Antibacterial Activity of *Bixa orellana* Compared with *Camellia sinensis* Against *Streptococcus mutans*: An *In Vitro* Comparative Study

Lindsay Granados-Jamanca[1], Sara Mercedes Medrano-Colmenares[2], Eloy Gamboa-Alvarado[1], Marysela Ladera-Castañeda[2], Luz Castañeda-Pérez[2], Luis Adolfo Cervantes-Ganoza[3], Alberto Cornejo-Pinto[2], César Félix Cayo-Rojas[1]

[1]Universidad Privada San Juan Bautista, School of Stomatology, [2]Universidad Nacional Federico Villarreal, Postgraduate School, "Grupo de Investigación Salud y Bienestar Global" and Faculty of Dentistry, [3]Universidad Inca Garcilaso de la Vega, Faculty of Stomatology, Lima, Peru

Abstract

Aim: *Bixa orellana* and *Camellia sinensis* are plant species cultivated in several South American countries such as Peru and used to combat diseases due to their antimicrobial properties. The aim of this study was to assess the antibacterial activity of the methanolic extract of *B. orellana* compared with the ethanolic extract of *C. sinensis* against *Streptococcus mutans* at 24, 48, and 72 h. **Materials and Methods:** This *in vitro* and longitudinal experimental study had a sample of 12 wells per group. The antibacterial activity was assessed at concentrations of 1000 mg/mL (100%), 750 mg/mL (75%), and 500 mg/mL (50%), by the well diffusion method on Müller Hinton Agar in two stages. In first stage, antibacterial activity of ethanolic extract of *C. sinensis* and methanolic extract of *B. orellana* was determined. In second stage, concentrations of both extracts with higher antibacterial activity were compared using 0.12% chlorhexidine as a control. In addition, antibacterial sensitivity was assessed according to Duraffourd's scale and the minimum inhibitory and bactericidal concentration (MIC and MBC) was determined. Statistical analysis was performed using Kruskall Wallis test and ANOVA test of one factor inter-group and intra-group with Tukey and Bonferroni post hoc, considering a significance level of 5%. **Results:** In first stage, ethanolic extract of *C. sinensis* (100% and 75%) and methanolic extract of *B. orellana* (100% and 75%) showed higher antibacterial activity against *S. mutans* at 48 h ($P < 0.001$ and $P < 0.05$, respectively). In second stage, at 48 h, highly sensitive activity was observed against *C. sinensis* (100% and 75%) and *B. orellana* at 100%. In addition, *C. sinensis* at 100% and 75% showed significantly higher antibacterial activity against *S. mutans* compared with *B. orellana* ($P < 0.05$) and chlorhexidine ($P < 0.05$). Likewise, a significant increase in antibacterial activity could be observed in all concentrations at 48 h ($P < 0.001$), decreasing significantly in all groups at 72 h ($P < 0.001$). The MIC of the ethanolic extract of *C. sinensis* was 250 mg/mL and the MBC was 500 mg/mL. In the methanolic extract of *B. orellana* the MIC was 125 mg/mL and the MBC was 500 mg/mL. **Conclusion:** Ethanolic extract of *C. sinensis* and methanolic extract of *B. orellana*, both at 100% concentration, presented their highest antibacterial activity against *S. mutans* at 48 h, with *C. sinensis* more effective compared with *B. orellana*. However, this antibacterial effect decreased in both extracts at 72 h. The MBC of *C. sinensis* and *B. orellana* against *S. mutans* was 500 mg/mL for both extracts, whereas the MIC was 250 mg/mL and 125 mg/mL, respectively, for both extracts.

Keywords: *Bixa orellana*, *Camellia sinensis*, Chlorhexidine, Inhibition Halos, Microbial Sensitivity Test, Minimum Bactericidal Concentration, Minimum Inhibitory Concentration, *Streptococcus mutans*

Received: 13-10-2022, **Revised:** 27-11-2022, **Accepted:** 03-12-2022, **Published:** 28-04-2023.

Introduction

Dental caries is an infectious disease of multifactorial origin resulting from a dysbiosis between bacteria that inhabit the oral cavity, giving rise to a predominance of

Access this article online

Quick Response Code:

Website: www.jioh.org

DOI: 10.4103/jioh.jioh_212_22

Address for correspondence: Dr. César Félix Cayo-Rojas, Universidad Privada San Juan Bautista, School of Stomatology, Av. Jose Antonio Lavalle Avenue No. 302–304 (Ex Hacienda Villa), Chorrillos, Lima, Peru.
E-mail: cesar.cayo@upsjb.edu.pe

How to cite this article: Granados-Jamanca L, Medrano-Colmenares SM, Gamboa-Alvarado E, Ladera-Castañeda M, Castañeda-Pérez L, Cervantes-Ganoza LA, *et al.* Antibacterial activity of *Bixa orellana* compared with *Camellia sinensis* against *Streptococcus mutans*: An *in vitro* comparative study. J Int Oral Health 2023;15:174-83.

more cariogenic microorganisms, mainly *Streptococcus mutans*.[1] This bacterium is a gram-positive coccus with cariogenic potential mainly due to its ability to synthesize large amounts of extracellular glucan polymers from sucrose, in addition to being able to transport and metabolize carbohydrates into organic acids, and to develop in low pH environmental conditions.[2,3]

Dental caries has high prevalence in Latin America and the Caribbean, affecting primary and permanent dentition, in addition to causing enamel and dentin demineralization that destroys the dental organ.[4] Many people's physical and emotional health is affected by this disease, which can generate from acute pain to definitive tooth loss.[5] Thorough hygiene, a balanced diet low in sugars, as well as regular visits to the dentist can help prevent and minimize the prevalence of this disease, since procedures to prevent tooth decay such as the application of fluoride varnish, silver diamine fluoride, application of sealants, among others, are becoming more and more common.[6]

While it is true that the population has different types of treatments to combat certain diseases, more and more people are relying on the phytotherapeutic properties of various plant species existing in their geographical environment. In developing countries, such as those in South America, where economic, cultural and social inequality prevails, there are native communities, peasants, and villagers who use natural resources as traditional medicine to combat various diseases, mainly infections. In the absence of medical personnel, the population places their health in the hands of healers, who have learned empirically how to combat illnesses naturally, as they have become experts in the use of traditional medicine.[7,8]

It is well known that many drugs have been elaborated from active principles extracted from plants. For this reason, the study of alternative and complementary medicine has been developing in a superlative way.[9] In addition, it has been reported that antibiotic resistance of some pathogenic microorganisms can be prevented by using new compounds that are not based on the same synthetic antimicrobial agents that already exist, but rather by using different plant species that provide a new antibiotic source.[10,11]

In a large part of South America, including Peru, there is wide diversity of plant species from which people have been extracting the maximum medicinal benefit, as in the case of *Bixa orellana*, which has antimicrobial, antioxidant, antidiabetic, anticonvulsant and cardioprotective activity.[12] It is also known that the methanolic extract of its leaves has antimicrobial activity against Gram-positive microorganisms and fungi such as *Candida albicans*.[13,14] Medina *et al*. reported the antibacterial effect of the methanolic extract of *B. Orellana*, using methanol to extract phenols and flavonoids from the plant.[15] In addition, Alim *et al*.[16] reported that the alkaloids, phenols, triterpenoids, glycosides and tannins of *B. orellana* presented good antimicrobial potential to inhibit oral microorganisms.

Another plant species that has gained importance in pharmacological field due to its properties linked to a lower incidence of oral cancer, dental caries, stroke, cardiovascular diseases and obesity is *Camellia sinensis*.[17-19] Its properties would be due to high concentrations of polyphenols such as epicatechin, epicatechin gallate and epigallocatechin,[20] which would inhibit bacterial growth of *Helicobacter pylori, Staphylococcus aureus, S. mutans, Streptococcus sobrinus, Salmonella typhi*, and *Shigella*.[21] These polyphenolic compounds also decrease acid production by Streptococcus and their ability to synthesize adherent glucan.[22,23] One study reported that the set of polyphenolic compounds from *C. sinensis*, called Sunphenon, has effects on *S. mutans* causing decreased cell viability, reduced cellular binding of the microorganism to the hydroxyapatite surface and suggested that a diet supplemented with Sunphenon would contribute to fewer carious lesions.[24] Thus, it became evident that *C. sinensis* polyphenols could be used as a slow-release source of catechins active against Streptococcus growth, and may also inhibit the preliminary adherence of *S. mutans* to the tooth surface.[17]

Based on these scientific bases, it is important to assess the antimicrobial activity of *B. orellana* and *C. sinensis* against *S. mutans* and thus discover which natural product is more effective against this oral pathogen in order to provide an alternative in dentistry for dental caries prevention and treatment, laying the groundwork for future randomized clinical trials such as mouthwashes or toothpastes based on these natural products. Therefore, aim of this study was to assess the antibacterial activity of methanolic extract of *B. orellana* compared with ethanolic extract of *C. sinensis* against *S. mutans* at 24, 48, and 72 h. The null hypothesis considered was that there are no significant differences when comparing the antibacterial activity of ethanolic extract of *C. sinensis* and methanolic extract of *B. orellana* against *S. mutans* at different times.

Materials and Methods

Type and delimitation of study

This *in vitro*, longitudinal and analytical experimental study was approved by an Institutional Research Ethics Committee of the Universidad Privada San Juan Bautista (UPSJB) in Peru, with resolution No. 1200-2021-CIEI-UPSJB, performing the experimental part in the Microbiology laboratory of the School of Stomatology UPSJB between February and March 2022.

Collection and processing of extracts

In the present experimental study, all methods were carried out in accordance with the relevant guidelines set

out in the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. Plants with leaves of *B. orellana* (1 kg) and with leaves of *C. sinensis* (1 kg) were collected and their taxonomic recognition was carried out in the herbarium of Natural History Museum of the Universidad Nacional Mayor de San Marcos (No. 17-USM-2021 and No. 18-USM-2021, respectively). Afterwards, leaves were selected from both plants and dried in an oven for 24 h at 60°C. Then, both samples were ground and placed in hermetic amber glass containers of 4 liters of capacity. Next, 1 liter of 96% ethyl alcohol was added to the prepared *C. sinensis* sample and 1 liter of absolute methanol (1:2, w/v) to the *B. orellana* sample until the product was completely covered. Both containers were shaken 3 times per 24 h, with a cold alcoholic maceration time of 7 days. After evaporation of both solvents, extracts were obtained at concentrations of 1000 mg/mL for each sample. To assess the MIC and MBC of methanolic extract of *B. orellana* and ethanolic extract of *C. sinensis*, serial dilutions of factor 2, from 1:2 to 1:128, were performed.

Procedure to assess antibacterial sensitivity

The *S. mutans* strain (ATCC 25175) was reactivated in TSA Agar contained in 94 x 16 mm Petri dishes, in controlled anaerobiosis, at 37°C and for 72 h according to the manufacturer's specifications. Subsequently, a hoe of bacterial colonies was isolated and used for assay standardization by preparing an inoculum at 0.5 on the McFarland scale. The bacterial suspension was homogeneously seeded in all the Petri dishes containing Mueller Hinton Agar previously prepared and to which 05 perforations of 6 mm in diameter were made with the help of a punch to assess antibacterial sensitivity of the extracts using the well technique. In each well 100 µl of methanolic extract of *B. orellana* or ethanolic extract of *C. sinensis* were added at concentrations of 1000 mg/mL (100%), 750 mg/mL (75%) and 500 mg/mL (50%), 0.12% Chlorhexidine as positive control and distilled water as negative control, according to established experimental study groups. The whole procedure was performed under a type II biosafety hood to avoid environmental and cross contamination. The plates were incubated in controlled anaerobiosis at 37°C for 24, 48, and 72 h. After each incubation time, diameters of the inhibition halos were measured in millimeters (mm) with the help of a Vernier digital caliper (Vogel, Ossenpadd, Kevelaer, Germany) and recorded in a Microsoft Excel 2019 spreadsheet.

To reduce measurement bias as much as possible, the double-blind technique was used (both the person who measured the inhibition halos and the person who performed the statistical analysis were unaware of the group assignment according to product used). On the other hand, a measurement calibration of the inhibitory halos was made to the principal investigator, both intra-examiner (LG) and inter-examiner (LG and EG) and a Pearson's R correlation coefficient of 0.91 (CI: 0.84–0.98) and 0.84 (CI: 0.79–0.89) was respectively obtained, demonstrating very good concordance.

Table 1: Duraffourd scale to determine antifungal sensitivity, according to diameter of inhibition halos

Duraffourd scale		
Classification	**Representation**	**Diameter (mm)**
Null	–	< 8
Sensitive	+	8–13.99
Very sensitive	++	14–20
Highly sensitive	+++	> 20

mm = millimeters

Finally, the Duraffourd scale[25,26] was used to assess antibacterial sensitivity of both extracts in their different concentrations, according to their inhibition halos and compared with 0.12% chlorhexidine (control) [Table 1].

Sample size calculation and sampling for comparison of inhibition halos

The sample size consisted of 12 replicates (n = 12) and was calculated from a pilot study by the mean comparison formula, considering an $\alpha = 0.05$ and a statistical power of $1–\beta = 0.80$ with variances $S_1^2 = 0.56$ and $S_2^2 = 0.81$ and a mean difference of 1.2 mm. In addition, the study units were selected by simple random sampling without replacement. The groups were formed as follows:

- 12 replicates with methanolic extract of *B. orellana* leaves (stock: 1000 mg/mL),
- 12 replicates with methanolic extract of *B. orellana* leaves (stock: 750 mg/mL),
- 12 replicates with methanolic extract of *B. orellana* leaves (stock: 500 mg/mL),
- 12 replicates with ethanolic extract of *C. sinensis* leaves (stock: 1000 mg/mL),
- 12 replicates with ethanolic extract of *C. sinensis* leaves (stock: 750 mg/mL),
- 12 replicates with ethanolic extract of *C. sinensis* leaves (stock: 500 mg/mL),
- 12 replicates with Chlorhexidine 0.12 % [Control (+)].
- 12 replicates with distilled water [Control (-)].

Determination of minimum inhibitory concentration and minimum bactericidal concentration

Reactivated strains of *S. mutans* (ATCC 25175) grown in the Microbiology laboratory of the Universidad Privada San Juan Bautista, Peru, were used.

MBC was considered as the minimum concentration of an antibiotic that, in a predetermined period of time, was able to induce the death *in vitro* of 99.9% of a bacterial population; while MIC was the minimum concentration

of the extract where there was no visible growth after an incubation period.[27]

BHI (Brain Heart Infusion) agar plates were prepared 48 h in advance. In sterile 1.5 mL microcentrifuge tubes, 100 µL of the extract was added for each dilution. The absorbance was measured using a DR 6000 UV-VIS spectrophotometer with light beam at a distance of 1 cm. The absorbance at 600 nm gave a value between 0.08 and 0.10. The inoculum suspension was then prepared so that it was adjusted to 1.5×10^8 UFC/mL, according to McFarland bd bbl turbidity standard of 0.5.

A volume of 5 µL of *S. mutans* was added to each microcentrifuge tube, homogenized by vortexing (Thermolyne®) and the turbidity absorbance of the stock solution and its dissolutions were measured with a spectrophotometer. The tubes were then incubated at 37°C for 24 h under anaerobic conditions, and the absorbance was measured again and compared with the first result to determine the MIC. Subsequently, 100 µL of the extract was inoculated and spread on BHI (Brain Heart Infusion) agar plates using sterile handles. The plates were incubated at 37°C for 24 h under anaerobic conditions and visual inspection for colony growth was performed. According to these results, MBC was determined.[27]

Statistical analysis

The data were collected on an ad hoc form and entered into a Microsoft Excel 2019 spreadsheet. Subsequently, they were exported and processed with the SPSS version 24 statistical package. For descriptive analysis, measures of central tendency and dispersion, such as mean and standard deviation, were used. The Shapiro–Wilk normality test and Levene's homoscedasticity test were performed, and their results showed that the data met the requirements for applying the intergroup one-factor ANOVA test with Tukey's post hoc in the case of *C. sinensis* extract. However, this was not met in the case of *B. orellana* extract, so the nonparametric Kruskal–Wallis test with Bonferroni's post hoc was used. In addition, to compare the related measures for both *C. sinensis* extract and *B. orellana* extract, the normal distribution and sphericity (Shapiro–Wilk test and Mauchly's *W* test, respectively) of differences in means between times were assessed. In order to verify compliance with statistical assumptions, an intragroup ANOVA test with Bonferroni's post hoc correction was used. In all statistical tests a significance level at 95 % ($P < 0.05$) was considered.

Results

At 24 and 72 h, *S. mutans* strains showed sensitivity to ethanolic extract of *C. sinensis* at 100%, 75% and 50%. However, at 48 h, highly sensitive activity was observed against the three concentrations of *C. sinensis* [Table 2].

Ethanolic extract of *C. sinensis* at 100% and 75% showed the highest average inhibitory halos at 24 h with 9.53 mm (95% CI: 9.16–9.89) and 9.73 mm (95% CI: 9.23–10.22) respectively. Likewise, at 48 h with 24.58 mm (95% CI: 23.75–25.42) and 23.52 mm (95% CI: 22.89–24.14) respectively. Likewise, at 72 h with 13.90 mm (95% CI: 13.55–14.25) and 11.99 mm (95% CI: 11.30–12.68) respectively. [Table 2].

On the other hand, at 24 h, the ethanolic extract of *C. sinensis* at 100% and 75% showed significantly higher antibacterial activity against *S. mutans*, compared with chlorhexidine 0.12% ($P = 0.002$ and $P < 0.001$, respectively). Furthermore, this effect increased at 48 h ($P < 0.001$ and $P < 0.001$, respectively). However, at 72 h only 100% ethanolic extract of *C. sinensis* showed significantly

Table 2: Descriptive values and comparison of inhibitory halos (mm) for antibacterial activity of three concentrations of the ethanolic extract of *C. sinensis*, according to time

Time	Solution	*n*	Mean	SD	SE	95%CI		Min	Max	*F*	*P*	S*
						UL	LL					
24 h	CS 100%	12	9.53[a,b]	0.57	0.16	9.16	9.89	8.4	10.3	8.82	<0.001	+
	CS 75%	12	9.73[a]	0.78	0.22	9.23	10.22	8.5	11.2			+
	CS 50%	12	8.99[b,c]	0.58	0.17	8.62	9.36	8.1	9.9			+
	CHX 0.12%	12	8.56[c]	0.5	0.14	8.24	8.88	7.8	9.3			+
48 h	CS 100%	12	24.58[d]	1.32	0.38	23.75	25.42	22.5	26.7	119.97	<0.001	+++
	CS 75%	12	23.52[d]	0.99	0.28	22.89	24.14	22.1	25.2			+++
	CS 50%	12	20.11[e]	0.85	0.25	19.57	20.65	18.5	21.7			+++
	CHX 0.12%	12	16.88[f]	1.21	0.35	16.12	17.65	15.2	18.7			++
72 h	CS 100%	12	13.90[g]	0.55	0.16	13.55	14.25	12.9	14.8	35.74	<0.001	+
	CS 75%	12	11.99[h]	1.08	0.31	11.3	12.68	10.9	14.2			+
	CS 50%	12	10.88[i]	0.66	0.19	10.46	11.29	10.2	12.2			+
	CHX 0.12%	12	12.89[k]	0.56	0.16	12.53	13.25	12.2	13.9			+

n: replicates (sample size); SD: Standard Deviation; SE: Standard Error of mean; 95% CI: 95% Confidence Interval; LL: Lower Limit; UL: Upper Limit; F: Based on one-factor intergroup ANOVA test ($P < 0.05$, significant differences). *Based on Duraffourd scale: sensitive (+), very sensitive (+ +) and highly sensitive (+ + +), CS: *C. sinensis*; CXH: chlorhexidine 0.12%. Different letters are different significances, according to Tukey's post hoc

Table 3: Descriptive values and comparison of inhibitory halos (mm) for antibacterial activity of three concentrations of methanolic extract of B. *orellana*, according to time

Time	Solution	*n*	Mean	DE	Median	IQR	Min	Max	KW	*P*	S*
24 h	BO 100%	12	10.22	1.33	10.10[a]	1.92	7.60	12.10	14.664	0.002	+
	BO 75%	12	9.05	0.43	8.90[a,b]	0.62	8.60	9.90			+
	BO 50%	12	8.51	0.71	8.35[b]	1.35	7.70	9.60			+
	CHX 0.12%	12	9.32	0.94	9.50[a,b]	1.00	7.50	10.90			+
48 h	BO 100%	12	22.70	3.68	23.35[a]	5.22	16.70	28.30	22.847	0.000	+++
	BO 75%	12	21.50	3.71	20.70[a]	6.30	16.30	28.80			+++
	BO 50%	12	19.19	1.66	19.30[a,b]	2.37	16.50	21.70			++
	CHX 0.12%	12	16.48	1.44	16.10[b]	2.40	14.40	18.40			++
72 h	BO 100%	12	17.22	2.46	16.05[a]	3.75	15.10	22.60	21.795	0.000	++
	BO 75%	12	16.28	2.15	16.15[a]	3.48	13.10	20.10			++
	BO 50%	12	12.09	4.10	13.35[b,c]	7.72	6.00	16.90			+
	CHX 0.12%	12	13.03	1.45	12.95[c]	1.70	10.70	15.20			+

n: replicates (sample size); IQR: Interquartile Range; KW: Based on Kruskall Wallis test ($P < 0.05$, significant differences). *Based on Duraffourd scale: sensitive (+), very sensitive (+ +) and highly sensitive (+ + +), BO: *B. orellana*; CXH: 0.12% chlorhexidine (control). Different letters are different significances, according to Dunnet's post hoc with Bonferroni correction

higher antibacterial activity than 0.12% chlorhexidine ($P = 0.010$) [Table 2].

At 24 h, *S. mutans* strains showed sensitivity to methanolic extract of *B. orellana* at 100%, 75% and 50%. However, at 48 h, highly sensitive activity was observed against this extract at 100% and 75% [Table 3].

Methanolic extract of *B. orellana* at 100% and 75% showed the highest average inhibitory halos at 24 h with 10.22 ± 1.33 mm and 9.05 ± 0.43 mm, respectively. Similarly, at 48 h with 22.70 ± 3.68 mm and 21.50 ± 3.71 mm, respectively. The same at 72 h with 17.22 ± 2.46 mm and 16.28 ± 2.15 mm, respectively [Table 3].

On the other hand, at 24 h, only the 100% methanolic extract of *B. orellana* showed significantly higher antibacterial activity against *S. mutans*, compared with the same extract at 50% ($P = 0.001$). However, at 48 and 72 h, the methanolic extract of *B. orellana* at 100% and 75% showed significantly higher antibacterial activity against *S. mutans* ($P < 0.05$), compared with chlorhexidine 0.12% [Table 3].

When assessing the two concentrations of ethanolic extract of *C. sinensis* (100% and 75%) and methanolic extract of *B. orellana* (100% and 75%) that showed the highest antibacterial activity in the first stage against *S. mutans*, it was observed at 24 h that the sensitivity was low. However, at 48 h, highly sensitive activity was observed against ethanolic extract of *C. sinensis* (100% and 75%) and methanolic extract of *B. orellana* at 100%. Finally, at 72 h, the *C. sinensis* extract (100% and 75%) and *B. orellana* (100% and 75%) showed very sensitive activity [Table 4].

Ethanolic extract of *C. sinensis* at 100% presented the highest average inhibitory halos at 24 h with 10.89 ± 0.91 mm. The same at 48 h with 22.25 ± 0.63 mm. Similarly, at 72 h with 17.12 ± 0.66 mm [Table 4].

On the other hand, at 24 h, there were no significant differences between chlorhexidine 0.12% (control), ethanolic extract of *C. sinensis* (100% and 75%), and methanolic extract of *B. orellana* (100% and 75%) against *S. mutans* ($P > 0.05$). Furthermore, at 48 h, ethanolic extract of *C. sinensis* at 100% and 75% showed significantly higher antibacterial activity against *S. mutans* compared with methanolic extract of *B. orellana* ($P < 0.05$) and chlorhexidine 0.12% ($P < 0.05$). Finally, at 72 h, significant differences were only observed between the ethanolic extract of *C. sinensis* and chlorhexidine 0.12% ($P = 0.002$) [Table 4].

When intragroup comparisons of ethanolic extract of *C. sinensis* and *B. orellana* were made at 24, 48 and 72 h, a significant increase in antibacterial activity was observed in all concentrations at 48 h ($P < 0.001$), and at the same time it was observed that antibacterial activity decreased significantly in all groups at 72 h ($P < 0.001$). It should be noted that the same pattern was observed with chlorhexidine 0.12% (control) both at 48 h ($P < 0.001$) and 72 h ($P < 0.001$) [Table 5] and [Figure 1].

Regarding ethanolic extract of *C. sinensis* leaf, it could be observed that the MIC against *S. mutans* at 24 h was the second dilution (250 mg/mL), according to turbidity absorbance in the tube. In addition, when visualizing the growth of colonies on a plate, it was observed that the MBC was at the first dilution (500 mg/mL) [Table 6]. On the other hand, in the methanolic extract of *B. orellana* leaf, it could be observed that the MIC against *S. mutans* at 24 h was in the third dilution (125 mg/mL), according to turbidity absorbance in the tube. In addition, when visualizing the growth of colonies on the plate, it could be observed that MBC was in the first dilution (500 mg/mL) [Table 6].

Table 4: Descriptive values and comparison of inhibitory halos (mm) for antibacterial activity of the ethanolic extract of *C. sinensis* at 100% and 75% versus the methanolic extract of *B. orellana* at 100% and 75%, according to time

Time	Solution	*N*	Mean	SD	Median	IQR	Min	Max	KW	*P*	S*
24 h	CS 100%	12	10.89	0.91	10.90[a]	1.53	9.80	12.50	9.90	0.042	+
	CS 75%	12	10.02	0.29	9.90[b]	0.40	9.60	10.50			+
	BO 100%	12	10.22	0.58	10.05[c]	0.67	9.50	11.40			+
	BO 75%	12	10.03	0.38	9.90[d]	0.73	9.40	10.60			+
	CHX 0.12%	12	10.26	0.37	10.30[e]	0.60	9.80	10.90			+
48 h	CS 100%	12	22.25	0.63	22.20[a]	0.85	21.50	23.40	48.35	<0.001	+++
	CS 75%	12	22.07	0.84	21.95[a]	1.48	21.10	23.70			+++
	BO 100%	12	20.84	0.81	20.75[a,b]	1.08	19.50	22.70			+++
	BO 75%	12	19.18	0.61	19.15[b,c]	0.85	18.30	20.20			++
	CHX 0.12%	12	18.38	0.83	18.25[c]	1.18	17.10	19.90			++
72 h	CS 100%	12	17.12	0.66	16.80[a]	0.80	16.40	18.60	14.39	0.006	++
	CS 75%	12	16.58	1.25	16.80[a,b]	2.20	14.50	18.30			++
	BO 100%	12	16.80	0.62	16.60[a,b]	1.15	16.10	17.80			++
	BO 75%	12	16.54	0.50	16.70[a,b]	0.70	15.50	17.20			++
	CHX 0.12%	12	15.93	0.53	15.90[b]	0.93	15.10	16.80			++

n: replicates (sample size); IQR: Interquartile Range; KW: Based on Kruskall Wallis test ($P < 0.05$, significant differences). *Based on the Duraffourd scale: sensitive (+), very sensitive (+ +) and highly sensitive (+ + +), CS: *C. sinensis*; BO: *B. orellana*; CXH: 0.12% chlorhexidine (control). Different letters are different significances, according to Dunnet's post hoc with Bonferroni correction

Table 5: Intragroup comparison of inhibitory halos (mm) at 24, 48 and 72 h, according to type of solution

Solution	Time		Difference of means	SE	95%CI		Z	W	*P	**P
					LL	UL				
CS 100%	24 h	48 h	-11,358	0.267	-12.111	-10.605	0.457	0.21	<0.001	<0.001
		72 h	-6,225	0.330	-7.156	-5.294	0.882			<0.001
	48 h	72 h	5,133	0.206	4.552	5.715	0.475			<0.001
CS 75%	24 h	48 h	-12,050	0.310	-12.925	-11.175	0.601	0.39	<0.001	<0.001
		72 h	-6,567	0.389	-7.663	-5.470	0.252			<0.001
	48 h	72 h	5,483	0.450	4.213	6.753	0.155			<0.001
BO 100%	24 h	48 h	-10,625	0.240	-11.301	-9.949	0.220	0.78	<0.001	<0.001
		72 h	-6,583	0.290	-7.402	-5.764	0.612			<0.001
	48 h	72 h	4,042	0.271	3.277	4.806	0.888			<0.001
BO 75%	24 h	48 h	-9,158	0.194	-9.705	-8.611	0.155	0.999	<0.001	<0.001
		72 h	-6,517	0.196	-7.070	-5.964	0.371			<0.001
	48 h	72 h	2,642	0.194	2.096	3.188	0.912			<0.001
CHX 0.12%	24 h	48 h	-8,125	0.274	-8.899	-7.351	0.534	0.084	<0.001	<0.001
		72 h	-5,675	0.186	-6.200	-5.150	0.600			<0.001
	48 h	72 h	2,450	0.341	1.488	3.412	0.356			<0.001

CS: *C. sinensis*, BO: *B. orellana*, CHX: chlorhexidine; Z: Shapiro Wilk test, normal distribution of mean differences ($P > 0.05$); W: Mauchly's Sphericity ($P > 0.05$); *Based on one-factor within-group ANOVA test ($P < 0.05$, significant differences); **Based on Bonferroni's Post hoc ($P < 0.05$, significant differences)

Discussion

Use of plants as natural medicine for treating and preventing various diseases is a topic of great relevance in terms of dental public health. Therefore, the purpose of this study was to evaluate the antibacterial activity of the methanolic extract of *B. orellana* in comparison with the ethanolic extract of *C. sinensis*, against *S. mutans* at 24, 48, and 72 h. According to the results obtained, the null hypothesis was rejected.

In this study, results showed that the ethanolic extract of *C. sinensis* leaf at 100% presented the highest antibacterial activity, followed by the methanolic extract of *B. orellana* leaf at 100% at 24 h, the ethanolic extract of *C. sinensis* leaf at 75% at 48 h, and the methanolic extract of *B. orellana* at 100% at 72 h, being its antibacterial activity higher than chlorhexidine 0.12% at different times. An increase in this activity was observed at 48 h and a decrease at 72 h. In addition, MICs were found for ethanolic extract of *C. sinensis* leaf (250 mg/mL) and methanolic extract of *B. orellana* leaf (125 mg/mL). MBC was 500 mg/mL for both extracts.

Antimicrobial activity of *C. sinensis* is attributed to catechins, mainly epigallocatechin-3-gallate (EGCG), which is found in abundance and represents 50% of the

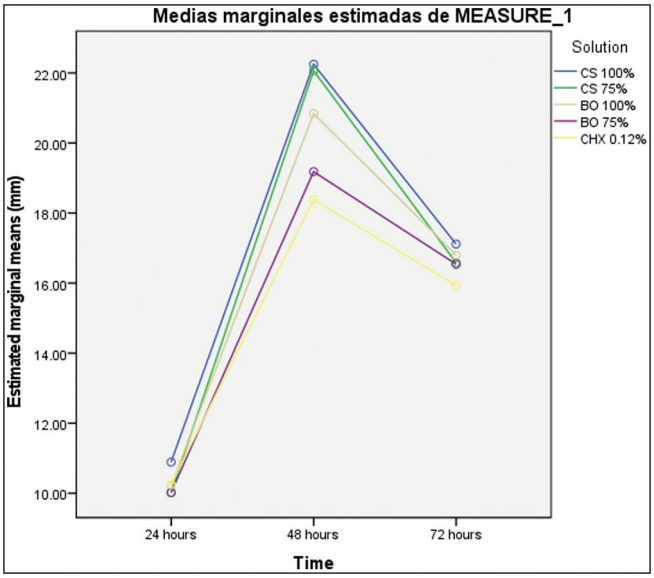

Figure 1: Marginal means of inhibitory halos of the antibacterial activity of ethanolic extract of *Camellia sinensis* (CS) and *Bixa orellana* (BO) against chlorhexidine 0.12% (CHX), at 24, 48 and 72 h

Table 6: Quantitative method of *S. mutans* growth in serial dilutions of ethanolic extract of *C. sinensis* leaf and methanolic extract of *B. orellana*

Growth	Stock 1000 mg/mL	500 mg/mL	250 mg/mL	125 mg/mL	62.5 mg/mL	31.25 mg/mL	15.625 mg/mL	7.812 mg/mL	PC	NC
In tube (CS)	–	–	–	+	+	+	+	+	+	–
In plate (CS)	–	–	+	+	+	+	+	+	+	–
In tube (BO)	–	–	–	–	+	+	+	+	+	–
In plate (BO)	–	–	+	+	+	+	+	+	+	–

PC: positive control (BHI agar with inoculation of *S. mutans* without ethanolic extract of *C. sinensis*), NC: negative control (BHI agar with ethanolic extract of pure *C. sinensis* without microbes). With growth (+), without growth (-). CS: *C. sinensis*, BO: *B. orellana*

total catechin pool.[17] EGCG has been shown to cause irreversible alteration of the bacterial membrane[28] and inhibit bacterial DNA gyrase, thus preventing DNA supercoiling and causing bacterial cell death.[29] Furthermore, the specific activity against *S. mutans* is based on the fact that EGCG interferes with bacterial adhesion to tooth enamel and suppresses the action of glucosyltransferase and amylase, which reduces the production of lactic acid on tooth surfaces.[30-32] However, it seems that the successful antibacterial effect of *C. sinensis* is not only due to this catechin alone, since according to a study by Sasaki *et al*,[33] the combination of EGCG, with gallocatechin gallate (GCG) and catechin gallate (CG) showed stronger antibacterial activity against *S. mutans* than the same catechins alone.

Similarly, antimicrobial action of *B. orellana* could be due to flavonoids and alkaloids it possesses, since its ability to form protein complexes, bacterial and lipophilic cell walls, as well as the rupture of bacterial membranes has been reported.[34] In addition, other studies report that the antioxidant and antimicrobial properties of *B. orellana* are attributed to the bioactive compounds found in its seeds as carotenoid bixin (bixin or 6-methyl hydrogen (9Z)-6,6′-diapocarotene-6) and polyphenolic compounds existing in its different parts as catechin, chlorogenic acid, chrysin, butein, hypoaletin, and xanthoangelol,[35] which would inhibit the production of free radicals and denature proteins present in cell structure of some microorganisms to prevent their proliferation.[36-39]

In present study, the ethanolic extract of *C. sinensis* at a concentration of 1000 mg/mL (100%) presented the highest antimicrobial activity against *S. mutans* compared with the methanolic extract of *B. orellana* and chlorhexidine 0.12% at 24, 48 and 72 h, and reached its maximum antibacterial activity at 48 h with an inhibition halo of 22.25 mm, classifying it as highly sensitive according to the Duraffourd scale.[25,26] These results are in agreement with those reported by Otake *et al*.,[40] who showed that a crude extract of *C. sinensis* called "Sunphenon" had the ability to prevent the adherence of *S. mutans* to the hydroxyapatite of tooth surface and to inhibit the enzyme glucosyltransferase. They also demonstrated *in vivo* that when rats were fed with a Sunphenon-enriched diet, caries formation was greatly inhibited. However, results of Otake *et al.*[40] differ from those reported by Cayo *et al.*,[41] who conducted a study with *C. sinensis* from same cultivation

site as this study, reporting that the 20% ethanolic extract showed its highest inhibition halo of 18.64 mm at 24 h on *S. mutans*, being sensitive against the extract according to the Duraffourd scale. These differences could be due to low percentages in concentrations used, since it has been frequently observed that the higher the concentration of extract, the greater the antimicrobial effect.[41]

On the other hand, methanolic extract of *B. orellana* at concentration of 1000 mg/mL (100%) presented the second largest inhibition halo at 24, 48 and 72 h, reaching its maximum antibacterial activity at 48 h with an inhibition halo of 20.84 mm, classifying it as highly sensitive according to the Duraffourd scale. These results are similar to those reported by Medina *et al*,[15] who conducted a study with methanolic extract of *B. orellana* leaves, producing an inhibition halo of 19.97 ± 1.31 mm against *S. mutans*, being classified between very sensitive to highly sensitive according to the Duraffourd scale, and MIC of 62.5 µg/mL. They also assessed the seed extract, which had an activity of 15.11 ± 1.03 mm against *S. mutans*, showing lower effect compared with leaf extract. Likewise, the MIC of seed extract was 31.25 µg/mL. According to Medina *et al*,[15] these differences in MICs of leaf and seed methanolic extracts could be related to the fact that antibacterial active principles obtained in leaves and seeds of plants can be isolated in different concentrations, and the higher efficiency in leaf extract compared with seed extract may be due to the absence of alkaloids in seeds. It should be noted that this study and the study conducted by Medina *et al,*[15] were carried out with *B. orellana* from Peru.

However, in a study carried out in Brazil by dos Santos *et al*[12] where crude ethanolic extract of *B. orellana* seeds was added to endodontic sealants, a considerable antibacterial effect was observed only after 24 h of incubation against *S. mutans*, with MIC of 3.12 mg/mL. This difference in greater effect and shorter time with this study could be due to the fact that dos Santos *et al*[12] used *B. orellana* cultivated in Brazil, and geographical area could influence the concentration of antibacterial chemical components.[42,43] In addition, seed extract was used, which is the *B. orellana* part with highest content of carotenoids[44] such as bixin, also isolated from leaves and bark, but with higher concentration in seeds.[45] In spite of this argument, these results differ from those reported by Medina *et al.*[15] and Rojas *et al.*,[7] who carried out a study in Colombia where the methanolic extract of *B. orellana* seeds had no greater effect against *Streptococcus β hemolyticus* compared with the positive control (clindamycin), giving more reason to the argument that the geographic zone would be determinant in composition of plant species.[42,43] In addition, dos Santos *et al*[12] used ethanol as solvent to extract the active compounds of *B. orellana*, being capable of extracting constituents such as terpenes, the most important being carotenoids, including Bixin, which is abundantly found in *B. orellana* seeds.[35,46] Finally, these differences could also be due to the fact that dos Santos *et al*[12] obtained the extract by freeze-drying, which would allow a better preservation of its active principles and phytochemical properties.[47,48]

It is also important to highlight that the presence of phenolic compounds in both plant species (*B. orellana and C. sinensis*), depending on their concentration, could influence stability of antimicrobial properties over time,[42,43] which could explain the decrease in antimicrobial effect of their extracts after 48 to 72 h, obtained in this study.

Chlorhexidine has a broad spectrum of activity against Gram-positive and Gram-negative bacteria, as well as high capacity to adsorb on dental tissue and mucous membranes with prolonged gradual release at therapeutic levels. It is on the World Health Organization's list of Essential Antiseptics and remains the treatment of choice in dental practice. It is also used as a control or reference in assessing the efficacy of mouthwashes, being considered the "gold standard" in clinical and *in vitro* trials, as well as to assess the potency of new antimicrobials. Despite this, undesirable side effects have been reported, which is why substances with similar therapeutic efficacy and fewer side effects should be studied and used in dentistry.[49,50] It is known that antibacterial action mechanism of chlorhexidine blocks free acid groups (sulfates, carboxyls and phosphates), preventing or decreasing bacterial adhesion and coaggregation. Chlorhexidine also binds to negative charges on the bacterial cell wall, thus hindering the adhesion mechanism between them.[41]

Currently, natural products derived from plants for management and control of diseases have gained importance due to their low-cost effectiveness.[16,41] In addition, *B. orellana* and *C. sinensis* dissolved in ethanol and methanol solvents, respectively; have shown to be effective against *S. mutans*, when these plants were cultivated in the Peruvian geographic area.[15,41] Therefore, this study is important because it provides relevant information on the antimicrobial properties of ethanolic extract of *C. sinensis* and methanolic extract of *B. orellana* against *S. mutans*, since this bacterium proved to be highly sensitive to both products, observing that at its maximum concentration, *C. sinensis* presented greater antimicrobial effectiveness than *B. orellana* against *S. mutans*. This lays the foundation for future research to enrich the knowledge for creating products based on these plant species, which can be used as mouthwashes or toothpastes, as well as additive ingredients in different dental materials for preventing and combating dental caries.

Among limitations of this study is not having specifically identified the chemical components of each extract, which would have allowed a clearer understanding of the component which contributes to antibacterial activity. Although a

minimum inhibitory and bactericidal concentration was determined, a minimum cytotoxicity concentration was not determined, which is relevant if the intention is to lay the foundations for a future study in humans.

It is recommended to assess antimicrobial activity of the active principles of *C. sinensis* such as catechins, especially EGCG, as well as the active principles of *B. orellana* in seed, leaf and root to determine which structure has the maximum antimicrobial potential. It is also recommended to further study the cytotoxicity concentration of *C. sinensis* and *B. orellana*, as well as assessing possible synergies with synthetic antibiotics in order to increase their effectiveness. Finally, it is recommended to assess both extracts in clinical samples from humans.

Conclusion

At 100% concentration, the ethanolic extract of *C. sinensis* and the methanolic extract of *B. orellana* showed the highest antibacterial activity against *S. mutans* at 48 h, with *C. sinensis* extract being more effective compared with *B. orellana* extract. However, this antibacterial effect decreased in both extracts at 72 h. On the other hand, the MBC of *C. sinensis* and *B. orellana* against *S. mutans* was 500 mg/mL, for both extracts. While the MIC was 250 mg/mL and 125 mg/mL, respectively.

Acknowledgements

We thank the "Biomaterials" research line team of the San Juan Bautista Private University, Academic Program of Stomatology, Lima and Ica, Peru, for their constant support in the preparation of this manuscript.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

Authors' contribution

LGJ, CCR conceived the research idea; LGJ, EGA and SMC elaborated the manuscript; ACP, LGJ and LCP collected and tabulated the information; ACP, LGJ, MLC and EGA carried out the bibliographic search; CCR and ACP interpreted the statistical results; SMC, LCP, LGJ and CCR helped in the development from the discussion; LCP, EGA, SMC, MLC, LCG and CCR performed the critical revision of the manuscript. All authors approved the final version of the manuscript.

Ethical policy and institutional review board statement

This research was approved by the Ethics and Research Committee of the Universidad Privada San Juan Bautista with approval letter mo. 1410-2021-CIEI-UPSJB.

Patient declaration of consent

Not applicable.

Data availability statement

The data that support the study results are available from the author (Dr. César Cayo-Rojas, e-mail: cesar.cayo@upsjb.edu.pe) on request.

References

1. Zhan L. Rebalancing the caries microbiome dysbiosis: Targeted treatment and sugar alcohols. Adv Dent Res 2018;29:110-6.
2. Ojeda JC, Oviedo E, Salas LA. *Streptococcus mutans* y caries dental. Rev CES Odont 2013;26:44-56.
3. Lemos JA, Palmer SR, Zeng L, Wen ZT, Kajfasz JK, Freires IA, Abranches J, *et al*. The Biology of *Streptococcus mutans*. Microbiology Spectrum 2019;7:1-26.
4. Sampaio FC, Malheiros Z, Benítez C, Stewart B, Bönecker M. Dental caries in latin american and caribbean countries: Urgent need for a regional consensus. Braz Oral Res 2021;35:e057.
5. Kudsi Z, Fenlon MR, Hill K, Baysan A. Developing a questionnaire to measure psychological disturbance associated with tooth loss. J Dent 2020;98:103353.
6. Cayo-Rojas C, Gerónimo-Nieto E, Aliaga-Mariñas A. Salivary pH changes caused by cariogenic and non-cariogenic food intake in preschoolers from Huaura, Peru. Rev Cuba Estomatol 2021;58:e3518.
7. Rojas JJ, Ochoa VJ, Ocampo SA, Muñoz JF. Screening for antimicrobial activity of ten medicinal plants used in colombian folkloric medicine: A possible alternative in the treatment of non-nosocomial infections. BMC Complement Altern Med 2006;6:2.
8. Maguiña Vargas Ciro. Infecciones nosocomiales. Acta méd Peru 2016;33:175-7.
9. Yoo S, Murata RM, Duarte S. Antimicrobial traits of tea- and cranberry-derived polyphenols against *Streptococcus mutans*. Caries Res 2011;45:327-35.
10. Shah PM. The need for new therapeutic agents: What is in the pipeline? Clinical Microbiology and Infection 2005; 11:36-42.
11. Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. Environ Health Perspect 2001;109 Suppl 1:69-75.
12. Dos Santos DC, da Silva Barboza A, Schneider LR, Cuevas-Suárez CE, Ribeiro JS, Damian MF, *et al*. Antimicrobial and physical properties of experimental endodontic sealers containing vegetable extracts. Sci Rep 2021;11:6450.
13. Fleischer TC, Ameade EP, Mensah ML, Sawer IK. Antimicrobial activity of the leaves and seeds of *Bixa orellana*. Fitoterapia 2003;74:136-8.
14. Irobi ON, Moo-Young M, Anderson WA. *In vitro* clonal propagation of annatto (*Bixa orellana* L.). Pharm Biol 1996;34:87.
15. Medina D, Ulloa G, Camere R, Caballero S, Mayta F, del Valle J. Antibacterial activity of *Bixa orellana* L. (achiote) against *Streptococcus mutans* and Streptococcus sanguinis. Asian Pacific Journal of Tropical Biomedicine 2016;6:400-3.
16. Alim S, Bairagi N, Shahriyar S, Kabir M. *In vitro* antibacterial potential of *Bixa orellana* L. against some pathogenic bacteria and comparative investigation on some standard antibiotics. Journal of Pharmacognosy and Phytochemistry 2016;5:178-81.
17. Chacko SM, Thambi PT, Kuttan R, Nishigaki I. Beneficial effects of green tea: A literature review. Chin Med 2010;5:13.
18. Schneider C, Segre T. Green tea: Potential health benefits. Am Fam Physician 2009;79:591-4.
19. Taylor PW, Hamilton-Miller JM, Stapleton PD. Antimicrobial properties of green tea catechins. Food Sci Technol Bull 2005;2:71-81.
20. Araghizadeh A, Kohanteb J, Fani MM. Inhibitory activity of green tea (*Camellia sinensis*) extract on some clinically isolated cariogenic and periodontopathic bacteria. Med Princ Pract 2013;22:368-72.

21. Matsumoto M, Minami T, Sasaki H, Sobue S, Hamada S, Ooshima T. Inhibitory effects of oolong tea extract on caries-inducing properties of mutans streptococci. Caries Res 1999;33:441-5.
22. Hirasawa M, Tanaka T, Otake S. Inhibition of acid production in dental plaque bacteria by green tea catechins. Caries Res 2006; 40:265-70.
23. Saito N. [Anti-caries effects of polyphenol compound from *Camellia sinensis*]. Nichidai Koko Kagaku 1990;16:154-63.
24. Ferrazzano GF, Amato I, Ingenito A, De Natale A, Pollio A. Anti-cariogenic effects of polyphenols from plant stimulant beverages (cocoa, coffee, tea). Fitoterapia 2009;80:255-62.
25. Duraffourd C, Lapraz JC. Clinical Phytotherapy Notebooks. Mexico: Editorial Masson; 1986.
26. Guevara L, Bonilla P, Caicedo M. Antimicrobial activity of orthodontic adhesive with silver nanoparticles on *Streptococcus mutans*. Revista Odontología 2020;22:33-44.
27. Parvekar P, Palaskar J, Metgud S, Maria R, Dutta S. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver nanoparticles against staphylococcus aureus. Biomater Investig Dent 2020;7:105-9.
28. Ikigai H, Nakae T, Hara Y, Shimamura T. Bactericidal catechins damage the lipid bilayer. Biochim Biophys Acta 1993;1147:132-6.
29. Gradisar H, Pristovsek P, Plaper A, Jerala R. Green tea catechins inhibit bacterial DNA gyrase by interaction with its ATP binding site. J Med Chem 2007;50:264-71.
30. Sakanaka S, Okada Y. Inhibitory effects of green tea polyphenols on the production of a virulence factor of the periodontal-disease-causing anaerobic bacterium porphyromonas gingivalis. J Agric Food Chem 2004;52:1688-92.
31. Hattori M, Kusumoto IT, Namba T, Ishigami T, Hara Y. Effect of tea polyphenols on glucan synthesis by glucosyltransferase from *Streptococcus mutans*. Chem Pharm Bull (Tokyo) 1990;38:717-20.
32. Hamilton-Miller JMT. Anti-cariogenic properties of tea (*Camellia sinensis*). J Med Microbiol 2001;50:299-302.
33. Sasaki H, Matsumoto M, Tanaka T, Maeda M, Nakai M, Hamada S, *et al*. Antibacterial activity of polyphenol components in oolong tea extract against *Streptococcus mutans*. Caries Res 2004;38:2-8.
34. Biswas SJ, Giri SK, Saha NC, Raha S, Pandey A. Phytochemical evaluation, acute toxicity studies and antimicrobial efficacy of seed extract of *Bixa orellana*: A plant grown in wild in Purulia district. J Pharma Phytochem 2018;7:2065-71
35. Quintero J, Naranjo AM, Silva M, Ciro GL, Rojas JJ. Ultrasound-assisted extraction of bioactive compounds from annatto seeds, evaluation of their antimicrobial and antioxidant activity, and identification of main compounds by LC/ESI-MS analysis. Int J Food Sci 2019;3721828.
36. Viuda M, Ciro GL, Ruiz Y, Zapata JE, Sendra E, Pérez JA, *et al*. *In vitro* antioxidant and antibacterial activities of extracts from annatto (*Bixa orellana* L.) leaves and seeds. J Food Saf 2012;32:399-406.
37. Chisté RC, Yamashita F, Gozzo FC, Mercadante AZ. Simultaneous extraction and analysis by high performance liquid chromatography coupled to diode array and mass spectrometric detectors of bixin and phenolic compounds from annatto seeds. J Chromatogr A 2011;1218:57-63.
38. Butnariu M. Methods of Analysis (Extraction, Separation, Identification and Quantification) of Carotenoids from Natural Products. J Ecosyst Ecography 2016;6:1-19.
39. Shahid-ul-Islam, Rather LJ, Mohammad F. Phytochemistry, biological activities and potential of annatto in natural colorant production for industrial applications—A review. J Adv Res 2016;7:499-514.
40. Otake S, Makimura M, Kuroki T, Nishihara Y, Hirasawa M. Anticaries effects of polyphenolic compounds from japanese green tea. Caries Res 1991;25:438-43.
41. Cayo C, Cervantes L. Antibacterial activity of *Camellia sinensis* versus propolis against *Streptococcus mutans*. Rev Cubana Estomatol 2020;57:e2967.
42. González L, Díaz R, Castillo C, Nieto A, Méndez D. Phenolic compounds: Presence, identification and antioxidant activity in plants and fruits. Mex J Bio Technol 2017;2:46-64.
43. Paucar E, Peltroche N, Cayo C. Antibacterial and antifungal activity of the essential oil of minthostachys mollis against oral microorganisms. Revista Cubana de Investigaciones Biomédicas 2021;40:e1450.
44. Melka B, Bisrat D, Babu GN. Isolation, characterization and biological activities of food colorants from *Bixa orellana*. Journal of Pharmacovigilance 2017;5:4.
45. Teixeira da Silva JA, Dobránszki J, Rivera-Madrid R. The biotechnology (genetic transformation and molecular biology) of *Bixa orellana* L. (achiote). Planta 2018;248:267-77.
46. Conrad OA, Dike IP, Agbara U. In vivo antioxidant assessment of two antimalarial plants-allamamda cathartica and *Bixa orellana*. Asian Pac J Trop Biomed 2013;3:388-94.
47. Bjelošević M, Zvonar Pobirk A, Planinšek O, Ahlin Grabnar P. Excipients in freeze-dried biopharmaceuticals: Contributions toward formulation stability and lyophilisation cycle optimisation. Int J Pharm 2020;576:119029.
48. Gonçalves O, Alves M, Grácio J, Nunes V. A comparative study of raspberry dehydration by lyophilisation or conventional drying. Int Adv Res Eng J 2018;2:267-72.
49. Castilho AL, Saraceni CH, Díaz IE, Paciencia ML, Suffredini IB. New trends in dentistry: Plant extracts against enterococcus faecalis. The efficacy compared with chlorhexidine. Braz Oral Res 2013;27:109-15.
50. Baena ES, Piloni J, Santos EM, Gómez CA, Rangel E, Castro J. Comparison of the antimicrobial activity of hibiscus sabdariffa calyx extracts, six commercial types of mouthwashes, and chlorhexidine on oral pathogenic bacteria, and the effect of hibiscus sabdariffa extracts and chlorhexidine on permeability of the bacterial membrane. Journal of Medicinal Food 2021;24:67-76.