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

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No, the protocol does not use either equipment.**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees self-record interview statements. JoVE can provide support for this option.

4. **Filming location:** Will the filming need to take place in multiple locations? **Yes, it will take place at the School of Dentistry and the Institute of Chemistry, Araraquara, São Paulo, Brazil**

   If **Yes**, how far apart are the locations? **About 1,74 miles.**

NOTE: This is an author produced video, all footage uploaded to FTP and slated.

Current Protocol Length

Number of Steps: 22
Number of Shots: 55

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Sabrina Marcela Ribeiro:** The presented protocol is useful for rapidly screening and analyzing bioactive candidates in NPs to control oral biofilms. It can also be adapted for applications in other biofilm research fields.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Sabrina Marcela Ribeiro:** This multi-target screening and analysis method makes it possible to screen multiple active components simultaneously.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Marlise I. Klein:** Dental caries is a biofilm-diet-derived, highly prevalent chronic disease globally. This protocol can help identify NPs to control biofilms and, consequently, prevent dental caries.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Introduction of Demonstrator on Camera

1.4. **Marlise I. Klein:** Demonstrating the procedure will be Érick Dante de Oliveira Fratucelli, an undergrad student from my laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator looks up from workbench or desk or microscope and acknowledges the camera.

Protocol

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

2.1. Begin by preparing the extraction solvent with a hydroalcoholic mixture **[1]**. Use sample concentrations between 50 and 100 milligrams per milliliter of the extraction solvent and perform 15-minute ultrasound assisted extractions in microtubes. Repeat the extraction 3 times **[2]**.

2.1.1. Talent preparing the extraction solvent

2.1.2. Talent performing UAE in batches

2.2. After each extraction step, centrifuge the sample **[1]** to decant the solid residue **[2]** and remove the supernatant **[3]**.

2.2.1. Talent centrifuging the sample after each extraction step

2.2.2. Talent decanting the solid residue

2.2.3. Talent removing the supernatant

2.3. Combine the supernatants from each extraction step **[1]** and filter them **[2]**. Save the aliquots for chemical analysis and bioassays **[3]**.

2.3.1. Talent combining the obtained supernatants

2.3.2. Talent filtering the combined supernatants

2.3.3. Talent making aliquots of the sample.

2.4. For fractionation, dilute the crude extract sample to obtain a 100-milligram per milliliter solution using the initial extraction solvent **[1]**. Then, transfer 1 milliliter of this sample to a pre-conditioned solid-phase extraction cartridge with 1 gram of adsorbent and a capacity of 6 milliliters **[2]**.

2.4.1. Talent adding the initial extraction solvent to the crude extract

2.4.2. Talent transferring the sample to SPE

2.5. Perform fractionation using around 3 dead volumes of each extraction eluent **[1]** and collect one fraction by eluent composition **[2]**. Save aliquots for chemical analysis and bioassays **[3]**.

2.5.1. Talent performing fractionation

2.5.2. Talent collecting fraction

2.5.3. Talent saving the aliquots for chemical analysis and bioassays.

2.6. Remove the solvent under vacuum, nitrogen flow, or lyophilization **[1]** and register the weight and yield **[2]**.

2.6.1. Talent removing solvent.

2.6.2. Talent weighing the sample.

3. Bioassay and Anti-microbial Activity

3.1. Reconstitute the dry matter with the best possible solvents **[1]**. Calculate the solvent concentration for the stock solution using the formula in the text manuscript **[2]**.

3.1.1. Talent adding solvent in the dry crude extract

3.1.2. Talent calculating the concentration of solution to be added

3.2. Reactivate the microbial strain of *S. mutans* UA159 on blood agar **[1]** and culture it in liquid culture medium **[2]**. Perform a 1 to 20 dilution of the initial culture using the same culture medium **[3]** and incubate it until it reaches the mid-log growth phase **[4]**.

3.2.1. Talent culturing microbial strains on blood agar

3.2.2. Talent adding the strain in culture medium.

3.2.3. Talent performing dilutions of the grown strain.

3.2.4. Talent incubating the culture.

3.3. Prepare the inoculum with a defined population in TYEg *(spell out 'T-Y-E-G')* for antimicrobial assays and TYE with 1% sucrose for biofilm assays **[1]**.

3.3.1. Talent preparing the inoculum.

3.4. For antimicrobial activity, define the concentration of the sample for analysis and add it to a 96-well plate **[1]**. Include a set of controls in each plate as described in the text manuscript **[2]**.

3.4.1. Talent adding the sample in 96-well plate.

3.4.2. Talent setting up different sets of controls.

3.5. Using TYEg, adjust the volume to 100 microliters **[1]**. Inoculate 100 microliters of microbial culture **[2]** and incubate the plate for 24 hours at 37 degrees Celsius in 5% carbon dioxide **[3]**.

3.5.1. Talent adding culture medium into the wells.

3.5.2. Talent adding bacterial inoculum in the wells

3.5.3. Talent incubating the plates.

3.6. Analyze the bacterial growth according to turbidity by visual inspection of the wells **[1]**. Inoculate an aliquot of the desired dilution in specific agar plates in duplicates **[2]** and incubate the plates **[3]**. After the incubation, perform colony counts **[4]**. *Videographer: This step is difficult!*

3.6.1. Talent looking at the wells.

3.6.2. Talent plating the desired dilution in duplicates.

3.6.3. Talent incubating the plates.

3.6.4. Talent counting the bacterial colonies.

4. Validation of Biological Activity and Biological Data Analysis

4.1. Weigh hydroxyapatite beads in microtubes **[1]** and sterilize them **[2]**, then wash the beads using adsorption buffer AB **[3]**.

4.1.1. Talent weighing HA beads

4.1.2. Talent sterilizing the HA beads

4.1.3. Talent washing the beads with AB buffer

4.2. Add 500 microliters of saliva into the microtubes to form a salivary film **[1]** and incubate at 37 degrees Celsius with 24 rotations per minute for 40 minutes **[2]**. *Videographer: This step is important!*

4.2.1. Talent adding saliva in the microtubes.

4.2.2. Talent incubating the microtubes.

4.3. Remove the saliva supernatant **[1]** and wash the beads three times with AB to prepare them for downstream assays **[2]**. *Videographer: This step is important!*

4.3.1. Talent removing the supernatant

4.3.2. Talent washing the beads.

4.4. Add 500 microliters of the sample or the control into each microtube containing beads with salivary pellicle **[1]** and incubate them at 24 rotations per minute and 37 degrees Celsius for 30 minutes **[2]**. Wash the beads 3 times with AB **[3]**. *Videographer: This step is important!*

4.4.1. Talent adding sample to the microtubes

4.4.2. Talent incubating the microtubes

4.4.3. Talent washing the beads with AB buffer.

4.5. Add 500 microliters of microbial culture to each microtube **[1]** and incubate the tubes in similar conditions for 1 hour **[2]**. Then, wash the unbound cells 3 times with AB buffer **[3]**. *Videographer: This step is important!*

4.5.1. Talent adding microbial culture to the microtube.

4.5.2. Talent incubating the microtube.

4.5.3. Talent washing the tube with AB buffer.

4.6. Resuspend each sample with 1 milliliter of AB buffer **[1]** and sonicate at 7 watts for 30 seconds **[2]**. Serially dilute aliquots of each suspension ten-fold **[3]** and plate them on specific agar plates **[4]**. *Videographer: This step is important!*

4.6.1. Talent adding AB buffer.

4.6.2. Talent sonicating the solution

4.6.3. Talent serially diluting the solution

4.6.4. Talent plating the diluted solution.

4.7. Incubate the plates for 48 hours at 37 degrees Celsius in 5% carbon dioxide **[1]** and count the colonies **[2]**. *Videographer: This step is important!*

4.7.1. Talent incubating the plates

4.7.2. Talent counting the colonies.

4.8. For data analysis, input the raw data for the bioassays into a spreadsheet **[1]** and calculate the log of microbial growth inhibition by each treatment and the log percentage of microbial growth inhibition compared with the control **[2]**.

4.8.1. SCREEN: 61773_4.8_t1.mp4. NOTE: Skip or speed through the parts where formulas are being entered into cells, showing this process once is enough.

4.9. Correct the optical density of the readings and calculate the percentage of biomass inhibition **[1]**.

4.9.1. SCREEN: 61773_4.9_t2.mp4. 1:10 - end. NOTE: Speed up as needed.

Results

5. The screening results of the twelve crude extracts from three varieties of *C. sylvestris* against *S. mutans*

5.1. The chemical profile of biological screening for the quantity of clerodane-type diterpenes and glycosylated flavonoids in three varieties of *C. sylvestris* extracts **[1]**, namely, *sylvestris* **[2]**, intermediate **[3]**, and *lingua,* are shown here **[4]**.

5.1.1. LAB MEDIA: Figure 7

5.1.2. LAB MEDIA: Figure 7. *Video Editor: Emphasize the bars labeled S.*

5.1.3. LAB MEDIA: Figure 7. *Video Editor: Emphasize the bars labeled I.*

5.1.4. LAB MEDIA: Figure 7. *Video Editor: Emphasize the bars labeled L.*

5.2. After analysis of the raw data, four extracts showed a favorable response. 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 and variety **[1]**.

5.2.1. LAB MEDIA: Figure 8

5.3. Calculated percent CFU of treated planktonic cells **[1]**, percent biomass of the treated biofilms **[2]**, and percent CFU of the treated biofilm are shown here **[3]**.

5.3.1. LAB MEDIA: Figure 8 A

5.3.2. LAB MEDIA: Figure 8 B

5.3.3. LAB MEDIA: Figure 8 C

5.4. No crude extract significantly affected the removal of *S. mutans* cells adhered to the salivary pellicle **[1]**.

5.4.1. LAB MEDIA: Figure 9A

5.5. The adhesion of *S. mutans* cells to the glucan matrix was weakened by three of the crude extracts **[1]**.

5.5.1. LAB MEDIA: Figure 9B

Conclusion

6. Conclusion Interview Statements

6.1. **Paula C. Pires Bueno:** Since each plant species requires optimized and specific chemical analysis methods, the best solvent extraction and the analytical method must be selected from previous reports or defined by experimental design.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.1*

6.2. **Marlise I. Klein:** It is critical to prepare formulations with the most active crude extracts and fractions and test them on complex models to prevent the development of biofilms and cavities.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.