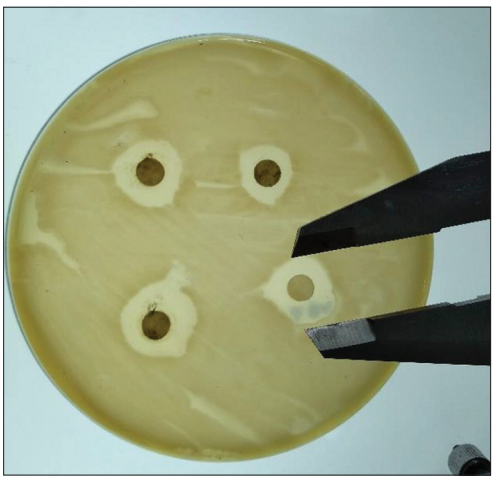


Figure 3: Measurement of inhibition halos

Results

We analyzed sixty discs inoculated with the experimental substances distributed into four groups (n = 15 each). No specimen was lost during execution. Table 1 shows that all the groups presented a normal distribution at both 24 h and 48 h ($P > 0.05$). In the 24-h group, the highest inhibition halos were achieved with chlorhexidine 0.12% with a mean of 12.8 ± 0.8 mm followed by EERO 75% with a similar mean of 12.1 ± 0.6 mm. Similarly, at 48 h, chlorhexidine 0.12% showed the highest antibacterial activity with 13.0 ± 0.7 mm followed by EERO 75% with 12.2 ± 0.5 mm [Graph 1].

When comparing the antibacterial activity of EERO according to time, Table 2 shows that statistically significant differences were only found with EERO 50% at 24 h and 48 h ($P = 0.027$). While EERO 25%, EERO 75% did not show a significant differences in inhibition halos at 24 h and 48 h ($P = 0.071$ and $P = 0.276$, respectively).

On *post hoc* analysis to determine the group which had the highest antibacterial activity, statistically significant differences were found between EERO 25% and EERO 75% groups at 24 h and 48 h ($P = 0.007$ at 24 h and $P = 0.006$ at 48 h) [Table 3].

Table 1: Evaluation of the antibacterial activity of the different concentrations of the ethanol extract of *Rosmarinus officinalis* according to time

Time	Groups	Mean	SD	Minimum	Maximum	P*
24 h	EERO 25%	10.8	0.8	9.8	12.5	>0.05
	EERO 50%	11.2	0.8	10.0	12.4	
	EERO 75%	12.1	0.6	11.5	13.1	
	CHX 0.12%	12.8	0.8	11.0	14.0	
48 h	EERO 25%	10.9	0.8	10.0	12.5	>0.05
	EERO 50%	11.3	0.8	10.0	13.0	
	EERO 75%	12.2	0.5	11.5	13.0	
	CHX 0.12%	13.0	0.7	11.5	14.0	

All groups were measured (mm). *Shapiro-Wilk test, $P>0.05$: All groups presented normality. EERO – Ethanol extract of *Rosmarinus officinalis L*., CHX – Chlorhexidine, SD – Standard deviation

Table 2: Comparison of the antibacterial activity of the ethanol extract of *Rosmarinus officinalis* according to the incubation time

Group	Time (h)	Mean	SD	P**
EERO 25%	24	10.8	0.8	0.071
	48	10.9	0.8	
EERO 50%	24	11.2	0.8	0.027**
	48	11.3	0.8	
EERO 75%	24	12.1	0.6	0.276
	48	12.2	0.5	
CHX 0.12%	24	12.8	0.8	0.063
	48	13	0.7	

All groups were measured (mm). **Student's *t*-test, $P<0.05$; statistically significant. EERO – Ethanol extract of *Rosmarinus officinalis L*., CHX – Chlorhexidine, SD – Standard deviation

Table 3: *Post hoc* analysis of the antibacterial activity of ethanol extract of *Rosmarinus officinalis* versus *Streptococcus mutans* ATCC 25175 among the study groups at 24 h and 48 h

Time	Group	EERO 25%	EERO 50%	EERO 75%	CHX 0.12%
24 h	EERO 25%	-	1.000	0.007*	<0.001
	EERO 50%	-	-	0.076	<0.001
	EERO 75%	-	-	-	0.590
	CHX 0.12%	-	-	-	-
48 h	EERO 25%	-	1.000	0.006*	<0.001
	EERO 50%	-	-	0.143	<0.001
	EERO 75%	-	-	-	0.444
	CHX 0.12%	-	-	-	-

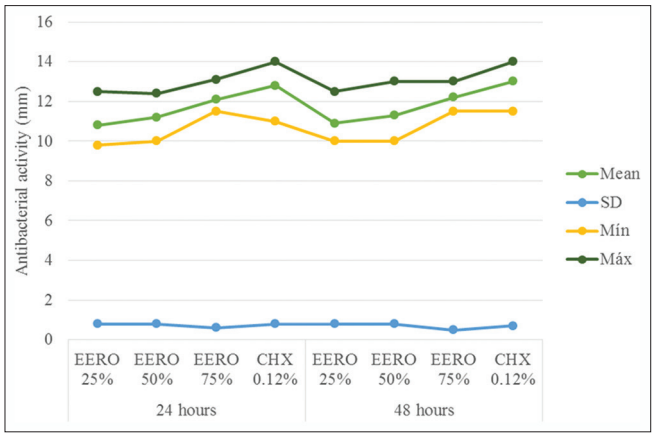
All groups were measured (mm). *Bonferroni *post hoc* test. $P<0.05$; statistically significant. EERO: Ethanol extract of *Rosmarinus officinalis L*., CHX – Chlorhexidine

Discussion

Medicinal plants play an increasingly important role as an alternative in dental treatments due to their antibacterial properties against microorganisms, causing disease at an oral level. To verify the antibacterial activity of medicinal plants,[14-21] disc diffusion agar methods have been used, which consist of inoculating the agar in a 5-mm well with experimental solution followed by 24 h and 48 h of incubation. Following incubation, bacterial activity is demonstrated by the formation of inhibitory halos.

The study by Silva *et al*.[1] evaluated the effectiveness of different concentrations of essential oils combined with calcium hydroxide against *E. faecalis*. These authors performed microdilution tests to define the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) and found that the essential oil made of *R. officinalis* showed an antimicrobial effect against this bacterium. On the other hand, the results of the study by Elmi *et al*.[5] showed that at a concentration of 0.4 mg/ml, the essential oils of both *Melaleuca alternifolia* and *R. officinalis* had antimicrobial activity similar to that of the antibiotics used as a control. These findings strengthen the hypothesis of the potential use of this natural resource as a potential antimicrobial agent for reproductive biotechnologies applied to dentistry.

Similarly, Amaral *et al*.[6] demonstrated that the MICs of *R. officinalis L*. were effective against standard strains of harmful Gram-positive and Gram-negative bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, and Bacillus cereus*). Another study showing similar antimicrobial results to those of the present study was that of Abdallah *et al*.[11] who described that the antibacterial activity of *R. officinalis L*. extract against the strain of *Xanthomonas oryzae pv. Oryzae* may be attributed to its inhibition of bacterial growth, biofilm formation, as well as the destruction of its cell wall.



Graph 1: Evaluation of antibacterial activity through inhibitory halos

Another study which showed similar results to those of our study was carried out by Zairi *et al*.[14] who evaluated the antimicrobial properties of *Rosmarinus officinalis*, *Thymus algeriensis,* and *Thymus capitatus,* which are widely used in traditional medicine in Tunisia. They described that the bioactivity of these plants is related to their essential oils and revealed that aqueous extracts made from these plants are not toxic compared to methanolic extracts. This study also reported the important bioactivities (antioxidant, antimicrobial, and safety potential) of these plants in traditional medicine.

For all of the above, there is currently a great interest in determining the medicinal potential that extracts from natural resources such as rosemary may have. Different studies have reported that the extracts of this plant have hepatoprotective, antifungal, insecticide, antioxidant, and antibacterial biological bioactivities. These properties are mainly due to phenolic compounds. However, there are multiple effects that can modify this activity.[1,5,6,11,13]

At present, oral bacterial infections are frequent, including tooth decay caused by certain microorganisms of the oral cavity. The results of our study on the antibacterial activity of *R. officinalis* provide important data related to the prevention of tooth decay, which is essential for dental professionals, teachers, and students, thus improving public health. In the current practice, different brands of mouthwashes are used as adjuvants to oral hygiene, and it is necessary to know the advantages and disadvantages of their use. In the present study, the antibacterial activity of *R. officinalis* compared to that of *S. mutans* was positive against this bacterium, demonstrating that *R. officinalis* can be used as a treatment to replace commercial mouthwashes. Furthermore, the cost of rosemary is low and is of easy access to the population and does not present adverse effects compared to chlorhexidine in relation to tooth staining and mucous sensitivity to taste.

The main limitations of this study were that the strain of *S. mutans* had to be imported from another country due to limited availability in Peru. Another limitation was that only the ethanolic extract was evaluated, and it is necessary to determine whether other types of extracts could increase or decrease the antimicrobial effect of this plant. Despite these limitations, the study is relevant to Peruvian dentistry. However, further studies on *R. officinalis* are needed to evaluate its effects on bacterial strains that interact at the level of the oral cavity and produce pathologies. In addition, studies are needed to determine the MICs and MBCs of the EERO. Finally, toothpastes and dental mouthwashes containing EERO should be studied to determine whether the effectiveness of this extract is maintained in these processed products.

Conclusions

According to the results of the study:

1. The EERO presented antibacterial activity against *S. mutans* ATC25175
2. Increasing the concentration of EERO increases the size of the inhibition halo; however, the action by chlorhexidine 0.12% is not exceeded
3. The inhibitory activity of EERO at 75 mg/ml had the greatest antibacterial effect and was similar to that of chlorhexidine 0.12%.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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