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Title: In Vitro Evaluation of the Potentiating Effects of Plant-Derived Molecules on Conventional Biocides

Authors and Affiliations:

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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**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 25

Number of Shots: 51 (7 SC)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.
Answers are slightly edited in accordance with journal guidelines.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Mariana Sousa:** Our research evaluates plant-derived compounds that enhance biocide efficacy, identifying synergistic combinations to improve microbial control while reducing toxicity and resistance development.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Mariana Sousa:** Existing methods lack dynamic and synergy analyses; this protocol integrates kinetic modeling, regrowth assessment, and interaction mapping for comprehensive antimicrobial evaluation.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Mariana Sousa:** This protocol enables measurement of bactericidal activity, synergistic interactions, regrowth potential, and disinfection kinetics of biocide–phytochemical combinations across varying concentrations and time points.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Mariana Sousa:** Following this procedure, researchers can perform biofilm assays, resistant strain testing, mechanism studies, formulation optimization, and predictive modeling of antimicrobial combinations.

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

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Mariana Sousa:** Future studies will explore novel phytochemicals, resistant strains, and biofilm models, with validation of antimicrobial combinations in healthcare, industrial, and environmental applications.

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

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Bacterial Culture Preparation and Calibration Curve Generation

Demonstrator: Mariana Sousa

2.1. To begin, remove the cryovials containing glycerol stocks of bacterial strains *Escherichia coli* CECT 434 and *Staphylococcus aureus* CECT 976 from the freezer [1] and place them on ice [2].

2.1.1. WIDE: Talent removing the cryovials from the minus 80 degrees freezer.
***Videographer:** Please obtain CU shot as well for this shot*

2.1.2. Talent placing labeled cryovials on ice.

2.2. Streak the bacteria on PCA plates [1-TXT] and incubate them for 24 hours at 25 degrees Celsius in an incubator [2].

2.2.1. Talent streaking or placing the bacterial culture onto plate count agar plates.
TXT: PCA: Plate Count Agar

2.2.2. Talent placing the plates in the incubator.

2.3. Inoculate 1 bacterial colony from the streaked plate into tryptic soy broth [1]. Incubate the culture in a shaking incubator at 100 revolutions per minute and 25 degrees Celsius [2].

2.3.1. Talent inoculating 1 bacterial colony from a streaked plate into tryptic soy broth.

2.3.2. Talent placing the inoculated broth in the shaking incubator.

2.4. After incubation, prepare 10-fold serial dilutions of the culture in sterile saline solution containing 0.85 percent weight per volume sodium chloride [2]. Using a spectrophotometer, measure the optical density at 600 nanometers of each dilution [3].

2.4.1. Talent preparing 10-fold serial dilutions of the bacterial culture in sterile saline solution.

2.4.2. Talent placing the sample and measuring the optical density at 600 nanometers in the spectrophotometer.

- 2.5. Next, plate the cells at the required dilutions on PCA plates [1] and incubate them at 25 degrees Celsius for 24 hours [2]. Select the plates presenting between 10 and 100 colonies [2] and determine the corresponding colony-forming units per milliliter values [4].
 - 2.5.1. Talent plating the appropriate dilutions on plate count agar.
 - 2.5.2. Talent placing the plates in the incubator.
 - 2.5.3. Talent examining the plates and selecting a plate with between 10 and 100 colonies.
 - 2.5.4. Talent counting colonies on the plate by marking them and writing a count in a notebook.
- 2.6. Plot the colony-forming units per milliliter values against the corresponding optical density readings within the linear range of 0.04 to 0.8 [1].
 - 2.6.1. SCREEN: 2.6.1.
- 2.7. With at least 2 independent assays, perform linear regression analysis to obtain the calibration equation [1]. Use the resulting equation to estimate bacterial concentrations in subsequent experiments [2].
 - 2.7.1. SCREEN: 2.7.1 00:01-00:08 .
 - 2.7.2. SCREEN: 2.7.1. 00:15-00:17 and 2.7.2
- 2.8. Then, centrifuge the inoculum at 3772 g for 10 minutes [1] and wash the cells with PBS [2].
 - 2.8.1. Talent placing the inocula in the centrifuge.
 - 2.8.2. Talent adding PBS to the tube and mixing.
- 2.9. Using a spectrophotometer, measure the optical density of the cells in PBS at 600 nanometers [1] and adjust the density of the inoculum to 1×10^8 colony-forming units per milliliter with fresh tryptic soy broth [1].
 - 2.9.1. Talent placing sample in spectrophotometer.
 - 2.9.2. Talent adding TSB media to the cells taken in a tube to adjust the optical density.

3. Antimicrobial Treatment and Quantification

3.1. Use 2 biocides, Benzalkonium chloride and peracetic acid, at 15 percent in ultrapure water, and 2 phytochemicals, salicylic acid and eugenol, at 99 percent in dimethyl sulfoxide [1-TXT].

3.1.1. Shot of the labelled vials with stocks of BAC, PAA, SAL, and EUG being arranged on desk for dilution. **TXT: BAC: Benzalkonium chloride**

PAA: Peracetic acid

SAL: Salicylic acid

EUG: Eugenol

3.2. Dilute the stock solutions in 15-milliliter tubes to prepare [1] the required concentrations of the compounds [2].

3.2.1. Talent aliquoting one stock to a new 15 ml tube containing water.

3.2.2. LAB MEDIA : Table 1.

3.3. For all assays, include 2 negative controls: bacteria without treatment [1] and bacteria exposed to 5 percent dimethyl sulfoxide [2].

3.3.1. Talent labelling a tube as the 'Untreated bacterial negative control'.

3.3.2. Talent labeling another tube as 'Negative control: exposed to 5% DMSO'.

3.4. Next, add 1 milliliter of cell suspension to 1 milliliter of ultrapure water and wait for 2 minutes [1].

3.4.1. Talent adding 1 milliliter of cell suspension to 1 milliliter of ultrapure water in a tube and keeping the tube aside.

3.5. Then, add 8 milliliters of the prepared biocide or phytochemical solution to the cells [1] and mix using a vortex [2]. Incubate the mixture in a shaking incubator at 100 revolutions per minute and 25 degrees Celsius for 30 minutes [3].

3.5.1. Talent adding 8 milliliters of biocide or phytochemical solution to the cells.

3.5.2. Talent vortexing the tube to mix the compounds with the cells.

3.5.3. Talent placing the tube in the shaking incubator.

- 3.6. Prepare the universal neutralizer to neutralize each biocide or phytochemical by dilution [1 and 2].
- 3.6.1. Talent mixing the neutralization solution. *Video editor: Show 3.6.1 and 3.6.2 using split screen*
- 3.6.2. **Text on plain background:**
Universal neutralizer:
30 g/L Polysorbate 80
30 g/L Saponin
1 g/L L-histidine
3 g/L Lecithin
5 g/L Sodium thiosulphate in 0.0025 M phosphate buffer
- 3.7. Add 1 milliliter of the sample being neutralized to 8 milliliters of universal neutralizer [1] and then add 1 milliliter of ultrapure water [2].
- 3.7.1. Talent adding 1 milliliter of the sample to 8 milliliters of universal neutralizer.
- 3.7.2. Talent adding 1 milliliter of ultrapure water to the mixture.
- 3.8. To perform a ten-fold serial dilution, first add 100 microliters of a specific dilution to 900 microliters of sterile saline solution [1]. Then, serially dilute the sample from 10 to the power of minus 1 to dilution 10 to the power of minus 7 [2]. Next, plate 10 microliters of each dilution onto PCA plate using the drop plate method to determine surviving cells [3]. Incubate the plates at 25 degrees Celsius without agitation for 24 hours [4].
- 3.8.1. Talent transferring 100 microliters into 900 microliters of sterile saline solution.
- 3.8.2. Talent continuing the serial dilution series up to 10 to the power of minus 7.
- 3.8.3. Talent plating 10 microliters of each dilution onto plate count agar using the drop plate method.
- 3.8.4. Talent placing the plates in the incubator.
- 3.9. Visually count colony-forming units on plates containing more than 10 and less than 100 colonies [1]. Record the concentration at which complete reduction in culturability occurs as the minimum bactericidal concentration [2]. Calculate the colony-forming units per milliliter using the Equation [3].

- 3.9.1. Talent examining plates and counting colonies within the specified range.
- 3.9.2. Talent recording the minimum bactericidal concentration based on observed results in a book or computer.
- 3.9.3. SCREEN: 3.9.3 00:05-00:11 and 00:15-00:22.

3.10. Then, assess the logarithmic reduction of bacteria [1].

- 3.10.1. SCREEN: 3.10.1. 00:05-00:09

4. Evaluation of Combination Treatments and Regrowth Analysis

4.1. Apply the previously described methodology to assess the activity of all possible dual and triple combinations to search for possible potentiation or synergistic effects [1]. Use 1 biocide and 1 phytochemical for dual combinations, or 1 biocide and 2 phytochemicals for triple combinations [2 and 3 side by side].

- 4.1.1. Talent aliquoting solutions to multiple treatment tubes labeled 'dual' and 'triple combinations'.

4.1.2. TEXT ON PLAIN BACKGROUND

Dual combination: 1 Biocide + 1 Phytochemical

Concentrations for dual combinations:

- BAC: 0.1, 0.5, 0.75 mg/L
- PAA: 0.1, 0.3, 0.5 mg/L
- SAL: 50, 100, 250 mg/L
- EUG: 100, 250, 750 mg/L

4.1.3. **Triple combination:** 1 Biocide + 2 Phytochemicals

Concentrations for triple combinations:

- BAC: 0.75 mg/L
- PAA: 0.5 mg/L
- SAL: 100, 250 mg/L
- EUG: 250, 750 mg/L

Video editor: Please show these 2.24.2 and 2.24.3 side by side using split screen and add a vertical line in between to divide these 2

- 4.2. Select the concentrations that were assessed in the dose-response assay for individual molecules that resulted in non-bactericidal effects [1]. To assess bacterial regrowth ability, allow the microbial cells to grow in liquid medium for 24 hours after treatment and neutralization at 25 degrees Celsius and 100 revolutions per minute [2].
 - 4.2.1. Talent looking at the previous calculation in a book or on screen and picking up a sample tube.
 - 4.2.2. Talent placing the samples in the shaking incubator at 25 degrees Celsius.
- 4.3. Perform ten-fold serial dilution, plating, and incubation of the PCA plates as demonstrated earlier to quantify the colony-forming units per milliliter [1].
 - 4.3.1. *Reuse 3.8.2, 3.8.3 and 3.8.4 using split screen.*
- 4.4. Finally, assess antimicrobial interactions and kinetics by combining the fractional bactericidal concentration index classification, Combenefit mapping, and time-response modeling [1].
 - 4.4.1. SCREEN: 4.4.1 00:04-00:07, 00:12-00:14, 00:17-00:20

Results

5. Results

5.1. Benzalkonium chloride, peracetic acid, salicylic acid, and eugenol showed a clear concentration-dependent reduction in culturability after 30 minutes of treatment [1].

5.1.1. LAB MEDIA: Figure 1. *Video editor: Serially highlight all 4 panels and emphasize the decreasing gray squares and black circles data points.*

5.2. Benzalkonium chloride and peracetic acid achieved complete inactivation at much lower concentrations [1] than salicylic acid and eugenol [1].

5.2.1. LAB MEDIA: Figure 1. *Video editor: Highlight A and B.*

5.2.2. LAB MEDIA: Figure 1. *Video editor: Highlight C and D.*

5.3. After 24 hours of regrowth, *Staphylococcus aureus* remained inactivated at the same concentrations used for the immediate effects for all compounds, indicating that the effectiveness of the four molecules was sustained [1].

5.3.1. LAB MEDIA: Figure 2. *Video editor: Highlight the gray square series in ABCD.*

5.4. In contrast, *Escherichia coli* showed some regrowth after 24 hours, requiring higher concentrations of Benzalkonium chloride, salicylic acid, and eugenol to achieve complete inactivation [1]. Only for peracetic acid, complete inactivation of *Escherichia coli* occurred at 1 milligram per liter, matching the concentration observed in the immediate effect data [2].

5.4.1. LAB MEDIA: Figure 2. *Video editor: Highlight the black circle series in A, C and D.*

5.4.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the black circle series in B and emphasize the black circle at the 1.0 mg/ml mark on the X axis*

5.5. Synergy maps revealed that antimicrobial interactions were highly concentration-dependent [1], showing both synergistic [2] and antagonistic regions [3].

5.5.1. LAB MEDIA: Figure 3.

5.5.2. LAB MEDIA: Figure 3. *Video editor: Show 2ND AND 3RD ROWS and point out blue zone across the surfaces.*

5.5.3. LAB MEDIA: Figure 3. *Video editor: Show 1st and last rows and point out red*

zones across the surfaces.

5.6. Benzalkonium chloride and peracetic acid generally demonstrated faster and stronger time-dependent killing compared to salicylic acid and eugenol [1].

5.6.1. LAB MEDIA: *Figure 4. Video editor: Highlight A and B.*

5.6.2. LAB MEDIA: *Figure 4. Video editor: Highlight C and D.*

5.7. Higher concentrations consistently resulted in faster bacterial inactivation over time for all compounds [1].

5.7.1. LAB MEDIA: Figure 4. *Video editor: Highlight the square and inverted triangle data points across A B C D.*

Escherichia coli

Pronunciation link: <https://www.merriam-webster.com/dictionary/Escherichia%20coli>

IPA: /ˌe-shə-ˈri-kē-ə-ˈkō-, lī/

Phonetic Spelling: esh·uh·REE·kee·uh KOH·lye

Staphylococcus aureus

Pronunciation link: <https://www.merriam-webster.com/dictionary/Staphylococcus%20aureus>

IPA: /ˌstaf-ə-lō-ˈkă-kəs ˈôr-ē-əs/

Phonetic Spelling: staf·uh·loh·KAH·kuhs OR·ee·uhs

Cryovials

Pronunciation link: <https://www.merriam-webster.com/dictionary/cryovial>

IPA: /ˈkrī-ə-, vī-əl/

Phonetic Spelling: KRY·oh·vye·uhl

Incubator

Pronunciation link: <https://www.merriam-webster.com/dictionary/incubator>

IPA: /ˈɪŋkjəˌbeɪtər/

Phonetic Spelling: ING·kyuh·bay·ter

Inoculate

Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculate>

IPA: /ɪˈnɑːkjəˌleɪt/

Phonetic Spelling: ih·NAHK·yuh·layt

Tryptic

Pronunciation link: <https://www.merriam-webster.com/dictionary/tryptic>

IPA: /'trɪp.tɪk/

Phonetic Spelling: TRIP·tik

Spectrophotometer

Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>

IPA: /,spek.troʊ.foʊ'tɑː.mə.tə/

Phonetic Spelling: SPEK·troh·foh·TAH·muh·ter

Optical density

Pronunciation link: <https://www.merriam-webster.com/dictionary/optical>

IPA: /'ɑːptɪkəl/

Phonetic Spelling: OP·tih·kuhl

Colony-forming units

Pronunciation link: <https://www.merriam-webster.com/dictionary/colony>

IPA: /'kɑːləni/

Phonetic Spelling: KAH·luh·nee

Linear regression

Pronunciation link: <https://www.merriam-webster.com/dictionary/regression>

IPA: /rɪ'grɛʃən/

Phonetic Spelling: rih·GRESH·uhn

Centrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>

IPA: /'sentrəˌfjuːdʒ/

Phonetic Spelling: SEN·truh·fyooj

PBS

Pronunciation link: No confirmed link found

IPA: /ˌpiː biː 'ɛs/

Phonetic Spelling: pee·bee·ESS

Benzalkonium chloride

Pronunciation link: <https://www.merriam-webster.com/dictionary/benzalkonium%20chloride>

IPA: /ˌben-zal-'kō-nē-əm 'klôr-īd/

Phonetic Spelling: ben·zal·KOH·nee·uhm KLOR·ide

Peracetic acid

Pronunciation link: <https://www.merriam-webster.com/dictionary/peracetic>

IPA: /ˌper-ə-'sē-tik/

Phonetic Spelling: pehr·uh·SEE·tik

Salicylic acid

Pronunciation link: <https://www.merriam-webster.com/dictionary/salicylic>

IPA: /,sələ'sɪlɪk/

Phonetic Spelling: sal·uh·SIL·ik

Eugenol

Pronunciation link: <https://www.merriam-webster.com/dictionary/eugenol>

IPA: /ju:'dʒi:nɔ:l/

Phonetic Spelling: yoo·JEE·nawl

Dimethyl sulfoxide

Pronunciation link: <https://www.merriam-webster.com/dictionary/dimethyl%20sulfoxide>

IPA: /daɪ'mɛθəl səl'fʊ:k,səɪd/

Phonetic Spelling: dye·METH·uhl sul·FOK·side

Biocide

Pronunciation link: <https://www.merriam-webster.com/dictionary/biocide>

IPA: /'baɪoʊ,səɪd/

Phonetic Spelling: BYE·oh·side

Phytochemical

Pronunciation link: <https://www.merriam-webster.com/dictionary/phytochemical>

IPA: /,fɑtoʊ'kemɪkəl/

Phonetic Spelling: FYE·toh·KEM·ih·kuhl

Vortex

Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>

IPA: /'vɔɪ,tɛks/

Phonetic Spelling: VOR·teks

Polysorbate

Pronunciation link: No confirmed link found

IPA: /,pɑ:li'sɔ:rbet/

Phonetic Spelling: pol·ee·SOR·bayt

Saponin

Pronunciation link: <https://www.merriam-webster.com/dictionary/saponin>

IPA: /'sæpənɪn/

Phonetic Spelling: SAP·uh·nin

L-histidine

Pronunciation link: <https://www.merriam-webster.com/dictionary/histidine>

IPA: /'hɪstə,di:n/

Phonetic Spelling: HIS·tuh·deen

Lecithin

Pronunciation link: <https://www.merriam-webster.com/dictionary/lecithin>

IPA: /ˈlesəθɪn/

Phonetic Spelling: LES·uh·thin

Sodium thiosulphate

Pronunciation link: <https://www.merriam-webster.com/dictionary/thiosulfate>

IPA: /ˌθaɪoʊˈsʌlfet/

Phonetic Spelling: THYE·oh·SUL·fayt

Microliters

Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>

IPA: /ˈmaɪkroʊˌliːtər/

Phonetic Spelling: MYE·kroh·lee·ter

Bactericidal

Pronunciation link: <https://www.merriam-webster.com/dictionary/bactericidal>

IPA: /bækˌtɪrəˈsaɪdəl/

Phonetic Spelling: bak·TEER·uh·SYE·duhl

Culturability

Pronunciation link: No confirmed link found

IPA: /ˌkʌl.tʃə.əˈbɪl.ə.ti/

Phonetic Spelling: KUL·chur·uh·BIL·uh·tee

Synergistic

Pronunciation link: <https://www.merriam-webster.com/dictionary/synergistic>

IPA: /ˌsɪnəˈdʒɪstɪk/

Phonetic Spelling: sin·ur·JIS·tik

Antagonistic

Pronunciation link: <https://www.merriam-webster.com/dictionary/antagonistic>

IPA: /ænˌtæɡəˈnɪstɪk/

Phonetic Spelling: an·TAG·uh·NIS·tik

Combeneft

Pronunciation link: No confirmed link found

IPA: No verified American IPA found

Phonetic Spelling: KOM·ben·uh·fit

Fractional bactericidal concentration index

Pronunciation link: No confirmed link found

IPA: /ˈfrækʃənəl bækˌtɪrəˈsaɪdəl ˌkɑːnsənˈtreɪʃən ˈɪndeks/

Phonetic Spelling: FRAK·shuh·nuhl bak·TEER·uh·SYE·duhl kon·suhn·TRAY·shuhn IN·deks

