Provisional chapter

Non-microbial Natural Products That Inhibit Drug-Resistant *Staphylococcus aureus*

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Additional information is available at the end of the chapter

Abstract

Drug resistance developed in human pathogenic bacteria is emerging and has become a global problem. Methicillin-resistant *Staphylococcus aureus* (MRSA) spreading in both hospital and community areas has posed a great impact to global public health. Current antibiotics used against these resistant strains are no longer efficacious and the search for new alternative is in urgent need. In the past decades, natural products have demonstrated multiple biological activities in biomedical areas including their antibacterial actions against various drug-resistant bacteria. More promisingly, some natural products could reverse the resistance of bacteria to the antibiotics, making the target bacteria susceptible to these drugs again. Numerous natural products have also exhibited potent synergism against the drug-resistant bacteria when used in combination with various types of antibiotics. Recently, several antibacterials derived from microbes have been developed and approved by Food and Drug Administration (FDA) for clinical use. In this chapter, we discuss the potential use of non-microbial natural products in controlling *Staphylococcus aureus* (*S. aureus*)'s growth, and the underlying challenges in developing the natural products into clinical applications.

Keywords: natural products, *Staphylococcus aureus*, methicillin-resistant, antibacterial, MRSA

1. Introduction

Staphylococcus aureus (S. aureus) is a coagulase-positive Gram-positive cocci bacterium, commonly found on human skin and mucous membranes. Up to 30% of the world population is colonised by this bacterium [1]. Despite being part of the human normal microbiota, it is known to be a pathogen causing various levels of diseases ranging from mild skin infections

such as boils and rashes, to life-threatening diseases such as persistent bacteraemia, sepsis, and pneumonia [2]. The pathogenicity of this bacterium is attributed to its vast arrays of virulence factors such as adhesins, production of enzymes and toxins, biofilm formation, and evasion of immunity strategies [3–5]. Apart from the known virulence factors, this opportunistic pathogen is best known for its formidable reputation due to its antibiotic-resistant phenotype. Methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) are among the two recognised health threats to the humans. As of now, MRSA is listed as a 'serious threat' by Centres for Disease Control and Prevent (CDC) and 'priority pathogen' by the World Health Organisation (WHO), while VRSA is listed as 'concerning threat' by CDC.

Despite the rapid advancement of modern medicine, *S. aureus* infections remain highly prevalent in the human populations as transmission of these pathogens can occur through direct contact [6, 7]. Drug-resistant *S. aureus* particularly MRSA can be defined either as healthcare- or community- associated, based on the '48-hour rule'. In the former, MRSA infections develop after 48 hours from hospital admission while the later develops within 48 hours of admission. In this classification system, there are three categories of MRSA infections, namely (i) healthcare-associated, hospital-onset, (ii) healthcare-associated, community onset and (iii) community-associated MRSA infections. Essentially, healthcare-acquired MRSA infections (HA-SA or HA-MRSA) lead to bacteraemia, infective endocarditis, and prosthetic-associated infections while community-acquired MRSA infections (CA-MRSA) often lead to skin and soft tissues infection as well as community-acquired pneumonia in healthy individuals [2, 8, 9]. Compounding to the situation, MRSA has been reported to infect livestock including pigs, poultry, and cattle. Livestock-associated MRSA (LA-MRSA) can be transmitted to individuals handling these infected livestock [10]. In the early days, *S. aureus* has already been recognised as the main culprit causing hospital-acquired infections (HAIs) such as surgical site infections, bloodstream infections, and pneumonia [11]. The epidemiology of *S. aureus* shifted in the 2000s with the observation of MRSA infections dominating HAIs. In fact, MRSA strains account for up to 75% of all *S. aureus* infections in different part of the world [12–17]. In the US, for instance, MRSA causes approximately 11,000 deaths annually [18].

Antibiotic-resistant *S. aureus* is known to be associated with higher morbidity and mortality rates as compared to antibiotic-susceptible strains [19–21]. In the last decade, studies show that MRSA alone causes more death in the US hospitals than of HIV/AIDS, viral hepatitis, and tuberculosis in combination [22, 23]. In addition to health burden, these antibiotic-resistant *S. aureus* also imposes economic burden in order to eliminate the associated infections [24, 25]. The bacterium develops resistance to nearly all antibiotics introduced to treat infections caused by the bacterium. In 2011, the Expert Panel of the Infectious Diseases Society of America (IDSA) presented an evidence-based guideline for the management of antibiotic-resistant *S. aureus* infections, including antibiotic choices in both adult and paediatric patients [26]. The key antibiotic choices are described below.

1.1. Vancomycin

Vancomycin, a glycopeptide, was first introduced in the 1960s and has been the most reliable therapeutic agent for MRSA infections, including bacteraemia and endocarditis [27]. This broad spectrum antibiotic is a cell wall synthesis inhibitor. It binds to the c-terminal

of D-Ala-D-Ala residues of the peptides of the N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM) murein subunits, preventing transpeptidases from forming the peptide bridge between peptidoglycan chain, leading to bacterial cell death. However, there is also a group of *S. aureus* resistant to vancomycin known as VRSA.

1.2. Daptomycin

This antibiotic is a promising alternative to vancomycin for infections caused by MRSA. This cyclic lipopeptide was approved for clinical use in the U.S in 2003 and Europe in 2006. Daptomycin targets only Gram-positive bacteria and is commonly used for complicated skin and skin-structure infections, bacteraemia, and right-sided endocarditis [27]. This antibiotic, however, is not recommended for the pneumonia caused by MRSA. Some studies indicated that daptomycin interacts with pulmonary surfactants present in the lung tissues, leading to the inhibition of daptomycin antibacterial activity [2, 28]. Daptomycin works by targeting the cytoplasmic membrane of bacteria in a calcium ion-dependent manner. In the presence of calcium ions, daptomycin aggregates and forms micellar structures. Daptomycin is then inserted into the membrane and binds strongly to phosphatidylglycerol headgroups leading to depolarisation and permeabilisation of the membrane. This then leads to cytoplasmic content leakage and cell death [29, 30]. The emergence of daptomycin-resistant *S. aureus* is relatively uncommon. However, increasing records of daptomycin-resistant *S. aureus* have been reported [31–33].

1.3. Linezolid

Linezolid, an oxazolidinone, was first approved by the FDA in 2000 for skin bacteraemia and pneumonia-origin *S. aureus* infections [1]. Linezolid is considered as a standard broad-spectrum intravenous therapies directed towards vancomycin- and teicoplanin-resistant Gram-positive pathogens, including MRSA. Linezolid inhibits protein synthesis by binding to the 23S subunit of the 50S ribosome. Linezolid-resistant *S. aureus* is relatively uncommon. Resistant strains have been previously reported in staphylococci involving mutations in the 23S rRNA and rRNA methyltransferase. These mutations prevent the binding of linezolid to the ribosome for interfering protein synthesis [34].

1.4. Ceftaroline

Ceftaroline is a fifth generation cephalosporin with a broad-spectrum bactericidal activity against both Gram-positive bacteria including MRSA and some Gram-negative bacteria. This antibiotic is used primarily for the treatment of acute bacterial skin and skin structure infections, and community-acquired bacterial pneumonia caused by *S. aureus* [35]. Ceftaroline has an enhanced affinity for penicillin binding protein 2a (PBP2a), thus is an ideal antibiotic choice for MRSA infections. This antibiotic is relatively new, and was approved for use in 2010 in the U.S, 2012 in Europe, and 2013 in Australia [36]. However, the emergence of ceftaroline resistance in different parts of the world with a demonstrated decrease of PBP2a binding affinity and heteroresistance, has been documented [37–39]. The associated mechanisms of resistance involve glutamic acid-to-lysine substitutions in the non-penicillin binding domain and the transpeptidase domain of the PBP2a [39, 40].

There is increasing evidence demonstrating that *S. aureus* is becoming resistant against all possible antibiotic choices used to treat the infections in the past. Hence, the search for and development of new antibacterials against drug-resistant *S. aureus* is of pivotal importance. Natural products represent an enormous reservoir of compounds that are diverse in structures and chemical properties. These compounds have been used as antibiotics, such as penicillin and streptomycin. The discovery and use of natural products as antibiotics led to the Golden Age of antibiotics in the 1950s to 1960s. In the past decades, many pharmaceutical companies moved away from natural products programmes partly due to a shift to both high-throughput screening and combinatorial synthesis that focus on small synthetic molecules [41, 42]. However, these approaches are proven to have limited successes [43]. In 2015, the Nobel Prize in Physiology or Medicine was awarded to William C. Campbell and Satoshi Omura, and Youyou Tu for their discovery of new anti-parasitic drugs of natural sources, Avermectin and Artemisinin, respectively. This marks the new milestone and brings optimism for natural product drug discovery. Antimicrobial properties of countless natural products have been tested on *S. aureus* and an earlier review summarises these research findings collected between 1995 and 2003 [44]. The purpose of this review is to provide an update on natural products that have been shown to demonstrate promising bactericidal effects against drug-resistant *S. aureus*, published in journal between 2014 and 2017. The resistance mechanisms of drug-resistant *S. aureus* will be discussed, followed by new anti- *S. aureus* agents collected from non-microbial natural products, their potential synergism with antibiotics, the molecular targets and mechanisms of these agents, and potential challenges in developing them into clinical trials.

2. Mechanism of *S. aureus* antimicrobial resistance

Generally, bacteria acquire resistance against antibiotics via different molecular mechanisms, including enzymatic inactivation of antibiotics, alteration of antibiotics target(s) leading to decreased affinity for the antibiotics, removing antibiotics via efflux pumps and changing membrane permeability [45, 46]. *S. aureus* is known to resist all the clinically approved antibiotics using various resistance mechanisms mentioned above. The detailed resistance mechanisms for important antibiotics, including penicillin, methicillin, and vancomycin are discussed below.

2.1. Penicillin resistance

Penicillin was first isolated from a soil fungus, *Penicillium* in the 1940s. This antibiotic was once thought to be a miracle drug as it could cure previously fatal infections. However, few years after its introduction, penicillin resistance including penicillin-resistant *S. aureus* was isolated from hospitals. Penicillin resistance of *S. aureus* is highly prevalent with up to 86% of clinical *S. aureus* isolates being resistant to the antibiotic in the US [47]. Meantime, far way in Australia, a similar observation was made as 80% of *S. aureus* isolates were resistant to penicillin [48]. Penicillin resistance in staphylococci is mediated by the production of enzyme penicillinase or beta-lactamase encoded by the *blaZ* gene. This enzyme inactivates the antibiotic by hydrolysis of the beta-lactam ring of the antibiotic [49]. Studies show that penicillinase genes can be present on either plasmid of the chromosome of *S. aureus* [50].

2.2. Methicillin resistance

Methicillin is a penicillinase-resistant beta-lactam. It was first introduced in 1950s and prescribed for *S. aureus* infection. The first MRSA was documented in 1961 in the UK while the first MRSA in the US was first reported in 1968. Since then, many MRSA clones spread to every corner of the globe. Methicillin resistance is usually encoded by *mecA* gene that is located in a mobile genetic element of *S. aureus*, known as the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*). *mecA* is responsible for the synthesis of low-affinity PBP2a which leads to decreased methicillin binding. Methicillin resistance confers broad spectrum of activity generally to the entire beta-lactam class of antibiotics including penicillins and cephalosporins [51]. The origin of SCC*mec* is thought to be originated from coagulase-negative staphylococcal species as there is no homologues of *mecA* present in methicillin-susceptible staphylococci. In recent years, a novel *mecA* homologue, *mecC* has been identified in both livestock and human in European countries. Similar to *mecA*, *mecC* codes for PBP2a with reduced affinity for methicillin and oxacillin, making them MRSA [52, 53].

2.3. Vancomycin resistance

As mentioned earlier, vancomycin is a gold standard antibiotic choice for MRSA infections. However, the emergence of vancomycin-intermediate *S. aureus* (VISA) (with a MIC value in the range of 3–8 μg/mL) and VRSA (with a MIC value ≥16 μg/mL) result in the failure of vancomycin treatment for MRSA infection. This antibiotic was first released in 1958. However, reduced vancomycin susceptibility in *S. aureus* was reported in 1997 in Japan [54]. VISA is also spreading to different parts of the world [38]. By comparison, the burden of VISA is relatively higher than VRSA, as the former is commonly associated with persistent infection, treatment failure and poor clinical outcomes. The molecular resistance of VISA is less-defined as compared to VRSA. Typically, VISA features increased cell wall thickness, reduced cross-linking rate, an increase of free D-alanyl-D-alanine residues in the peptidoglycan layers which provides more vancomycin binding, leading to an increased consumption of vancomycin while VISA remains unharmed [27, 55–57]. It is suggested that VISA involves accumulation of mutations, or rather, adaptation mechanisms in coping with the challenge of vancomycin. VRSA acquires complete resistance to vancomycin by obtaining plasmid(s) from vancomycin-resistant *Enterococcus* spp. that harbours *vanA* operon encoded on transposon Tn1546. VRSA maintains the resistance by retaining the original plasmid or by integrating Tn1546 from the enterococcal plasmid into staphylococcal resident plasmid. The *vanA* operon facilitates the synthesis of D-Ala-D-lactate instead of D-Ala-D-Ala peptidoglycan precursors. In doing so, vancomycin fails to bind hence leading to resistance observed in VRSA [57].

3. Bactericidal properties of natural products against drug-resistant *S. aureus*

Standard antibiotics treatment against drug-resistant *S. aureus* has failed in the clinical setting due to several causes as abovementioned. Interestingly, these resistant clinical isolates can be

killed by various naturally derived compounds and more promisingly, the antibiotic resistance exhibited by the bacteria can be reversed, and making them susceptible to the antibiotics again. In this section, we discuss the non-microbial natural products that showed bactericidal action against drug-resistant *S. aureus* and their potential to be used in combination with current antibiotics for its synergistic effects.

3.1. Potent natural products against drug-resistant *S. aureus*

Numerous natural products have shown potent antibacterial effects against *S. aureus*. Interestingly, these antibacterial actions are not limited to drug-sensitive wild-type *S. aureus*, but also extended to antibiotic-resistant *S. aureus*, including MRSA [58, 59], VISA [60], and VRSA [61]. Some of these natural compounds that showed promising bactericidal effects against drug-resistant *S. aureus* are summarised in **Table 1**. Due to the extensive repertoire of natural compounds against drug-resistant *S. aureus*, **Table 1** shows only those that are extracted from Pub-Med indexed publications, from year 2014 to 2017. These research articles reported the minimal inhibitory concentration (MIC) of the natural products against the drug-resistant *S. aureus* mainly MRSA using Clinical & Laboratory Standards Institute (CLSI) standard broth microdilution assay. As shown in **Table 1**, the MICs mostly range from micro to milligramme per millilitre. The bactericidal non-microbial natural products are derived from various sources including, plants, insects, animals, and fungi. These natural compounds have been reported to target and act on multiple bacterial targets such as cell wall [62, 63], pyruvate kinase [64], cell division [65], DNA topoisomerase [66], and efflux pump [67, 68]. These pharmacological targets are further discussed in Section 4.

3.2. Synergism of natural products and antibiotics

While serving as potent antibacterial agents alone, several studies have been carried out to investigate the potential of natural products to be used in combination with current antibiotics. This is particularly important against drug-resistant *S. aureus* which have shown resistance against several antibiotics. Natural compounds have been shown to reverse the antibiotic resistance. For instances, Akilandeswari and coworkers demonstrated that apigenin (AP) reversed the bacterial resistance of MRSA when used in combination with ampicillin and ceftriaxone [123]. The resulting MIC for ampicillin was shifted from 800 to 107 μg/mL, and the MIC for ceftriaxone was shifted from 58 to 2.6 μg/mL. Similarly, Mun and colleagues also showed that a plant-derived flavonol, morin reversed the oxacillin- and ampicillin-treated MRSA [124]. Essential oils derived from *Pituranthos chloranthus, Teucrium ramosissimum* and *Pistacia lentiscus* also reduced the resistance of MRSA to various antibiotics in Penicillins' group such as amoxicillin, piperacillin, and oxacillin [125].

Cumulative studies highlight the role of natural compounds in decreasing the reliance on antibiotics in bacterial treatment particularly in MRSA's management, hence preventing the emergence of antibiotic resistance. In addition, production of antibacterial agents from natural products might be more cost-effective than antibiotics production. With advent of modern biotechnology, mass production of these antibacterial products is feasible. More importantly, the manufacturing process allows genetic modifications (e.g. to improve biological activity,

Extract/ compound	Test strain	MIC	References
Curcumin	MRSA	217 μg/mL	[69]
Quinolone alkaloids	MRSA	8–128 μg/mL	[70]
Bee venom	MRSA	0.085–0.11 μg/mL	[71]
Magnolol and honokiol	MDR MRSA, MRSA	8–16 ppm	[72]
Quercus infectoria gall extracts	MRCoNS, MRSA	80–630 μg/mL	[73]
Kaempferia pandurata rhizome extracts	MRCoNS, MRSA	4–16 ppm	[74]
Mulinum spinosum extracts	MRSA	500–1000 μg/mL	[75]
Isothiocyanates from cruciferous plants	MRSA	2.9–110 μg/mL	[76]
Lichen	MRSA	3.9–500 μg/mL	[77]
Marinopyrrole A	MRSA	0.19–0.78 μM	[78]
6,6′-dihydroxythiobinupharidine	MRSA	1–4 μg/mL	[66]
Mature carpels of *Manglietiastrum sinicum*	MRSA	0.016–0.14 μM	[79]
Pentacyclic triterpenoids	MRSA	2–512 μg/mL	[80]
Psoralea corylifolia fruit constituents	MRSA	8–32 μg/mL	[81]
Thai longan honey	MRSA	12.5% (v/v)	[82]
Rubiaceae, Fabaceae, and Poaceae leaves extract	MRSA	5.5–388.4 μg/mL	[83]
Cinnamomum extracts	MRSA	19.5 μg/mL	[84]
Garcinia mangostana pericarp extracts	MRSA	17–20 μg/mL	[85]
MFM 501	MRSA	15.6–31.3 μg/mL	[86]
3′-demethoxy-6-*O*-demethylisoguaiacin	MRSA	12.5 μg/mL	[87]
Essential oils from *Schinus areira* leaves and fruits	MRSA	6–30 μg/mL	[88]
Spiromastixones A-O	MRSA	0.125–8 μg/mL	[89]
Hypericum riparium leaves extract	MRSA	6.54–18.5 μM	[90]
Demethyltexasin	MDR MRSA, MRSA	16–128 μg/mL	[91]
Oleoresin	MRSA	18.2–30 μg/mL	[92]
Thymoquinone	MRSA	8–16 μg/mL	[93]
Phenanthrene fraction	MRSA	8–64 μg/mL	[94]
Rhamnus californica and *Umbellularia californica* extracts	MRSA	3.3–6 mg/mL	[95]
Juncus and Luzula species	MRSA	9.75–156 μg/mL	[96]
E23 marine compound	MRSA	0.5–2 μg/mL	[97]
Piper betle extracts	MRSA	78–625 μg/mL	[98]
Letharia vulpine extracts	MRSA	31.25 μg/mL	[65]
Verrucosispora MS100047	MRSA	3.125–12.5 μg/mL	[99]
Micromonohalimanes B	MRSA	40 μg/mL	[100]
Pterospartum tridentatum extracts	MRSA	78.1 μg/mL	[101]

Extract/ compound	Test strain	MIC	References
N-3 substituted thiazolidine-2,4-dione derivatives	MRSA	6.25–12.5 μg/mL	[102]
Sapotaceae extracts	MRSA	45–97 μg/mL	[103]
Thymus daenensis	MRSA	25 mg/mL	[104]
Compositae extracts	MRSA	31.25 μg/mL	[105]
Roemerine	MRSA	32–64 μg/mL	[106]
Couroupita guianensis extracts	MRSA	62.5–156 μg/mL	[107]
Cotinus coggygria leaf extracts	MRSA	0.313–0.625 mg/mL	[108]
Eremophila alternifolia extracts	MRSA	10–20 μM	[109]
Baicuru	MRSA	39 μg/mL	[110]
Thymus bovei essential oil	MRSA	0.5 mg/mL	[111]
Formicamycins	MRSA	0.625–80 μM	[112]
Rumex aquaticus extracts	MRSA	192.3–463 μM	[113]
Endophenzine G	MRSA	2–128 μg/mL	[114]
Greek oregano isolates	MRSA	160–640 μg/mL	[115]
Macrocyclic bis(bibenzyl)s	MRSA	0.5–16 μg/mL	[116]
Acylquinic acids	MRSA	0.63–1.25 mg/mL	[117]
Houttuynia cordata poultice extracts	MRSA	0.11–1.76 mg/mL	[118]
Dandelion root extracts	MRSA	62.5–500 μg/mL	[119]
Solanioic acid	MRSA	1 μg/mL	[120]
Emodin	MRSA	32–64 μg/mL	[62]
Rhizoma coptidis	MRSA	1.2–2.84 mg/mL	[121]
Macrocyclic bis(bibenzyl)s	MRSA	0.5–32 μg/mL	[122]

MIC—minimal inhibitory concentration; MDR—multidrug-resistant; MRCoNS—methicillin-resistant coagulase negative *Staphylococcus aureus*; MRSA—methicillin-resistant *Staphylococcus aureus*.

Table 1. Antibacterial natural products against drug-resistant ***Staphylococcus aureus***: An update from publication year 2014 to 2017.

Compound	Combination drug	Test strain	References
Curcumin	Oxacillin, ampicillin, ciprofloxacin, norfloxacin	MRSA	[127]
Curcumin	Gentamicin, amikacin, ciprofloxacin	MRSA	[128]
Lippia origanoides extracts	Neomycin, amikacin	MRSA	[129]
Grape pornace extracts	Oxacillin, ampicillin, nalidixic acid, ciprofloxacin, norfloxacin, levofloxacin, tetracycline, chloramphenicol	MRSA	[130]
Artocarpin	Ampicillin, norfloxacin, tetracycline	MRSA	[131]
Apigenin	Ampicillin, ceftriaxone	MRSA	[123]
Morin	Oxacillin	MRSA	[124]

Compound	Combination drug	Test strain	References
Bioactive fraction from *Duabanga grandiflora*	Ampicillin	MRSA	[132]
Bee venom	Ampicillin, penicillin, gentamicin, vancomycin	MRSA	[71]
Diosmetin	Erythromycin	MRSA	[64]
Brazilin	Amikacin, etimicin, gentamicin, streptomycin	MRSA	[133]
Sophora moorcroftiana genistein	Norfloxacin	MRSA	[134]
*Sophora moorcroftiana d*iosmetin	Norfloxacin, streptomycin, ciprofloxacin	MRSA	[134]
Medihoney	Rifampicin	MRSA	[135]
Magnolol and Honokiol	Oxacillin	MRSA	[63]
9EA-FC-B	Ampicillin	MRSA	[136]
Oxyresveratrol	Ciprofloxacin, gentamicin	MRSA	[137]
Zanthoxylum capense constituents	Ciprofloxacin	MRSA	[138]
Poncirus trifoliate extract	Oxacillin	MRSA	[139]
Juglans regia	Oxacillin	MRSA	[140]
Glabridin	Norfloxacin, oxacillin, vancomycin	MDR MRSA	[141]
Coumarins	Chloramphenicol, gentamicin, fosfomycin, levofloxacin, minocycline, piperacillin/ tazobactam, teicoplanin, vancomycin	MRSA	[142]
Polycarpol	Oxacillin, amoxicillin, vancomycin	MRSA, VISA	[143]
Linoleic and oleic acids	Erythromycin	MRSA	[144]
Salvianolate	Fosfomycin, erythromycin, piperacillin-tazobactam, clindamycin	MRSA	[145]
Phellinus baumii extracts	Oxacillin, cefazolin, cefepime, penicillin	MRSA	[146]
Epigallocatechin gallate	Oxacillin, tetracycline, ciprofloxacin	MRSA	[147]
Clerodane diterpene	Norfloxacin	MRSA	[67]
Carnosic acid	Gentamicin	MRSA	[148]
Ursolic acid	Ampicillin, tetracycline	MRSA	[149]
Anadenanthera colubrine extracts	Neomycin, amikacin	MRSA	[150]
Herbal extracts	Oxacillin, gentamicin	MRSA	[151]
Essential oils	Amoxicillin, tetracycline, piperacillin, ofloxacin, oxacillin	MRSA	[125]
Coumarin derivatives	Tetracycline, norfloxacin	MRSA	[68]

MDR—multidrug-resistant; MRSA—methicillin-resistant *Staphylococcus aureus*; VISA—vancomycin-intermediate *Staphylococcus aureus*; VRSA—vancomycin-resistant *Staphylococcus aureus*.

Table 2. Synergistic anti-***Staphylococcus aureus*** effects of natural products and drugs.

solubility, stability, toxicity, production method, production cost and time, etc.) [41, 126]. Some of the challenges and limitation of using natural products as therapeutic modalities are discussed in Section 5.

4. Bacterial targets of *S. aureus* by natural products

In previous section, we discussed the anti-staphylococcal activities by various natural products alone and in combination with multiple types of antibiotics. As some of the mechanisms of antibiotic resistance have already been studied and reported, such as enzymes inactivation, antibiotics trapping, and efflux pumps, this information enables the anti-staphylococcal molecular targets of the natural products to be elucidated. These particular section summaries the molecular targets of natural products against drug resistant *S. aureus* such as bacterial cell wall and membrane, cell division protein FtsZ, pyruvate kinase, DNA topoisomerase, efflux pump proteins, and PBP2a. The reported pharmacological targets are depicted in **Figure 1**.

4.1. Cell wall and membrane

Cell wall of *S. aureus* is a popular pharmacological target of various antibiotics such as penicillins, cephalosporins, vancomycin, bacitracin, and others [152]. These antibiotics interfere with the cell wall biosynthesis and leading to death of the bacteria. Among all, peptidoglycan is the major cell wall components and has been targeted by various drugs [153]. Other cell wall components including adhesins, teichoic acids, immunodominant antigens, and cell wall enzymes are also being targeted by multiple antibiotics [154].

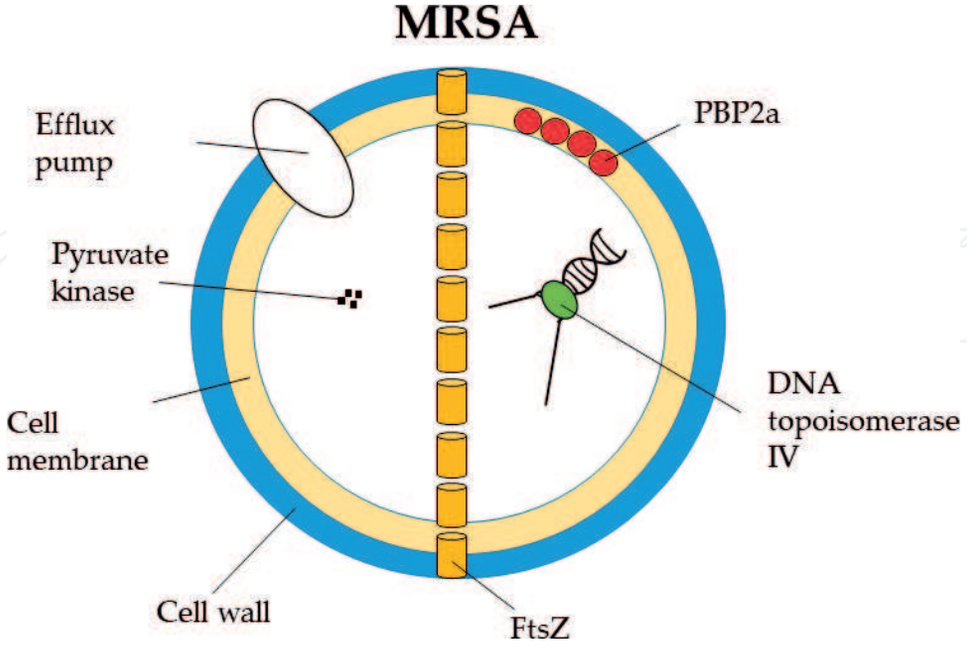

Figure 1. Pharmacological targets of drug-resistant *Staphylococcus aureus* from reported bactericidal natural products.

Similarly, a variety of bactericidal natural compounds also act on bacterial cell wall. For instances, Cao and coworkers showed that a natural compound emodin that targets MRSA could damage the cell wall and compromise the intracellular components. The cellular morphology was altered after the treatment when observed under transmission electron microscopy (TEM) [62]. Kim and colleagues also showed that magnolol targeted cell wall components to exert its pharmacological effect. In a mechanistic study, it has been shown that magnolol inhibited *mecI*'s pathway [63]. In addition, magnolol also targets various resistant genes, such as *mecA*, *femA*, and *femB* in mRNA form. It has also been shown that *Juglans regia* (English walnut) targeted the bacterial cell wall and resulted in the anti-staphylococcal effects [140]. While showing synergism in combination with antibiotics, apigenin was shown to compromise the cell membrane followed by subsequent leakage of intracellular constituents. This finding was demonstrated using TEM which showed significant morphological change of bacterial cell wall, shape, and plasma membrane [123].

4.2. Efflux pump

The function of efflux pumps of bacteria is to eliminate metabolites or materials that are potentially toxic and stress-inducing to the cells including antimicrobial compounds [155]. Hence, the bacterial efflux pumps have been known to contribute significantly to antimicrobial resistance by extruding a large number of antibiotics or drugs. They are often known as multidrug resistance (MDR) efflux pumps [155]. For decades, MDR efflux system has served as an excellent antibacterial target. Numerous promising candidates have previously demonstrated their potencies in targeting efflux pumps as the major mechanism to killing the bacteria [156, 157]. For examples, Wang and colleagues showed that genistein killed the MRSA by inhibiting NorA efflux protein when used in combination with drugs [134]. Mechanistic studies have also shown that various bactericidal natural compounds such as coumarin derivatives [68], linoleic and oleic acids [144], clerodane diterpene [67], and *Anadenanthera colubrina* (Cebil/Vilca) [150] acted on MRSA's efflux pump or proteins.

4.3. Penicillin-binding protein 2a (PBP2a)

PBP2a is encoded by *mecA* resistance gene and this gene can be acquired across different species for methicillin resistance [158]. Both PBP2a protein and *mecA* gene are emerging antimicrobial targets for therapeutics development [159, 160]. Various type of natural products targeting *mecA* gene or PBP2a have also been reported, these compounds include curcumin [161], tiliroside, pinoresinol, magnatriol B, and momorcharaside B [151], *Acalypha wilkesiana* (evergreen shrub) extract [136], and *Poncirus trifoliata* extract [139]. In combination with antibiotics, several natural compounds have also reduced the expression of PBP2a. For instances, Mun and colleagues showed that the combination of morin and oxacillin synergistically killed the MRSA depending on the PBP2a-mediated resistance mechanism [124]. Another study demonstrated that the combination of ampicillin and *Duabanga grandiflora* extract inhibited the PBP2a protein [132]. Hong and colleagues also showed that β-lactams and *Phellinus baumii* extracts synergistically killed the MRSA by targeting PBP2a [146].

4.4. Cell division protein FtsZ

FtsZ is a tubulin-like GTPase that recruit cell division proteins for new cell wall formation [162, 163]. Due to its pivotal role in cell division, it has been recognised as an important target for various antibacterial compounds or drugs including natural products. Liu and colleagues successfully developed several phenolic compounds targeting FtsZ of MRSA using a computer-aided simulation. These natural compounds showed potent bactericidal activities against MRSA [72]. It has also been shown that *Letharia vulpina* (lichen) extract possess antimicrobial activity by damaging cell membrane of MRSA, as well as disrupting cell division processes, possibly targeting FtsZ [65].

4.5. Other targets

Other bacterial proteins that are being targeted by natural products for antimicrobials discovery are pyruvate kinase (PK) and DNA topoisomerase IV. Pyruvate kinase serves as a catalyst to catalyse pyruvate and regulate carbohydrate metabolism [164] whereas DNA topoisomerase IV relaxes supercoiled DNA and performs decatenation events during DNA replication [165]. When used in combination with erythromycin, diosmetin drastically suppressed the MRSA PK activities in a dose-dependent manner. Chan and colleagues also speculated that the inhibition of PK could result in ATP deficiency and efflux pump malfunction [64]. Furthermore, Okamura and group demonstrated that a compound derived from *Nuphar japonicum* (water-lily) inhibited DNA topoisomerase IV of MRSA, but not DNA gyrase which is also carrying an important role in DNA replication [66].

5. Challenges and limitations

Despite great potentials shown by natural products of botanical origin, there is still a long way for them to be used for clinical application. Majority of these products function as supplements for their nutritional and immune-enhancing values, but none of these non-microbial derived natural products is FDA-approved, nor being used for treating bacterial infections. Several natural products antibacterials of microbial origins have been approved since 2010, including fidaxomicin, ceftaroline, dalbavancin, oritavancin, ceftolozane-tazobactam and ceftazidime-avibactam [166, 167]. Between 1980 and 2014, 59% of the total of 140 the FDA-approved antibacterials are originated from natural products or their derivatives, but none of them is originated from plants [166], despite the increasing evidences suggesting that plants may be promising antibacterials as discussed in this review. A few key challenges and limitations are highlighted and discussed in this section, including (a) design of antibacterial screening; (b) solubility and bioavailability of natural compounds; and (c) research directions towards clinical trials.

5.1. Design of antimicrobial screening

Antibacterial screening generally involves phenotypic screening relying on both Kirby-Bauer disc diffusion or broth micro-dilution methods. These methods are commonly used until

today due to the cost-effective and ease of preparation nature [168]. In disc diffusion method, antibacterial activity of an extract or compound is determined based on the presence of inhibitory zone on agar plates seeded with susceptible bacteria while broth micro-dilution method examines the MIC of the antibacterial that inhibits bacterial growth [169]. Very often, these methods are used in the initial antibacterial screening of crude extracts, which may comprise up to hundreds of compounds. This complexity may jeopardise the identification of true antimicrobial effects, leading to false negative results, as some active components may be of low abundance nature [170]. The exclusion of extracts and compounds that have high MIC values following initial screening means giving up on potential novel antibacterials. To overcome this, if crude extract is used, pre-fractionate followed by antibacterial screening to identify the most potent fraction is recommended. These fractions with promising results can be further sub-fractionated until potent compounds are identified. The fractionation technique usually involves the use of HPLC coupled to mass spectrophotometry [171]. By doing this, it reduces the chances of losing potent antibacterial during the screening step

In addition to the use of disc diffusion and broth-dilution methods, various techniques are currently used in the antibacterial studies. One such technique is the time-kill assay (also known as time-kill curve). In this technique, following the broth-dilution method, the bactericidal effects of different concentrations of the antibacterial agents (usually covering the ½ x MIC, 1 x MIC and 2 x of the MIC) at different time points, e.g. 0, 4, 6, 8, 10, 12 and 24 h, are assayed, revealing a time-dependent or a concentration-dependent antibacterial effects of these antibacterials [172]. At the moment, there is a lack of such studies in most of the reviewed articles. As the time-kill assay is able to provide a wealth of information on the dynamic interaction between antibiotics and the microbial strains, specifically *S. aureus* in this context, the inclusion of time-kill assay will further verify the antibacterial activity observed in natural products.

5.2. Solubility and bioavailability of natural products

One of the main limitations of adopting natural products for clinical applications is its solubility and bioavailability [161, 173]. This is highly related to the chemical properties, in particular aqueous solubility of the natural compounds. For examples, curcumin which is a polyphenolic compound, is known to have poor solubility in water, and the main solvents used are usually DMSO, DMF or ethanol [161]. The water insolubility has significant impact on its antibacterial effect and the reported biological action is further reduced under the physiological conditions [161]. There have been several studies demonstrating the reduced antimicrobial effects of natural compounds in the presence of normal human serum. Marinopyrrole A, which has previously shown potent antibacterial action against MRSA, showed approximately 256-fold higher MIC when tested in the presence of 20% serum [72]. The reduced activity could be due to the non-specific serum protein bindings and protein degradation due to metabolic enzymes and complements that largely affect the bioavailability. It has also been reported that curcumin showed reduced antibacterial activities against *S. aureus* when tested in the presence of human plasma and whole blood [174]. Similarly, human serum albumin has significantly decreased the bactericidal properties of curcumin [174, 175].

Numerous methods have been developed to overcome the solubility and bioavailability issues. Natural products loaded into nanocarriers such as nanoparticles, microemulsions,

micelles, *etc.* have improved the overall stability and bioavailability [176]. Incorporation of natural compounds such as resveratrol and thymol into liposomes has also increased the solubility and stability for their medicinal uses [173]. Furthermore, development of bioconjugates and nanoformulations also greatly improves the pharmacological action of natural products. This has been extensively reviewed for curcumin [161, 177].

5.3. Clinical trials

In the past decades, research organisations are de-prioritising natural products in their drug discovery programmes because of the costs associated with the development and licensure. For instance, between 1995 and 2001, Glaxo Smith Kline conducted 70 HTS campaigns, each worth approximately USD 1 million to identify only five potential antibacterial leads [178]. This early screening does not guarantee marketing and launching of these potential antibacterials as only approximately 30% of drugs, including natural products used as anti-infectives receive FDA approval [179]. Following initial *in vitro* testing, clinical (phase I to III) testing is required to ensure the efficacy and safety of new antibacterials on human subjects. The complexity of clinical trial adds another barrier to the development of new antibacterials [180, 181]. On one hand, pharmaceutical companies are faced with multiple regulatory bottlenecks such as increased stringency of trial design, increased demands regarding the design of phase III studies, and increased stringency of safety requirements for pre-licencing and post-licencing procedures of drugs [181]. On the other hand, bacteria are acquiring resistance at fast pace. It complicates clinical trials as these trials cannot be completed without a substantial number of the enrolled patients being infected with new, highly resistant strains. Clinical trials involving rare infectious diseases such as meningitis or endocarditis are most affected as these trials may take years and require multiple centres to complete [180]. Upon completion and success of clinical trials, pharmaceutical companies are required to file for approvals from the relevant agencies such as FDA in the US and European Medicines Agencies in the Europe. The entire process may take up to 15 years for the drug discovery to the launching stage [178]. The lack of interest and investment in antibacterial of natural sources reflects in the identification of only one such antibacterial agent, New Mexico Honey, as a decolonization agent for CA-MRSA abscess in the phase II clinical trial phase (ClinicalTrials.gov identifier number NCT00532324, accessed on the Dec 18, 2017).

In recognition of a lack of novel antibacterials in the clinical pipelines, FDA launched incentives such as Generating Antibiotics Incentives Now (GAIN) Act to foster the research and development of new antibacterial. For instance, granting five additional years of exclusivity to new antibacterials to the pharmaceutical companies, providing incentives for drugs used for treating serious and life-threatening infections, including *S. aureus*, and reducing new antibacterial drug application time to 6 months [182].

5.4. Future directions

The search for new antibacterial agent in natural products remains an exciting yet challenging task. Evidences show that regulatory agencies are working collaboratively with pharmaceutical companies in improving the development of new antibacterials from natural sources. The

combined efforts are the key in shaping the development and marketing of potent antibacterials in the coming years. Scientists working in the field, however, may play a bigger role in the discovery of novel antibacterials by addressing technical shortcomings of the screening of natural products for novel antibacterials.

One such aspect for consideration is to expand the antibacterial screening to include anti-virulence screening such as quorum sensing systems, biofilm formation and pilus adhesins. The investigation of anti-virulence rationalises that because anti-virulence drugs do not kill bacterial cells and thus exerting less selective pressure for resistance. It is believed that the development of resistance is slower compared to bactericidal agents. Anti-virulence would constitute a valuable alternative to bactericidal agents [183, 184]. Anti-virulence of natural product such as anti-quorum sensing of goldenseal (*Hydrastis canadensis* L.) [185], anti-biofilm of dihydrocelastrol and dihydrocelastryl acetate present in many plants [186] in MRSA have been reported. This area of research is still lacking, in-depth investigation on anti-virulence potentials and solid evidence of slow resistance rate is still required.

Another challenging aspect of natural product not mentioned earlier is low bioavailability of natural products, creating inconsistent results between preclinical and clinical studies [187–189]. To overcome this challenge, scientists are exploring the incorporation of nanoparticles into a delivery system for natural products in order to increase therapeutic effects of natural products [190]. Preclinical successes of curcumin-nanoparticles in inhibiting *in vitro* growth of *S. aureus* [191] and MRSA and enhancing wound healing in in vivo murine wound model [192] have been documented thus far. This emerging field holds promises for natural products in treating bacterial infections. However, drug targeting using nanoparticles remains a challenge, toxicity and safety needs further in-depth evaluations.

6. Conclusions

Non-microbial natural products have shown promising bactericidal activities against drug-resistant *S. aureus*. The mechanisms of bacterial killings are under investigation and great efforts are being made to evaluate their antibacterial activities in clinical trials. This chapter provides an important update on the anti-staphylococcal activity of natural products against *S. aureus* and the underlying challenges are highlighted. These issues need to be addressed in order to transform the antibacterial natural products into clinically useful antibiotics in the future.

Acknowledgements

We thank Sunway Internal Research Grant (INT-2018-SHMS-SIHD-01 and INTS-2017-SST-DBS-02) from Sunway University and National Cancer Council Malaysia (MAKNA) Cancer Research Award (CRA) 2016 (EXT-SIDS-SIHD-MAKNA-2017-01) for partly supporting this work.

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