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Antibacterial and Antibiofilm Activities of *Sesbania grandiflora* Against Foodborne Pathogen *Vibrio cholerae*

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ABSTRACT

The progressing threat of antimicrobial resistance to global public health remains a problem yet to be solved. Hence, new natural products and novel strategies in combating bacteria are continuously being discovered. In this study, *Sesbania grandiflora* was tested for its antibacterial and antibiofilm activities against the pathogenic *Vibrio cholerae*. *S. grandiflora* was able to yield growth inhibition at 7.81 mg/mL and was bactericidal at 15.63 mg/mL. However, it was only able to start to actively inhibit growth at 125 mg/mL. *S. grandiflora* ethanolic extract was also able to significantly inhibit biofilm formation at concentration as low as 0.98 mg/mL. Hence, the results showed the concentration-dependent antibiofilm activity of *S. grandiflora* that it was able to inhibit biofilm formation without completely eradicating the microorganism at lower concentrations. These activities were due to their phytochemical composition which exhibit antibacterial and antibiofilm activities. Isolation and characterization of their bioactive compounds may enhance the efficacy of their activities.

INTRODUCTION

The density-dependent bacterial cell-to-cell communication, termed as quorum sensing (QS), results to the transcription of genes responsible for a variety of bacterial mechanisms including pigmentation, production of virulence factors, and biofilm formation (Miller and Bassler, 2001). Biofilm is defined as a sessile community of microbial cells that are attached to a surface or embedded to extracellular polymeric substances (EPS) (Donlan and Costerto, 2002). It results in the creation of microenvironments which have conditions different from its "external" environment such as pH, availability of oxygen, and temperature, among others. These conditions allow the microbial cells involved in this complex community to be protected against external substances such as antibiotics which results to their resistance. Furthermore, biofilm allows the immediate interactions between cells which result to the easy transport of substances responsible for a variety of physiological mechanisms as well as autoinducers that may result to the continuity of their communication (Paraje, 2011). Therefore, this communication mechanism as well as the physiological functions dependent on it has been a target for the discovery of new bioactive compounds against bacterial infections. The inhibition of quorum sensing, a population density-dependent bacterial cell-to-cell communication system involving signalling molecules (Borges *et al.*, 2013), results to inhibition of biofilm formation and serves as a novel strategy to effectively combat bacteria without the risk of resistance due to the little to no pressure it poses to the bacteria (Givskov, 2012). On a wet surface, such as clinical, industrial and food processing

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environments and drinking water distribution systems, biofilms usually create a sticky gel composed of polysaccharides, proteins and other organic components. Due to the presence of biofilms in food processing, a possibility of disease transmission and food spoilage because of contamination may occur (Salta *et al.*, 2013).

Among the most common food pathogens is *Vibrio cholerae.* It is also one of the most medically important species among the *Vibrio* species due to its ability to produce heat-labile enterotoxins and cause pandemic and endemic cholera, cholera-like diarrhea, and extraintestinal infections (Caroll *et al.*, 2016). *V. cholerae* favorably inhabits nutrient-rich environments that are warm and abundant with aquatic macrophytes, phytoplanktons, zooplanktons, fishes, molluscs, and crustaceans (Borroto, 1997). Several researches also suggest that *V. cholerae* particularly favors attachment to planktons and other inanimate surfaces (Matz, *et al.*, 2006) as means of survival against unfavorable conditions such as acidity, temperature and salinity changes in their environment (de Magny *et al.*, 2011).

Natural products have been crucial to the discovery and development of new drugs against bacterial infections. One of the rich sources of natural products is *Sesbania grandiflora* (L.) Poir., a member of the Family Fabaceae. It is native to the Southeast Asia including Thailand, Indonesia, Malaysia, Laos, and the Philippines and has since been introduced to other parts of the world. It is commonly known in the Philippines as *Katuray* and is used as an ingredient in a number of recipes, as well as a traditional medicine (Bureau of Plant Industry, 2014). The leaves of *Sesbania grandiflora* have been used in local traditional medicine since ancient times as a tea out of dried leaves and are considered to have antibacterial, antihelminthic, antitumor, and contraceptive properties (Arfan *et al.*, 2016). Almost every part of *S. grandiflora* is used as traditional medicine to treat diseases such as fever, small pox, dysentery, headache, and sore throat among others (Ghani, 2002). Several reports mention the isolation of tannins, sterols, saponins, flavonoids, and alkaloids from the leaves of the plant (Fojas *et al*., 1982; Roy *et al.*, 2014; Arfan *et al.*, 2016). These bioactive constituents have potential health benefits that possess antimicrobial and antifungal properties (Goun *et al.*, 2003).

In this study, the potential antibacterial and antibiofilm activities of *Sesbania grandiflora* against the pathogenic *Vibrio cholerae* have been elucidated to serve as an additional source of natural products harnessing two different mechanisms against bacterial infections caused by *V. cholerae*.

MATERIALS AND METHODS

Collection of *Sesbania grandiflora*

Young and healthy *Sesbania grandiflora* leaves were collected from Bicutan, Taguig City. The leaves in the apical part of the twigs were preferred since they possess the highest concentration of phytochemicals (Bhakta and Ganjewala, 2009). The collected leaves were sent to the laboratory for extraction, and to the Botany Division, National Museum of the Philippines for the identification of the leaves.

Extraction of *Sesbania grandiflora*

The leaves of *Sesbania grandiflora* were washed with water to remove the debris, and then air-dried for five days at room temperature. The dried leaves were then pulverized using a blender. Approximately 500 grams of powdered leaves were steeped in 2 L of absolute ethanol for 48 hours. The ethanolic extracts were filtered using Whatman #1 filter paper. Then, the collected filtrates were subjected to rotary evaporation at 60°C to remove the solvent from the extract. The remaining solvent was further evaporated using water bath set at 60°C. The crude extracts were then stored in properly-labelled amber bottles at 5°C prior to use.

Test Organism

Vibrio cholerae (ITDI 0063) from the DOST - Industrial Technology Development Institute Culture Collection was used as the test organism. The culture was first streaked onto TCBS Agar to ensure that the culture obtained was pure, and then maintained in Tryptic Soy Agar supplemented with 2% NaCl (Merck, Germany).

Preparation of Inoculum

A loopful of 24-hour old confirmed *Vibrio cholerae* culture was inoculated in 9 mL sterile distilled water supplemented with 2% NaCl. The inoculum was compared with 0.5 McFarland Standard to obtain a standardized inoculum for the conduct of assays (Tenover, 2009).

Antibacterial Activity

Determination of Minimum Growth Inhibitory Concentration (MGIC)

In a 96-well microtitre plate, a two-fold serial dilution of the extract was made. Afterwards, a uniform amount (100 µL) of inoculum was placed on each well. Similarly, antibacterial positive controls ampicillin (10 µg) (YSS Laboratories), and gentamicin (10 µg) (YSS Laboratories) as well as negative control absolute ethanol (Ajax FineChem) were also used. The antibiotics used were chosen from the list of antibiotics commonly used for antibiotic susceptibility testing for *Vibrio* spp. in CLSI M-45 Guidelines (2010). The plates were incubated at 30°C for 24 hours. The presence of white pellets on the bottom of the wells indicated growth. The lowest concentration that showed growth inhibition is the Minimum Growth Inhibitory Concentration (Quinto and Santos, 2005).

Determination of Minimum Bactericidal Concentration (MBC)

For confirmatory testing, the contents of the wells used to determine the MGIC were streaked onto freshly-prepared TSA with 2% NaCl to determine the Minimum Bactericidal Concentration, which is the lowest concentration that completely showed no growth. The presence of at least a single colony, which indicates growth, confirmed the results for the MGIC. Otherwise, the absence of growth confirmed the MBC.

Agar Well Diffusion Assay

The agar well diffusion method was used to determine the antibacterial activity of the ethanolic extract of *Sesbania grandiflora* against *Vibrio cholerae*. Using a sterile cotton swab, standardized cell suspension was inoculated by spreading evenly over the surface of the TSA (with 2% NaCl). The plates were allowed to dry and a sterile cork borer (5 mm) was used to cut

uniform wells in the agar. Each well was filled with 100 µL of different concentrations of the *S. grandiflora* extract. Similarly, antibacterial controls ampicillin (10 µg) (YSS Laboratories), and gentamicin (10 µg) (YSS Laboratories) as well as negative control absolute ethanol (Ajax FineChem) were also subjected to agar well diffusion assay. After incubation at 30°C for 24 hrs, the plates were observed and their zones of growth inhibition (ZOGI) were measured.

Antibiofilm Activity

Biofilm Quantification Assay

The *Sesbania grandiflora* extract was tested against *Vibrio cholerae*. In a 96-well microtitre plate, a two-fold serial dilution of the extract was made resulting to 100 µL amount of extract per well. Absolute ethanol served as the negative control. Afterwards, a uniform amount (100 µL) of inoculum was placed on each well. The plates were incubated at 30°C for 24 hours. The contents of the wells were discarded and washed to remove planktonic cells. The biofilms adhering on the wells were stained with 125 µL 0.1% crystal violet for 15 minutes and then washed to remove excess dye. The biofilms stained with crystal violet were dissolved with 125 µL 30% acetic acid and then incubated for 15 minutes at room temperature to allow the crystal violet to solubilize. Afterwards, 125 µL of the solubilized crystal violet was transferred onto new microtitre plates for quantification using a spectrophotometer at OD 550 nm to measure its absorbance (O'Toole, 2011).

Statistical Analysis

Statistical analysis was done through IBM SPSS Statistics 2.0 software. One-way Analysis of Variance (ANOVA) was used to determine if a significant difference exists among the zones of inhibition and absorbance values of the different concentrations of *Sesbania grandiflora* as well as against the controls. Tukey's *post hoc* test was employed to determine between which concentrations the significant difference exists.

RESULTS AND DISCUSSION

Minimum Growth Inhibitory Concentration (MGIC)

Different concentrations *of Sesbania grandiflora* ethanolic extract were prepared by two-fold serial dilution. At the starting concentration (500 mg/mL) up to 7.81 mg/mL, no visible white pellets were observed on the bottom of the well. This indicated that the concentrations used were growth inhibitory. Moreover, the results showed that 7.81 mg/mL is the Minimum Growth Inhibitory Concentration since it was the lowest concentration that yielded observable growth inhibition. This suggests that as the concentration of the extract increased, the growth inhibition also increased. The growth inhibitory activity of *S. grandiflora* leaves may be credited to the phytochemicals present on the ethanolic extract (Fojas *et al.*, 1982; Roy *et al.*, 2014; Arfan *et al.*, 2016).

Minimum Bactericidal Concentration (MBC)

To confirm the results of the determination of MGIC through serial dilution, the Minimum Bactericidal Concentration (MBC) of the extracts were also determined. The contents of the wells treated with *S. grandiflora* ethanolic extract were streaked on TSA with 2% NaCl plates. The results showed that at the starting concentration (500 mg/mL) up to 15.63 mg/mL, no growth was observed. Hence, 15.63 mg/mL is the MBC of the *S. grandiflora* ethanolic extract against *V. cholerae*. The MBC agrees with the results of the MGIC (7.81 mg/mL) which showed that at concentration lower than the MBC, the extract was not able to completely eradicate *V. cholerae* but was able to inhibit its growth.

Quantitative Antibacterial Activity

Sesbania grandiflora ethanolic extract was able to inhibit the growth of *Vibrio cholerae* at concentration as low as 7.81 mg/mL which makes it the MGIC (Figure 1). Moreover, no significant difference ($p > 0.05$) was observed among the concentrations 7.81 mg/mL, 15.63 mg/mL, and 31.25 mg/mL, but a significant difference was found between the MGIC and at 62.5 mg/mL concentration. Nonetheless, these concentrations only exhibited antibacterial activities which are considered inactive (Quinto and Santos, 2005). Partial antibacterial activity was observed at 125 mg/mL, whereas, the extract was considered active with no significant differences ($p > 0.05$) at concentrations 250 mg/mL and 500 mg/mL with the mean diameter of its zones of growth inhibition (ZOGI) at 14.33 mm and 17.67 mm, respectively. However, only at the highest concentration (500 mg/mL) with a mean ZOGI of 17.67 mm did the antibacterial activity of *S. grandiflora* crude ethanolic extract not exhibit significant difference ($p > 0.05$) with a commercial antibiotic, gentamicin (18.33 mm). Therefore, at the concentration of 500 mg/mL, the antibacterial activity of *S. grandiflora* crude ethanolic leaf extract is comparable with gentamicin against *V. cholerae*. However, a significantly higher activity was observed with ampicillin (37.33 mm) than the most effective concentration of *S. grandiflora* extract. This activity is attributed to the presence of phytochemicals with antibacterial properties at high concentrations such as alkaloids and tannins (Fojas *et al.*, 1982; Roy *et al.*, 2014; Arfan *et al.*, 2016).

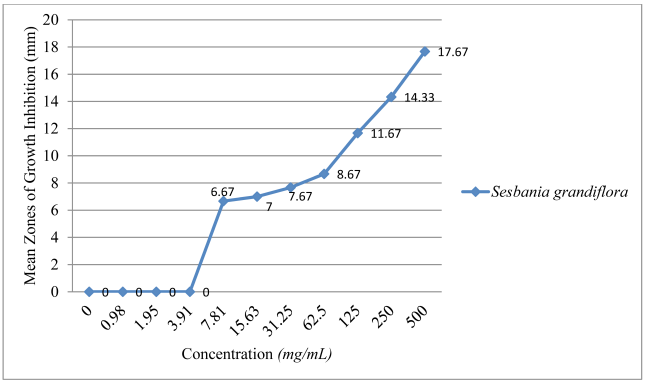

Fig. 1: Antibacterial Activity of *Sesbania grandiflora* based on zones of growth inhibition.

Biofilm Inhibition

Similarly, *Sesbania grandiflora* ethanolic leaf extract was subjected to biofilm inhibition assay in a 96-well microtitre plate. Different concentrations of the extract were prepared using

two-fold serial dilution and the wells were inoculated with a uniform amount of *V. cholerae*. Absolute ethanol served as the negative control. The results showed that the absorbance values of concentrations starting from 500 mg/mL up to 15.63 mg/mL were significantly lower than that of the negative control, an indication that biofilm formation was inhibited at these concentrations. However, the results of the preceding assays indicate that these concentrations were bactericidal and no growth was observed in the determination of MBC. Thus, the lack of biofilm formation observed were due to the eradication of *V. cholerae.* Moreover, the concentration of 7.81 mg/mL yielded significant biofilm inhibition as compared with the negative control ($p < 0.05$), but was shown to be growth inhibitory as per the preceding assays. Interestingly, the concentrations 3.91 mg/mL, 1.95 mg/mL, and 0.98 mg/mL were able to yield absorbance values lower than the negative control (0.21 AU). The results of the previous assays showed that these concentrations were not able to inhibit the growth of *V. cholerae*. No significant differences were observed between the three concentrations ($p > 0.05$), however, significant differences with the negative control only exist with concentrations 1.95 mg/mL ($p < 0.05$), and 0.98 mg/mL ($p < 0.05$). Hence, the lowest concentration used in this study (0.98 mg/mL) was the Minimum Biofilm Inhibitory Concentration as well as the most effective concentration.

The results suggest that *S. grandiflora* extract is growth inhibitory at high concentrations but is biofilm inhibitory at lower concentrations. This also agrees with the previous studies on biofilm inhibition which show concentration-dependent antibiofilm activity (Figure 2) (Taganna *et al.*, 2011; Vasavi *et al.*, 2013). This observation also proved that the antibacterial and antibiofilm activities of *S. grandiflora* leaf extract work on two different and independent mechanisms, and that biofilm inhibition may be attained without affecting bacterial growth. This biofilm inhibitory activity of *S. grandiflora* is due to its phytochemical composition that includes tannins and alkaloids (Fojas *et al.*, 1982; Roy *et al.*, 2014; Arfan *et al.*, 2016), which were previously shown to inhibit biofilm formation by either binding with the receptors involved in the quorum sensing system or with the enzymes responsible for the synthesis of autoinducers (Taganna *et al.*, 2011).

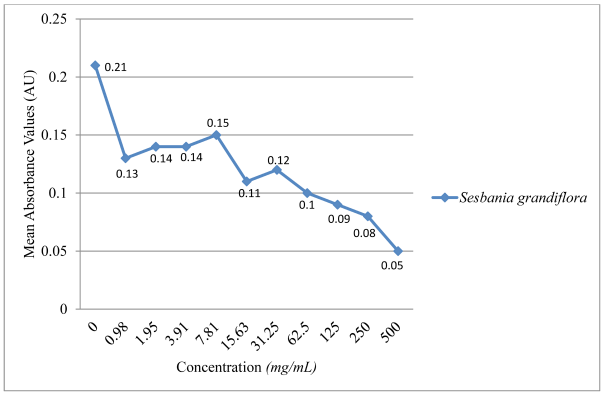

Fig. 2: Antibiofilm Activity of *Sesbania grandiflora* based on absorbance values.

CONCLUSION

The search for natural products that exhibit antibacterial and antibiofilm activities has been of interest since the rise of antimicrobial resistance. In this study, *Sesbania grandiflora* ethanolic extracts were tested for their antibacterial and antibiofilm activities against the pathogenic *Vibrio cholerae. S. grandiflora* was antibacterial until 7.81 mg/mL but was only active at concentrations starting from 125 mg/mL and above. Biofilm inhibition assays showed that *S. grandiflora* leaf extract exhibited significant antibiofilm activity at concentrations as low as 0.98 mg/mL which also demonstrate its concentration-dependent activity. The activities may be attributed to the phytochemicals present in the extract. Further isolation and characterization of the bioactive compounds may enhance the efficacy of the extracts. These observations may lead to the development of this plant as a potential drug against bacterial infections.

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Nil.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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