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Research Article

Methanolic Extract Of *Swietenia macrophylla* Exhibits Antibacterial And Antibiofilm Efficacy Against Gram-Positive Pathogens

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ABSTRACT

Gram-positive pathogens cause infections such as pneumonia, skin infections, anthrax, and sinusitis. The objective of this study was to determine the phytochemical profile, antibacterial and antibiofilm efficacy of *Swietenia macrophylla* methanolic extract (SMME) against Gram-positive pathogens. The secondary metabolites of SMME were analyzed using GC-MS while the antibacterial efficacy of SMME against *Staphylococcus aureus* ATCC 33862, *Bacillus cereus* ATCC 11778, *Streptococcus pneumonia* ATCC 19615, and *Clostridium sporogenes* ATCC 13124 was assessed using MIC and MBC assays. Biofilm biomass assay and time-kill assay were performed to determine the antibiofilm activity of SMME against the pathogens. Results demonstrated that six common antibacterial secondary metabolites were present in the SMME. The major compound was found to be β-amyrin (22.8%). The SMME showed the lowest MIC values against *B. cereus* (31.25 µg/mL) and *C. sporogenes* (31.25 µg/mL) and the lowest MBC value against *S. aureus* (1000 µg/mL). The SMME also significantly (p<0.05) inhibited all the biofilms. It started to inhibit *S. pneumonia* and *C. sporogenes* biofilms after 12 h of exposure. On the other hand, the BIC_{50} value showed that the SMME was most effective against *B. cereus*. In conclusion, the secondary metabolites in the SMME may contribute to the antibacterial and antibiofilm efficacy against Gram-positive pathogens.

Key words: Antibacterial activity, antibiofilm activity, Gram-positive bacteria, phytochemical compounds, *Swietenia macrophylla*

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INTRODUCTION

Tropical plants have long served as an important repository of medicinal plants for many countries (Mitra *et al*., 2007). One of the most popular medicinal plants used is *Swietenia macrophylla*, which is also known as the "sky fruit tree". The secondary metabolites of *S. macrophylla* include alkaloids, terpenoids, anthraquinones, cardiac glycosides, saponins, phenols, flavonoids, volatile oils, phospholipids, and long-chain unsaturated acid (Ayyappadhas *et al*., 2012). Due to the rich secondary metabolites in *S. macrophylla*, many people use this plant to treat a wide range of diseases. Traditionally, the leaves have been used for the treatment of diarrhea, febrifuge, colds, and cataract (Ayyappadhas *et al*., 2012). Maiti *et al*., (2007) have also reported the antibacterial and antifungal effects of *S. macrophylla* seeds.

Diseases transmitted by microbes such as bacteria and fungi remain the major health problems in many countries worldwide. *Staphylococcus aureus, Bacillus cereus, Streptococcus pneumonia*, and *Clostridium sporogenes* are Gram-positive bacteria that commonly infect the human body. *S. aureus* is known to cause clinical infections such as endocarditis, osteoarticular, skin and soft tissue infection, and pleuropulmonary and device-related infections (Sathasivam *et al*., 2022). *S. pneumoniae* is responsible for a wide range of infections which include invasive and non-invasive such as pneumonia, meningitis, septicemia, and otitis media (Ordóñez & Ordóñez, 2023). On the other hand, *B. cereus* and *C. sporogenes* are frequent infectious agents responsible for foodborne diseases (Brunt *et al*., 2015; Glasset *et al*., 2018). The World Health Organization has announced that medicinal plants would be the best source to obtain a variety of drugs. Over the past few decades, the potential use of natural products or plant-derived substances to combat biofilm infections has been well documented (Yahya *et al*., 2014; Johari *et al*., 2020; Zawawi *et al*., 2020). Recently, *S. macrophylla* possesses has been demonstrated to be effective against

biofilms formed by Gram-negative pathogens (Man *et al*., 2022). However, its antibacterial activity and efficacy on the biofilm formation by Gram-positive pathogens remain not well investigated. Thus, the present work was performed to determine the secondary metabolites of SMME using GC-MS and to determine the antibacterial and antibiofilm efficacy of *S. macrophylla* methanolic extract (SMME) against four types of Gram-positive pathogens.

MATERIALS AND METHODS

Preparation of SMME

The leaves of *S. macrophylla* were collected from MyGeneBank, Serdang, Selangor, Malaysia. Identification of plant species of *S. macrophylla* was performed by the Institute of Biology, Universiti Putra Malaysia (UPM). The leaves were cleaned by washing them with running tap water and were then allowed to dry at 27 °C for two weeks. Then, the leaves were crushed by using an electric blender into coarse powder form and were extracted using 80% (v/v) methanol by using a maceration method (Begashaw *et al*., 2017). The extract was filtered using filter paper (Whatman No. 1) and the organic solvent was removed under low pressure at a temperature of < 40 °C by using a rotary evaporator. The SMME obtained was kept at 4 °C for further analysis.

GC-MS analysis

GC-MS analysis was performed using a Hewlett-Packard 6890N gas chromatography system with mass spectrometry (Hewlett-Packard 5973 inert mass selective detector). One µL of SMME was injected with a split ratio of 30:1. Helium gas was used as a carrier at 1.5 mL/min. The temperature of the HP-5MS column (length 30.0 m, internal diameter 0.25 mm, film-0.25 µm) was set at 150 °C for one min after the sample injection while the temperature was maintained at 290 °C with a 10 °C/min rate. The mass spectra generated during GC-MS analysis were interpreted using the National Institute of Standards and Technology (NIST) database.

Preparation of bacterial culture

Staphylococcus aureus (ATCC 33862), *S. pneumoniae* (ATCC 19615), *B. cereus* (ATCC 11778), and *C. sporogenes* (ATCC 13124) were kindly provided by the Institute of Science, Universiti Teknologi Mara (UiTM), Shah Alam. Gram staining and biochemical tests were performed to determine the purity of the bacterial culture. All the bacteria were maintained in nutrient broth (NB) and incubated at 37 °C. The bacterial inocula were adjusted to an optical density (OD) of 0.7 at 600 nm for both broth microdilution and biofilm biomass assays.

Broth microdilution assay

MIC determination was performed using the microtiter broth dilution assay method in a sterile 96-well microplate with a flat bottom as previously reported (Man *et al*., 2022). In general, the SMME was serially diluted in distilled water to obtain 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, and 31.3 µg/mL concentrations respectively. A volume of 120 µL of bacterial inoculum and 80 µL of SMME were loaded into the wells of the microplate in triplicates. Fresh NB and vancomycin (Sigma-Aldrich, Germany) were used as negative and positive controls, respectively. The microplate was incubated at 37 °C for 24 h and the resulting turbidity was visually inspected. The lowest concentration of the SMME showing no visible bacterial growth or no turbidity was recorded as the MIC value of the extract. To determine MBC, a loopful of the sample in each well showing no visible growth in MIC determination was streaked on agar plates and incubated at 37 °C for 24 h.

Biofilm biomass assay

The assay was done as previously described by Yahya *et al*. (2017) with minor modifications. Briefly, a volume of 120 µL of the bacterial inoculum and 80 µL of SMME were loaded into the wells of the microplate. Fresh NB and IP-protected antibiofilm cocktails were used as the negative and positive controls, respectively. The microplate was incubated at 37 °C for 24 h. The media containing planktonic fractions were discarded while the biofilm fractions were rinsed once using phosphate saline buffer pH 7.4 and heat-fixed at 60 °C for 20 min. The biofilm staining was performed using 1% (w/v) crystal violet for 10 min at room temperature. The biofilms were then destained using the same buffer, dissolved in 70% (v/v) ethanol, and measured by a microplate reader (ThermoFisher Scientific, USA) at 600 nm. The mean absorbance of the samples was determined, and the percentage inhibition of biofilm was calculated using the equation as shown below (Famuyide *et al*, 2019):

$$\text{Percentage (\%) inhibition} = (\text{OD negative control} - \text{OD experimental})/(\text{OD negative control}) \times 100$$

Time-kill assay

The concentrations of SMME showing the highest percentage of biofilm inhibition against each bacterium were chosen for the time-kill assay. Preparation of the microplates and crystal violet staining were performed as described in the biofilm biomass assay above. The assays were performed at 0 h, 6 h, 12 h, 18 h, 24 h, and 30 h respectively.

Data analysis

The experimental results were expressed as the mean ± standard error of the mean (SEM) of three triplicates. Where applicable, the differences between the samples and control were determined by an independent T-test using Statistical Package for the Social Sciences (SPSS) software version 22.0. The result was considered significant if $p<0.05$. The half-maximum biofilm inhibitory concentration (BIC_{50}) values for the inhibition of Gram-positive bacterial biofilms were calculated by using GraphPad Prism software version 8.0.

RESULTS AND DISCUSSION

Yield of SMME

Table 1 shows the total yield of SMMEs. The yield of SMME was found to be 3.37%. The present study used 80% (v/v) methanol due to its high polarity solvent. A previous study by Mallik and Banik (2012) used ethanol solvent to extract *S. macrophylla* leaves and the extraction yield (3.85%) was slightly higher than that produced in the present study.

Table 1. The yield of SMME

Plant species	Plant part used	Dried materials (g)	Amount yield (g)	Extraction yield %
S. macrophylla	Leaves	200	6.73	3.37%

Secondary metabolites

Table 2 shows the secondary metabolites in the SMME. They were identified in the retention time between 16.51 min to 28.51 min. The major secondary metabolite in the SMME was found to be β-amyrin (22.8%). Other secondary metabolites included Heptadecane (5.75%), Eicosane (5.75%), Heneicosane (5.75%), Hexacosane (2.9%), and Octadecane (2.9%). Chakraborty *et al*. (1971) demonstrated the presence of triterpenoids such as β-amyrin in *Swietenia mahogany* leaves. This compound has also been shown to be present in n-hexane and methanol extracts of *Bombax malabaricum* flowers which have antimicrobial properties (El-Hagrassi *et al*., 2011). β-amyrin is known to be effective against some fungi (Jabeen *et al*., 2011). Heptadecane represents a major component in the hexane extract of *Temnopleurus alexandri* which possesses antibacterial activity against *B. subtillis, S. aureus, and Pseudomonas aeruginosa* (Uma & Parvathavartini 2010). Similarly, *Nectaroscordum tripedale* extract which exhibits antifungal activity also contains heptadecane, decadienal, and hexadecanoic acid (Sepahvand *et al*., 2019). A previous study performed by El-Shafay *et al*. (2015) reported that diethyl ether extract of *Sargassum fusiforme* showed high inhibitory activity against *S. aereus* and *K. pneumonia* due to the high percentage of eicosane (9.9%). Eicosane has also been reported by Ahsan *et al*. (2017) as an antifungal agent. Heneicosane was also found in *Scorzonera undulata* in a high ratio and its antibacterial activity against Gram-positive and Gram-negative bacteria is possibly due to the synergistic effect with the major phytochemicals (Boussaada *et al*., 2008). Adesalu *et al*. (2016) reported that heneicosane was found in green alga *Oedogonium* and it was useful to produce biopesticides. In a previous study, the ethyl acetate of *Sansevieria liberica* root extract shows high inhibitory activity against test microbes which is due to the presence of hexacosane (Rukaiyat *et al*., 2015). Octadecane, heptadecane, and eicosane can be classified into alkenes groups. According to Rouis-Soussi (2014), some compounds in the alkenes group have a great antimicrobial effect, especially against *S. aureus* and *E. coli*. Girija *et al*. (2014) showed that there was a synergistic antibacterial effect between heptadecane, eicosane and octadecane in *Loligo duvauceli* against *E. coli, K. pneumonia, and Candida albicans*.

Table 2. Secondary metabolites identified in the SMME

Retention Time	Area (%)	Name of Compound	Chemical Formula	Molecular Weight g/mol	Biological Activity/Uses	Quality (%)
16.51	5.75	Heptadecane	$C_{17}H_{36}$	240.47	Antibacterial activity (Uma and Parvathavartini, 2010) and Antifungal activity (Sepahvand *et al*., 2019)	97
16.51	5.75	Eicosane	$C_{20}H_{42}$	282.55	Antimicrobial activity (El-Shafay *et al*., 2015)	97
16.51	5.75	Heneicosane	$C_{21}H_{44}$	296.57	Antimicrobial activity (Boussaada *et al*., 2008)	96
20.02	2.90	Hexacosane	$C_{26}H_{54}$	366.71	Antibacterial activity (Rukaiyat *et al*., 2015)	98
20.02	2.90	Octadecane	$C_{18}H_{38}$	254.49	Antibacterial activity (Girija *et al*., 2014)	95
27.29	22.80	β-amyrin	$C_{30}H_{50}O$	426.72	Antimicrobial activity (El-Hagrassi *et al*., 2011)	97

Antibacterial potential

Table 3 depicts the results for the MIC and MBC of SMME against tested pathogens. The lowest MIC value of 31.3 µg/mL was recorded against *B. cereus* and *C. sporogenes.* The MBC values against *S. pneumoniae, B. cereus,* and *C. sporogenes* were found to be greater than 1000 µg/mL while the MBC value against *S. aureus*

was 1000 µg/mL. A previous study by Gopalan *et al*. (2019) showed the lowest MIC value of *S. macrophylla* seed extract was recorded against *B. subtilis* (1.56 mg/mL), followed by *B. cereus* (3.13 mg/mL). In 2009, Tan *et al*. showed a good inhibitory effect of methanolic extracts of *S. macrophylla* leaves against *B. subtilis* and *S. aureus* with larger inhibition zones as compared to gentamicin. Meanwhile, Ushie *et al*. (2016) also reported that the lowest MIC values (50 mg/mL) of methanolic extracts of *S. macrophylla* leaves were recorded against *S. aureus, E. coli,* and *Salmonella typhi*. Tan *et al*. (2009) reported the MBC values of *S. macrophylla* seed extract ranging from 3.13 mg/mL to 25 mg/mL. On the other hand, Sahgal *et al*. (2009) reported that the methanolic *Swietenia mahogany* seed extracts were effective against *S. aureus* and *Enterococcus faecalis* with MBC values of 12.5 mg/mL.

Table 3. MIC and MBC values of SMME against Gram-positive pathogens

Bacterial Species	Antibiotics	Methanol Extract (µg/mL)	
	Vancomycin (8 µg/mL)	MIC	MBC
S. aureus	No growth	250	1000
S. pneumonia	No growth	1000	>1000
B. cereus	No growth	31.3	>1000
C. sporogenes	No growth	31.3	>1000

Biofilm inhibition

Figure 1 shows the inhibitory effect of SMME against *S. aureus* biofilm. The SMME at all test concentrations significantly (p<0.05) hindered the biofilm formation by *S. aureus*. The percentage of inhibition against *S. aureus* biofilm ranged from 41.49% to 61.65 %. Figure 2 shows the impact of SMME against *S. pneumoniae* biofilm. Five concentrations of SMME (31.3 µg/mL, 62.5 µg/mL, 125 µg/mL, 500 µg/mL, 1000 µg/mL significantly (p<0.05) inhibited *S. pneumoniae* biofilm. The percentage of inhibition against *S. pneumoniae* biofilm ranged from 8.27% to 66.34%. Figure 3 shows the inhibitory action of SMME against *B. cereus* biofilm. Four concentrations of SMME (31.3 µg/mL, 62.5 µg/mL, 250 µg/mL, 500 µg/mL) significantly (p<0.05) inhibited *B. cereus* biofilm. The percentage of inhibition against *S. pneumoniae* biofilm ranged from 13.76% to 44.59%. Figure 4 shows the inhibition of SMME against *C. sporogenes* biofilm. All six concentrations of SMME significantly (p<0.05) inhibited *C. sporogenes* biofilm. The percentage of inhibition against *C. sporogenes* biofilm ranged from 68.93% to 82.50%.

Biofilm refers to a sessile, densely packed, and chemically heterogeneous microbial community (Yaacob *et al*., 2021a). It is known to express multiple essential proteins underlying complex protein interaction networks (Othman & Yahya 2019; Yaacob *et al*., 2021b). Further investigations of existing natural products, antifungals, and antibiotics are needed to better fight against a wide spectrum of biofilm infections (Johari *et al*., 2020; Isa *et al*., 2022; Rashid *et al*., 2022). In the present study, SMME displayed a non-concentration-dependent antibiofilm effect against all four tested pathogens at different concentrations. The antibiofilm activity of SMME might be due to the presence of typical antimicrobial compounds. To the best of our knowledge, the present study provides evidence of the antibiofilm activities of SMME against *S. aureus, S. pneumoniae, B. cereus,* and *C. sporogenes.* However, the inhibition of these biofilms by other medicinal plants has previously been reported (Quelemes *et al* 2015; Minami *et al*. 2017; Famuyide *et al*. 2019).

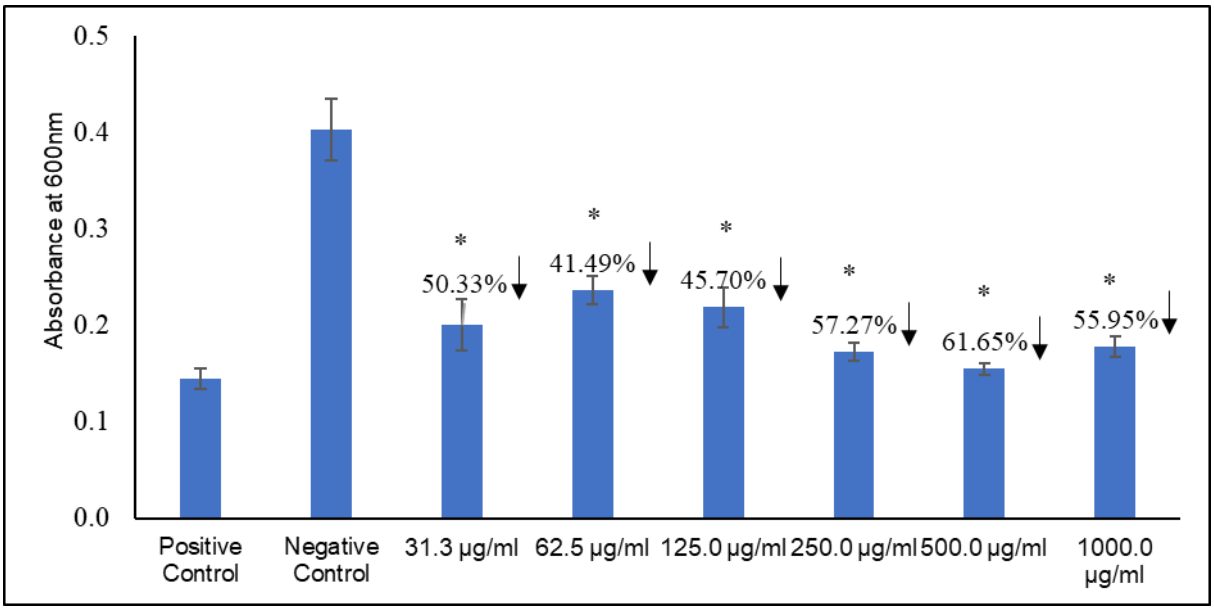

Fig. 1. The antibiofilm activity of SMME against *S. aureus* ATCC 33862. Positive control: bacterial inoculum with IP-protected antibiofilm cocktail. Negative control: bacterial inoculum with fresh broth. Each bar represents the mean ± SD of three replicates. Asterisk (*) indicates a significant (p<0.05) between the control group and the test group. The percentage value (%) indicates the biofilm inhibition compared with the negative control.

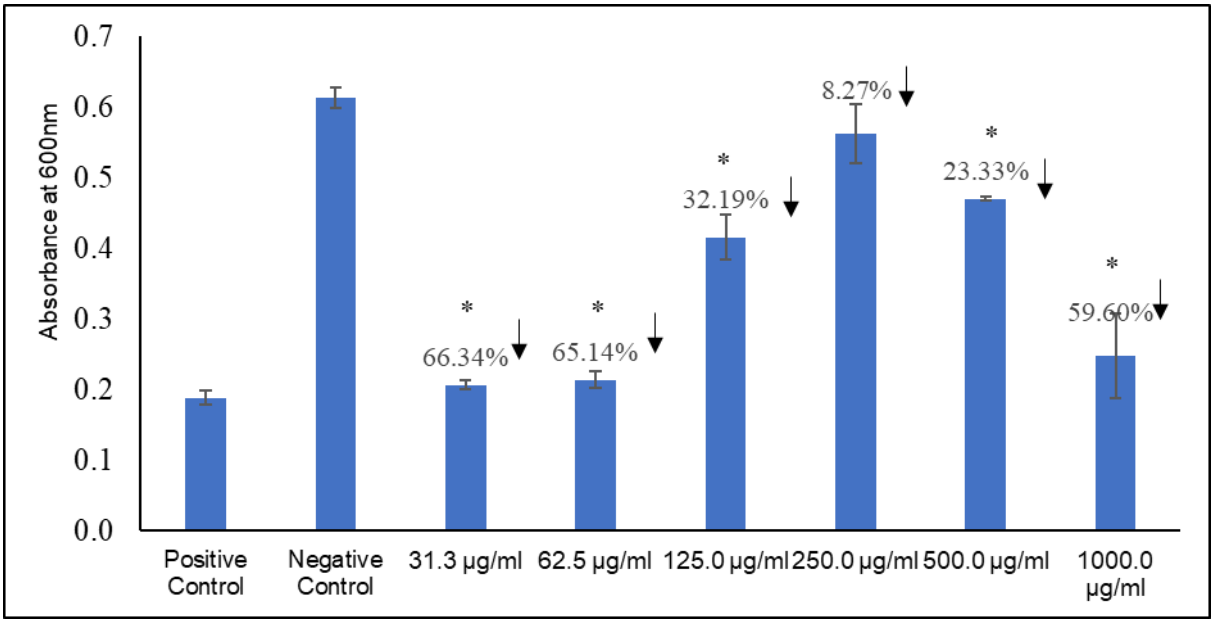

Fig. 2. The antibiofilm activity of SMME against *S. pneumoniae* ATCC 19615. Positive control: bacterial inoculum with IP-protected antibiofilm cocktail. Negative control: bacterial inoculum with fresh broth. Each bar represents the mean ± SD of three replicates. Asterisk (*) indicates a significant (p<0.05) between the control group and the test group. The percentage value (%) indicates the biofilm inhibition compared with negative control.

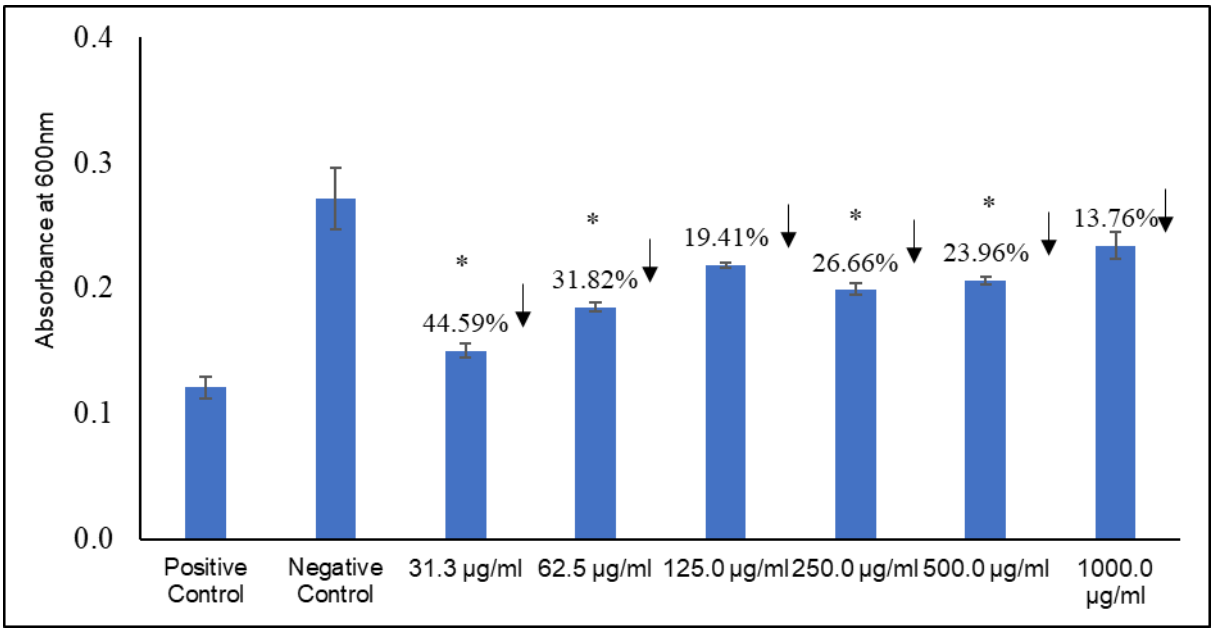

Fig. 3. The antibiofilm activity of SMME against *B. cereus* ATCC 11778. Positive control: bacterial inoculum with IP-protected antibiofilm cocktail. Negative control: bacterial inoculum with fresh broth. Each bar represents the mean ± SD of three replicates. Asterisk (*) indicates a significant (p<0.05) between the control group and the test group. The percentage value (%) indicates the biofilm inhibition compared with negative control.

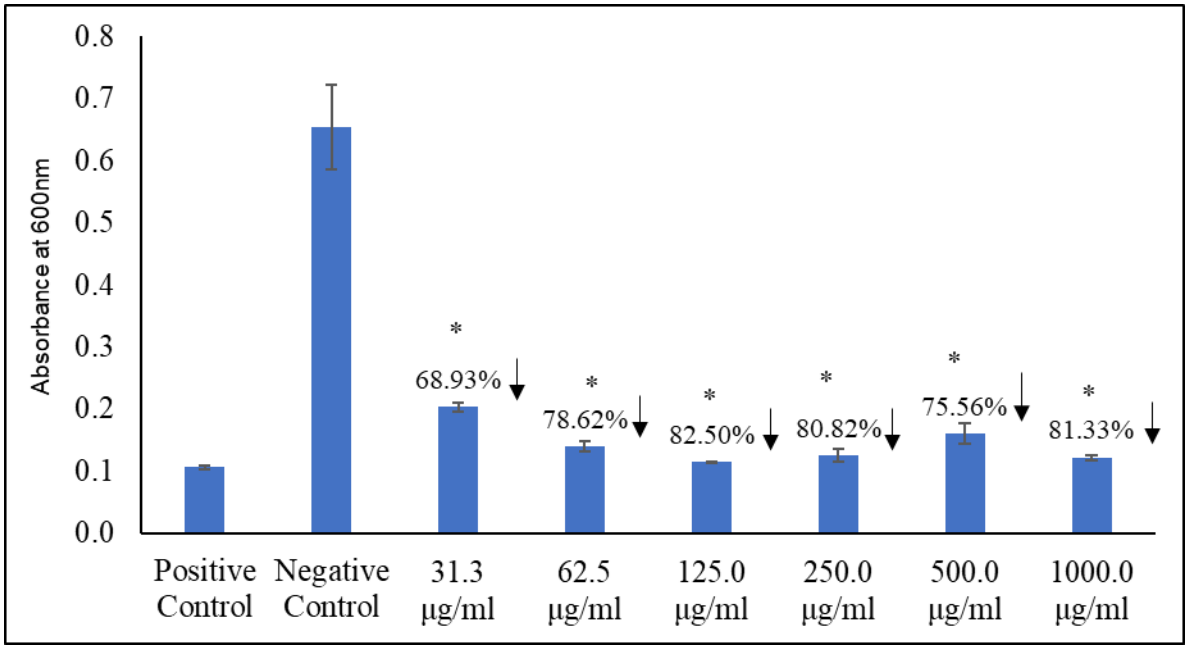

Fig. 4. The antibiofilm activity of SMME against C. sporogenes ATCC 13124. Positive control: bacterial inoculum with IP-protected antibiofilm cocktail. Negative control: bacterial inoculum with fresh broth. Each bar represents the mean ± SD of three replicates. Asterisk (*) indicates a significant ($p<0.05$) between the control group and the test group. The percentage value (%) indicates the biofilm inhibition compared with the negative control.

Time-kill kinetics

The test concentrations of SMME with the highest biofilm inhibitory effect (Figure 1 - Figure 4) were used in this assay. Figure 5 to Figure 8 show the time-kill curves of SMME against *S. aureus, S. pneumoniae, B. cereus,* and *C. sporogenes* respectively.

Figure 5 displays the time-kill curve of SMME against *S. aureus* at 500 µg/mL. The treated (500 µg/mL) time-kill curve showed an increment over the first 6 hr and 12 h followed by a decline to 24 h. The lowest absorbance was recorded at 24 h indicating the best incubation time for the inhibitory effect of SMME against *S. aureus*. This result is in agreement with a time-kill kinetic study by Appiah *et al*. (2017) reporting that methanol extracts of *Trametes gibbosa*, *Trametes elegans*, *Schizophyllum commune,* and *Volvariella volvacae* exhibited bacteriostatic actions against *S. aureus* and most of it showed a rise up after 6 h up to 24 h.

Figure 6 shows the time-kill curve of SMME against *S. pneumoniae* at 31.3 µg/mL. The treated (31.3 µg/mL) time-kill curve showed an increment for the first 12 hr and started to decrease at 18 h. The lowest absorbance was recorded at 6 h indicating the best incubation time for the inhibitory effect of SMME against *S. pneumoniae*. This result is in contrast with Limsuwan *et al*. (2012). The study reported that *Rhodomyrtus tomentosa* extracts which belong to the family Myrtaceae showed a killing effect against *Streptococcus pyogenes* after 16 h at 8 X MIC while 4 X MIC and 2 X MIC inhibited the growth of the organism.

Figure 7 shows the time-kill curve of SMME against *B. cereus* at 62.5 µg/mL. The treated (62.5 µg/mL) time-kill curve showed an increment for the first 12 h and started to decrease after that. The lowest absorbance was recorded at 30 h indicating the best incubation time for the inhibitory effect of SMME against *B. cereus*. Kang *et al*. (2018) stated that when thyme essential oil was present at the MIC level, the number of viable cells of *B. cereus* decreased by about 44.4% within a 4 h period, and when thyme essential oil was doubled maximum bactericidal was observed. The complete inhibition was achieved at 8 h exposure. Meanwhile, Ramli *et al*. (2018) reported that *Syzygium polyanthum* L. extract was effective in inhibiting *B. cereus* ATCC 33019 and *B. cereus* BC-NP.8, and the time taken for completely killing bacteria was recorded at 4 h.

Figure 8 shows the time-kill curve of SMME against *C. sporogenes* at 125 µg/mL. The treated (125 µg/mL) time-kill curve showed fluctuation and increment occurred at the first 12 hr and started to decrease until 30 hr. The lowest absorbance was recorded at 30 hr indicating the best incubation time for the inhibitory effect of SMME against *C. sporogenes*. The study of time kinetics of *C. sporogenes* inhibition by the plant extracts remains limited.

Biofilm Inhibitory Concentration (BIC_{50}) value

Table 4 displays the BIC_{50} value of SMME against the pathogens. The observed values of BIC_{50} showed that SMME was most effective against *B. cereus* (33.86 µg/mL), followed by *S. pneumoniae* (85.44 µg/mL), *S. aureus* (228.3 µg/mL) and *C. sporogenes* (2033 µg/mL). The BIC_{50} value was calculated to determine the concentration that is required to inhibit 50% of biofilm. The lower BIC_{50} value indicates a higher antibiofilm activity. In 2019, Famuyide *et al*. reported that acetone leaves extract of *Syzygium masukuense*, *Syzygium gerrrardii*, *Sygygium legatii*, and *Eugenia erythrophobia* possessed high antibiofilm activity against *B. cereus*. Meanwhile, Bazargani and Rohloff (2016) stated that lemongrass essential oil could inhibit *S. aureus* biofilm at 1.25 µg/mL.

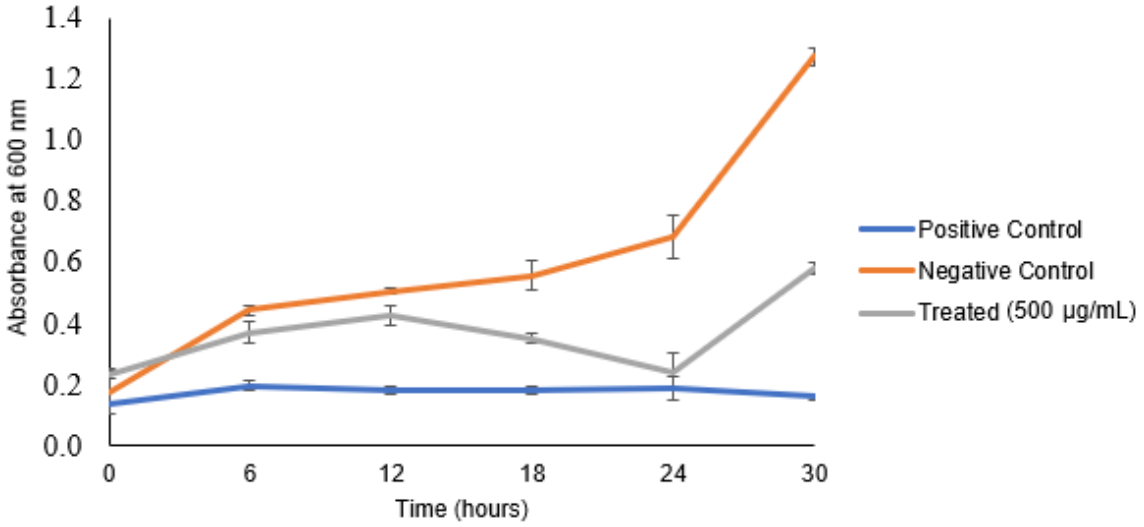

Fig. 5. Time-kill kinetics of SMME against *S. aureus* ATCC 33862 biofilm at 500 µg/mL. Positive control: bacterial inoculum with IP-protected antibiofilm cocktail. Negative control: bacterial inoculum with fresh broth.

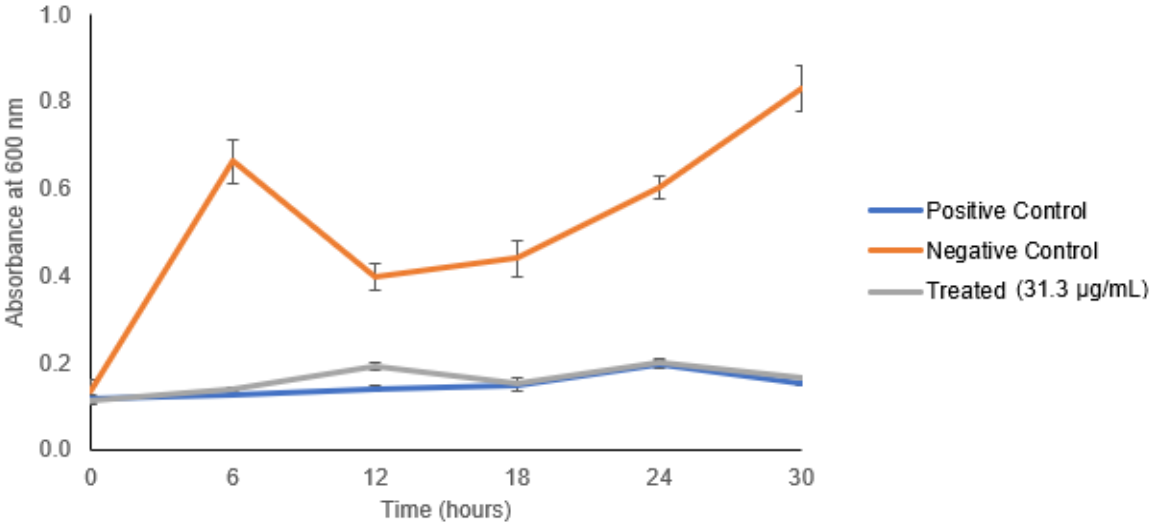

Fig. 6. Time-kill kinetics of SMME against *S. pneumoniae* ATCC 19615 biofilm at 31.3 µg/mL. Positive control: bacterial inoculum with IP-protected antibiofilm cocktail. Negative control: bacterial inoculum with fresh broth.

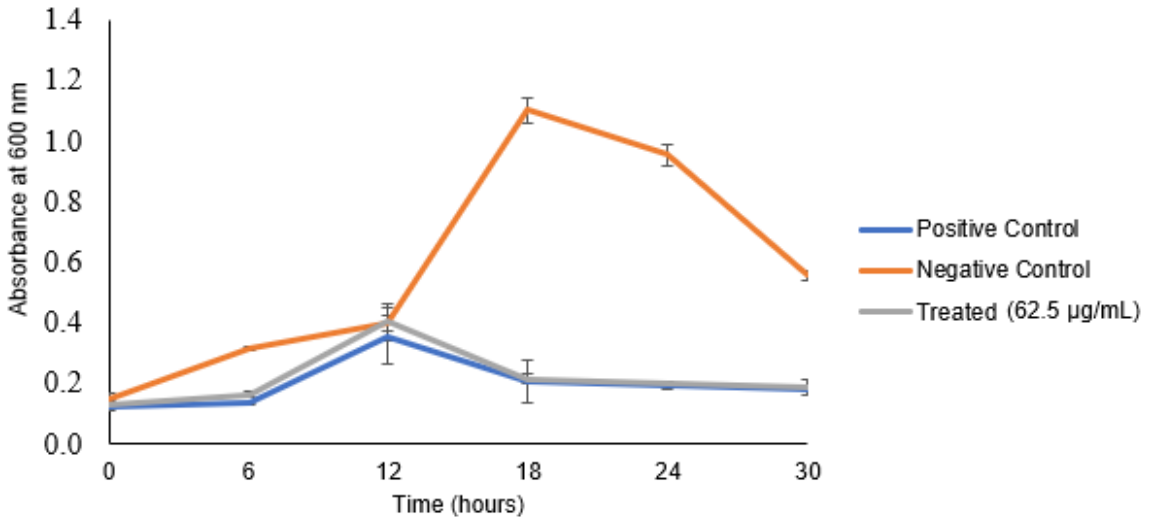

Fig. 7. Time-kill kinetics of SMME against *B. cereus* ATCC 11778 biofilm at 62.5 µg/mL. Positive control: bacterial inoculum with IP-protected antibiofilm cocktail. Negative control: bacterial inoculum with fresh broth.

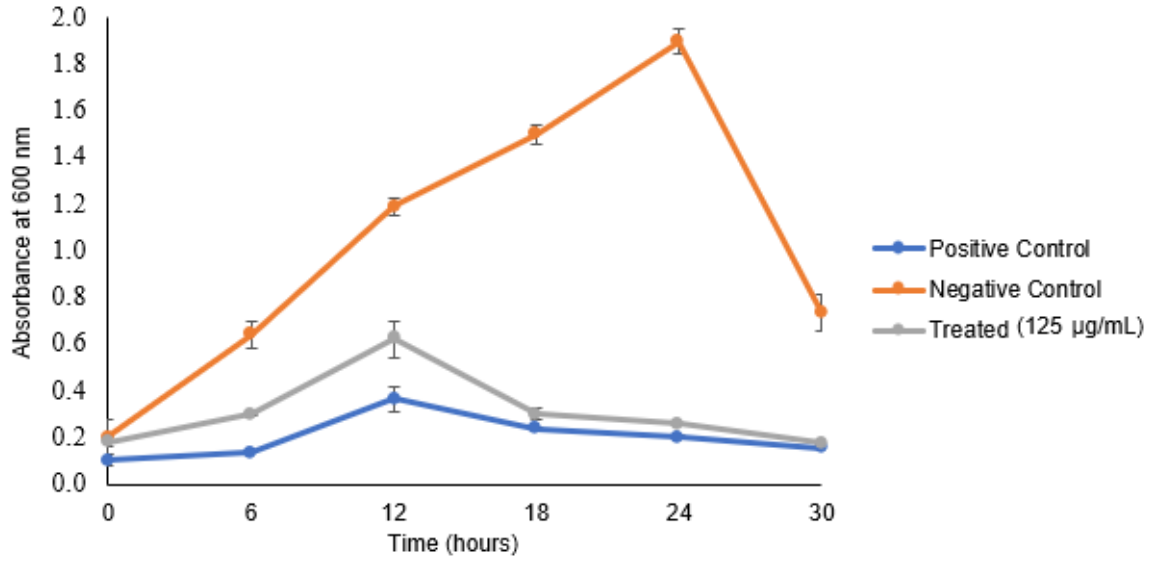

Fig. 8. Time-kill kinetics of SMME against *C. sporogenes* ATCC 13124 biofilm at 125 µg/mL. Positive control: bacterial inoculum with IP-protected antibiofilm cocktail. Negative control: bacterial inoculum with fresh broth.

Table 4. BIC_{50} values of SMME against Gram-positive pathogens

Bacterial species	BIC_{50} value (µg/mL)
S. aureus	228.3
S. pneumonia	85.44
B. cereus	33.86
C. sporogenes	>1000

CONCLUSION

The major secondary metabolite in the SMME was β-amaryn which is classified as triterpenoid. The SMME exhibited the lowest MIC value against *B. cereus* (31.3 µg/mL) and *C. sporogenes* (31.3 µg/mL) and the same MBC value against all tested microorganisms (>1000 µg/mL) except *S. aureus* (1000 µg/mL). The antibiofilm activity of SMME against all the tested pathogens was found to be non-concentration dependent. The biofilm-killing kinetics shown by SMME were found to be different among the pathogens. The SMME showed the highest efficacy against *B. cereus*, followed by *S. pneumoniae, S. aureus, and C. sporogenes*. The findings from the present study may be useful for the management of infectious diseases caused by Gram-positive pathogens.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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