Investigating the antimicrobial and antioxidative properties of *Santalum album*

Group 1-28

ABSTRACT :

This project investigated the antibacterial, antioxidant, and antifungal effects of *Santalum album*, also known as sandalwood. It has been used since ancient times and is prevalent as a folk remedy in both Chinese and Indian folk medicine. We carried out a well diffusion assay, a DPPH (2,2-diphenyl-1-picrylhydrazyl) test and an antifungal growth test to test the aqueous extract of *Santalum album*'s antibacterial, antioxidative and antifungal properties respectively. We found out that the aqueous extract of sandalwood extract has significant antifungal properties, weak antioxidative properties and no antibacterial properties. From our results, we believe that the active compounds of the sandalwood extract have the potential of being developed into new antifungal drugs.

INTRODUCTION

Santalum album has been considered as a medicinal plant since ancient times and is used in both Chinese and Indian folk medicine. In traditional Indian medicines, diseases treated by *S. album* oil range from common colds and burns to urinary tract and mouth inflammations (De Groot & Schmidt, 2016). In traditional Chinese medicine, *S. album* is most often used in incense sticks to treat a variety of problems ranging from skin diseases to even anxiety (Kirtikar & Basu, 1933). The folk wisdom inherent in its widespread use caused us to theorise that it may contain certain antioxidative, antifungal and antibacterial substances.

Invasive bacterial infections cause a large number of disabilities and deaths. Concurrently, the rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives. Decades after the first patients were treated with antibiotics, bacterial infections have again become a threat. Methicillin-Resistant *Staphylococcus aureus* (MRSA) was first identified five decades ago, only 2 years after the invention of penicillin (Spellberg *et al.*, 2014). MRSA infections can be very serious and are among the most frequently occurring of all antibiotic-resistant threats (Centre for Disease Control [CDC], 2018). In the U.S. alone, 11, 285 deaths have been attributed to MRSA per year (Gross, 2013). Vancomycin-Resistant *Enterococci* (VRE) also presents a major therapeutic challenge (Rossolini *et al*., 2014). *Enterococci* cause a wide range of illnesses, mostly among patients in hospitals or other health care settings, including bloodstream, surgical-site, and urinary tract infections. Overall, 20, 000 of hospital-acquired *Enterococcal* infections per year are vancomycin-resistant, leading to 1, 300 deaths (Toda *et al*., 2019). Few antimicrobial options are available to treat VRE. The threat of increasing antibiotic resistance across a wide range of bacterial strains demands the development of new antibiotics to combat this problem.

Invasive fungal infections can also cause disability and death. *Candida* is currently the leading cause of healthcare-associated bloodstream infections in U.S. hospitals (Brown *et al*., 2012). These infections are also costly for patients and healthcare facilities. Each case of *Candida* bloodstream infection is estimated to result in an additional 3 to 13 days of hospitalization and between $6,000 to $29,000 (Rivero-Menendez *et al*., 2016). Although antifungals can be used to treat most types of *Candida*, the most common cause of fungal infections, some types of *Candida,* such as *Candida glabrata* and *Candida parapsilosis* are becoming increasingly resistant to the antifungal medications most commonly used for treatment, such as the

echinocandins, the preferred treatment for many *Candida* infections (Brown *et al*., 2012). On the other hand, *Aspergillus* is the leading cause of invasive mold infections, with an estimated 200,000 cases worldwide every year. The preferred treatments for these infections are voriconazole and certain other azole drugs (Matthaiou *et al*., 2015). However, 12% of *Aspergillus* infections are estimated to be resistant to azole medications (Rivero-Menendez *et al*., 2016). Due to both the high costs of antifungal and the threat antifungal-resistant fungi pose, there is also a need for alternative antifungal drugs.

Chronic wounds, which are wounds that do not heal normally in 30 days, are also a cause for concern as these chronic wounds are more likely to become infected (Couch, 2019). If inadequate treatment is provided, the results can be severe, even fatal sometimes. This problem is severe as chronic wounds affect 6.5 million people in the US alone (Nelson, 2017). Since integrity of wound healing depends on low levels of reactive oxygen species and oxidative stress (Fitzmaurice *et al*., 2012). Antioxidants are therefore postulated to help control wound oxidative stress and thereby accelerate wound healing. As such, if *S. album* does indeed have strong antioxidant effects, it could be used in things such as wound dressings.

We believed that extracts from *Santalum album (S. album)* had the potential to be developed as antibacterial and antifungal drugs, and therefore investigated its antimicrobial properties and antioxidative properties.

OBJECTIVES

This study aimed to examine the efficacy of *S. album* extracts in limiting bacterial and fungal growth along with the antioxidative properties of *S. album*. The results of these experiments would potentially pave the way for new antimicrobial drugs.

HYPOTHESIS

We believed that the *S. album* extracts would display antibacterial, antifungal and antioxidative properties.

METHODOLOGY

Extraction

S. album heartwood purchased from a local Chinese Traditional Medicine shop was brought to the lab and ground into fine powder using a metal grinder with the help of a lab technician. The powder was then mixed with a deionised water so that a 5% (mass/volume) extract could be obtained. The mixture was then blended for 5 minutes. The blended mixture was then centrifuged at 9500 rpm for 10 mins at 5˚C for removal of debris. The aqueous mixture was then decanted from the suspension. The mixture was then filtered through a microfilter (pore size 0.45um) for removal of all bacteria and ensured sterility in downstream tests. The supernatant was then aliquoted and stored at -4˚C until required for further usage.

Microorganisms for Antimicrobial tests

For the antimicrobial screening, two species of bacterial isolate and one species of fungus isolate were selected. *Escherichia coli* (ATCC 25922) and *Staphylococcus epidermidis* (ATCC 12228) strains were used. *Aspergillus niger* (Carolina) fungus was used as the test organism. The bacterial cultures were maintained on nutrient agar while the fungus was maintained on potato dextrose agar, both at 4°C. The fresh cultures were obtained by growing the bacteria at 37˚C overnight while growing the fungus at 28°C for 72 h.

Well Diffusion Test

Escherichia coli (ATCC 25922) and *Staphylococcus epidermidis* (ATC 12228) were cultured in nutrient broth at 37˚C overnight. The resultant suspension was then used for the well diffusion test. Using a sterile cotton swab, the bacteria were spread evenly onto agar plates. Sterile plastic borders were used to punch 3 wells (6mm) into each of these inoculated agar plates. Using a sterile pipette, 80µL of either *S. album* extract, 10% bleach (positive control) or sterile water (negative control) was introduced into each well. The set-up was then incubated at 37˚C and the resultant bacterial growth was observed 24 hours later. Any resulting zones of inhibition generated were measured with a 15cm ruler and the mean diameter of the zone of inhibition was recorded (Fig 1A, Fig 1B).

DPPH test

1.9 mL of methanol was mixed with 0.1 mL of *S. album* extract and 1 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. A negative control containing 2 mL of methanol and 1mL of DPPH solution was prepared. The tubes were placed in the dark for 10 minutes before spectrophotometric measurements were taken at 517 nm wavelength of light. The measurements were compared with those obtained from the negative control. By calculating the percentage of DPPH left using the formula below, the antioxidant effects of the extract was determined.

$$\left(\frac{A_{517}\text{ of control} - A_{517}\text{ of sample}}{A_{517}\text{ of control}}\right) \times 100$$

Fungal Growth Test

Aspergillus niger (Carolina) was cultured on potato dextrose agar for a few days at room temperature. 5ml of *S. album* extract was then added to 95ml of potato dextrose agar (PDA) and left to solidify. After it solidified, a small layer of *A. niger* was cut out with a sterile scalpel and placed in the middle of both the petri dishes containing PDA (negative control) and the petri dishes containing the PDA which had *S. album* extract mixed in it. The resultant diameter of fungal surface growth was then monitored over a week.

RESULTS AND DISCUSSION

Compounds in aqueous extract of *S. album* do not inhibit Gram-positive or Gram-negative bacterial growth

As seen in Figures 1A and 1B, the aqueous extract of *S. album* did not inhibit the growth of either Gram-positive or Gram-negative bacteria. This tells us that there are little to no compounds present in the aqueous extract of *S. album* with antibacterial properties. This disproves our hypothesis and tells us that the aqueous extract of *S. album* contains little compounds with the potential of becoming new antibacterial drugs.

Our data is corroborated by another study which found that the aqueous extract of *S. album* displayed no antibacterial effects (Chanda & Parekh, 2006). However, the study also found that the methanol extract of *S. album* displayed antibacterial effects against *B. subtilis*. As such, we theorise that the antibacterial compounds in *S. album* are likely to be membrane-associated, non-polar molecules which did not manifest in our aqueous extracts.

Compounds in aqueous extract of *S. album* inhibit fungal growth

The *A. niger* on the PDA containing 5% aqueous *S. album* extract inhibited the growth of *A. niger* by about 13% (Fig 3), meaning that the aqueous extract of *S. album* has antifungal properties. This proves our hypothesis correct and tells us that *S. album* contains compounds with the potential of becoming new antifungal drugs.

Compounds in aqueous extract of *S. album* extract are weak antioxidants

The 5% aqueous extract of *S. album* was able to scavenge 11% of DPPH activity (Fig 2). On the other hand, a 5% vitamin C extract is able to scavenge 71% of DPPH activity. This demonstrates that *S. album* extract is a weak antioxidant. Therefore, while it might ameliorate the detrimental complications of chronic wounds and promote healing, the extent of its potential contribution remains to be further elucidated.

LIMITATIONS

Eue to the biological safety considerations of our lab, we were only able to obtain the fungus *A. niger*. It has been reported that the *Aspergillus* family is only the 2nd leading cause of fungal whereas *Candamia* species are the most common cause of fungal infections (Brown *et al*., 2012). As such, the range of *S. album's* antifungal impact in a clinical setting is not fully elucidated.

CONCLUSION

From the results of the experiments, it is possible that novel molecules for human therapeutic purposes can be acquired from the aqueous extract of *S. album*. This study showed that the aqueous extract of *S. album* displayed potent antifungal properties, weak antioxidative properties and no antibacterial properties. This means that *S. album* has the potential to be developed as a new antifungal drug, as stated in our introduction.

FUTURE WORK

Tests on more strains of fungi could be carried out to further examine *S. album*'s antifungal properties. Research could also be carried out to find out if the slight antioxidant effect of *S. album* in its aqueous form is sufficient to effectively promote wound healing.

References

Nelson, H. (2017). Chronic Wounds Affect 6.5 Million in U.S. Retrieved 20 August 2020, from https://intermountainhealthcare.org/blogs/topics/live-well/2017/04/chronic-wounds-affect-65-million-in-us/

Chronic Wounds. (2020). Retrieved 20 August 2020, from https://www.woundsource.com/patientcondition/chronic-wounds

De Groot AC, Schmidt E (2016). Sandalwood oil. In: De Groot AC, Schmidt E, editors. Essential Oils, Contact Allergy and Chemical Composition. Boca Raton, FL: CRC Press.

Kirtikar KR and Basu (1933); Santalum album Indian MedicinalPlants, Vol.3, II ed.,

Spellberg, B., & Gilbert, D. (2014). The Future of Antibiotics and Resistance: A Tribute to a Career of Leadership by John Bartlett. *Clinical Infectious Diseases*, *59*(suppl 2), S71-S75. doi: 10.1093/cid/ciu392

The biggest antibiotic-resistant threats in the U.S. (2018). Retrieved 6 January 2020, from https://www.cdc.gov/drugresistance/biggest-threats.html?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fdrugresistance%2Fbiggest_threats.html

Gross, M. (2013). Antibiotics in crisis. *Current Biology*, *23*(24), R1063-R1065. doi: 10.1016/j.cub.2013.11.057

Rossolini, G., Arena, F., Pecile, P., & Pollini, S. (2014). Update on the antibiotic resistance crisis. *Current Opinion In Pharmacology*, *18*, 56-60. doi: 10.1016/j.coph.2014.09.006

Magill, S., Edwards, J., Bamberg, W., Beldavs, Z., Dumyati, G., & Kainer, M. *et al*. (2014). Multistate Point-Prevalence Survey of Healthcare–Associated Infections. *New England Journal Of Medicine*, *370*(13), 1198-1208. doi: 10.1056/nejmoa1306801

Morgan, J., Meltzer, M., Plikaytis, B., Sofair, A., Huie-White, S., & Wilcox, S. *et al*. (2005). Excess Mortality, Hospital Stay, and Cost Due to Candidemia: A Case-Control Study Using Data From Population-Based Candidemia Surveillance. *Infection Control & Hospital Epidemiology*, *26*(6), 540-547. doi: 10.1086/502581

Toda, M., Williams, S., Berkow, E., Farley, M., Harrison, L., & Bonner, L. *et al*. (2019). Population-Based Active Surveillance for Culture-Confirmed Candidemia — Four Sites, United States, 2012–2016. *MMWR. Surveillance Summaries*, *68*(8), 1-15. doi: 10.15585/mmwr.ss6808a1

Brown, G., Denning, D., Gow, N., Levitz, S., Netea, M., & White, T. (2012). Hidden Killers: Human Fungal Infections. *Science Translational Medicine*, *4*(165), 165rv13-165rv13. doi: 10.1126/scitranslmed.3004404

Rivero-Menendez, O., Alastruey-Izquierdo, A., Mellado, E., Cuenca-Estrella, M. (2016). Triazole Resistance in Aspergillus spp.: A Worldwide Problem?. *Journal Of Fungi*, *2*(3), 21. doi: 10.3390/jof2030021

Matthaiou, D., Christodoulopoulou, T., & Dimopoulos, G. (2015). How to treat fungal infections in ICU patients. *BMC Infectious Diseases*, *15*(1). doi: 10.1186/s12879-015-0934-8

Chanda, S., & Parekh, J. (2006). Screening of aqueous and alcoholic extracts of some Indian medicinal plants for antibacterial activity. *Indian Journal of Pharmaceutical Sciences, 68*(6), 835. doi:10.4103/0250-474x.31032

APPENDIX

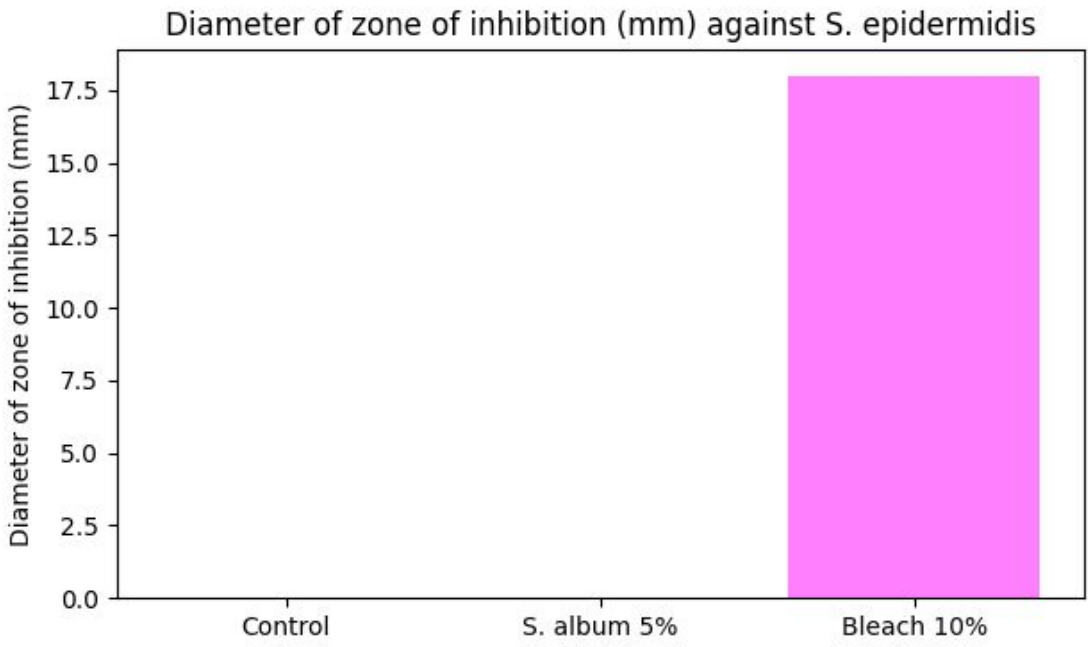

Fig 1A: Well Diffusion Test shows that the 5% aqueous extract of *S.album* demonstrated no antibacterial activity against the Gram-positive bacteria *S. epidermidis*.

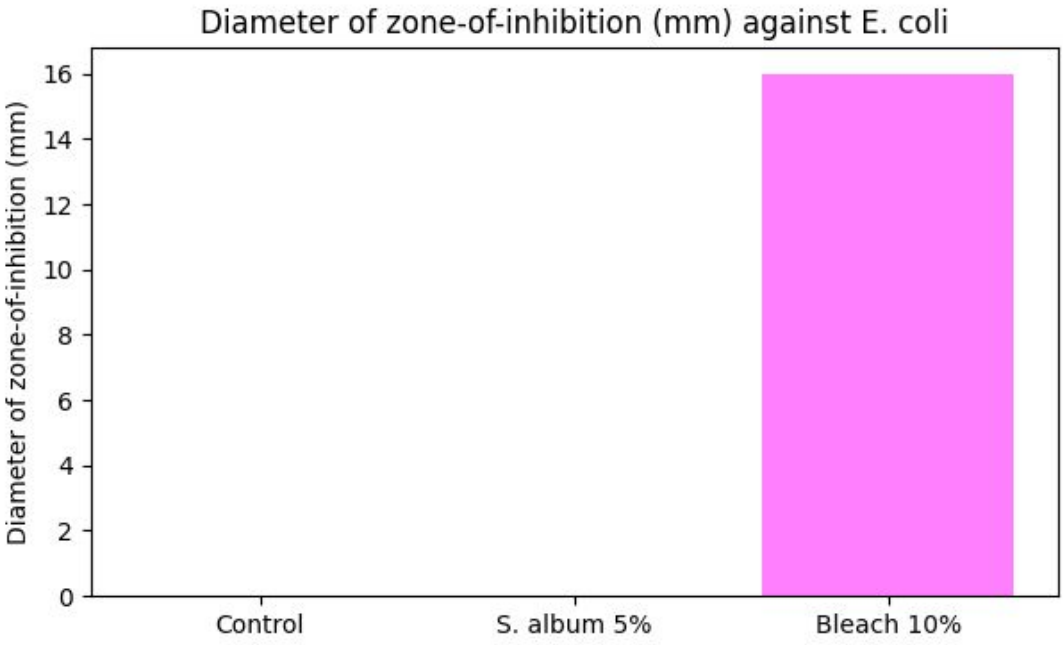

Fig 1B: Well Diffusion Test showed that the 5% aqueous extract of *S. album* demonstrated no antibacterial activity against the Gram-negative bacteria *E. coli*.

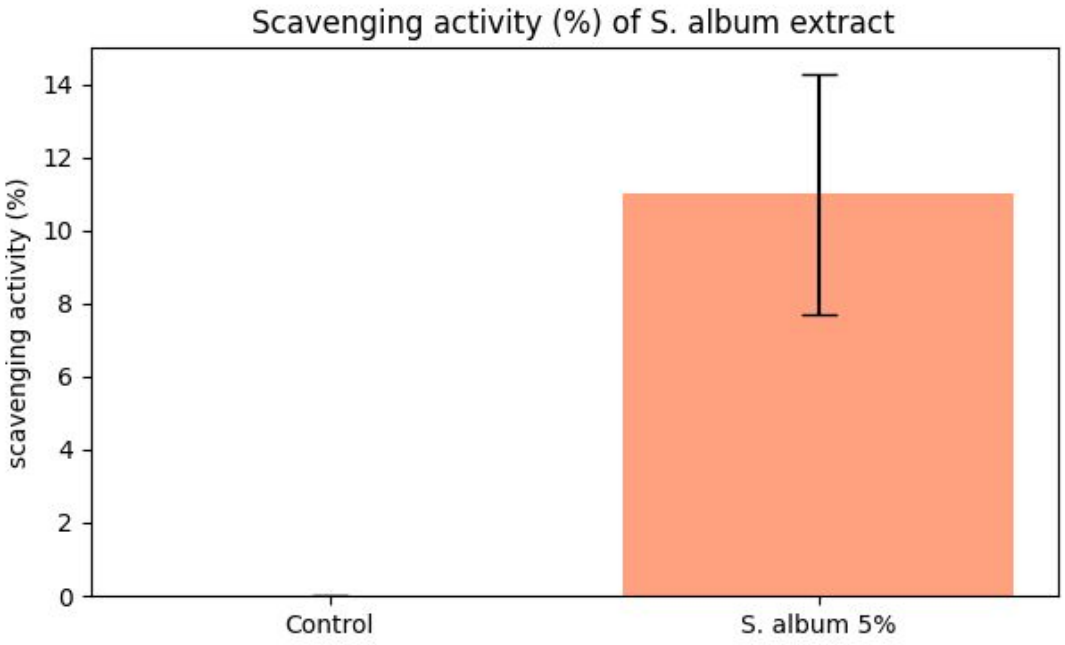

Fig 2: DPPH test showed that the 5% aqueous extract of *S. album* extract displayed some antioxidant effects.

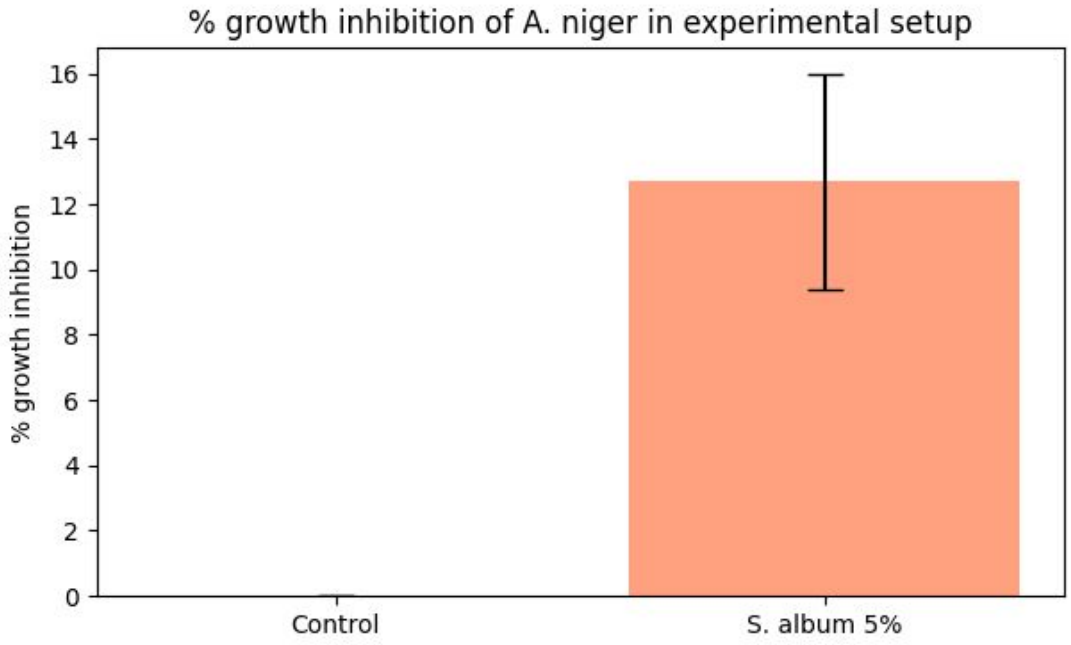

Fig 3: Fungal Growth Test showed that the 5% aqueous extract of *S. album* inhibited the growth of *A. niger* by about 12.7%.