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# Extraction and Antibacterial Activity of *Alpinia conchigera* Rhizome Extract and 1'*S* -1'-Acetoxychavicol Acetate (ACA) against *Streptococcus pneumoniae*

(Pengekstrakan dan Aktiviti Antibakteria Ekstrak Rizom *Alpinia conchigera* dan 1'*S* -1'- Asetoksikavikol Asetat (ACA) terhadap *Streptococcus pneumoniae*)

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ABSTRACT

The study aimed to investigate the antibacterial effects of *Alpinia conchigera* rhizome extract and 1'*S*-1'-acetoxychavicol acetate (ACA) against *Streptococcus pneumoniae* strain successfully isolated from oral rinse of older adults from an old folks' home. The grounded rhizomes of *A. conchigera* were macerated in hexane solvent to yield crude hexane extract. Then, *A. conchigera* rhizome extract was subjected to column chromatography to isolate ACA. Both *A. conchigera* rhizome extract and ACA were screened for the antibacterial activity against *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate by disc diffusion test, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time kill assay. The highest inhibition zone recorded by *A. conchigera* rhizome extract (100 mg/mL) and ACA (100 mg/mL) were 36.83 ± 0.85 mm and 30.67 ± 0.94 mm, respectively against *S. pneumoniae* isolate. The MIC and MBC values recorded for *A. conchigera* rhizome extract and ACA against both *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate ranging from 12.50 mg/mL to 50.00 mg/mL. Based on the time kill curve, both extracts with the concentration of 2 × MIC showed killing properties against *S. pneumoniae* isolate. Besides that, the morphology of *S. pneumoniae* isolate treated with ACA (12.50 mg/mL) was observed under scanning electron microscope and showed structural changes such as cell wall disruption and morphological disorder. Moreover, several virulence genes of *S. pneumoniae* were studied using RT-qPCR. It is observed that two genes, *pavA* and *psaA* function in adherence and attachment of *S. pneumoniae* to the host cells were downregulated while *nanA* which that responsible for biofilm production was upregulated. Therefore, both *A. conchigera* rhizome extract and ACA showed great potential as alternative antibacterial agent against *S. pneumoniae*.

Keywords: *Alpinia conchigera*; pneumonia; rhizome extract; *Streptococcus pneumoniae*; 1'*S*-1'-acetoxychavicol acetate

ABSTRAK

Penyelidikan ini bertujuan untuk mengkaji kesan antibakteria ekstrak rizom *Alpinia conchigera* dan 1'*S* -1'-asetoksikavikol asetat (ACA) terhadap strain *Streptococcus pneumoniae* yang berjaya dipencilkan daripada bilasan mulut orang dewasa yang diperoleh daripada rumah orang tua. Serbuk rizom *A. conchigera* direndam di dalam pelarut heksana untuk menghasilkan ekstrak heksana. Kemudian, ekstrak rizom *A. conchigera* tersebut dikenakan kolumn kromatografi bagi memencilkan ACA. Ujian resapan cakera (DDA), kepekatan perencatan minimum (MIC), kepekatan bakterisidal minimum (MBC) dan asai kadar masa membunuh telah dijalankan untuk menentukan sifat antibakteria kedua-dua ekstrak rizom *A. conchigera* dan ACA terhadap *S. pneumoniae* ATCC 49619 dan pencilan *S. pneumoniae*. Berdasarkan prosedur DDA, ekstrak rizom *A. conchigera* (100 mg/mL) dan ACA (100 mg/mL) masing-masing merekodkan zon perencatan tertinggi iaitu 36.83 ± 0.85 mm dan 30.67 ± 0.94 mm apabila diuji terhadap pencilan *S. pneumoniae*. Nilai MIC dan MBC yang direkodkan oleh ekstrak rizom *A. conchigera* dan ACA terhadap ATCC 49619 *S. pneumoniae* dan pencilan *S. pneumoniae* adalah daripada julat 12.50 mg/mL sehingga 50.00 mg/mL. Berdasarkan lengkung kadar masa membunuh, kedua-dua ekstrak dengan kepekatan 2 × MIC menunjukkan sifat bakterisidal terhadap strain *S. pneumoniae*. Selain itu, morfologi strain *S. pneumoniae* yang telah dirawat dengan ACA (12.50 mg/mL) telah diperhatikan melalui mikroskop elektron imbasan dan menunjukkan perubahan struktur seperti pemecahan dinding sel dan kerosakan morfologi. Tambahan lagi, beberapa gen virulens *S. pneumoniae* telah dikaji menggunakan RT- qPCR. Hasil mendapati bahawa dua gen, *pavA* dan *psaA*, yang berfungsi untuk pelekatan *S. pneumoniae* terhadap sel perumah dikawal atur turun, manakala gen *nanA* yang berfungsi untuk penghasilan

biofilem telah dikawal atur naik. Oleh itu, kedua-dua ekstrak rizom *A. conchigera* dan ACA menunjukkan potensi yang baik sebagai agen antibakteria alternatif melawan *S. pneumoniae.*

Kata kunci: *Alpinia conchigera;* ekstrak rizom; pneumonia; *Streptococcus pneumoniae*; 1'*S* -1'-asetoksikavikol asetat

## 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). In Malaysia, pneumonia is a worrying disease, as it ranks among the top three diseases that lead to death in adults and children (Department of Statistics Malaysia 2022). Pneumonia occurs when an infection in the respiratory tract causes the small sacs known as alveoli to be filled with pus and fluid, resulting in breathing difficulty due to limited oxygen intake (WHO 2021). Bacterial infection is one of the main causes of pneumonia, and the bacteria that is commonly isolated is *Streptococcus pneumoniae*. There are several genes that are responsible for the pathogenicity of this bacteria. Fortunately, antibiotic treatment can reduce the mortality and morbidity associated with bacterial infections. However, the usage of antibiotics as a treatment to combat oral opportunistic pathogens has brought concern regarding the emergence of multidrug-resistant pathogens (Sharma et al. 2024). It is crucial to look for an alternative treatment that uses natural products that can act as antibacterial agents against *S. pneumoniae.*

A lot of previous research has used plant extracts as interventions against *S. pneumoniae.* For example, a medicinal plant in Indonesia, *Lawsonia inermis*, has been proven to inhibit the growth of *S. pneumoniae* ATCC 49619 by causing cell wall disruption, decreasing cell membrane integrity, and causing morphological disorder (Tafroji et al. 2022). Besides that, leaf extract of *Anredera cordifolia* (Ten.) Steenis collected from Bogor, West Java, Indonesia also showed antibacterial activity against *S. pneumoniae* ATCC 49619 and clinical isolate *S. pneumoniae* PU 067 (Nasution, Artika & Safari 2020). In another study by Mwale, Makunike and Mangoyi (2019), leaves of *Melia azedarach* collected from the University of Zimbabwe that were originally used to treat typhoid fever were evaluated for their medicinal properties. It was proven that a low concentration of the leaf extract can exhibit antibacterial effects against *S. pneumoniae.*

*Alpinia conchigera* belongs to the family of Zingiberaceae and is known as *Languas conchigera* Burkill. Locally, this plant is recognized as '*lengkuas ranting'*, '*lengkuas kecil'*, '*lengkuas padang'*, '*lengkuas getting'* and '*chengkenam'* which is native to Peninsular Malaysia (Ibrahim, Chooi & Hassan 2000). This plant consists of several different parts, which include the rhizomes, leaves, and pseudostems. Different parts of the plant have been studied for their potential benefit against microorganisms and fungi (Islam 2019).

1'*S*-1'-acetoxychavicol acetate (ACA) is the major bioactive compound found in the rhizome of *A. conchigera* and has been proven to exhibit good antimicrobial properties against various types of pathogens and multidrug-resistant bacteria such as *S. aureus*, *S. typhi*, *E. coli*, *P. aeruginosa*, and *B. cereus* (Latha et al. 2009; Zhang et al. 2021). ACA also demonstrates anti-inflammatory and anti-cancer properties, for example, against human cancer cells. Based on a study by Awang et al. (2010), ACA demonstrated enormous potential for future development as a chemotherapeutic drug against five human tumour cell lines, which were breast adenocarcinoma (MCF-7), oral squamous carcinoma (HSC-2), oral squamous carcinoma (HSC-4), hepatocyte carcinoma (HepG2), and epidermoid cervical carcinoma (CaSki). This bioactive compound was also found to cause cell death through apoptosis, and followed by secondary necrosis. It was reported that ACA was non-toxic to the normal cell (Liew et al. 2017).

Therefore, this study aimed to investigate the antibacterial properties of *A. conchigera* rhizome extract and its bioactive compound, ACA, against *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate. Besides that, the regulation of three main virulence genes of *S. pneumoniae* was also investigated upon treatment of ACA, which are *nanA*, *pavA*, and *psaA*. The *nanA* gene encodes neuraminidase A that functions in cleaving sialic acids from host glycoproteins and glycolipids. This action helps *S. pneumoniae* to adhere and invade epithelial cells in the respiratory tract, as it also promotes biofilm formation (Mitchell & Mitchell 2010). *PavA* is also an important virulence factor for *S. pneumonia*, as this gene promotes adherence to epithelial cells and tissue invasion, while *psaA* plays an essential role in defending this bacterial strain against oxidative stress and promoting the attachment of *S. pneumoniae* to the host cells (Nieto et al. 2013).

## MATERIALS AND METHODS

### PLANT MATERIALS

*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. This specimen was identified by Prof. Dr Halijah Ibrahim from the Institute of Biological Sciences at the University of Malaya (Taib et al. 2020).

### EXTRACTION OF *Alpinia conchigera* RHIZOME EXTRACT

The rhizomes were washed, sliced, and air-dried until constant mass was obtained. The dried rhizomes were grounded, and 10 kg of powdered rhizomes were extracted with hexane (RM, Malaysia) for 72 h at room temperature using the maceration method. The powdered rhizomes and the solvent were gently mixed using a glass rod periodically to ensure complete extraction. The extracts were filtered using Whatman filter paper No. 1, and the filtrate was collected in a 1000 mL conical flask and dried *in vacuo* using a rotary evaporator (EYELA, USA) at 10 °C to obtain the concentrated crude extracts. The *A. conchigera* rhizome extract was then transferred into vials and stored at -20 °C until further use. The residue was subjected to the same extraction process for another two times to maximize the extraction yield (Mohd Salleh 2014).

### ISOLATION OF 1'*S*-1'-ACETOXYCHAVICOL ACETATE (ACA)

The *A. conchigera* rhizome extract was subjected to column chromatography (CC) on silica gel using a stepwise gradient system (*n*-hexane to MeOH) to fractionate the compounds presence in ACRE-*n*-hexane. The ACRE-*n*-hexane (8 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), which was used as the stationary phase (approximately 30:1, silica gel to sample ratio). The column was eluted with a stepwise gradient of *n*-hexane: ethyl acetate (100:0 to 50:50). The elutes were collected separately using a conical flask and combined based on the thin layer chromatography (TLC) profile and the $R_f$ value when compared with the reference ACA.

### ORAL RINSE SAMPLE PREPARATION

Oral rinse samples were obtained from Pusat Jagaan Al-Fikrah, Selangor, with ethical approval (USIM/JKEP/2021-184) from the Ethics Committee, USIM. Twelve participants were recruited based on the inclusion criteria: Residents aged more than 60 years old, not edentulous, and not on antibiotics for the past three months (Poudel et al. 2024). The participants were briefed, and consents were obtained. The samples were taken after an oral rinse with 10 mL of 0.1M sterile phosphate-buffered saline (PBS) at pH 7.2 for around 60 s (Samaranayake, MacFarlane & Williamson 1987). Samples were stored in an icebox and transported within six hours to the Oral Biology Laboratory, Faculty of Dentistry, USIM.

### ORAL RINSE SAMPLE PROCESSING

The samples were centrifuged at 4,000 rpm for 15 min at 4 °C in a refrigerated centrifuge (Beckman Coulter Life Science, Indiana, USA). The supernatant was discarded, and the pellet obtained was suspended in 1 mL 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 four times ($10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$) to isolate pure colonies of *Streptococcus pneumoniae.* A volume of 50 µL aliquots of each dilution were inoculated using the spread plate technique on blood agar (Oxoid, UK). The agar plates were then incubated in anaerobic conditions for 24 h at 37 °C.

### ISOLATION AND IDENTIFICATION OF CLINICAL STRAIN OF *Streptococcus pneumoniae*

After 24 h of incubation, the morphology and characteristics of *S. pneumoniae* were observed on the blood agar and through the Gram stain procedure. The blood agar was used to isolate the genus of *Streptococci*, to detect and determine the haemolytic properties, which can be classified into three groups, which are beta, alpha, and gamma haemolysis. The Optochin test was also conducted to further isolate the *S. pneumoniae* strain (Ranjan et al. 2014). The isolated presumptive *S. pneumoniae* strain was then further confirmed for identification using the DNA barcoding protocol provided by Molecular Biology Services (1st BASE, Singapore). The pure single colony of *S. pneumoniae* strain that has been confirmed was then transferred into Brain Heart Infusion Broth (BHIB) and incubated for 24 h at 37 °C in anaerobic conditions, and the turbidity of the overnight culture was standardized according to 0.5 McFarland standards (Thermo Fisher Scientific, USA) equal to $1.5 \times 10^8$ CFU/mL.

### ANTIBACTERIAL ASSAYS

#### DISC DIFFUSION TECHNIQUE

Disc diffusion assay was carried out to screen the antibacterial effect of *A. conchigera* rhizome extract and ACA based on the guidelines from the Clinical Laboratory Standard Institute (CLSI) (Humphries et al. 2023). The *A. conchigera* rhizome extract and ACA were dissolved in 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) to the series of concentrations: 100.0, 25.0 and 12.5 mg/mL. The controls of this assay include 10% DMSO as the negative control and 30 µg cefepime antibiotic disc (Oxoid, UK) as the positive control.

A volume of 100 µL of *S. pneumonia* ATCC 49619 and *S. pneumoniae* isolate bacterial suspension adjusted to 0.5 McFarland standards was spread thinly and evenly on Mueller Hinton Agar (MHA) surface using sterile cotton swabs to obtain a confluent bacterial growth. Sterile filter paper discs with a diameter size of 6 mm were placed onto the inoculated MHA and were impregnated with 10 µL *A. conchigera* rhizome extract and ACA at three different concentrations (100.0, 50.0 and 25.0 mg/mL), 30 µg cefepime antibiotic disc, and 10% DMSO. The agar plates were incubated for 24 h at 37 °C in anaerobic conditions. The diameter of the inhibition zone with different concentrations (100, 50 and 25 mg/mL) of *A. conchigera*

rhizome extract, ACA and negative control was measured in millimetres (mm). The disc diffusion assay was carried out in three replicates. One-way ANOVA was used to analyze the differences using the SPSS software (IBM, USA).

### MINIMUM INHIBITORY CONCENTRATION

Minimum inhibitory concentration (MIC) was carried out to determine the lowest concentration of *A. conchigera* rhizome extract and ACA required to inhibit *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate based on the CLSI protocol of antimicrobial susceptibility testing (Parvekar et al. 2020). MIC was performed using the broth microdilution technique in 96-well microtiter plate. In this procedure, cefepime solution and 10% DMSO were used as positive and negative controls, respectively. Two-fold serial dilutions of *A. conchigera* rhizome extract and ACA ranging from 0.2 to 50 mg/mL were prepared in a sterile 96-well plate using Mueller Hinton Broth (MHB) as the diluent. A total of 50 µL of the adjusted overnight cultures of *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate were inoculated into each well. The total volume in each well was 100 µL. The 96-well microtiter plates were incubated for 24 h at 37 °C in anaerobic conditions. After the incubation time, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Thermo Scientific, MA, USA) solution with the concentration of 2 mg/mL was added to each well, and the 96-well microtiter plates were incubated for 2 h. 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

Minimum bactericidal concentration (MBC) was carried out to determine the lowest concentration of *A. conchigera* rhizome extract and ACA that can kill *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate (Abalaka et al. 2012). A volume of 10 µL from each well containing *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate was incubated with *A. conchigera* rhizome extract and ACA at concentrations ranging from 50 mg/mL to 0.2 mg/mL and inoculated on MHA using the streak plate method. The agar plates were incubated for 24 h at 37 °C in anaerobic conditions. The growth of bacteria in each plate was observed, and MBC values were recorded by the lowest concentration of the samples that showed no bacterial growth on MHA after the incubation period.

### TIME KILL ASSAY

The time kill assay was performed to assess the bacteriostatic or bactericidal properties of *A. conchigera* rhizome extract and ACA and the relationship between the concentrations used and the incubation period (Bouacha, Besnaci & Boudiar 2023). The concentrations of *A. conchigera* rhizome extract and ACA used for time-killing measurement were ½ MIC, 1 × MIC and 2 × MIC and were evaluated at six-time intervals: 0, 4-, 8-, 12-, 16-, and 24-h using the broth microdilution technique in 96-well microtiter plates. In this procedure, only *S. pneumoniae* isolate was selected to further observe the effect of *A. conchigera* rhizome extract and ACA when tested against a virulence strain.

An aliquot of 50 µL sterile MHB was added into each well, and then 50 µL of *A. conchigera* rhizome extract and ACA were serially diluted across the wells, starting from the highest concentration (2 × MIC) to the lowest concentration (½ MIC) based on MIC determination results. 50 µL of standardized 0.5 McFarland overnight culture of *S. pneumoniae* isolate was added into each well. The positive control included was 0.2% chlorhexidine, 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 h) at 37 °C in anaerobic conditions.

At each time interval, the *A. conchigera* rhizome extract, ACA, and positive control were diluted using ten-fold serial dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$), while for inoculum control ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$) using sterile saline water as the diluent. Then, 10 µL from each dilution factor were inoculated on MHA using the streak plate technique and incubated for 24 h at 37 °C in anaerobic conditions. The colonies formed were counted to determine colony forming unit per mL (CFU/mL). Bactericidal and bacteriostatic activities were demonstrated as a decrease of 99.9% ($\geq 3 \log_{10}$) and a 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).

### SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) was done to observe the morphological changes of *S. pneumoniae* isolate upon the treatment given at certain concentrations and incubation times. The morphology of *S. pneumoniae* isolate was observed after being treated with 12.5 mg/mL (MIC value) of ACA and incubated for 4 h based on the time kill profile. The results were compared with the negative control, which was the non-treated *S. pneumoniae* isolate.

A total of 5 mL sterile BHIB were added into centrifuge tubes (Beckman Coulter Life Science, Indiana, USA). Then, 5 mL of prepared ACA with the concentration of 12.5 mg/mL were added into the sterile BHIB. The mixtures were vortexed until homogenous solutions were obtained. The overnight culture was adjusted to 0.5 McFarland, which equals $1.5 \times 10^8$ CFU/mL. Next, 5 mL of the adjusted overnight culture was added into the centrifuge tubes

and mixed by pipetting up and down. The non-treated *S. pneumoniae* isolate was prepared similarly without any ACA added. The Beckmann centrifuge tubes were incubated at two time points, which are 4 h (bacteriostatic level) and 5 h (bactericidal level) at 37 °C in anaerobic conditions. After the incubation period, the samples were centrifuged at 12,000 rpm for 20 min at 4 °C. The pellet was collected, and the supernatant was discarded.

The samples were fixed using 4% glutaraldehyde for 4 to 6 h at 4 °C. Then, the pellets were washed with 0.1 M sodium cacodylate buffer for three times of 10 min each. After that, post fixation was done using 1% osmium tetroxide for 2 h at 4 °C. The washing steps were repeated three times of 10 min each. Next, the pellets were dehydrated using a series of acetones (35%, 50%, 75%, 95%, and 100%). The dried pellets were then transferred onto aluminium foil (1 cm diameter) coated with albumin. The specimens were transferred into the specimen basket and put into a critical dryer for about 30 min to completely dry the samples. This was done to preserve the surface structure of the specimens, which could otherwise be damaged due to surface tension when changing from the liquid to gaseous state. The samples were mounted on the stubs using conductive carbon adhesive tape. The specimens were viewed under the scanning electron microscope (SEM, Jeol JSM-6400, Tokyo, Japan) after being coated with gold using a sputter coater to increase the conductivity of the samples.

## GENE EXPRESSION STUDY

### PRETREATMENT AND RNA EXTRACTION

*S. pneumoniae* isolate was grown in BHIB overnight at 37 °C in anaerobic conditions. Then, the turbidity of the overnight culture was adjusted to 0.5 McFarland. The concentration of ACA at 6.25 mg/mL (½ MIC) was added into the mixture and incubated at 37 °C for 4 h and 30 min. The pellets of treated and non-treated *S. pneumoniae* isolates were prepared and harvested by centrifugation at 16,000 × g for 3 min prior to RNA extraction. The RNA was extracted using the PrimeWay Total RNA Extraction Kit (1st BASE, Singapore).

### REAL-TIME QUANTITATIVE PCR (RT-qPCR)

The expression changes of selected genes of the *S. pneumoniae* isolate treated with ACA was investigated using quantitative Real-Time Quantitative PCR (RT-qPCR). The GoTaq 2-Step RT-qPCR system facilitates detection and quantification of RNA expression levels using GoScript™ Reverse Transcriptase and GoTaq qPCR Master Mix (Promega, USA). The primers of target and reference genes were designed using Primer3Plus and their specificity and quality were determined using OligoCalc. The target and reference genes include *nanA, pavA, psaA* and 16S rRNA *S. pneumoniae.* Table 1 shows the forward (F) and reverse (R) primer sequences for the targeted and reference genes. The cycling parameter had been optimized based on the average annealing temperature of all primers as shown in Table 2.

TABLE 1. Primer sequences for targeted and reference genes

| Target gene | Primer sequence | References |
|---|---|---|
| 16S rRNA *S. pneumoniae* | F: 5′-CCACACTGGGACTGAGACAC-3′<br>R: 5′-TCAACCTTGCGGTCGTACTC-3′ | (Nieto et al. 2013; Sakai et al. 2013) |
| *nanA* | F: 5′-AAACTACCTGAAGGAGCGGC-3′<br>R: 5′-CCCTTGTCGCTATAGGCTGG-3′ | |
| *pavA* | F: 5′-GTGCCTAACGACCAAGACCA-3′<br>R: 5′-TGCGTCTGTCTTGACTGCAT-3′ | |
| *psaA* | F: 5′-GCGACGGCGTTGATGTTATC-3′<br>R: 5′-AGCTGTCGCCTTCTTTACCT-3′ | |

TABLE 2. Cycling parameters for RT-qPCR procedures

| Step | Cycles | Temperature | Duration |
|---|---|---|---|
| DNA polymerase activation | 1 | 95 °C | 2 min |
| Denaturation | 40 | 95 °C | 15 s |
| Annealing and extension | 40 | 52 °C | 1 min |

The cycle threshold (Ct) values obtained from the RT-qPCR cycles for each gene were used to calculate the relative expression of gene between treated and non-treated samples in the form of fold change. The value of the fold change was calculated using the following formula: ($\Delta CT_D$ - $\Delta CT_B$) – ($\Delta CT_C$ - $\Delta CT_A$) (Rao et al. 2013), where $\Delta CT_D$ represents the reference gene, 16S rRNA from non-treated samples, $\Delta CT_B$ represents reference gene 16S rRNA from samples treated with ACA, while $\Delta CT_C$ refers to the gene of interest from treated samples, and $\Delta CT_A$ refers to the gene of interest from non-treated samples. This procedure was repeated twice with three replicates for each trial. The relative fold change of the genes on treated *S. pneumoniae* isolate were compared with the non-treated *S. pneumoniae* isolate using t-test. Statistical analysis was done using the SPSS 26 software (IBM, USA).

## RESULTS AND DISCUSSION

### *Alpinia conchigera* RHIZOME EXTRACT AND 1'*S*-1'-ACETOXYCHAVICOL ACETATE (ACA)

A total of 21.391 g of *A. conchigera* rhizome extract was obtained from the extraction procedure. Then, a total of 16 g of the crude *A. conchigera* rhizome extract was subjected to column chromatography (CC) to isolate 3.7573 g of ACA. The remaining crude hexane extract was used for antibacterial testing. The isolated ACA was confirmed by TLC analysis. The $R_f$ value of the isolated ACA was compared with the reference ACA. Figure 1 shows the TLC profile of the isolated and reference ACA. The retention factor ($R_f$) value of ACA isolated was 0.69.

### *S. pneumoniae* ISOLATE FROM ORAL RINSE SAMPLE

Only one *S. pneumoniae* strain was successfully isolated. On blood agar, *S. pneumoniae* isolate formed whitish colonies in circular shape with a pinpoint size (0.5 - 1.0 mm). The colonies formed were transparent and surrounded by an area of green discoloration that indicated α-haemolytic reaction as shown in Figure 2(a). In addition to that, optochin test conducted showed zone of inhibition ≥ 14 mm which indicated *S. pneumoniae* isolate as it is sensitive towards optochin (Figure 2(b)). *S. pneumoniae* isolated was further confirmed using DNA Barcoding using Molecular Biology Service (1st BASE, Singapore).

### ANTIBACTERIAL ASSAYS OF HEXANE EXTRACT AND ACA AGAINST *S. pneumoniae*

#### DISC DIFFUSION ASSAY (DDA)

*S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate were successfully inhibited at different concentrations of *A. conchigera* rhizome extract and ACA when compared

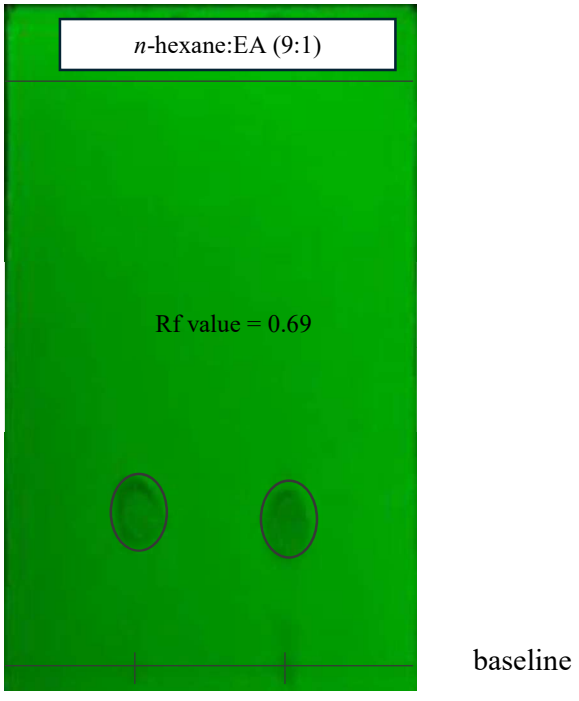


FIGURE 1. TLC profile of (a) reference ACA and (b) isolated ACA, stationary phase = silica gel $F_{254,}$ mobile phase = n-hexane: ethyl acetate (9:1)

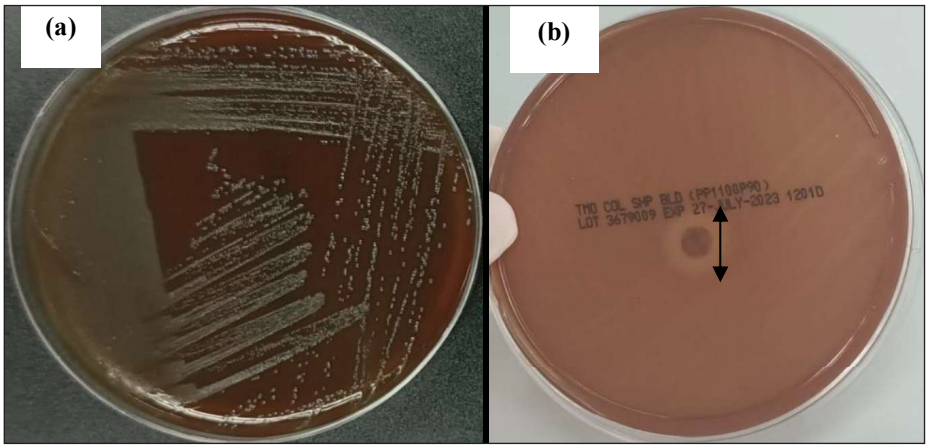


FIGURE 2. (a) The Streptococcus pneumoniae isolate on blood agar, (b) The inhibition zone of S. pneumoniae isolate when tested against optochin disc

to the negative control (10% DMSO). Table 3 shows the summary for the diameter of the inhibition zone of *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate after being treated with *A. conchigera* rhizome extract and ACA at the concentrations of 100, 50, and 25 mg/mL. Under both treatments, the zone of inhibition showed a decreasing pattern following the concentration. The higher the concentration of the tested samples, the bigger the inhibition diameter. The largest zones of inhibition for *A. conchigera* rhizome extract and ACA against *S. pneumoniae* isolate at 100 mg/mL were 36.83 ± 0.85 mm and 30.67 ± 0.94 mm, respectively.

The disc diffusion assay was performed as a preliminary testing of the *A. conchigera* rhizome extract and ACA against *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate. This assay has been commonly used to screen for the antimicrobial properties of plant extracts. However, both *A. conchigera* rhizome extract and ACA used in this study were non-polar compounds. This can lead to false positive results as the polarity of compounds could affect the diffusion rate of the compounds into the filter paper discs and agar. Plus, this technique cannot determine the minimum concentration of the extract required to inhibit or kill the pathogens (Balouiri, Sadiki & Ibnsouda 2016). Nonetheless, the disc diffusion assay still should be considered as preliminary testing before performing broth microdilution to screen for the antimicrobial activity of a compound.

### MINIMUM INHIBITORY AND BACTERICIDAL CONCENTRATION (MIC & MBC)

Further screening was conducted through the broth microdilution technique to determine the minimum inhibitory and bactericidal concentration (MIC and MBC) of *A. conchigera* rhizome extract and ACA against *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate. This procedure used broth media instead of agar that caused the non-polar extracts easier to be dissolved. The MIC and MBC were conducted to further determine the lowest concentration of *A. conchigera* rhizome extract and ACA to inhibit or kill *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate.

Based on the results of MIC and MBC, the bacteriostatic and bactericidal effect can be determined. Bacteriostatic is shown when MBC value is more than four times of MIC value (MBC > 4 MIC) meanwhile if MBC value is less than four times of MIC (MBC < 4 MIC), the result indicates bactericidal effect. Based on the results, both *A. conchigera* rhizome extract and ACA showed bactericidal properties. Table 4 shows the MIC and MBC values of *A. conchigera* rhizome extract and ACA against *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate. The concentration of *A. conchigera* rhizome extract required to inhibit and kill *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate was slightly higher with the value of 25.00 mg/mL compared to ACA. This may be caused by the antagonistic activity of several bioactive compound presence in the rhizome extract (Uduwana, Abeynayake & Wickramasinghe 2023). From the results, it was observed that, ACA with the concentration of 12.50 mg/mL was able to exhibit inhibitory and killing effects towards both *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate.

### TIME KILL CURVE OF *A. conchigera* RHIZOME EXTRACT AND ACA AGAINST *S. pneumoniae* ISOLATE

The bacteriostatic and bactericidal effects of *A. conchigera* rhizome extract and ACA can be further observed using the time kill assay. The concentrations of *A. conchigera* rhizome extract used against *S. pneumoniae* isolate were ½ MIC (12.50 mg/mL), MIC (25.00 mg/mL) and 2 × MIC

TABLE 3. Inhibition diameters of *A. conchigera* rhizome extract and ACA against *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate in disc diffusion assay

| Samples | Mean inhibition diameter (mm) | |
|---|---|---|
| | *S. pneumoniae* ATCC 49619 | *S. pneumoniae* isolate |
| *A. conchigera* rhizome (100 mg/mL) | 31.33 ± 0.47* | 36.83 ± 0.85* |
| *A. conchigera* rhizome (50 mg/mL) | 24.00 ± 0.82* | 7.33 ± 0.47 |
| *A. conchigera* rhizome (25 mg/mL) | 18.67 ± 0.47* | 7.00 ± 0.00 |
| ACA (100 mg/mL) | 27.33 ± 0.47* | 30.67 ± 0.94* |
| ACA (50 mg/mL) | 14.67 ± 0.47* | 7.33 ± 0.47 |
| ACA (25 mg/mL) | 13.00 ± 0.82* | 7.33 ± 0.47 |
| 30 µg cefepime | 31.33 ± 0.47 | 39.67 ± 0.47 |
| 10% DMSO | 6.00 ± 0.00 | |

*significant difference ($p<0.05$) when compared to negative control

TABLE 4. MIC and MBC values of *A. conchigera* rhizome extract and ACA against *S. pneumoniae* ATCC 49619 and *S. pneumoniae* isolate

| Bacteria | *A. conchigera* rhizome extract | | ACA | | Cefepime solution | |
|---|---|---|---|---|---|---|
| | MIC (mg/mL) | MBC (mg/mL) | MIC (mg/mL) | MBC (mg/mL) | MIC (mg/mL) | MBC (mg/mL) |
| *S. pneumoniae* ATCC 49619 | 25.00 | 50.00 | 12.50 | 12.50 | 0.25 | 0.50 |
| *S. pneumoniae* isolate | 25.00 | 50.00 | 12.50 | 12.50 | | |

(50.00 mg/mL) while ACA used were ½ MIC (6.25 mg/mL), MIC (12.50 mg/mL) and 2 × MIC (25.00 mg/mL). According to the results of the time kill profiling, as shown in Figure 3(a), it is observed that *A. conchigera* rhizome extract at the concentration of 2 × MIC showed bactericidal effect at the early hours of the incubation period (8$^{th}$ h) while *A. conchigera* rhizome extract at the concentration of MIC showed the same effect as 2 × MIC but took a longer duration at 12$^{th}$ h but showed regrowth of *S. pneumoniae* isolate. ACA showed total killing effect at 4$^{th}$ and 8$^{th}$ h when ACA with the concentration of 2 × MIC and MIC were tested against *S. pneumoniae* isolate respectively (Figure 3(b)). However, both *A. conchigera* rhizome extract and ACA at the concentration of ½ MIC did not exhibit any inhibitory or killing effect against the bacteria but the growth of *S. pneumoniae* isolate was slightly reduced compared to the inoculum control over 24 h. Based on the time kill profile, both extracts exhibited bactericidal effect with the reduction of 99.9% ($\geq 3$ $\log_{10}$) of total number of colonies forming units per mL compared to the inoculum control. It can also be said that both extracts were dose dependent because at higher concentration, the extracts took shorter time to kill the *S. pneumoniae* isolate.

### MORPHOLOGICAL CHANGES OF *S. pneumoniae* ISOLATE AFTER ACA TREATMENT

Based on the time kill profiling results, ACA with the concentration of 12.50 mg/mL (MIC value) reached bacteriostatic level after incubation for 4 h with the reduction of < 3 $\log_{10}$ CFU/mL from initial growth. The morphology of *S. pneumoniae* isolate treated with ACA was compared to the non-treated (Figure 4). It is observed that the *S. pneumoniae* isolate was in lancet shape, diplococci, and arranged in a chain after 4 h of incubation (Figure 4(a)). However, the morphology and structure of *S. pneumoniae* isolate changed after ACA treatment (Figure 4(b)). Upon 4 h of exposure, *S. pneumoniae* isolate showed wrinkled cell walls. Some of the bacteria lose their lancet shape and diplococci characteristics, as well as shrinking and lysed cells. This result indicated that ACA has the function to disrupt the cell wall of *S. pneumoniae* isolate, which can significantly impact the pathogenicity of this bacterial strain. The cell wall disruption can lead to modification of peptidoglycan and teichoic acid that play important roles in bacterial resistance towards antibiotics and antibacterial agents. Besides that, the cell wall of *S. pneumoniae* also helps in the adherence and attachment to the host cells (Rajagopal & Walker 2017; Zhou et al. 2022).

EXPRESSION ANALYSIS

RT-qPCR was performed on selected virulence genes that play a major role in the pathogenicity of *S. pneumoniae*. The selected genes were compared to the reference gene 16S rRNA of *S. pneumoniae*. The relative expression is the fold change of target genes in *S. pneumoniae* isolate treated with 6.25 mg/mL of ACA to non-treated *S. pneumoniae* isolate with the normalization to the reference gene, 16S rRNA. The relative expression of selected genes in treated samples, *nanA, pavaA,* and *psaA* were recorded in Table 5. The results showed that both genes, *pavA* and *psaA* were significantly downregulated ($p<0.05$), whereas the *nanA* gene, which involved in biofilm formation was significantly upregulated ($p<0.05$).

Biofilm is one of the important virulence factors of *S. pneumoniae* that reduced the susceptibility of *S. pneumoniae* to antimicrobial agents and increase the ability to evade host immune system (Domenech, García & Moscoso 2012). *S. pneumoniae* exhibited its first line of defence by upregulating the *nanA* gene to increase the formation of biofilm (Zhou et al. 2015). However, based on the observed morphological changes in the *S. pneumoniae* isolate, the cell wall of these bacteria was disrupted upon treatment with 6.25 mg/mL of ACA. This suggests that the upregulation of the *nanA* gene may be due to biofilm formation as a byproduct, as the bacteria were inhibited and killed by the treatment, leading to the release of biofilm components to the outer cell membrane. Other two selected genes, *pavA* and *psaA* were downregulated upon treatment to ACA. Both genes play important roles in the adherence and attachment of *S. pneumoniae* to the host cells (Novick et al. 2017). These results were in the agreement with morphology study that displayed the cell wall disruption of *S. pneumoniae* when treated with ACA.

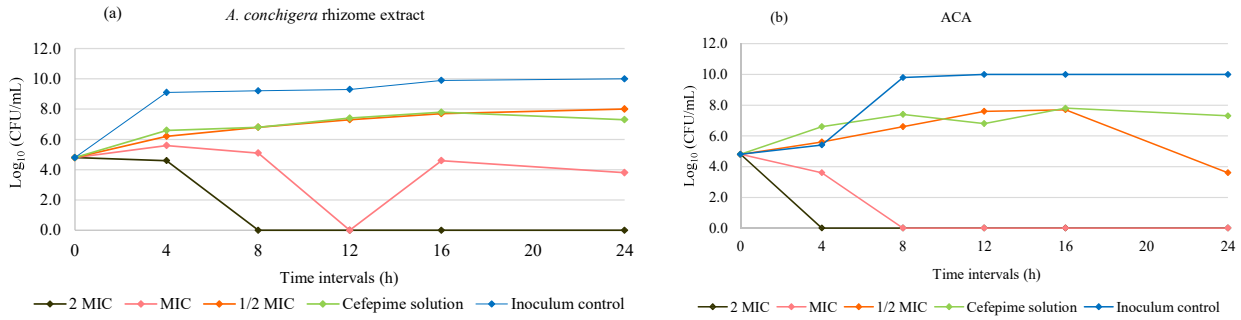


FIGURE 3. Time-kill kinetic graph of $\log_{10}$ CFU/mL versus time for (a) A. conchigera rhizome extract and (b) ACA against S. pneumoniae isolate

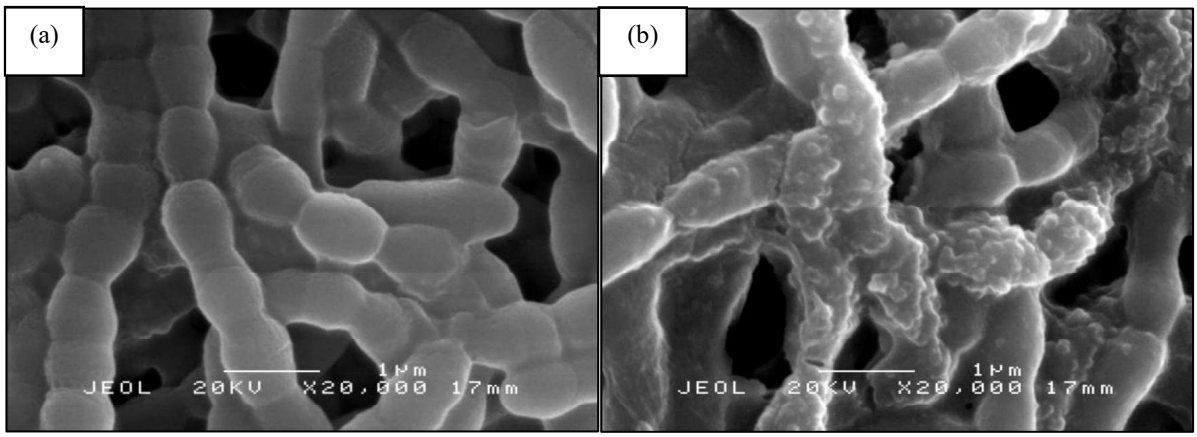


FIGURE 4. (a) Non-treated S. pneumoniae isolate, and (b) S. pneumoniae isolate treated with ACA (12.50 mg/mL)

TABLE 5. Selected genes that displayed altered expression after treated with ACA (6.25 mg/mL)

| Gene of interest | Description | Fold change ± SD | *p*-value |
|---|---|---|---|
| *nanA* | Biofilm formation | 5.33 ± 0.21 | 0.007 |
| *pavA* | Adherence of *S. pneumoniae* to the host cell | -0.87 ± 0.54 | 0.009 |
| *psaA* | Attachment of *S. pneumoniae* to the host cell | -0.52 ± 0.14 | 0.001 |

## CONCLUSION

In conclusion, *A. conchigera* rhizome extract and ACA possess antibacterial activity which can inhibit and kill the growth of *S. pneumoniae* isolate. Besides that, ACA showed the ability to cause structural and morphological changes such as cell wall disruption when treated against *S. pneumoniae* isolate. The result of RT-PCR analysis suggests that ACA inhibit the expression of *pavA* and *psaA* genes, which are responsible in the adherence and attachment of the bacteria to the host cells. These findings provide new insight that both *A. conchigera* rhizome extract and ACA have a great potential to be used as alternative antibacterial treatment against *S. pneumoniae.*

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