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Aqueous Extract of *Rubus ulmifolius* Schott (Rosaceae) against Pathogenic Gram-Positive Oral Bacteria: an in Vitro Evaluation

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Abstract: Different Gram-positive bacteria, *S. mutans*, *S. intermedius*, *S. anginosus*, and *E. faecalis*, are often associated with different oral diseases, such as tooth decay, endodontic infections, and the occurrence of abscesses. A set of *in vitro* evaluations has been performed to assess the aqueous leaf extract of *Rubus ulmifolius* Schott as antimicrobial/antibiofilm activity against the above-mentioned pathogens by agar diffusion method and using a Standard antibiofilm model. The antioxidant activity was measured with the ABTS test. The extract's phenolic and flavonoid quantitative profiles were determined using the Folin-Ciocalteu reagent and a colorimetric method. The results suggested an interesting antimicrobial profile with a formulate range concentration amounting to (6.25 - 0.78) %. The antioxidant activity was with an EC_{50} = (0.0087 ± 0.0009) mg/mL. The total phenolic content, expressed as the equivalent mass of gallic acid with respect to the dry mass of the extract, was (149 ± 17) mg/g, and the total flavonoid concentration, calculated using quercetin as a reference compound, was (60 ± 7) mg/g. Considering that until now, *R. ulmifolius* has not been sufficiently tested for its biological proprieties, these preliminary results encourage additional investigations for its use in the field of oral infections.

Keywords: antibacterial activity; *Enterococcus faecalis*; flavonoid content; oral streptococci; phenolic content; *Rubus ulmifolius*.

1. Introduction

Concerning the dynamics of the human microbiota, the *Streptococcus* and *Enterococcus* genera can show a multitasking pathogenic profile. For example, many species of this genus exhibit opportunistic behavior that can be modulated by the host's immunological and epigenetic status, as well as the eubiotic or dysbiotic status of the tissue microbiota that contains, for example, the *Streptococcus* genera [1]. In this context, these gram-positive strains represent interesting pathogenic bacteria normally isolated in the oral cavity. *Streptococcus intermedius* is the etiological agent implicated in severe human infections, including endocarditis, pneumonia, tissue abscesses, and dentoalveolar infections, and it plays an important role in the initial adhesion to the tooth surface and the assembly of the biofilm in dental implant [2,3]. At the same time, Streptococcus anginosus is normally represented in the normal oral bacterial flora but could cause various infectious diseases, from mild localized infections to life-threatening invasive infections. A failure in infection treatment can result in postinfectious illnesses such as acute rheumatic fever and post-streptococcal glomerulonephritis [4,5].

Streptococcus mutans is involved in dental caries, a chronic multifactorial disease caused by different microorganisms associated with the supragingival plaque microbiota [6–8].

Furthermore, *Enterococcus faecalis* is frequently associated with endodontic infections. It is reported that endodontic treatment failure is due to drug resistance to various antimicrobial compounds [9,10].

These microorganisms are organized into a complex biofilm, producing acids that can destroy tooth enamel, resulting in the complete weakening of tooth structure. In developing countries, this disease is the primary cause of tooth loss. In fact, it is reported that at least 3 billion people worldwide are affected by this infection in the permanent or primary teeth [11]. It's also worth noting that the global prevalence of this oral disease is steadily rising due to dietary changes that include increased consumption of sugary foods. New antibacterial-antibiofilm formulations against these oral pathogens are strongly requested [12]. One of the prophylactic treatments for overcoming these oral infections is the use of mouthwashes and toothpaste [13]. Formulations derived from botanical sources are commonly used to prevent oral infections caused by streptococci. These products, obtained from different vegetable matrices, contain many antibacterial agents such as phenolic compounds, tannins, and flavonoids [10,14,15]. Compared with synthetic-based formulations, they can be less toxic and act on oral cavity pathogens with fewer adverse reactions [16]. For example, synthetic antimicrobial agents such as chlorhexidine (CHX), cetyl pyridinium chloride (CPC), and delmopinol used in the control of oral plaque can cause side effects [17,18]. In addition, other new antimicrobial compounds have recently been reported in the literature to be present in various plant extracts [19] and their antimicrobial-antibiofilm activity against oral microorganisms [20]. For example, using lentisk berry oil, the researchers demonstrated different antibacterial activity patterns within streptococcal species (including *S. mutans* and *S. intermedius*) [5]. Rubus is an intriguing and promising plant genus of the Rosaceae family, with nearly 700 species ranging from sea level to 1100 m in height [21,22]. The perennial shrub *Rubus ulmifolius* Schott is native to Europe and North America [23]. In Sardinia Island, the flowering season occurs between April and June. The fruit's ripening and development form an aggregate of several fleshy drupes, which change color from green to black during ripening [24]. Extracts of this species' leaves and fruits have important biological properties [25–27], and various antimicrobial molecules, such as specific flavonoids and tannin compounds, have been identified [28,29].

In this study, we investigated the antimicrobial activity of these four streptococcal-Enterococcal species as well as the total amount of compound classes, such as phenolics and flavonoids, in the *R. ulmifolius* aqueous extract.

2. Materials and Methods

2.1. Plant material.

Leaves of *Rubus ulmifolius* were collected from the wild in Masullas (OR) (39.7089 N, 8.7739 E) in Southern Sardinia in May 2019. Prof. Andrea Maxia, Department of Life and Environmental Sciences, University of Cagliari, confirmed the identification of plants. The fresh material was air-dried at room temperature for a week. Before being subjected to extraction, the plant material was ground at a Malavasi mill (Bologna, Italy).

2.2. Preparation of plant extract.

About 10 g of ground, dried leaves were added to 120 ml of distilled water. The mixture was left to macerate in the dark, at room temperature, for 48 h. The extract was filtered through Whatman filter paper no. 42, then stored at -20°C until use. The obtained aqueous fraction was lyophilized; then, the powder was diluted in distilled water to 10 mg/mL for total polyphenol and flavonoid content quantification and antioxidant activity.

2.3. Total polyphenol and flavonoid contents.

The total polyphenol content (TPC) in the extract was determined by the Folin-Ciocalteu (FC) method, as previously reported by [30]. The extracted sample at an initial concentration of 10 mg/mL, the 5 % Folin-Ciocalteu's reagent, and the 20 % (w/v) sodium carbonate solutions were prepared with deionized water. Briefly, 0.8 mL of distilled water, 0.005 mL of extract, and 0.05 mL of FC reagent were mixed. Likewise, the blank sample was prepared without the addition of extract samples. After 1 min, a sodium carbonate solution was added to the mixture. The absorbance was measured at a wavelength λ = 750 nm, in a Cary 50 Varian UV–VIS spectrophotometer. The total phenolic content was determined thanks to a calibration curve constructed using gallic acid as the reference compound.

The phenols' content in the extract is presented in terms of gallic acid equivalent (GAE) with respect to the dried weight (dw) of the extract and expressed in mg/g.

The total flavonoid content (TFC) was determined using a colorimetric method. The extract (0.5 mL) was added to 0.02 mL of 10 % aluminum nitrate, 0.02 mL of 1 M sodium acetate solution, and 0.855 mL of 80 % EtOH in water. After 40 min, at room temperature, the absorbance was measured at λ = 415 nm. Quercetin was used as a reference compound to generate a standard calibration curve. The results are expressed in mg/g of quercetin equivalents (QE) with respect to the dried weight of the extract.

2.4. Determination of antioxidant capacity.

The antioxidant activity was expressed by the Free Radical Scavenging Ability using ABTS•+ (derived from 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay, as previously described [31]. 6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (Trolox) was used as the standard reference. The concentration range of extract used for the antioxidant tests was from (0 to 0.010) mg/mL. The antioxidant activity is given as EC_{50} value, indicating the extract concentration required to decrease the ABTS•+ radical cation by 50 %.

2.5. Antibacterial activity.

In this work, we have used (i) *S. intermedius* DSM 20573 (German Collection of Microorganism and Cell Culture), (ii) *S. mutans* CIP103220 (Institut Pasteur Collection), (iii) *S. anginosus*, CCUG 35782, isolated from human dental plaque, Culture Collection University of Gothenburg (CCUG), (iv) *E. faecalis* was a clinical isolate GO2, from endodontic specimens isolated from the Department of Dental Disease Prevention (University of Cagliari).

The experiments followed the EUCAST (http://www.eucast.org) procedures [10].

2.5.1. Agar diffusion test.

A first evaluation was performed using the agar diffusion test (Kirby-Bauer) described in the literature [32]. In brief, each strain (5 10^7 CFU mL) was inoculated onto a Petri dish surface that contained an agarized medium (Schaedler agar- Microbiol, Uta, Cagliari). 0.1 mL of *R. ulmifolius* aqueous extract was put into a well contained in the dish. The Petri plates were then incubated in 5% CO_2, at 37°C, for 24 h. After incubation, the diameter of the inhibition alone was measured. Each experiment was performed in triplicate.

2.5.2. Antibiofilm assay.

Minimum biofilm inhibitory concentration (MBIC) was evaluated following the crystal violet staining protocol described by the Montana University Center for Biofilm Engineering [32]. Each strain was cultured in triplicate on 96-well microplates with different concentrations of aqueous leaf extract in Schaedler Broth; all formulations were analyzed in a dilution range from 50 % to 0.39 %. After 4 days at 37°C in an atmosphere containing 5 % CO_2, the medium was discarded, and the wells were washed three times with a 0.9 % NaCl solution; then 0.1 mL of a 0.4 % crystal violet solution was added to each well; after 10 min the dye was discarded, followed by three washes with 0.9 % NaCl. After an air-drying procedure at 25°C for 15 min, 0.2 mL of 30 % acetic acid was added to each well. The plates were read with a microplate reader at λ = 620 nm (SLT-Spectra II, SLT Instruments, Germany). The MBIC represented the lowest concentration, expressed as a percentage of the ratio mass of extract over total volume (w/v), showing an absorbance comparable with the negative control in the same concentration series. The data showing a standard deviation (SD) within 10 % of the mean value were considered significant.

2.6. Statistical analyses.

Three distinct biological replicas were created for each analysis, and quantitative data were expressed as mean ±SD. All results were compared to a positive control, which was the bacterial strain growing without formulation.

Statistical differences were evaluated using the Graph Pad INSTAT software (GraphPad Software, San Diego, CA, USA). Comparison between data groups was assessed by one–way analysis of variance (ANOVA) followed by the Bonferroni Multiple Comparisons Test (*post hoc* test). The statistical significance of differences was evaluated using Pearson’s chi-square test on antimicrobial activity data.

3. Results and Discussion

3.1. Total phenol content and antioxidant activity.

The total phenolic content, with respect to GAE, was measured to be 149 ± 17 mg/g. The total flavonoid content, with respect to quercetin, was 60 ± 7 mg/g. The *R. ulmifolius* extract contains a considerable amount of total phenols and flavonoids, which could be related to its antimicrobial and antibiofilm activities. The extract showed good antioxidant activity by exhibiting an EC_{50} = 0.0087 ± 0.0009 mg/mL, a value comparable to that obtained for Trolox 0.0013 ± 0.0004 mg/mL. This is a promising result that confirms the potential of the application of *R. ulmifolius*.

3.2. Antimicrobial assays.

After the diffusion susceptibility test, the formulation obtained by maceration in water showed an interesting inhibition profile against orally evaluated Gram-positive bacteria, Table 1. These results could also be related to the phenolic and flavonoid profile of the extract; the data are in accordance with previous studies that link the antibacterial profile of *R. ulmifolius* to phenolic compounds such as ellagic acid [22], gallic acid, ferulic acid, and tiliroside [29,33], even if further investigation could be interesting to evaluate the broad chemical composition of this natural formulate, for example, through an adequate chemical characterization of the extract by using LC-MS/MS.

The antibiofilm analysis, evaluated in microplates through the use of a liquid medium, partially reflected the results suggested by Kirby-Bauer.

Three distinct biological replicas were created for each analysis, and quantitative data were expressed as mean ±SD. All results were compared to a positive control, the bacterial strain growing without formulation.

Figure 1 shows that there was significant biofilm reduction until a limiting concentration (w/v) of 0.025 %; complete biofilm inhibition activity was observed until concentrations ranging from (0.78 to 6.25 %), Table 1.

Table 1. The mean diameter, *ϕ*, of the inhibition zone was determined by agar diffusion test and minimum biofilm inhibitory concentration, MBIC, for *R. ulmifolius* water extract against different bacterial strains.

Strain	Kirby Bauer mm inhibition Ø		MBIC % (w/v)
	Formulation	Positive control*	
S. mutans CIP103220	18	0	0.78
S. intermedius DSM 20573	14	0	1.56
S. anginosus CCUG *35782*	0	0	6.25
E. faecalis GO2	10	0	3.12

Legend: * = bacterial culture without formulation.

In a solution concentration of 0.025 %, the biofilm residue was 47 %, 69 %, 95 %, and 80 % for *S. mutans*, *S. intermedius*, *S. anginosus*, and *E. faecalis*, respectively. This was compared to the positive control (100 %), with an effective reduction range of approximately 53 % to 5 %, respectively. This preliminary work shows a different response inside this bacterial group to the *R. ulmifolius* extract, and further investigations/questions could be interesting to raise, for example, if different cell targets are involved, etc.

Controlling growth inhibition and biofilm formation of these Gram-positive bacteria are important measures to prevent dental caries, drug-refractory endodontic infections, and other severe oral diseases. In this study, the aqueous extract was found to be particularly effective in inhibiting the biofilm of these pathogenic bacteria. Therefore, this extract has the clinical potential to be the active ingredient in mouthwash, toothpaste, and other preparations to prevent oral infections.

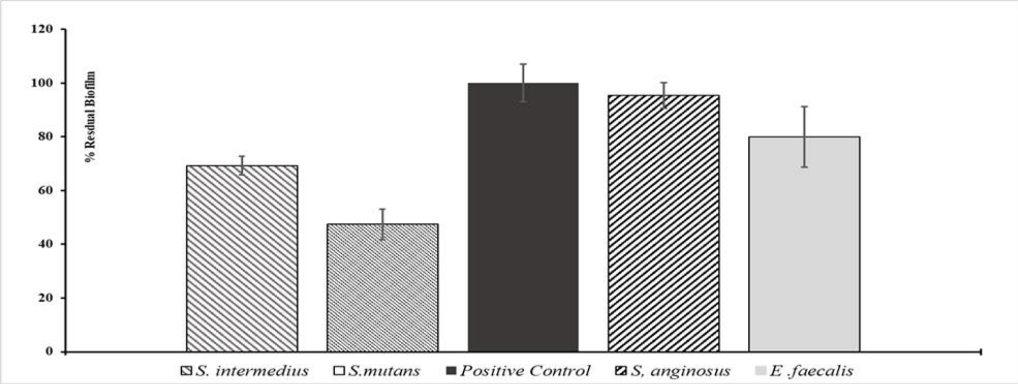

Figure 1. Residual biofilm was determined by using 0.025 % of *R. ulmifolius* water extract. The positive control data represents the mean behavior of each strain grown without formulation.

4. Conclusion

In conclusion, this work showed the antimicrobial and antibiofilm activities of the aqueous extract of *R. ulmifolius* leaves against some Gram-positive pathogenic bacteria. The study demonstrates that the extract is potentially useful as an antimicrobial and anti-biofilm agent in the prevention of oral pathologies caused by *Streptococcus spp*. and *E. faecalis*. The high content of phenols and flavonoids in the extract could be related to its antimicrobial and antibiofilm activities and good antioxidant capacity. These preliminary results can be defined as promising and worthy of further studies in the field of the prevention of dental diseases. However, we consider our work to be a preliminary stage of further investigations, i.e., after a strong chemical-biological characterization of the extract by using plants recruited in different geographical areas and in different months. However, this primary experimental study suggests the potential activity/use of a little-studied plant against oral infections, now considered the basis of severe oral and degenerative systemic diseases.

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Conflicts of Interest

We declare the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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