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Antibacterial activity of *Myrciaria dubia* (*Camu camu*) against *Streptococcus mutans* and *Streptococcus sanguinis*

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ABSTRACT

Objective: To evaluate the antibacterial and cytotoxic effect of *Myrciaria dubia* (*Camu camu*) (*M. dubia*) methanol extract, against *Streptococcus mutans* (ATCC 25175) (*S. mutans*) and *Streptococcus sanguinis* (ATCC 10556) (*S. sanguinis*).

Methods: Two methanol extracts of *M. dubia* were prepared *in vitro*, from the seeds and pulp. Ten independent tests were prepared for each type of extract, using 0.12% chlorhexidine solution as positive control. Agar diffusion test was used by preparing wells with the experimental solutions cultivated in anaerobic conditions for 48 h at 37 °C. Meanwhile, the minimum inhibitory concentration and the cytotoxic effect over MDCK cell line was found.

Results: A higher antibacterial effect was observed with the methanol seed extract with an inhibitory halo of (21.36 ± 6.35) mm and (19.21 ± 5.18) mm against *S. mutans* and *S. sanguinis*, respectively. The methanol extract of the pulp had an effect of (16.20 ± 2.08) mm and (19.34 ± 2.90) mm, respectively. The minimum inhibitory concentration of the pulp extract was 62.5 μg/mL for both strains, whereas for the seed antibacterial activity was observed even at low concentrations. The CC_{50} of the seeds extract was at a higher concentration than 800 μg/mL and 524.37 μg/mL for the pulp extract.

Conclusions: The experimental findings demonstrated the antibacterial effect of the methanol extract of *M. dubia* against *S. mutans* and *S. sanguinis*. These extracts were not cytotoxic at high concentrations.

1. Introduction

The high prevalence of dental caries in the population suggests that a vast majority of patients does not have an adequate oral hygiene, as consequence of a bad tooth-brushing technique, lack of awareness of oral health or a lack of motivation [1]. The dental caries is caused by a dental plaque or oral biofilm, which is the principal etiological factor, containing diverse microorganisms that adhere to the tooth walls creating a microbiota unbalance in the host and consequent bacterial overgrowth [2]. Therefore, oral antiseptics are used for proliferation control of these microorganisms.

In Peru, dental caries prevalence is around 95% in the general population per the Pan American Health Organization. The most predominant microorganisms in the oral flora are *Streptococcus mutans* (*S. mutans*) and *Streptococcus sanguinis* (*S. sanguinis*) [2,3]. Diverse publications have demonstrated that plant or fruit extracts can improve symptoms or diminish the prevalence of dental caries, as well as to inhibit the pathogens growth in the dental plaque with less toxicity [4]. In the last years, the use of natural products is increasing as an alternative treatment for dental disease, due to their antibacterial, anti-inflammatory and antioxidant effects [4,5].

The World Health Organization has reported that more than 80% of the world population uses some short of alternative medicine or phytotherapy for therapeutic purposes in dental caries [6,7]. In that sense, Peru is a privilege country regarding the use of alternative medicine, with more than 80 000 species with the potential in the development of new therapeutic drugs [5].

The *Myrciaria dubia* (*M. dubia*), commonly known as "*camu camu*", is a native bush in the amazonic regions. It's chemical components have antibacterial, anti-inflammatory and antioxidant properties [8–10]. However, their properties and clinical relevant use have not been fully studied in the odontological field.

Therefore, the main purpose of this study was to evaluate the *in vitro* antibacterial and cytotoxic effect of methanol extracts of both seeds and pulp of *M. dubia* on *S. mutans* (ATCC 25175) and *S. sanguinis* (ATCC 10556) strains.

2. Materials and methods

2.1. Plant material and extracts

The *M. dubia* fruits were obtained from a naturist house in Lima, Peru. The *camu camu* pulp and seeds were separated and immersed independently in absolute methanol (1:1, w/v). The samples were incubated at room temperature and covered from sunlight by 7 days. The mixtures were filtered through a Whatman No. 4 filter paper and the filtrates were evaporated at 50 °C [11]. All extracts were stored at 4 °C until used.

2.2. Bacterium strain

Strains of *S. mutans* and *S. sanguinis* were used (Genlab del Peru S.A.C., Peru). The cultivation medium was brain heart infusion (BHI) agar (Oxoid, Hampshire, UK). Cultures were grown anaerobically for 72 h at 37 °C. For antibacterial activity assay, three or four isolated colonies were inoculated in 3 mL of BHI broth and incubated in anaerobically conditions for 72 h at 37 °C. The cultures were later diluted with fresh medium to approximate the density of 0.5 McFarland standard, which represents an estimated concentration of 1.5×10^8 CFU/mL.

The McFarland standard was prepared by inoculating colonies of the bacterial test strain in sterile saline and adjusting the cell density to the concentration specified before [12].

2.3. Antibacterial screening of the methanol extracts

2.3.1. Determination of antibacterial activity

To determinate the antibacterial activity of the studied extracts, the cup-plate agar diffusion method was used [13]. BHI agar was autoclaved for 15 min at 121 °C and cooled to 55 °C. The medium was then inoculated with the prepared bacterial suspension, mixed gently and finally poured into sterile Petri dishes, sugar tubes containing molten agar (10 mL) were sterilized and cooled to 40–42 °C. The tubes were then inoculated with 0.1 mL of the appropriate culture suspension of bacterium. These agar plates were incubated under sterile conditions for 8 h at room temperature. Three wells per plate of 6 mm in diameter and 4 mm in depth were made with a sterile cork borer, preserving a distance of 3 cm

between them. The wells were filled with 100 μL of the corresponding methanol extract. The chlorhexidine at 0.12% was used with positive controls [14]. The Petri dishes were incubated under the same growth conditions mentioned above. At the end of the period, the inhibition zones formed were measured in millimeters using a vernier. The inhibition zones with less than 12 mm in diameter were not considered for the antibacterial activity analysis. For each extract, 12 replicates were assayed.

2.3.2. Determination of minimum inhibitory concentration (MIC)

The MIC was determined using the microdilution method as described by Jayaraman *et al.* [15] and Clinical and Laboratory Standards Institute [16]. Serial two-fold dilutions of all the extracts were prepared with sterile saline in a 96-well microtiter plate, obtaining a concentration range from 500 to 0 μg/mL. Then, 5 μL of *S. mutans* or *S. sanguinis* suspension (OD 550 = 0.6) were added to the wells containing the dilutions. Each dose was assayed in quadruplicate. Uninoculated wells containing sterile saline or saline and extract were used as controls. After incubation in anaerobic conditions for 72 h at 37 °C, the samples were observed and MIC was recorded as the lowest concentration of each plant extract that inhibited the bacterial growth as detected by the absence of visual turbidity.

2.4. Cytotoxicity assay of M. dubia

2.4.1. Cell lines

MDCK cells were obtained from ATCC (American Type Culture Collection, USA). The cells were grown in minimum essential medium with Earle's salts (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 25 μg/L gentamicin and 200 mmol/L L-glutamine (growth medium). Infected cells were maintained in minimum essential medium with 1% fetal bovine serum, 25 μg/L gentamicin and 200 mmol/L L-glutamine (maintenance medium). All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2–95% air.

2.4.2. Cytotoxicity assay

Cytotoxicity of *M. dubia* extract was assessed using an assay based on the color change subsequent to the reduction of MTT by mitochondrial enzymes [17–20]. The assays were performed using 1×10^4 cell/well in 200 μL of medium which were cultured in 96-well plates and incubated at 37 °C in a humidified atmosphere with 5% CO_2–95% air. When cell cultures were confluent, the culture medium was removed from the wells, which were replenished with 0.2 mL of the maintenance medium containing *M. dubia* extract prepared by dilution. *M. dubia* concentrations had a range from 0 to 800 μg/mL. Each dose was assayed in quadruplicate. The wells with 0.2 mL maintenance medium but without *M. dubia* extract were used as cell controls. All cultures were incubated at 37 °C for 6 days. Later, 20 μL of MTT stock solution (3 mg/mL in phosphate buffered saline) was added to each well and after 3 h of incubation under culture conditions, the medium was carefully removed and formazan crystals were solubilized by adding 200 μL of dimethyl sulfoxide. Finally, cell viability was expressed as the percentage of the absorbance value determined for the control cultures.

Absorbance (A_{570} nm) was measured in an ELISA reader [17–20]. Cytotoxic concentration 50 (CC_{50}) is defined as the concentration of compound that reduces the viability of MDCK cells by 50%.

3. Results

3.1. Antibacterial activity of the plant extracts

This study was to determine the *in vitro* antibacterial effectivity of two methanol extracts of *M. dubia* against *S. mutans* and *S. sanguinis* strains. The methanol extracts were prepared with the seeds and pulp of *M. dubia* and 0.12% chlorhexidine solution was used as control.

Table 1 shows the median of the methanol extracts inhibition halos. For both *S. mutans* and *S. sanguinis*, the seeds extract of *M. dubia* had a major antibacterial effect if compared with the pulp extract (Table 1).

The antibacterial effect of seeds and pulps methanol extracts of *M. dubia* was compared on *S. mutans* and *S. sanguinis* strains. Shapiro Wilk test showed that there is not a normal distribution in the seeds group extract for both strains.

viability at a 50% with a concentration of 424.37 µg/mL. These values were confirmed by microscopic observation of the cytopathic effect. Thus, the methanol extract can contain the active compounds of *M. dubia* to produce biological effects (Figure 1).

Figure 1. Cytotoxic effect of methanol extracts of seed and pulp of *M. dubia* on MDCK cells.
Values are mean ± SD of three independent experiments.

Table 1
In vitro antibacterial effect of *M. dubia* methanol extract on *S. mutans* and *S. sanguinis* strains.

Microorganism	Group	Average (mm)	Median (mm)	SD (mm)	Minimum (mm)	Maximum (mm)	Normality[*]
S. mutans	Seeds	21.36	17.93	6.35	15.24	32.18	0.000
	Pulp	16.20	16.15	2.08	13.50	20.04	0.252
	Chlorhexidine	23.97	23.41	1.75	21.12	27.82	0.330
S. sanguinis	Seed	19.21	16.50	5.18	14.48	27.42	0.000
	Pulp	19.34	18.61	2.90	15.68	26.64	0.180
	Chlorhexidine	22.75	22.56	2.52	19.14	28.22	0.675

[*]: Shapiro Wilk test, level of statistical significance, ($P < 0.05$).

By comparing the antibacterial effect of seed and pulp extracts against both microorganisms, statistically significant differences were observed in strains of *S. mutans* with $P = 0.004$, whereas for strains of *S. sanguinis*, no statistically significant differences were found with $P = 0.214$.
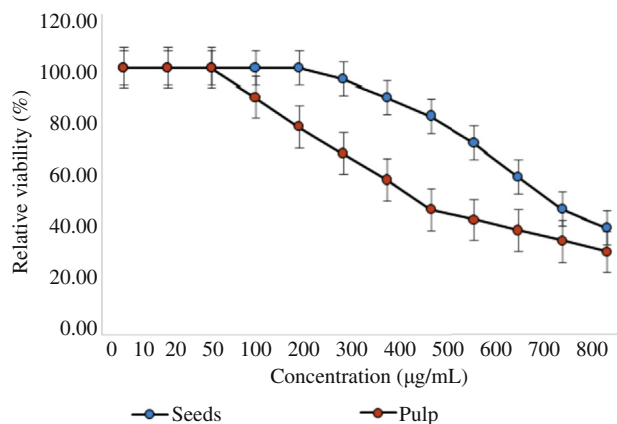
3.2. MIC

The MIC of the methanol extract of the seeds and pulp *M. dubia* on strains of *S. mutans* and *S. sanguinis* was studied. The MIC antibacterial effect of the methanol seeds extract against both strains, could not determine due to an antibacterial activity even at very low concentrations of the extract. However, for the pulp extract, and MIC with a range of 50–75 µg/mL (62.5 µg/mL) was observed for both strains.

3.3. Toxicity of M. dubia extract

The toxicity of the methanol extracts of *M. dubia* was determined using MDCK cell. MDCK cell were incubated with increasing amounts (0–800 µg/mL) of *M. dubia* seeds and pulp extracts and cell viability was determined by MTT method.

Our results showed that the methanol seeds extract could inhibit 50% of the cellular viability at 725 µg/mL, whereas the methanol extract from *M. dubia* pulp can inhibit cellular

4. Discussion

Traditional medicine has been used in several countries over the years, due to its low cost and high effectivity for certain bacterial diseases [5]. However, their clinical relevance and their impact on dentistry have not been fully studied.

Several authors have described the methods used to evaluate *in vitro* susceptibility of bacteria to different agents or extracts. The diffusion technique (on paper or well) is widely used to assess natural extracts with antimicrobial activity and has the advantage that its results are highly reproducible. However, the paper technique has some disadvantages, one of which is the hydrophilic surface, which interferes with some compounds of natural extract and prevents the diffusion of these in the agar. For the well diffusion technique, the extract has a direct contact with the agar, so it is considered as a more sensitive technique and can facilitate the assessment of potential antibacterial agents or any substance interest [13,21]. These features are important and should be considered especially in performing pioneering studies with natural extracts.

Agar diffusion techniques have showed that the methanol extract of *M. dubia* has antimicrobial activity against *S. mutans* and *S. sanguinis*, which demonstrate that methanol is an organic and effective solvent, with a high capacity to extract more phenols and flavonoids as previously described by Leyva *et al.* [22].

In a recent study by Myoda *et al.*, the antibacterial activity of acetone seed and pulp extracts of *M. dubia* was evaluated against strains of *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces*, showing halos of inhibition lower than 4-mm diameter for both acetone extracts, besides no antimicrobial activity against strains of *Escherichia coli* and *Saccharomyces cerevisiae* was observed [9].

On the other hand, in our study, the MIC of the seeds methanol extract of *M. dubia* could not be determined due to an antibacterial activity even at low concentrations; unlike the study mentioned above where the MIC of acetone extract was identified less than 6.0 mg/mL [9].

Today, in the pharmacological field and in the phytotherapeutic medicine, it is important to evaluate the toxicity of natural compounds for their potential impact on public and environmental health [17,18,23]. Therefore, cytotoxicity was determined on seed and pulp extracts of *M. dubia*.

There are several tests for the toxicity determination of various substances. But the MTT assay is an effective method to determine cell viability and has the advantage of being a quantitative method, unlike colorimetric methods, which are qualitative [11,17,24]. Regarding the sensitivity and efficiency of this method, we found studies such as those by Fattahi, who used this method to assess cell viability of cells infected with herpes simplex virus, demonstrating the sensitivity of this method and the simplicity of the assay procedures, therefore allowing the study of a larger number of compounds [19]. Vijayarathna *et al.* used the MTT assay showed that the *Elaeis guineensis* methanol extract had significant cytotoxic effects on MCF-7 cells [25]. We can conclude that it is important to perform cytotoxic studies on natural extracts as a requirement for their use as an alternative medicine in patients.

The methanol seed and pulp extracts of *M. dubia* showed no cytotoxicity against MDCK cell line. These results agree with the study of Rondán *et al.*, who evaluated the subacute toxicity of *camu camu* in an *in-vivo* study, and found no evidence of subacute toxicity in any of the groups exposed on a histopathologic examination [26].

Currently, no studies evaluating the antibacterial activity of *M. dubia* against bacteria of the oral cavity are known. With the results obtained in this study, we observed that methanol extract of seeds and pulp have the antibacterial effect against high prevalence microorganisms in the oral cavity, like *S. mutans* and *S. sanguinis*.

The limitation found in this study was the precarious information regarding the properties and clinical relevance of the *M. dubia* fruit in dental use. Our results point out the importance of conducting more studies for the development of toothpastes containing active compound of *M. dubia* at low cost and easy access, due the abundance of the fruit in the Peruvian Amazon. In addition, the use of the extract as a raw material in the manufacture of drugs is an alternative to the use of synthetic chemicals.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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