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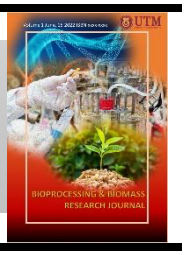

Research Article

Antibacterial and Antibiofilm Properties of *Zingiber officinale* and *Kaempferia parviflora* Against *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is a pathogenic bacterium whose biofilm-forming ability contributes to resistance against antibacterial treatments, highlighting the need for alternative strategies using plant extracts. This study evaluated the antibacterial and antibiofilm properties of *Zingiber officinale* (normal ginger) and *Kaempferia parviflora* (black ginger) ethanolic extracts against *S. aureus*, focusing on extraction yield, total phenolic content (TPC), functional group characterization (ATR-FTIR) and their efficacy in inhibiting *S. aureus* growth (disc diffusion assay) and biofilm formation (biofilm assay). *K. parviflora* yielded 3.05% extract compared to 0.57% for *Z. officinale* but *Z. officinale* had a slightly higher TPC (43.03 mg GAE/100 g) compared to *K. parviflora* (39.83 mg GAE/100 g). ATR-FTIR analysis predicted the presence of phenols, alkane and aromatic functional group in both extracts. Antibacterial assay showed that only *K. parviflora* extract inhibited *S. aureus*, with an 8.33 mm zone of inhibition at the tested concentrations. For biofilm assay, *K. parviflora* extract effectively inhibited *S. aureus* biofilm formation after seven days, with the highest inhibition of 59.92%. Overall, *K. parviflora* demonstrated promising potential as a plant-based antibacterial and antibiofilm agent against *S. aureus*.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive pathogenic bacterium that are associated with various infections in humans. Infections caused by *S. aureus* are challenging to manage because of its ability to form biofilm (Idrees et al., 2021). *S. aureus* secretes extracellular polymeric substances (EPS) to withstand and reduce the effects of antibacterial drugs, as biofilm formation hinders drug diffusion and prevents drugs from reaching cells within biofilm (Kaplan et al., 2018; Donlan, 2000). *S. aureus* can form biofilms on medical devices like urinary catheters and implants like pacemakers (Suresh et al., 2019). Improper sanitization and treatment can cause infections in patients, leading to an increase in treatment costs due to frequent replacement of catheters or implants. In recent years, implants and catheters have been treated by coating them with antiseptics and antibiotics to prevent *S. aureus* biofilm development, however this approach can contribute to the emergence of resistant strains (Suresh et al., 2019).

Over the years, plant extracts including *Zingiber officinale* have been studied for their antibacterial and antibiofilm properties against various bacterial species. *Z. officinale* is belongs to the Zingiberaceae family, has long been valued as an aromatic, pungent spice and historically employed in Chinese, Indian and Middle Eastern medicine to treat various diseases (Agnaou et al., 2021). *Z. officinale* contains phytochemicals like gingerol that are involved in antibacterial and antibiofilm activity (Ohaegbu et al., 2022). There were numerous works on the antibacterial and antibiofilm activities of *Zingiber officinale* against *S. aureus*. For example, the ethanolic extract of *Z. officinale* was shown to inhibit the growth of *S. aureus* at a concentration of 1.25 mg/mL (Saeloh & Visutthi, 2021). Furthermore, Das et al. (2019) reported that *Z. officinale* essential oil exhibited a

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94% inhibition rate against *S. aureus* biofilms, compared to other bacteria tested.

However, there is an imbalance in existing literature, where *Z. officinale* has been well-studied for its antibacterial and antibiofilm activities, while there are limited reports on the antibiofilm effects of *Kaempferia parviflora* against *S. aureus*. Belonging to the same family, *K. parviflora* or black ginger contains methoxyflavones involved in various biological activities like anti-allergic, anti-cancer, anti-inflammatory and antimicrobial activity (Chen *et al*., 2018). *K. parviflora* demonstrated notable antibacterial activity against *S. aureus*. As reported by Leswara & Larasati (2024), *K. parviflora* ethanolic extracts at 25%, 50%, 75% and 100% showed inhibition rates of 10.33%, 12.07%, 12.13% and 13.37%, respectively against *S. aureus* ATCC 25923. Nevertheless, its antibiofilm efficacy has been demonstrated against *Streptococcus mutans*, with concentrations ranging from 0.46 to 30 mg/mL were found to inhibit biofilm mass by 66% to 82% (Mala et al., 2018). Therefore, investigating the effects of *Z. officinale* and *K. parviflora* on *S. aureus* can help to identify effective plant extracts with the potential to inhibit bacterial growth and biofilm formation.

MATERIALS AND METHOD

Materials

Zingiber officinale (normal ginger) and *Kaempferia parviflora* (black ginger) were purchased from local market in Johor and Selangor. Stock culture of *Staphylococcus aureus* ATCC 6538 was retrieved from -80°C freezer at laboratory of Faculty of Science (T02), Universiti Teknologi Malaysia, Skudai, Johor. Nutrient agar (NA) (Merck, Germany) and Nutrient broth (NB) (Merck, Germany) were used as medium for bacterial streaking, growth and biofilm assay while Mueller-Hinton agar (MHA) (Condalab, Spain) and Mueller-Hinton broth (MHB) (Oxoid, UK) were used for disc diffusion assay. Sodium carbonate, Na_2CO_3 (R & M Chemicals, Malaysia) and Folin-Ciocalteu reagent were used in total phenolic content (TPC) assay.

Z. officinale and *K. parviflora* ethanolic extraction

Z. officinale and *K. parviflora* were extracted by maceration based on method described by Ridzuan et al. (2018) with modifications. After the rhizomes were washed, peeled, cut, submerged in liquid nitrogen and ground, 50 g of ground rhizomes were immersed in 250 mL of 95% ethanol and macerated at 60 °C and 200 rpm for 2 h. The mixtures were filtered, concentrated by a rotary evaporator (IKA RV10 rotary evaporator) at 55 °C and lyophilized to obtain crude extracts.

Total phenolic content (TPC) assay

Folin-Ciocalteu method was performed according to Ridzuan (2013) with modifications. For gallic acid standard, 40 μL of 50 mg/L working standard solution was mixed with 3160 μL of distilled water and 200 μL of Folin-Ciocalteu reagent. Then, 600 μL of Na_2CO_3 were added into the tube. The tube was shaken vigorously to homogenize the mixture. The same procedure was repeated for other concentrations; 100, 250, 375 and 500 mg/L, as well as *Z. officinale* and *K. parviflora* extract working solutions. All tubes were incubated for 2 hours, in the absence of light. The absorbances relative to the blank were recorded at 765 nm using a UV-Vis spectrophotometer. The TPC were expressed as Gallic Acid Equivalent (GAE) mg/100 g extraction from the calibration curve of gallic acid standard solution, ($y = 0.0008x – 0.0037$) where y is absorbance.

Characterization of *Z. officinale* and *K. parviflora* using ATR-FTIR

The ATR-FTIR analyses of *Z. officinale* and *K. parviflora* were carried out following methods of Terouzi & Oussama (2016) with modifications. The FTIR spectra were acquired using a FTIR Spectrophotometer equipped with a universal Attenuated Total Reflectance (ATR) accessory, operated at a resolution of 8 cm^{-1} and 8 scans. The spectral data were collected in the absorbance mode within the wave number range of 4000 to 650 cm^{-1}. A drop of each *Z. officinale* and *K. parviflora* extract was directly placed on an ATR cell provided with a diamond crystal.

Disc Diffusion Assay

A few colonies of overnight grown *S. aureus* were inoculated into 100 mL MHB and incubated at 37 °C for 24 h at 150 rpm. The suspension was adjusted to an optical density of approximately 0.1 at OD_{600nm} using a UV-Vis spectrophotometer. A 100 μL of the suspension was pipetted onto each MHA plate and swabbed using sterile cotton swab. Sterile blank discs (Oxoid, UK) were loaded with 10 μL *Z. officinale* extracts (100 mg/mL and 50 mg/mL), *K. parviflora* extracts (100 mg/mL and 50 mg/mL), negative control (100% DMSO) and positive control (10 μg/disc Ampicillin, Amp) (Glentham Life Sciences, UK). The discs were placed on the swabbed MHA plates. All the plates were sealed with parafilm and incubated at 37 °C for 24 h in an upright position. The antibacterial activities were determined by measuring the diameter of the inhibition zone (ZOI) in mm.

Biofilm Assay

Microtiter assay was conducted according to Yunus et al. (2024) with modifications. A Few colonies of *S. aureus* were inoculated into 100 mL of NB and incubated at 37 °C for 18 h at 150 rpm. 10 mL of the culture was sub-cultured into 90 mL of NB and incubated at 37 °C at 150 rpm until reaching the late exponential phase. Meanwhile, a two-fold serial dilution of *K. parviflora* extract was done in concentrations ranging from 50 mg/mL to 0.098 mg/mL in a sterile 96-well plate. Subsequently, 100 μL of *S. aureus* culture was added to each well. The plate was incubated at 37 °C at 100 rpm. Four different 96-well plates were prepared, each was incubated for 1, 3, 5 and 7 days. After incubation, the supernatant was discarded. The wells were rinsed with sterile distilled water and dried at 60 °C for 1 h. The plates were air-dried overnight in an inverted position at room temperature. Then, 200 μL of crystal violet solution was added to each well and left for 30 min. The wells were rinsed with sterile distilled water and 200 μL of 30% glacial acetic acid were added. The absorbance was measured at OD_{600nm} by using a microplate reader (ELISA). The biofilm inhibition percentage was calculated, and biofilm formation was interpreted based on the criteria of Stepanović et al. (2007).

RESULTS AND DISCUSSION

Z. officinale and *K. parviflora* ethanolic extraction

After lyophilization, the weight obtained for *K. parviflora* crude ethanolic extract (1.53 g) was higher than *Z. officinale* (0.29 g), as shown in **Table 1**. The percentage yield for *K.*

parviflora and *Z. officinale* extracts were 3.05% and 0.57%, respectively. Previous research reported higher yields for both extracts compared to the present study. It was found that *Z. officinale* extract yields ranged from 2.4% to 3.6% (Al-Areer et al., 2023; Yassen & Ibrahim, 2016). For *K. parviflora*, previous studies reported values at 7.22% (Aidiel et al., 2024), 13.06% (Sitthichai et al., 2022) and 34.84% (Leswara & Larasati, 2024). The lower yield in the present study may be due to the moisture content of the fresh ginger rhizomes used for extraction. According to Al-Areer et al. (2023), the presence of water molecules in fresh ginger may alter the extraction yield of its active compounds.

This is evident during the pre-extraction process, as the fresh rhizomes appeared more watery and mushy compared to ginger that had been dried and ground into a powdery form. Not only that, the sample size used for extraction is also an important factor, since this study utilized ground fresh ginger which have a larger particle size instead of the finer dried powdered ginger that are typically used. This is important as particle size reduction is necessary in the pre-extraction process to enhance the surface area interaction between the plant material and the extraction solvent (Manousi et al., 2019). Grinding produced coarser smaller samples, while powdered samples exhibit a more uniform and finer particle size, resulting in improved surface interaction with extraction solvents (Azwanida, 2015).

Table 1 Weight of crude ethanolic extracts of *Z. officinale* and *K. parviflora*

Type of ginger	Weight of crude ethanolic extract (g)	Percentage yield (%)
Z. officinale	0.29 ± 0.06	0.57 ± 0.11
K. parviflora	1.53 ± 0.06	3.05 ± 0.13

Total phenolic content (TPC) evaluation

Figure 1 shows the gallic acid standard curve that was used to determine the TPC of *Z. officinale* and *K. parviflora* extracts. The TPC values were expressed in milligram gallic acid equivalent per hundred gram (mg GAE/100 g) using gallic acid standard curve equation: y = 0.0008x – 0.0037. The TPC of *Z. officinale* was 43.03 mg GAE/100 g while for *K. parviflora*, the TPC value was 39.83 mg GAE/100 g, as shown in **Table 2**. Overall, the TPC values for both gingers were lower compared to previous studies. According to Sharif & Bennett (2016), the *Z. officinale* freeze-dried extract showed a TPC of 263 mg GAE/100 g, while Abdul Qadir et al. (2017) reported a TPC value of 98.36 mg GAE/100 g for *Z. officinale* dried extract. For K. *parviflora*, Sitthichai et al. (2022) reported that the TPC value of *K. parviflora* dried extract was 5228 mg GAE/100 g. The high moisture content in the fresh rhizomes used in the present study might have contributed to the lower TPC values. Dried ginger has been reported to exhibit higher TPC than fresh ginger, due to the drying process that aids in breaking down the cell walls of the food matrix, thereby releasing more phenolic compounds (Mustafa & Chin, 2023).

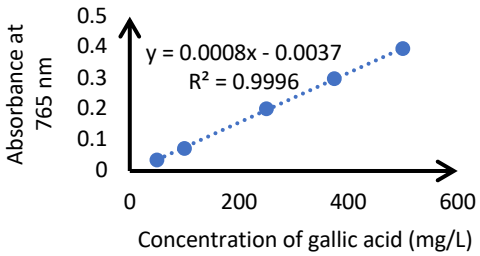

Figure 1 Gallic acid standard curve

Table 2 TPC value of *Z. officinale* and *K. parviflora* extracts

Type of ginger	Total phenolic content (mg GAE/100g)
Z. officinale	43.03 ± 2.02
K. parviflora	39.83 ± 1.52

Data was expressed as mean ± standard deviation (n=3).

Characterization of *Z. officinale* and *K. parviflora* using ATR-FTIR

Figure 2 shows the FTIR spectrum of *Z. officinale* extract. The first peak at 3370.01 cm^{-1} was due to the hydroxyl group stretch while the two peaks (1406.02 and 1313.70 cm^{-1}) could be due to the OH bend, indicating the possibility of phenol present (Coates, 2000). The peak at 1152.53 cm^{-1} corresponds to the C-O stretch, which closely aligns with almost similar peak identified in *Z. officinale* ethanolic extract found by Edo et al. (2024). In their study, the extract used showed antibacterial effects against various bacterial strains. The spectrums at 819.42, 779.85, 702.17 and 669.03 cm^{-1} were due to the aromatic ring. This could be contributed by the presence of benzene ring in the compounds, which showed the presence of phenols and flavonoids in the extract (Pharmawati & Wrasiati, 2020).

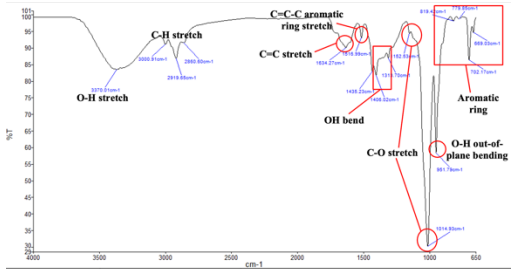

Figure 2 FTIR spectra of *Z. officinale* extract

Figure 3 shows the FTIR spectrum of *K. parviflora* extract. The first peak (3390.00 cm^{-1}) showed the presence of O-H stretch (hydroxyl group). The absorptions at 1348.84 and 1310.06 cm^{-1} could be due to the OH bend, indicating the presence of phenol (Coates, 2000). The band at 1639.51, 1604.51 and 1348. 84 cm^{-1} were almost the same with the previous finding by Krongrawa et al. (2023), where spectrum 1638, 1604 and 1350 cm^{-1} were found in the extract. The spectrum at 835.47, 770.85, 700.84 and 671.17 cm^{-1} were associated with aromatic ring, possibly due to the presence of benzene ring in the compounds, suggesting the presence of phenols and flavonoids in the extract (Pharmawati & Wrasiati, 2020).

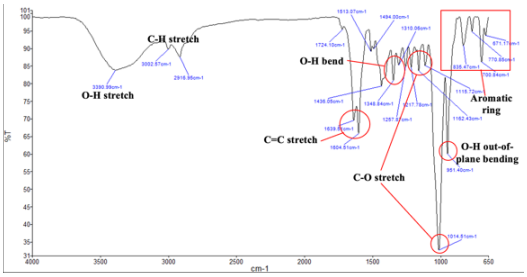

Figure 3 FTIR spectra of *K. parviflora* extract

Disc Diffusion Assay

Based on **Table 3**, *S. aureus* was only susceptible to *K. parviflora* extracts whereas no inhibition zone was recorded for both concentrations of *Z. officinale* extracts. No inhibition activity by *Z. officinale* and low inhibition activity by *K. parviflora* could be due to moisture content in the fresh ginger rhizomes used in ethanolic maceration. This is because drying plant material prior to extraction inhibits metabolic processes, thereby preventing changes in the chemical composition of the plant (Krakowska-Sieprawska et al., 2022). Since enzymes in plant need water to function properly, removal of moisture or water can help to stop the process. The elevated temperatures and absence of water contribute to the suppression of enzymes responsible for breaking down the active compounds (Krakowska-Sieprawska et al., 2022). Drying could prevent the water from interfering with the extraction of plant and bioactive compounds by the solvent. Moreover, excess moisture in fresh ginger produces a diluted and less concentrated extract.

Table 3 Zone of inhibition diameter (mm) of *Z. officinale* and *K. parviflora* extracts against *S. aureus*

Concentration (mg/mL)	Zone of inhibition diameter (mm)	
	Z. officinale	*K. parviflora*
50	-	8.33 ± 0.58
100	-	8.33 ± 0.58

Data was expressed as mean ± standard deviation (n=3).
The diameter of the discs (6 mm) was included in the zone of inhibition.

Apart from the high moisture content, the lack of inhibition by *Z. officinale* and low inhibition by *K. parviflora* may be attributed to the extraction method used, namely maceration. According to Azwanida (2015) as cited in Bitwell et al. (2023), maceration is limited by its low extraction efficiency and prolonged processing time. This suggested the possibility that antibacterial active compounds were not efficiently extracted, along with additional interference caused by the high moisture content. Contrarily to maceration method, other extraction techniques such as the Soxhlet method is more efficient and produce higher extract yield (Bitwell et al., 2023). This aligns with the findings by Al-Areer et al. (2023), who reported that the percentage yield of fresh ginger rhizomes obtained through maceration was lower than extract obtained using the Soxhlet method, with extraction yields of 2.4% and 3.3%, respectively.

The antibacterial ability of *K. parviflora* extract could be attributed to the presence of methoxyflavones. According to Chen et al. (2018), *K. parviflora* including its main effective methoxyflavones involved in anti-microbial activity. Interestingly, Asamenew et al. (2019) reported that methoxyflavones were identified exclusively in *K. parviflora* but not in *Z. officinale*. This distinction may be associated with the functional groups detected in this study, which correspond with those reported for one of the methoxyflavones in *K. parviflora* by Eze et al. (2023). Specifically, the absorption bands observed at 1604.51, 1436.05, 1014.51 and 835.47 cm^{-1} were nearly identical to those of 3,5,7,3',4'-pentamethoxyflavone, reported at 1604, 1436, 1014 and 836 cm^{-1}.

Biofilm Assay

Based on **Table 4**, the biofilm production capability of *S. aureus* was fluctuated after 1 and 3 days of incubation. After just 1 day of incubation, *S. aureus* formed strong biofilm at most concentrations. Instant exposure of *S. aureus* to *K. parviflora* extract might not be enough to hinder the initial adhesion of *S. aureus* to the wells or interfere with biofilm formation. Moreover, the inhibition percentage were strongly negative at all concentrations, up to -2400%, as shown in **Figure 4**. At this stage, *K. parviflora* extract did not show an inhibitory effect and even at lower concentrations, it actually enhanced biofilm formation.

Table 4 Biofilm production capability of *S. aureus* after the incubation with *K. parviflora* extract based on Stepanović et al. (2007) characterization

Concentration (mg/mL)	Incubation period (day)			
	1 (OD_c = 0.452)	3 (OD_c = 1.089)	5 (OD_c = 1.882)	7 (OD_c = 3.625)
50	Weak (OD_c < 0.856 ≤ 2OD_c)	No (0.327 ≤ OD_c)	No (1.067 ≤ OD_c)	No (-0.946 ≤ OD_c)
25	Moderate (2OD_c < 1.027 ≤ 4OD_c)	Weak (OD_c < 1.339 ≤ 2OD_c)	No (0.956 ≤ OD_c)	No (-0.811 ≤ OD_c)
12.5	No (0.432 ≤ OD_c)	Weak (OD_c < 1.622 ≤ 2OD_c)	No (1.051 ≤ OD_c)	No (-0.898 ≤ OD_c)
6.25	Strong (4OD_c < 2.316)	Weak (OD_c < 1.653 ≤ 2OD_c)	No (0.875 ≤ OD_c)	No (-0.892 ≤ OD_c)
3.125	Strong (4OD_c < 2.457)	Weak (OD_c < 1.693 ≤ 2OD_c)	No (0.770 ≤ OD_c)	No (-1.101 ≤ OD_c)
1.563	Strong (4OD_c < 2.503)	No (1.078 ≤ OD_c)	No (1.079 ≤ OD_c)	No (-0.719 ≤ OD_c)
0.781	Strong (4OD_c < 2.566)	Weak (OD_c < 0.086 ≤ 2OD_c)	No (-0.444 ≤ OD_c)	No (-0.790 ≤ OD_c)
0.391	Strong (4OD_c < 2.619)	No (-0.065 ≤ OD_c)	No (-0.855 ≤ OD_c)	No (-2.120 ≤ OD_c)
0.195	Strong (4OD_c < 2.689)	No (0.185 ≤ OD_c)	No (-0.830 ≤ OD_c)	No (-1.500 ≤ OD_c)
0.098	Strong (4OD_c < 2.634)	No (0.069 ≤ OD_c)	No (-0.598 ≤ OD_c)	No (-0.389 ≤ OD_c)

Result interpretation as the following; OD ≤ ODc = no biofilm; ODc < OD ≤ 2ODc = weak biofilm; 2ODc < OD ≤ 4ODc = moderate biofilm; 4ODc < OD = strong biofilm.

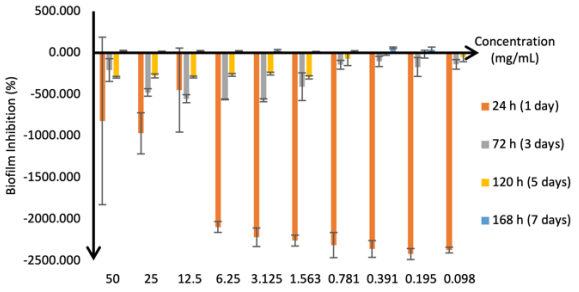

Figure 4 Percentage of biofilm inhibition of *S. aureus* by *K. parviflora* extract incubated for 1, 3, 5 and 7 days.

By day 3, biofilm formation began to weaken across all concentrations, with only 'no' or 'weak' biofilm observed. Although *K. parviflora* extract still did not inhibit biofilm, the enhancement effect on biofilm formation was reduced. The negative inhibition also lessens and moved closer to 0%. Starting from day 5, all concentrations showed no biofilm formation. However, the inhibition percentage was still

negative but approached 0%, indicating minimal influence of *K. parviflora* on *S. aureus*. Presumably, prolonged exposure allowed sufficient action of active compounds in the extract to disrupt the biofilm formation by *S. aureus*.

Finally, after 7 days of incubation, *K. parviflora* was effective in inhibiting biofilm formation of *S. aureus*. All concentrations showed positive inhibition percentages, ranging from 4.3% to 59.92%, as shown in **Figure 5**. This can be associated with the biofilm-forming capability of *S. aureus* on the wells of 96-well plates under shaking condition. Yunus et al. (2024) previously reported that *S. aureus* formed weak biofilm at day 3, moderate biofilm starting at day 4 and strong biofilm at day 6 and day 7. Presumably, the longer the incubation period, the higher the potential of *K. parviflora* extract to inhibit biofilm formation of *S. aureus*. The present study did not depict the action of inhibition process by the *K. parviflora* extract at molecular level but eventually, the compounds in the extract might need time to act and inhibit the biofilm formation of *S. aureus*. Only strong biofilm formation (day 7) showed positive readings, indicated that there was inhibition of biofilm.

The antibiofilm activity of *K. parviflora* against *S. aureus* may be attributed by the presence of methoxyflavones. Methoxyflavones have been associated with antibiofilm action; for example, 3,5,7,8,3',4'-hexamethoxyflavone extracted from *Citrus miaray* was found to inhibit *Vibrio harveyi* biofilm formation (Uckoo et al., 2015). This suggested that methoxyflavones, which are specific to *K. parviflora* but not to *Z. officinale,* might play an important role in the antibiofilm activity of *K. parviflora* against *S. aureus*. Nonetheless, further research should investigate the antibiofilm abilities of isolated methoxyflavones from *K. parviflora* against *S. aureus*.

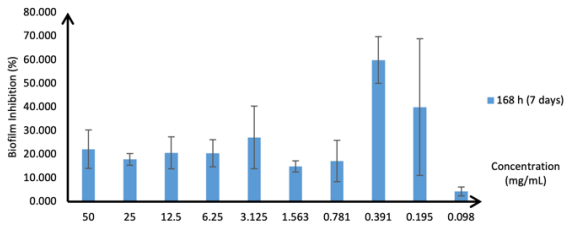

Figure 5 Percentage of biofilm inhibition of *S. aureus* using *K. parviflora* ethanolic extract incubated for 7 days.

CONCLUSION

In conclusion, the ethanolic extract of *K. parviflora* showed great potential in inhibiting the growth and biofilm formation of *S. aureus*. Nevertheless, the use of fresh rhizomes in this study may have restricted the extraction yield, phenolic content as well as antibacterial and antibiofilm activities, most likely due to their high moisture content. Therefore, future studies should consider pre-treatment methods such as drying and powdering ginger rhizomes to minimize moisture interference and improve extraction efficiency. In addition, employing alternative extraction approaches such as Soxhlet or ultrasound-assisted extraction could enhance extraction yield and efficiency, as well as facilitate greater extraction of phenolic compounds, which may result in higher TPC and stronger antibacterial and antibiofilm activities.

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Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this paper.

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