Biointerface Research in Applied Chemistry
Platinum Open Access Journal (ISSN: 2069-5837)

Article
Volume 12, Issue 1, 2022, 25 - 33
https://doi.org/10.33263/BRIAC121.025033

Fatty Acid Profile and Antimicrobial Activity of *Rubus ulmifolius* Schott Extracts Against Cariogenic Bacterium *Streptococcus mutans*

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Received: 10.03.2021; Revised: 6.04.2021; Accepted: 10.04.2021; Published: 19.04.2021

Abstract: The wild edible species *Rubus ulmifolius* is normally known as a source of several functional-natural compounds used in the traditional diet in several parts of the world. At present, few data are available in the literature about the biological property of its leaves, normally rich in phenolic acids, fatty acids, and other organic compounds with potential antimicrobial activity. Following this hypothesis, we have investigated the antibacterial activity of different dried leaved extracts against the main cariogenic bacterium, *Streptococcus mutans*. Standard antimicrobial-antibiofilm methods (MIC, MBC, MBIC) were performed to evaluate each extract's antimicrobial profile. In addition, the fatty acids (FA) quali-quantitative profile of *R. ulmifolius* leave extracts was assessed by reversed-phase HPLC-DAD/ELSD analysis. The results showed that the behavior of this bacterium with different extracts was strictly related to extraction method type, even though it was not related to fatty acid amount and composition, in fact, all the extracts showed similar, qualitative FA patterns, characterized by a concentration in the range from (25 to 36) % of saturated compounds. The methanolic extract showed the better result as antibacterial MIC 6.25 %. These preliminary results encourage further studies for the use of *R. ulmifolius* in mouthwashes or toothpaste with great anticaries activity.

Keywords: *Rubus ulmifolius;* dental caries; *Streptococcus mutans*; antimicrobial activity; fatty acids.

1. Introduction

Dental caries are an unsolved public health problem. Its etiology is again poorly understood due to complex interaction with host-cariogenic microorganisms. In fact, the oral cavity is the natural home to various microorganisms with potential cariogenic activity. Dental caries is a chronic infectious disease of the oral cavity that is predominantly responsible for tooth loss in children, adults, and tooth-root breakage in the elderly [1,2]. Caries is a biofilm and diet-dependent, and it is characterized by acid damage to the enamel, with a progressive localized demineralization of the enamel with subsequent cavitation and destruction of the teeth

[3]. *Streptococcus mutans* is considered one of the primary etiologic agents for dental caries, and it is responsible for the synthesis of insoluble extracellular matrix through glucans [4]. This pathogen is a Gram-positive facultative anaerobic, which plays an important role in the initial attachment to the tooth surface and biofilm assembly. It produces an abundant insoluble glucan, contributing to dental plaque's physical appearance and biochemical virulence [5,6]. At present, an anticaries drug is strictly required inside the medical and dentistry community because the biofilm structure in the supragingival plaque is high drugs waterproof. In this manner, the cariogenic bacteria are currently protected for antimicrobials and immune system cells [7-10]. In traditional oral-health practice, mouth hygiene is an essential tool to prevent tooth decay and plaque buildup. These prophylactic procedures are associated with antibacterial compounds in addition to mouthwashes and toothpaste. In fact, these formulations regarding aqueous solutions with refreshing, antiseptic properties, some of them for plaque control as it reduce the microbial load in the oral cavity [11-14]. Herbal mouthwashes are in high demand compared to mouthwashes containing synthetic compounds, as the first ones act on pathogens and microbes present in the mouth, reduce pain, and have fewer side effects [11]. It is known in the literature that extracts obtained from plants are characterized by compounds with antimicrobial activity, such as phenolic acids, tannins, and flavonoids [15]. These natural remedies are considered effective substitutes for chemical drugs in minor and medium problems releasing chemical antibacterial for main clinical problems. The active ingredients obtained from plants were currently the subject of in vitro studies to better understand their therapeutic properties and effect on the human body [16-19]. These plant extract's beneficial effect on supra and subgingival microorganisms is proven by various antimicrobial tests [20]. Besides, many oral microorganisms are reported as resistant to antibiotics. There are clinically relevant data on these drug-resistant bacteria residents in the oral cavity. For this reason, different dental associations are providing baseline information to guide antibiotic prescription in dentistry [21]. This serious problem is also reported for *S. mutans* [20]. The *Rubus spp.* includes several species and is the largest of the Rosaceae family [22]. *R. ulmifolius* is a perennial bush, it is largely distributed throughout the world since it has been colonized from Europe to North Africa and spreads from sea level to heights up to 1100 m [22,23]. Some compounds with antimicrobial properties, such as flavonoids and tannins, in this species' leaves and fruits are already known and reported in the current literature [22]. This work aims to investigate the FA profile and the antibacterial propriety of different extracts obtained from leaves of *R. ulmifolius*.

2. Materials and Methods

2.1. Plant material.

Leaves of *R. ulmifolius* were collected in the area of Masullas (OR) (39.7089 N, 8.7739 E) in May 2019. The plant was identified by Prof. Andrea Maxia, Department of Life and Environmental Sciences, University of Cagliari. The plant material was air-dried at room temperature for seven days; then, it was ground in a blender. Vegetable material was subjected to three different extraction methodologies.

2.2. Chemicals.

All the chemicals used in this study were of analytical grade. Standards of fatty acids (FA) and high purity solvents were purchased from Sigma–Aldrich (Milan, Italy).

2.3. Supercritical fluid extraction.

Supercritical Fluid Extraction (SFE) was performed in a laboratory apparatus. Extraction – in the semi-batch mode – was carried out by means of a continuous flow of CO_2 (purity 99 % - Air Liquide Italia, Cagliari, Italy) through a fixed bed (V = 320 mL) of the vegetable material. About 136 g of *R. ulmifolius* leaves were charged in each run. Operative conditions were: 300 bar and 40 °C in the extraction section and 20 bar and 15 °C in the separator. The obtained extract was labeled F1.

2.4. Solvent extraction.

Solvent extractions were performed using 10 g of each sample. The plant powder was transferred into a cellulose extraction thimble and inserted into a Soxhlet assembly fitted with a 100 mL flask. A 60 mL portion of *n*-hexane was added, and the whole assembly was heated for 6 h at 69 °C, the boiling temperature of the solvent. The extracts were concentrated using a rotary evaporator at 40 °C, and the dry extracts obtained, labeled F2. Plant materials (10 g) were macerated in 120 mL of ethanol or methanol for 48 h at room temperature. After filtration, the ethanol and methanol extracts were concentrated, using a rotary evaporator to give extract F3 and F4, respectively. All samples intended for chemical and biological characterization were stored at +4 °C until their use.

2.5. Oil saponification and analysis of oil fatty acids.

Extracts F1-F4 (2 mg, in EtOH solution) obtained from *R. ulmifolius* leaves by different extraction methods were subjected to mild saponification as previously reported [24]. Analyses of total FA after oil saponification were carried out with an Agilent Technologies 1100 HPLC (Agilent Technologies, Palo Alto, CA) equipped with a Diode-Array Detector (DAD) and an Infinity 1260 Evaporative Light Scattering Detector (ELSD). Unsaturated FA (UFA), detected at a wavelength λ, of 200 nm, and saturated FA (SFA) were eluted with $CH_3CN/H_2O/CH_3COOH$ (75/25/0.12, v/v/v) as mobile phase at a flow rate of 2.3 mL/min, using an XDB-C18 Eclipse column as previously reported. Recording and integration of the chromatogram data were carried out through an Agilent OpenLAB Chromatography data system. The identification of FA was performed using standard compounds and UV spectra (for UFA). Calibration curves of FA were constructed using standards and were found to be linear (DAD) and quadratic (ELSD) (correlation coefficients > 0.995). The FA quali-quantitative profile of *R. ulmifolius* leaves extracts was assessed by reversed-phase HPLC-DAD/ELSD analysis.

2.6. Antibacterial activity.

2.6.1. Agar diffusion test.

Streptococcus mutans CIP103220 (Institut Pasteur Collection) was used in this work. Prior to use, this strain was stored at -80 °C in a tube containing Schaedler Broth with 20 % glycerol. The experiment was performed in triplicate following the EUCAST (http://www.eucast.org) procedures [13]. A first evaluation, as antibacterial property, was performed by using the agar diffusion test (Kirby-Bauer), already previously described in the literature [10]. Briefly, the strain (5 10^5 CFU mL) was inoculated onto a Petri dish surface that contained an agarized medium (Schaedler agar- Microbiol, Uta, Cagliari). 0.05 mL of extract

was put into a well contained in the culture medium. The Petri dishes were incubated in 5 % CO_2 at 37 °C. After 24 h incubation time, the inhibition diameter was measured. The experiment was performed in triplicate for each extract.

2.6.2. Broth dilution tests, MIC, and MBC.

MIC (minimum inhibitory concentration) and (CMB) minimum bactericide concentration were assessed by a procedure described in the Clinical & Laboratory Standards Institute (CLSI) protocols [25]. We have used sterile Nunc™ Microwell™ 96-well microplates (Thermo Fisher Scientific) and in these conditions, each leave extract was diluted by two-fold serial dilutions with Schaedler Broth from (50 to 0.04) %. For this bacterium, 10^7 CFU/mL liquid suspension was prepared from the standardized inoculum in Schaedler broth; 0.02 mL of this suspension was put into each well to obtain a final concentration of 10^6 CFU/mL. The culture was incubated at 37 °C and 5 % CO_2 for 24 h. The minimum bactericidal concentration (MBC) was determined by culturing in Schaedler agar of 0.15 mL of two or more concentrated dilutions from MIC. After 24-48 hours of incubation at 37 °C with 5 % CO_2 the colony-forming units (CFUs) were enumerated. The MBC was the lowest concentration able to reduce the bacterial growth of 99.9 % in CFU/mL, when compared to the MIC dilution, Table 1.

2.6.3. Antibiofilm assay.

Minimum biofilm inhibitory concentration (MBIC) was evaluated following the "crystal violet staining protocol" published by the Montana University Center for Biofilm Engineering [10]. The *Streptococcus* strain was cultured on 96-well microplates with different concentrations of *R. ulmifolius* leave extracts in Schaedler Broth. After 4 days of incubation at 37 °C with 5 % CO_2, the liquid medium was discarded. Each sample was gently washed three times with a NaCl 0.9 % solution Then, a 0.1 mL of crystal violet solution (0.4 %) was added to each well. After 10 min, the colorant was discarded. Following three washes with 0.9 % NaCl and an air-drying procedure at 25 °C for 15 min, an addition of 0.2 mL of 30 % acetic acid was finally performed. The plates were read with a microplate reader at λ = 620 nm by Multiskan™ FC Microplate Photometer (Thermo Scientific™). The MBIC represented the lowest concentration showing an absorbance comparable with the negative control (sample without bacteria). Were considered significant, in the same concentration series, the data showing a Standard deviation (SD) within 10 % of the mean value (experiment performed in triplicate).

2.7. Statistical analyses.

Graph Pad INSTAT software (GraphPad Software, San Diego, CA, USA) was used to evaluate statistical differences. Comparison between data groups was assessed by one–way analysis of variance (ANOVA) followed by the Bonferroni Multiple Comparisons Test (post hoc test). The values with $p < 0.05$ were regarded as statistically significant. The statistical analysis was performed by using Pearson's chi-square test for antimicrobial activity. All experiments were performed in triplicate.

3. Results and Discussion

3.1. Fatty acid composition of leave extracts.

Values of concentration (expressed as mg/g of dried extract) of the main saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) FA measured in F1-F4 extracts are shown in Figure 1. All the extracts showed similar qualitative FA patterns, characterized by a concentration in the range from (25 to 36) % of saturated FA (SFA), mainly palmitic acid (16:0) and stearic acid (18:0); from (3 to 5) % of monounsaturated FA (MUFA), oleic acid (18:1 n-9); and from (60 to 70) % of polyunsaturated FA (PUFA), consisting of the essential FA linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3). Significant differences among different extracts were observed in the absolute FA amounts. The main fatty acid of all *R. ulmifolius* leave extracts was 18:3 n-3 that averaged (185 ± 16) mg/g, (64 ± 18) mg/g, (8.05 ± 0.09) mg/g, and (53.98 ± 0.02) mg/of dry weight in F1, F2, F3, and F4 extracts, respectively. F1 extract obtained by SFE extraction showed the highest total FA amount (352.58 mg/g of dry weight), followed by F2>F4>F1, Figure 1. Fatty acid (FA) composition (expressed as mg/g of died extract) determined by HPLC-DAD/ELSD analysis of *R. ulmifolius* leave extracts F1-F4 after saponification. The oil analysis was performed in triplicate, and all data are expressed as mean values ± standard deviations (SD); (n = 3). Different letters (^{a,b,c}) reported on each mean value of FA represent statistically significant differences ($P < 0.05$) between extracts (One–way ANOVA followed by the Bonferroni Multiple Comparisons Test).

3.2. Antibacterial activity.

3.2.1. Agar diffusion test.

Following the diffusion-susceptibility test, three formulates obtained by (i) supercritical extraction, (ii) Soxhlet in *n*-hexane, and (iii) methanol extract, showed a significative inhibition profile against *S. mutans*. But this Kirby-Bauer analysis showed that ethanol extract did not demonstrate appreciable antimicrobial activity against this pathogen, Figure 2. These results seem not to be correlated to the formulates fatty acid profile. As shown in Figure 1, the most active methanolic extract contained fatty acid concertation from 50-to 150-fold lower respect other analyzed formulates. These data are in accordance with previous studies mainly reporting the antibacterial profile of *R. ulmifolius* to phenolic compounds [26], i.e., ellagic acid [27], quercetin-3-O-beta-D-glucuronide; kaempferol-3-O-beta-D-glucuronide, gallic acid, ferulic acid, tiliroside [28], and Rubanthrone A [29]. For this reason, the ethanol extract was excluded for MIC-CMB and MBIC valuation.

3.2.2. MIC & MBC values of active *R. ulmifolius* extracts.

Bacterial inhibition and bactericidal activity were observed for an extract concentration >50 % for supercritical and Soxhlet *n*-hexane procedures. Only the methanolic extract demonstrated interesting MIC and MBC values, 6.25 % and 25 %, respectively, Table 1. This experiment is in accordance with Tabarki *et al*., which described the activity of leaves extracted with methanol against different nonoral human pathogens [26], and with Martini *et al*., which cited an activity versus *Helicobacter pylori* [30]. However, these results are innovative in this context because *R. ulmifolius* extracts are not mentioned yet in literature as anti-*S. mutants*. In the same way, no existing data on *R. ulmifolius* leaves and their antibiofilm activity.

3.2.3. Minimum biofilm inhibition concentration (MBIC).

Results shown in Table 1 demonstrated that a complete Biofilm inhibition activity was observed until 1.56 % for all three formulates. But an appreciable biofilm reduction was observed until the extract concentration of 0.05 %, Figure 3. This evaluation has again shown the methanol base extraction is most effective compared to other studied methods. In this case, the biofilm residual was 28 % compared to the positive control (100 %), with an effective reduction of about 72 %. The other formulates demonstrated a biofilm residual of 56 % and 62 %, respectively, for supercritical and Soxhlet *n*-hexane extraction methods. Following this study's aim, an "antibiofilm formation inhibition evaluation" for an anticaries candidate is strictly crucial. In fact, the carious disease could be represented as a "Biofilm related disease", and normally the medical – dentistry practice provides for three different steps of prophylaxis: (i) supragingival plaque growth control by using antimicrobials, i.e., mouthwashes, toothpaste (ii) resilient plaque mechanical remoting (iii), avoid the plaque reformation by antiplaque, antibacterial toothpaste, etc. [31,32].

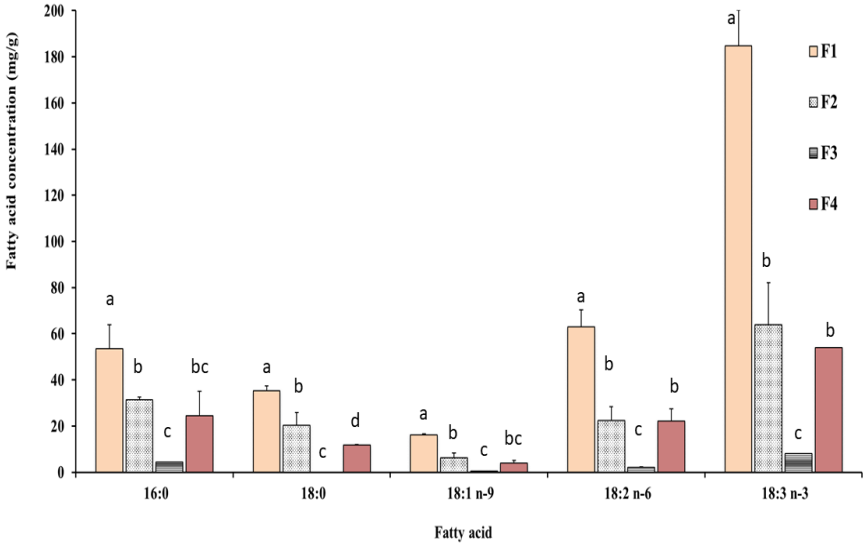

Figure 1. Fatty acid concentration related to *R. ulmifolius* extracts studied in this work. Supercritical extraction (F1), solvent extraction by maceration with methanol extract (F3) and ethanol extract (F4) and by Soxhlet apparatus in n-hexane (F2).

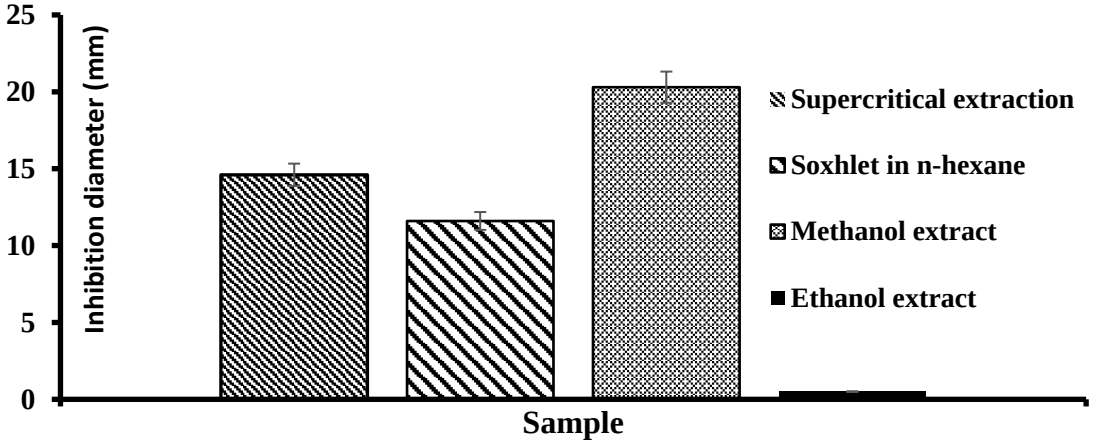

Figure 2. Antimicrobic sensitivity testing (Kirby Bauer) of 4 different *R. ulmifolius* extracts against cariophatogen bacterium *S. mutans*.

Table 1. *S. mutans* MIC, MBC, and MBIC values observed with active *R. ulmifolius* extracts.

Strain	Supercritical extraction			Soxhlet in *n*-hexane			Methanol extract		
	% W/V			% W/V			% W/V		
	MIC	MBC	MBIC	MIC	MBC	MBIC	MIC	MBC	MBIC
S. mutans CIP103220	>50	>50	1.56	>50	>50	1.56	6.25	25	1.56

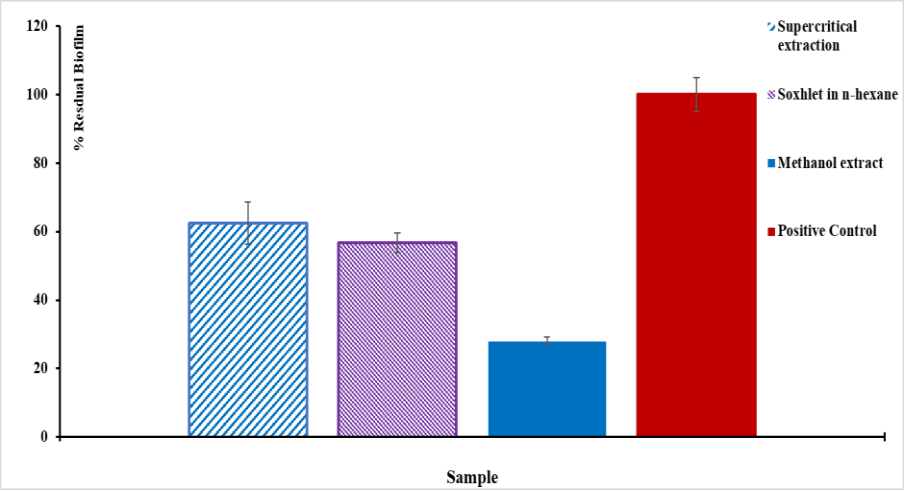

Figure 3. Biofilm residual by using 0.05 % of 3 different *R. ulmifolius* extracts.

4. Conclusions

Tooth caries is often related to bad alimentary and lifestyle habits and, on the other hand, to poor socio-economic condition. Many pieces of research aim at the discovery or synthesis of an ideal anticaries drug. At present, such pathology, related to a polymicrobial biofilm, imposes mechanical removing as the only temporary effective therapy against this health problem, and an antimicrobial procedure with innovative antibacterial compounds is strongly requested for tooth plaque control.

In this work, we report the activity of leave extracts of *R. ulmifolius* – obtained by different extraction methods – against the cariopathogen bacteria *S. mutants*, the main microorganism involved in caries formation and progression. The experimental results showed that methanolic extraction was related to a better antimicrobial activity, even if all extracts showed an interesting antibiofilm activity. The FA profile showed similar qualitative patterns between different extracts. Significant differences among different extracts were observed only in the absolute FA amounts. In this context, the preliminary results obtained can be defined as promising and worthy of further study in the field of oral microbiology.

Funding

This research received no external funding.

Acknowledgments

The authors acknowledge Dr. Sonia Casu for the fundamental contribution to the retrieval and collection of plant material.

Conflicts of Interest

The authors declare that they have no competing interests

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