



SHORT COMMUNICATION

An assessment of the acetone extract from the leaves of *Canarium odontophyllum* (Miq.) for antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes nosocomial and community infections and is a global health concern. This study evaluated the antibacterial activity of acetone extract from *Canarium odontophyllum* leaves against MRSA ATCC 33591 and Mu50 strains. The MIC/MBC ratio, determined using the broth micro dilution method, revealed the bacteriostatic effect of the extract against both strains. Time-kill assay against the Mu50 strain showed that the extract inhibited the growth of MRSA at low concentration but exhibited a concentration-dependent bacterial killing effect at 4x MIC. These findings confirm that an acetone extract from *C. odontophyllum* leaves inhibited growth of MRSA at low concentrations and could be utilised as an alternative anti-MRSA agent.

Keywords: Bacteriostatic, *C. odontophyllum*, MBC, MIC, MRSA

INTRODUCTION

Transmission of infectious diseases is a significant burden to the economy and to community health. Most pathogens that are involved in the transmission of infectious diseases represent multi-drug resistant strains (Jones *et al.*, 2008). Pathogenic bacterial infections are the major cause of increased mortality and morbidity in hospitals (Rice, 2006). *Staphylococcus aureus* frequently colonises the surface of the outer skin and upper respiratory tract, especially the nasal tract (Stapleton *et al.*, 2002). As a virulent pathogen, *S. aureus* accounts for the high mortality rate in patients with pneumonia, endocarditis, sepsis and urinary tract infection (Kollef *et al.*, 2006).

In the 1950s, methicillin was introduced to treat *S. aureus* infection. Unfortunately, after several years, resistance of *S. aureus* to methicillin was discovered (Crum *et al.*, 2006). Methicillin is a β -lactam antibiotic that interferes with the activity of penicillin-binding protein required for the synthesis of peptidoglycan in *S. aureus* (Shurland *et al.*, 2007). The *mecA* gene is located within the bacterial chromosome which allows the cell to transfer it to other cells and is regulated by two recombinase genes, the *ccrA* and *ccrB*. Methicillin resistance in *Staphylococcus aureus* is observed when the *mecA* gene is present along with *ccrA* and *ccrB* which facilitate

the expression of the *mecA* gene, the latter inhibits penicillin-binding protein (PBP) production and makes antibiotic binding more difficult. The presence of *mecA* gene that encodes for PBP2a is used as a benchmark for detection of methicillin-resistant *S. aureus* (Rachman *et al.*, 2017).

The significance of methicillin-resistant *Staphylococcus aureus* (MRSA) infection cannot be underestimated, as treatments are ineffective and it is associated with increased morbidity, mortality, hospital admission and healthcare cost (Cosgrove *et al.*, 2005). MRSA also shows high resistance rates against tetracycline, clindamycin, cotrimoxazole, rifampicin, macrolides and fluoroquinolones (Kaleem *et al.*, 2010).

Therefore, many studies have been carried out to identify alternative treatments to curb the problem of MRSA resistance, especially on the use of natural products. Plants offer a diverse reservoir of biologically active components as potential therapeutic agents, including as antimicrobials. *Canarium odontophyllum* is locally known as 'Borneo olive' or 'dabai'. It belongs to the Burseraceae family and the genus is *Canarium L.* (Mogana and Wiart, 2011). The tree can be found in Sumatra, Borneo and the Philippines (Khoo *et al.*, 2012). Secondary metabolites contained in the extract of *C. odontophyllum* leaves, such as tannins, flavonoids, terpenoids, saponins and phenols, contribute to

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antibacterial (Basri and Nor, 2014a) including anti-MRSA activities (Basri and Sandra, 2016a).

An acetone extract of *C. odontophyllum* leaves was previously shown to exhibit bacteriostatic action with prolonged and persistent antimicrobial effect against MRSA ATCC 33591 (Basri *et al.*, 2016b). Therefore, in the present study, different concentrations of the extract were used to investigate the pharmacodynamics of the active compounds in the extract, and to give insight into the mode of action of the acetone extract of *C. odontophyllum* leaf against MRSA.

MATERIALS AND METHODS

Plant material

The leaves of *C. odontophyllum* were purchased from Kuching, Sarawak and assigned the voucher specimen number UKMB 40052. The whole leaf was used to prepare the extract (Basri and Nor, 2014a). The stock solution was prepared by completely dissolving the residue of the acetone extract of 100 g of *C. odontophyllum* leaves in absolute acetone using a vortexer. A stock solution of the test material was prepared at a concentration of 20 mg/mL and stored at 4 °C. The working solution was prepared by calculating the appropriate dilution of the stock solution. The working solution was sterilised using a 0.45 µm pore sized membrane filter before it was tested.

Chemicals

Vancomycin and linezolid powdered reagents were obtained from Sigma Aldrich (St. Louis, MO, USA). The antibiotic stock solutions were prepared at a concentration of 10 mg/mL. The vancomycin powder was dissolved in sterile distilled water, and linezolid was dissolved in 10% DMSO and stored at 4 °C. The working solution was prepared by calculating the appropriate dilution of the stock solution, and the solution was sterilised using a 0.2 µm pore sized membrane filter prior to the test.

MRSA strains

Reference (ATCC 33591) and clinical (MRSA Mu50) strains were obtained from the collection of Biomedical Science Programme, the Faculty of Health Sciences, UKM and stored in the Microbiology Laboratory at University Kebangsaan Malaysia Medical Centre. The stock cultures were grown on Mueller-Hinton agar (MHA) and incubated at 37 °C for 24 h to obtain isolated colonies. The bacterial inoculums were prepared by transferring one or two single colonies of the same morphology into Mueller-Hinton broth (MHB) using a sterile wire loop followed by 24 h incubation at 37 °C. The estimated concentration of 10⁸ CFU/mL was determined at an optical density of 0.08 at a wavelength of 625 nm. The inoculum suspension was adjusted to obtain 10⁶

CFU/mL. The bacterial suspension was used within 30 min.

Determination of the minimum inhibitory concentration (MIC)

The MIC value of the extract was determined using the microbroth serial dilution method, with a final inoculum of bacteria of approximately 10⁶ CFU/mL. First, 50 µL MHB was added to each well of a 96-well microtiter plate. Then, 50 µL of the analyte working solution was added to the first well and diluted two-fold. Finally, 50 µL of bacterial suspension was added to each well so that the final volume in each well was 100 µL. Negative controls were wells containing only MHB and the analyte, whereas the positive control wells contained only MHB and the bacterial suspension. The MIC value was the lowest concentration of the analyte that did not show any growth of the bacteria after incubation at 37 °C for 24 h (Amman *et al.*, 2011). For confirmation, 20 µL triphenyl tetrazolium chloride (TTC) was added to each well. TTC at a concentration of 2 mg/mL and a volume of 20 µL was added to each well and incubated for 20 min. Wells that appeared pink were interpreted as positive for bacterial growth, whereas colourless wells were interpreted as negative for bacterial growth (CLSI, 2012). The tests were conducted in triplicate.

Determination of minimum bactericidal concentration (MBC)

The MBC value of the analyte was the lowest concentration that did not show any bacterial growth when subcultured on agar. The suspension in the wells that showed no visible growth of bacteria in the microtiter plate was transferred to MHA plates and incubated for 24 h at 37 °C (CLSI, 2012). The tests were conducted in triplicate.

Time-kill assay (TKA) analysis

The TKA analysis was conducted using the broth macro-dilution technique. A universal bottle that contained 10 mL of bacteria with approximately 10⁶ CFU/mL of inoculum was treated with the agents at different concentrations. Untreated bacteria were used as the growth control. Viable counts were performed at 0, 2, 4, 6, 8 and 24 h (incubation at 37 °C) after adding the treatment agent. In each subsequent hour, 100 µL of sample was taken from the universal bottle and serially diluted ten-fold with normal saline (0.9% NaCl). Then, 50 µL was dispensed as five evenly spaced drops, 10 µL per drop, onto the designated quadrant of MHA agar plate in duplicate. After the drops on the agar were completely dry, the plates were incubated at 37 °C for 24 h. Bacterial colony count between 3-30 CFU/cm² for each drop was determined (Herigstad *et al.*, 2001). Time-kill curves were constructed by plotting the log₁₀ CFU/cm² on the x-axis and time (h) on the y-axis. A compound was considered as bactericidal if it reduced bacterial concentration by 3 log₁₀

CFU/cm² during the incubation period or killed 99.9% of the starting inoculum (Barber *et al.*, 2014).

RESULTS

Determination of MIC and MBC

The MIC and MBC values of the acetone extract, vancomycin and linezolid against MRSA ATCC 33591 and MRSA Mu50 are shown in Table 1. The MIC and MBC values of the acetone extract of *C. odontophyllum* against MRSA Mu50 were 312.5 µg/mL and 625 µg/mL respectively, whereas against MRSA ATCC 33591 the values were 625 µg/mL and 1250 µg/mL respectively. Vancomycin had MIC equal to MBC, which were 3.91 µg/mL against MRSA Mu50 and 0.98 µg/mL against MRSA ATCC 33591. The MIC and MBC values of linezolid against both strains were 1.56 µg/mL and 6.25 µg/mL, respectively.

Table 1: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the acetone extract of *C. odontophyllum* leaves, vancomycin and linezolid against MRSA ATCC 33591 and MRSA Mu 50.

Antimicrobial agent	MRSA strain	MIC (µg/mL)	MBC (µg/mL)
Acetone extract	ATCC 33591	625	1250
	Mu 50	312.5	625
Vancomycin	ATCC 33591	0.98	0.98
	Mu 50	3.91	3.91
Linezolid	ATCC 33591	1.56	6.25
	Mu 50	1.56	6.25

TKA analysis

The time-kill curves are presented in Figure 1. MRSA Mu50 treated with 1/2x MIC of the acetone extract of *C. odontophyllum* leaves showed a slight increase in bacterial colony count that was lower than the growth control after a 24 h incubation period. MRSA Mu50 showed inhibited growth after the first 8 h in response to 1x MIC; however, the reduction was < 3 log₁₀ CFU/cm². The 2x MIC resulted in a < 3 log₁₀ CFU/cm² colony count throughout the 24 h incubation. However, the acetone extract of *C. odontophyllum* leaf at 4x MIC showed a bactericidal effect by a 3 log₁₀ CFU/cm² reduction after exposure to MRSA Mu50 for 24 h. Linezolid and vancomycin showed static curves with reductions of < 3 log₁₀ during the incubation period.

DISCUSSION

MRSA are strains of *S. aureus* that are resistant to methicillin and most β-lactam antibiotics, such as penicillin, cephalosporins and carbapenems (APIC, 2010). Increased antibiotic resistance has caused a huge clinical challenge to treat infectious diseases (Ansari *et*

al., 2014). Therefore, natural products have been used as alternatives to treat infections over the years (Wright *et al.*, 2007). Some natural products have antimicrobial potential against MRSA (Razmavar *et al.*, 2014; Ishak *et al.*, 2016).

C. odontophyllum is a candidate for the control of MRSA infection (Basri *et al.*, 2014b). The *in vitro* antimicrobial activity of drugs is usually assessed by determining their MIC and MBC values after incubation with a bacterial inoculum. For bactericidal drugs, the MBC is usually the same as their MIC. In contrast, the MBCs of bacteriostatic drugs are many-fold greater than their MICs (Levison and Levison, 2009). In a previous study, the acetone leaf extract of *C. odontophyllum* showed bactericidal activity against MRSA ATCC 33591 with an MBC value equivalent to MIC (Basri and Sandra, 2016a) which was in agreement with Basri *et al.* (2016b), who showed bactericidal activity against MRSA ATCC 33591. The finding of the present study however, disputed the previous literature (Basri and Sandra, 2016a; Basri *et al.*, 2016b) as the acetone leaf extract from *C. odontophyllum* demonstrated bacteriostatic effect against both MRSA strains. The difference in antibacterial activity in this study may be due to variability of the strains. According to Monte *et al.* (2014), some phytochemicals contained in extracts exhibit significant potential to alter antibiotic resistance. Secondary metabolites, such as saponins, terpenoids, tannins, flavonoids and phenolic compounds, contained in *C. odontophyllum* leaves have activity against MRSA (Basri and Nor, 2014a). MRSA is a Gram-positive bacterium comprised of a mesh-like peptidoglycan layer that allows permeation (Biswas *et al.*, 2013).

The MIC assay results indicated that MRSA Mu50 was more susceptible towards the extract than MRSA ATCC 33591. The acetone extract was less potent compared to the standard antibiotic, which may be due to the variety of bioactive compounds such as flavonoid, tannin and terpenoid that are present in the crude extract compared to standard antibiotics that contain the active, pure compound (Gatsing *et al.*, 2010).

The TKA is utilized in microbiological studies to assess the *in vitro* antimicrobial activity of compounds in relation to time. In the present study, the drop plate method was used instead of the streak plate method in which log₁₀ CFU/cm² was used instead of log₁₀ CFU/mL to count the bacterial colonies (Barber *et al.*, 2014). The 0.5x MIC of *C. odontophyllum* extract showed partial inhibition of MRSA Mu50 in the colony count. The 1x MIC and 2x MIC inhibited the bacterial growth but did not exhibit bactericidal activity because a reduction of 3 log₁₀ CFU/cm² was not observed. This finding was supported by Basri *et al.* (2016b) who reported that an acetone extract of *C. odontophyllum* leaves showed no bactericidal effect at 1x MIC. The 1x MIC and 2x MIC exhibited bacteriostatic activity by maintaining bacterial growth or killing < 99.9% of the bacteria (Silva *et al.*, 2011). Interestingly, the 4x MIC of the extract reduced the colony count by 3 log₁₀ CFU/cm² (Barber *et al.*, 2014).

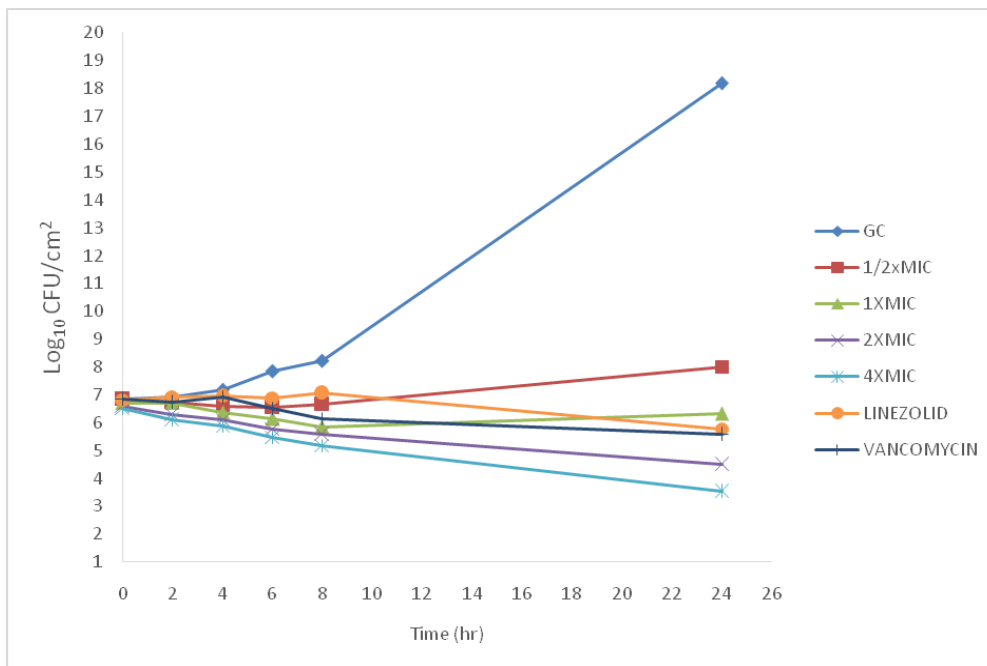


Figure 1: Time-kill curve for acetone extract at 1/2x, 1x, 2x, 4x minimum inhibitory concentration (MIC) of *C. odontophyllum* leaves, vancomycin, linezolid and growth control (GC) against MRSA Mu50. (GC, growth control indicating untreated MRSA).

In the present study, vancomycin and linezolid showed bactericidal activity against MRSA. Leekha *et al.* (2011) reported that time-dependent killing often slows the bactericidal action and vancomycin and linezolid have been proven to be agents that exhibit time-dependent killing (Quintiliani, 2012). The acetone extract of *C. odontophyllum* leaves exhibited concentration-dependent bacterial killing effect at 4x MIC against MRSA Mu50 strain.

CONCLUSION

In conclusion, an attempt has been made to evaluate the properties of *C. odontophyllum* leaves that have anti-MRSA property, which has been attracting considerable global interest in recent years. From this investigation *C. odontophyllum* has clearly demonstrated that its extract possesses compounds with antimicrobial properties and serves as an important source for treating diseases caused by MRSA infection.

ACKNOWLEDGEMENTS

This project was funded by the Ministry of Higher Education, Government of Malaysia under Research Code Grant No. FRGS /2/2014/SG05/UKM/02/3.

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