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Effectiveness of *Dioscorea hispida* Dennst as antibacterial and antibiofilm agent

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ABSTRACT

Aims: Antimicrobial resistance (AMR) is a growing threat to public health, where treatments using conventional drugs are becoming ineffective. One viable but underexplored alternative is through the use of *Dioscorea hispida*, a wild plant that exhibits antimicrobial properties. This study aims to explore *D. hispida* effectiveness as an antibacterial and antibiofilm agent against selected pathogenic and non-pathogenic bacteria.
Methodology and results: Different concentrations of *D. hispida* crude extracts (0 – 2.5 mg/mL) were tested against the growth of planktonic bacterial cells over 24 h incubation, and the half maximal effective concentration (EC_{50}) obtained was used in the antibiofilm test over 24 and 48 h. All bacteria treated with *D. hispida* showed significant ($P<0.05$) reduction in planktonic cell and biofilm densities against the negative control starting at 0.3 mg/mL. However, in comparison to the antibiotic, only certain bacteria were significantly affected by *D. hispida*, implying the plant has a 'moderate' biocidal activity in general. Furthermore, Atomic Force Microscopy imaging of *S. aureus* biofilm with *D. hispida* revealed increased height and width of cell clusters despite reduction in volume compared to the negative control, suggesting unique biofilm resistance behaviour against the plant.
Conclusion, significance and impact of study: This study demonstrated *D. hispida* capability as a natural antimicrobial and antibiofilm agent. The plant could complement current antimicrobials to maximise killing efficiency and minimise occurrences of resistance. Unique biofilm behaviour against *D. hispida* also warrants further investigation on the effect of biocides towards biofilm structure. Overall, this research provides new insights into a traditional plant-based antimicrobial activity in combating infectious diseases and AMR.

Keywords: Dioscorea hispida, antimicrobial, biofilm, traditional medicine, Atomic Force Microscopy

INTRODUCTION

Infections and diseases caused by microorganisms are a constant threat to the society, requiring medical intervention including the application of antimicrobial drugs (e.g. antibiotics, antifungals and antivirals). However, the misuse and overuse of these antimicrobials have given rise to a number of antimicrobial resistant (AMR) microorganisms, leading to the evolution of "superbugs". These superbugs, such as those coming from a group coined "ESKAPE" (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter* species) are multidrug resistant, causing serious consequences to public health particularly to the more vulnerable groups within society (e.g. small children, elderly and immune-compromised patients). This phenomenon is predicted to worsen due to the uncontrolled use of antimicrobials and the rapid capability of these bacteria to develop resistance (Unemo and Jensen, 2017). To combat this problem, developing viable alternatives to the commonly used antimicrobials is necessary apart from efforts to control antimicrobial over prescription and consumption.

Dioscorea hispida Dennst, or locally known as Ubi Gadong, is a promising natural alternative to the commonly used antimicrobials. *D. hispida* are edible wild tubers that are currently under-exploited but potentially effective as an antimicrobial due to their high content of bioactive alkaloids (Hron *et al.*, 1997). These alkaloids (e.g. dioscorine, found in the rhizome) are poisonous and therefore, they are removed from the plant prior to consumption. Despite the toxicity, *D. hispida* is primarily consumed in some tropical regions and used as a therapeutic drug to alleviate inflammation and combat indigestion (Mat Lazim *et al.*, 2016). Miah *et al.* (2018) has recently shown that certain fractions of phenolic compounds in *D. hispida* possess high antioxidant and

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thrombolytic activities, making the plant a potent traditional therapeutic. The free radical scavenging property in *D. hispida* was also observed in other *Dioscorea* species, e.g. *D. batatas*, that was linked to its dioscorine content (Hou *et al.*, 2001; Ohizumi *et al.*, 2009).

Previous studies have demonstrated *D. hispida* as a potential antimicrobial agent and insecticide whilst showing acceptable tolerance in vertebrates (Bhamarapravati *et al.*, 2003; Otake *et al.*, 1995). The starch was tested as one of the disinfectants against *Helicobacter pylori*, a Gram-negative bacterium that causes several gut diseases including stomach ulcer (Bhamarapravati *et al.*, 2003). Furthermore, through disk diffusion tests, *D. hispida* showed the ability to inhibit the growth of certain groups of bacteria and fungi (Azman *et al.*, 2015; Miah *et al.*, 2018). *Dioscorea hispida* has also been used as a coating material on rubber wood and demonstrated repelling activity against white-rot fungi and termites (Mat Lazim *et al.*, 2016). Interestingly, toxicity tests against zebrafish as a vertebrate model showed the organism's tolerance towards the starch at an acceptable concentration, implying the suitability of *D. hispida* as an antimicrobial agent with little negative impact to vertebrates (Azman *et al.*, 2015).

Whilst the above studies on *D. hispida* are useful, there is still a large gap in knowledge and understanding to establish *D. hispida* as an effective antimicrobial agent. For example, the maximal effective concentration (EC) of *D. hispida* against bacteria was not determined from the limited range of concentrations tested in previous studies. Additionally, it has not been established whether *D. hispida* effectively targets a broad range of bacteria based on the limited number of species used. Furthermore, the potential use of *D. hispida* as an antibiofilm agent has never been explored. Biofilms are known to be more resistant towards environmental stress (e.g. starvation, toxicity, predation) than its free-living, planktonic form and have been the root cause of many infectious diseases (Donlan and Costerton, 2002). Therefore, including biofilms as part of antimicrobial research is imperative. This study explored the effectiveness of *D. hispida* as an antibacterial and antibiofilm agent by testing the plant starch against selected microorganisms in their planktonic and biofilm forms. Further, through Atomic Force Microscopy, the biofilm morphology of a bacterium in the presence of *D. hispida* was compared against that of control biofilms without *D. hispida* or with an antibiotic.

MATERIALS AND METHODS

Discorea hispida starch and liquid stock preparation

Starch extraction was performed according to method described by Mat Lazim *et al.* (2016), in which the wild tuber was washed, pulverised, separated and dried. To obtain liquid stock of *D. hispida* (50 mg/mL), the prepared starch (50 g) was mixed with 100 mL 5% (w/v) NaOH, with agitation at 200 rpm for 2 h.

D. hispida antibacterial test against planktonic bacterial cultures

Escherichia coli UKMCC1006, *Staphylococcus aureus* UKMCC1016, *Pseudomonas aeruginosa* UKMCC1011, *Klebsiella pneumoniae* UKMCC1007 and *Bacillus subtilis* UKMCC1002 (obtained from the Universiti Kebangsaan Malaysia Culture Collection, Bangi, Malaysia), both in free-living (planktonic) and biofilm forms were used to test the effectiveness of *D. hispida* starch as an antibacterial agent.

Overnight bacterial cultures were prepared by incubation in Nutrient Broth (NB) (per L of dH_2O: 5 g peptone and 3 g meat extract, pH 7) at 37 °C. Bacterial cells were diluted to approximately 10^7 cells/mL before being tested against *D. hispida* at the following concentrations (mg/mL): 0.16, 0.31, 0.63, 1.25 and 2.5 for 24 h at 37 °C. Cell growth inhibition was measured by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a ratio of 2:15 (MTT: cell culture) before incubating at 37 °C for 1 h and Optical Density (OD) was measured at 595 nm. Gentamicin (150 µg/mL) served as the positive control for inhibition, whilst bacterial cultures only (i.e. without *D. hispida*) were used as a negative control. All tests were conducted in at least three independent replicates and three technical replicates for each bacterium.

The half maximal effective concentration (EC_{50}, i.e. that of which inhibits 50% of the bacterial population) of *D. hispida* was determined through plotting the percentage of bacterial inhibition against *D. hispida* concentration using the GraphPad Prism 7 software. The EC_{50} value obtained for each bacterium was then used in the antibiofilm experiment. The percentage of bacterial inhibition was calculated using the following formula:

$$OD_{sample} - OD_{extract} / OD_{control} \times 100$$

where OD_{sample} = OD of the tested bacterial culture with *D. hispida* at a specific concentration, $OD_{extract}$ = OD of *D. hispida* at concentration used in OD_{sample} without bacteria and $OD_{control}$ = OD of bacteria without *D. hispida* but with NaOH (used as solvent for *D. hispida*) at concentration used in OD_{sample}.

D. hispida treatment against bacterial biofilms

Biofilm for each bacterium was prepared and its biomass quantified according to the previously described method by O'Toole (2011), with or without the addition of *D. hispida*. An overnight culture of each bacterium was prepared in NB at 37 °C and inoculated (1:100 dilution) into fresh M9 medium (per L of dH_20: 64 g Na_2HPO_4-$7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5.0 g NH_4Cl, 2 mL of 1 M $MgSO_4$, 100 µL of 1 M $CaCl_2$ and 20 mL of 20% (w/v) D-glucose solution) with 0.25% (w/v) of casamino acids (CAA). *D. hispida* was added into each bacterial culture at EC_{50} previously obtained from the antibacterial test and the culture was incubated at 37 °C for 24 and 48 h. Biofilm density for each bacterium was measured at 550

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nm using Crystal Violet staining. Gentamicin (150 µg/mL) was used as positive control, whilst negative control contained zero amount of *D. hispida* starch. All tests were conducted in at least three independent replicates and three technical replicates for each bacterium.

Atomic Force Microscopy (AFM) analysis of *S. aureus* biofilm in the presence of *D. hispida*

The effect of *D. hispida* against a bacterial biofilm was further observed using AFM. *S. aureus* was chosen due to its pathogenicity and relatively higher susceptibility to *D. hispida* than other tested bacteria based on the biofilm assay. Mica sheets (1 cm × 1 cm each, used as base for biofilm), were sterilised in 0.5% $NaOCl_3$ for 2 h and rinsed 3 times with sterile distilled water prior to putting each into a well of a 12-well plate that contained M9 media with glucose and CAA. An overnight culture of *S. aureus* was inoculated (1:100) into a well containing the M9 medium, mica sheet and *D. hispida* at 0 or 2.5 mg/mL (the highest concentration used in this study). Streptomycin (150 µg/mL) was used as positive control in replacement of *D. hispida.* The cultures were incubated at 37 °C for 24 h in stagnation with at least three independent replicates for each test.

After incubation, the mica sheets containing the biofilms were gently rinsed in sterile M9 media three times before air-dried in a laminar flow cabinet for 4 h at room temperature. Each biofilm was visualised using an atomic force microscope (Dimension V, Veeco Instruments Inc., New York) operated by the Research NanoScope 7.20 software (Veeco Instruments Inc., New York). The cantilever used had nominal spring constants between 20-80 N m^{-1} and frequency of 335 kHz. Scans were conducted in tapping mode at 20 × 20 µm. At least three scans per independent replicate was performed for each sample. Further data analysis was conducted using Gwyddion version 2.5 software (Nečas and Klapetek, 2012).

RESULTS AND DISCUSSION

In this study, we explored the effectiveness of *D. hispida* as an antibacterial and antibiofilm agent, serving as an alternative to the over prescribed and over consumed antimicrobials such as antibiotics. The quest for an effective substitute is timely due to the emergence of multidrug resistant microorganisms such as the 'ESKAPE' pathogens, partly caused by an increasing dependency on the same class of drugs to treat infections. *Dioscorea hispida* Dennst is a wild, tuberous plant available in Malaysia and Southeast Asia and its known toxicity makes it of high potential as a locally sourced biocide. As shown in this study and past research, the tuber was able to target a considerable range of microorganisms and invertebrates, whilst showing an acceptable tolerance level in vertebrates. Here, we elaborated further the effect of *D. hispida* against a selected group of bacteria that are Gram negative (*E. coli*, *P. aeruginosa* and *K. pneumoniae*) and Gram positive (*S. aureus* and *B. subtilis*) in both planktonic and biofilm forms.

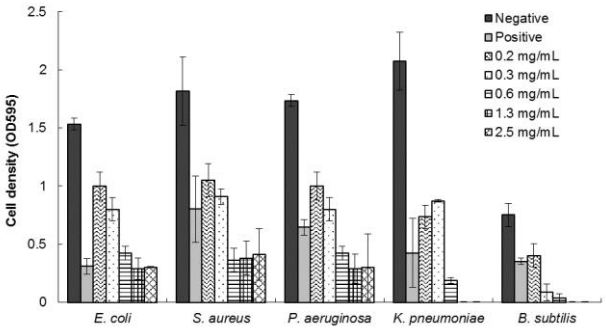

Figure 1: Effect of different *D. hispida* concentrations (0.2, 0.3, 0.6, 1.3 and 2.5 mg/mL) on planktonic cell density (OD_{595}) of *E. coli*, *S. aureus*, *P. aeruginosa*, *K. pneumoniae* and *B. subtilis* at 24 h of incubation. Gentamicin (150 µg/mL) was used as positive control. All tests were conducted in three independent replicates.

Table 1: Half maximal effective concentration (EC_{50}) of *D. hispida* against *E. coli*, *S. aureus*, *P. aeruginosa*, *K. pneumoniae* and *B. subtilis*.

Bacteria	EC_{50} (mg/mL)
E. coli	0.4226 ± 0.08
P. aeruginosa	0.4088 ± 0.09
K. pneumoniae	0.4223 ± 0.04
S. aureus	0.3101 ± 0.02
B. subtilis	0.3041 ± 0.04

Overall, the presence of *D. hispida* significantly ($P<0.05$) reduced bacterial growth compared to those without any treatment. This was clearly shown by the decrease in bacterial cell density (OD_{595}) as *D. hispida* concentration increased after 24 h of incubation (Figure 1). At the lowest *D. hispida* concentration (0.2 mg/mL), all five microorganisms showed approximately two-third to half the cell density of negative control (i.e. without *D. hispida*). Cell density was further reduced to more than half at 0.6 mg/mL *D. hispida* for all bacteria. However, as *D. hispida* concentration increased further (> 0.6 mg/mL), the cell density remained relatively constant for *E. coli*, *S. aureus* and *P. aeruginosa* but declined to almost zero for *K. pneumoniae* and *B. subtilis*, implying higher resistance in the former bacterial group than the latter. The varied susceptibility amongst bacteria towards *D. hispida* is expected and in line with previous findings, where the starch worked more effectively against certain bacteria over others (Azman *et al.*, 2015; Miah *et al.*, 2018). The mechanism of action by *D. hispida* against microorganisms is still largely unknown; however, the tuber contains a high amount of phenolic compounds that have been previously demonstrated to be antibacterial based on a number of mechanisms, including membrane permeabilisation and disruption, redox imbalance

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induction and catalase inhibition (Taylor *et al.*, 2004; Greenberg *et al.*, 2008; Araya-Cloutier *et al.*, 2018; Ajiboye *et al.*, 2018; Carvalho *et al.*, 2018). Thus, phenolics such as the alkaloid dioscorine found in *D. hispida* may work in a similar manner to that of other phenolic compounds, but further research into *D. hispida* mechanism of action is necessary to ascertain this. Interestingly, the two bacteria (*K. pneumoniae* and *B. subtilis*) in which *D. hispida* was most effective against were Gram negative and positive, respectively, therefore implying that cell wall variation holds little importance to the starch mode of action.

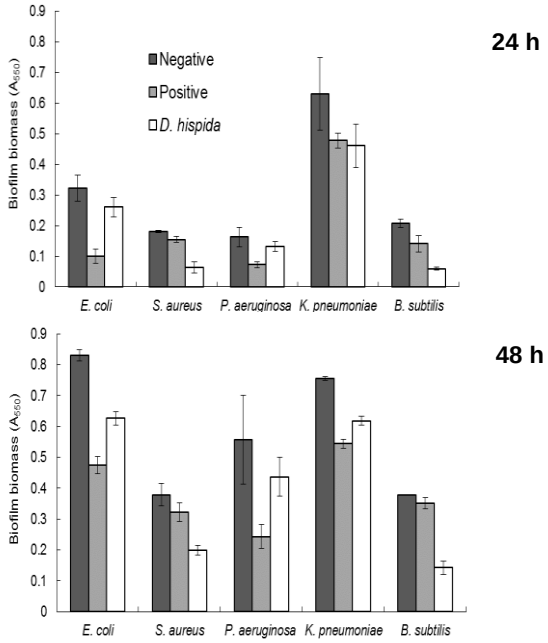

Figure 2: Effect of *D. hispida* (at EC_{50}) on biofilm biomass (OD_{550}) of *E. coli*, *S. aureus*, *P. aeruginosa*, *K. pneumoniae* and *B. subtilis* at 24 and 48 h of incubation. All microorganisms showed significant (P<0.05) reduction in biofilm density against the negative control (without *D. hispida*), however, performance against the positive control (150 µg/mL gentamicin) varied. All tests were conducted in three independent replicates.

Despite the significant reduction in bacterial cell density with *D. hispida*, a higher dosage of the starch (i.e. > 0.6 mg/mL) was still necessary to render it as or more effective than the antibiotic control. From the results shown (Table 1), *D. hispida* needed to be at least 0.3 mg/mL for *B. subtilis* and 0.6 mg/mL for other bacteria to be significantly more effective than gentamicin at 0.15 mg/mL (P<0.05). This shows *D. hispida* has a 'moderate' antibacterial activity compared to antibiotics, which are still more potent than the starch. Miah *et al.* (2018) showed similar trend (where antibiotics outperform *D. hispida*) through disk diffusion tests of *D. hispida* phenolic extracts (400 µg/disc) and ciprofloxacin (30 µg/disc) against a number of pathogenic bacteria. Although *D. hispida* is effective as an antibacterial agent (despite its weaker performance than antibiotics), the plant could also act as a complement to antimicrobials to achieve maximum killing of pathogens and minimise the emergence of resistant microorganisms. However, the question of whether or not the performance of the antibiotic with *D. hispida* is more effective than that of the antibiotic alone still needs to be addressed with further research.

The effectiveness of *D. hispida* was explored further by observing its impact on biofilms. In general, microorganisms are able to grow as two different phenotypes, i.e. 1) planktonic, free-living cells; or 2) biofilms, where cells congregate and are attached to a surface or each other, encased by a layer of extracellular polymer matrix. Biofilms have been shown to be the more predominant form than planktonic cells in causing infections due to their higher resistance to antimicrobials and other environmental stresses. The sessile lifestyle also increases the microorganism's capability to invade host cells, which also, together with host extracellular matrix proteins, act as substrata for microbial cell attachment (Bjarnsholt, 2013; Høiby *et al.*, 2010). For examples, *P. aeruginosa* biofilm has been known to be the causative agent for lung infection (pneumonia) in cystic fibrosis patients, whereas *S. aureus* in its sessile form causes nosocomial infection, particularly from the contamination of medical devices (e.g. catheters, tubes) (Gellatly and Hancock, 2013; Lister and Horswill, 2014). Thus, it is of paramount importance that the biofilm form, apart from the cell free-living lifestyle, is addressed in this study. Biofilm biomass was quantified after 24 and 48 h of incubation with half maximal concentration of *D. hispida* obtained from the planktonic assay (Table 1).

Similar to the overall trend with planktonic cells, *D. hispida* caused significant (P<0.05) reduction in biofilm density for all bacteria in comparison to the negative control (without *D. hispida*) (Figure 2). Amongst the five bacteria tested, *B. subtilis* and *S. aureus* were the most affected by *D. hispida*, with biofilm densities reducing approximately half or more than half of the negative and positive controls after 24 and 48 h incubation (P<0.05). However, for *E. coli*, *P. aeruginosa* and *K. pneumoniae*, *D. hispida* was less effective than gentamicin in reducing their biofilm growth. Furthermore, the fact that free-living *K. pneumoniae* (Figure 1) could be more effectively eradicated than its biofilm (Figure 2) shows a higher resistance by the bacterium in sessile mode. It is known that these three bacteria (*E. coli*, *P. aeruginosa* and *K. pneumonia*) produce 'strong' biofilms, resistant to biological and chemical antibiofilm agents, including antibiotics (Donlan and Costerton 2002). Various mechanisms of biofilm resistance have been found in these bacteria, including quorum sensing systems that regulate the expression of resistance-conferring genes and induction of rpoS-mediated stress response, apart from the polymer matrix and biofilm architecture themselves as protective measurement (Mah and O'Toole, 2001; Gellatly and Hancock, 2013; Vital-Lopez *et al.*, 2015). Hence, due to the biofilm higher resistance

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than its planktonic cells, increased concentration of either *D. hispida* or the antibiotic is deemed necessary to eradicate biofilms. Alternatively, the synergy between *D. hispida* and other antimicrobials against biofilms should be explored, as suggested earlier.

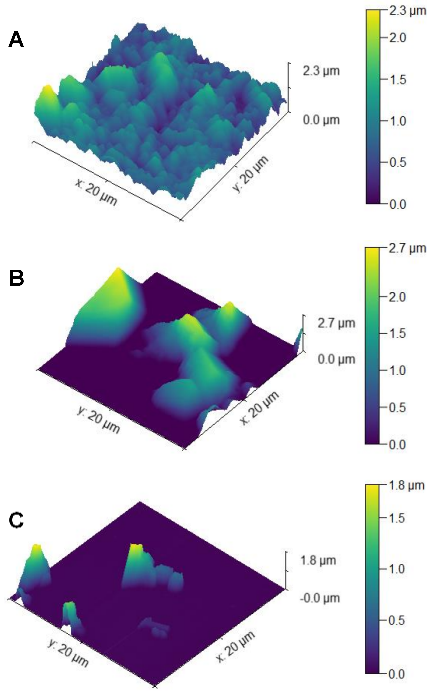

Figure 3: Representative Atomic Force Micrographs (20 × 20 µm scan) of *S. aureus* biofilms. Treatment: A) without *D. hispida*; B: with *D. hispida* at 2.5 mg/mL and C: with streptomycin at 150 µg/mL.

The action of *D. hispida* starch against biofilms was further explored through AFM imaging, using *S. aureus* as the bacterial model and streptomycin as the antibiotic control (Figure 3). The micrographs showed full surface coverage and relative evenness of *S. aureus* biofilm without any treatment (Figure 3A), in contrast to the increasing sparseness of the biofilm with *D. hispida* (Figure 3B) and antibiotic (Figure 3C). Treatment with *D. hispida* markedly caused a reduction in biofilm volume (193 ± 12 µm^3) compared to the negative control (312 ± 20 µm^3). However, treatment with streptomycin reduced the biofilm volume even further (29 ± 7 µm^3) than treatment with *D. hispida*, contrasting the trend obtained in the biofilm quantification assay (where the antibiotic performed weaker than *D. hispida*) shown in Figure 2. This was likely due to the change of antibiotic used (i.e. streptomycin instead of gentamicin), indicating varied responses from *S. aureus* biofilm against different antibiotics. Interestingly, despite the volume reduction and lesser surface coverage, treatment with *D. hispida* caused the biofilm to appear taller (Figures 3 and 4) compared to the negative control, with the average height of 1.72 ± 0.5 and 0.71 ± 0.2 µm, respectively. Biofilm height distribution (Figure 4) also indicates that biofilm with *D. hispida* generated peaks of between 1.6-2 µm, which were not found in either control with a lower height average. This behaviour may signify a mechanism of *S. aureus* biofilm resistance towards *D. hispida* at 2.5 mg/mL by congregating more cells and/or extracellular polymer matrix to form larger and taller biofilm clusters in order to protect itself from *D. hispida* biocidal activity. However it is not known whether cells in the biofilm with *D. hispida* were mostly alive or dead, or if *D. hispida* was able to penetrate into the deeper area of biofilm through voids and channels, warranting further investigation into the plant antibiofilm mechanism of action. It is anticipated that a higher concentration of *D. hispida* would lessen biofilm formation further, causing a similar reduction effect observed in the antibiotic treatment (Figure 3C).

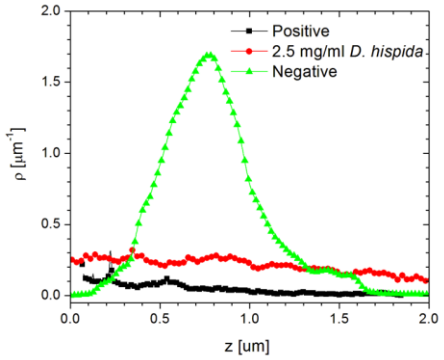

Figure 4: Height (z) distribution (µm) of *S. aureus* biofilms with *D. hispida* at 2.5 mg/mL (red circle), positive control (streptomycin) at 150 µg/mL (black square) and negative (no treatment) control (green triangle) obtained from the AFM scans.

CONCLUSION

This study shows that *D. hispida* significantly inhibited planktonic cell growth and biofilm density of bacteria compared to untreated samples. However, compared to antibiotics, only certain bacteria were significantly affected by *D. hispida*, implying a “moderate” action of this plant as an antimicrobial. Thus, we suggest that *D. hispida* is used concurrently with another antimicrobial agent to enhance killing efficiency and further reduce the chances of resistance from occurring. Additionally, treatment with *D. hispida* at a certain concentration resulted in taller and larger biofilm clusters despite the reduced overall volume. This finding was in contrast to biofilms without *D. hispida*, highlighting unique biofilm mechanism of resistance against the plant. Overall, this study provides further insights into the effectiveness of traditional plants as a natural alternative in combating infectious diseases and antimicrobial resistance.

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