INTERNATIONAL JOURNAL OF CURRENT MEDICAL AND PHARMACEUTICAL RESEARCH

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RESEARCH ARTICLE

TIME-KILL ASSAY AND POST-ANTIBIOTIC EFFECT OF ACETONE EXTRACT OF CANARIUM ODONTOPHYLUM LEAVES AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA)

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ARTICLE INFO

Article History:

Received 19th June, 2016
Received in revised form 16th July, 2016 Accepted 18th August, 2016 Published online 19th September, 2016

Key words:

Canarium odontophyllum, oxacillin, Antibacterial activity, Methicillin-resistant Staphylococcus aureus, MRSA, MIC, MBC, TKA, PAE

ABSTRACT

Methicilin-resistant *Staphylococcus aureus* (MRSA) infection is a major nosocomial infection that has emerged as community-acquired MRSA worldwide. The resistance of MRSA towards ß-lactam antibiotic had led to research on natural product as novel target for MRSA treatment. *Canarium odontophyllum*(CO*)* Miq.locally known as dabai has been considered as an alternative phytotherapeutic treatment against MRSA. The aim of this study is to determine the time-kill assay (TKA) and post-antibiotic effect (PAE) of acetone extract of *C. odontophylum* leaves in comparison to oxacillin against MRSA ATCC 33591. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extract against MRSA were determined using broth microdilution method and streak-plate technique. Results showed that acetone extract of *C. odontophylum* leaves was bactericidal against MRSA ATCC 33591 with equal MIC and MBC value of 1250 µg/ml. As expected, oxacillin was also bactericidal with MIC value that equal MBC value of 7.81 µg/ml. From TKA analysis, acetone extract of *C. odontophylum* leaves did not display bactericidal effect against MRSA throughout the 24 hours incubation period. While, oxacillin has shown bactericidal activity by a reduction of ≥3 log in colony count at 2.6 hours. Interestingly, the acetone extract of *C. odontophyllum* leaves showed longer PAE time (0.85 ± 1.74 hour) against MRSA ATCC 33591 compared to oxacillin at 0.18± 2.43 hour. In conclusion, despite the bacteriostatic action of acetone extract for *C. odontophyllum* leaves it exhibited a prolonged and persistent antimicrobial effect. These findings indicated that acetone extract of *C. odontophyllum* could give some potential insight on pharmacodynamics information against MRSA.

INTRODUCTION

Staphylococcus aureus come from Micro coccaceae family (Lowy, 1998).It is gram positive cocci that have grape-like structure under microscope (Lisa and Kevin, 2006). *S. aureus* is a normal flora for human it commonly colonized on outer skin surface and upper respiratory tract usually nasal passages. Individual without health problem only suffer from minor skin infection like abscesses and boil. *S. aureus*is an opportunistic pathogen that can cause serious infection such as pneumonia bacteremia, osteomyelitis, endocarditis, septicemia and meningitis (Stapleton and Taylor, 2002).

In 1950s the use of methicillin was introduce to treat *S. aureus*. Methicillin, nafcilin, oxacillin and dicloxacilin are anti-staphylococcal penicillin. This therapeutic agent prevent the development of β-lactamase that being produce by *S. Aureus* (Tacconelli *et al*, 2008). However, methicillin-resistance *Staphylococcus aureus* (MRSA) was first known in 1961 in United Kingdom (Joven, 1961). MRSA is S. *aureus* strain that resistance to action of methicillin and most of ß-lactam antibiotic like penicillin, cephalosporin and carbapenems (APIC, 2010). *S. aureus* produces a mechanism that helps resistivity as methicillin-hydrolysing β-lactamase, alter form of PBP2 and PBP2a (Stapleton and Taylor, 2002).MRSA infection appears as serious pathogen in nosocomial and community (Ippolito *et al*, 2010).

MRSA strain could be hospital-associated methicillin-resistance *Staphylococcus aureus* and community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA). HA-MRSA infections are risky to previous hospitalization, longer length of stay before infection, surgery, enteral feeding, used of invasive instrument and longer use an tibiotic (Graffunder and Venezia, 2002). While, CA-MRSA commonly infect a healthy and young patient. CA-MRSA in fection is risky through direct contact with infected wounds, sharing items or

are in an area crowded with people who cause skin contiguity with skin (CDC, 2013).

Vancomycin is an effective antibiotic before, unfortunately there is a restriction that led to the failure of this treatment include an increase in the minimum inhibitory concentration, a decrease in the death rate of bacteria, poor tissue penetration and potential toxic effects (Kollef, 2007). Not only that, now there are also reports of vancomycin-resistance *S. aureus* (VRSA) (CDC, 2014). Linezolid have been an alternative to treat VRSA but there are also reports of *S. aureus* resistance to linezolid in some countries (Micek, 2007; Gu *et al*, 2012). Resistance to antimicrobials is associated with high mortality rates and high medical costs (Jean and Hsueh, 2011).

Mainly natural product especially plant is the best source of biological compounds for medical purposes and help in overcoming the problem of multi drug resistance microorganisms (Lahlou, 2013; Ncube, 2008). Plant extracts has been proved contain of chemical constituents that are synthesized and stored in the plant including the leaves, branches, fruit, seeds and flowers (Paiva *et al*, 2010). Secondary metabolism of plants such as terpenoids, alkaloids, lectins and phenolic namely phenols, quinones, flavonoids and tannins can act as a defense mechanism against microorganisms, insects and herbivore (Cowan, 1999).

Canarium odontophyllum known as "dabai" or "zaitun Borneo". It is a member of Burseraceae family (Khoo *et al*, 2012) and genus *Canarium L* . (Mogana *et al*, 2011). This tree can be found in the tropical rainforests of Sarawak, especially in rural areas such as Sarikei, Sibu and Kapit. This tree has been shown to have antibacterial (Basri *et al*, 2014a), antifungal (Basri *et al*, 2014b), antioxidants (Shakirin *et al*, 2010), anti-cholinesterase (Shakirin *et al*, 2012) and anti-cancer (Basri *et al*, 2015). Extracts of the leaves of this plant contain chemical constituents such as tannins, flavonoids, terpenoids, saponins and phenolic (Basri and Nor, 2014). Based on a previous study, leave extract of this plant has antibacterial activity (Basri andNor, 2014) and anti-cancer (Basri *et al*, 2015). This plant also has potential as a source of anti-MRSA (Basri *et al*, 2014).

The problem of the emergence of resistant mutant strains triggers the present study to be carried out that can reduce the dose regimen, undesirable effects on drugs and may lead to discovery of a novel, more effective treatment for combating MRSA infection. This present study is to analysis the time kill assay (TKA) and post-antibiotic effect (PAE) of acetone extracts of *C. odontophyllum* against MRSA.

MATERIALS AND METHODS

Preparation of extract solution

The leaf of *C. odontophyllum* was purchased from Kuching, Sarawak with voucher specimen number UKMB 40052.The whole leaf was used for preparation of extract by previous student. The stock solution for test material were prepare by dissolve the crystals acetone extract of *C. odontophyllum* leaf in absolute acetone then vortex until the solution is dissolve completely. Stock solutions are prepared at a concentration of 20 mg/ml and stored at 4^{o}C. The working solution is prepared by calculating the dilution of stock solution then sterilized using a membrane filter of 0.45µm pore size before the test is conducted.

Preparation of Oxacillin Solution

Oxacillin powder was obtained from Sigma Aldrich (USA). The stock solution for oxacillin was prepare at concentration of 20 mg/ml. Oxacillin powder was dissolve with sterile distilled water then stored at 4°C. The working solution is prepared by calculate the dilution of stock solution and sterilized using a membrane filter with pore size of 0.2µm prior to the test.

Preparation of Bacteria Inoculum

Bacterial strains used in this study is a reference strain of MRSA ATCC 33591 and MRSA ATCC 43300 obtained from the collection of Biomedical Sciences, the Faculty of Health Sciences, UKM stored in Microbiology laboratories. The stock culture were grown on Muller-Hinton agar (MHA) then incubated at 37°C for 24 hours to obtain isolated colony. One or two single colony which has the same morphological is transfer into 10 ml Muller-Hinton broth (MHB) using sterile wire loop before being incubated for 24 hours at 37°C. The concentration of the inoculum was determined by 0.08 optical density (OD) under wavelength of 625 nm using a spectrophotometer which is equivalent to 0.5 McFarland standard turbidity that is estimated to 10^8 CFU/ml. If OD exceeds 0.08, MHB will be added to the bacterial suspension to reach OD reading 0.08. The inoculation then is dilute by dilution of 1: 100 to obtain inoculation size 10^6 CFU/ml. Bacterial suspension should be used within 30 minutes.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined for both MRSA ATCC 33591 and MRSA ATCC 43300 in MHB by broth microdilution method, using final inoculation of approximately 10^6 CFU/ml. The MIC value were taken from the lowest concentration of extract and oxacillin in the well of microtiter plate that inhibited the visible growth of organism after 24 hour incubation of incubation at 37°C (Amman *et al*, 2011). For conformation, Triphenyl tetrazolium chloride a (TTC) was added to each well. Well that appear pink were interpreted as positive results for presence of bacteria growth, while colorless were interpreted as negative results which is no growth of bacteria (CLSI, 2012).

Determination of Minimum Bactericidal Concentration (MBC)

MBC values were taken at the lowest concentration that does not show any growth in subculture agar. The wells show no visible growth of the organism on MIC microtiter plate will be transferred to MHA.Then, the plate will be incubate for 24 hours at 37°C (CLSI, 2012).

Time kill Assay Analysis

The time-kill curve of acetone extract of *C. odontophyllum* leaf was evaluated using broth microdilution assay. Eppendorf tube that contains 40µl MHB will be added 10 µL extract or oxacillin then 50 µL of bacterial suspension with inoculum approximately 10^6 CFU/ml will be inoculates. Eppendorf tube that contains only bacteria and 50 µL of MHB will be growth control. Eppendorf tube is then incubated at 37^{o}C and viable counts were performed at 0, 4, 8, 12 and 24 hour after the addition of the treatment agent. To avoid contamination each sample in eppendorf tube will in aliquots into five eppendorf tube. In each following hour, 10 µL of sample will be taken from eppendorf tube and diluted with ten-fold serial dilution

with normal saline (0.9% NaCl) and spread on MHA plates and incubate for 24 hours at temperature of 37°C. Bacterial colony counts between 30-300 CFU/ml for each plate was determined to construct time-kill curve by plotting the log $_{10}$ CFU/ml on the x-axis and the time (hours) on the y-axis (Basri and Khairon, 2012). *C. odontophyllum* leaf extract is considered bactericidal if it results in a reduction $3\log_{10}$ during incubation period denoting > 99% killing (Jacqueline *et al*, 2003).

Figures and Legends

Statistical analysis

Each experiment will be carried out in triplicate for MIC, MBC, TKA and PAE. Results for PAE will be stated in average ± standard deviation.

RESULT

Determination of MIC

The MIC and MBC value of acetone extract against MRSA ATCC 33591 and MRSA ATCC 43300 were shown in Table1.

Table 1 The MIC and MBC value of acetone extract against MRSA ATCC 33591 and MRSA ATCC 43300.

Concentration of Acetone Extract (µg/ml)	MIC Test Result		Control		MBC Test Result		Control	
	ATCC 33591	ATCC 43300	Positive	Negative	ATCC 33591	ATCC 43300	Positive	Negative
5000	-	-	+	-	-	-	+	-
2500	-	-	+	-	-	-	+	-
1250	-	-	+	-	-	-	+	-
625	+	-	+	-	+	+	+	-
312.5	+	+	+	-	+	+	+	-
156.25	+	+	+	-	+	+	+	-
78.13	+	+	+	-	+	+	+	-
39.06	+	+	+	-	+	+	+	-
19.53	+	+	+	-	+	+	+	-
9.77	+	+	+	-	+	+	+	-

(+) Presence of bacterial growth, (-) Absence of bacterial growth, Positive control: MHB and Bacterial Suspension, Negative control: MHB and Acetone Extract

Table 2 The MIC and MBC value of oxacillin against MRSA ATCC 33591 and MRSA ATCC 43300.

Concentration of Oxacillin (µg/ml)	MIC Test Result		Control		MBC Test Result		Control	
	ATCC 33591	ATCC 43300	Positive	Negative	ATCC 33591	ATCC 43300	Positive	Negative
500	-	-	+	-	-	-	+	-
250	-	-	+	-	-	-	+	-
125	-	-	+	-	-	-	+	-
62.5	-	-	+	-	-	-	+	-
31.25	-	-	+	-	-	-	+	-
15.63	-	-	+	-	-	-	+	-
7.81	-	-	+	-	-	-	+	-
3.91	+	+	+	-	+	+	+	-
1.95	+	+	+	-	+	+	+	-
0.98	+	+	+	-	+	+	+	-

(+) Presence of bacterial growth, (-) Absence of bacterial growth, Positive control: MHB and bacterial suspension, Negative control: MHB and acetone extract

Determination of Post-antibiotic Effect

The PAE of MRSA ATCC 33591 was determined with acetone extract of the leaves of *C. odontophyllum* and oxacillin using viable plate count method. The treatment group consisted of acetone extract or oxacillin at concentration of 10 X MIC and the bacteria suspension at inoculation concentration approximately10^6 CFU/ml while the control groups consist of MHB and bacteria suspension. Dilution at 1:1000 was carried out using MHB after incubating both the treatment and control
group for 1 h at 37°C.Then, 2 µl of the diluted sample was streaked on Mueller Hinton agar at 0,2,4,6,8,10 and 24 h in order to count the number of colonies present after 24 h of incubation at 37°C and it was performed in triplicate. Graph $\log_{10}$ CFU/ml against time was plotted, where the duration of PAE were obtained from the graphs (Basri *et al*, 2013).

PAE Calculation (Craig and Gudmundsson, 1996)

PAE = T-C
Where:

T =Time required for the treated organism to increase 1 $\log_{10}$CFU/ml following dilution at 1:1000
C= Time required for the control organism to increase 1 $\log_{10}$CFU/ml following dilution at 1:1000

The MIC ofacetone extract of *C. odontophyllum* against ATCC 33591 was 1250 µg/ml while against ATCC 43300 was 625 µg/ml. The MBC value for acetone extracts against ATCC 33591 and ATCC 43300 were equal which is 1250 µg/ml.

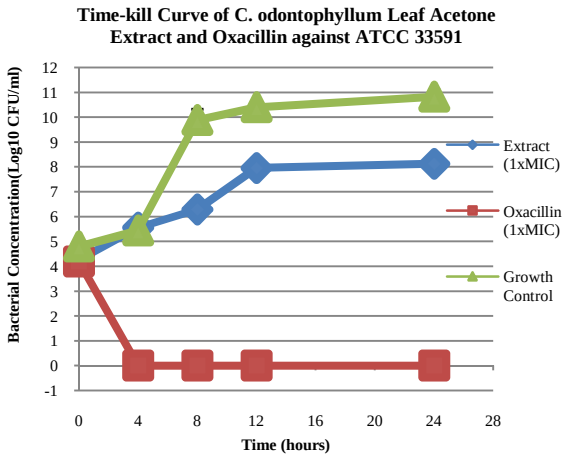
The MIC and MBC values of oxacillin against MRSA ATCC 33591 and MRSA ATCC 43300 were shown in Table 2. The MIC and MBC values for oxacillin against MRSA ATCC 33591 and ATCC 43300 were equal which is 7.81µg/ml.

Analysis of Time-kill assay

Time-kill curves was shown in Figure 1. Acetone extract of *C. odontophyllum* leaf at 1X MIC did not display bactericidal effect by showing slight increase in bacteria concentration during the 24 hour period incubation. Whereas, oxacillin showed bactericidal effect by reduction $3\log_{10}$ CFU/ml at2.6 hours then, showed no bacterial growth from 4 to 24 hours.

Determination of PAE

The duration of PAE obtained for acetone extract of *C. odontophyllum* leaf against ATCC 33591 was 0.85 ± 1.74 hours while the duration of PAE for oxacillin against ATCC 33591 was 0.18 ± 2.43 hours. This shows that the duration PAE for acetone extract is longer than the duration of PAE for oxacillin.



me-kill curve for acetone extract, oxacillin and growth control.

DISCUSSION

Medicinal plants have been used over the years to treat infection (Wright and Sutherland, 2007).Secondary metabolites contained in the plant area significant source that helps in overcoming the problem of antibiotic resistance (Ncube *et al*, 2008).

An antibacterial susceptibility test is conduct using broth microdilution method. This method can help in distinguishing bacteriostatic and bactericidal effect in addition to quantitatively determine the MIC (Ncube *et al*, 2008). For antimicrobial with equal MBC and MIC values or not more than one dilution MIC was interpreted as a bactericidal and antimicrobial resulted in MBC two or more dilution of the MIC was interpreted as bacteriostatic (King, 1996). Therefore, acetone extract of *C. odontophyllum* leaf against ATCC 33591 is bactericidal but bacteriostatic against ATCC 43300. This study similar toBasri *et al* (2014a) and Basri and Sandra (2016) that reported the bactericidal effect of acetone extract of *C. odontophyllum* leaf against ATCC 33591.However, the MIC and MBC for acetone extract of *C. odontophyllum* leaf against ATCC 33591 was higher than reported by Basri and Sandra (2016) which is 156.25 µg/ml. Increased MIC values of antibacterial agents may be due to decrease in susceptibility of acetone extract of *C. odontophyllum* leaf against MRSA.

The present study showed oxacillin exhibit bactericidal effect against both MRSA strain. Oxacillin has been proven as a ß-lactam antibiotic that showed bactericidal effect which acts as an inhibitor of cell wall (Harvey *et al*, 2012). However based on CLSI (2007) for oxacillin MIC test against *S. aureus*, MIC ≥4µg/ml is interpreted as oxacillin resistant. This means the MRSA strain used in this study have been resistant to oxacillin. Oxacillin resistance mechanism is similar to methicillin (Katzung and Trevor, 2015). Oxacillin resistance against *S. aureus* was reported by Bell *et al* (2002) in which *S. aureus* against oxacillin resistance has been detected in the Asia-Pacific region and South Africa (APAC).

Secondary metabolites contained in the plant plays an important role in acting as a defense mechanism against predators such as microorganisms, insects and herbivores (Cowan, 1999). The antibacterial activity of acetone extracts of *C. odontophyllum* leaf is due to secondary metabolite which is saponins, terpenoids, tannins, flavonoids and phenolic compounds that contained in plant (Basri and Nor,2014).

The TKA analysis, at 1 X MIC acetone extract of *C. odontophyllum* leaf showed an increase in the growth of bacteria. This mean 1 XMIC concentration acetone extract of *C. odontophyllum* leaf is not effective to give a bactericidal effect against MRSA ATCC 33591. Bactericidal effect is determined when there is a reduction of $3\log_{10}$ CFU/ ml in colony count during incubation period (Jacqueline *et al*, 2003). However, bactericidal effect can be seen on oxacillin at 2.86 hour. Bactericidal effect may be due to oxacillin act to inhibit the final step of peptidoglycan synthesis of the bacterial cell wall (Katzung and Trevor, 2015). This study supportby Basriand Sandra (2016) that reported methanol extract of leaves of *C. odontophllum* did not exhibit bactericidal effect when alone, but contradict to studies that claimed oxacillin also display no bactericidal effect.

Bactericidal effect cannot be seen in this extract may be due to acetone extract of *C. odontophyllum* leaf is a crude extract which contain various bioactive compounds which display different pharmacological actions. According Efferth and Koch (2011) interactions between various phytochemicals found in a plant extract may show a synergistic pharmacological effects or antagonistic in phytotherapy. Compared to oxacillin that containing an active compound to certain therapy.

The absence of bactericidal effect on the extract at a concentration of 1 XMIC may be due to extract is a compound that shows concentration dependent killing. Concentration dependent killing will show different events at different concentrations (Vogelman and Craig, 1986).Based on Chatterjee *et al*. (2009) the death rate of bacteria varies depending on the concentration of the extract, the duration of exposure and strain of bacteria tested. For oxacillin, ß-lactam antibiotics typically show time dependent killing, the increase in the concentration rarely show an increase in the rate of bactericidal activity (Turnidge, 1998; Vogelman and Craig, 1986).According Leekha *et al* (2011) time dependent killing often show a slow bactericidal action, this is in contrast to present study that showed quite fast time-kill rate.

Post-antibiotic effect (PAE) was applied to determine the effect of dose regimen of antibacterial agents. It also helps to reduce and prevent the occurrence of toxic effects and resistance to antibacterial agents (Amman *et al*, 2011).This present study of PAE showed longer duration of PAEof acetone extract of *C. odontophyllum* compare to PAE duration for oxacillin.

The longer duration of PAE for extractthan oxacillin in this study is accordance with a study conducted by Basri *et al* (2013) who found the gall *Q. infectoria* acetone extract showed longer PAE duration than vancomycin. Bioactive compounds in acetone extract may be the cause of consistent antimicrobial effect for *C.odontophyllum*. Tannins inhibit the growth of bacteria by interacting with the enzyme and protein or acting indirectly on the membrane of bacteria (Scalbert, 1991). The presence of tannins in gall extract of *Q. infectoria* and acetone extract of *C. odontophllum* could be a factor for prolonged period for PAE.

In this study, oxacillin showed a short period of PAE. ß-lactam antibiotics exhibit bactericidal action depending on the time. Most of the ß-lactam does not have or has a short PAE. Therefore, the frequency of drug intake is important to determine the effectiveness of this drug (Levison, 2004).The

discovery of the extracts from *C. odontophylum* as antibacterial agents can help in overcoming the problem of resistance of *S. aureus* to antibiotics. The use of this natural extract not only help to reduce medication costs but also reduce dose toxicity effects of drug taken.

CONCLUSION

Despite showing bactericidal activity from broth microdilution method, the acetone extract from *C. Odontophylum* leaf at 1XMIC did not exhibit killing effect against MRSA ATCC 33591 from TKA analysis. However, the prolonged PAE time of the acetone extract from *C. Odontophylum* leaf compared to oxacillin against ATCC 33591 suggests that it has the potential to be developed as an alternative treatment to combat MRSA infection.

Acknowledgement

This project was funded by Universiti Kebangsaan Malaysia under the Research University Grant Code Grant GUP-2014-059.

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