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Potential of anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity of the marine-associated bacterial extracts

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global health threat, highlighting the urgent need for new treatments as antibiotic effectiveness wanes. Marine microbes are valuable sources of potential anti-MRSA compounds. In this study, marine bacteria were isolated, cultured in marine broth, and extracted with ethyl acetate. The extracts were tested against clinical MRSA 142. Among four bacteria isolated from sponges and sediment, isolates S6.2 (sponge-derived) and SK3 (sediment-derived actinobacteria) showed the strongest anti-MRSA activity, with MIC values of 0.156 mg/mL and 0.078 mg/mL, respectively. HPLC analysis revealed key peaks at Rt 25.58 and 29.02 minutes for S6.2 and a prominent peak at Rt 29.18 minutes for SK3. The SK3 isolate exhibited robust growth and high metabolite production on marine agar, ISP no. 2, 5, and 7, indicating it a promising candidate for anti-MRSA compound production.

Keywords: anti-MRSA, marine associated bacteria, extracts, culture, potential

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INTRODUCTION

Staphylococcus aureus is a pandemic pathogen that causes a wide spectrum of pyogenic lesions involving several organs, resulting in nosocomial outbreaks and community-acquired infections. In the 1940s, the medical treatment of *S. aureus* infection became successful and routine due to the discovery of antibiotic agents such as penicillin[1]. Nonetheless, *S. aureus* has rapidly developed resistance to penicillin and methicillin, an antibiotic commonly used for *S. aureus* treatment since the late 1950s. In 1961, scientists identified the first methicillin-resistant *S. aureus* (MRSA) in England[2]. MRSA strains are highly variable in different geographical areas and can rapidly mutate to acquire resistance to commercially available antibiotics, with the exception of vancomycin and teicoplanin[3]. A recent report by both the World Health Organization and the Centers for Disease Control highlighted that MRSA infection accounts for over 94,000 cases and approximately 18,650 deaths annually in the United States[4]. In European countries, MRSA has been reported in more than 25% of infections, and its prevalence in African countries (except South Africa) has increased since 2000[5].

Since the 1980s, MRSA detection in healthcare settings in asian nations currenly has increased[6]. MRSA virulence and spread in Thailand have led to the infection rate increasing to 45% since 1999; furthermore, the national antimicrobial resistance surveillance centre surveyed 46 hospitals and found vancomycin resistance in 98% of MRSA strains in 2023[7]. The emergence of vancomycin-resistant *S. aureus* and intermediate MRSA strains has recently been recognized and leaves physicians with few available options for antibiotics to treat MRSA infection[8]. Thus, the discovery of new anti-MRSA antibiotic agents is urgent. Bioactive natural products are one of the main sources of antibiotic drugs[3]; many antimicrobial agents and resistance-modifying compounds have been the prototype of molecules isolated from natural resources, particularly marine environments, including microorganisms and other invertebrates. Among microorganisms, marine bacteria are a rich source of anti-MRSA compounds[8,9].

Herein, we report the isolation and cultivation of marine-derived microorganisms and screened the anti-MRSA properties of their extracts, aiming to identify the promising candidates for the development of new antimicrobial compounds.

METHODOLOGY

Marine sponges and sediment collection

Samples were collected from the intertidal zone of Sarai Island (6°39′.97″ N, 99°51′.32.01″ E), Satun Province, Thailand (Figure 1), during low tide. Sponge and sediment samples were collected by hand and kept in iceboxes at 4°C during transfer to the laboratory and immediate isolation of the associated microorganisms.

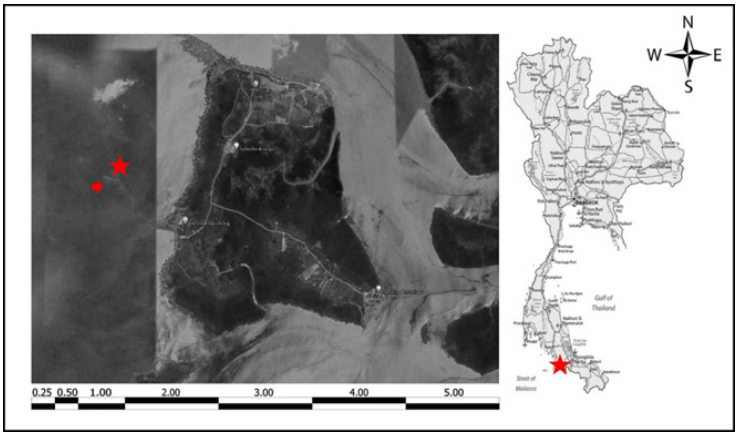

Figure 1. Area of sample collection

Isolation, cultivation and purification of marine-associated bacteria

Sponge-associated bacteria

The sponge samples were washed with sterile seawater until clean, and then their surfaces were sterilized by rapidly swabbing with 70% ethanol, followed by immersion in sterile seawater for 2 min. Approximately 1 cm^3 samples of the central core tissues were cut and ground in 3 mL of sterile sea water. The ground tissue was serially diluted in sterile seawater to 10^{-6} before 100 µl aliquots were spread on Zobell Marine Agar (Himedia, New Delhi, India). The plates were placed in the incubation chamber at 30°C for 7 days, with observation on each day. Clear colonies with different characteristics were picked with a needle, restreaked on other MA plates to obtain pure strains, and maintained in a marine agar slant at 4°C for further experiments. For long-term preservation, 15% glycerol was added, followed by freezing at -80°C. The sponge samples were identified by Dr. Pedpradab S. using the standard reference guide for sponge identification[10].

Sediment-associated bacteria

Three grams of sediment were collected using a gravity core sampling instrument (Dormer, Sydney, Australia). The samples were stored in sterile sample bags made of low-density polyethylene (LDPE) and kept at 4°C during transport to the laboratory for subsequent experiments. Marine-associated bacteria were isolated using the serial dilution method. Sediment samples were serially diluted (10^{-1} to 10^{-9}), and the dilutions were plated onto marine agar medium. The inoculated plates were incubated at 37°C for 24 to 72 hours. Morphologically distinct bacterial colonies were observed and picked from the 10^{-5} to 10^{-7} dilution plates. These colonies were further purified using the streak plate technique. All experiments were conducted in triplicate. A pure bacterial strain was subsequently cultivated in marine broth medium for 72 hours before extraction with ethyl acetate.

Cultivation of bacteria and crude extract preparation

Seed cultures were prepared by inoculating pure colonies of the isolated bacteria in 5 mL of marine broth and incubating them at 30°C on a shaker for 10 days. The seed cultures were examined for anti-MRSA activity by the agar well diffusion and agar overlay method[11]. The cultures that showed anti-MRSA properties were transferred to 180 mL of marine broth in a 250 mL conical flask and incubated in the previously described conditions for 10 days. The cell suspension was lysed using an ultrasonic bath at 30°C for 5 minutes, followed by centrifugation at 5,000 rpm for 5 minutes. The supernatant (liquid part) was extracted three times with ethyl acetate in a separatory funnel. This extraction process resulted in the formation of two distinct liquid layers: the organic phase (ethyl acetate) and the aqueous phase (broth). The organic phase was then concentrated by evaporation using a rotary evaporator set to a water bath temperature of 45°C, yielding the crude extract.

The organic layer was separated and concentrated under a vacuum to yield the crude extract. The extract showing the most potent anti-MRSA activity was further studied for growth in different media, including ISP no. 1-7, Mueller Hinton Agar (MHA), Marine Agar, and Luria Bertani (LB).

Isolation and purification of methicillin-resistant *Staphylococcus aureus*

The MRSA strain was isolated from Thasala Hospital, Thailand, using the method[12,13]. The method consisted of two procedures. First, the cefoxitin disc screen test method was used for the detection of MRSA. The susceptibility of *S. aureus* isolates to 30 µg of cefoxitin was determined by the disc diffusion

method on Mueller-Hinton agar plates using a bacterial suspension equivalent to a 0.5 McFarland standard. The MRSA-inoculated plates were incubated at 35°C for 24 hrs. The results were interpreted according to CLSI guidelines. For MRSA sensitivity, the inhibition zone was ≤21 mm. Second, for multiplex PCR for the SCC*mecA* gene, DNA was extracted from MRSA strains using an extraction kit (Qiagen, USA) according to the manufacturer's instructions. Multiplex PCR was performed to detect three loci (A, B and C) of the *mecA* gene for SCC types I, II, and III, respectively. The primers and amplification method[12].

Anti-MRSA activity determination

The agar well diffusion and agar overlay methods were used to examine the extracts' anti-MRSA activity. The agar well diffusion method was performed by using MHA. The MRSA suspension was equally diluted to a 0.5 McFarland standard with sterile sodium chloride and spread on an MHA medium. Then, wells with a diameter of 6 mm were punched aseptically with a sterile borer. Eighty microlitres of MRSA suspension was transferred to the wells and incubated at 37°C for 24 h to measure the inhibition zone. The agar overlay method was performed by spotting marine-associated strains on Zobell marine agar plates and allowing them to grow in an incubation chamber for 3-5 days. The tested MRSA strain in a soft medium was gently overlaid on the marine strain and then incubated at 37°C for 24 h to measure the inhibition zone. Medium and vancomycin were used as negative and positive controls, respectively. Mass culturing of bacteria was performed in 500 mL conical flasks on a shaker, and the cultured organisms were screened daily for anti-MRSA properties.

Bioautography analysis

Direct thin-layer chromatography (TLC) bioautography was examined by following the method[14]. Briefly, the developed chromatogram was sprayed with bacterial suspension (10^6 CFU/mL), incubated at 25°C for 48 h under humid conditions, and then sprayed with tetrazolium salt solution and reincubated at 25°C for 24 h. The antimicrobial activity was visualized as a clear, bright zone against a purple background on the TLC image.

Chromatographic analysis of the crude extract

Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were used to analyse the components in crude extracts. TLC was performed by using an ALUGRAM® Xtra SIL G/UV_{254} precoated TLC sheet (Macherey-Nagel, Düren, Germany). A mixture of chloroform, ethyl acetate, and methanol (6:3:1, v/v) was used as an eluent (mobile phase). HPLC chromatograms were obtained by a Dionex UltiMate 3000 (Thermo Scientific,

Waltham, MA, USA). The separation column was achieved using an Acclaim 120 C18 reversed-phase column (5 μm, 4.6 x 150 mm, Thermo Scientific, Waltham, MA, USA). Gradient elution was programmed to progress from 100% water to 100% methanol within 30 min with a flow rate of 1 mL/min, and a photodiode array detector was set at wavelengths of 230, 254, 285 and 366 nm.

RESULTS and DISCUSSION

MRSA isolation

Three MRSA strains (MRSA 142, MRSA 1096, and MRSA 2468) were isolated from patients in Thasala Hospital. All strains were examined for *mecA*-mediated oxacillin-resistant *Staphylococcus* and inducible clindamycin-resistant *Staphylococcus* (Table 1). All clinically isolated MRSA strains showed oxacillin resistance and retained *mecA*; only strain 142 exhibited a D-shaped zone in the clindamycin inducible resistance examination. On the basis of the results, MRSA 142 was selected as the index strain for further experiments.

Table 1. The isolated MRSA and drug-resistant examination

Strain code	Oxacillin resistance	mecA gene	Clindamycin resistance
			Inducible resistance (D-zone of inhibition)
MRSA 142	✓	✓	✓
MRSA 1096	✓	✓	-
MRSA 2468	✓	✓	-

Isolation and culture of marine-associated bacteria and anti-MRSA assay

Several marine sponges (Figure 2) were used to isolate associated bacteria. Three of them, namely, *Cacospongia* sp., *Mycale grandis*, and *Paratetilla bacca*, yielded bacterial strains S1.2, S6.2, and SL9, respectively.

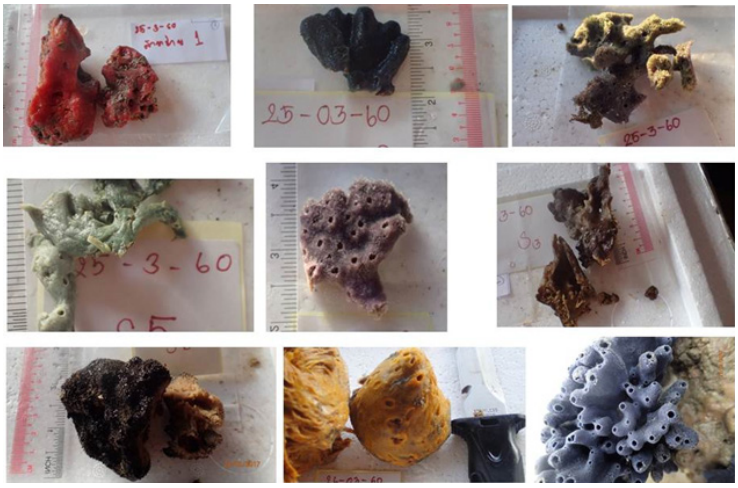

Figure 2. Marine sponge samples using for isolating associated bacteria

In addition, a starin of bacteria, namely SK3 (Figure 3), was isolated from marine sediment. The isolated bacteria were cultivated in marine broth for crude extract preparation.

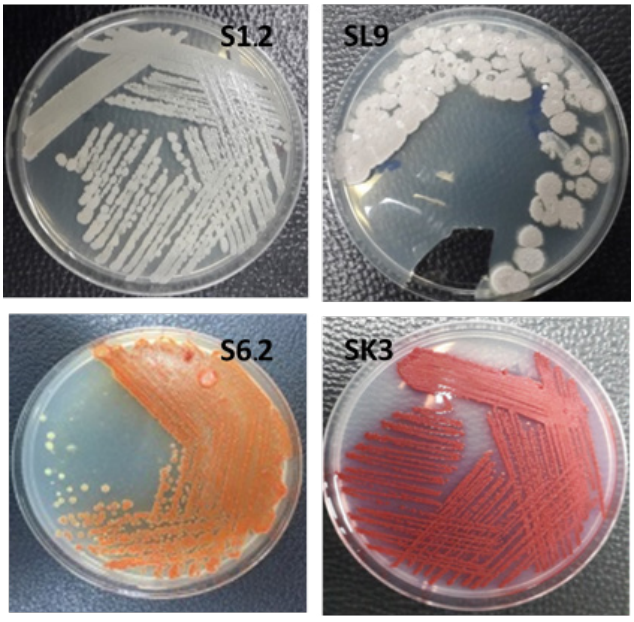

Figure 3. Colony feature on solid media (marine agar) of the isolated bacteria

The cultures were harvested in the late lag phase and then extracted with ethyl acetate to obtain the crude extracts. The anti-MRSA bioactivity of the extracts was subsequently determined (Table 2 and Figure 4).

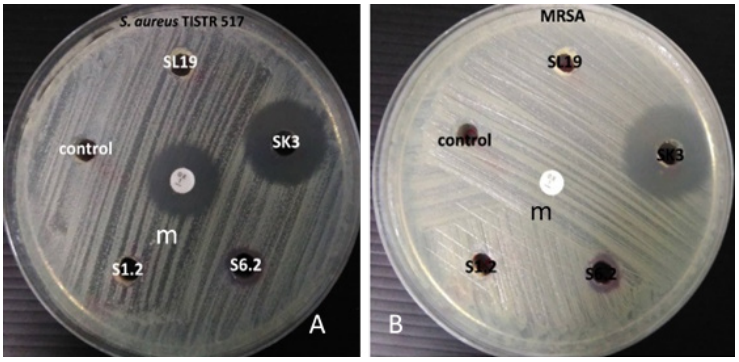

Figure 4. Inhibition zones of bacterial extracts against S. aureus (A) and MRSA (B). Marine broth and methicillin (m) were used as negative and positive control, respectively.

Table 2. Anti-*S.aureus* and anti-MRSA screening of the bacteria extracts

Test organisms	Extract code	Inhibition zone (cm)
S. aureus TISTR 517	SK3	0.032 ± 0.01
	S6.2	0.007 ± 0.01
	S1.2	0.003 ± 0.02
	SL19	0.003 ± 0.05
	control	0.002 ± 0.01
MRSA	SK3	0.037 ± 0.01
	S6.2	0.0060 ± 0.02
	S1.2	0.002 ± 0.01
	SL19	0.001 ± 0.04
	control	0.002 ± 0.01

The S6.2 extract exhibited weak inhibition against the tested microorganisms, whereas the SK3 extract showed strong inhibition. The SK3 extract was further checked for potential anti-MRSA bioactivity by using MIC and MBC methods, and the colony features were studied using different types of media. The SK3 extract exhibited the greatest potential for anti-MRSA bioactivity, indicated using MIC and MBC values (0.156 ± 0.001 and 0.625 ± 0.003 mg/mL, respectively; Table

3). Moreover, our study revealed that the SK3 strain displayed different colony features and colours when cultured on various solid media. The colonies were pink with white spots on ISP media No. 2, 3, and 4, whereas they appeared dark orange on ISP No. 5 and 7 and marine agar. Furthermore, cream-coloured colonies were observed when the SK3 strain was cultured on ISP No. 6, MHA, and LB media.

Table 3. MIC and MBC values of the extracts from marine associated bacteria

Codes	Test organisms	MIC mg/mL	MBC (mg/mL)
S6.2	MRSA 142	0.156	0.625
	S.aureus 517	0.312	1.25
SK3	MRSA 142	0.0078	>0.031
	S.aureus 517	0.0625	>0.25

Chemical analysis of the extract

The SK3 extract was subsequently analysed for the allocation of active compounds by using TLC-directed bioautography and for its chemical constituents by using HPLC methods. According to TLC-directed bioautography, the positions of the bright spots (Figure 5) indicated the presence of anti-MRSA metabolites within the extract.

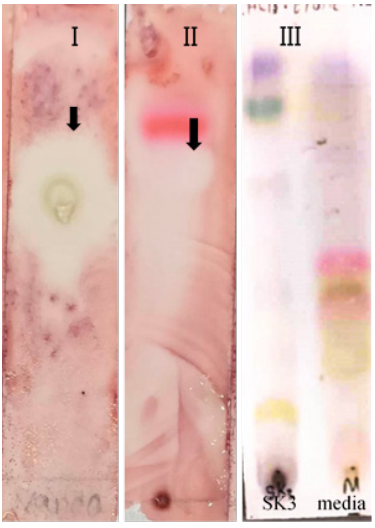

Figure 5. Bioautogram (I, II) and TLC (III) of the extract from a bacterium SK3 demonstrate inhibition against MRSA and chemical profile. I represent a positive control (Vancomycin), while II and III are the extract and its chemical profile, respectively. The brilliant spots observed on II correspond to the active secondary metabolites containing.

These active metabolites did not react with the anisaldehyde reagent, suggesting that they were not steroids or terpenoid compounds present in the extract. According to HPLC (Figure 6), the main active compounds were observed at an Rt of 29.187 min in the SK3 extract, with the remaining peaks identified as components of the culture media. The S6.2 extract contained minor metabolites at Rt values of 25.583 min and 29.023 min, with the other peaks attributed to components of the culture media.

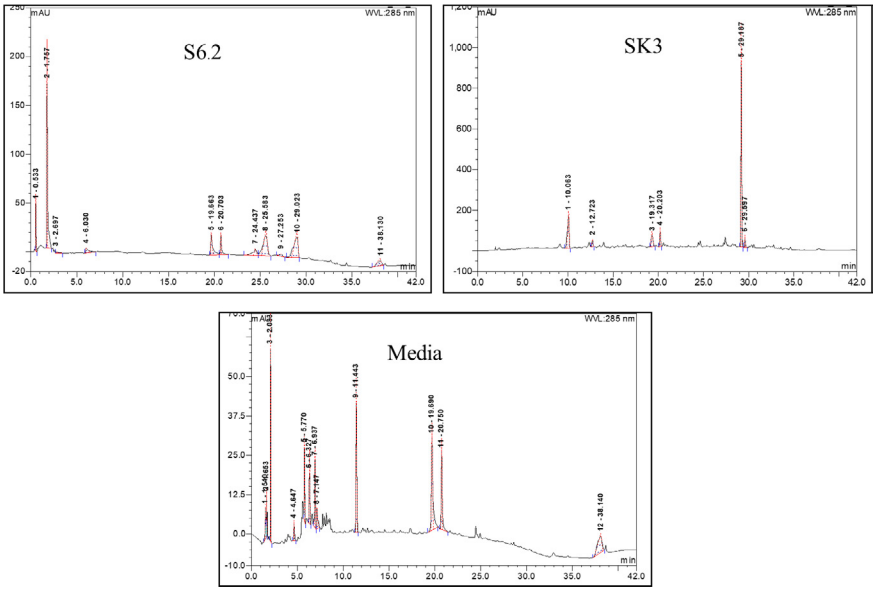

Figure 6. HPLC chromatograms of the active extracts show peaks at Rt ranging 0 - 20.750 minute, which are attributed to the media composition. The active metabolites of S6.2 are expected at Rt 25.583 and 29.023 minute, while SK3 exhibits a main peak at Rt 29.187.

A MRSA outbreak in Thailand was observed 15 years ago. Since 2012, the National Antimicrobial Resistance Surveillance Center (NARSC) has reported that the rate of MRSA vancomycin resistance has increased to 98%[7]. In this work, three MRSA strains were isolated from the mucous of patients in Thasala Hospital, Nakhon Sri Thammarat Province, Thailand. The isolated MRSA strains were determined for their drug resistance induction by using the *mec*A gene, oxacillin, and clindamycin as indicators (Table 1). All strains tested positive for anti-oxacillin resistance and carried the *mec*A gene, but only MRSA142 showed a D-shaped zone on clindamycin resistance testing. A D-shaped zone on agar well plates can indicate the presence of inducible antibiotic resistance. It occurs when bacteria carry genes that can be turned on or off, depending on the type of certain antibiotics. In this case, MRSA142 exhibited inducible

resistance to clindamycin. This information is essential when considering bacterial strains for drug susceptibility screening, and as a result, MRSA142 was selected for further use as a test organism in an anti-MRSA drug discovery program. We isolated 4 marine-associated bacterial strains from several marine sponges and sediment; three of the strains showed anti-MRSA activity (Table 3 and Figure 4), including S6.2, which was isolated from a red Demospongia sponge, *Mycale grandis* (No. 1, Figure 2), and SK3, which was isolated from sediment. Normally, sponges are rich sources of diverse associated microorganisms, some of which produce antimicrobial metabolites, while sediment contains mainly antibiotic-producing actinobacteria[15]. However, this also depends on geographic variation and cultured media, even for the same species and hosts[15-17]. For example, 1234 bacterial strains were isolated from sponges in South Australian marine environments, of which 21% showed antimicrobial activity against MRSA[18]. A total of 460 strains were isolated from 18 sponges in Vietnam water, of which 90 strains exhibited antimicrobial activity, and in particular, 21 strains exhibited activity against *S. aureus*[19]. Actinobacteria are well distributed in marine sediment and show antimicrobial activity against several drug-resistant pathogens. According to this work, 92 actinobacteria, mainly *Streptomyces* spp., were isolated from Philippine marine sediment and exhibited anti-multidrug-resistant *S. aureus* activity[20], while 6 actinobacterial strains isolated from Nicobar Island, the Andaman Sea, also showed anti-*S. aureus* activity[21]. The above data indicate the high diversity of antimicrobial compounds produced by associated marine bacteria; accordingly, intensive surveys and screening for their antimicrobial activity should be performed on bacteria in various regions. Assessment of the anti-*staphylococcal* activity of the extracts from marine-associated bacteria showed that strains SK3 and S6.2 created the largest zone of inhibition (Figure 4 and Table 3).

Various sponges have been reported as sources of anti-MRSA compound-producing bacteria, including *Haliclona* sp., *Axinella* sp., *Dysidea* sp., *Epipolasis* sp., *Neopretosia* sp., and *Mycale* sp.[22]. In this study, we isolated an unidentified bacterium. The extract (coding as 6.2) from this bacterium exhibited anti-MRSA activity with a MIC value of 0.156 mg/mL. Previous studies have shown that the genus Mycale hosts a diverse array of associated bacteria, particularly *Bacillus* sp., *Vibrio* spp., *Streptomyces* sp., *Cobetia* sp., *Pseudomonas*, and *Nocardiopsis* sp.[23]. Some of these bacteria produce various classes of anti-MRSA compounds, including brominated biphenyldiols, peptides, terpenoids, alkaloids, and molecules containing five-membered lactone rings[24]. For instance, a novel linear peptide, bogorol A, demonstrates high anti-MRSA potency with an MIC value of 2.5 μg/mL, while 3,3′,5,5′-tetrabromo-2,2′-biphenyldiol ex-

hibits MIC values ranging from <0.25 to 2 mg/mL.[25-26]. Additional examples have been summarized by Liang et al. (2023)[23]. In this work, we analyzed the preliminary data of the active extract. However, the pure active compounds responsible for the observed anti-MRSA activity have not yet been identified.

HPLC was primarily used for the analysis of active components in the extracts (Figure 6). The HPLC chromatograms revealed that the active constituents contained in the S6.2 extract were located at retention times (Rt) of 25.583 and 29.023 min, while SK3 showed only one peak at 29.18 min. Other peaks (at Rt 0 - 20.703 min) in both the S6.2 and SK3 extracts were components of the medium (chromatogram of pure medium was used as a reference), which did not exhibit activity against MRSA. However, the type of active compounds still remains unidentified until they are purified and characterized. The MIC and MBC values (Table 3) revealed that strain SK3 contained the highest potency of anti-MRSA constituents, which was observed as a peak at Rt 29.187 min and probably a very minor compound at Rt 29.597 min on HPLC chromatogram. SK3 was accordingly selected for further study. SK3 is a gram-negative bacterial strain that formed a circular, shiny colony on marine agar (Figures 3 and 7). It was cultured in 9 different solid media for one week, including marine agar (MA), MHA (Mueller-Hinton Agar), LB (Luria Bertani), and ISP No 2-7, to observe colony features and anti-MRSA activity potential. Its colony features and anti-MRSA activity potential differed according to the culture medium, as shown in Figure 7.

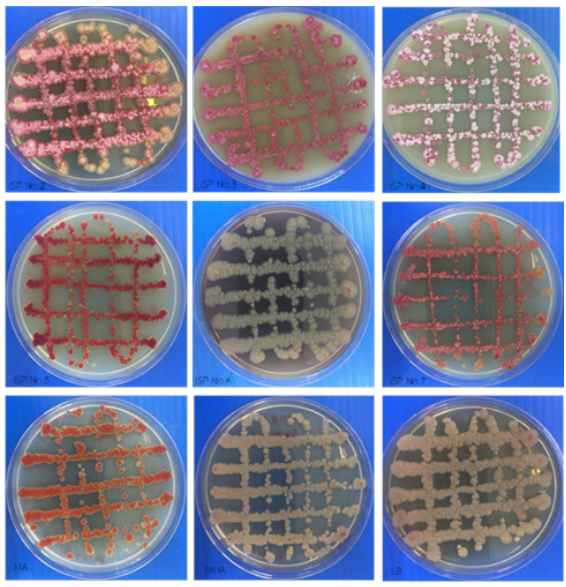

Figure 7. Colony feature of SK3 on different kind of solid media

The growth and potential of bioactive compound synthesis of marine actinobacteria varies on different culture media, as found in some previous reports. ISP no. 2 promoted a high degree of antimicrobial compound production in many marine actinomycetes (also found in SK3), but yeast extract peptone (YP) and starch yeast extract peptone (SYP) did not support active compound production[27,19]. Based on the principle that many marine bacteria produce secondary metabolites that are released into the environment, the potential for secondary metabolite production of SK3 was examined by the agar plug diffusion technique. A portion of the individual solid medium on which bacteria had grown was excavated and then transferred into drilled wells of MRSA discs. We found that SK3 produced the strongest anti-MRSA metabolites when cultured with marine agar (MA), ISP No. 2, 5 and 7 (Figure 7). This indicated that nutrient composition and concentration were related to the growth form of SK3 and also their secondary metabolite synthesis. Generally, appropriate concentrations of elemental ions, monosaccharides, peptone, and yeast extract are essential components for the growth of marine bacteria and antibacterial metabolite synthesis[27]. Sodium and potassium ions are involved in oxidative metabolism processes and biologically active metabolite production, while peptone and yeast extract are sources of organic carbon and monosaccharides[28]. Minor concentrations of amino acids such as L-asparagine and L-tyrosine are still essential cofactors for producing secondary metabolites and/or growth promoters[29-30], as found in ISP No. 5. It is worth noting that starch or complex carbohydrates contained in ISP No. 3 and 4 may have a negative effect on bioactive compound synthesis, as estimated by the inhibition zone.

STATEMENT OF ETHICS

This study does not require any ethical approval.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Rachow K: Acquisition, analysis of data, and statistical analysis; Patchara P: Design project, data analysis, and drafting manuscript; Monthon L: Design project and critical review manuscript.

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