

Antimicrobial activity of tropical soft corals found in the Northern Straits of Malacca

Running title: Antimicrobial activity of tropical soft corals

¹Hana Marican, ⁴Raihana Edros*, ^{1,3}Mahadi Mohammad, ²Sazlina Salleh

¹*School of Biological Sciences, Universiti Sains Malaysia, Penang, 11800, Malaysia*

²*Centre for Policy Research, Universiti Sains Malaysia, Penang, 11800, Malaysia*

³*Centre of Marine and Coastal Studies, Universiti Sains Malaysia, Penang 11800, Malaysia*

⁴*Faculty of Technology, Universiti Pahang Malaysia, Gambang, Pahang 26300, Malaysia*

Corresponding author: rzahirah@ump.edu.my

Abstract

*The study was conducted to obtain and test crude extracts from soft corals found at Pulau Payar, an island off the northern Straits of Malacca. Specimens were extracted in an equal volume of methanol and dichloromethane. The samples were then concentrated to obtain crude extracts. Anti-bacterial activities of the crude extracts were tested by disc diffusion method, minimum inhibition concentration (MIC) and minimum bacterial activities (MBC) assays. Out of twelve crude extracts tested, 50 percent of them showed inhibition for *Staphylococcus aureus* and MRSA; and approximately 8 percent have showed inhibition for *Enterococcus raffinosus*. The MIC of each extract is stated in the tables below. Only one of them showed bactericidal properties against MRSA. Soft corals around the world, have been found to contain beneficial chemical compounds, however not much research has been done in the region. This shows that soft coral extracts found in the Northern Straits of Malacca do have antibacterial potential, and this potential can be harnessed for future pharmaceutical exploration.*

Keywords: Antibacterial, octocoral, secondary metabolites, ESKAPE, extract, Malaysia, disc diffusion, MIC, MBC

Abbreviation: ESKAPE- *Enterococcus raffinosus*, *Staphylococcus aureus*, *Klebsilla pneumoniae*, *Acinobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*

MRSA- Methicillin-resistant *Staphylococcus aureus*

MIC- Minimum inhibition concentration, **MBC-** Minimum bactericidal concentration

I. INTRODUCTION

Ever since the 1960s, there has been a growing need for drugs to control new illnesses and to combat resistant strains of microorganisms. This has encouraged the search for new sources of natural products. Marine environments are one of the richest and most complex ecosystems in terms of biological and chemical diversity [1]. In 2012, approximately 1241 new biochemical compounds were reported, which clearly identifies the marine environment as a bountiful source of bioactive compounds [2,3,4]. Bioactive natural products have been isolated from both marine macro- and micro-organisms. The advances of science and technology in the past 50 years, have resulted in the isolation of approximately 24,662 unique new bioactive compounds from marine organisms [5]. Out of this, marine invertebrates are known to be major producers of these natural products which serve several biological and ecological roles [6].

Antimicrobial activity of secondary metabolites has been reported extensively for several groups of marine invertebrates, such as ascidians [7], byozoans [8], sponges [9,10], hard corals [11], gorgonian octocorals [12] and alcyonacean soft corals [13]. Bioactive secondary metabolites from marine organism can be divided into several groups; steroids, terpenoids, isoprenoids, nonisoprenoids, quinones, brominated compounds, nitrogen heterocyclics and nitrogen sulphur heterocyclics [14,4].

Soft corals such as Coelenterates (class: Anthozoa, subclass: Octocorallia) have been studied and are known to produce a range of valuable compounds [15]. Most of these compounds are categorised to be sesquiterpenoids and diterpenoids [4]. These compounds have a direct use for the octocorals. For example, 3 extracts of *Sinularia* species displayed defence mechanism against predators [16]. Soft coral extracts have also been discovered to aid in interspecific competition for space [17,18] and in prevention of fouling on the coral tissue [19]. In addition, a range of secondary metabolites play an important role solely in reproduction of soft corals. Diterpenes such as thunbergol and epi-thunbergol, are present in the eggs of *Lobophytum compactum* and *Lobophytum crissum*, but do not appear to be effective in deterring reef fish, and they do not possess effective antibacterial properties [20,21]. Besides benefitting the corals themselves, secondary metabolites are known to have properties that are beneficial to humans such as antifungal, antiviral and antibacterial activities [5,22].

Soft corals around the world, have been found to contain beneficial chemical compounds, however not much research has been done in the region. This is a cause for concern as coral reefs in Southeast Asia have the highest degree of biodiversity and most extensive coastlines worldwide. Total coral reef area is almost 100,000 km², covering nearly 34 percent of the world's total coral reef area [23]. Southeast Asian coral reefs hold more than 75 percent of the global coral species (over 600 of the world's nearly 800 reef-building coral species) and more than 33 percent of the global reef fish [23]. Thus, a fundamental study on the coral reefs; not only ecology, but also taxonomy, and other biochemical studies are necessary for exploration. Most researches have been conducted on the temperate species, therefore we have sought to study whether there is a similarity between the secondary metabolites of temperate climate and those are found in our region; and if climate (mainly sea surface temperature) might play a role in the physiology of the soft corals.

Little is known on the antimicrobial activity of octocorals in this region, and this is surprising considering our location in the coral triangle. Therefore, the aim of the current study was to test the antimicrobial activity of crude extracts of various soft coral species from the northern Straits of Malacca, more specifically, Pulau Payar and Pulau Songsong against a range of clinical strains of bacteria.

II. METHODOLOGY

2.1 Collection

Samples were collected by SCUBA from Pulau Payar Marine Park, and Pulau Songsong, two islands off the coast of Kedah, Malaysia. The average sea surface temperature of the two islands was 32°C. Specimens were collected as they were readily available in the study area. Whole specimens were collected between depths of 5m and 20m, and frozen at -20°C for further analysis.

2.2 Extraction

Samples were defrosted and weighed to 100g. Samples were macerated and extracted in 1:1 ratio of 90% methanol and dichloromethane for 24 to 48 hours. Extracts were then filtered using a vacuum filter and 0.45µm filter membrane. Filtrate was concentrated in a rotary evaporator. Crude extracts were stored at -20°C to prevent the degradation of secondary metabolites.

2.3 Antimicrobial Assays

Disk diffusion, minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) tests were performed on twelve soft coral crude extracts. Tests were conducted based on the Clinical Laboratory Standard Institute (CLSI) protocols for antimicrobial assays. Tests were done in duplicates, and repeated twice. Mueller Hinton agar (MHA) and Mueller Hinton broth (MHB) were prepared for the tests. Bacteria used were the 'ESKAPE' strains which consist of *Enterococcus raffinosus* ATCC 49464, *Staphylococcus aureus* ATCC 25923, *Klebsilla pneumoniae*, *Acinobacter baumannii*, *Pseudomonas aeruginosa* ATCC 27853 and *Enterobacter aerogenes* ATCC 51697, as well as Methicillin-resistant *Staphylococcus aureus* (MRSA). *E. raffinosus*, *S. aureus* and MRSA are gram-positive bacteria, while *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. aerogenes* are gram-negative bacteria. All tests were done in a Biosafety Cabinet Esco class II type A2, and incubation was done in lab companion incubator.

Extracts which had inhibition zones on the disc diffusion assays, were then tested to find the minimum inhibition concentration (MIC). Wells that showed positive results for MIC were plated to find out if the extracted was bactericidal or bacteriostatic (MBC test).

2.3.1 Disc Diffusion test

3 to 5 well-isolated colonies were selected from the MHA plate culture and transferred into a universal tube containing 4 mL of sterilised MHB. The inoculated broths were incubated at 37°C for 18 to 24 hours. The inoculum was then prepared by adjusting the turbidity of growth culture with sterile broth to achieve 0.5 OD at 625nm; which means that the suspension contained approximately 1 to 2 x 10⁸ CFU/mL. Test plates were inoculated by dipping sterile swab into the adjusted suspension and streaking the swab over the entire dried surface area of MHA plates. The concentration of crude extracts used for disc diffusion was set at 4mg/mL. 6mm discs were prepared with the crude extracts, and placed on MHA plates inoculated with the respective bacteria strains. Plates were then incubated at 37°C for 18-24 hours. The inhibition zones were measured to the nearest whole millimetre.

2.3.2 Minimum inhibition concentration (MIC) test

MIC tests were done in 96-well plates. 50 μ L of sterile MHB was aliquoted into all wells, except that of the 1st row where 90 μ L of sterile MHB was added. 10 μ L of extract (400mg/mL concentration) was added into the 1st row well and mixed thoroughly. Subsequently, 50 μ L from the 1st row wells were added into the 2nd row wells and mixed well by pipetting. This continued up until the 12th row of wells, where a final 50 μ L of mixture was discarded. 50 μ L of bacteria (1×10^6 CFU/mL) was added into the wells. This made the final concentrations in wells for the MIC test to be, starting from the 1st well to 12th well was a 2 fold dilution; with the concentrations being 20mg/mL, 10mg/mL, 5mg/mL, 2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.3125mg/mL, 0.156mg/mL, 0.0781mg/mL, 0.039mg/mL, 0.0195mg/mL, 0.0098mg/mL respectively. Each plate had positive and negative controls, where the positive controls were Ampicillin, Vancomycin and Polymycin B; and the negative control was sterile MHB. The concentration of positive control antibiotics (Ampicillin, Vancomycin and Polymycin B), also for the MIC test were 2mg/mL, 1mg/mL, 0.5mg/mL, 0.25mg/mL, 0.125mg/mL, 0.0625mg/mL, 0.03125mg/mL, 0.0156mg/mL, 0.0078mg/mL and 0.0039mg/mL respectively.

The 96 well plates were incubated at 37°C for 18-24 hours. Wells in which there was no growth were considered to have inhibition, and the MIC was the well with the lowest concentration of no bacterial growth.

2.3.3 Minimum bactericidal concentration (MBC) test

MBC tests were done on MHA plates. Each MHA plate was divided into 8 sections. 10 μ L was taken from each well that did not show growth in the MIC test and aliquoted on a section of MHA. Plates were left to dry for 3-5 minutes, and then incubated at 37°C for 18 to 24 hours. Sections where there was growth meant that the extracts were bacteriostatic (cell inhibition), while sections with no growth showed bactericidal (cell death) activity.

III. RESULTS

Antimicrobial assays were performed with extracts of 12 soft corals. The data revealed considerable variability in natural extract concentration and in antimicrobial activity as tabulated in Table 1. Out of 12 crude extracts tested, 6 showed inhibition for *Staphylococcus aureus* and MRSA; and 2 showed inhibition for *Enterococcus raffinosus*. *Klebsilla pneumoniae*, *Acinobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes* did not yield any positive inhibition zones in the disk diffusion tests. From the active soft coral species examined S3 exhibited the highest antimicrobial activity (Table 2).

3.1 Antibacterial Screening

Extracts from samples S1, S2, S3, S7, S8 and S13 showed inhibition in the disc diffusion tests against *Staphylococcus aureus* and MRSA. The extracts came from specimens *Sarcophyton sp.*, *Carijoa sp.*, *Coelogorgia sp.*, *Junceella sp.* and *Virgularia sp.* respectively. Extracts from S2 and S3 showed inhibition against *Enterococcus raffinosa*. It is important to note that the extracts only exhibited resistance against gram-positive bacteria.

Table 1 depicting the disc diffusion inhibition zones of the crude extracts. Those left blank showed no inhibition activity against the bacteria tested

Specimen	Extracts	Disc diffusion inhibition zone (mm)						
		<i>E.raffinosis</i>	<i>S.aureus</i>	<i>K.pneunomiae</i>	<i>A.baumanii</i>	<i>P.aeruginosa</i>	<i>E.aerogenes</i>	MRSA
<i>Sarcophyton sp.</i>	S1	-	9	-	-	-	-	7
<i>Carijoa sp.</i>	S2	8	10	-	-	-	-	12
<i>Coelogorgia sp.</i>	S3	9	11	-	-	-	-	11
<i>Viminella sp.</i>	S6	-	-	-	-	-	-	-
<i>Junceella sp.</i>	S7	-	12	-	-	-	-	8
<i>Junceella sp.</i>	S8	-	9	-	-	-	-	7
<i>Echinogorgia sp.</i>	S9	-	-	-	-	-	-	-
<i>Dendronepthea sp.</i>	S10	-	-	-	-	-	-	-
<i>Dendronepthea sp.</i>	S11	-	-	-	-	-	-	-
<i>Ellisella sp.</i>	S12	-	-	-	-	-	-	-
<i>Virgularia sp.</i>	S13	-	8	-	-	-	-	8
<i>Melithaea sp.</i>	S14	-	-	-	-	-	-	-

Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of the positively screened extracts are depicted in table 2. S3 had the lowest MIC against *S. aureus*. Only extract S1 showed bactericidal properties against MRSA.

Table 2: depicting the MIC and MBC values of the crude extracts which exhibited positive results during the disc diffusion screening test

Extracts	Minimum Inhibition Concentration (MIC) in mg/mL			Minimum Bactericidal Concentration (MBC) in mg/mL		
	<i>S. aureus</i>	MRSA	<i>E. raffinosus</i>	<i>S. aureus</i>	MRSA	<i>E. raffinosus</i>
S1	5	10		-	10	
S2	10	20	-	-	-	-
S3	2.5	10	-	-	-	-
S7	20	20		-	-	
S8	20	-		-	-	
S13	-	-		-	-	

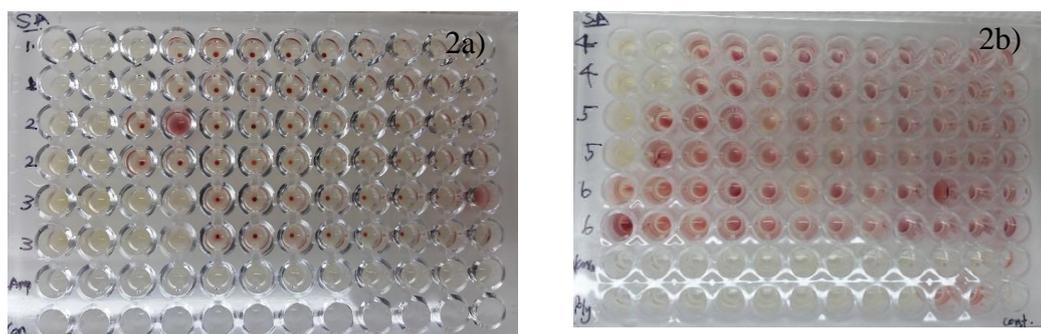


Figure 2a and b depicting MIC test results for *Staphylococcus aureus*, where wells containing bacteria are stained red with triphenyl-tetrazolium chloride solution (TTP).

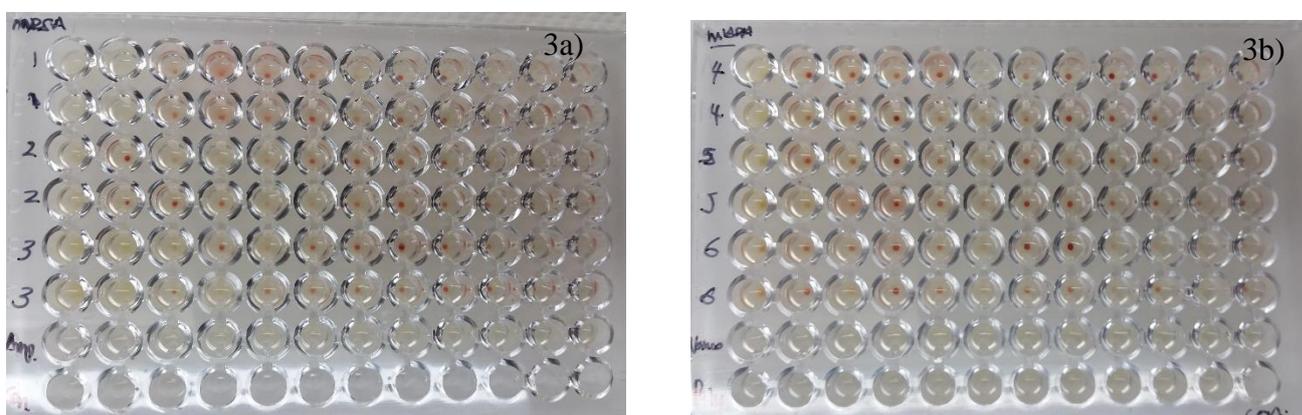


Figure 3a and b depicting MIC test results for MRSA, where wells containing bacteria are stained red with triphenyl-tetrazolium chloride solution (TTP).

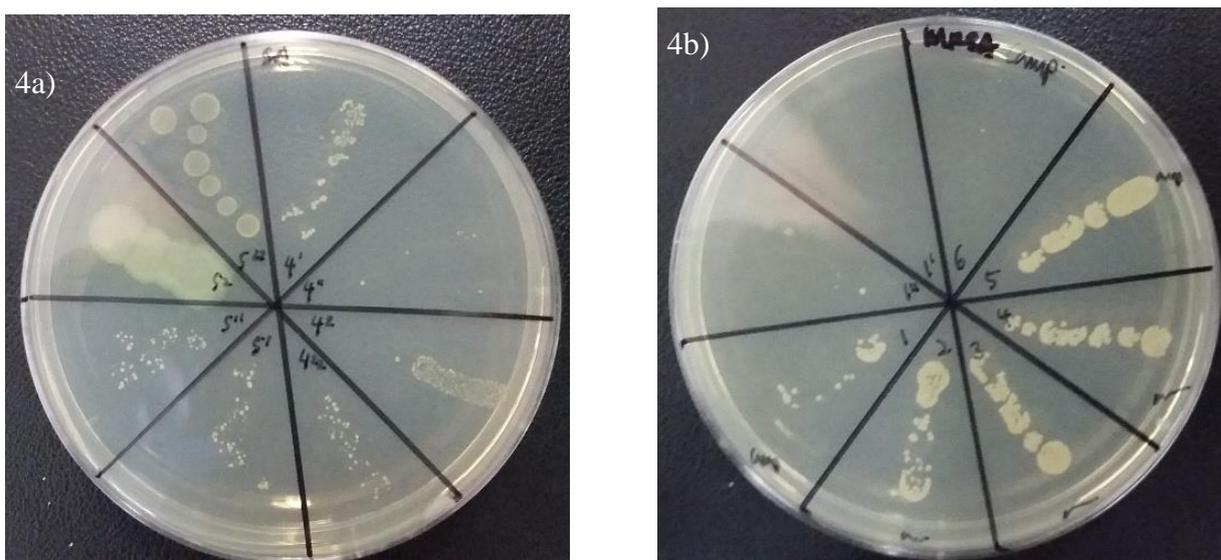


Figure 4a and b depicting MBC results. In figure 4b, section labelled 1¹ and 1¹¹ display bactericidal activity of the extract

IV. DISCUSSION

Due to the fact that a large proportion of marine bioactive metabolites with different biological activities are provided by marine organisms, the coral reef ecosystems have become sources of great interest to natural product discovery. The current study was conducted to screen the crude extracts of ten soft coral species collected from the coral reef ecosystems in the northern Straits of Malacca and test their potential to inhibit the growth of clinical bacteria strains.

Our results indicated that extracts from *Sarcophyton sp.*, *Carijoa sp.*, *Coelogorgia sp.*, *Junceella sp.* and *Virgularia sp.* possessed antibacterial activity against the tested bacterial strains. Only gram positive bacteria were susceptible to the soft coral extracts. Gram negative bacteria have a unique outer membrane which excludes certain drugs and antibiotics from entering the cell [24]. Therefore, gram negative bacteria are more resistant.

In a study done on *Sarcophyton trocheliophorum* from the Red Sea [25,26], the extract was found to inhibit the same set of bacteria tested in the current study; where the ethyl acetate and hexane extracts were more effective against gram negative and gram positive bacteria respectively. *Sarcophyton* is well known as a producer species of sarcophine derivatives [27,28]. Sarcophine was isolated from the *S. glaucum* collected from the Red Sea [29]. Approximately two hundred terpenes had been previously isolated from 16 species of the genus *Sarcophyton* demonstrating various biological features, such as antiviral, antifouling activities, predator deterrence, and anti-inflammatory activities [30]. In a study done in Indonesia on *Lobophytum sp.* (which is similar to genus *Sarcophyton*), it was found that crude fractions of the specimen had moderate anti-bacterial activity against test bacteria from both gram positive and gram negative bacteria [31]. Purified ethanolic extracts of *Carijoa sp.* from the Indo-pacific yielded 4 compounds in which displayed cytotoxic activity against human hepatoma Bel-7402 and human normal embryonic lung fibroblast MRC-5, as well as antibiotic resistance against a range of bacteria [32].

A study on Vietnamese octocorals by Kapustina *et al.* (2014)[33], found an extract from *Junceella sp.* exhibited antibacterial activity against *Escherichia coli*, but was inactive against *Bacillus megaterium*. This might have been due to the difference in cell wall, as *E.coli* is gram negative, and *B. megaterium* is gram positive. This is in contrast with the results of this study as *Staphylococcus aureus* is a gram positive bacteria. The extract also showed moderate toxicity against brine shrimp *Artemia salina*. More evidence to the antibacterial properties of *Junceella sp.* can be found in a study done in India [34], where the methanolic extract of *Junceella juncea* inhibited growth of bacterial strains such as *Salmonella typhii* and *Vibrio cholera*.

V. CONCLUSION

In conclusion, the soft corals of the northern Straits of Malacca potentially have medicinal value, more specifically that of antibacterial properties. This study provides evidence that there is indeed potential of soft corals in the area.

Future studies should include testing of different solvent extracts, as well as a wider range of bacteria. Other properties such as anti-cancer should also be tested, to study the effectiveness of the crude extracts. Purification of these extracts can be done to isolate the individual effective secondary metabolites.

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