Tropical Biomedicine 42(2): 130-140 (2025)
https://doi.org/10.47665/tb.42.2.005

RESEARCH ARTICLE

In vitro antibacterial potential of 1'*S*-1'-acetoxychavicol acetate (ACA) on oral opportunistic pathogens

M Zahidan, N.S.[1], Sabri, N.U.A.[1], Adnan, S.N.A.[2], Awang, K.[3], Ab Malik, N.[4*]

[1]Faculty of Dentistry, Universiti Sains Islam Malaysia, Kuala Lumpur, Malaysia
[2]Department of Basic Science, Faculty of Dentistry, Universiti Sains Islam Malaysia, Kuala Lumpur, Malaysia
[3]Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia
[4]Department of Conservative Dentistry and Prosthodontics, Faculty of Dentistry, Universiti Sains Islam Malaysia, Kuala Lumpur, Malaysia
*Corresponding author: liza_amalik@usim.edu.my

ARTICLE HISTORY

Received: 27 November 2024
Revised: 3 March 2025
Accepted: 7 March 2025
Published: 30 June 2025

ABSTRACT

Oral opportunistic pathogens resulting from poor oral health can lead to serious issues in elderly and immunocompromised individuals, including lower respiratory tract infections and aspiration pneumonia (AP). The main objective of this study is to evaluate the antibacterial effect 1'*S*-1'-acetoxychavicol acetate (ACA) isolated from *Alpinia conchigera* rhizome extract against selected oral opportunistic pathogens which are *Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. In this study, a total of 13 respondents were recruited to obtain the clinical isolates of selected oral opportunistic pathogens. From these samples, 3 strains of *S. aureus*, 1 strain of *S. pneumoniae,* 3 strains of *K. pneumoniae* and 1 strain of *P. aeruginosa* were obtained and further tested. To achieve the objective, disc diffusion assay (DDA), minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time kill assay were carried out to determine the antibacterial properties. Based on DDA results, ACA displayed good antibacterial activity against clinical isolates and ATCC strains of *S. aureus, S. pneumoniae* and *P. aeruginosa* with the zone of inhibition recorded at 25.07 ± 0.09 mm, 36.83 ± 0.85 mm and 14.00 ± 0.82 mm respectively while clinical isolates and ATCC strains of *K. pneumoniae* did not show any inhibition diameter. The range of MIC and MBC values for ACA recorded were between 0.39 mg/mL until 12.50 mg/mL. ACA exhibited both bacteriostatic and bactericidal properties mostly when treated with concentration of 2 × MIC and MIC at different time intervals. In conclusion, ACA possesses antibacterial effect against clinical isolates and ATCC strains of *S. aureus*, *S. pneumoniae*, *K. pneumoniae* and *P. aeruginosa*.

Keywords: 1'*S*-1'-acetoxychavicol acetate; *A. conchigera* rhizomes; antibacterial activity; oral opportunistic pathogens.

INTRODUCTION

The World Health Organization (WHO) reported that pneumonia is one of the largest infectious diseases that cause death among adults and children worldwide (WHO, 2021). Pneumonia occurs when an infection in the respiratory tract causes the alveoli to be filled with pus and fluid resulting in breathing difficulty due to limited oxygen intake (WHO, 2021). One type of pneumonia is aspiration pneumonia (AP). It occurs when foreign materials or any substances such as saliva, gastric contents, food residuals, or oral microbiome from the laryngopharynx, oropharynx, upper gastrointestinal tract, or oral cavity are aspirated and inhaled into the lungs that will lead to inflammation and infection (Ishii, 2020).

The source of infection may be caused by opportunistic pathogens that can invade people with weakened immune systems like the elderly, cancer patients, and people with AIDS or HIV (Vaillant & Naik, 2023). The most common oral opportunistic pathogens isolated from AP are those originating from the oropharynx and oral cavity, for example, *S. aureus*, *S. pneumoniae*, *K. pneumoniae*, and *P. aeruginosa* (Mandell & Niederman, 2019). Poor oral hygiene is one of many factors that causes the invasion of these oral opportunistic pathogens (Sedghizadeh *et al*., 2017).

Therefore, physicians had suggested the use of mouthwash that can reduce the prevalence of opportunistic pathogens (Ab Malik *et al*., 2018; do Nascimento *et al*., 2018). However, the chemical ingredients incorporated in the products such as chlorhexidine gluconate has several drawbacks because it may stain the teeth, reduce the taste sensation, and increase supragingival calculus formation (Sakaue *et al*., 2018). In a study reported that 9.8% of treatment group which received chlorhexidine complained of having mucosal irritation compared to 1% of the control group (Shi *et al*., 2010). Other chemotherapeutic agents, such as hydrogen peroxide and carbamide peroxide, were also claimed to be carcinogenic and decrease the microhardness of the enamel (Fernandes *et al*., 2017; Argemí *et al*., 2020). Therefore, there is a need to seek for alternative treatment that use plant extracts because most natural-

based medicines may not require the use of synthetic chemicals and drugs (Bachtel & Israni-Winger, 2020).

In this study, ACA isolated from *Alpinia conchigera* rhizome extract is used as an antimicrobial agent against the selected oral opportunistic pathogens which are *S. aureus*, *S. pneumoniae*, *K. pneumoniae,* and *P. aeruginosa*. *A. conchigera* is a medicinal herbal tree that can be found and easily grown on the east coast of Peninsular Malaysia (Taib *et al*., 2020). It is also known as '*lengkuas ranting*' and different parts of this plant which include the rhizomes, leaves, and pseudo stems have been studied for their potential benefit against microorganisms and fungi (Islam, 2019). The rhizomes of this plant contain the most abundant bioactive compounds (Ibrahim *et al*., 2009). *A. conchigera* rhizome extract contains many bioactive compounds such as 1'*S*-1'-acetoxychavicol acetate (ACA), *trans*-*p*-coumaryl diacetate, *p*-hydroxybenzaldehyde.

Based on a previous study, ACA isolated from *Alpinia galanga* showed promising antibacterial properties against five multidrug-resistant *S. aureus* strains. This is due to the ability of this compound to alter the bacterial morphology and damaged the bacterial cell membrane integrity (Zhang *et al*., 2021). Therefore, this study focused on the antibacterial activity of ACA which have a high potential to be used as an alternative medicine against oral opportunistic pathogens.

MATERIALS AND METHODS

Extraction of *Alpinia conchigera* Rhizomes

A. conchigera rhizomes were obtained from Hulu Langat, Selangor. A voucher specimen (KL 5831) was deposited in the Herbarium of Chemistry Department, Faculty of Science, University of Malaya. The rhizomes were washed, sliced, and air-dried until constant mass was obtained. The dried rhizomes were ground, and the powdered rhizomes (10 kg) were extracted with *n*-hexane for 72 hours at room temperature using maceration method. The extracts were filtered and then dried in vacuo using rotary evaporator.

Isolation of 1'*S*-1'-acetoxychavicol acetate (ACA)

The *n*-hexane of *A. conchigera* rhizome extract was subjected to column chromatography (CC) on silica gel using stepwise gradient system (*n*-hexane to MeOH) to isolate ACA. The *n*-hexane of rhizome extract (16 g) was loaded on a 23.5 cm × 90 cm glass column packed with 240 g of silica gel with the size of 0.043-0.063 mm (Merck, Germany) as the stationary phase. The column was eluted with a stepwise gradient of *n*-hexane : ethyl acetate, (100:0 to 50:50). The eluates were collected and combined based on the thin layer chromatography (TLC) profile.

Clinical Sample Preparation

Clinical samples were obtained from Pusat Jagaan Al-Fikrah, Selangor with ethical approval (USIM/JKEP/2021-184) from Ethics Committee, USIM. A total of 13 participants were recruited for oral rinse collection to ensure the investigated pathogens can be retrieved. The inclusion criteria for the sample collection were residents aged more than 60 years old, not edentulous and not on antibiotics for the past three months. The oral rinses were collected using 10 mL of 0.1M sterile phosphate-buffered saline (PBS) at pH 7.2 for around 60 seconds (Samaranayake *et al*., 1987). Samples were stored in an icebox and transported to the Oral Biology Laboratory, Faculty of Dentistry, Universiti Sains Islam Malaysia.

Clinical Sample Processing

The samples were centrifuged at 4 000 rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet obtained was suspended in 1mL of sterile PBS as the main stock. A total of 100 µL aliquot from the main stock was used to dilute the samples using a serial dilution technique. The samples were diluted by a factor of 10 for four times (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) to isolate the selected oral opportunistic pathogens. A volume of 50 µL aliquots of each dilution were inoculated using spread plate technique on selective-differential growth media such as Mannitol Salt Agar (MSA), MacConkey Agar (MCA) and blood agar. The agar plates were then incubated in both aerobic and anaerobic conditions for 24 hours at 37°C to culture the pure colonies of *S. aureus*, *S. pneumoniae*, *K. pneumoniae* and *P. aeruginosa.*

Identification of Clinical Strain of Oral Opportunistic Pathogens

The isolated oral opportunistic pathogens from the oral rinse sampling were further confirmed using DNA Barcoding protocol by Molecular Biology Services (1st BASE, Singapore).

Antibacterial Assays

The single colony of clinical isolates and ATCC strains of ATCC 25923 *S. aureus,* ATCC 49619 *S. pneumoniae*, ATCC 700603 *K. pneumoniae* and ATCC 27853 *P. aeruginosa* were transferred into Brain-Heart Infusion Broth (BHIB) and incubated for 24 hours at 37°C in aerobic condition while *S. pneumoniae* was incubated in anaerobic condition. The turbidity of the inoculums was standardized according to 0.5 McFarland standards which equals to 1.5 10^8 CFU/mL.

Disc Diffusion Assay (DDA)

Disc diffusion assay was carried out to screen the antibacterial effect of ACA based on the guidelines from the Clinical Laboratory Standard Institute (CLSI) (Humphries *et al*., 2023). The ACA was dissolved in 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) to the series of concentration: 100, 50 and 25 mg/mL. 10% DMSO was used as the negative control while 0.2% chlorhexidine (CHX) (Helitoz Plus, Malaysia) and 30 µg cefepime antibiotic disc (Oxoid, UK) were incorporated as the positive controls. A volume of 100 µL standardized clinical isolates and ATCC strains of *S. aureus*, *S. pneumoniae*, *K. pneumoniae* and *P. aeruginosa* bacterial suspension were spread thinly and evenly on Mueller Hinton Agar (MHA) surface using sterile cotton swabs to obtain a confluent bacterial growth. Then, the surface of the media was allowed to dry for 3 to 5 minutes for absorption of excess moisture. Sterile filter paper discs with a diameter size of 6 mm were placed onto the inoculated and dried MHA using sterile forceps and were impregnated with 10 µL ACA at three different concentrations respectively (100.0, 50.0 and 25.0 mg/mL), 0.2% CHX and 10% DMSO. A 30 µg cefepime disc was also placed onto the MHA agar. All agar plates were incubated for 24 hours at 37°C in aerobic conditions while *S. pneumoniae* was incubated in anaerobic condition. The diameter of the inhibition zone was measured in millimetres (mm). The disc diffusion assay was carried out in three replicates.

Minimum Inhibitory and Bactericidal Concentration (MIC & MBC)

Minimum inhibitory concentration (MIC) was carried out to determine the lowest concentration of ACA required to inhibit the clinical isolates and ATCC strains of *S. aureus*, *S. pneumoniae*, *K. pneumoniae* and *P. aeruginosa* based on the CLSI protocol of antimicrobial susceptibility testing (Parvekar *et al*., 2020). MIC was performed using broth microdilution technique in 96-well microtiter plate. In this method, 0.2% CHX and cefepime solution were used as positive controls while 10% DMSO as negative control, sterile Mueller Hinton Broth (MHB) as sterility check, and mixture of sterile MHB and adjusted overnight culture as inoculum control. The total volume in each well was 100 µL.

The ACA was diluted to the highest concentration of 25.0 mg/mL. A total of 50 µL of Mueller Hinton Broth (MHB) (HiMedia, India) was distributed to each row of the wells. Then, 50 µL of ACA solution was serially diluted across the wells in duplicate, starting from a concentration of 25.0 to 0.2 mg/mL. A total of 50 µL of the adjusted overnight cultures of clinical isolates and ATCC strains of

selected oral opportunistic pathogens were inoculated into each well containing the serially diluted ACA. The plates were then incubated for 24 hours at 37°C in aerobic conditions while *S. pneumoniae* was incubated in anaerobic condition.

One mg/mL of sterile filtered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Thermo Scientific, MA, USA) solution was prepared by adding 1 mL PBS solution to 1 mg MTT powder. Then, 20 µL of the MTT solution were added to each well and the 96-well microtiter plates were incubated for 2 hours. The colour changes were assessed visually and recorded. The MIC values were recorded by the lowest concentration of the samples that showed no changes from yellow to blueish purple after the addition of MTT solution.

Minimum bactericidal concentration (MBC) was carried out to determine the lowest concentration of ACA that can kill the clinical isolates and ATCC strains of *S. aureus*, *S. pneumoniae*, *K. pneumoniae* and *P. aeruginosa* (Abalaka *et al*., 2012). A volume of 5 µL of the samples from each well consist of the oral opportunistic pathogens incubated with ACA from highest to the lowest concentration were inoculated on MHA using the streak plate method. The agar plates were incubated for 24 hours at 37°C in aerobic conditions while *S. pneumoniae* was incubated in anaerobic condition. MBC values were recorded by the lowest concentration of the samples that showed no bacterial growth on MHA.

Time Kill Assay

Time Kill Assay was performed to assess the bacteriostatic or bactericidal properties of ACA and the relationship between the concentrations of ACA used and the incubation period (Bouacha *et al*., 2023). In this study, two different concentrations of ACA: 2 MIC and MIC were evaluated at six-time intervals: 0, 4, 8, 12, 16 and 24 hours. In this method, broth microdilution technique using 96-well microtiter plates were used.

An aliquot of 50 µL of sterile MHB were added into each well, then 50 µL of ACA with the concentration of 2 MIC and MIC based on MIC and MBC determination results. A total of 50 µL of standardized 0.5 McFarland overnight culture were added into each well. Positive controls used were 0.2% CHX and cefepime solution while 50 µL sterile MHB mixed with 50 µL adjusted overnight culture was used as inoculum control. Then, the 96-well microtiter plates were incubated according to the time intervals (0, 4, 8, 12, 16 and 24 hours).

At each time interval, ACA and positive controls were diluted using ten-fold serial dilutions to (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) while for inoculum control to (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) using sterile saline water as the diluent. Then, 10 µL from each dilution factor were inoculated on MHA using streak plate technique and incubated for 24 hours at 37°C in aerobic conditions while *S. pneumoniae* was incubated in anaerobic condition. The colonies formed were counted to determine colony forming unit per mL (CFU/mL). Plates with 30-300 colonies were selected for colony-forming units counts. Time kill curve was designed by plotting $\log_{10}$ of colony forming units per mL (vertical axis) versus time intervals (horizontal axis). In this procedure, representative clinical and ATCC strains were tested. Bactericidal and bacteriostatic activities were demonstrated as decrease of 99.9% (≥ 3 Log_{10}) and decrease of less than 99.9% (< 3 Log_{10}) respectively of total number of colonies forming units per mL compared to the inoculum control (Israyilova *et al*., 2022).

RESULTS

***Alpinia conchigera* Rhizome Extract and 1'*S*-1'-acetoxychavicol acetate (ACA)**

From the extraction process, a total of 21.39 g of *A. conchigera* rhizome extract was obtained. Out of this, 16 g was subjected to column chromatography (CC), resulting in the isolation of 3.75 g of ACA. The isolated ACA was confirmed using TLC analysis, where its R_f value was compared with that of the reference ACA. Figure 1 presents the TLC profile of both the isolated and reference ACA. The Rf value of the isolated ACA was found to be 0.69.

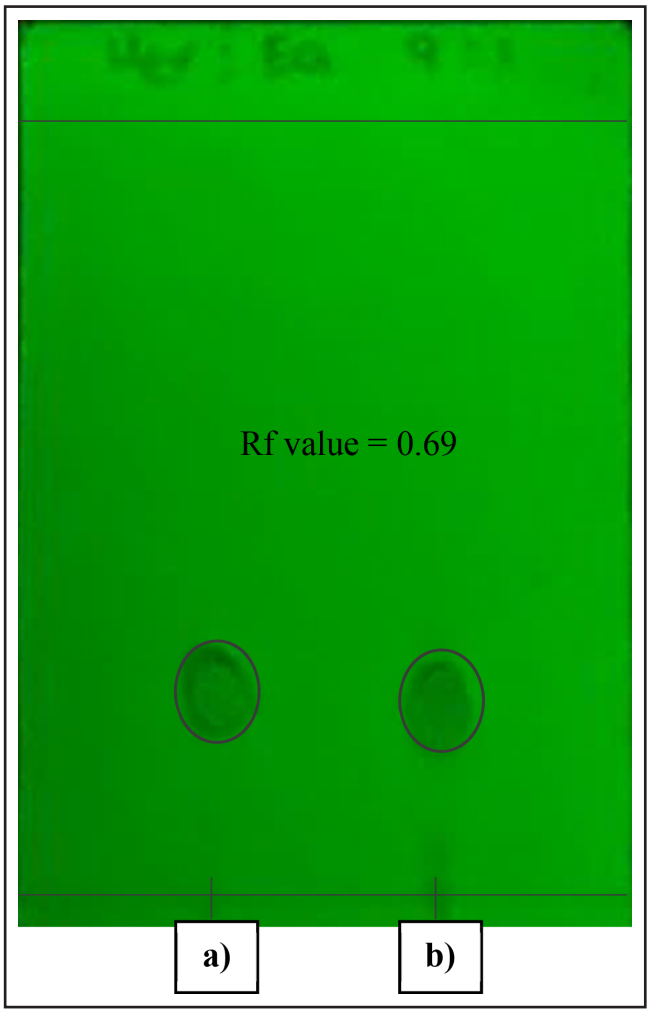

Figure 1. TLC profile of **a)** reference ACA **b)** isolated ACA, stationary phase = silica gel $F_{254,}$ mobile phase = *n*-hexane: ethyl acetate (9:1).

Clinical Samples

A total of 13 clinical samples were successfully retrieved from Pusat Jagaan Al-Fikrah, Selangor. The respondents included nine males and four females. From these samples, 3 strains of *S. aureus* (respondents 009, 012, and 013) were isolated using MSA where the colonies formed are circular, 2 to 4 mm in size, convex, smooth, opaque,

and yellow, with a surrounding yellow opaque zone. Only one *S. pneumoniae* strain (respondent 005) was successfully isolated using blood agar that produces small, circular, whitish colonies, measuring between 0.5 and 1.0 mm in size. The colonies were transparent and surrounded by a greenish discoloration, indicating an ב-haemolytic reaction. Additionally, the optochin test revealed a zone of inhibition of 14 mm or more, confirming the strain as *S. pneumoniae*, as it is sensitive to optochin. On MCA, *K. pneumoniae* and *P. aeruginosa* colonies are circular, convex, and measure 2 to 3 mm in size. *K. pneumoniae* colonies have a mucoid surface, are opaque, and appear pink to red, while *P. aeruginosa* colonies initially have a smooth surface when freshly isolated but develop a mucoid layer due to slime production. Three isolates of *K. pneumoniae* (008, 009, and 012) and one strain of *P. aeruginosa* (010) were successfully isolated from the clinical samples. A representative from each species was further confirmed using DNA barcoding method. The identification percentage of similarity are between 99% to 100% for all selected oral opportunistic pathogens (Figure 2).

```
>Staphylococcus aureus strain ATCC 12600 16S ribosomal RNA, partial sequence
Sequence ID: NR_115606.1 Length: 1476
Range 1: 1 to 1476

Score:2663 bits(2952), Expect:0.0,
Identities:1476/1476(100%),  Gaps:0/1476(0%), Strand: Plus/Plus
```

```
>Streptococcus pneumoniae strain ATCC 33400 16S ribosomal RNA, partial sequence
Sequence ID: NR_028665.1 Length: 1515
Range 1: 12 to 1497

Score:2646 bits(2934), Expect:0.0,
Identities:1478/1486(99%),  Gaps:0/1486(0%), Strand: Plus/Plus
```

```
>Klebsiella pneumoniae strain DSM 30104 16S ribosomal RNA, partial sequence
Sequence ID: NR_117686.1 Length: 1530
Range 1: 10 to 1488

Score:2645 bits(2933), Expect:0.0,
Identities:1474/1479(99%),  Gaps:0/1479(0%), Strand: Plus/Minus
```

```
>Pseudomonas aeruginosa strain DSM 50071 16S ribosomal RNA, partial sequence
Sequence ID: NR_117678.1 Length: 1527
Range 1: 11 to 1488

Score:2666 bits(2956), Expect:0.0,
Identities:1478/1478(100%),  Gaps:0/1478(0%), Strand: Plus/Plus
```

Figure 2. Identification percentage of similarity.

Antibacterial Assays

Disc Diffusion Assay (DDA) of 1'S-1'-Acetoxychavicol Acetate (ACA)

Only ACA at 100 mg/mL showed antibacterial effect against two clinical isolates of *S. aureus* 009 and 012 with significant inhibition recorded 15.00 ± 0.00 mm and 10.67 ± 0.47 mm respectively while *S. aureus* isolate 013 did not show any inhibition diameter. *S. aureus* ATCC 25923 recorded the zone of inhibition of 6.83 ± 0.24 mm. However, ACA showed a larger inhibition diameter when treated against *S. pneumoniae* isolate 005 and *S. pneumoniae* ATCC 49619. ACA at three different concentrations used (100, 50 and 25 mg/mL) showed significant inhibition zone of 27.33 ± 0.47 mm, 14.67 ± 0.47 mm and 13.00 ± 0.82 mm respectively while *S. pneumoniae* isolate 005 showed significant inhibition zone of 30.67 ± 0.94 mm when treated with 100 mg/mL ACA. However, ACA at 50 and 25 mg/mL showed the same size of inhibition diameter of 7.33 ± 0.47 mm.

All clinical strains of *K. pneumoniae* 008, 009 and 012 and *K. pneumoniae* ATCC 700603 strain did not show any zone of inhibition after treated ACA at all concentration range. Meanwhile, ACA also inhibited *P. aeruginosa* ATCC 27853 at all concentrations, with the highest zone of 9.67 mm at 100 mg/mL. *P. aeruginosa* isolate 010 only showed inhibition when treated with ACA at 100 mg/mL, with a zone of 6.50 mm. Table 1 shows inhibition diameter for all clinical isolates and ATCC strains of selected oral opportunistic pathogens.

Minimum Inhibitory and Bactericidal Concentration of 1'S-1'-Acetoxychavicol Acetate (ACA)

The MIC and MBC were conducted to further determine the lowest concentration of ACA to inhibit or kill the clinical isolates and ATCC strains of *S. aureus, S. pneumoniae, K. pneumoniae* and *P. aeruginosa*. Besides that, the bacteriostatic and bactericidal properties of the tested compound can be determined through this method. *S. aureus* isolate 013, *S. pneumoniae* isolate 005, *S. pneumoniae* ATCC 49619, *K. pneumoniae* isolates 008, 009, 012, *K. pneumoniae* ATCC 700603, *P. aeruginosa* isolate 010 and *P. aeruginosa* ATCC 27853 recorded the same MIC and MBC values of 12.5 mg/mL. The lowest MIC and MBC values recorded were 0.4 mg/mL and 0.8 mg/mL respectively by *S. aureus* isolate 009, 012 and *S. aureus* ATCC 25923. Table 2 showed the MIC and MBC values of the samples.

Table 1. Inhibition diameter for all clinical isolates and ATCC strains of selected oral opportunistic pathogens

Bacteria	Inhibition Diameter Mean ± SD (mm)					
	ACA 100 mg/mL	ACA 50 mg/mL	ACA 25 mg/mL	0.2% chlorhexidine	30 µg cefepime	10% DMSO
S. aureus 009	15.00 ± 0.00*	6.00 ± 0.00	6.00 ± 0.00	11.67 ± 0.47	18.67 ± 0.47	6.00 ± 0.00
S. aureus 012	10.67 ± 0.47*	6.00 ± 0.00	6.00 ± 0.00	12.00 ± 0.00	16.67 ± 0.47	6.00 ± 0.00
S. aureus 013	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	8.00 ± 0.82	29.00 ± 0.82	6.00 ± 0.00
S. aureus ATCC 25923	6.83 ± 0.24	6.00 ± 0.00	6.00 ± 0.00	12.33 ± 0.47	20.67 ± 0.47	6.00 ± 0.00
S. pneumoniae 005	30.67 ± 0.94*	7.33 ± 0.47	7.33 ± 0.47	9.67 ± 0.47	39.67 ± 0.47	6.00 ± 0.00
S. pneumoniae ATCC 49619	27.33 ± 0.47*	14.67 ± 0.47*	13.00 ± 0.82*	10.67 ± 0.47	31.33 ± 0.47	6.00 ± 0.00
K. pneumoniae 008	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	9.33 ± 0.47	30.00 ± 0.82	6.00 ± 0.00
K. pneumoniae 009	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.50 ± 0.00	27.00 ± 0.82	6.00 ± 0.00
K. pneumoniae 012	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	7.33 ± 0.47	25.67 ± 0.47	6.00 ± 0.00
K. pneumoniae ATCC 700603	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.50± 0.00	21.67 ± 0.47	6.00 ± 0.00
P. aeruginosa 010	6.50 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	7.33 ± 0.47	29.00 ± 0.82	6.00 ± 0.00
P. aeruginosa ATCC 27853	9.67 ± 0.47	7.67 ± 0.47	7.00 ± 0.00	8.67 ± 0.47	31.00 ± 0.82	6.00 ± 0.00

Table 2. MIC and MBC values of ACA against all clinical isolates and ATCC strains of selected oral opportunistic pathogens

Bacteria	ACA	
	MIC (mg/mL)	MBC (mg/mL)
S. aureus 009	0.39	0.78
S. aureus 012	0.39	0.78
S. aureus 013	12.50	12.50
S. aureus ATCC 25923	0.39	0.78
0.2% chlorhexidine	0.002	0.006
Cefepime solution	0.008	0.008
S. pneumoniae 005	12.50	12.50
S. pneumoniae ATCC 49619	12.50	12.50
0.2% chlorhexidine	0.05	0.20
Cefepime solution	0.25	0.50
K. pneumoniae 008	12.50	12.50
K. pneumoniae 009	12.50	12.50
K. pneumoniae 012	12.50	12.50
K. pneumoniae ATCC 700603	12.50	12.50
0.2% chlorhexidine	0.025	0.20
Cefepime solution	0.008	0.008
P. aeruginosa 010	12.50	12.50
P. aeruginosa ATCC 27853	12.50	12.50
0.2% chlorhexidine	0.10	0.20
Cefepime solution	0.008	0.008

Time Kill Curve of 1'S-1'-Acetoxychavicol Acetate (ACA)

A time-kill assay was done to study how ACA interact with oral opportunistic pathogens over different time intervals. In this test, each pathogen species included an ATCC strain and a representative sample from clinical isolate of each species. The results showed $\log_{10}$ of colony forming units per mL was plotted against time to construct time kill curves. ACA exhibited bactericidal properties against selected oral opportunistic pathogens. It also revealed that the effect of ACA was dependent on concentration. The 0.2% chlorhexidine (CHX) killed all strains within 4 hours, while cefepime (CFP) did not stop or kill the bacteria but slightly reduced their presence compared to the control.

ACA at the concentration of 2 MIC exhibited total killing effect against both *S. aureus* isolate 012 and *S. aureus* ATCC 25923 at 16 hours of incubation while ACA at 1 MIC did not show any inhibitory effect against both clinical and ATCC strains of *S. aureus* (Figure 3). However, ACA at both 2 MIC and 1 MIC showed bactericidal properties against *S. pneumoniae* isolate 005 and *S. pneumoniae* ATCC 49619 at two different time intervals which were at 4 hours (2 MIC) and 8 hours (1 MIC) (Figure 4) while *K. pneumoniae* isolate 009, *K. pneumoniae* ATCC 700603, *P. aeruginosa* isolate 010 and *P. aeruginosa* ATCC 27853 were totally killed as early as 4 hours when treated with ACA at both 2 MIC and 1 MIC concentration (Figure 5 and Figure 6).

DISCUSSION

Disc diffusion assay (DDA) was conducted as a preliminary test to assess the antibacterial properties of 1'*S*-1'-acetoxychavicol acetate (ACA) extracted from *A. conchigera* rhizome extract, against selected oral opportunistic pathogens. These pathogens included both clinical isolates and ATCC strains of *S. aureus*, *S. pneumoniae*, *K. pneumoniae*, and *P. aeruginosa*. The results indicated that ACA showed zones of inhibition for all tested pathogens, except for the clinical isolates and ATCC strains of *K. pneumoniae*. As this was the preliminary screening, high concentrations of ACA were tested (100 mg/mL, 50 mg/mL, and 25 mg/mL) to observe the potential antibacterial activity of ACA. It is proven that the highest concentration of ACA (100 mg/mL) exhibited the inhibitory properties as the treatment of ACA at 100 mg/mL recorded the largest zone of inhibition for most of clinical isolates and ATCC strains of *S. aureus* and *S. pneumoniae* when compared to the other two concentrations tested.

Based on the result, two species of oral opportunistic pathogens which are clinical isolates and ATCC strains of *K. pneumoniae* and *P. aeruginosa* did not show significant inhibition zone when treated with ACA at all concentration range when compared with clinical isolates and ATCC strains of *S. aureus* and *S. pneumoniae*. This is because gram-negative bacteria are more resistant towards natural bioactive compound due to the complex cell wall structure which are compact and lipopolysaccharide-rich, which makes it harder for hydrophobic compounds to diffuse through the cell membrane (Breijyeh *et al*., 2020). Although gram negative bacteria possess thin cell wall, the presence of the outer membrane that act as protective layer limits the penetration of many antibiotics and antimicrobial agents (Saxena *et al*., 2023). However, DDA is not the ideal method for determining the antibacterial activity of plant extracts, as the assay relies on the ability of the extract to diffuse through the filter paper disc and agar (Bubonja *et al*., 2020). Since the bioactive compound used in this study is non-polar, the rate of diffusion into the disc and agar was limited.

Therefore, MIC and MBC tests were conducted to further evaluate the antibacterial activity of ACA. These tests used broth media instead of agar, which made it easier for ACA to dissolve. This study confirmed that ACA exhibited both inhibitory and bactericidal effects against clinical isolates and ATCC strains of selected oral opportunistic pathogens. The lowest MIC and MBC values were observed with *S. aureus* clinical isolate 009, 012 and *S. aureus* ATCC 25923 in response to ACA. Other strains resulted in similar MIC and MBC values of 12.50 mg/mL. This indicates that ACA possessed bactericidal properties, as the MBC value was less than four times the MIC value (MBC < 4 ' MIC) (Hanafiah *et al*., 2019).

The MIC and MBC values observed in this study were slightly higher than those reported in previous research using ACA extracted from the rhizomes of *A. conchigera* (Aziz *et al*., 2013; Taib *et al*., 2020). This variation may be attributed to factors such as differences in the plant's growth region, plant age, and extraction efficiency. Additionally, different bacterial strains exhibited varying MIC and MBC values, which can be explained by factors such as genetic variability (Li *et al*., 2017). Variations in genes responsible for cell wall synthesis, membrane permeability, and drug targets influence bacterial susceptibility to antimicrobial agents. Furthermore, bacteria employ diverse resistance mechanisms, such as enzymatic degradation of antimicrobials, modification of membrane permeability, and the use of efflux pumps to expel antimicrobial agents. The structure and composition of bacterial cell walls also play a significant role in determining susceptibility to these agents (Reygaert, 2018).

MIC and MBC procedures were important especially in clinical aspects because the findings of these procedures help clinicians to provide appropriate dosage regimen to treat the bacterial infections (Kowalska & Dudek-Wicher, 2021). Besides that, the information on the susceptibility of bacteria towards specific antibiotics allow to maximize the efficacy and minimize the development of antibiotic resistance (Lee *et al*., 2013). However, the *in vitro* findings of MIC and MBC procedures cannot be directly interpreted into *in vivo* because antibiotics and antimicrobial agents act differently *in vivo* due to factors such as absorption and metabolism. Besides that, pathogens that caused infection on the host cell may exhibit different physiological characteristics compared to pathogens in the *in vitro* settings (Levison & Levison, 2009).

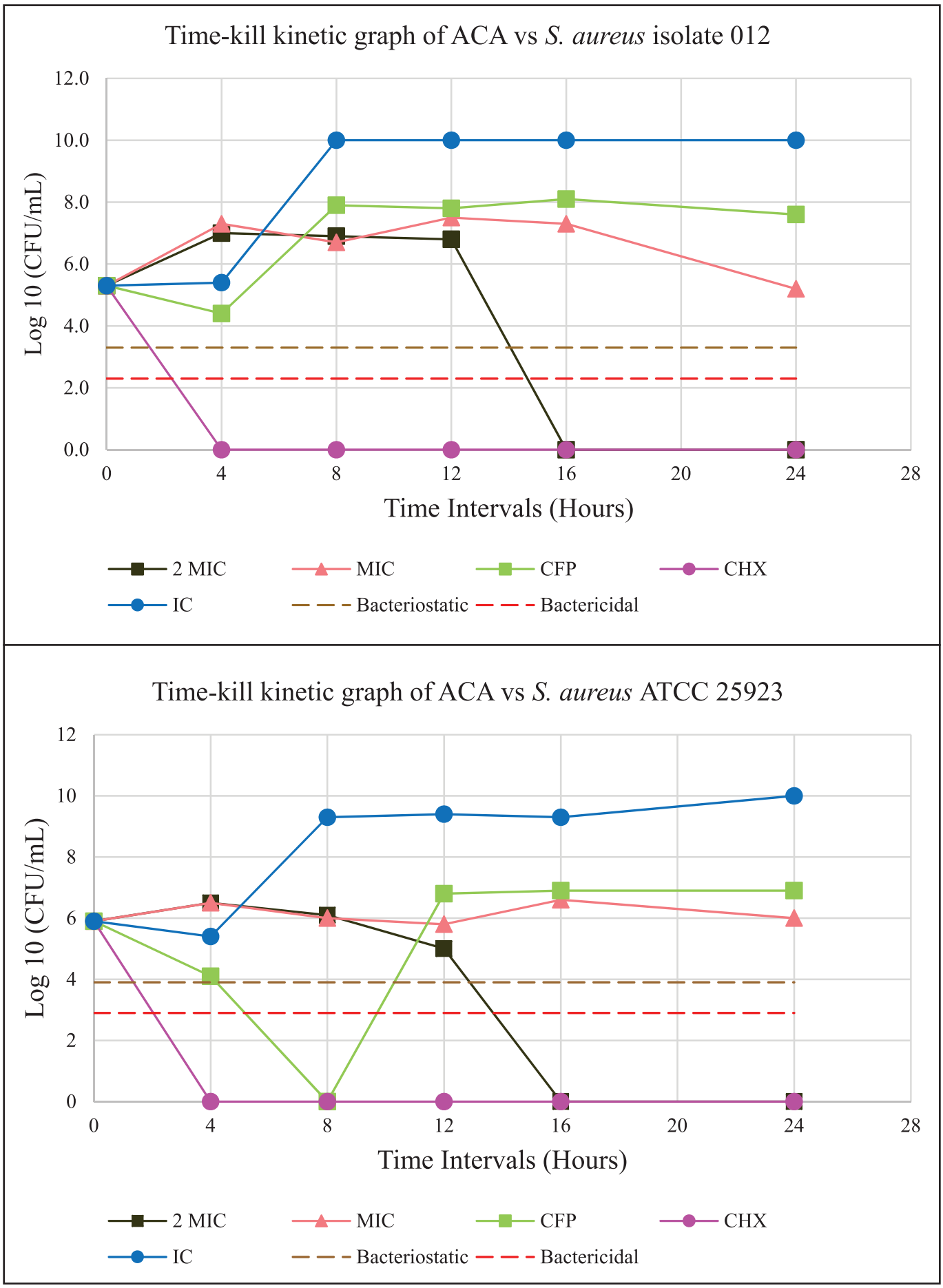

Figure 3. Time-kill kinetic graph of Log_{10} CFU/mL versus time for ACA against *S. aureus* isolate 012 and *S. aureus* ATCC 25923. CFP= 0.008 mg/mL. CHX= 0.2%. IC is untreated sample. CFU is colony-forming unit.

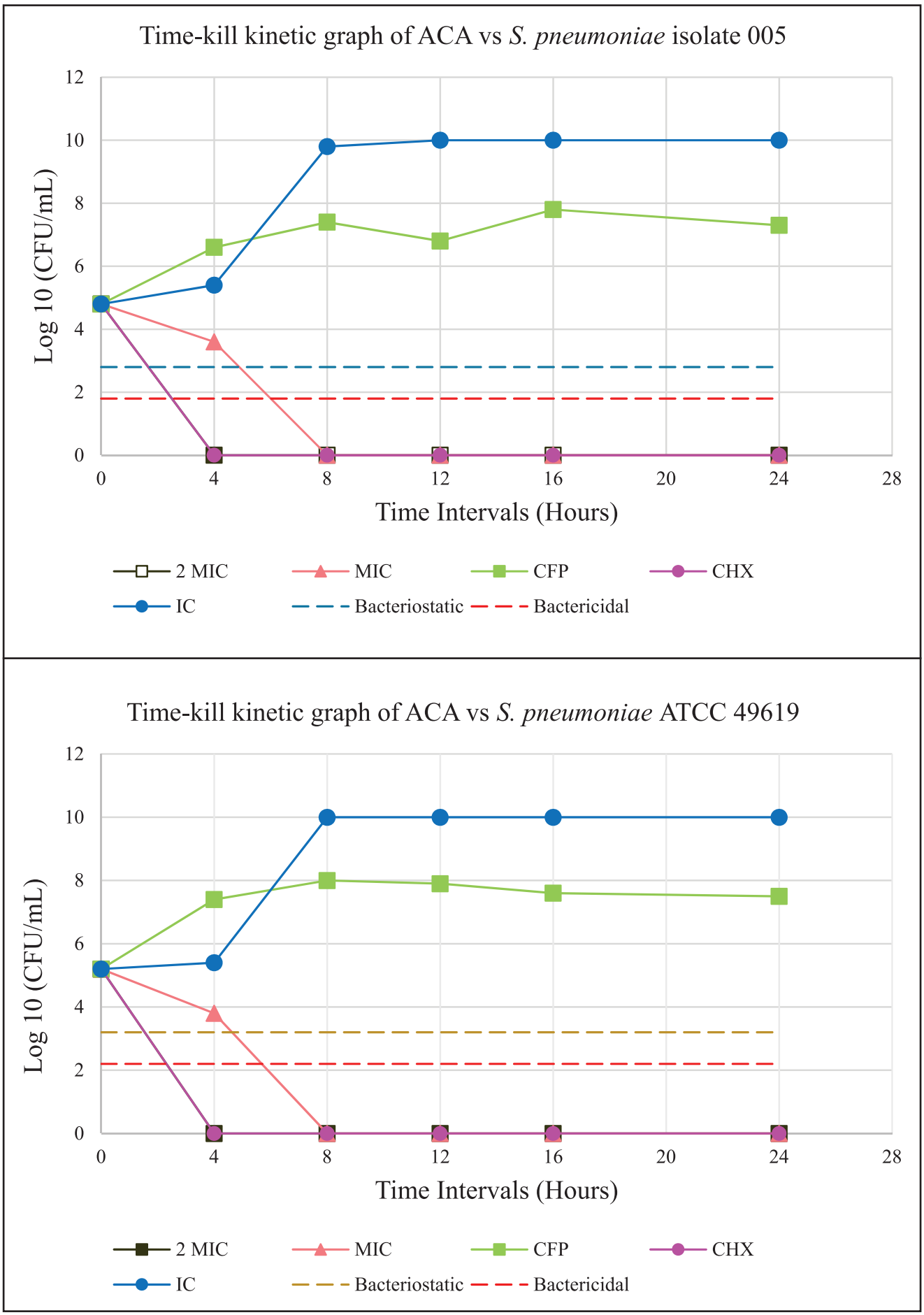

Figure 4. Time-kill kinetic graph of Log_{10} CFU/mL versus time for ACA against *S. pneumoniae* isolate 005 and *S. pneumoniae* ATCC 49619. CFP= 0.25 mg/mL. CHX= 0.2%. IC is untreated sample. CFU is colony-forming unit.

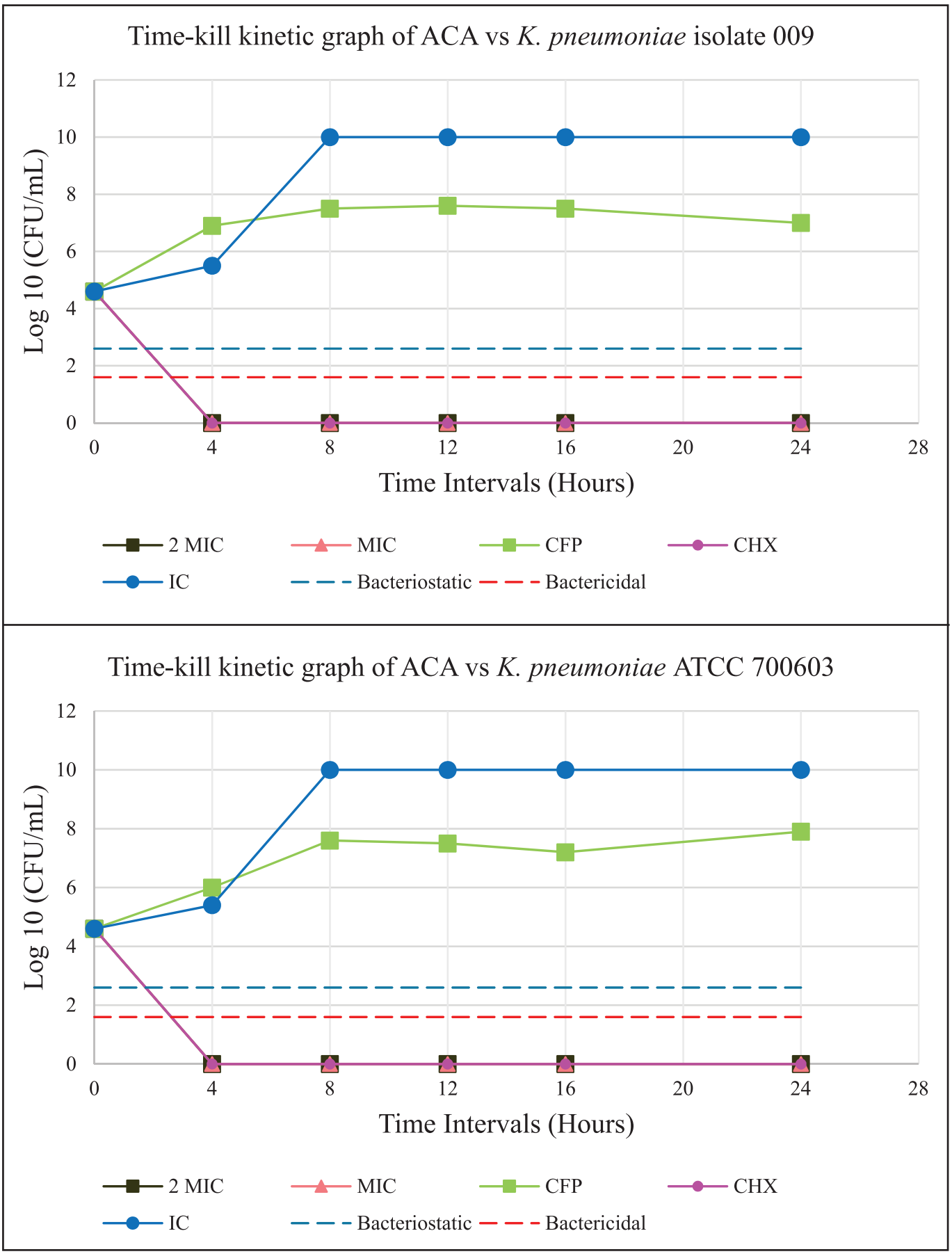

Figure 5. Time-kill kinetic graph of Log_{10} CFU/mL versus time for ACA against *K. pneumoniae* isolate 009 and *K. pneumoniae* ATCC 700603. CFP= 0.008 mg/mL. CHX= 0.2%. IC is untreated sample. CFU is colony-forming unit.

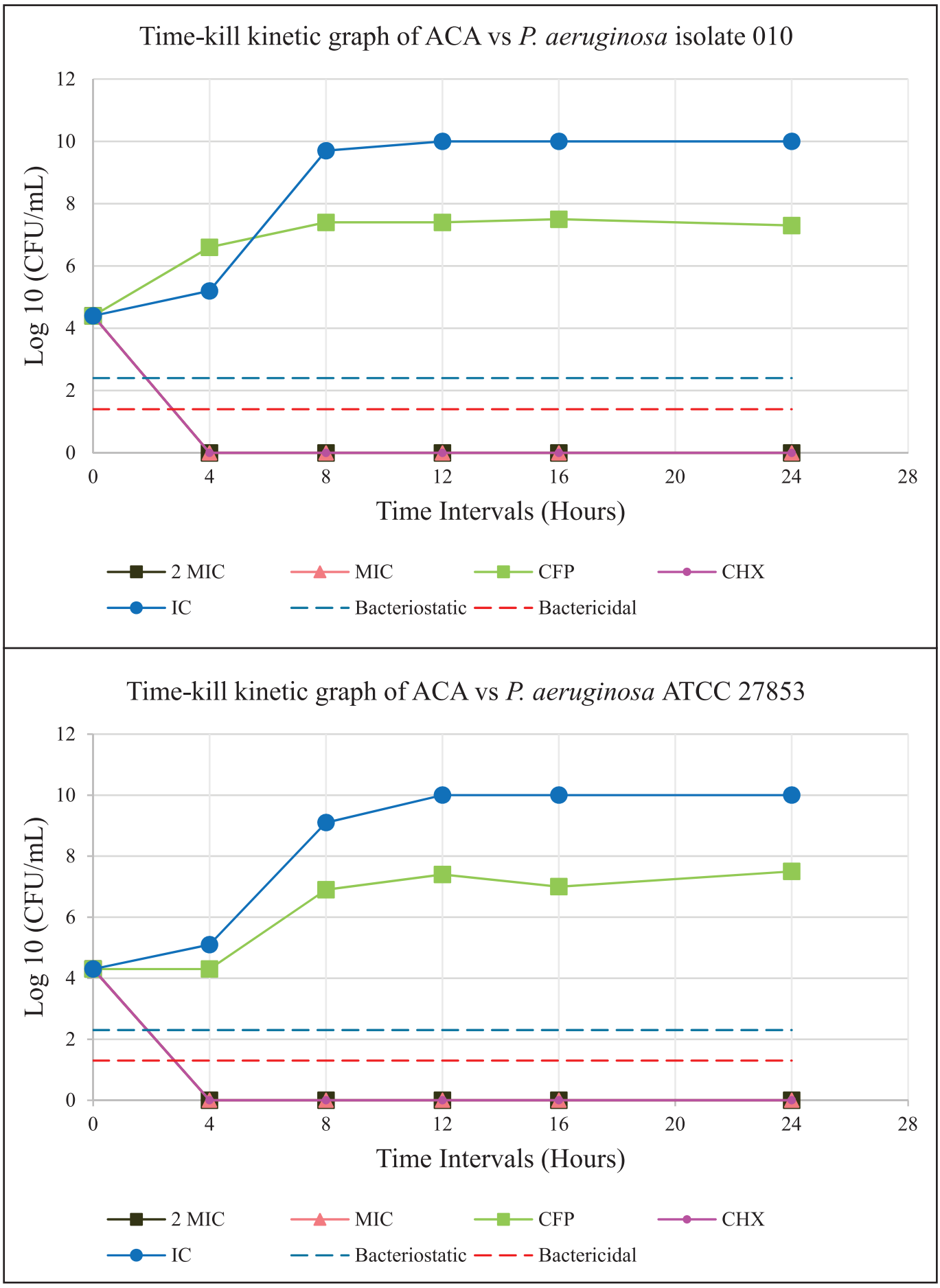

Figure 6. Time-kill kinetic graph of Log_{10} CFU/mL versus time for ACA against *P. aeruginosa* isolate 010 and *P. aeruginosa* ATCC 27853. CFP= 0.008 mg/mL. CHX= 0.2%. IC is untreated sample. CFU is colony-forming unit.

The findings from MIC and MBC procedures were unique that lead to the next step of this study which is time kill assay. MIC and MBC studies were limited to the incubation time of 24 hours (Parvekar *et al*., 2020). This indicated that the inhibitory effects were observe after 24 hours treatment of ACA against the oral opportunistic pathogens. Along the duration of 24 hours, the real time of inhibitory and killing effects of ACA cannot be determined.

Therefore, time kill assay was conducted to observe the kinetic profile of antibacterial activity of ACA at each time intervals which were 0,4,8,12,16 and 24 hours. The concentrations of ACA used were based on the result of MIC and MBC procedures. The concentrations included were 2 MIC and 1 MIC. ACA at the concentration of 2 MIC exhibited bactericidal properties towards the oral opportunistic pathogens as early as 4 hours of incubation with the reduction of > 3 $\log_{10}$ CFU/mL from initial growth. At the concentration of MIC, *S. pneumoniae*, *K. pneumoniae*, and *P. aeruginosa* that recorded the same MIC and MBC values showed bactericidal properties but at different time intervals. Based on the results, ACA can be categorized as concentration-dependent antimicrobial agents as the higher concentration of the extracts used, the greater the rate and extent of the antibacterial properties. Based on the findings, it can be concluded that ACA exhibited promising antibacterial activity towards the selected oral opportunistic pathogens tested in this study which consist of *S. aureus, S. pneumoniae, K. pneumoniae* and *P. aeruginosa*.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGEMENT

This work was funded by the Ministry of Higher Education (MOHE) of Malaysia under the Fundamental Research Grant Scheme (FRGS) FRGS/1/2021/STG05/USIM/02/4.

REFERENCES

Ab Malik, N., Abdul Razak, F., Mohamad Yatim, S., Lam, O.L.T., Jin, L., Li, L.S.W. & McGrath, C. (2018). Oral health interventions using chlorhexidine – effects on the prevalence of oral opportunistic pathogens in stroke survivors: A randomized clinical trial. *Journal of Evidence Based Dental Practice* **18**: 99-109. https://doi.org/10.1016/J.JEBDP.2017.08.002

Abalaka, M.E., Daniyan, S.Y., Oyeleke, S.B. & Adeyemo, S.O. (2012). The antibacterial evaluation of *Moringa oleifera* leaf extracts on selected bacterial pathogens. *Journal of Microbiology Research* **2**: 1-4. https://doi.org/10.5923/j.microbiology.20120202.01

Argemí, R.A., Navarro, B.G., García-Seisdedos, P.O., Devesa, A.E. & López-López, J. (2020). Mouthwash with alcohol and oral carcinogenesis: systematic review and meta-analysis. *Journal of Evidence Based Dental Practice* **20**: 2, 101407. https://doi.org/10.1016/j.jebdp.2020.101407

Aziz, A.N., Ibrahim, H., Rosmy Syamsir, D., Mohtar, M., Vejayan, J. & Awang, K. (2013). Antimicrobial compounds from *Alpinia conchigera*. *Journal of Ethnopharmacology* **145**: 798-802. https://doi.org/10.1016/J.JEP.2012.12.024

Bachtel, N. & Israni-Winger, K. (2020). Focus: plant-based medicine and pharmacology: Introduction. *The Yale Journal of Biology and Medicine* **93**: 227-228.

Bouacha, M., Besnaci, S. & Boudiar, I. (2023). An Overview of the most used methods to determine the *in vitro* antibacterial activity of honey. *Acta Microbiologica Bulgarica* **39**: 23-30.

Breijyeh, Z., Jubeh, B. & Karaman, R. (2020). Resistance of gram-negative bacteria to current antibacterial agents and approaches to resolve it. *Molecules* **25**: 1340. https://doi.org/10.3390/MOLECULES25061340

Bubonja-Šonje, M., Knezević , S. & Abram, M. (2020). Challenges to antimicrobial susceptibility testing of plant-derived polyphenolic compounds. *Archives of Industrial Hygiene and Toxicology* **71**: 300-311. https://doi.org/10.2478/AIHT-2020-71-3396

do Nascimento, N.P.G., Gonçalves, P.F., Douglas-de-Oliveira, D.W. & Flecha, O.D. (2018). Aspiration Pneumonia and oral health: a critical review of literature. *Revista Brasileira de Odontologia* **75**: 1-7. https://doi.org/10.18363/rbo.v75.2018.e1058

Fernandes, F.P., Turssi, C.P., França, F.M.G., Basting, R.T. & do Amaral, F.L.B. (2017). Whitening mouthwash containing hydrogen peroxide decreases enamel microhardness *in vitro*. *Brazilian Journal of Oral Sciences* **16**: 1-9. https://doi.org/10.20396/BJOS.V16I0.8650504

Hanafiah, R.M., Kamaruddin, K.A.C., Saikin, N.A.A., Abdul, W.N.A.B.W., Yakop, M.F., Lim, V. & Said, S.M. (2019). Antibacterial properties of *Clinacanthus nutans* extracts against *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*: An *in-vitro* study. *Journal of International Dental and Medical Research* **12**: 401-404.

Humphries, R.M., Miller, L., Zimmer, B., Matuschek, E. & Hindler, J.A. (2023). Contemporary considerations for establishing reference methods for antibacterial susceptibility testing. *Journal of Clinical Microbiology* **61**: e0188622. https://doi.org/10.1128/JCM.01886-22

Ibrahim, H., Aziz, A.N., Syamsir, D.R., Ali, N.A.M., Mohtar, M., Ali, R.M. & Awang, K. (2009). Essential oils of Alpinia conchigera Griff. and their antimicrobial activities. *Food Chemistry* **113**: 575-577. https://doi.org/10.1016/J.FOODCHEM.2008.08.033

Ishii, M. (2020). The Role of Anaerobes on the Pathogenesis of Aspiration Pneumonia: Anaerobes May Be Involved in the Pathogenesis? In: Aspiration Pneumonia. Respiratory Disease Series: Diagnostic Tools and Disease Managements, Teramoto, S. & Komiya, K. (editors) Singapore: Springer, pp. 105-110.

Islam, M.T. (2019). A literature-based phyto-pharmacological review on *A. conchigera* Griff. *Oriental Pharmacy and Experimental Medicine* **19**: 379-392. https://doi.org/10.1007/s13596-019-00397-7

Israyilova, A., Shoaib, M., Ganbarov, K., Huseynzada, A., Hajiyeva, S. & Ismiyev, A. (2022). Antimicrobial activity and time kill curve study of newly synthesized dialkyl carboxylate cyclohexane derivative: A novel anti-*Pseudomonas aeruginosa* compound. *Acta Scientiarum. Technology* **44**: e58868. https://doi.org/10.4025/actascitechnol.v44i1.58868

Kowalska-Krochmal, B. & Dudek-Wicher, R. (2021). The minimum inhibitory concentration of antibiotics: methods, interpretation, clinical relevance. *Pathogens* **10**: 165. https://doi.org/10.3390/PATHOGENS10020165

Lee, C.R., Cho, I.H., Jeong, B.C. & Lee, S.H. (2013). Strategies to minimize antibiotic resistance. *International Journal of Environmental Research and Public Health* **10**: 4274-4305. https://doi.org/10.3390/IJERPH10094274

Levison, M.E. & Levison, J.H. (2009). Pharmacokinetics and pharmacodynamics of antibacterial agents. *Infectious Disease Clinics of North America* **23**: 791. https://doi.org/10.1016/J.IDC.2009.06.008

Li, J., Xie, S., Ahmed, S., Wang, F., Gu, Y., Zhang, C., Chai, X., Wu, Y., Cai, J. & Cheng, G. (2017). Antimicrobial activity and resistance: Influencing factors. *Frontiers in Pharmacology* **8**: 364. https://doi.org/10.3389/FPHAR.2017.00364

Mandell, L.A. & Niederman, M.S. (2019). Aspiration Pneumonia, *New England Journal of Medicine* **380**: 651-663. https://doi.org/10.1056/NEJMRA1714562

Parvekar, P., Palaskar, J., Metgud, S., Maria, R. & Dutta, S. (2020). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver nanoparticles against *Staphylococcus aureus*. *Biomaterial Investigations in Dentistry* **7**: 105-109. https://doi.org/10.1080/26415275.2020.1796674

Reygaert, W.C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology* **4**: 482. https://doi.org/10.3934/MICROBIOL.2018.3.482

Sakaue, Y., Takenaka, S., Ohsumi, T., Domon, H., Terao, Y. & Noiri, Y. (2018). The effect of chlorhexidine on dental calculus formation: An *in vitro* study. *BMC Oral Health* **18**: 1-7. https://doi.org/10.1186/s12903-018-0517-3

Samaranayake, L.P., MacFarlane, T.W. & Williamson, M.I. (1987). Comparison of Sabouraud dextrose and Pagano-Levin agar media for detection and isolation of yeasts from oral samples. *Journal of Clinical Microbiology* **25**: 162-164. https://doi.org/10.1128/JCM.25.1.162-164.1987

Saxena, D., Maitra, R., Bormon, R., Czekanska, M., Meiers, J., Titz, A., Verma, S. & Chopra, S. (2023). Tackling the outer membrane: facilitating compound entry into Gram-negative bacterial pathogens. *npj Antimicrobials and Resistance* **1**: 17. https://doi.org/10.1038/s44259-023-00016-1

Sedghizadeh, P.P., Mahabady, S. & Allen, C.M. (2017). Opportunistic oral infections. *Dental Clinics of North America* **61**: 389-400. https://doi.org/10.1016/j.cden.2016.12.007

Shi, Z., Xie, H., Wang, P., Wu, Y., Chen, E., Ng, L., Worthington, H.V., Singer, M. & Needleman, I. (2010). Oral hygiene care for critically ill patients to prevent ventilator associated pneumonia [Intervention Protocol]. *Cochrane Database of Systematic Reviews* **2**: CD008367. https://doi.org/10.1002/14651858.CD008367

Taib, M.N.A.M., Anuar, N., Hanafiah, K.M., Al-Shammary, A.A.K., Saaid, M. & Awang, K. (2020). Chemicals constituents isolated from cultivate *Alpinia conchigera* Griff. and antimicrobial activity. *Tropical Life Sciences Research* **31**: 159. https://doi.org/10.21315/TLSR2020.31.1.10

Justiz Vaillant AA, Naik R. HIV-1–Associated Opportunistic Infections. [Updated 2023 Jan 27]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing.
Available from: https://www.ncbi.nlm.nih.gov/books/NBK539787/

WHO (World Health Organization). (2021). Pneumonia. World Health Organization. https://www.who.int/health-topics/pneumonia. Accessed 27 January 2022.

Zhang, D., Zou, L., Wu, D.T., Zhuang, Q.G., Li, H. Bin, Mavumengwana, V., Corke, H. & Gan, R.Y. (2021). Discovery of 1'-acetoxychavicol acetate (ACA) as a promising antibacterial compound from galangal (*Alpinia galanga* (Linn.) Willd). *Industrial Crops and Products* **171**: 113883. https://doi.org/10.1016/J.INDCROP.2021.113883