Eradication of Staphylococcus aureus Biofilms By Synerigistic Action of Basil Oil and Vancomycin

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Abstract – The purpose of this study is to quantify the in vitro synergistic activity of basil oil and Vancomycin. A microtiter plate assay was used to determine the anti-microbial activity of the combination of both compounds against *Staphylococcus aureus* biofilms. The pre-formed biofilms were exposed to three different treatments: (1) Vancomycin, (2) basil oil, (3) and the combination of both. Water was used as the negative control. The three treatments were all active in eradicating the pre-formed biofilms with Vancomycin being the most effective followed by the combination of the two compounds. However, total biofilm removal was not achieved in any of the three treatments. The resulting action of the combination is less than that of vancomycin alone, but higher than that of basil oil alone, suggesting that there is an antagonistic interaction between the two compounds.

Introduction. – Staphylococcus aureus is a gram-positive cell that may be observed as grape-like clusters. By 1961, a short time after penicillin was introduced, certain Staphylococcal strains have evolved and developed resistance to most common antibiotics (i.e., Vancomycin, Linezolid and Daptomycin). Staphylococcus aureus can cause skin infections in addition to many other types of infections. Spread of this organism to the bloodstream is known as bacteremia or sepsis. This can result to serious complications like pneumonia, endocarditis which can lead to heart failure, and osteomyelitis [1]. Aside from the serious effects of S. aureus, data gathered in 2013 [2] suggested that Methicillin-Susceptible Staphylococcus aureus, or just S. aureus was common in the Philippines. Out of the 637 patients of with S. aureus growth, Methicillin-Susceptible Staphylococcus aureus accounted for 61.4% of it.

The problem of S. aureus not only lies with its prevalence but also with its decreased susceptibility to antibiotics because of the bacterial biofilm it forms. Bacterial biofilms are sticky mass of bacteria embedded in an extracellular matrix. Bacteria in a biofilm are 101000 times more resistant to the effects of antimicrobial agents and account for more than 80% of all microbial infections in humans [3]. Biofilms cause certain antibiotics and other external agents ineffective in killing this bacterium [4]. Thus, several alternatives have been studied by researchers as novel solutions against S. aureus biofilms.

One study in particular is the use of essential oils to eradicate these biofilms. An essential oil or a volatile oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants. An oil is "essential" in the sense that it contains the "essence of the plant's fragrancethe characteristic fragrance of the plant from which it is derived. Essential oils are generally extracted by distillation, often by using steam. Other processes include expression, solvent extraction, absolute oil extraction, resin tapping, and cold pressing. They are used in perfumes, cosmetics, soaps and other products, for flavoring food and drink, and for adding scents to incense and household cleaning products.

Essential oils have been used medicinally in history. Medical applications range from skin treatments to reme-

dies for cancer and even to antimicrobial effects. Additionally, many essential oils are relatively easy to obtain, have low mammalian toxicity and degrade quickly in water and soil making them environmentally friendly. However, essential oils contain very concentrated properties of the herb or plant from which they are derived from. So, essential oils must be used with care, with proper education and in safe amounts. Essential oils are a rich source of biologically active compounds. There has been an increased interest in looking for antimicrobial properties of extracts from these compounds in aromatic plants particularly essential oil. The physical barrier of biofilms composed of exopolymeric substances (EPS) can account for their increased antibiotic resistance. Bacterial EPS are molecules released in response to the physiological stress encountered in the natural environment. Eugenol is a phenylpropanoid, a group of plant secondary metabolites with a wide variety of functions both as structural and signaling molecules. Eugenol is found in Ocimum Basilicum basil oil and other essential oil and plays a significant role in dental and oral hygiene preparations [5].

Synergism is a correlated activity of two or more structures so that the combined action is greater than when each structure is working independently. The study of Yadav et al. [6] suggests that Eugenol exhibits significant anti-biofilm activity against Methicillin-Resistant Staphylococcus aureus biofilms when it was synergised with the antibiotic Carvacrol. Eugenol concentration of 0.5Minimum Inhibitory Concentration (lowest concentration of an antimicrobial that will inhibit the growth of a microorganism after overnight incubation) decreased the biomass of biofilm by more than 50%. Eugenol was also synergistic with Fluconazole, a cell-wall synthesis inhibitor antibiotic against Candida albicans biofilm. Since a synergistic activity was observed when Eugenol was combined with some antibiotic, it is also possible that it will exhibit synergism when tested with other antibiotics.

A potential antibiotic can be a cell-wall synthesis inhibitor. Cell-wall synthesis inhibitor antibiotics showed high ratios between the susceptibility of free flowing bacteria in suspension (planktonic cells) and biofilm cells. The ratios were: 7.0 for Cefazolin, 6.4 for Vancomycin and 5.6 for Dicloxacillin [7]. Cefazolin showed the highest ratio but it has poor stability and in the presence of a mutated S. aureus that synthesizes -lactamase and possesses mecA to facilitate its survival [8]. The next potential antibiotic would be vancomycin. Vancomycin resistance is higher in the biofilm mode of growth than in the planktonic mode of growth [9]. In the study of Singh et al. [10], Vancomycin penetration was also significantly reduced (P¡0.05) through S. aureus biofilms. This study aims to eradicate S. aureus biofilms by synergizing the compounds basil oil and Vancomycin.

Methods. – The methods is composed of five main parts; Acquisition, Media preparation, Growing, Exposure, and Analysis. Different combinations were used to treat the bacterial biofilms. Minimum inhibitory concentrations were used for the exposure of the pre-formed biofilms to the different combination treatments, the bacterial biofilms were treated in 96-well microtiter plates and was analyzed using a microplate reader.

Acquisition of S. aureus bacterial strains and compounds. A strain of S. aureus pure bacterial culture was obtained from University of the Philippines - The National Institute of Molecular Biology and Biotechnology, Los Baos, Laguna. Basil oil was purchased from an herbal store in a mall. Vancomycin was bought from a local drug store. The culture was stored in a refrigerator (Condura CSD230SA) at 20C. S. aureus was streaked directly to a petri plate with Nutrient Agar in order to obtain a subculture. The plate was wrapped and incubated at 30C for 24 hours. The subculture was then assessed for purity by examining the similarities in cell morphology of the bacterial colonies that formed.

Media Preparation. The glasswares (petri plates, test tubes, micropipette tips, stirring rods) were autoclaved (Delixi LS-B35L) for 15 minutes at the optimal pressure of 15 psi. Using a 500 mL media bottle, 20 grams of Tryptic Soy Agar was mixed with 500 mL distilled water. The mixture was cooked in the hot plate (LMS HTS-1003) and continually stirred until the mixture cleared up. Using a separate 500 mL media bottle 2.4 grams of Nutrient Broth was suspended in 500 mL of distilled water. The solution was mixed using a sterile stirring rod until the solid was dissolved. Peptone water was prepared with 0.5 grams of Bacteriological Peptone, and 4.8 grams of Sodium Chloride. It was then suspended in 500 mL of distilled water in a 500 mL media bottle. The media was autoclaved for 15 minutes at 15 psi and was cooled down afterwards. In four sterile test tubes, 15 mL of Nutrient Broth mixture was pipetted. Peptone water of 10 mL was pipetted into two test tubes. Nutrient agar (20-30 ml) was poured to each agar plate. After the agar has solidified it was stored in the refrigerator at 20C.

Growing of S. aureus. An inoculating loop was used to transfer an isolated colony from the agar plate to a test tube filled with 15 mL of Tryptic Soy Broth with 0.4 grams of glucose to create a subculture. The test tube containing the subculture was incubated for 18 to 24 hours at 30C. A McFarland procedure was conducted to compare the optical densities of the cultures, using a bond paper with black and white stripes.

Growing of S. aureus biofilm. A 96-well microtiter plate was used to grow the biofilms of S. aureus. 200 L of the

previously diluted liquid media was transferred to columns 2, 4, 6, 8 and 10 of the plate using a micropipette. The microtiter plate was sealed and placed in the incubator for 24 hours at 30C. (Yadav, 2015). This was conducted inside the laminar flow hood.

Exposure of biofilm to Basil Oil and Vancomycin. The microtiter plates seeded with S. aureus was removed from the incubator and was placed in the laminar flow hood. The biofilms formed in the microtiter plates was exposed to 5% v/v basil oil, 128 g per mL Vancomycin, and a combination of 12 g per mL Vancomycin and 5% v/v Basil oil. water and broth for controls. Since the oil wouldnt mix completely with water, a 100 L of emulsifier Tween 20 was used. 150 L of each treatment was transferred to each well of columns 2, 4, and 6 and rows B-G. Column 8 acte was pipetted with water for a negative control. After incubation, the plates were gently washed two times with sterile Phosphate Buffer Saline. Thereafter, the plates were stained with 125 L of crystal violet (0.1%) for 15 minutes. Excess stain was removed by washing with Phosphate Buffer Saline. The crystal violet (CV) attached to the biofilm samples was dissolved with 125 L of ethanol. The absorbance at 600 nm (OD600) was measured using a Microplate Reader.

Evaluation and Analysis. The wells of the microtiter plate were checked for absorbance using a Microplate Reader. The absorbance values are the measure of the cells of S. aureus biofilm. An ANOVA statistical analysis with a level of confidence of 0.05 was performed in order to compare the means of the absorbance values of the untreated cells in different treatments.

Disposal of Used Media. Concentrated Lysol was diluted in a beaker containing water and be poured into the used agar plates, and test tubes. At optimal pressure for autoclaving, 15 psi, the plates were autoclaved to ensure the killing of the bacterial cells. After 15 minutes of autoclaving, the plates were cooled down for disposal of used media. All the glasswares that were used were washed using an antimicrobial liquid dishwashing soap and were properly and safely stored. Used microtiter plates were soaked in 70% ethanol for 5 minutes for continued sterilization and disposed into the hazardous/used media waste can.

Results. – The anti-biofilm activity of basil oil and cell-wall synthesis inhibitor Vancomycin was determined against gram-positive Staphylococcus aureus with water as the negative control. 128g/mL Vancomycin and 5% (v/v) basil oil and the combination of both showed antimicrobial activity against S. aureus biofilms.

In microplate reading, the absorbance value of each well corresponds to the un-eradicated biofilm (biofilm mass) in it. The absorbance mean values (see Fig 1) for the biofilms treated with Vancomycin and basil oil and their combination were lower than that of the controls. Among the three treatments, Vancomycin had the lowest biofilm mass followed by the combination and basil oil alone.

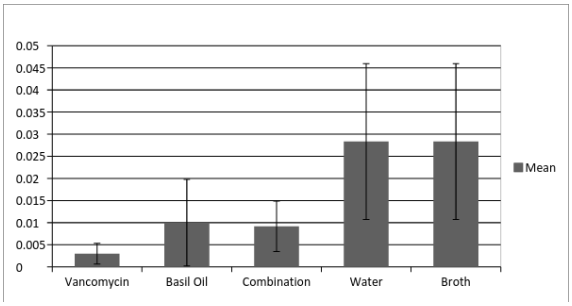

Fig. 1: Absorbance mean values of *S. aureus* after exposure to treatments

	Sum of Squares	DF	Mean Square	F	p(same)
Between Groups	0.001813	3	0.000604333	20.13	2.953E-06
Within Groups	0.000600333	20	3.00167E-05		

Fig. 2: ANOVA table

In the One-Way ANOVA, the calculated p value ($p = 6.56x10^{-7}$) of the absorbance values of the biofilms is less than 0.05 which means that the three treatments have significant differences in eradicating biofilm when compared to the control. This indicates that all treatments were effective in eradicating S. aureus biofilm. The three treatments, however, had no significant difference when compared to each other.

Discussion. – The absorbance values represent the amount of uneradicated biofilms left on the surface of the wells of the microtiter plate. The three treatments, Vancomycin, basil oil and the combination of both showed no significant difference amongst each other but they were significantly lower than that of the control.

Statistically speaking, all of the treatments were effective in eradicating biofilms. The insignificant difference between the three treatments states that there is no synergism between Vancomycin and basil oil. Although there is no significant difference, the absorbance value of Vancomycin was lesser than that of the other two treatments, making it the most effective treatment. The Minimum Biofilm Eradication Concentration (MBEC) used was the highest that was found and may be one of the probable extraneous variable that led to the penetration of the antibiotic.

In vitro susceptibility of a microorganism to a particular treatment does not guarantee the success of the clinical use of the therapeutic agent. The main disadvantage of the results of in vitro studies is that it is difficult to carry out comparison among each of the studies because of the different test methods, test assays, and variation in chemical phytoconstituents in essential oils (Yap et al 2013).

Basil oil constituents include phenylpropanoids that are potential biofilm treatments. Three of the major components are eugenol, geraniol, and linalool [11]. The bactericidal effect of eugenol on Streptococcus agalacticae seems to be dependent on changes in cell envelope, as judged by alterations in the morphology and structure observed in treated cells [12]. Studies report that eugenol induce cell lysis through protein and lipid leakage leading to the disruption of cytoplasmic contents in membranes. The anti-bactericidal component of Eugenol wasnt identified yet. In this study, the absorbance value of basil oil was low, which means that it effectively eradicated biofilm.

In a study conducted by Yap et al. [13], out of the 35 essential oils-antibiotic pairs, only four of them showed synergistic effect. Another study also shows no synergism between essential oils, namely; cinnamon and oregano essential oils and antibiotics (not mentioned specifically) indicating that not all combinations of antibiotic and essential oils will show synergistic effects. The interaction between Essential oils and antibiotics can produce four possible types of effects: indifferent, additive, antagonistic, or synergistic effects. An additive effect is observed when the combined effect is equal to the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects while the absence of interaction is defined as indifference [14].

The release of cellular content in the treated bacteria led to the hypothesis that the first effect of an essential oil is membrane disruption [15]. Although, it should not be ignored that interaction with other targets in the bacterial cell might play a key role with the observed antibacterial effects. Essential oils are composed of a large number of chemical constituents and these constituents rely on the genetic make-up of the plant where it came from. Due to the complex composition of essential oils, it is likely that their antibacterial activity is because of the different mechanisms of action that implies to target the cell. It is because of this reason that bacteria rarely develop resistance mechanisms for essential oils.

Chemical polymorphism is also a characteristic of essential oils. The genus Ocimum, in which basil oil belongs to, is known to have a high degree of chemical polymorphism [16]. Genetic factors, as well as environmental factors can be attributed to such influence on the chemical composition of the essential oil, it was established that the production of phenolic compounds is favoured in warmer and drier climatic zones, while the other, Nonphenolic compounds usually accumulate in higher quantities in cooler and damper areas [15]. Thus, this volatility exhibited by the essential oil can be a cause for variations in its composition, functional groups, and active components that can affect their synergistic interactions with the antibiotic. Most of the antimicrobial activity in EOs is found in the oxygenated terpenoids (e.g., alcohols and phenolic terpenes), Different terpenoid components of EOs can interact to either reduce or increase antimicrobial efficacy [14].

A study conducted by van Vuuren et al. [17], suggests that a combination of an essential oil, M. alternifolia, with ciprofloxacin against a gram-positive bacteria S. aureus showed antagonistic effect. Antagonism is a phenomenon where the effect of the combinations of two or more substance is less compared to their effect individually. The antagonistic effect was postulated to be due to the combination of the essential oil and the antibiotic raising the dose to an unacceptable therapeutic level. Thus, when combining the two it is also important to take note of the ratio at which the two components exist [17]. This antagonistic interaction recommends that natural treatments using essential oils must be monitored carefully when combined with antibiotics.

Conclusion. – Vancomycin and basil oil are both effective in eradicating biofilms but there is no synergism between the two agents.

Acknowledgment. – We would like to thank the following people and institutions for lending us help in the conduct of our research:

Mr. Harold P. Mediodia, our adviser during the conduct of our study who sternly guided us all throughout.

To Ms. Jeanne Claudeen Arona, for helping us acquire the microtiter plates that we needed for our study.

University of the Philippines Los Baos - Naitonal Institute of Molecular Biology and Biotechnology, for the bacterial cultures.

University of the Philippines Visayas - Naitonal Institute of Molecular Biology and Biotechnology, for accommodating us in their laboratory and allowing us to use their equipment.

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