Anti-MRSA activity of ethyl acetate crude extract from endophytic fungus *Ceratobasidium ramicola* IBRLCM127 isolated from rhizome of *Curcuma mangga* Valeton & Zijp

Anti-MRSA activity of ethyl acetate crude extract from endophytic fungus *Ceratobasidium ramicola* IBRLCM127 isolated from rhizome of *Curcuma mangga* Valeton & Zijp

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Abstract. The study was conducted to investigate the effects of ethyl acetate crude extract of *C. ramicola* IBRLCM127, an endophytic fungus which was previously isolated from rhizome of *C. mangga* against a common human bacterial pathogen, methicillin-resistant *Staphylococcus aureus* (MRSA) in order to develop a new MRSA treatmet. The efficiency of antimicrobial compounds in inhibiting or killing the bacterial cells was evaluated by adopting minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The effect of ethyl acetate crude extract on the growth profile of MRSA was examined using time-kill assay. Scanning electron microscope (SEM) observation was opted to investigate the ultrastructural effect of MRSA cells. The results revealed that fungal extract demonstrated a bactericidal effect on MRSA with the ratio of MBC/MIC was 1. Both MIC and MBC values of the extract were 0.5 mg/mL. The result obtained from the time-kill study disclosed that the bactericidal activity of fungal extract under investigation was both time and concentration-dependent. After 12 hours of exposure to the extract, the formation of cavities and a few cell debris can be observed on the bacterial cells, indicating the failure of cell wall and cell membrane to maintain their rigid structure due to the rupture caused by the extract. Prolonged exposure to the extract for up to 48 hours caused the bacterial cell wall to lyse and release its cytoplasmic content into the surrounding which led to cell death. Based on the SEM observation, the fungal ethyl acetate extract of *C. ramicola* IBRLCM127 exhibited a prominent anti-MRSA activity particularly against cell membrane of MRSA cells. This report was the first report concerning the antimicrobial potential of endophytic fungus *C. ramicola* recovered from local medicinal plant, *C. mangga*.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* is an established pathogenic strain originated from *S. aureus* which developed resistance to methicillin and causes community and nosocomial-acquired infections globally [1]. The resistance of *S. aureus* infections to methicillin is caused by the acquisition of mecA or mecCgene, a gene that responsible for protein encoding of which can overcome the challenge by a wide array of β-lactam antibiotics [2]. This human pathogenic bacterial strain infected skins, joints, bones and soft tissues as well as patients with implant such as prostheses or catheters and caused high morbidity and mortality rate with poorer outcomes clinically despite the improvement in identification and prevention techniques of MRSA [3]. Local medicinal herb, *Curcuma mangga* or also known as 'temu pauh' in Malaysia is one of the understudied species

compared to other Curcuma species and it possess hidden potential of pharmacological activities such as analgesic, antioxidant, anticancer, anti-inflammatory, antiproliferation and antimicrobial effects [4, 5]. Since potential microbial community such as endophytes have been reported to harbor in most of terrestrial plant species, these endophytes have been depicted as hidden source of novel bioactive compounds with antimicrobial activity that can overcome the dilemma of bacterial resistance to antibiotics. The capability of endophytic fungi to mimic their host plant bioactive metabolites can be exploited to upscale the production of these valuable metabolites in a laboratory. Thus, in the current study, the inexhaustible supply of endophytic fungus *Ceratobasidium ramicola* isolated from rhizome of *C. mangga* was chosen over its host plant due to the plant biodiversity conservation issue. The ethyl acetate extract of this endophytic fungus that was reported previously to possess an outstanding anti-candidal activity [6] was also expected to demonstrate a promising antibacterial activity since endophytic fungus itself was capable to produce the similar bioactive metabolites as their host plant [7]. Ethyl acetate was chosen as a solvent of extraction for the fungal extract as it is capable of attracting hydrophilic and lipophilic compounds [8]. To the best of our knowledge, none of the report available on antibacterial potential of endophytic fungus *C. ramicola.* Thus, this study was intended to investigate the effect of ethyl acetate crude extract derived from endophytic fungus *C. ramicola* IBRLCM127 which was previously isolated from rhizome of *C. mangga* towards the growing of MRSA cells.

2. Materials and Methods

2.1. Fungal culture maintenance

The fungal isolate of *C. ramicola* IBRLCM127 was provided by the Industrial Biotechnology Research Laboratory (IBRL) culture collection, Universiti Sains Malaysia, Penang, Malaysia. The isolate was cultured and maintained routinely once a month on potato dextrose agar (PDA) (Oxoid, UK) incorporated with host plant powder (2.0 g/L) and incubated for 7 days at 30 ^{o}C.

2.2. Culture medium

Yeast extract sucrose broth (YES) (Oxoid, UK) consists of (g/L): yeast extract, 20; magnesium sulphate, 0.5 and sucrose, 40 was mixed with 1000 mL host plant extract [9]. The culture medium mixture was then adjusted to 5.8 ± 0.2 and autoclaved for 15 minutes at 121 ºC prior to be used as a medium to cultivate fungal isolate in a shake-flask system.

2.3. Cultivation and extraction

Two mycelial of 7 days old agar plugs (diameter = 1.0 cm, thickness = 4.0 mm) were excised from the periphery plate and transferred into 100 mL of YES broth in Erlenmeyer flask followed by incubation according to the designated conditions of temperature (30^{0} C), agitation speed (120 rpm) and incubation time (20 days) in dark condition [9]. The fungal biomass and fermented broth were then separated out using filter paper (Whatman, No. 1). Filtered broth was extracted using ethyl acetate thrice using the similar volume (1:1, v/v) and only the organic phase was chosen to be dried and concentrated using rotary evaporator to yield fungal ethyl acetate extract.

2.4. Test microorganisms

The isolate of MRSA ATCC 33591 was obtained from IBRL, Universiti Sains Malaysia, Penang, Malaysia. The bacterial culture was grown on nutrient agar (Oxoid, UK) and incubated for 24 hours at 37^{o} C. The preparation of bacterial inoculum was done by picking up 2-5 single colonies from the freshly cultured plate above and suspended into 0.85% sterile physiological saline (5.0 mL) (w/v) and turbidity was adjusted to match 0.5 McFarland standard.

2.5. MIC and MBC values

Broth microdilution was used to obtain the value of minimal inhibitory concentration of fungal extract [10] using a sterile 96-well microtiter plate (TPP, Switzerland). Mueller Hinton Broth (Oxoid, UK) was used in the preparation of extract with single-fold dilution. The extract concentration ranges were 20 to 1 000 µg/mL. A total amount of 100 µL of the prepared extract and 100 µL of bacterial inoculum was then dispensed into each well to make up a final volume of 200 µL with a bacterial final concentration in each well was 1x 10^7 CFU/mL. A reference drug and control used in this study were chloramphenicol (Sigma-Aldrich, USA) and the mixture of 5% methanol and bacterial inoculum [11]. The microtiter plate was incubated for 24 hours at 37^0 C followed by the addition of preprepared 40 µL of 0.2 mg/mL ethanolic p-iodonitrotetrazolium violet (INT) (Sigma-Aldrich, USA) into each well as a growth indicator. The MIC value refers to the fungal extract with the smallest value of concentration that able to retard the bacterial growth after 24 hours [12]. Determination of minimal bactericidal concentration was done upon obtaining the MIC value. The enumeration of viable cells using Mueller Hinton Agar (MHA) (Oxoid, UK) was conducted according to standard viable plate count after incubation period of 24 hours at 37^0 C. The MBC was recorded as the lowest concentration of extract that caused 99.9% of bacterial growth reduction relative to the control.

2.6. Time-kill curve study

The time-kill curve study was conducted based on the method proposed by Taufiq and Darah [11]. The extract concentration of 1/2MIC, MIC and 2MIC values of MRSA were employed in this study. Prior to that, MIC value of MRSA (500 µg/mL) was used. Bacterial suspension (approximately 1x 10^8 CFU/mL) with a volume of 0.1 mL was added into 19.9 mL of MHB in Erlenmeyer Flask with different extract concentrations to yield the initial concentration of 1 x 10^7 CFU/mL of bacterial inoculum. Bacterial inoculum and 1% methanol (methanol; v/v) in MHB served as a control. The prepared cultures were then incubated in an orbital shaking incubator (Lab-Companion, Korea) for optimum aeration using temperature, incubation period and agitation speed of 37 ^{o}C, 48 hours and 150 rpm, respectively. A total volume of 0.1 mL of the aliquot was taken out every 4 hours for 48 hours for viable cell count. A curve for time-kill study (log CFU/mL vs time) was then plotted for all extract concentration used in the study including the control. The growth reduction (time to reduce 50%, 90% and 99.9% of bacterial cells) was calculated using equation 1. The experiment was performed in three replicates on separate occasions.

$$\text{Growth reduction} = \frac{V_i - V_z}{V_z} X\ 100 \quad (1)$$

whereby, V_i = initial viable cell count, V_z = viable cell count at z time

2.7. Scanning electron microscope (SEM) study

A treatment bacterial sample was prepared by transferring 0.5 mL of 1 x 10^8 CFU/mL of bacterial inoculum into Erlenmeyer flask with 9.0 mL of MHB in it and incubated in a shaker according to temperature, agitation speed and incubation period of 37^o C, 150 rpm and 18 hours, respectively. After that, 0.5 mL of extract at 2MIC concentration (bactericidal concentration) (1 000 µg/mL) was added into the bacterial culture to obtain a final concentration of 1000 µg/ mL with volume of 10 mL. A control was also included consisting of a mixture of 0.5 mL of 20% DMSO (v/v) and 9.5 mL of bacterial culture. The mixtures were then subjected to incubation at 150 rpm and 37^o C for 36 hours. During that time, the cultures were subjected to the harvesting process at 0, 12, 24 and 36 hours followed by SEM study [13].

2.8. Statistical analysis

ANOVA test was chosen in this study to determine the statistical difference of different fungal extract concentrations effect on the growth of MRSA in time-kill assay. All tests involved were conducted

separately in three replicates. The results from time kill assay were then recorded as the mean ± standard deviation (SD) and $p<0.05$ were accepted as statistically significant.

3. Results

3.1. MIC and MBC values

The ethyl acetate crude extract result for susceptibility test is shown in Table 1. Based on the result, the values of MIC and MBC are similar (500 µg/mL), showing that the similar extract concentration was required either to inhibit bacterial growth or kill their cells. The ratio of MBC/MIC was 1 (less than 4), thus the fungal extract showed bactericidal effect against MRSA.

Table 1. The values of MIC and MBC of fungal ethyl acetate crude extract towards MRSA.

Test microorganisms	MIC (µg/mL)	MBC (µg/mL)	Ratio (MBC/MIC)
Methicilin-resistant *S. aureus* (MRSA ATCC33591)	500	500	1 (Bactericidal)

3.2. Time-kill study

The time-kill study result of fungal crude extract of *C. ramicola* IBRLCM127 against MRSA ATCC 33591 at concentrations under investigation (1/2MIC=250 μg/mL; MIC=500 μg/mL; 2MIC=1000 μg/mL) and control are depicted in Figure 1. The control curve of MRSA exhibited a typical bacterial growth curve consisting lag, exponential and stationary phases. Bacterial cells were experiencing a lag phase within 4 hours of incubation time and then increased dramatically (exponential phase) until 16 hours where it began to enter the stationary phase that had been maintained up till 48 hours.

When the MRSA cells treated with ½ MIC of fungal extract, the similar growth curve pattern to the control was also observed but with a reduction in the viable cell counts. The cells growth was delayed for the first 16 hours (lag phase) probably due to the low concentration of extract used and after that it started to increase steadily (exponential phase) until 24 hours. After 24 hours of treatment, the MRSA cells were entering a stationary phase until 32 hours followed by dead phase thereafter. Meanwhile, for the killing curve at the MIC values, the curve was found to be almost flat and the viable cells at the end of incubation was statistically similar as in initial inoculum. For 2MIC, there was a rapid decline in viable cell count where no bacterial regrowth observed after 44 hours exposed to the fungal extract. The result reflected that at higher concentration, the fungal extract demonstrated bactericidal effect against MRSA ATCC 33591 as well as concentration and time of exposure dependent.

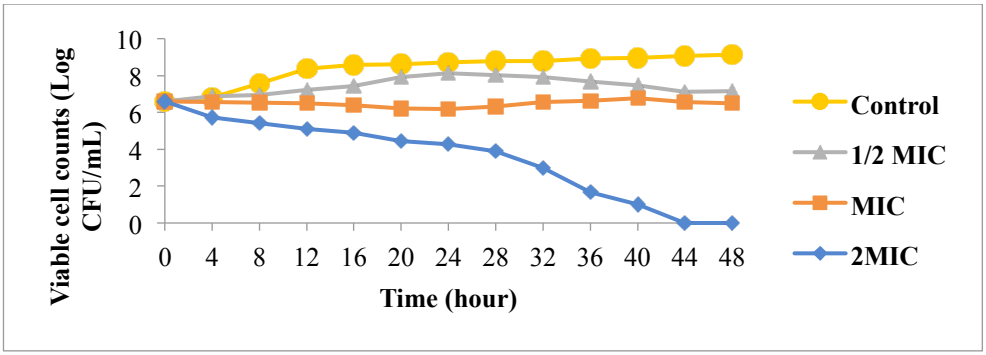

Figure 1. Time-kill study of the ethyl acetate crude extract of *C. ramicola* IBRLCM127 towards MRSA ATCC33591.

The time taken to achieve 25, 50, 90, 99, and 99.9% growth reduction in initial inoculum of bacterial cells is shown in Table 2. The longer incubation period was needed to achieve a higher percentage of reduction in bacterial growth. The higher concentration of fungal extract was also

required to inhibit or kill the bacterial cells with twice MIC concentration showed the reduction in cell viability. Extract at 2MIC concentration was also able to kill the bacterial cells for up to 99.9%.

Table 2. Time taken to achieve 25, 50, 90, 99, and 99.9% growth reduction in initial inoculum of MRSA ATCC33591.

Percentage of reduction (%)	Time (h)			
	Control	½ MIC	MIC	2MIC
50	NR	NR	16-20	0 - 4
90	NR	NR	NR	4 – 8
99	NR	NR	NR	16 – 20
99.9	NR	NR	NR	28 – 32

3.3. Structure degeneration and morphological changes of the extract treated bacterial cells

SEM study was adopted to observe the effects of bacterial cell membrane structure degeneration after ethyl acetate extract treatment. The SEM images revealed that the fungal fungal extract was significantly ($p<0.05$) affected morphologically of the microbial cells (Figure 2), based on the micrograph comparison between treated cells and control (untreated) cells. Figure 2A shows the control cells of MRSA where the intact coccal shaped and smooth cell surface still maintained their rigidity. However, after 12 hours exposed to the fungal extract at 2MIC concentration, cavities formation (dotted arrow) and a few cell debris (white arrow) can be observed on the bacterial cells (Figure 2B). Figure 4C shows that the bacterial cells were started to clump together (dotted arrows) after 24 hours of exposure time to the extract. A total damage of the bacterial cell structures can be observed after 48 hours of exposure time (Figure 4D). At this stage (36 hours), most of the bacterial cells lost their coccal shape, underwent lysis, shrunk suddenly and totally collapsed with the invagination of the cell which could subsequently led to the leakage of cytoplasm. The lysed bacterial cell wall could possibly cause the cytoplasmic content to be released into the surrounding which caused the total cell collapsed and left only the cell debris (dotted arrows). Ultimately, the bacterial cell death occurred completely.

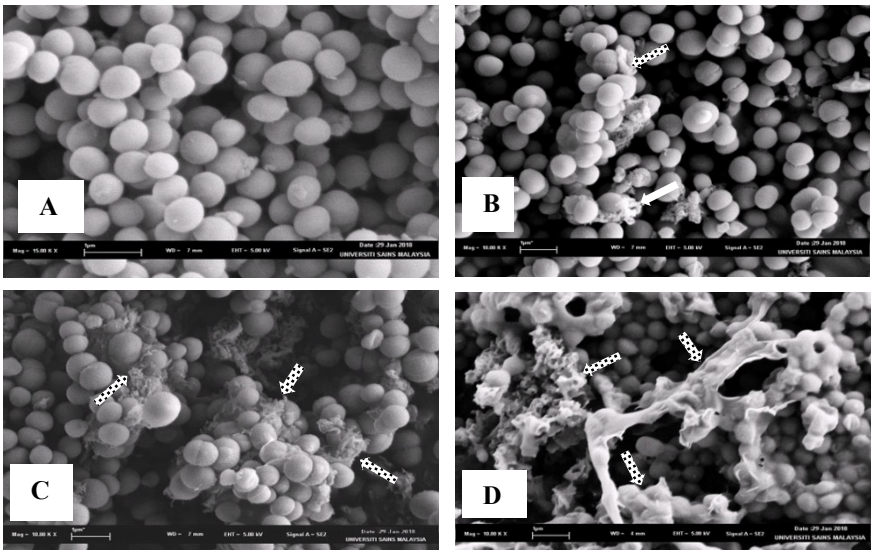

Figure 2. SEM photomicrographs of the MRSA ATCC 33591 cells treated with fungal extract of *C. ramicola* IBRLCM127 at different times of exposure, (A) 0 h, (B) 12 h, (C) 24 h, (D) 36 h. Scale bars: 200 nm.

4. Discussion

The emergence of resistant pathogenic bacteria to the existing commercial antibiotics due to the excessive prescription and incorrectly prescribed antibiotics is the greatest public health threats [14, 15]. Thus, a novel source of antibiotic production particularly by manipulating new and underexplored niches and habitats is needed to tackle this growing problem to replace the existing antibiotics which bacteria has become resistant to them [16]. In search of novel anti-MRSA compounds, endophytic fungus *C. ramicola* IBRLCM127 residing in the rhizome of *C. mangga,* Malaysian medicinal herb was isolated. Medicinal herbs are documented to be an arsenal of biologically active compounds and thus very well protected chemically [17]. Any endophytes that capable to withstand plant chemical weapons for their colonisation purpose are expected to possess unusual metabolites as it can overcome the plant defense system and subsequently forming a symbiotic relationship with the host plant [18]. Thus, these potential metabolites can be further explored for novel antibiotic source. The selection of endophytic fungus over its host plant was due to the fungal growth can be mass propagate in a laboratory and the fermentation conditions can be manipulated to yield an inexhaustible supply of fungal bioactive metabolites.

MIC and MBC are used to determine the *in vitro* antibacterial activity by incubating microtiter plate aerobically overnight according to standard protocols [10]. MIC is defined as the minimal concentration of antimicrobial compounds that keep a clearing view of 10^5 colony forming units (CFUs) of bacteria/mL and preventing the suspension from becoming turbid after aerobically incubation overnight. MBC is complementary to MIC and it refers to the smallest value of concentration of antimicrobial compounds with bactericidal effect. MBC is determined by subculturing broth dilutions that prevents the bacterial cell growth either at or above MIC level by streaking it onto agar followed by incubation at 37^0 C for 24 hours. Fungal ethyl acetate crude extract of *C. ramicola* IBRLCM127 was found to be bactericidal against MRSA cells due to the low ratio of MBC/MIC (equivalent to 1), indicating that MBC value was one-fold higher than MIC [11]. Bactericidal drugs are the drugs with MBC values not exceeding four-fold of the MIC values [19].

Time kill study serves as a tool to provide an important information on the rate, concentration and potential actions of antimicrobial drugs *in vitro* [20] and also provides additional information about the time-dependent or concentration-dependent drugs [21]. Current results corroborate with previous studies [22, 23] where 2MIC demonstrated high killing effect at longer exposure period to the extract.In the current study, longer exposure time was required to completely killed the bacterial cell because the extract used was actually non-purified extract. Thus, the exact concentration of extract used could be lower than 2MIC value. Significant reduction of cell counts (p<0.05) for 2MIC after 4 hours of incubation also indicates that the fungal extract was strongly bactericidal, congruent with the study conducted by Perim et al. [24]. The current result suggests that time kill analysis provides a quantitative assessment of pharmacodynamic parameters beyond that of MIC and can be used to evaluate the effect of new candidate of antimicrobial agent or combination of agents in antibiotic therapy.

Crude natural product extracts have been widely reported to possess antimicrobial potential where its main target of action are microbial cell membrane permeability and cell wall biosynthesis [11]. SEM photomicrographs from the current study revealed that the effect of fungal extract on MRSA cells were time dependents, of which longer exposure to the extract causes the cavities formation or craters on microbial cells' surfaces which indicating cell wall and cell membrane failure in maintaining their rigidity structure. The severity of the cells' deformation worsen with the increasing of time of exposure to the extract . In this stress condition, Ron [25] emphasized the secretion of sticky mucus occurred which could caused the bacterial cells to clump together. Bible et al. [26] also added that the clumping of bacterial cells was a special metabolic scavenging strategy of the cell as a preparation to withstand further stress condition. As a result, the normal cellular functions such as cell division and replication, respiration and metabolism were started to be affected [27]. Generally, cell envelop of Gram-positive bacteria consists of cell wall and cell membrane [28]. The disruption of

bacterial inner membrane that is responsible for many crucial functions such as the process of osmoregulation, transport respiration, lipids synthesis and biosynthesis and cross linking of peptidoglycan can lead to the metabolic dysfuntion, formation of pores and cell death [29]. Additionally, the absence of outer membrane in Gram positive bacteria such as MRSA also cause them to become more sensitive towards the extract [30, 31].

5. Conclusion

The current result disclosed that fungal ethyl acetate extract of *C. ramicola* IBRLCM127 demonstrated antibacterial potential towards MRSA ATCC 33591 with concentration and time dependents. The main targets of fungal extract were bacterial cell wall and cell membrane that ultimately led to the total cell death completely. The findings may be important for the production of naturally derived anti-MRSA agents on an industrial scale.

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