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Article

Myrtaceaes as antimicrobial agents against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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SUMMARY

Anti-microbial resistance causes more than 700,000 deaths annually, a figure that is predicted to grow to 10 million by 2050 and is one of the leading contributors to the Global Burden of Disease. The six leading contributors to the surge of antimicrobial resistance are named the ESKAPE pathogens. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two of these bacteria. More than 80% of the active ingredients in antimicrobial drugs are based on natural products. Botanical species are the largest contributors of these active ingredients, yet they remain largely unstudied. Our research aimed to explore the antimicrobial properties of the Myrtaceae family. We collected, dried, and extracted the flowers of six native Australian Myrtaceae species. We tested these crude extracts at ten different concentrations on *S. aureus* and *P. aeruginosa*, to determine percentage inhibition, which was used as an indicator of the efficiency of the crude extract in causing bacterial death. The minimum inhibitory concentration (MIC) determines the viability of an extract for further drug development. For *S. aureus*, all six tested Myrtaceae species exceeded MIC80 (concentration needed for 80% inhibition), with Corymbia ficifolia being the most potent, exceeding MIC80 at 0.04 mg/mL. For *P. aeruginosa* four out of the six tested Myrtaceae species reached MIC80. Xanthostemon chrysanthus was most effective against *P. aeruginosa* with a MIC80 of 5.00 mg/mL. Myrtaceae species were shown to be highly effective against *S. aureus* and even *P. aeruginosa*, suggesting that further investigation into Myrtaceae species, could be revolutionary in drug development for newer, more effective antimicrobial drugs.

INTRODUCTION

One of the greatest modern global concerns is the increasingly evident Antimicrobial Resistance (AMR) crisis, which has resulted in an increasing number of infectious diseases affecting the population which are untreatable with currently used antimicrobial medication (1). The AMR crisis is a significant contributor to the global disease burden resulting in longer hospital stays, increased mortality, and higher medical expenses, putting strain on medical systems (1).

AMR occurs naturally with genetic mutation or with the misuse and overuse of antimicrobial drugs (2). Poor infection control, inadequate sanitary conditions, and poor access to appropriate medicines alongside the increasingly globalized distribution of pathogens increase the frequency of AMR (2,3). Drug resistance can be caused by the modification of the drug target, the inactivation of the drug, or changes in cell permeability which reduce drug accumulation within the cell, preventing the drug from being effective (4,5).

The six leading contributors to the surge of AMR are named the ESKAPE pathogens and include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and the *Enterobacter* species (6). The greatest contributors to the global burden of disease are methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *E. faecium* (VRE), and fluoroquinolone-resistant *P. aeruginosa* (7).

P. aeruginosa is a gram-negative, aerobic rod, and monoflagellated bacterium and is highly resistant to external factors (8). Due to the opportunistic nature of *P. aeruginosa*, it rarely infects uncompromised tissue however invades immunodeficient tissue, making infections difficult to treat (9). For example, patients with conditions such as severe burns, tuberculosis, cancer, cystic fibrosis, or AIDS have increased susceptibility to *P. aeruginosa* with a 50% fatality rate (9).

S. aureus is a gram-positive and cocci-shaped bacterium and is naturally found on the skin and mucous membranes (10, 11). If *S. aureus* is allowed to enter internal tissue or the bloodstream, it can cause infections throughout the body (12). *S. aureus* causes one of the largest ranges of infection, and different strains have independent pathophysiological properties, making it difficult to treat (12).

The World Health Organization estimates that more than 700,000 deaths annually are caused solely by AMR (13). The mortality of many conditions increases dramatically after contracting an infection from an antimicrobial resistant strain (13). The number of deaths caused by antimicrobial resistant pathogens is expected to be more than 10 million by 2050 (14). With this rapidly increasing rate of AMR, researchers have begun to focus on natural products as a new source of active ingredients to develop more effective antimicrobial drugs (15). More than 80% of the active compounds in antimicrobial drugs are based on natural products (15). Botanical species are the largest contributors of these active ingredients, however, due to the large biodiversity of Plantae species, many botanical species remain unstudied (15).

The pharmacological properties of botanical species depend on their phytochemical constituents, which are classified into two categories. Primary plant metabolites contribute to functions of life such as cell division, growth, respiration, and reproduction; thus, they are essentially the same across all botanical species (16). Secondary metabolites are bioactive compounds that are characterized by their chemical structures and differ between species (17). Unlike synthetic treatments, secondary metabolites have developed and evolved with exposure to different pathogens over time,

thus these bioactive compounds have high specificity against the metabolic processes of foreign agents, which may be effective on ESKAPE pathogens (17).

Myrtaceae is a family of Plantae that contains an estimated 150 genera and 3,300 species (18). In Australia, there are 70 genera and 1646 species of Myrtaceae, most of which are endemic to Australia (18,19). Research into Myrtaceae suggests that they contain different types of alkaloids, flavonoids, terpenes, and tannins, which are classes of secondary metabolites (20).

With such a large range of secondary metabolites, we hypothesized that the crude extracts of *Xanthostemon chrysanthus* (golden penda), *Syzygium australe* (creek lilly-pilly), *Melaleuca leucadendra* (weeping paperbark), *Angophora bakeri* (narrow-leaved apple gum), *Corymbia ficifolia* (red flowering gum), *and Callistemon viminalis* (weeping bottle brush) would show varying antimicrobial properties, because there is a large probability that the crude extracts contain at least one effective bioactive compound against the bacteria (21). Eucalyptus is the most frequently used Myrtaceae genus in antibiotic development. Thus, we expected that *A. bakeri* and *C. ficifolia* would be most effective against *S. aureus* and *P. aeruginosa* because Angophora and Corymbia genera are closely related to Eucalyptus in structure and metabolism (22). Finally, we predicted that *P. aeruginosa* will be less susceptible to crude extracts because gram-negative bacteria contain two outer layers: the second outer membrane being an effective barrier, preventing the passage of large molecules such as secondary metabolites or antibiotics (23). In contrast, *S. aureus* is a gram-positive bacterium and only has a peptidoglycan layer, which is relatively porous, allowing secondary metabolites access to intercellular metabolic processes, making gram-positive bacteria more susceptible to these bioactive compounds (23).

We collected, dried, and extracted crude extracts from the flowers of six Myrtaceae species, and we tested ten concentrations of each crude extract in a cell viability bioassay against *S. aureus* and *P. aeruginosa* to explore the antimicrobial properties of the crude extracts. We recorded the results in percentage inhibition, which we used as an indicator of the effectiveness of the crude extract in causing bacterial death. In drug development, crude extracts are evaluated based on the concentration at which percent inhibition exceeds 80%, which is called the minimum inhibitory concentration 80 (MIC80). Extracts seemed more effective on *S. aureus* than on *P. aeruginosa*. For *S. aureus*, all six test species exceeded MIC80 at relatively lower concentrations. In comparison only four out of the six tested Myrtaceae reached MIC80 on *P. aeruginosa* at much higher concentrations. For further experimentation, we can isolate and retest the phytochemical constituents of effective species to identify which bioactive compounds caused inhibition. The efficiency of crude extracts on both gram-negative and gram-positive bacteria species suggests that the tested species and other Myrtaceae species could be effective against other bacteria and multi-drug resistant strains.

RESULTS

We created extractions from the dried flowers of six Myrtaceae species and tested ten concentrations of these crude extracts against *S. aureus* and *P. aeruginosa* via a cell viability bioassay using resazurin as our indicator of cell viability. In active cells, resazurin is reduced to resoflurin and fluorescence can be measure using a plate reader. All experiments were carried out in triplicate.

All six investigated Myrtaceae species reached MIC80 for *S. aureus* (**Figure 1, Table 1**). *C. ficifolia* reached MIC80 with the lowest concentration at 0.04 mg/mL suggesting that it is the most potent against *S. aureus* out of the six tested species. *M. leucadendra* and *C. viminalis* reached MIC80 at 0.08 mg/mL, followed by *A. bakeri* which reached MIC80 at 0.63 mg/mL. *X. chrysanthus* reached MIC80 at 2.50 mg/mL. *S. australe* showed the least potency on *S. aureus* with a MIC80 of 5.00 mg/mL (**Table 1**). Only four plant species reached MIC80 for *P. aeruginosa*. *X. chrysanthus* and *S. australe* reached MIC80 at 5.00 mg/mL. *A. bakeri* and *C. viminalis* reached MIC80 at 10.00 mg/mL (**Figure 2, Table 1**). *M. leucadendra* and *C. ficifolia* did not inhibit 80 percent of *P. aeruginosa* and thus did not reach MIC80 (**Table 1**).

Some wells of the *P. aeruginosa* plates appeared yellow, suggesting that resazurin may have reduced into the non-fluorescent dihydroresorufin. To investigate the natural reduction of resazurin by the crude extracts we prepared an additional microplate without bacteria. Without bacteria,

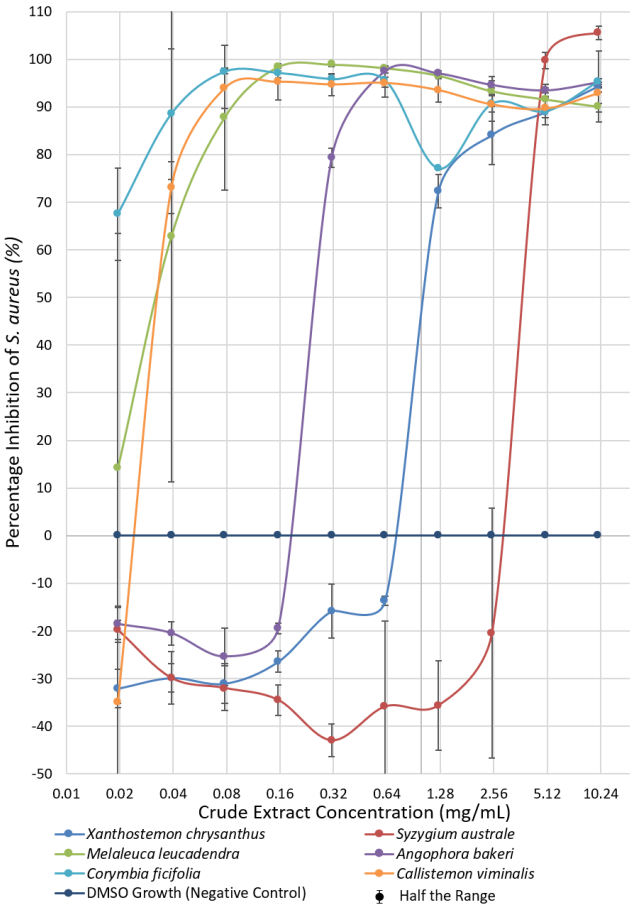

Figure 1: The mean percentage inhibition of *S. aureus* caused by Myrtaceae crude extracts. Inhibition was measured at ten different concentrations after 21 hours of incubation in a cell viability bioassay. Percentage inhibition was calculated from Relative Fluorescence Units (RFU), presented in mean percentage inhibition ± half the range, represented by the error bars (n=3).

Species	Against *S. aureus*	Against *P. aeruginosa*
Xanthostemon chrysanthus	2.50 mg/mL	5 mg/mL
Syzygium australe	5.00 mg/mL	5 mg/mL
Melaleuca leucadendra	0.08 mg mL	-
Angophora bakeri	0.63 mg/mL	10 mg/mL
Corymbia ficifolia	0.04 mg/mL	-
Callistemon viminalis	0.08 mg/mL	10 mg/mL

Table 1: Minimum Inhibitory Concentration of 80% (MIC80). Concentration (mg/mL) at which MIC80 was reached against *S. aureus* and *P. aeruginosa*.

wells should remain blue, but *S. australe* naturally reduces the resazurin into resorufin. To confirm the inhibition of *P. aeruginosa* from *S. australe*, we prepared an agar plate with *P. aeruginosa* and placed a sample of the 5.00 mg/mL *S. australe* crude extract before the agar plate was incubated. There was no measurable zone of inhibition, suggesting that MIC80 for *S. australe* was closer to 10.00 mg/mL than 5.00 mg/mL.

We found a significant difference between the percentage inhibition of *S. aureus* and *P. aeruginosa* for the *S. australe* (p = 0.001219), *M. leucadendra* (p = 0.000003), *A. bakeri* (p = 0.027883), *C. ficifolia* (p = 0.000000) and *C. viminalis* (p = 0.001230) conditions (**Table 2**). *X. chrysanthus* illustrated little inhibition in both *S. aureus* and *P. aeruginosa* thus there was no significant difference between the two bacterial conditions (p = 0.257339) (**Table 2**). In concentrations greater than 0.63 mg/mL, p-values were less than 0.05 (**Table 3**). The most significant difference between inhibition of *S. aureus* and *P. aeruginosa* was at 2.50 mg/mL (p = 0.008925) (**Table 3**). At low concentrations, there was less inhibition, thus percentage inhibition was similar, regardless of the concentration Therefore, the difference between *S. aureus* inhibition and *P. aeruginosa* inhibition was statistically insignificant for concentrations lower than 1.25 mg/mL as the p-values were greater than 0.05 (**Table 3**).

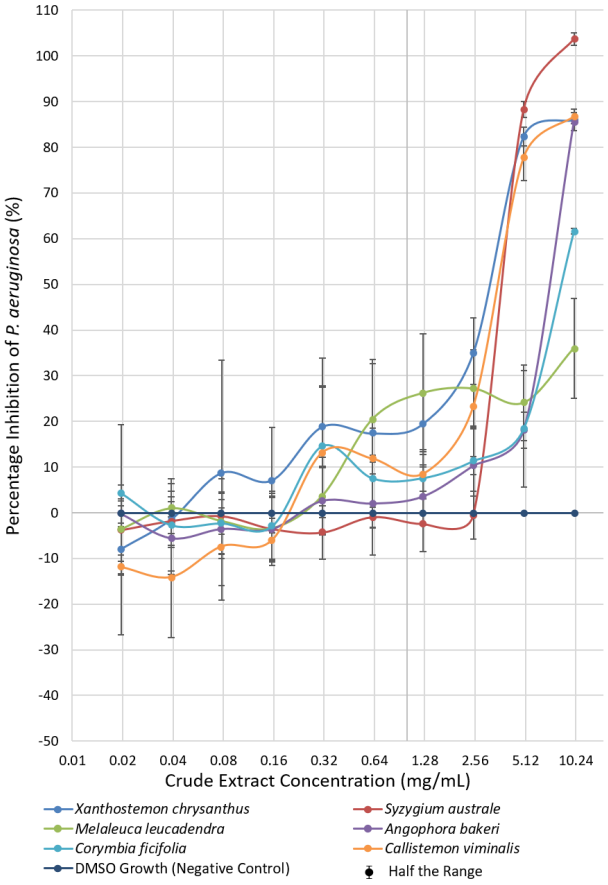

Figure 2. The mean percentage inhibition of *P. aeruginosa* caused by Myrtaceae crude extracts. Inhibition was measured at ten different concentrations after 18 hours of incubation in a cell viability bioassay. Percentage inhibition was calculated from Relative Fluorescence Units (RFU), presented in mean percentage inhibition ± half the range, represented by the error bars (n=3).

Myrtaceae Species	p-value
Xanthostemon chrysanthus	0.257339
Syzygium australe	0.001219
Melaleuca leucadendra	0.000003
Angophora bakeri	0.027883
Corymbia ficifolia	0.000000
Callistemon viminalis	0.001230

Table 2: *P*-values for the six tested Myrtaceae species calculated from one-way t-test. *P*-values < 0.05, the inhibition observed for that crude extract was significantly different between *S. aureus* and *P. aeruginosa*.

Concentration of Crude Extract (mg/mL)	Calculated *p*-value
10.0	0.036736
5.00	0.015959
2.50	0.008925
1.25	0.015108
0.63	0.071548
0.31	0.074167
0.16	0.126159
0.08	0.151644
0.04	0.144153
0.02	0.496901

Table 3: *P*-values for the ten concentrations of crude extract calculated from one-way t-test. *P*-values < 0.05, the inhibition observed at that concentration was significantly different between *S. aureus* and *P. aeruginosa*.

DISCUSSION

Our investigation aimed to explore the antimicrobial properties of native Australian Myrtaceae species against *S. aureus* and *P. aeruginosa*. Our results suggest that all the Myrtaceae species demonstrate varying antimicrobial properties. These results suggest that native Australian Myrtaceae are a viable and large source of secondary metabolites, whether that be as phytochemical adjuvants which increase immunity or as a direct defence against pathogens. All six species exceeded MIC80 for *S. aureus* and four species exceed MIC80 for *P. aeruginosa*. The species with the most potential are *C. ficifolia* for *S. aureus* (**Figure 1**). *X. chrysanthus* was the most potent against *P. aeruginosa* **(Figure 2)**. The six Myrtaceae species illustrated inhibition to varying extent, supporting the hypothesis. For five out of the 6 species *P. aeruginosa* was significantly less inhibit than *S. aureus* ($p < 0.027883$) (**Table 2**)**.** *X. chrysanthus* was the only extract were *P. aeruginosa* and *S. aureus* had similar percentage inhibition ($p = 0.257339$) (**Table 2**).

Statistical indicators of variability often require large datasets to be an accurate representation of variation. In this case, half of the range is the best metric to measure variability because there are few apparent outliers and as range only looks at the two most extreme values of a data set, therefore the fact there are only 3 repeats is less significant to the calculation of variability. Generally, the variance is higher for the lower concentrations, as the concentration increases the variance decreases. The clear trend observed regardless of the range of variance suggests that the variation between repeats is largely insignificant to the accuracy of the results and conclusions.

The yellow undertones in some of the wells suggest that resorufin was reduced to the non-fluorescent dihydroresorufin, which the plate readers recognize as inhibition. The misreading of fluorescence can result in an inaccurate representation of inhibition. We prepared an additional microplate to evaluate the reduction of resazurin. Resazurin is a blue dye which is nonfluorescent, it reduces to resorufin which is pink in medium cell activity. Resorufin further reduces to dihydroresorufin. Without bacteria, the wells should remain blue, but *S. australe* naturally reduces the resazurin into resorufin and possibly into dihydroresorufin. The reduction caused by extracts was only substantially evident in *S. australe*. Therefore, our *S. australe* data has lower confidence compared to the other tested species.

Even with the reduction caused by *S. australe*, the overall interpretation of our data remains unaffected, because for the *S. aureus* condition there is a clear trend that illustrates inhibition occurred regardless of the natural reduction of resazurin (**Figure 1**). Although the extent that *S. australe* reduces resazurin is the same in both bacteria conditions, it is only significant for *P. aeruginosa*. Reduction only appeared relevant for 10.00 mg/mL and 5.00 mg/mL concentrations of *S. australe* crude extract. In the *S. aureus* condition, there are clear trends in inhibition, even at lower concentrations where the crude extract would not naturally reduce the resazurin, which suggests that even though reduction may occur in the higher concentrations, it is secondary to inhibition (**Figure 1**). In the *P. aeruginosa* condition inhibition in the four extracts that reached MIC80 occurred at relatively high concentrations, which coincides with the reduction that occurs naturally (**Figure 2**). Therefore, supplementary testing was only necessary for *P. aeruginosa*.

To test the implication of the error, we prepared an agar plate as a supplementary test. If MIC80 for *S. australe* was truly 5.00 mg/mL, the contact sites with the extract should be distinctly less opaque than the bacterial culture surrounding it, creating clear zones of inhibition. The little inhibition we observed suggests that MIC80 for *S. australe* was closer to 10.00 mg/mL than 5.00 mg/mL. Thus, we interpreted MIC80 for *S. australe* at 10.00 mg/mL after the supplementary test.

Some of the stock plates indicate possible contamination in isolate wells. These isolated wells were considered outliers and excluded from the mean percentage inhibition calculations because they were reflective of contamination rather than the inhibition itself. The fact that they are isolated suggests that the contamination is most likely a handling error, rather than a systematic error.

The tested Myrtaceae species appear to be effective antimicrobial agents against *S. aureus*, suggesting that the secondary metabolites found within the crude extracts have high potential in antibiotic development against *S. aureus* infections and potentially Methicillin-resistant *S. aureus*. *C. ficifolia* ($p = 0.000000$), *M. leucadendra* ($p = 0.000003$) and *C. viminalis* ($p = 0.001230$) had the most significant difference in percentage inhibition between *S. aureus* and *P. aeruginosa* and were the most effective against *S. aureus* (**Table 2**). This suggests that within the crude extracts, there are highly effective secondary metabolites that either work in isolation or collectively with other secondary metabolites to inhibit the metabolic processes of the bacteria. Thus, these species may contain highly potent bioactive compounds that could be used to develop antibiotics in the future.

P. aeruginosa appeared less susceptible to the tested crude extracts, which we hypothesized because *P. aeruginosa* is a gram-negative bacterium and typically is less affected by antimicrobial agents, suggesting that penetrability of the crude extracts is an important factor in how effective extracts are against bacteria (23). There is significant difference in inhibition in concentrations higher than 0.63 mg/mL between *S. aureus* and *P. aeruginosa* ($p < 0.015148$) (**Table 3**). Although *P. aeruginosa* was not as susceptible to crude extracts, it may be because effective bioactive compounds were in small concentrations within the extracts or higher concentrations of extracts were needed to inhibit the more resistant *P. aeruginosa*. Other Myrtaceae species, especially species closely related to *X. chrysanthus*, *S. australe*, *A. bakeri*, and *C. viminalis,* which were effective on both bacteria*,* may have highly effective secondary metabolites.

There is some evidence regarding the antimicrobial properties of Myrtaceae species which supports the findings of this investigation. *Syzygium aromaticum* and *Melaleuca alternifolia* essential oils are effective against *P. aeruginosa* and *S. aureus* biofilms (24). There is also substantial evidence that extracts of Myrtaceae species are less effective on gram-negative bacteria. *Myrcia alagoensis* essential oils appear less effective on gram-negative bacteria (25). Such observations were explained to be due to the lack of permeability of the outer membrane or efflux pumps that export antimicrobial agents providing protection against antibiotic action (25). Evidence suggests that most gram-positive bacteria illustrate similar degrees of inhibition to Myrtaceae species. For example, gram-positive bacteria *S. aureus, S. pyogenes*, *E. faecalis* and *E. faecium* exhibit similar MIC50 and MIC90

values (26). However gram-negative bacteria indicate more variability. *Myrcianthes hallii* was tested against *E. coli* and *P. aeruginosa*. The MIC value was considerably higher than the four gram-positive bacteria, but E. coli had a much higher MIC50 and MIC90 value compared to *P. aeruginosa* (26). Although gram-negative bacteria appear less susceptible, there is significant evidence that Myrtaceae species inhibit these bacteria. *Callistemon citrinum* leave and flower extracts were found to cause significant zones of inhibition against gram-negative bacteria such as *Vibro alginolyticus* and *Aeromonas hydrophila* (27). When extracts of Eucalyptus were tested against *P. aeruginosa*, MIC was reached at 25 mg/mL, which suggests that higher concentrations of extracts are needed to be effective against highly resistant bacteria like *P. aeruginosa* (28).

To fully evaluate the potential of Myrtaceae species in antimicrobial drug development, the scope of the investigation needs to be extended. Further experimentation includes isolating the secondary metabolites in the crude extracts that were able to meet MIC80. Secondary metabolites are largely responsible for the pharmacological properties of botanical species (17). Existing chemical analyses of common Myrtaceae species suggests that extracts may include compounds that damage cell walls, cause metabolic failure, or inhibit the production of essential biological compounds (29-31). High Performance Liquid Chromatography (HPLC) can be used to isolate and identify these bioactive compounds. HPLC is a process that involves ratios of solvents with different polarities, where crude extracts can be separated into their individual constituents; the molecules are identified and quantified within the extract (32). For drug development, molecules must be isolated, and the same methodology must be conducted to find MIC80 for each pure molecule. There is evidence that leaf and flower extracts contain different secondary metabolites, thus demonstrating different degrees of inhibition (33).

The bioassay provides a quantitative analysis of cell viability; however, it is not indicative of the mechanisms or interactions between secondary metabolites and microorganisms which causes inhibition. Understanding and investigating these mechanisms could reveal how effective secondary metabolites inhibit microorganisms, this knowledge could be used to guide the search for more secondary metabolites that specifically target these aspects of a pathogen. Furthermore, we only tested the effectiveness of Myrtaceae species at inhibiting two bacterial strains. Although *S. aureus* and *P. aeruginosa* provide insight into ESKAPE pathogens and gram-positive and gram-negative bacteria, respectively, we would need to perform experiments on other ESKAPE pathogens to determine if our results generalize to these pathogens. Further investigation of the effective species should include isolation of secondary metabolites, exploring leaf extracts, and testing extracts on a broader spectrum of bacteria.

MATERIALS AND METHODS

Flower collection

X. chrysanthus, S. australe, M. leucadendra, A. bakeri, C. ficifolia and *C. viminalis* flowers were obtained from trees around the Southeast Queensland region during December, which is the blooming season for most Myrtaceae. Flowers were collected straight from wild trees to limit the effect of contamination from substances such as pesticides which could influence the bioactive compounds within the flowers. All leaves and stems were removed from the specimens. Flowers were left in a shaded, dry room at 26˚C for 3 months, to avoid the denaturation and breakdown of bioactive compounds that can occur with direct sunlight or heat (34). An electric grinder was used to grind the dry samples and 1.00 g (± 0.01 g) was measured into a test tube.

Extraction Process

Each ground flower was put into a test tube and dissolved in 6 mL of 100% methanol. Methanol, a polar substance, extracts polar bioactive compounds; however, it has the capacity to extract some non-polar compounds (34). Methanol also has a relatively low evaporation temperature at 64.7˚C, making it efficient for extraction processes (34). Samples were sonicated for 20 minutes to agitate particles or discontinuous fibers in a liquid, in order to accelerate the extraction process (35). Sonicated samples were filtered through Pasteur pipettes containing filter paper, and the filtered methanol was collected into the empty test tube which was previously measured for its empty mass.

Ground flowers were then re-saturated with an additional 8 mL of methanol and the same sonication and filtering process was completed five times (using new methanol each time) to fully extract the methanol soluble compounds. Discoloration of the methanol was used as an indicator of the degree of extraction with each repetition.

Methanol extracts were placed in a Genevac to evaporate the methanol. The temperature was set at 40˚C, the pressure slowly decreased from 300 Pa to 20 Pa and rotated at 1390 rpm. The mass of the dried extracts was measured, and the concentration of the crude extract was calculated. Dried extracts were redissolved in 10 mL of methanol and different volumes of each extract was put in the rotatory evaporator to obtain 40 mg of each dried extract. The final concentration of the first column in the serial dilution needed to be 200 mg/mL, 40 mg of dried extracts were dissolved in 200 µL of dimethyl sulfoxide (DMSO). DMSO was used as the solvent for the bioassay crude extracts because methanol has antimicrobial properties and thus could compromise the accuracy of the bioassay (36).

Bioassay

Bacterial stocks were kept frozen at -81˚C and then streaked on agar plates, under sterile condition, and incubated at 37˚C prior to experiments. *S. aureus* and *P. aeruginosa* were incubated for their optimum growth periods, approximately 20 hours and 16 hours, respectively, (37, 38). The same streak plates were used for all three repeats of each bacterium and were placed in the fridge (4˚C) in between experiments. One colony was collected from the streak plate using a sterile micropipette tip and put into 10 mL of single strength LB broth, two broth cultures were made per bioassay alongside a blank. To form the inoculate for each bioassay, 1 mL of the overnight culture was put into 20 mL of single strength LB Broth. Spectrophotometry was used to estimate the starting concentration of bacteria for each experiment. To normalize for differences in starting amount of bacteria the raw data was converted to percentage growth and then percentage inhibition. DMSO blanks were used as an indicator of bacterial growth and used to calculate the slope

of growth for each repeat.

Bioassays allow for quantitative data on the efficiency of agents against target cells in vitro to be collected (39). We used resazurin (7-Hydroxy3H-phenoxazin-3-one 10-oxide), which in the absence of cell activity it remains in its original form (blue), which is non-fluorescent if cells are viable and metabolic processes continue the dye reduces into resorufin which is pink and fluoresces (40), thus RFU can be used as an indicator of cell viability. 5 µL of the extract was placed into each well which contained 25 µL of H_2O, 25 µL of LB Double Strength Broth, and 50 µL of bacterial inoculate.

To control for and easily identify the source of any contamination, each plate contained five control conditions. We used antibiotics to check if the plate was contaminated with other bacteria. Erythromycin was the antibiotic used against *S. aureus* and Tobramycin was the antibiotic used against *P. aeruginosa*. Wells that contained only broth and inoculate ensured that growth could occur in our bioassays and the blank condition which not contain inoculate checked for contamination of our reagents. Additionally, we had controls that contained DMSO (our solvent for dried extracts) to check for any inhibition of growth caused directly by DMSO (DMSO + inoculate) as well as DMSO-blank which checked for contamination of our DMSO stock.

After the bioassays were incubated according to the bacterium's incubation period, 10 µL of resazurin was added to all wells. The *S. aureus* microplates were incubated for an additional 1 hour at 37˚C and *P. aeruginosa* was incubated for an additional 2 hours (41). Fluorescence was measured by BMG LABTECH, FLUOstar Omega fluorescent plate reader with excitation at 544 nm and emission at 590 nm. For the supplementary test, a volume of 10 µL of resazurin was added to each sample well, and the plate was put into the incubator for 1 hour, no bacterium was added. In addition, the agar plate spread with *P. aeruginosa*, aimed to test the inhibition of 5.00 mg/mL *S. australe* crude extract was incubated for 16 hours at 37˚C.

Data Processing

Raw data was measured in Relative Fluorescence Units (RFU). The mean RFU of the DMSO Blank condition was taken as 0% growth. The mean RFU of the DMSO growth condition was taken as 100% growth. Excel was used to calculate the slope and intercept of the regression between 0% and 100% growth. The slope and intercept were calculated for each repeat to normalize the data to overcome the difference in starting bacterial concentrations. Percentage growth was calculated based on the equation: *Percentage Growth = ((RFU x Slope) + Intercept) x 100*). Percentage inhibition was calculated by the equation: *Percentage Inhibition = 100 – Percentage Growth*. Variance was measured in the form of mid-range, with the equation *Variation = Range / 2*. Error bars represented this variance (**Figure 1,2**). For a species to be considered active, the percentage inhibition must be greater than 80% (42). Percentage inhibition between 50% and 80% is considered to be partially active, and percentage inhibition less than 50% is characterized as inactive (42). Therefore, minimum inhibitory concentration (MIC) is calculated in reference to 80% inhibition (MIC80). As percentage inhibition was calculated from percentage growth some percentage inhibition values appear negative, this indicates that growth has occurred (42).

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REFERENCES

1. "WHO | About The Global Burden Of Disease (GBD) Project". *Who.Int*, 2012, www.who.int/healthinfo/global_burden_disease/about/en/?
2. Hay, Simon . *et al*. "Measuring And Mapping The Global Burden Of Antimicrobial Resistance". *BMC Medicine*, vol 16, no. 1, 2018, p. 78. *Springer Science And Business Media LLC*, doi.org/10.1186/s12916-018-1073-z.
3. "Antimicrobial Resistance". *Who.Int*, 2020, www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance?
4. C Reygaert, Wanda. "An Overview Of The Antimicrobial Resistance Mechanisms Of Bacteria". *AIMS Microbiology*, vol 4, no. 3, 2018, pp. 482-501. *American Institute Of Mathematical Sciences (AIMS)*, doi.org/10.3934/microbiol.2018.3.482.
5. Santajit, Sirijan, and Nitaya Indrawattana. "Mechanisms Of Antimicrobial Resistance In ESKAPE Pathogens". *Biomed Research International*, vol 2016, 2016, pp. 1-8. *Hindawi Limited*, doi.org/10.1155/2016/2475067.
6. Rice, Louis . "Federal Funding For The Study Of Antimicrobial Resistance In Nosocomial Pathogens: No ESKAPE". *The Journal Of Infectious Diseases*, vol 197, no. 8, 2008, pp. 1079-1081. *Oxford University Press (OUP)*, doi.org/10.1086/533452.
7. Boucher, Helen W. *et al*. "Bad Bugs, No Drugs: No ESKAPE! An Update From The Infectious Diseases Society Of America". *Clinical Infectious Diseases*, vol 48, no. 1, 2009, pp. 1-12. *Oxford University Press (OUP)*, doi.org/10.1086/595011.
8. Wu, W *et al*. *Molecular Medical Microbiology*. 2nd ed., Elsevier Science Publishing Co Inc, 2014, pp. 753-767.
9. Wu, Min, and Xuefeng Li. *Molecular Medical Microbiology (Second Edition)*. 2nd ed., Elsevier Science Publishing Co Inc, 2014, pp. 2097-2145.
10. "Pseudomonas Aeruginosa Infection | HAI | CDC". *Cdc.Gov*, 2019, www.cdc.gov/hai/organisms/pseudomonas.html.
11. Medveov, Albeta, and Ubomr Valk. "Staphylococcus Aureus: Characterisation And Quantitative Growth Description In Milk And Artisanal Raw Milk Cheese Production". *Structure And Function Of Food Engineering*, vol 1, no. 1, 2012, pp. 71-102. *Intech*, doi.org/10.5772/48175.
12. Taylor, Tracey, and Unakal CG. "Staphylococcus Aureus". *Pubmed*, 2020, pubmed.ncbi.nlm.nih.gov/28722898/.
13. Tayler, Liz. "Antimicrobial Resistance: Global Burden". *Who.Int*, 2016, https://www.who.int/medicines/technical_briefing/tbs/TBS2016_AMR_GAP.pdf.
14. Friedman, Eric. "Behind The Headlines: 10 Million Deaths From Antimicrobial Resistance By 2050 (Or Not?) - O'neill". *O'neill Institute*, 2020, oneill.law.georgetown.edu/behind-the-headlines-10-million-antimicrobial-deaths-by-2050.
15. Harvey, Alan. "Natural Products In Drug Discovery". *Drug Discovery Today*, vol 13, no. 19-20, 2008, pp. 894-901. *Elsevier BV*, doi.org/10.1016/j.drudis.2008.07.004.
16. Katiyar, Chandrakant *et al*. "Drug Discovery From Plant

Sources: An Integrated Approach". *AYU (An International Quarterly Journal Of Research In Ayurveda)*, vol 33, no. 1, 2012, p. 10. *Medknow*, doi.org/10.4103/0974-8520.100295.
17. "SECONDARY METABOLITES AND THEIR EFFECT ON HUMAN HEALTH". *MONQ*, monq.com/eo/secondary-metabolites/human-health/.
18. "Myrtaceae". *Encyclopedia Britannica*, 2015, www.britannica.com/plant/Myrtaceae/additional-info.
19. "Myrtaceae | Encyclopedia.Com". *Encyclopedia.Com*, 2018, www.encyclopedia.com/science-and-technology/technology/technology-biographies/myrtaceae.
20. Cock, Ian, and Matthew Chessman. *Bioactive Compounds Of Medicinal Plants*. 1st ed., Apple Academic Press, 2018, pp. 35-84.
21. Ynalvez, R.A., and K.L. Compean. "Antimicrobial Activity Of Plant Secondary Metabolites: A Review". *Research Journal Of Medicinal Plant*, vol 8, no. 5, 2014, pp. 204-213. *Science Alert*, doi.org/10.3923/rjmp.2014.204.213.
22. Walters, Brian. "Eucalypts...But Not Eucalyptus". *Anpsa.Org.Au*, anpsa.org.au/APOL2/jun96-5.html.
23. "Why Do Gram Positive And Gram Negative Bacteria Show Different Antibiotic Susceptibility Patterns?". *AIMED - Let's Talk About Antibiotics*, 2015, aimed.net.au/2015/08/12/why-do-gram-positive-and-gram-negative-bacteria-show-different-antibiotic-susceptibility-patterns/.
24. Kavanaugh, Nicole L., and Katharina Ribbeck. "Selected Antimicrobial Essential Oils Eradicate Pseudomonas Spp. And Staphylococcus Aureus Biofilms". *Applied And Environmental Microbiology*, vol 78, no. 11, 2012, pp. 4057-4061. *American Society For Microbiology*, https://doi.org/10.1128/aem.07499-11.
25. Silva, Aline do N. *et al*. "Chemical Composition And Antibacterial Activity Of Essential Oils From Myrcia Alagoensis (Myrtaceae)". *Natural Product Communications*, vol 8, no. 2, 2013, pp. 1934578X1300800. *SAGE Publications*, doi.org/10.1177/1934578x1300800235.
26. Chavez Carvajal, Patricia *et al*. "Chemical Characterization And In Vitro Antibacterial Activity Of Myrcianthes Hallii (O. Berg) Mcvaugh (Myrtaceae), A Traditional Plant Growing In Ecuador". *Materials*, vol 9, no. 6, 2016, p. 454. *MDPI AG*, doi.org/10.3390/ma9060454.
27. Larayetan, Rotimi A. *et al*. "Terpene Constituents Of The Aerial Parts, Phenolic Content, Antibacterial Potential, Free Radical Scavenging And Antioxidant Activity Of Callistemon Citrinus (Curtis) Skeels (Myrtaceae) From Eastern Cape Province Of South Africa". *BMC Complementary And Alternative Medicine*, vol 17, no. 1, 2017. *Springer Science And Business Media LLC*, doi.org/10.1186/s12906-017-1804-2.
28. Dawoodi, Vida, and Zeynab Golshani. "Anti-Pseudomonal Activity Of Leaf Extracts Of Myrtaceae Plants Against B- Lactamase-Producing Strains". *Zahedan Journal Of Research In Medical Sciences*, 2013, brief.land/zjrms/articles/1340.html.
29. Mickymaray. "Efficacy And Mechanism Of Traditional Medicinal Plants And Bioactive Compounds Against Clinically Important Pathogens". *Antibiotics*, vol 8, no. 4, 2019, p. 257. *MDPI AG*, doi.org/10.3390/antibiotics8040257.
30. Niinemets, Ülo. "Storage Of Defense Metabolites In The Leaves Of Myrtaceae: News Of The Eggs In Different Baskets". *Tree Physiology*, vol 38, no. 10, 2018, pp. 1445-1450. *Oxford University Press (OUP)*, doi.org/10.1093/treephys/tpy115.
31. Kohanski, Michael *et al*. "How Antibiotics Kill Bacteria: From Targets To Networks". *Nature Reviews Microbiology*, vol 8, no. 6, 2010, pp. 423-435. *Springer Science And Business Media LLC*, doi.org/10.1038/nrmicro2333.
32. Gonelimali, Faraja D. *et al*. "Antimicrobial Properties And Mechanism Of Action Of Some Plant Extracts Against Food Pathogens And Spoilage Microorganisms". *Frontiers In Microbiology*, vol 9, 2018. *Frontiers Media SA*, doi.org/10.3389/fmicb.2018.01639.
33. Larayetan, Rotimi A. *et al*. "Terpene Constituents Of The Aerial Parts, Phenolic Content, Antibacterial Potential, Free Radical Scavenging And Antioxidant Activity Of Callistemon Citrinus (Curtis) Skeels (Myrtaceae) From Eastern Cape Province Of South Africa". *BMC Complementary And Alternative Medicine*, vol 17, no. 1, 2017. *Springer Science And Business Media LLC*, doi.org/10.1186/s12906-017-1804-2.
34. Teles, Aline Soares Cascaes *et al*. "Effect Of Temperature On The Degradation Of Bioactive Compounds Of Pinot Noir Grape Pomace During Drying". *Brazilian Journal Of Food Technology*, vol 21, no. 0, 2017. *Fapunifesp (Scielo)*, doi.org/10.1590/1981-6723.5917.
35. Gillespie, Claire. "How Does Sonication Work?". Sciencing, 2018, sciencing.com/sonication-work-5171302.html.
36. Sullivan, Dexter W. *et al*. "A Review Of The Nonclinical Safety Of Transcutol®, A Highly Purified Form Of Diethylene Glycol Monoethyl Ether (DEGEE) Used As A Pharmaceutical Excipient". *Food And Chemical Toxicology*, vol 72, 2014, pp. 40-50. *Elsevier BV*, doi.org/10.1016/j.fct.2014.06.028.
37. Cendra, Maria del Mar *et al*. "Optimal Environmental And Culture Conditions Allow The In Vitro Coexistence Of Pseudomonas Aeruginosa And Staphylococcus Aureus In Stable Biofilms". *Scientific Reports*, vol 9, no. 1, 2019. *Springer Science And Business Media LLC*, doi.org/10.1038/s41598-019-52726-0.
38. "Pseudomonas Aeruginosa - Information And Epidemiology Services". *Ehagroup.Com*, www.ehagroup.com/resources/pathogens/pseudomonas-aeruginosa/.
39. Hoskins, W. M., and R. Craig. "Uses Of Bioassay In Entomology". *Annual Review Of Entomology*, vol 7, no. 1, 1962, pp. 437-464. *Annual Reviews*, doi.org/10.1146/annurev.en.07.010162.002253.
40. "CA035-S | Resazurin Cell Viability Assay Clinisciences". *Clinisciences.Com*, www.clinisciences.com/en/other-products-186/resazurin-cell-viability-assay-681000216.html.
41. Artini, Marco, *et al*. "Evaluation of Contact Lens Multipurpose Solutions on Bacterial Biofilm Development." Eye & Contact Lens Science & Clinical Practice, vol. 41, no. 3, May 2015, pp. 177–182., https://doi.org/10.1097/icl.0000000000000105.
42. "Table Of Substrates, Inhibitors And Inducers". *U.S. Food And Drug Administration*, 2020, www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers.