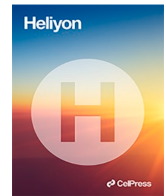
Heliyon 9 (2023) e17892

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Alpha-amyrin as an anti-biofilm agent against methicillin-resistant and vancomycin-intermediate *Staphylococcus aureus*

Pooi Yin Chung [a,*], Pey Lin Narissa Loh [a], Hui-min Neoh [b], Ramliza Ramli [c]

[a] *School of Medicine, International Medical University, Kuala Lumpur, Malaysia*
[b] *Universiti Kebangsaan Malaysia Molecular Biology Institute, Kuala Lumpur, Malaysia*
[c] *Universiti Kebangsaan Malaysia Medical Center, Kuala Lumpur, Malaysia*

A R T I C L E I N F O

Keywords:
Biofilm
Alpha-amyrin
Methicillin-resistant *Staphylococcus aureus*
Vancomycin-intermediate *Staphylococcus aureus*

A B S T R A C T

Staphylococcus aureus has caused life-threatening infections and developed resistance against conventional antimicrobials, posing a significant threat to human health worldwide. Biofilms that surround the bacteria cells act as a protective layer, allowing cells inside the biofilm to be resistant to external stresses such as antimicrobials. Therefore, biofilms further complicate treatment available for infections caused by multi-drug resistant *Staphylococcus aureus*. A previous study on alpha-amyrin (AM), derived from ursane, was reported to significantly reduce the biomass and inhibit the metabolic activity of reference strain methicillin-resistant and methicillin-sensitive *S. aureus* (MRSA and MSSA, respectively). In this study, the antibiofilm activity of AM was extended to include clinical isolates of MSSA and MRSA, and laboratory-generated vancomycin-intermediate *S. aureus* (VISA) collected from University Kebangsaan Malaysia Medical Center (PPUKM) and Universiti Kebangsaan Malaysia Medical Molecular Biology Institute (UMBI). Pre-formed biofilms of biofilm-forming isolates identified from the Congo Red Agar (CRA) assay were then exposed to AM, vancomycin and oxacillin, and evaluated using the crystal violet and resazurin assays. The results showed that AM reduced the biofilm biomass of three isolates of MSSA, eight isolates of MRSA and four isolates of VISA but increased the metabolic activity in certain MSSA, MRSA and VISA isolates, indicating AM may possess biofilm reduction effects but not bactericidal effects. Based on these findings, AM could be further studied and developed as a potential therapeutic agent for chronic *S. aureus* infections.

1. Introduction

Staphylococcus aureus is a Gram-positive pathogen that causes potentially life-threatening nosocomial- and community-acquired infections. The ability of this pathogen to rapidly develop and acquire antibiotic resistance has led to the emergence of multidrug-resistant strains such as methicillin-resistant *S. aureus* (MRSA). At present, vancomycin (VAN) remains the last resort for the treatment of MRSA infections [1]. However, reports regarding MRSA strains that have developed resistance to VAN have emerged in many parts of the world, with the first such strain reported in USA two decades ago [2]. While complete resistance towards VAN has not been reported in Malaysia in published literature, the emergence of a vancomycin-intermediate *S. aureus* (VISA) isolate from a female patient who was first admitted for leptospirosis in an unnamed referral hospital has been reported [3].

* Corresponding author.
E-mail address: katrina_chung@imu.edu.my (P.Y. Chung).

https://doi.org/10.1016/j.heliyon.2023.e17892
Received 20 January 2023; Received in revised form 23 June 2023; Accepted 30 June 2023
Available online 5 July 2023
2405-8440/© 2023 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

In addition to multidrug resistance, *S. aureus* also has the ability to form biofilm, a characteristic associated with infectious diseases and inert surfaces, including medical devices for internal or external use. The number of biofilm-associated infections will most likely increase with improvements in medical care and the increased use of prosthetic and biomedical implants. Bacteria in biofilms are encased in a polysaccharide glycocalyx, which provides them with protection against host defenses and antimicrobial drugs, and survive in hostile environmental conditions. Thus, biofilm-associated infections do not respond consistently to current antibiotics as these antibiotics are developed to treat infections caused by planktonic bacterial populations in acute infections.

Pentacyclic triterpenoids have been shown to exhibit antimicrobial and antibiofilm activities against different strains of bacteria. Two friedelane-based pentacyclic triterpenoids, dihydrocelastrol and dihydrocelastryl diacetate were found to inhibit the biofilm formation and disrupt the mature biofilms of MRSA strains. Glycyrrhetinic acid, ursolic acid and betulinic acid affected the exopolysaccharide (EPS) contents and reduced the EPS-associate extracellular enzymatic activities in *Vibrio cholerae* C6709 biofilms [4]. At sub-minimum inhibitory concentrations (MIC), beta-amyrin acetate and oleanolic acid significantly decrease the adhesion of *S. aureus* ATCC 43300, *Klebsiella pneumoniae* and *Enterococcus faecium* [5]. From the transcriptomic analysis, the response mechanisms of planktonic MSSA and MRSA to AM, betulinic acid and betulinaldehyde showed that these compounds regulate multiple desirable targets including pathways which are involved in the regulation of biofilm formation [6,7].

In a preliminary study using the crystal violet assay, AM which is an ursane derivative with five six-membered rings and methyl groups on C-19 and C-20, has been shown to significantly reduce the biomass of MRSA at 16 μg/mL. AM could also significantly reduce mature biofilms of MRSA with no cytotoxic effects on normal mammalian cells, further supporting its potential use as an antimicrobial and antibiofilm agent against MRSA [8]. However, the anti-biofilm activity of AM has not been demonstrated in clinical isolates of MSSA and MRSA, as well as VISA strains. Hence, the aim of the study is to evaluate the effects of AM, oxacillin (OXA) and VAN on the biofilm of these *S. aureus* isolates.

2. Materials and methods

2.1. Bacteria isolates

Reference strains *S. aureus* ATCC 29213 and ATCC 43300, representing non-biofilm-forming MSSA and biofilm-forming MRSA were used as controls in this study. The clinical isolates of MRSA and MSSA were collected from University Kebangsaan Malaysia Medical Center (PPUKM) while the laboratory-generated VISA strains were obtained from Universiti Kebangsaan Malaysia Medical Molecular Biology Institute (UMBI). A total of 19 isolates, i.e. nine clinical isolates of MRSA and 10 clinical isolates of MSSA, were collected from the Bacteriology Unit, Department of Laboratory Diagnostic Services, PPUKM, Malaysia. Four laboratory-generated VISA isolates were collected from UMBI UKM, Malaysia. The identity of these isolates was confirmed using standard bacteriological methods such as Gram stain, colony morphology, coagulase test and antimicrobial susceptibility test.

2.2. AM and antibiotics

Antibiotics OXA and VAN were used as controls in this study. These antibiotics and AM were obtained commercially from Sigma-Aldrich (USA). Stock concentrations of the antibiotics and AM were prepared, filter-sterilized and stored at −20 °C in aliquots of 500 μL till further use as frequent freezing and thawing may reduce their potency.

2.3. Evaluation of biofilm production in S. aureus isolates

The biofilm production for *S. aureus* clinical isolates was assessed using the Congo Red Agar plates assay (CRA) [9]. All clinical isolates of MSSA and MRSA, and VISA strains to be tested for biofilm formation were inoculated on CRA plates, maintained at room temperature for 24 h and incubated at 35 °C for the next 24 h. Black colonies on the CRA plate represent *S. aureus* biofilm producers, in contrast to the red colonies, which represent *S. aureus* non-biofilm producers. The assay was carried out thrice in five replicates ($n = 3 \times 5$).

2.4. Evaluation of minimum biofilm eradication concentration (MBEC) of AM and antibiotics in biofilm cultures

All the identified biofilm-forming isolates were grown for 18 h in tryptic soy broth (TSB). A volume of 100 μL bacterial suspension in TSB was placed into wells of the 96-well microtiter plates. The plates were incubated at 37 °C for 24 h under static conditions for the bacteria cells to form biofilms in the wells.

Twofold serial dilutions of AM (16–256 μg/mL) and antibiotics (OXA and VAN at 1–16 μg/mL) were added to the prepared biofilms in the microtiter plates. After incubation, the biofilms were evaluated using the optimized crystal violet (CV) and resazurin (RZ) assays reported previously [8]. The assays were carried out twice in three replicates ($n = 2 \times 3$) for each concentration. The lowest concentration of AM, OXA and VAN that showed a significant reduction in the biomass and metabolic activity of the cells in the biofilm were recorded as the effective minimum biofilm eradication concentration (MBEC).

2.5. Statistical analysis

All values were expressed as means ± standard deviation from replicates of the experiments. A one-way analysis of variance was

used to determine the differences in biofilm formation and metabolic activity between the control (without treatment) and each test group (SPSS software version 17.0). Differences achieving a confidence level of 95% were considered significant.

3. Results

3.1. Biofilm production

Black dots appeared on the colonies in 16 of the isolates, which included nine MRSA, three MSSA and four VISA isolates, indicating these isolates were biofilm-formers (Table 1). The presence of glucose (20 g/L) present in the Congo red agar could stimulate the production of the biofilm which combines with the Congo red dye to yield the black color [10].

3.2. Evaluation of biofilm biomass – crystal violet assay

The MBEC values, the lowest concentration which showed the most significant reduction of the biofilm in the bacteria isolates compared to the control (without treatment), are shown in Table 2. AM, OXA and VAN were able to significantly decrease the biomass of biofilm in three isolates of MSSA (BD 1105, BN 0156 and SW 2150), six isolates of MRSA (BD 0295, BO 19382, SPU 640, TIS 1723, TIS 1732 and TIS 1768) and four VISA strains (261, 332, 377 and 775). Although AM could reduce the biomass in BD 5426 and TIS 1670, OXA and VAN seem to increase their biomass. The MSSA, MRSA and VISA isolates that have the lowest MBEC values are shown in Figs. 1(A) and 2(A) and 3(A), respectively.

3.3. Evaluation of the metabolic activity of bacteria cells in biofilm

The metabolic activity of the MSSA, MRSA and VISA isolates in response to treatment with AM, OXA and VAN were varied. The metabolic activity of MSSA SW 2150 decreased significantly with AM, OXA and VAN [Fig. 1(B)]. However, despite decreasing the biomass of the biofilm, AM seemed to increase the metabolic activity in MRSA TIS 1732 [Fig. 2(B)] and VISA 775 [Fig. 3(B)]. This contrasting effect on the biomass of biofilm and metabolic activity suggests AM could possess antibiofilm activity but not bactericidal properties.

4. Discussion

S. aureus has been recognized as a prominent human pathogen that causes infections associated with catheters and other indwelling medical devices characterized by biofilm formation. The bacteria within the matrix of exopolysaccharide glycocalyx are protected from the defenses in the host and antimicrobials. The emergence of MRSA isolates exhibiting decreased susceptibility to glycopeptides presents a crucial challenge for antimicrobial therapy and infection control.

In this study, AM could significantly reduce the biomass of biofilms in clinical isolates of MSSA and MRSA, and laboratory-generated VISA strains in concentrations ranging from 32 to 256 μg/mL. This finding is consistent with a previous study that reported AM, along with two other pentacyclic triterpenoids betulinic acid and betulinaldehyde, could significantly reduce the biomass in reference strains of MRSA and MSSA [8]. Although the mechanism of action of AM against biofilms is still not known, betulinic acid derivatives which have a substitution of a triterpenoid at position C-3 with electronegative groups, such as acetyl ester, propyl ester, dichloroacyl ester, trifluoracyl ester and ketone, possessed high percentage of biofilm inhibition against *S. aureus* compared to the precursor, betulinic acid [11]. A study has reported that ursolic acid, an ursane derivative similar to AM, inhibited biofilm formation of *S. aureus*, while AM inhibited biofilm formation and promoted the planktonic lifestyle in *Pseudomonas aeruginosa* PAO1 [12].

The clinical isolates MSSA and MRSA in this study demonstrated different responses to OXA, VAN and AM. For example, MRSA TIS 1732 showed a significant reduction in biomass compared to MRSA TIS 1768 and BD 5426 when treated with OXA and VAN. This difference could be due to the different genes expressed in each *S. aureus* isolate, such as the *SCCmec*, *ica* and *fnb*, resulting in varying degrees and composition of the biofilms formed.

A recent study showed that biofilms formed by type IV SCC*mec* gene which are expressed by community-acquired MRSA (CA-MRSA) are more mature compared to biofilms formed by type II SCC*mec* gene found in hospital-acquired MRSA (HA-MRSA) [13]. The different SCC*mec* genes have resulted in different susceptibility of the MRSA isolates to non-beta lactam antibiotics, especially aminoglycosides, macrolides, lincosamides and fluoroquinolones, to which CA-MRSA is susceptible while HA-MRSA is resistant [14]. However, there is no reported literature on the difference in susceptibility of CA-MRSA and HA-MRSA to vancomycin. Hence, the co-existence of CA-MRSA and HA-MRSA in PPUKM could not be determined although the MRSA isolates in this study exhibited

Table 1
Identification of biofilm-forming isolates.

Clinical isolates	ID of biofilm-forming isolates	Biofilm-forming isolates (%)
MSSA	BD 1103, BN 0156, SW 2130	30
MRSA	BD 0295, BD 5426, BO 19382, SPU 640, TA 1040, TIS 1670, TIS 1723, TIS 1732, TIS 1768	100
VISA	VISA 261, VISA 332, VISA 377, VISA 775	100

Number of replicates, n = 5 × 3.

Table 2
MBEC values of AM, OXA and VAN for MSSA, MRSA and VISA isolates.

Bacteria	Isolates	MBEC (μg/ml)		
		AM	OXA	VAN
MSSA	BD 1103	32	16	1
	BN 0156	32	8	16
	SW 2130	32	1	1
MRSA	BD 0295	128	16	4
	BD 5426	32	–	1
	BO 19382	128	1	16
	SPU 640	64	1	1
	TA 1040	–	4	2
	TIS 1670	128	–	–
	TIS 1723	128	16	4
	TIS 1732	32	1	1
	TIS 1768	256	16	8
VISA	VISA 261	128	1	1
	VISA 332	128	16	8
	VISA 377	256	8	8
	VISA 775	32	1	4

- Cannot be determined from the concentrations tested, n = 3 × 5.

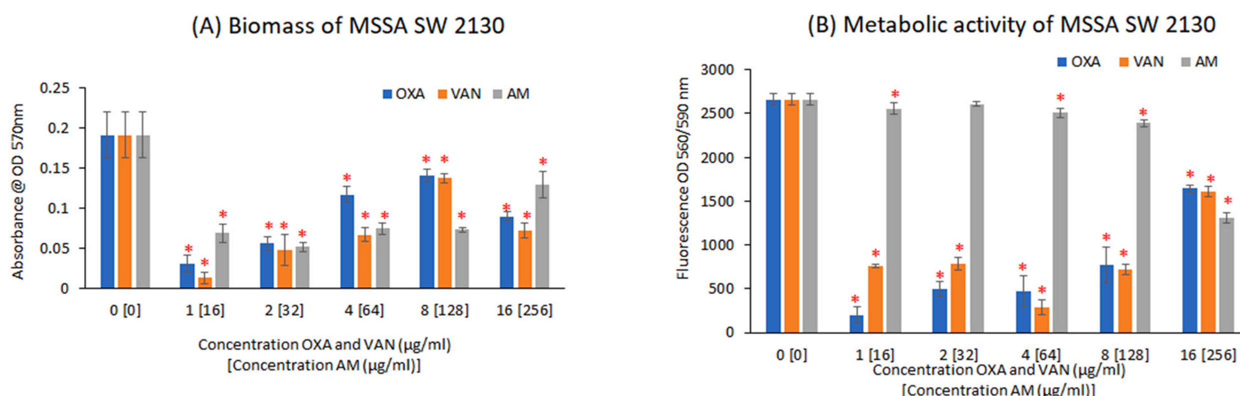


Fig. 1. The (A) biomass and (B) metabolic activity of MSSA SW 2130 when treated with OXA, VAN and AM.

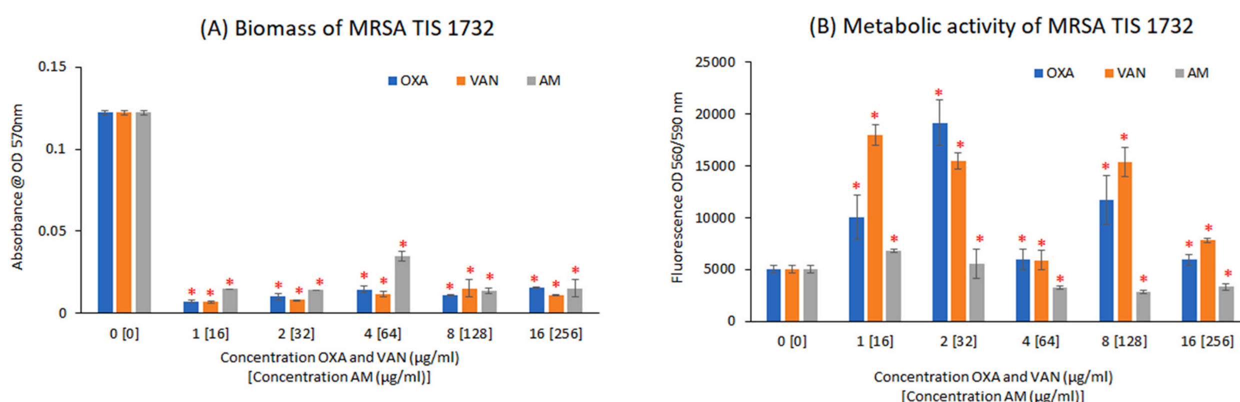


Fig. 2. The (A) biomass and (B) metabolic activity of MRSA TIS 1732 when treated with OXA, VAN and AM.

different susceptibility to vancomycin.

The principal role of *ica*A gene is to synthesize extracellular polymeric substances of staphylococcal biofilms, namely polysaccharide intercellular adhesin (PIA) proteins, which are essential for *S. aureus* biofilm accumulation and adhesion [15]. Sub-inhibitory concentrations of oxacillin trigger *ica*A gene expression [16] while sub-inhibitory doses of vancomycin induce membrane vesicles which increase cell surface adhesion, bacterial hydrophobicity and intercellular aggregation [17]. Hence, low doses of

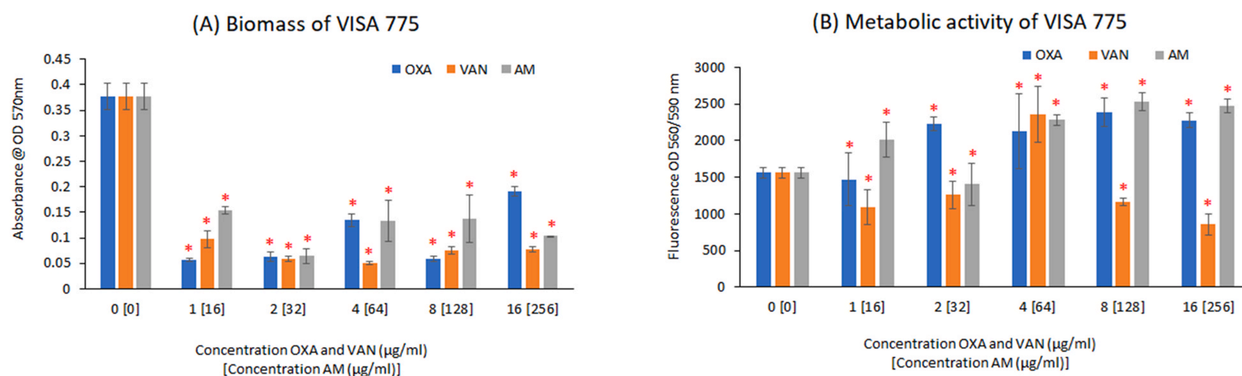


Fig. 3. The (A) biomass and (B) metabolic activity of VISA 775 when treated with OXA, VAN and AM.

antibiotics could lead to enhanced biofilm formation and complications of biofilm eradication [18]. Other than *S. aureus*, membrane vesicles have also been shown to play a role in the formation and maturation of biofilms of *Helicobacter pylori, Pseudomonas aeruginosa, Vibrio cholerae* and *Pseudomonas putida* [19].

Comparing the MSSA and MRSA isolates, the biofilms formed by MSSA are *ica*-dependent while MRSA produces *ica*-independent biofilms [20]. Currently, the best understood mechanism of the *ica* gene is that the *ica* gene produces an extracellular polysaccharide adhesin, polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine. PIA proteins are essential for staphylococci biofilm adhesion and accumulation [15]. The same study reported that in four clinical MRSA isolates, the MRSA still formed biofilms even after the deletion of the *ica* gene [20]. Although the mechanism of *ica*-independent biofilm formation is not clearly understood, *ica*-independent staphylococci have been reported to possess the accumulation-associated protein (*aap*) and Bap homolog protein (*bhp*) genes that induce an alternative PIA-independent mechanism during biofilm formation [21]. Hence, the responses of MSSA SW 2130 and MRSA BD 5426 were different in the same concentration range tested.

Another gene that is involved in biofilm formation is the fibronectin-binding (fnb) gene which expresses proteins that promote biofilm adhesion and accumulation. This gene which is regulated by the SaeRS two-component system is found in MRSA and VISA isolates, producing fibronectin-binding protein A (FnBPA) and fibronecting-binding protein B (FnBPB), respectively [22]. The decrease in the biomass of biofilm of the MRSA isolates and VISA strains showed that AM could possibly inhibit the expression of the *fnb* gene.

In a previous study using reference strains of MRSA, AM did not exhibit cytotoxic effect on the MRC5 cell line at the concentrations tested, even at the highest concentration of 256 μg/mL. This finding indicated that the compound could significantly reduce the biomass of biofilm without threatening the normal cells of the human host and supports its potential use as an antimicrobial and antibiofilm agent against MRSA and VISA [8].

In summary, the biofilm formed by each MRSA, MSSA and VISA isolates used in this study differs from one another due to the inhibition of genes that encode the synthesis of biofilm components in each *S. aureus* isolate. Thus, the same concentration of antimicrobial compounds (AM in this study) could give different responses in the treatment of biofilm-associated *S. aureus* infections. Based on the different responses, the presence and inhibition of the *SCCmec*, *ica* and *fnb* genes that affect biofilm formation upon treatment by AM could be further evaluated to elucidate the mechanism of action and target sites of AM. Other genes that are involved in the formation of biofilm in *S. aureus* such as the expression of the collagen-binding protein (cna), elastin binding protein (Ebps), laminin-binding protein (eno), and serine-aspartate repeat (Sdr) family of proteins sdrC and sdrD could also be evaluated as possible antibiofilm targets of AM.

5. Conclusion

AM has been shown to significantly reduce the biofilm of the clinical isolates of MRSA and MSSA as well as the laboratory-generated VISA strains. Based on these findings and its non-cytotoxic effect, AM could be further developed as a potential therapeutic agent for biofilm-associated *S. aureus* infections.

Author contribution statement

Pooi Yin Chung: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Pey Lin Narissa Loh: Performed the experiments.
Hui-min Neoh; Ramliza Ramli: Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supplementary material/referenced in article.

Funding statement

This work was supported by the International Medical University (IMU), Kuala Lumpur, Malaysia [grant number BMS1/2020(24)].

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17892.

References

[1] K.A. Rodvold, K.W. McConeghy, Methicillin-resistant *Staphylococcus aureus* therapy: past, present and future, Clin. Infect. Dis. 58 (2014) 20–27.
[2] Center for Disease Control, *Staphylococcus aureus* resistant to VAN-United States, Morb. Mortal. Wkly. Rep. 51 (2002) 565–567.
[3] R. Hashim, H.H. Hamzah, N.A. Dahalan, F. Amran, N. Ahmad, N.Z. Hazwani, S. Baharudin, S. Zainal, A.S.S. Raj, VAN-intermediate *Staphylococcus aureus* in Malaysia: a case study, J. Clin. Case Rep. 8 (2018) 1178.
[4] S.P. Bhattacharya, A. Bhattacharya, A. Sen, A comprehensive and comparative study on the action of pentacyclic triterpenoids on *Vibrio cholerae* biofilms, Microb. Pathog. 149 (2020).
[5] J.J. Kiplimo, N.A. Koorbanally, H. Chenia, Triterpenoids from *Vernonia auriculifera Hiern* exhibit antimicrobial activity, Afr. J. Pharmacy Pharmacol. 5 (2011) 1150–1156.
[6] P.Y. Chung, L.Y. Chung, P. Navaratnam, Transcriptional profiles of the response of methicillin-resistant *Staphylococcus aureus* to pentacyclic triterpenoids, PLoS One 2 (2013), e56687.
[7] P.Y. Chung, L.Y. Chung, P. Navaratnam, Identification, by gene expression profiling analysis, of novel gene targets in *Staphylococcus aureus* treated with betulinaldehyde, Res. Microbiol. 164 (2013) 319–326.
[8] P.Y. Chung, M.Y. Gan, B.Y. Chin, Pentacyclic triterpenoids as antibiofilm agents against methicillin-resistant and biofilm-forming *Staphylococcus aureus* (MRSA), Curr. Pharmaceut. Biotechnol. 23 (2022) 828–834.
[9] D.J. Freeman, F.R. Falkiner, C.T. Keane, New method for detecting slime production by coagulase negative staphylococci, J. Clin. Pathol. 42 (1989) 872–874.
[10] H.R.V. Rajkumar, R. Devaki, V. Kandi, Evaluation of different phenotypic techniques for the detection of slime produced by bacteria isolated from clinical specimens, Cureus 8 (2016) e505.
[11] G.N.S. da Silva, S.C.B. Gnoatto, M. Primon-Barros, A.J. Macedo, Triterpene derivatives as relevant scaffold for new antibiofilm drugs, Biomolecules 9 (2019) 58.
[12] T. Rasamiravaka, J. Ngezahayo, L. Pottier, O.R. Sofia, F. Souard, L. Hari, et al., Terpenoids from *Platostoma rotundifolium* (Briq.) A. J. Paton alter the expression of quorum sensing-related virulence factors and the formation of biofilm in *Pseudomonas aeruginosa* PAO1, Int. J. Mol. Sci. 18 (2017) 1270.
[13] M. Hamada, T. Yamaguchi, A. Sato, D. Ono, K. Aoki, C. Kajiwara, et al., Increased incidence and plasma-biofilm formation ability of sccmec type IV methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from patients with bacteremia, Front. Cell. Infect. Microbiol. 11 (2021), 602833.
[14] P.K. David, F. Bwanga, J. Seni, R. Mayanja, E. Kigozi, B. Mujuni, et al., CA-MRSA and HA-MRSA coexist in community and hospital settings in Uganda, Antimicrob. Resist. Infect. Control 8 (2019) 1–9.
[15] M. Omidi, F. Firoozeh, M. Saffari, H. Sedaghat, M. Zibaei, A. Khaledi, Ability of biofilm production and molecular analysis of *spa* and *ica* genes among clinical isolates of methicillin-resistant *Staphylococcus aureus*, BMC Res. Notes 13 (2020) 19.
[16] Z.A. Mirani, M. Aziz, M.N. Khan, I. Lal, Nu Hassan, S.I. Khan, Biofilm formation and dispersal of *Staphylococcus aureus* under the influence of oxacillin, Microb. Pathog. 61–62 (2013) 66–72.
[17] X. He, F. Yuan, F. Lu, Y. Yin, J. Cao, Vancomycin-induced biofilm formation by methicillin-resistant *Staphylococcus aureus* is associated with the secretion of membrane vesicles, Microb. Pathog. 110 (2017) 225–231.
[18] J.B. Kaplan, Antibiotic-induced biofilm formation, Int. J. Artif. Organs 34 (2011) 737–751.
[19] W. Wang, W. Chanda, M. Zhong, The relationship between biofilm and outer membrane vesicles: a novel therapy overview, FEMS Microbiol. Lett. 1 (2015) 362.
[20] J.P. O'Gara, *Ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*, FEMS Microbiol. Lett. 270 (2007) 179–188.
[21] R.A. Nasr, H.M. AbuShady, H.S. Hussein, Biofilm formation and presence of icaAD gene in clinical isolates of staphylococci, Egypt. J. Med. Hum. Genet. 13 (2012) 269–274.
[22] M.F. Cortes, C.O. Beltrame, M.S. Ramundo, F.A. Ferreira, A.M. Figueiredo, The influence of different factors including fnbA and mecA expression on biofilm formed by MRSA clinical isolates with different genetic backgrounds, Int. J Med. Microbiol. 305 (2015) 140–147.