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Corresponding Author:	Marlise Klein, Ph.D. Universidade Estadual Paulista Julio de Mesquita Filho Araraquara, Sao Paulo BRAZIL
Corresponding Author's Institution:	Universidade Estadual Paulista Julio de Mesquita Filho
Corresponding Author E-Mail:	marlise.klein@unesp.br
Order of Authors:	Sabrina Marcela Ribeiro
	Érick D. O. Fratucelli
	Júlia M. Fernandes
	Paula C. P. Bueno
	Alberto José Cavalheiro
	Marlise Klein, Ph.D.
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TITLE:

Systematic Approach to Identify Novel Antimicrobial and Antibiofilm Molecules from Plants' Extracts and Fractions to Prevent Dental Caries

AUTHORS AND AFFILIATIONS:

Sabrina M. Ribeiro[1]*, Érick D. O. Fratucelli[1]*, Júlia M. Fernandes[2]*, Paula C. P. Bueno[2,3], Alberto José Cavalheiro[2], Marlise I. Klein[1]

[1] Department of Dental Materials and Prosthodontics, São Paulo State University (UNESP), School of Dentistry, Araraquara, São Paulo, Brazil

[2] Department of Organic Chemistry, São Paulo State University (UNESP), Institute of Chemistry, Araraquara, São Paulo, Brazil

[3] Faculty of Pharmaceutical Sciences of Ribeirão Preto (FCFRP-USP), Department of Physics and Chemistry, University of São Paulo, Ribeirão Preto/SP, Brazil.

* These authors contributed equally.

Email addresses of co-authors:

Sabrina M. Ribeiro (sabrina.ribeiro@unesp.br)
Érick D.O. Fratucelli (erickdante121@gmail.com)
Júlia M. Fernandes (fernandesjm@outlook.com)
Alberto José Cavalheiro (alberto.j.cavalheiro@unesp.br)
Paula C. P. Bueno (paulabueno@yahoo.com)

Corresponding authors:

Marlise I. Klein (marlise.klein@unesp.br)
Alberto José Cavalheiro (alberto.j.cavalheiro@unesp.br)

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Microbiology, biofilm, natural products, mutans streptococci, antimicrobials, dental caries, drug discovery, biological approaches

SUMMARY:

Natural products represent promising starting points for the development of new drugs and therapeutic agents. However, due to the high chemical diversity, finding new therapeutic compounds from plants is a challenging and time-consuming task. We describe a simplified approach to identify antimicrobial and antibiofilm molecules from plant extracts and fractions.

ABSTRACT:

Natural products provide structurally different substances, with a myriad of biological activities. However, the identification and isolation of active compounds from plants are challenging because of the complex plant matrix and time-consuming isolation and identification procedures. Therefore, a stepwise approach for screening natural compounds from plants, including the isolation and identification of potentially active molecules, is presented. It includes the collection

of the plant material; preparation and fractionation of crude extracts; chromatography and spectrometry (UHPLC-DAD-HRMS and NMR) approaches for analysis and compounds identification; bioassays (antimicrobial and antibiofilm activities; bacterial "adhesion strength" to the salivary pellicle and initial glucan matrix treated with selected treatments); and data analysis. The model is simple, reproducible, and allows high-throughput screening of multiple compounds, concentrations, and treatment steps can be consistently controlled. The data obtained provide the foundation for future studies, including formulations with the most active extracts and/or fractions, isolation of molecules, modeling molecules to specific targets in microbial cells and biofilms. For example, one target to control cariogenic biofilm is to inhibit the activity of *Streptococcus mutans* glucosyltransferases that synthesize the extracellular matrix' glucans. The inhibition of those enzymes prevents the biofilm build-up, decreasing its virulence.

INTRODUCTION:

The earliest models of medicine used in societies were based on natural products (NPs). Since then, humans have been searching for new chemicals in nature that can be transformed into drugs[1]. This search caused a continuous improvement of technologies and methods for ethnobotanical screening[1-3]. NPs offer a rich source of structurally diverse substances, with a wide range of biological activities useful for developing alternative or adjuvant therapies. However, the inherent complex plant matrix makes the isolation and identification of the active compounds a challenging and time-consuming task[4].

NPs-based drugs or formulations can be used to prevent and/or treat several conditions affecting oral, including dental caries[4]. Dental caries, one of the most prevalent chronic diseases globally, derives from the interaction of sugar-rich diet and microbial biofilms (dental plaque) formed on the tooth surface that leads to demineralization caused by organic acids derived from microbial metabolism, and if not treated, leads to teeth loss[5,6]. Although other microorganisms may be associated[7], *Streptococcus mutans* is a critical cariogenic bacterium because it is acidogenic, aciduric, and an extracellular matrix builder. This species encodes multiple exoenzymes (e.g., glycosyltransferases or Gtfs) that use sucrose as a substrate[8] to build the extracellular matrix rich in exopolysaccharides, which are a virulence determinant[9]. Also, the fungus *Candida albicans* can drive up the production of that extracellular matrix[7]. Albeit fluoride, administered in various modalities, remains the basis for preventing dental caries[10], new approaches are needed as adjuvants to increase its effectiveness. In addition, the available anti-plaque modalities are based on the use of broad-spectrum microbicidal agents (e.g., chlorhexidine)[11]. As an alternative, NPs are potential therapies for controlling biofilms and preventing dental caries[12,13].

The further advance in the discovery of new bioactive compounds from plants includes necessary steps or approaches such as: (i) the use of reliable and reproducible protocols for sampling, considering that plants often show intraspecific variability; (ii) the preparation of comprehensive extracts and their respective fractions in small scale; (iii) the characterization and/or dereplication of their chemical profiles thought the acquisition of multidimensional data such as GC-MS, LC-DAD-MS, or NMR, for example; (iv) the use of viable and high-yield models to assess bioactivity; (v) the selection of potential new hits based on multivariate data analysis or other statistical tools; (vi) to perform the isolation and purification of the targeted compounds or

promising candidates; and (vii) the validation of the corresponded biological activities using the isolated compounds[2,14].

Dereplication is the process of rapidly identifying known compounds in crude extract and allows differentiating novel compounds from those that have already been studied. Besides, this process prevents isolation when bioactivity has already been described for certain compounds, and it is particularly helpful to detect "frequent hitters". It has been used in different untargeted workflows ranging from major compound identification or the acceleration of activity-guided fractionation up to the chemical profiling of collections of extracts. It can be fully integrated with metabolomic studies for the untargeted chemical profiling of CE or the targeted identification of metabolites. All of this ultimately leads to prioritizing extracts before the isolation procedures[1,15,16,17].

Therefore, in the present manuscript, we describe a systematic approach to identify antimicrobial and antibiofilm molecules from plant extracts and fractions. It includes four multidisciplinary steps: (1) collection of plant material; (2) preparation of crude extracts (CE) and fractions (CEF), followed by their chemical profile analysis; (3) bioassays; and (4) biological and chemical data analyses (**Figure 1**). Thus, we present the protocol developed to analyze of the antimicrobial and antibiofilm activities of *Casearia sylvestris* extracts and fractions against *Streptococcus mutans* and *Candida albicans*[13], as well as the procedures for the phytochemical characterization and data analysis. For simplicity, the focus here is to demonstrate the approach for screening natural compounds using the bacterium.

[Place Figure 1 here].

PROTOCOL:

1. Collection of Plant Material

1.1. Plant material

1.1.1. Record the access to the plant material on electronic platforms that regulate access to genetic heritage in the country where the collection will take place. For example, in Brazil, register with the National System for the Management of Genetic Heritage and Associated Traditional Knowledge – SisGen (website https://sisgen.gov.br/paginas/login.aspx).

1.1.2. Collect samples of the plant material of interest (e.g., leaves, stems, roots, flowers, fruits). Register if the material was collected during the reproductive or the vegetative phase.

1.1.3. Record the collection parameters (date, georeferencing, average annual temperature, and mean humidity percentage).

1.1.4. Identify the samples precisely, and a taxonomist must confirm the authenticity.

1.2. Plant samples stabilization and storage

1.2.1. Separate plant organs in individual plastic bags or flasks immediately after collections.

1.2.2. Inactivate potential enzymatic reactions by (i) freezing immediately in liquid nitrogen, (ii) dehydrating in a circulating air oven (40 °C), or (iii) freeze-drying the samples by lyophilization.

1.2.3. Store the stabilized material in hermetically sealed bags at room temperature or in a freezer until use (-20 or -80 °C, depending on the storage period or intended use).

1.2.4. Grind the samples in an analytical mill (knife or ball, depending on the tissue type or availability) and standardize particle size using standardized sieves.

1.2.5. Weigh the samples individually for the subsequent extraction steps.

2. Preparation of Crude Extracts (CE) and Fractions (CEF) to Chemical Profile Analysis and Bioassays

2.1. Preparation of Crude Extracts (CE)

NOTE: The steps are illustrated in the flow-chart in **Figure 2A**.

2.1.1. Prepare an extraction solvent with a hydroalcoholic mixture (e.g., ethanol (EtOH) 70% or ternary mixtures of water, EtOH, and other modifiers, defined by the experimental design of according to previous reports).

2.1.2. Use the ratio sample weight (dry weight, mg)/ extraction solvent (mL) varying from 50 to 100 mg for each mL of solvent.

2.1.3. For fast and reproducible extractions, use batch extractions using microtubes.

NOTE: At least three replicates should be used at this point to allow statistical analysis.

2.1.4. Perform ultrasound-assisted extractions (UAE) to make it quick, easy, and cheap.

2.1.5. Repeat the procedure three times (15 min each) for the best efficiency.

2.1.6. After each extraction step, decant the solid residue by centrifugation and remove the supernatant.

2.1.7. Combine the supernatants individually, filter, and save aliquots for simultaneous chemical analysis and bioassays. If necessary, remove the extracting solvent under vacuum, nitrogen flow, or lyophilization, and register the weigh and yield.

2.1.8. Store at -20 °C protected from light.

NOTE: The protocol can be paused here to screen the CE and select the ones that present the desired activity.

2.2. Fractionation of Crude Extracts (CEF)

NOTE: The steps are illustrated in the flow-chart in **Figure 2B**.

2.2.1. Use cartridges with at least 1 g of adsorbent. If alkaloids are potentially present in the CE, use solvents containing 0.1% of formic acid (FA).

2.2.2. Dilute sample in the most suitable solvent (or solvent mixture) to obtain a 100 mg/mL sample solution. Then, transfer 1 mL of the sample solution to a pre-conditioned a solid-phase extraction cartridges - SPE (1 g of adsorbent, 6 mL of capacity).

2.2.3. Perform the fractionation using around three dead volumes of each extraction eluent (to a cartridge of 1 g, it will correspond to 2 mL of each solvent). If alkaloids are potentially present in the CE, use solvents containing 0.1% of FA.

2.2.4. Collect one fraction by eluent composition and save aliquots for simultaneous chemical analysis and bioassays.

2.2.5. Remove the solvent under vacuum, nitrogen flow, or lyophilization and register the weigh and yield.

NOTE: If the CE is difficult to dissolve in the initial elution mixture, disperse the CE in a solid phase (e.g., C18 or celite) in 1:1 (w/w) proportion before loading the material on the top of the cartridge.

CAUTION: Weigh the microtubes in advance to calculate the mass yield of extracts and fractions.

2.3. **Chemical profiling analysis**

NOTE: Considering that each plant species requires optimized and specific methods for its chemical analysis, in the following sections, we describe the most common analytical approaches used to analyze plant materials. As a practical example, a reverse-phase liquid chromatography was developed and validated for the simultaneous analysis of phenolic compounds and clerodane-type diterpenes differentially biosynthesized by two varieties of *Casearia sylvestris* Swartz (Salicaceae). The UPLC-DAD apparatus was equipped with a degasser, a quaternary pump, an automatic sampler, a UV-Vis photodiode array detector, and an oven (see details in Bueno et al. 2015[18]). Similar approaches described in the following examples can be optimized according to other plant species and/or plant materials.

2.3.1. Chromatographic analysis and hyphenation possibilities

2.3.1.1. For separations using ultra-high-performance liquid chromatography (UPLC), use a C_{18} chromatographic column (e.g., 150 × 2.1 mm, 2.6 µm, 100 Å) protected by a compatible pre-column.

NOTE: Other column phases or chromatographic modes can be used depending on the plant species/material. Conventional HPLC can also be used; in that case, a suitable chromatographic column must be chosen. To achieve excellent separations, adjust the chromatographic conditions considering the flow rate (µL/min), column temperature (°C), and injection volume (µL). The mobile phase usually consists of water (A) and acetonitrile or methanol (B) using linear or multistep elution gradients or isocratic elution. Modifiers such as buffers, acids, bases, or others can also be used.

2.3.1.2. Perform the plant material analysis (CE and/or CEF) and register all related data, such as spectral data (using a UV-Vis and/or, preferentially, MS detectors), retention time (min), and others, depending on the available hyphenation.

NOTE: Liquid chromatography (LC) is usually hyphenated (coupled) with high-resolution mass spectrometry (HRMS), as LC−HRMS, and is commonly use for the rapid annotation of metabolite in CE ou CEF[15].

2.3.1.3. If qualitative data is required and quantitative data is necessary, carefully prepare and inject calibration curves, following the same protocol.

NOTE: The development of the best chromatographic conditions can be performed with the assistance of the design of experiments, as described by Bueno et al. 2015[18], or similar literature. It is important to consider the inclusion of internal standards during the method development. They are very much appreciated since they allow correct technical deviations during sample preparation and injections, and further normalization for data analysis.

2.4. Univariate and multivariate data analysis

2.4.1. Export the registered chromatograms in a suitable format (e.g., ASCII, .txt. or .csv format). A single data matrix can be set by joining and aligning the chromatograms if several samples are being analyzed, and comparisons are required. The resulting matrices must be normalized the chromatograms according to the internal standard used.

2.4.2. Analyze the plant metabolomics data using multivariate and univariate methods. Explore and visualize metabolomics datasets through the simultaneous analysis of multiple variables using multivariate statistical methods, including unsupervised principal component analysis (PCA) and hierarchical clustering analysis (HCA), or supervised partial least squares analysis (such as PLS, OPLS, PLS-DA). Univariate methods, such as ANOVA, Student's, Tukey and Welch's t-test, are especially interesting for the precise analysis of quantitative differences among samples[19].

2.5. **Dereplication and compounds annotation**

NOTE: The objective of this step is dedicated to rapid on-line identification of known NPs to avoid tedious isolation that can be performed simultaneously to the uni- or multivariate data analysis.

2.5.1. Perform the identification levels of the detected or target compounds:

(i) Identified compound, including full 3D structure and stereochemistry (level 0);

(ii) Identification achieved by two orthogonal parameters, such as retention time and MS/MS spectrum (level 1);

(iii) Putatively annotated compounds and compound classes (levels 2 and 3);

(iv) Unidentified or unclassified metabolites that can be differentiated based on analytical data (level 4)[19,20].

2.5.2. Characterize the known compounds using the commercial or public databases. Among the most important databases, it can be highlighted: NIST (https://www.nist.gov), Wiley (https://www.sisweb.com/software/ms/wiley.htm), MassBank (https://massbank.eu/MassBank/), GMD (http://gmd.mpimp-golm.mpg.de/), METLIN (https://metlin.scripps.edu), and the Global Natural Products Social Molecular Networking – GNPS database (https://gnps.ucsd.edu)[19].

NOTE: There are different levels of annotations and they depend on the hyphenated technique employed during the study and may include: the assistance of MS- (or NMR) based spectral databases, and in silico spectral prediction algorithms.

2.5.3. Isolation, purification, and complete compound identification

NOTE: If a given compound (whose identity was suspected by the statistical methods) needs complete structural identification, the first step to accomplish this task is to isolate and purify the desired compounds in a greater scale. It can be accomplished by scaling up the already developed protocols.

2.5.3.1. Perform the rapid and direct isolation of the target compound (s) by preparative chromatographic techniques well established and optimized. Semi-preparative HPLC with dry load injection can be used to avoid the compromises that generally need to be made between high loading and sample solubilization [1].

2.5.3.2. Accomplish the complete structural characterization and identification of isolated compounds. This can be made through the combination of different techniques:

(i) Nuclear Magnetic Resonance (NMR);

(ii) Mass spectrometry (MS);

(iii) Spectrometric techniques in the ultraviolet (UV) and infrared (IR) regions are also very useful for the characterization of the functional groups;

(iv) The use of chiroptical spectroscopy such as electronic and vibrational circular dichroism

(ECD and VCD, respectively), Raman optical activity (ROA), and X-ray crystallography are important techniques for absolute configuration characterization.

3. Bioassays

NOTE: Biological screening: To quickly assess CE and CEF's potential bioactivity, the initial screening of natural substances should be organized and straightforward.

3.1. Preparation of CE and CEF for bioassays

3.1.1. Reconstitute the dry matter with the best possible solvents (which can be determined experimentally). The experimental design[21, 22] define the stock solution and the concentration of solvents.

3.1.2. Calculate the solvent concentration of the stock solution. To do this, use the formula: $C_1 \times V_1 = C_2 \times V_2$, where C_1 represents the stock solution (mg) of CE and/or CEF; V_1 represents the volume of solvent; C_2 is the weight of the CE and/or CEF; V_2 is the final volume (mL) of the stock solution.

NOTE: For example, we selected 84.15% EtOH and 15% dimethyl sulfoxide (DMSO) as the solvent concentration of the stock solution. We prepared the stock concentration of the CE to 6 mg/mL and the CEF to 1 mg/mL[13]. The solvent to dilute the CE and CEF will depend on the method of evaluation of the biological activity. The solvent used as a vehicle must not interfere with biological and toxicological activity. Typically, water, DMSO, EtOH, or an aqueous solvent-based on EtOH is used to solubilize plant extracts or plant derivatives[4,13].

3.2. Preparation of test organisms

3.2.1. Reactivate a microbial strain, for example *S. mutans* UA159 on blood agar (48 h, 37 °C, 5% CO_2), and culture it in liquid culture medium (e.g., tryptone-yeast extract broth [TYE: 2.5% (w/v) tryptone with 1.5% (w/v) yeast extract] containing 1% glucose (w/v) (TYEg) for 16 h, 37 ^{o} C, 5% CO_2.

3.2.2. Perform a 1:20 dilution of the initial culture of the microorganism in the same culture medium (the dilution ratio of the initial culture may change according to the experimental design).

3.2.3. Incubate until it reaches the mid-log growth phase.

3.2.4. Prepare the inoculum for bioassays with a defined population (e.g., $2x10^6$ colony-forming units per milliliter - CFU/mL) in TYEg for antimicrobial assays and TYE with 1% sucrose (w/v) (TYEs) for biofilms assays.

NOTE: The growth conditions will depend on the microorganism tested.

3.3. Antimicrobial activity

NOTE: The steps are illustrated in **Figure 3**.

3.3.1. In a 96-well plate, add an aliquot (µL) of the CE and/or CEF stock solution (treatments). The volume of the aliquot is defined by the test concentration, which must be selected based on previous studies. For example, to test CE at the 0.5 mg/mL test concentration, use a 16.67 µL aliquot of the stock solution at 6 mg/mL. For this calculation, use the formula: $C_1 \times V_1 = C_2 \times V_2$, where C_1 is the stock concentration, V_1 is the volume of the stock solution aliquot, C_2 is the test concentration, and V_2 is the volume of the 96-well plate (which corresponds to 200 µL). In this experimental condition, the test concentration of the solvents (vehicle) will be 7% EtOH and 1.25% DMSO.

3.3.2. Include a set of controls for each plate: a column with treatments, without the inoculum (blank control per treatment, help to differentiate turbidity by the treatment used itself from microbial growth); a column with vehicle and inoculum (diluent of CE or CEF or 0 mg/mL control); a column with only the culture medium (culture medium control) and a column with only inoculum (microbial growth control).

3.3.3. Using TYEg, adjust the volume to 100 µL. Next, incubate, for example, 24 h, 37 °C, 5% CO_2 (depending on the microorganism tested).

3.3.4. Inoculate 100 µL of microorganism inoculum (1x 10^6 CFU/mL) into the 96-well plate.

3.3.5. Analyze the bacterial growth according to turbidity by visual inspection of the wells (clear or cloudy). Clear: means that there is no visual growth of the microorganism. Cloudy: means that there is visual growth of the microorganism.

3.3.6. Measuring the absorbance (optical density or O.D.) of the bacterial culture in each well (ELISA reader using 540 nm). Next, transfer 100 µL of the cultures to microtubes containing 900 µL of saline solution (0.89% NaCl), mix well by vortexing. Next, continue performing a ten-fold serial dilution until the desired value.

3.3.7. Inoculate an aliquot of the desired dilution in specific agar plates (in duplicate). For example, 10 µL of a specific dilution on blood agar plates.

3.3.8. Incubate. Conditions can change between microorganisms, for example, *S. mutans*: 48 h, 37 °C, 5% CO_2.

3.3.9. Perform colony counts on the plates for later transformation into CFU/mL as ($A_{\text{number of colonies}} \times 10^n$)/q. In this formula, **n** equals the absolute value of the dilution (0, 1, 2, or 3), and **q** equals the amount, in mL, pipetted for each dilution plated on the agar plate. Also, the CFU/mL can be converted to log values.

NOTE: When plant extracts are added to the culture medium, precipitation of particles from the extracts may occur. This fact can make it difficult to interpret the results. The same occurs when a microplate reader measures the turbidity as, in some cases, cells clump on the bottom of the microplate. In addition, depending on the extract used, the color of plant leaf extracts can make it difficult to quantify turbidity[23,24]. An alternative method uses dyes that reveal whether the microbial cells are metabolically active or not[24].

3.4. Antibiofilm activity

NOTE: The steps to evaluate the effect of treatments on biofilm formation are illustrated in **Figure 4**.

3.4.1. Formation and processing of biofilms

3.4.1.1. Dilute treatments in culture medium (TYEs) in a 96-well plate as described in the steps of the Antimicrobial Activity protocol.

3.4.1.2. Incubate the plate. In the example with *S. mutans,* the incubation is performed during 24 h, at 37 °C, and 5% CO_2.

3.4.1.3. After incubation, place the plates on an orbital shaker (5 min, 37 °C, 75 rpm) to loosen the cells not adhered to the biofilm. Then, discard the culture medium containing the not adhered cells, and wash the remaining biofilms three times with 0.89% NaCl to remove non-adherent cells.

3.4.2. Quantification of biomass from treated biofilms

NOTE: The steps are illustrated in the flow-chart in **Figure 4A**.

3.4.2.1. Keep the biofilms washed on the plate and add 50 µL of 1% crystal violet aqueous solution to each well.

3.4.2.2. Incubate the plate at room temperature for 35 min.

3.4.2.3. Wash the stained wells with MilliQ water (three times) and then air dry them for 60-90 min.

3.4.2.4. Elute the crystal violet from the stained wells with 200 µL of 99% EtOH by incubating the plate in an orbital shaker (5 min, 37 °C, 75 rpm).

3.4.2.5. Transfer a 150 µL aliquot from each well with the eluted dye to another plate and quantify the sample biomass (ELISA reader 570 nm).

3.4.3. Quantification of the viable microbial population (CFU/mL) of the treated biofilms

NOTE: The steps are illustrated in the flow-chart in **Figure 4B**.

3.4.3.1. Remove the washed biofilms from the plate with a pipette and 200 µL of NaCl 0.89% and transfer the resulting suspension individually to sterile microtubes.

3.4.3.2. Use an additional 200 µL of NaCl 0.89% per well and transfer it to the corresponding tube, already containing 200 µL of the initial biofilm suspension. Perform this process until reaching a total suspension of 1 mL biofilm per original well.

3.4.3.3. Use an aliquot from each tube to perform a ten-fold serial dilution.

3.4.3.4. Inoculate an aliquot of the desired dilution in specific agar plates (in duplicate). For example, 10 µL of a specific dilution on blood agar plates.

3.4.3.5. Incubate agar plates (e.g., 48 h, 37 °C, 5% CO_2), then count the colonies to determine the CFU/mL as described above.

3.5. Biological Activity Validation Phase

3.5.1. Salivary pellicle formation

3.5.1.1. Use Hydroxyapatite (HA) beads (Macro-Prep Ceramic Hydroxyapatite Type I 80 µm) as a surface to form the salivary film[25]. These beads surface mimic dental enamel.

3.5.1.2. Weigh the HA beads (e.g., 10 mg) in microtubes and sterilize. Then, use adsorption buffer (AB buffer: 50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, in dd-H2O, pH 6.5][25] containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.02% sodium azide (NaN_3) to wash the beads.

3.5.1.3. Collect and prepare human saliva[26]. It is necessary to have institutional ethics committee approval.

3.5.1.4. Add 500 µL of saliva into microtubes and incubate (40 min, 37 °C, 24 rpm).

3.5.1.5. Next, remove the saliva supernatant and wash the beads (three times with AB buffer containing PMSF and NaN_3). The *sHA* beads (HA bead with salivary pellicle) are now ready for downstream assays.

NOTE: Saliva is collected from healthy volunteers. After collection, dilute the saliva (1: 1 v/v) with AB buffer and centrifuge (1699 x g, 20 min, 4 °C). Sterilize by filtration (polyethersulfone membrane filter with low binding to 0.22 µm proteins)[26]. The Institutional Ethics Committee must approve the study. In our case, the Institution's Ethics Committee approved the study (CAAE:

68161417.0.0000.5416).

3.5.2. Detachment of *S. mutans* after adhesion to the film salivary and glucans treated with selected extracts

3.5.2.1. Cultivate the microorganism until the mid-log growth phase, as described above.

3.5.2.2. When cultures reached the desired O.D., centrifuge (4000 × g for 20 min), wash with 0.89% NaCl solution and resuspend the pellet with 0.89% NaCl using the same initial volume of the culture medium.

3.5.2.3. If using a streptococci, such as *S. mutans*, sonicate the cultures with a probe to dechain (30 s, 7 W, three times). If using a single cell organism, this step can be skipped.

3.5.2.4. Check the O.D. (540 nm) to adjust the concentration to 2 x 10^6 CFU/mL.

3.5.3. Adhesion of *S. mutans* to the salivary pellicle *(sHA)* and detachment of adhered cells

NOTE: The steps are illustrated in the flow-chart in **Figure 5**.

3.5.3.1. Obtain the *sHA* samples as described above.

3.5.3.2. Add an aliquot (in the example, we add 500 µL) of selected treatments (at the test concentration; for example, 0.5 mg/mL) or controls in microtubes containing samples of *sHA*.

3.5.3.3. Incubate the *sHA* samples with treatments or controls (30 min, 37 °C, 24 rpm); then, wash the beads three times with AB buffer (containing PMSF and NaN_3).

3.5.3.4. Add the microorganism culture. In the example, we add 500 µL of *S. mutans* culture (2 x 10^6 CFU/mL) to each microtube.

3.5.3.5. Incubate (1 h, 37 °C, 24 rpm) and then remove unbound cells by washing three times with AB buffer.

3.5.3.6. Resuspend each sample with an aliquot (in the example, we add 1000 µL) of AB buffer and sonicate with a probe (30 s, 7 W).

3.5.3.7. Use an aliquot of each suspension for a ten-fold serial dilution to determine the number of viable colonies by plating on specific agar plates (48 h, 37 °C, 5% CO_2). Next, count the colonies to determine the CFU/mL as described above.

NOTE: The step of sonicate is performed to detach cells adhered to *sHA*.

3.5.4. Adhesion of *S. mutans* to the initial glucan matrix (*gsHA*) and detachment of adhered cells

NOTE: The steps are illustrated in the flow-chart in **Figure 6**. The GtfB enzyme was purified from the culture supernatant *Streptococcus milleri* KSB8 engineered to produce GtfB. The purification was performed with a chromatography column containing hydroxyapatite beads using buffers containing two protease inhibitors (0.1 mM PMSF and 0.02% NaN_3)[27,28]. Then, the enzyme was checked on acrylamide gel (SDS-PAGE) and stained with silver nitrate. Aliquots of the enzyme were stored at -80 °C until use.

3.5.4.1. Obtain the *sHA* samples as described above. Next, add an aliquot (in the example, we add 500 µL) of GtfB enzyme to each tube and incubate in a homogenizer (40 min, 37 °C, 24 rpm). Then, wash three times with AB buffer (containing PMSF and NaN_3).

3.5.4.2. Add an aliquot (e.g., 500 µL) of sucrose substrate (100 mmol of sucrose) containing the treatments (or controls-at the test concentration, e.g., 0.5 mg/mL) to each microtube.

3.5.4.3. Incubate the samples in a homogenizer (4 h, 37 °C, 24 rpm). Then, perform three washes with AB buffer (with PMSF and NaN_3) to remove the treatments and excess of sucrose not incorporated in the synthesized glucans (samples of *gsHA*).

3.5.4.4. Add an aliquot (in the example, we add 500 µL) of *S. mutans* inoculum (2 x 10^6 CFU/mL) to each microtube.

3.5.4.5. Incubate in a homogenizer (1 h, 37 °C, 24 rpm) and wash three times with AB buffer (with PMSF and NaN_3) to remove unbound cells.

3.5.4.6. Resuspend each sample with an aliquot (e.g., 1000 µL) of AB buffer (with PMSF and NaN_3) and sonicate with a probe to detach cells adhered to *gsHA* (30 s, 7 W).

3.5.4.7. Use an aliquot of each suspension for a ten-fold serial dilution to determine the number of viable colonies by plating on specific agar plates (48 h, 37 °C, 5% CO_2). Next, count the colonies to determine the CFU/mL as described above.

4. Biological Data Analysis

4.1. Bioassays Data

4.1.1. Input raw data for the bioassays in a spreadsheet. Calculate the log of microbial growth inhibition by each treatment as ($A_{CFU/mL\ of\ treatments}$ + 1) x $\log_{10}$. Then, calculate the log percentage of microbial growth inhibition, compared to vehicle control using ($A_{log10\ CFU/mL\ of\ treatment}$/mean $A_{log10\ CFU/mL\ of\ vehicle\ control}$) x 100%.

4.1.2. Correct O.D. of planktonic cultures and biomass treated by treatments (CE and CEF treated groups) and by vehicle control (negative control). For correction, subtract the absorbance

of treated wells from that obtained in wells containing only culture medium (Ablank) as ($A_{\text{treated groups medium}}$ /$A_{\text{negative control medium}}$) x 100%.

4.1.3. After this correction, calculate the percentage of the biomass inhibition, compared to vehicle control as ($A_{\text{treated biomass}}$/mean $A_{\text{vehicle control}}$) x 100%.

4.1.5. Submit the raw data generated for statistical analysis of the data using specific software.

NOTE: The interpretation of the effectiveness of a given treatment is determined using breakpoints, such as the IC_{50}/IC_{90}. These values are defined as the minimum concentration of a treatment capable of inhibiting 50% and 90%, respectively, of bacterial growth or biofilm formation[24]. These parameters can help interpret the data and provide a basis for selecting compounds with better activity[13, 29].

REPRESENTATIVE RESULTS:

We provide an example of using a systematic approach to screen the biological activity of plant extracts and fractions to identify potentially active molecules for possible new anti-caries therapies: antimicrobial and antibiofilm activities of *Casearia sylvestris* extracts from distinct Brazilian biomes against *Streptococcus mutans* and *Candida albicans*[13].

Background

Complex interactions between specific oral microorganisms-host factors-diet rich in sucrose and starch can modulate the formation of pathogenic biofilms and initiate a cariogenic process[30,31]. *S. mutans* orchestrates the pathogenicity of biofilms associated with the development of dental caries because it produces Gtfs responsible for exopolysaccharides synthesis, besides its acidogenicity and aciduricity[31]. In addition, Gtfs enables the adhesion of *Candida albicans* (and other microorganisms), increasing the virulence of the biofilm[32,33]. We conducted a screening of the antimicrobial and antibiofilm activities of *C. sylvestris* leaf extracts and fractions from different Brazilian biomes, belonging to the *lingua and sylvestris* varieties against *S. mutans* and *C. albicans*[13]. *C. sylvestris* ("guaçatonga") is part of popular and traditional use in Brazil, and other countries of South America and Asia[34,35]. This plant is cited in the "National List of Medicinal Plants of Interest to SUS" (RENISUS), which contains 71 species that could treat the diseases with a high incidence in Brazil[36]. The chemical profile of leaf extracts of var. *sylvestris* presents a rich phytochemical composition, with abundant diterpenes[35], while phenolic compounds (mainly flavonoids) predominate in var. *lingua*[18].

We use the approach described in the protocol to identify which extracts and fractions of *C. sylvetris* are most active for the microorganisms evaluated, and, based on the results using simplistic models, we select which treatments will be tested in vitro complex models (hydroxyapatite discs, microcosms)[37,38]. Here, we present the results of the screening of the twelve CE against *S. mutans*. The focus is to demonstrate the usefulness of this approach for screening natural compounds instead of interpreting and discussing the data.

We collected the leaves of individuals from the two varieties of *C. sylvestris* from twelve different

populations in Brazil, comprising different formations of Brazilian biomes (please, see details in Ribeiro et al. 2019[13]). The collection was carried out between June and September 2012 and 2013 (SisGen; Register #A00892A). We recommend collecting representative samples, including individuals of different chemotypes and from different biomes, to address the chemical variability of secondary metabolites. If available, at least 3 to 5 individuals should be collected. Previous information concerning plant infraspecific chemical variability should also be considered as described by Ribeiro et al. 2019 and Bueno et al. 2015[13,18]. The chemical composition of the CE was examined by the chromatographic cited in Step 2 and we provide the chemical profile in **Figure 7**. The analysis of the chemical profile is essential to integrate the interpretation of data obtained in biological screening.

The CEs were fractionated in Hex, AcOEt, and MeOH fractions. The fractionation of CE allows the simplification of the mixture to increase the concentration of the potentially active compounds and decrease the possibilities of synergisms and antagonisms between compounds. Additionally, in more simple mixtures (fractions), it is easier to obtain spectral data of the compounds than in CE and to perform dereplication analysis[2]. Usually, fractionation can be done by liquid-liquid extraction or solid-phase extraction cartridges (SPE) containing preferentially reversed-phase adsorbent like C_{18} (40 µm, 100 Å). Other adsorbents or mixtures of adsorbents can be chosen, depending on the study purposes or chemical nature of the desired compounds. If the chosen technique is the SPE, the cartridges must be previously activated with pure organic solvent (e.g., EtOH) and conditioned with the initial eluent. Standardized protocols are available. Thus, the reader can consult and adapt them according to the intended study and plant material of interest. The twelve CE were solubilized with 84.15% EtOH and 15% DMSO to achieve 6 mg/mL (stock solution). Before the screening tests, we tested the diluent concentration (vehicle) that does not interfere with microbial growth. This step is important because it prevents the antimicrobial and antibiofilm actions of the solvents from affecting the results when testing treatments. The tests can be performed on a 96-well plate by treating the culture of the microorganism of interest with different concentrations of solvents (associated and/or isolated). Thus, we began our screening with CE at 0.5 mg/mL and vehicle at a concentration of 7% EtOH and 1.25% DMSO.

For screening antimicrobial and antibiofilm activity, 96-well plates were treated as described above. For this purpose, the volume of 16.67 µL of stock solution CE (6 mg/mL) was added to test each CE at the concentration of 0.5 mg/mL. The biofilms formed were processed as described in Step 3. The extracts effective against *S. mutans* (IC_{50} or 3 logs) were used to evaluate the "adhesion strength" of this bacterium to the salivary pellicle and initial glucan matrix, as described in Step 3.

The raw data obtained from biological assays were organized in Excel (as described in Step 4) and analyzed with appropriate statistical treatment[13]. The cutoff point to identify the extracts with the best activity was the IC_{50} inhibition (3 logs). From this parameter, four extracts showed a favorable response (**Figure 8**). The chromatographic data of these four extracts show the simultaneous presence of clerodane-type diterpenes and glycosylated flavonoids. In addition, they include the same biome (Atlantic Forest) and variety (*sylvestris*). To help interpret the biological data, we compared the chromatographic profile of the four extracts with the best

activity with the other screened extracts. Compared to the others, the extracts with the best activity have a higher amount of clerodane-type diterpenes and, simultaneously, glycosylated flavonoids. This observation indicates that it is likely that the effectiveness of these extracts is due to a synergistic interaction between the two secondary metabolites, thus increasing their biological activity. That is, the combined effect of clerodane-type diterpenes and glycosylated flavonoids is greater than the sum of their separate effects[13].

To confirm the data obtained in the screening, we evaluated the detachment of *S. mutans* after adhesion to the salivary pellicle and glucans treated with selected CE. The assays use biofilm models of in vitro single-species to evaluate better the biological activity of the selected crude extracts and identify possible action targets. The first analysis verifies whether the treatments used are capable of inhibiting the adherence of *S. mutans* to the salivary pellicle, but mainly, whether the cells of the microorganism that have adhered to the treated pellicle can be removed from the surface more easily by the mechanical stimulus, thus interrupting the first stage of biofilm formation. The addition of CE (with better activity) during the synthesis of glucans did not modify the salivary pellicle because no CE significantly affected the removal of cells adhered to the salivary pellicle (**Figure 9A**).

The adhesion of the initial glucan matrix (*gsHA*) investigates whether the treatments can inhibit the adhesion of *S. mutans* to the initial glucan matrix. Still, this methodology verifies if the microorganism cells that have adhered to the treated glucans can be removed by the mechanical stimulus more easily of the surface, thus interrupting the stage biofilm formation. Three CE affected the quality of glucans formed by GtfB and therefore weakened the adhesion of *S. mutans* to the initial glucan matrix (most *S. mutans* cells were removed after adhesion for glucans; **Figure 9B**). We believe that this behavior is related to the synergism between the secondary metabolites[13].

The Systematic Approach has helped us identify and select active crude extracts to halt the formation of cariogenic biofilms. Once selected and based on the chromatographic profile, we have the basis for elucidating the molecular mechanisms of action in complex models.

FIGURE AND TABLE LEGENDS:

Figure 1: Flow-chart of the Systematic Approach to identify active molecules from plants extracts and fractions.

Figure 2. Flow-chart of the plant material extraction and fractionation. The illustration shows the experimental design to prepare the crude extracts (A) and fractionation of crude extracts (B). UAE: Ultrasound Assisted Extraction; SPE: Solid Phase Extraction.

Figure 3. Experimental design for assessing antimicrobial activity in 96-well plates. The illustration depicts treatments (crude extracts or fractions) and controls. For the screening of multiple treatments, use a single concentration (mg/mL) in each well. CFU/mL: colony forming units per milliliter. O.D.: optical density.

Figure 4. Experimental design for antibiofilm assay in 96-well plates. The illustration shows treatments (crude extracts and fractions) and controls. For the screening of various treatments, use a single concentration (mg/mL) in each well. In **A**, the steps to quantify biomass of treated biofilms are illustrated. In **B**, is shown the steps to determine the population (CFU/mL) of the treated biofilms.

Figure 5. Experimental design to evaluate the adhesion to the salivary pellicle, followed by the detachment of adhered cells. The illustration shows the steps to be performed. Treatments: selected based on biological screening.

Figure 6. Experimental design to evaluate the adhesion to the initial glucan matrix (*gsHA*) followed by the detachment of adhered cells. The illustration shows the steps to be performed. Treatments: selected based on biological screening. Sucrose substrate: 100 mmol of sucrose.

Figure 7. **Quantity of clerodane-type diterpenes and glycosylated flavonoids in *C. sylvestris* extracts from Brazilian biomes.** The letters *S*, I, and *L* indicate the varieties *sylvestris*, intermediate, and *lingua*, respectively. Personal communication by Dr. Paula Carolina Pires Bueno. This figure has been modified from Ribeiro et al.[13].

Figure 8. Antimicrobial and antibiofilm activity of *C. sylvestris* crude extracts from Brazilian biomes against *S. mutans*. A. % CFU (log_{10}) of treated planktonic cells; **B.** % biomass of treated biofilms. **C.** % CFU (log_{10}) of treated biofilm. The data described are median (traces) and interquartile (boxes). The error bars represent the maximum and minimum values. The asterisks denote a statistically significant difference of a specific extract versus vehicle control (V), where: ****$p \leq 0.0001$; ***$p \leq 0.001$; ** $p \leq 0.01$; and *$p \leq 0.05$ (Kruskal-Wallis test, followed by Dunn's multiple comparisons test). Each species growth control (without treatment) is represented as Sm for *S. mutans*. The colors of the bars in each graph represent the variety to which the extracts belong, being, in dark gray color: var. *sylvestris*; light gray: var. intermediate and white: var. *lingua*. This figure has been modified from Ribeiro et al.[13].

Figure 9. *S. mutans* detachment after adhesion to the treated salivary pellicle and initial matrix of glucans. Post-release data of *S. mutans* to the treated salivary pellicle and glucans are shown in **(A)** and **(B)**, respectively. There was no difference between the control vehicle (V), and the extracts tested for both analyses. The percentage of CFU/mL was obtained considering the vehicle control (V) as 100%. The data described are median (traces) and interquartile (boxes). The error bars represent the maximum and minimum values. The asterisks denote a statistically significant difference of a specific extract versus vehicle control (V), where ****$p = 0.0001$ and **$p < 0.0031$ (Kruskal-Wallis test, followed by the multiple comparison test of Dunn). The growth control is represented by Sm for *S. mutans*. The colors of the bars of the graph represent the variety to which the extracts belong, being the color dark gray to var. *sylvestris.* This figure has been modified from Ribeiro et al.[13].

DISCUSSION:

The main challenges related to the work with natural crude extracts comprise their complex

749 composition and the inadequacies of classic bio-guided isolation studies. Although this process is
750 slow, it is effective and has led to major findings in NP research. To rationalize, prioritization-
751 driven studies are needed to rationalize. Thus, the use of modern chemical profiling approaches
752 for the analysis of CE and dereplication before isolation are important to characterize the studied
753 material and especially useful to avoid re-isolation of known compounds with already described
754 biological activity[2,15]. Besides, the acquisition of multidimensional data is necessary to perform
755 further multivariate data analysis to find the potential hits candidates responsible for the
756 observed biological activities. Consequently, the researcher can focus on the isolation (on a grand
757 scale) of these potential candidates.
758
759 Here, we present a systematic approach for bioassay-guided identification (in vitro) of active
760 compounds from plant extracts and fractions. This protocol allows a multi-target screening and
761 analysis method so that multiple active components can be screened and analyzed
762 simultaneously. The bioassays are on a small scale and of high yield, are fast, cost-effective, easy
763 to reproduce, and consume fewer reagents than traditional methods (e.g., initial isolation of
764 compounds of interest)[39]. For any natural product research, it is paramount the analytical tools
765 (for example, HPLC‑UV, HPLC‑DAD, LC‑MS). Recently, the approach is more structure-oriented
766 (chemistry-based) utilizing, to a higher degree, the power of analytical and elucidation platforms
767 (LC-HRMS and HRMS/MS, high-field NMR) and dereplication strategies, which consist in the rapid
768 identification of already known molecules. This step helps to understand the chemical profile
769 relationship with the biological response resulting in more focused isolation of candidate active
770 metabolites[3]. There are several well-established methods for chromatographic analysis. For
771 unknown NPs, it may sometimes be necessary to perform pilots to find the best possible method.
772 For example, we use an analytical method of chromatography validated for the simultaneous
773 analysis of secondary metabolites produced by two varieties of *C. sylvestris*[13,18]. When choosing
774 a chromatography method, we suggest considering a protocol based on the target compound (s),
775 and the advantages and disadvantages of all available methods, focusing particularly on their
776 efficiency and, of course, the total cost involved[40].
777
778 Although the systematic approach has proved useful for rapidly screening and analyzing bioactive
779 candidates in NPs, there remain important limitations. For example, the visual and O.D. readings
780 of the biomass and planktonic cultures may reproduce false-positive results[13,23,24]. Determining
781 the turbidity of the cultures with a microplate reader may fail when used for natural
782 compounds[23,24]. These failures occur because (i) in some test organisms, the cells are clustered
783 at the bottom of the well, and, in other organisms, the cells remain in suspension; (ii) solid
784 particles present in the CE precipitate and cause turbidity in the wells[23,24]. The pH of NPs is a
785 factor that must also be considered. The pH of the treatment solution is related to the chemical
786 composition of the secondary metabolites and, therefore, influences the biological response.
787 Although pH is not a limitation, it should be assessed with caution, depending on the purpose of
788 the study. For example, in biofilm models on tooth enamel surfaces, acidity can cause unwanted
789 enamel demineralization. In these cases, it is necessary to adjust the pH with the aid of suitable
790 buffers. Therefore, it is necessary to conduct tests to verify whether the pH adjustment affected
791 the biological response observed previously.
792

The classic tests to assess the activity of new compounds include determining the minimum inhibitory concentration (MIC) and the minimum bactericidal or fungicidal concentration (MBC or MFC)[29,41]. However, when the goal is to screen many plant extracts and fractions, the technique becomes exhaustive. Bioscreening can be performed with a single concentration of multiple treatments (e.g., plant extracts from distinct places)[13,42,29]. For these situations, the systematic approach is a method with high performance that returns consistent results in less work time. The treatments selected in the biological screening can be evaluated using refined models (clinically relevant, viable, and reproducible) to confirm the biological activity. For control of cariogenic biofilm, laboratory studies should focus mainly on biofilms formed on hydroxyapatite (tooth enamel substitute) or enamel surfaces placed in an upright position coated with a salivary pellicle[37]. It also allows the screening of plant samples of different varieties and biomes in a reproducible and fast way. The inclusion of these two variables (biome and variety) is vital because they influence the variability of the chemical composition of secondary metabolites[18] and modulate the biological response. This systematic approach can be adapted/modified for applications outside of oral biofilm research. For example, it can be particularly useful for other fields related to biofilm, using other species of interest.

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DISCLOSURES:

No conflicts of interest declared.

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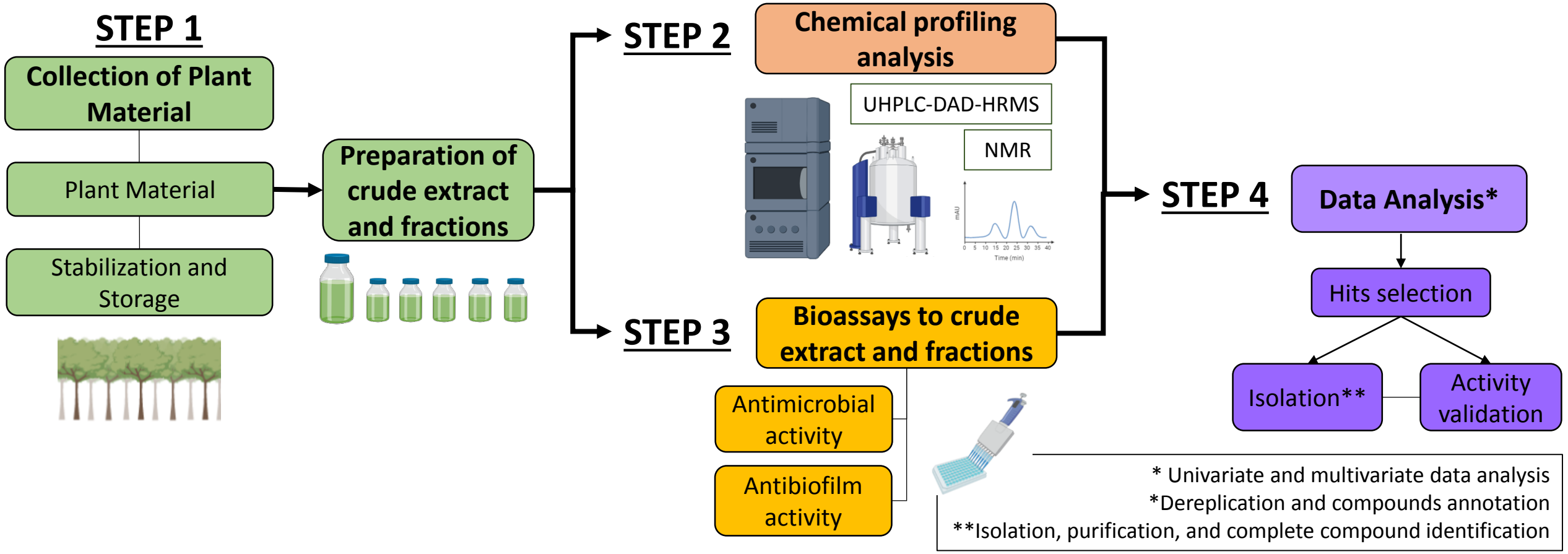


Figure2

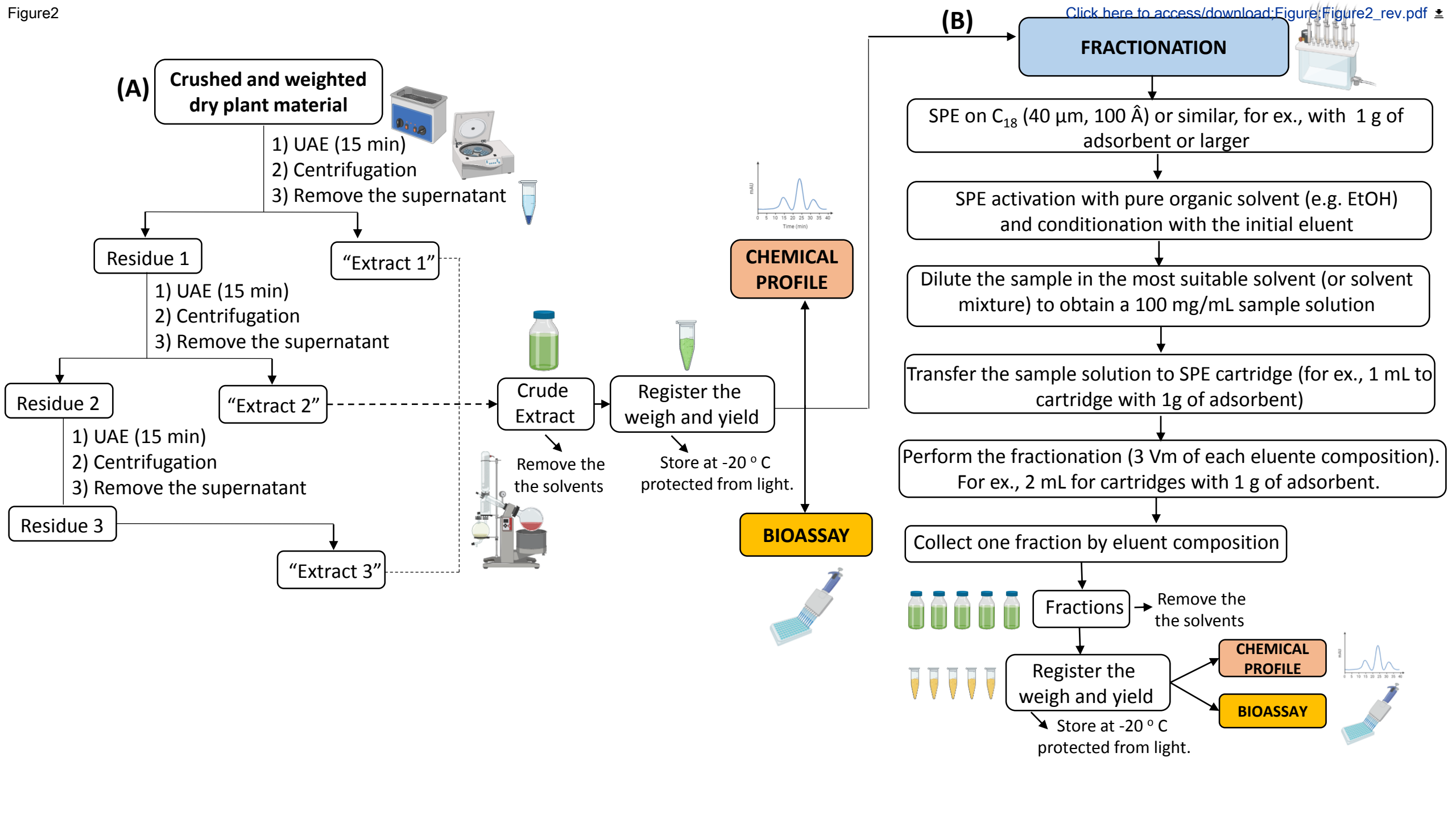
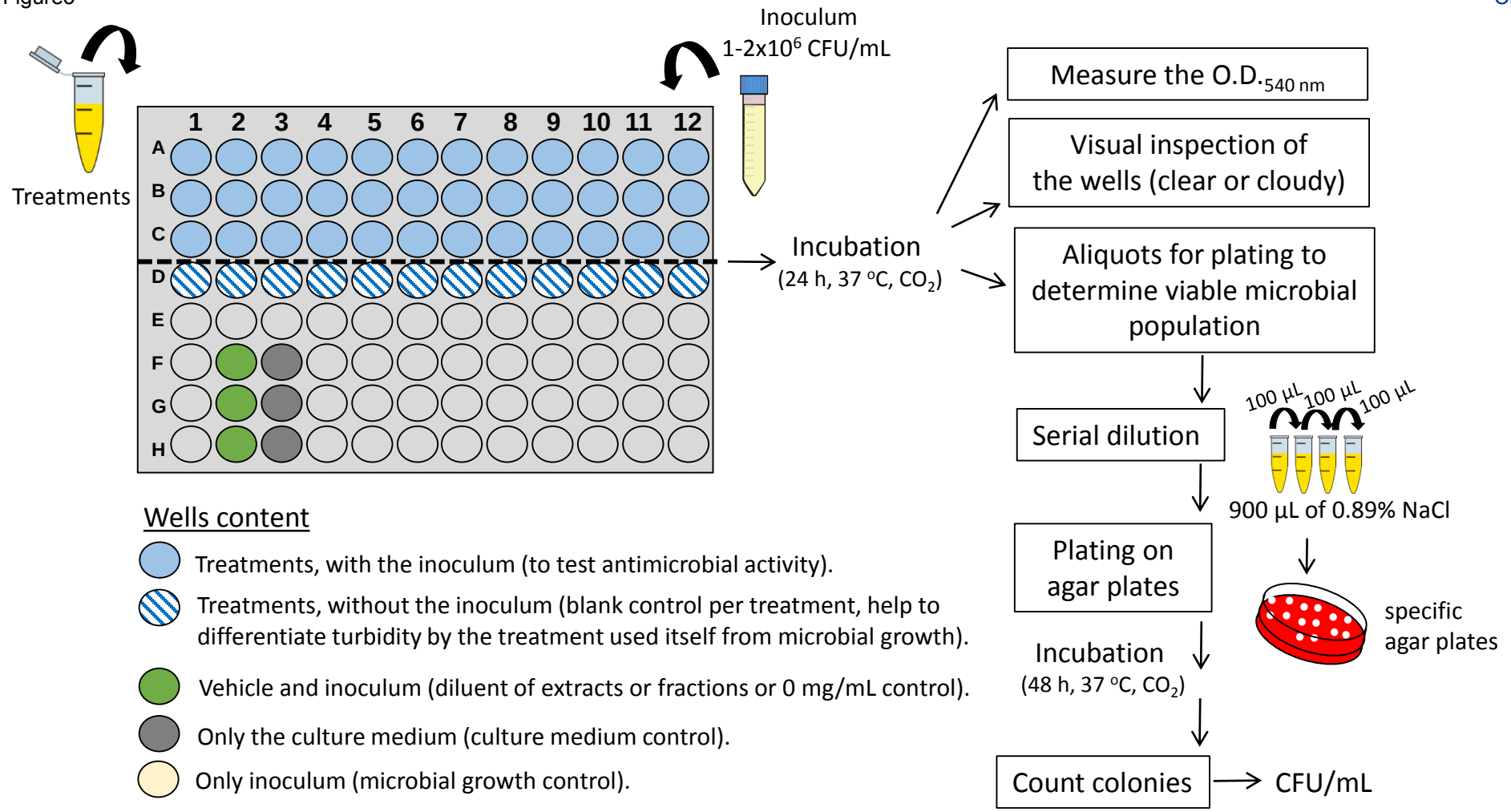


Figure3



Wells content

- Treatments, with the inoculum (to test antimicrobial activity).
- Treatments, without the inoculum (blank control per treatment, help to differentiate turbidity by the treatment used itself from microbial growth).
- Vehicle and inoculum (diluent of extracts or fractions or 0 mg/mL control).
- Only the culture medium (culture medium control).
- Only inoculum (microbial growth control).

Figure4

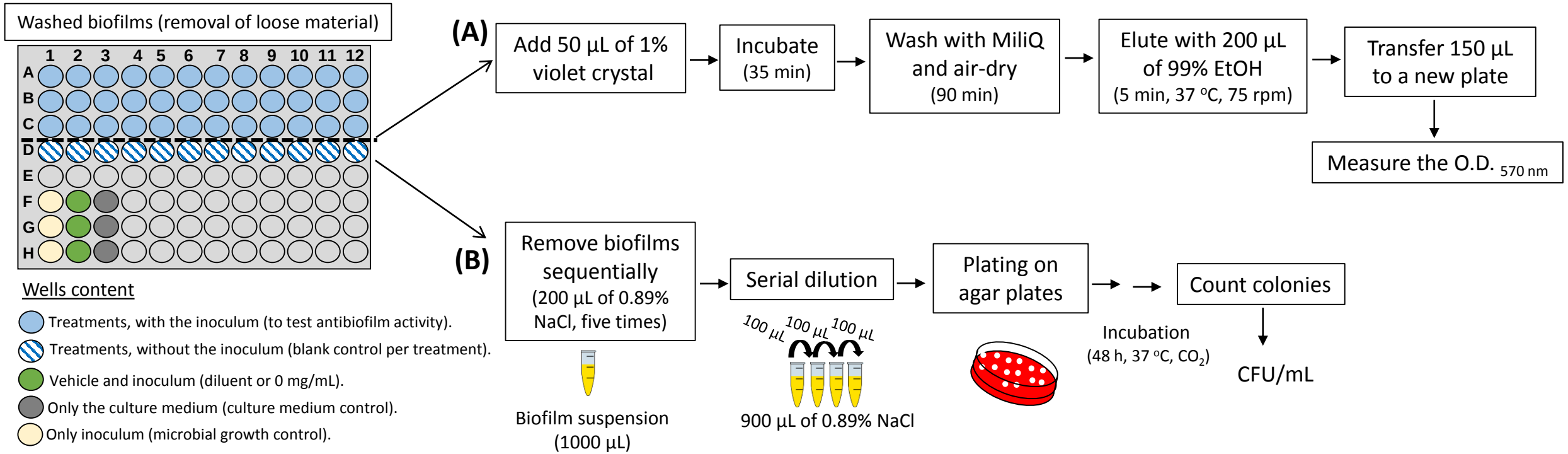


Figure5

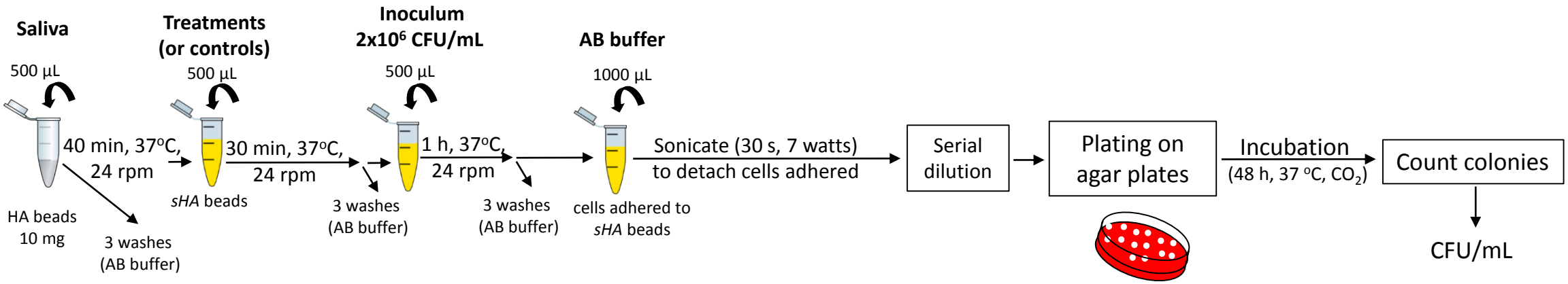


Figure6

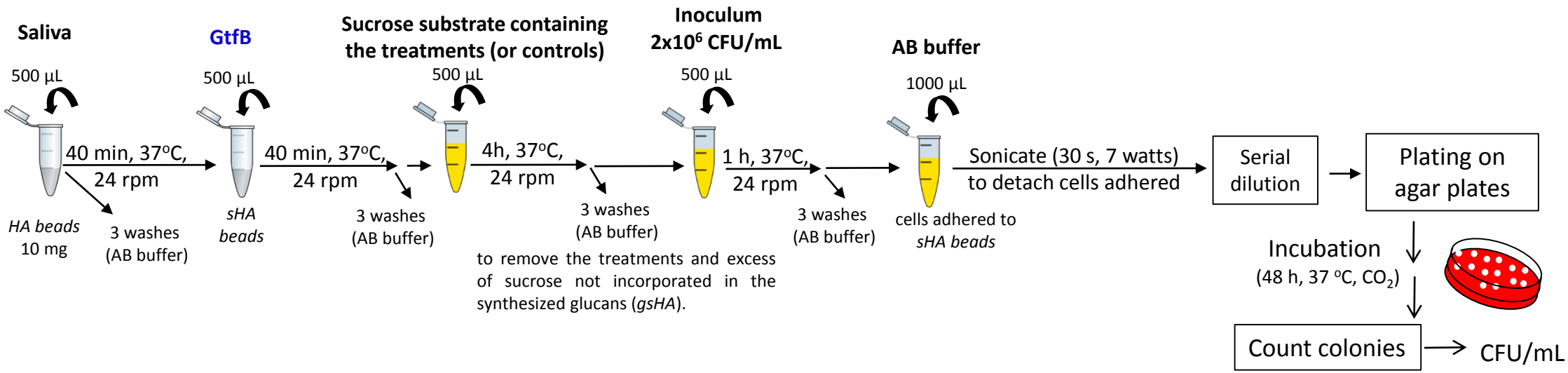


Figure7

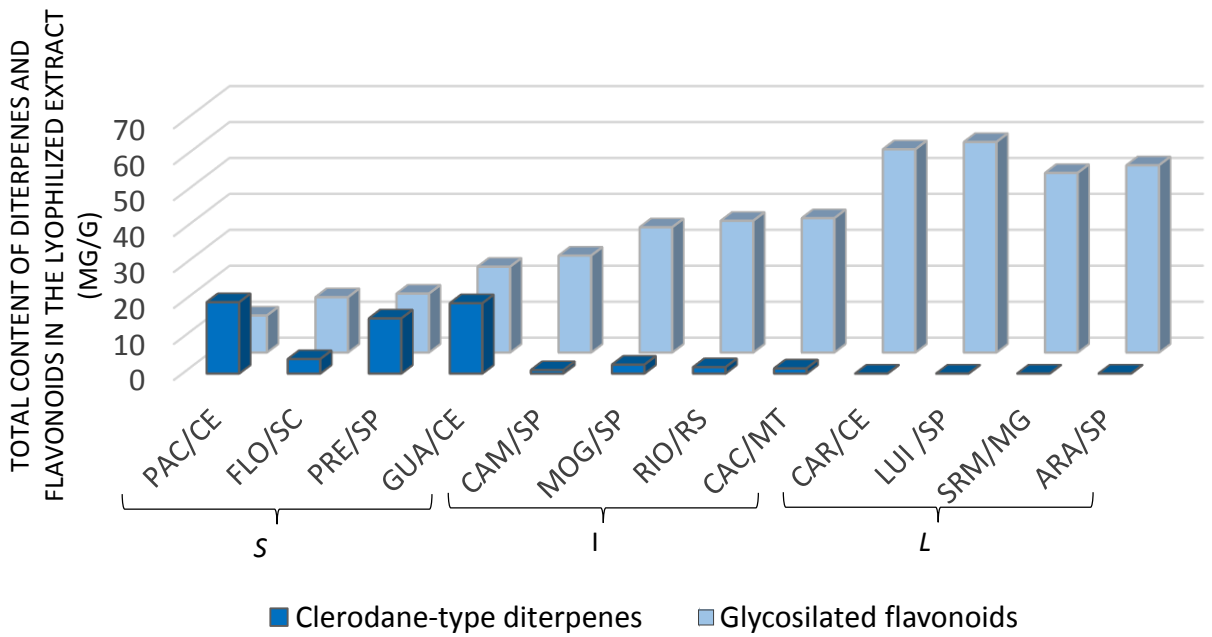


Figure8

Click here to access/download;Figure;Figure8_rev.pdf

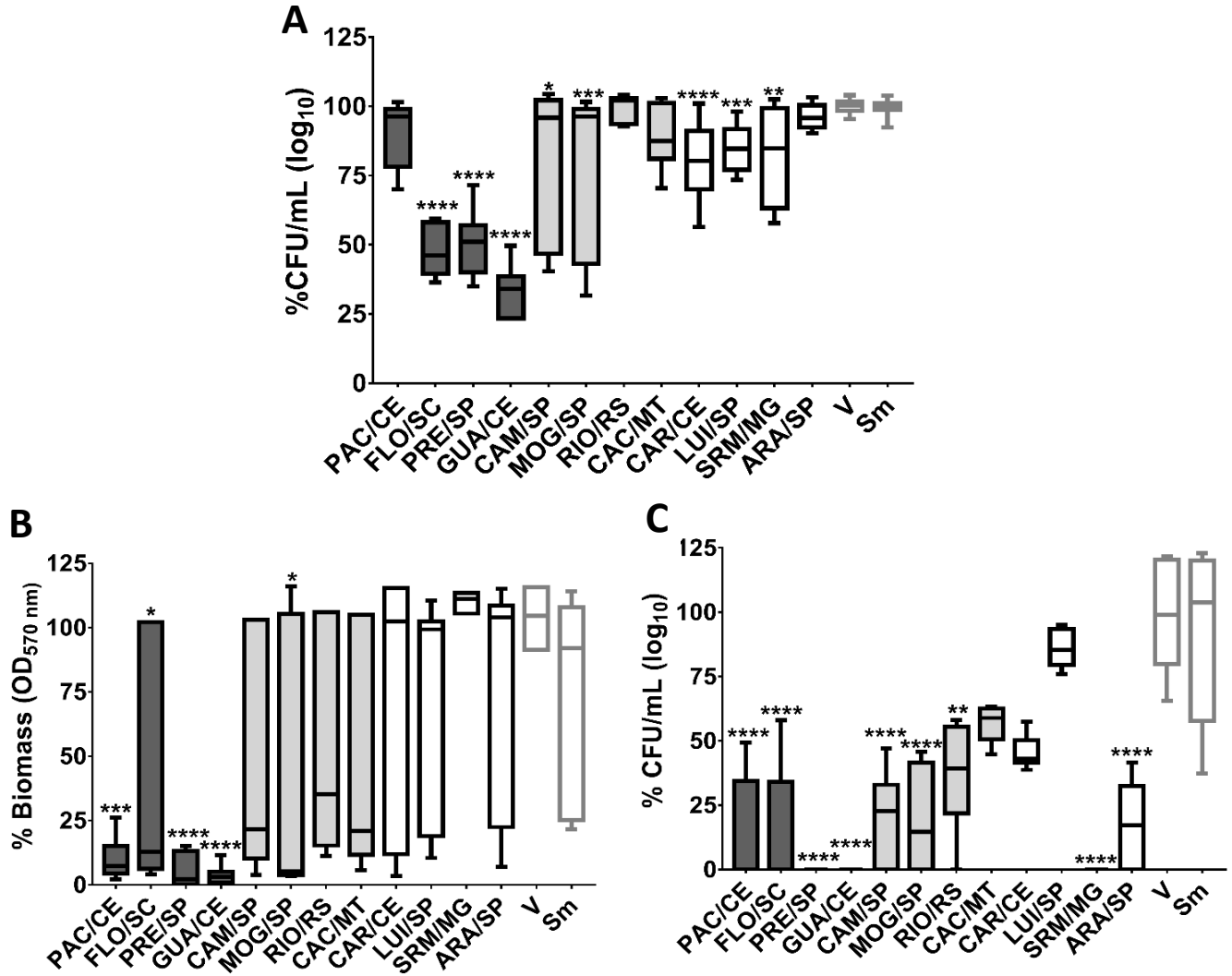


Figure9

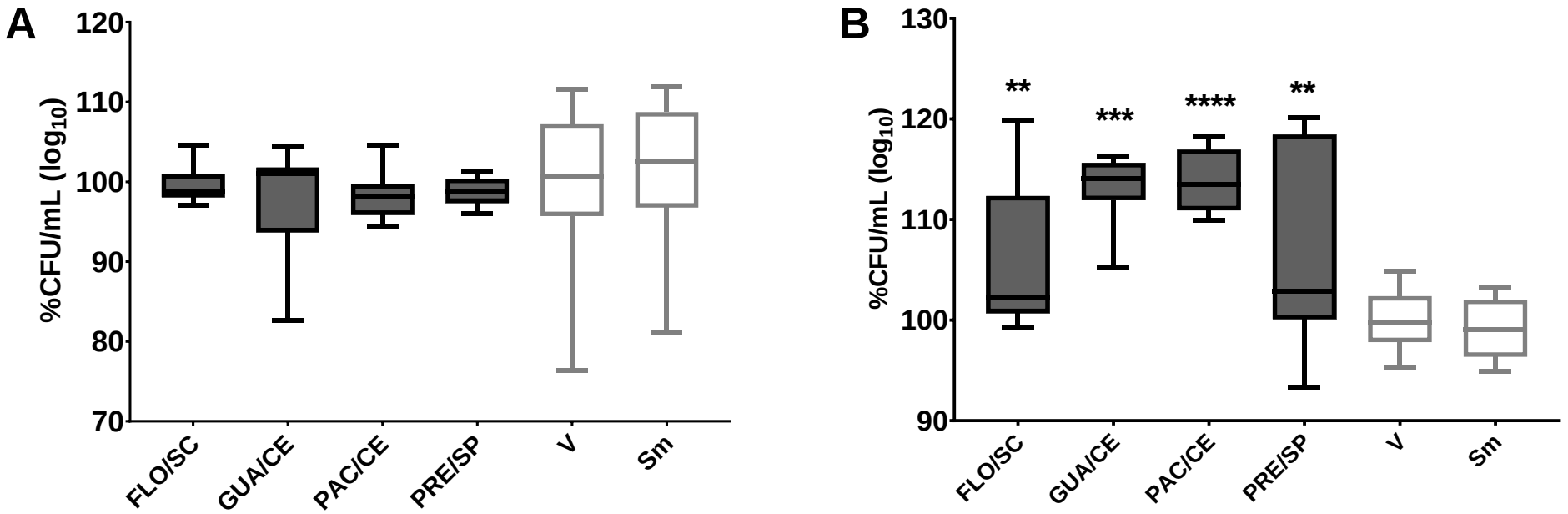


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_2020_08_09_rev.xls

Name of Material/ Equipment	Company
96-well microplates	Kasvi
Activated carbon	LABSYNTH
Analytical mill	Ika LabortechniK
Blood agar plates	Laborclin
Chromatographic column C18	Phenomenex Kinetex
Dimethyl sulfoxide	Sigma-Aldrich
ELISA plate reader	Biochrom Ez
Ethanol	J. T. Baker
Ethanol	Sigma-Aldrich
Ethyl acetate	J. T. Baker
GraphPad Software	La Jolla
Hexane	J. T. Baker
Incubator	Thermo Scientific
Isopropanol	J. T. Baker
Lyophilizer (a freeze dryer)	Savant
Nylon Millipore	LAC
Orbital shaker	Quimis
Polyamide solid phase extraction cartridge	Macherey-Nagel
Silica gel	Merck
Sodium Chloride (NaCl)	Synth
Solid phase extraction cartridges (SPE)	Macherey-Nagel
Tryptone	Difco
UHPLC-DAD	Dionex
Ultrasonic bath	UNIQUE
Yeast extract	Difco

Comments/Description

Flat bottom
Clean up and/or fractionation step
Model A11 Basic

150 × 2.1 mm, 2.6 µm, 100Â
Vehicle solution

For extraction and fractionation steps, and mobile phase composition
Vehicle solution
Fractionation step
GraphPad Prism7
Fractionation step

For extraction step
Modulyo
0.22 µm x 13 mm
Model G816 M20
Clean up and/or fractionation step
40–63 µm, 60 Â
0,89% in water
Clean up and/or fractionation step

Ultimate 3000 RS
Model USC 2800

Dear Vineeta Bajaj,

Enclosed is our revised manuscript "Systematic approach to identify novel antimicrobial and antibiofilm molecules from plants' extracts and fractions to prevent dental caries" (JoVE61773). We thank the Editorial and the Reviewers for the valuables suggestions to improve our manuscript. We have modified the manuscript to address each of the Editors´ and Reviewers' comments as detailed below in the line-by-line response. We have uploaded the revised manuscript version with each specific changes marked as a colored text (blue). We hope that our revised manuscript is now acceptable for publication in JoVE.

Sinceraly,

Marlise I. Klein and Alberto José Cavalheiro (Corresponding Authors)

Editorial comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
Answer: We have revised all the text, and any changes are highlighted.

• **Textual Overlap:** Parts of the manuscript show overlap with previously published work. Please re-write the text on lines 237-243, 246-264, 407-413, 420-427, 437-444, 450-456, 459-487, 631-635, to avoid this overlap.
Answer:
237-243: The text was rewritten (now Lines 217-223)
246-264: The text was rewritten (now Lines 224-244)
407-413: The text was rewritten (now Lines 372-377)
420-427: The text was rewritten (now Lines 385-392)
437-444: Line 437 now in Line 391-392
438-441: The text was rewritten in **Representative results** section (now Lines 561-565)
443-444: The text was rewritten in Lines 393-396.
450-456: The text was rewritten; now Lines 397-399.
459-487: Line 459 now in Lines 419-420.
Lines 461-464: The text was rewritten in **Representative results section**; Lines 553-556
Lines 465-487: The text was rewritten now in Lines 427-443)
631-635: The sentence was rewritten (now Lines 616-622)

• **Protocol Language:** The JoVE protocol should be almost entirely composed of numbered short steps (2-3 related actions each) written in the imperative voice/tense (as if you are telling someone how to do the technique, i.e. "Do this", "Measure that" etc.). Any text that cannot be written in the imperative tense may be added as a brief "Note" at the end of the step (please limit notes). Please edit your protocol section accordingly. Descriptive sections of the protocol can be moved to Representative Results or Discussion. The JoVE protocol should be a set of instructions rather a report of a study. Any reporting should be moved into the representative results.
1) Some examples NOT in the imperative: 1.1.1, 1.1.3., 2.1.1 etc
2) Split up long steps (e.g., 1.1.2).
3) For example: Lines 184-206 will need to re-written entirely or merged into the discussion.
Answers:
1) We revised the text and used imperative in all the steps. In addition, we have modified some steps, as listed below:

Step 1.1, item 1.1.1.: The sentence "*The access to the plant material must be registered on electronic platforms that regulate access to genetic heritage in the country where the collection will take place. For example, in Brazil, register with the National System for the Management of Genetic Heritage and Associated Traditional Knowledge – SisGen (website https://sisgen.gov.br/paginas/login.aspx).*" was rewritten in imperative voice (now Lines 114-118).

Step 1.1, item 1.1.2.: The sentence "*To address the chemical variability of secondary metabolites, collect representative samples, including individuals of different chemotypes and from different biomes. If available, at least 3 to 5 individuals should be collected. Previous information concerning plant infraspecific chemical variability should also be considered as described by Ribeiro et al., 2019 and Bueno et al., 2015[13], [15]. This information must be registered*." was rewritten and moved to the **Representative results section** (now Lines 496-500).

Step 1.2: We rewrote this step using imperative tense and added more items (now Lines 125-134).

Step 2.1.: We rewrote this step (now Lines 138-156). The sentence *"can be performed with the assistance of temperature, an ultrasound bath, or an automatic shaker"* was removed.

Step 2.2.: We rewrote this step. The sentence *"The fractionation of CE allows the simplification of the mixture to increase the concentration of the potentially active compounds and decrease the possibilities of synergisms and antagonisms between compounds. Additionally, in more simple mixtures (fractions), it is easier to obtain spectral data of the compounds than in CE, and to perform dereplication analysis[2]. Usually, fractionation can be done by liquid-liquid extraction or solid-phase extraction cartridges (SPE) containing preferentially reversed-phase adsorbent like C_{18} (40 µm, 100 Â). Other adsorbents or mixtures of adsorbents can be chosen, depending on the study purposes or chemical nature of the desired compounds. If the chosen technique is the SPE, the cartridges must be previously activated with pure organic solvent (e.g., EtOH) and conditioned with the initial eluent. Standardized protocols are available, and the reader can consult and adapt them according to the intended study and plant material of interest"* was moved to the **Representative results section** (now Lines 504-515).

Step 2, item 2.2.1.: We moved the sentence *"If the CE is difficult to dissolve in the initial elution mixture, disperse the CE in a solid phase (e.g., C18 or celite) in 1:1 (w/w) proportion before loading the material on the top of the cartridge."* to **NOTE** (now Lines 172-174).

Step 3, item 3.1..: The sentence *"Previously, we selected 84.15% EtOH and 15% dimethyl sulfoxide (DMSO) as the solvent concentration of the stock solution, and used the stock concentration of the CE to 6 mg/mL, and the CEF to 1 mg/mL[13]."* was rewrote and moved to **NOTE 1** (now Lines 273-275).

Step 3, item 3.3.: The sentence *"The classic tests to assess antimicrobial activity include determining the minimum inhibitory concentration (MIC) and the minimum bactericidal or fungicidal concentration (MBC or MFC). […] Bioscreening can be performed with a single concentration of multiple treatments (e.g., plant extracts from distinct places (Figure 3)."* was moved to the **Discussion section** (now Lines 655-657).

Step 3, Item 3.3.: The sentence *"In a static model using 96-well plates, biomass and microbial population are quantified during the formation and/or development of*

biofilms[21]*."* was removed.

Step 3, Item 3.5.: The sentence *"We evaluated the detachment of S. mutans after adhesion to the salivary pellicle and glucans treated with selected CE. The assays using biofilm models of mono in vitro species to evaluate the biological activity of the selected crude extracts better and identify possible action targets."* is now in the **Representative results section** (now Lines 543-546).

Step 3, Item 3.5.: The sentence *"The biological activity must be confirmed; in this step, the treatments selected in the biological screening are evaluated using refined models (clinically relevant, viable, and reproducible). For example, for control of cariogenic biofilm, laboratory studies should focus mainly on biofilms formed on hydroxyapatite (tooth enamel substitute) or enamel surfaces placed in an upright position coated with a salivary pellicle."* is now in the **Discussion section** (now Lines 663-665).

Step 3, Item 3.5.3: The sentence **"***This evaluation verifies whether the treatments used are capable of inhibiting the adherence of S. mutans […] the first stage of biofilm formation."* was rewritten in the **Representative results section** (now Lines 546-550).

Step 3, Item 3.5.4: The sentence *"This evaluation verifies whether the treatments used can inhibit the adhesion of S. mutans to the initial glucan matrix, […] biofilm formation"* was rewritten in the **Representative results section** (553-556).

2) We rewrote the long steps as recommended. Now we divide the steps of the items.

3) Lines 184-206: We rewrote this step using imperative tense and added more items (now Lines 177-210).

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video.
1) **Currently your protocol steps are fairly general. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. You may use specific example to focus your steps a bit.
2) For example, in Line 144, mention specifications of settings and device use.
3) Avoid using general statements starting with "Usually", "generally", etc.
Answers:
1) and 2) The protocol steps described are very straightforward, and specific settings were provided when available.
3) We removed the word "usually" before "-20 ºC" (Line 131) and the word "generally" before "hydroalcoholic" (Line 140).

• Protocol Numbering:
1) Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary.
2) All steps should be lined up at the left margin with no indentations.
3) Please add a one-line space after each protocol step.
4) Please ensure that the protocol section is under 10 pages long.
Answer: We adjusted the protocol numbering as recommended. Now, the steps are aligned on the left margin, and one-line space was added after each step. The current section of the protocol has 9 pages.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Answer: We highlighted the sections of the protocol steps for filming, maintaining the recommended limit of a maximum of 2.5 pages.

• **Results:** Avoid subheadings in this section. Remove any numbered lists.
Answer: We revised the result section was revised and removed the numbered lists.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
Answer: We revised the Discussion section and believe that it addresses all the required items.

• **Figure/Table Legends:** Please remove the figure/table legends from the figure files and place them directly below the Representative Results text.
Answer: We removed the figure/table legends from the figure files, as recommended.

• **Table of Materials:** Please sort in alphabetical order.
Answer: We adjusted the table of Materials as requested.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
Answer: We included the document regarding re-print permission in "Supplementary files" and adjusted the citations in the figures. In the first version of the document, we had already cited each figure's reference source appropriately in the legend.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The use of natural products as therapeutic agents to prevent or modulate oral diseases like dental caries is attracting increasing attention from researchers across the world. The authors are commended for a well-written manuscript describing a compact but comprehensive step-wise approach to identify anti-microbials and anti-biofilm molecules from plant extracts and fractions. The Introduction, various Methodologies and Discussion have been presented with a lot of clarity and scientific accuracy. The chemical profiling analysis described in the paper is to be specially commended. I believe the systematic approach described in the paper will be of great benefit in furthering research of natural products for oral care.

Major Concerns:

None

Answer: We thank **Reviewer #1** for the compliments. We have modified the manuscript to address the reviewer's minor concerns, as follows.

Minor Concerns:

1. Most plant extracts are soluble in solvents like 70% EtOH or DMSO - can the authors specifically comment on how to avoid the antimicrobial/antibiofilm actions of the solvents themselves from affecting results when testing extracts.

Answer: As we mentioned in the **Representative results section** (Line 516-523), the extracts were solubilized with 84.15% EtOH and 15% DMSO to reach 6 mg/mL (stock solution). We performed experiments using different concentrations of EtOH and DMSO (associated and isolated) to certify the final concentration of these reagents that did not interfere with the microbial viability of one bacterial and one fungal strain. Thus, it was possible to select the appropriate final vehicle concentration to assess the treatments' biological activity: 7% EtOH and 1.25% DMSO. The combination of EtOH and DMSO at those concentrations was used in all experiments, including the vehicle treated samples compared to the growth control samples, to ensure that the effects observed were due to the compounds in the extrats and fractions per se and not the solvents used. We appreciate the Reviewer's concern and include a sentence (Lines 518-521) commenting on the method used and the importance of this previous step.

2. For the antimicrobial activity assays, as the authors themselves mention in the manuscript, it is very common for the color of plant extracts or their precipitation to affect turbidity and thereby interfere with microplate O.D readings. How can researchers correct for this and get accurate results of plant extract antimicrobial activity?

Answer: Visual analysis will not always be the best response variable due to the color or precipitation of the extracts or fractions in the culture medium. Thus, we always use a set of controls on each assay (as described in Lines 305-309). Among these controls, we use a blank control per treatment (without microbial inoculation), which helps differentiate turbidity by the treatment per se or microbial growth. In addition to visual inspection, on all occasions, we read the optical density with an ELISA plate reader (as described in item 3.3, Lines 316-319). As precipitation can interfere with this reading, we also use an aliquot of cultures from each well to assess microbial viability via CFU/mL. One alternative is to use specific dyes that reveal whether the microbial cells are metabolically active or not (Eloff, 2019). However, we did not use this strategy because we prefer to check the microbial cell viability via plating on agar. This information was added to the discussion section.

Eloff JN. Avoiding pitfalls in determining antimicrobial activity of plant extracts and publishing the results. BMC Complement Altern Med. 2019;19(1):106. doi:10.1186/s12906-019-2519-3

Reviewer #2:

Manuscript Summary:

The manuscript shows a simplified approach to identify antimicrobial and antibiofilm molecules from plant extracts and fractions. And the manuscript is well written and the experimental design is reasonable.

We thank **Reviewer #2** for the inputs and concerns. We hope to have clarified the issues for each of the Reviewer' concerns, as detailed in the answers below.

Major Concerns:

In experimental case, the author want to test the antimicrobial and antibiofilm activities of *Casearia sylvestris* extracts from distinct Brazilian biomes against Streptococcus mutans and *Candida albicans*. But in the following manuscript, there is not any experiment about the *Candida albicans* including the data analysis. Please make a description it.

Answer: In our previous work, we used this screening approach to assess the biological activity of *C. sylvestris* extracts and fractions and to identify which were active against *S. mutans* and *C. albicans*. In this Protocol, we would like to keep just the results and discussion for the data obtained for *S. mutans* because the focus is to demonstrate the usefulness of this approach for screening natural compounds instead of interpreting and discussing the previously published data.

Minor Concerns:

In legend of figure 8, what is the meaning of "** ≤ p < 0.01"? Please clarify it.

Answer: There was a typo, and we corrected it to "** p ≤0.01".

Reviewer #3:

Manuscript Summary:

This manuscript describes a methodical approach to isolate and identify natural product compounds in plant extracts that inhibit microbial growth or biofilm formation, in this case of the cariogenic bacterium Streptococcus mutans. Human tooth decay remains a major health problem around the globe, hence novel therapies are required.

We thank **Reviewer #3** for the kind feedback. We modified the manuscript and hopefully have addressed the Reviewer's concerns.

Major Concerns:

The approach is mainly one of common sense. The authors describe the approach they have taken for *S. mutans* in a sequential fashion as an example. One concern is regarding the target audience and level of expertise of the reader. Naive readers would have a difficult time reproducing the results from the info given, which is a mixture of highly specific and defined information regarding reagents, concentrations, and volumes intermingled with broad sweeping statements stating that a given step, for example best solvents for extraction, would have to be optimized with little detail of where to start.

Answer: Thanks for the suggestion. We improved the step descriptions as suggested by the Editorial Comments. We hope that with the video there is better clarity of the protocol and the steps can be easily reproduced.

It would be helpful to provide a list or table of abbreviations or acronyms at the beginning of the paper. It was cumbersome in some places to have to stop and go chasing down the meaning of an abbreviated term. Please clearly define all up front.

Answer: We thank the Reviewer for this suggestion and have added a list with all abbreviations as a supplementary file. Still, we keep all abbreviations after the first mention of the word.

The discussion of Streptococcal-Candida interactions in the paper seemed more of a distraction than an important focus. I don't believe dual species or cultures or biofilms were evaluated in this paper, or even Candida monocultures. Were they? This was not entirely clear and easy to follow. Unless you are going to show examples of specific data of how this approach helps identify therapies that target this microbial pair specifically, it may be best not to veer off track.
Answer: We would like to keep this information in the text because previous evidence demonstrated the importance of these interactions for highly pathogenic cariogenic biofilm (e.g., Falsetta *et al.*, 2014). Previously, we evaluated both species in a single-species model (Ribeiro *et al.*, 2019). Thus, the data obtained before could guide which treatments can be tested in a biofilm model with both species (i.e., a dual-species biofilm model).

It would be helpful to define what is meant by dereplication in the introduction, and describe precisely how it is done and why.
Answer: We added a paragraph in the introduction with the description and justification for the use (Lines 91-99).

It was unclear to me what is meant by hyphenation. I am presuming the authors are referring to a tandem coupling of sequential methods, but am unsure. This should be clarified.
Answer: Liquid chromatography (LC) is usually hyphenated (coupled) with high-resolution mass spectrometry (HRMS), as LC−HRMS, for commonly use for the rapid annotation of metabolite in CE ou CEF (Bueno *et al.*, 2015). To make it clear, we added this sentence on lines 201-203.

The word strain should not be used synonymously with species, or even genus and species.
Answer: We have replaced the word "strain" for the appropriate terms, as listed below:
Line 293: replaced by “microorganism”.
Line 311: replaced by “microorganism”.
Line 670: replaced by “species”.

Information should be presented in the order it is most useful to the reader. For example, the description of the source and method of preparation of GtfB should precede the call for its use.
Answer: We removed the **NOTE** about source and method of preparation of GtfB before the steps (Lines 419-424), as recommended.

Minor Concerns:
line 267. Perhaps replace the word "aroused" with "suspected."
Answer: We replaced “aroused” by “suspected” in Line 245.

line 578 Change "halter" to "halt."
Answer: The word "halter" was replaced by "halt" in Line 561.

SUPPLEMENTAL FILE 2

List of abbreviations

C. sylvestris **-** *Casearia sylvestris*

CAD - Charged aerosol detectors

C. albicans **-** *Candida albicans*

CE - Crude extracts

CEF - Fractions

CFU/mL - Colony forming units per milliliter

DAD - UV/Vis diode array detector

DMSO - Dimethyl sulfoxide

ECD - Electronic circular dichroism

ELSD - Hyphenation with light scattering

EtOH - Ethanol

FA - Formic acid

gsHA **-** HA bead with initial glucan matrix

Gtfs - Glycosyltransferases

HA - Hydroxyapatite

IC_{50}/IC_{90} - The minimum concentration of a treatment capable of inhibiting 50 % and 90 %, respectively, of bacterial growth or biofilm formation

IR - Infrared

MS - Mass spectrometry

NaN_3 - 0.02% Sodium azide

NMR - Magnetic resonance

NPs - Natural products

O.D. - Pptical density

PMSF - 0.1 mM phenylmethylsulfonyl fluoride

ROA - Raman optical activity

sHA **-** HA bead with salivary pellicle

SisGen - National System for the Management of Genetic Heritage and Associated Traditional Knowledge

SPE - Solid-phase extraction cartridges

***S. mutans* -** *Streptococcus mutans*

TYE - Tryptone-yeast extract [2.5% (w/v) tryptone with 1.5% (w/v) yeast extract]

TYEg - Tryptone-yeast extract containing 1 % glucose

TYEs - Tryptone-yeast extract containing 1 % sucrose

UAE - ultrasound

UPLC - Ultra-high-performance liquid chromatography

UV - Ultraviolet

var. - variety

VCD - Vibrational circular dichroism

SPRINGER NATURE

Antimicrobial and antibiofilm activities of Casearia sylvestris extracts from distinct Brazilian biomes against Streptococcus mutans and Candida albicans

Author: Sabrina M. Ribeiro et al

Publication: BMC Complementary and Alternative Medicine

Publisher: Springer Nature

Date: Nov 12, 2019

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