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Screening for antibacterial metabolites in marine sponges collected from the coastline of Sri Lanka.

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Abstract

Natural products and their derivatives have and are still used by humans for various health ailments due to their rich sources of drug discovery. New biologically active compounds from natural products play a key role in drug development. Marine sponges and their associated microbes contain a lot of bioactive compounds that are potential for drug development. These compounds produce chemical compounds with useful pharmaceutical properties such as antitumor, anti-infective, anti-inflammatory and antibacterial properties. The main focus of this project was on the antibacterial activity of six different sponge specimens. The aim was to screen the antibacterial activity of the sponge specimen's extracts. In order to do so Minimum Inhibitory Concentration assay performed to screen the sponge's antibacterial activity against *E. coli* and *S. aureus*. Analytical HPLC was used for separation and Solid Phase Extraction (SPE) used for determining the effect of salts towards the inhibition of anti-bacterial activity for two selected extracts. Ethanolic extract of *Stylissa massa* showed an antibacterial activity against *S. aureus.* SPE would be a rapid purification step to remove the salts present in sponges at a high concentration but it has not shown a significant effect on the inhibition of antibacterial activity. However, further separation and purification need to be done to be able to completely screen for all the six different sponge specimens.

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1. Introduction

1.1. Natural products

Natural products and their derivatives have and are still used by humans for various health ailments due to its rich sources of drug discovery (Molinari, 2009). The use of natural products has been and still is an excellent way to find new therapeutic agents. Many of today's pharmaceutical products are based on natural products and their derivatives. During the 1940s to the end of 2014, 131 of 175 of the approved small molecules with 75% were directly derived from natural products. This shows how quickly a significant number of herbal medicines are produced and how it can potentially be therapeutically applicable in several different diseases (Newman & Cragg, 2016).

New biologically active compounds from natural products produced in nature have been discovered by performing various biological analyzes on them. They are identified as leads and can then be selected as candidates for further drug development. Today, more than 60% of all medicines available on the market consist of natural sources. Every year, new applications for natural products are discovered that show great interesting biological activities. These compounds produced as substances produced by a living organism and are called secondary metabolites (Molinari, 2009). These secondary metabolites have survival functions for the organisms that produce them. They act as a protection against various types of threats such as bacteria, fungi, plants, insects, large animals and microbial infections. They also act as metal transporters for symbiosis between microbes, plants and higher animals (Demain & Fang, 2000).

The oceans consist of more than 70% of the earth's surface and natural products in the oceans are enormously abundant. Marine biodiversity is an aggregation of highly interconnected ecosystem components (Costello and Chaudhary 2017). More than 90% of all living organisms are found in the marine environment. Therefore, the marine environment is a huge product source for secondary metabolites that are very unique to the aqueous environment (Lazcano-Pérez et al., 2012).

For research of natural products with adaptation of this study, a bioanalysis-controlled method is used for isolation of bioactive compounds from the crude extract. This is a liquid chromatograph (HPLC) (Ory et al., 2019). A solid phase extraction (SPE) is a method used to prevent the interference of salts on the recovery of secondary metabolites in the sponge extracts used in the study (Cutignano et al., 2015). The lowest inhibitory concentrations (MIC) were used in the study to determine the minimum bactericidal concentrations and screen whether the fractions contain any antibacterial activity (Beesoo et al., 2017).

1.2. Marine Sponges

Marine sponges are invertebrates found in temperate, polar and tropical areas. They are aquatic animals found in marine environments. Sponges are classified within four classes that are calcarea sponges (Calcarea), glass sponges (Hexactinellida), demosponges (Demospongiae) and the newly recognized sponges (Homoscleromorpha) (Soest et al., 2012). The sponges can be found on the seabed or attached to, for example, rocks, corals, shells and marine organisms. Sponges have different habitats such as tidal areas and coral reefs to the deep sea. They are also found in seas and freshwater lakes (Kennedy, 2019). Marina sponges have a skin of T-shaped or flattened cells and live by sucking in water and filtering food particles from it (Soest et al., 2012). Filtration is an active process involving choanocyte feed chambers that generate the water streams necessary for the filtration activity. Each choanocyte has a central flagellum that

strikes actively to create a water stream. This has a collar of cilia that captures food particles such as plankton, bacteria and detritus (Hooper et al. 2008). Sponges also have the ability to absorb dissolved organic material and have an enzymatic ability to break down almost all biological materials. It thus constitutes an important cleaning role in seas and lakes. Some marine sponges can break down organic products even in the absence of oxygen and they therefore do not contribute to the oxygen reduction (Tibell et al. 2016). Marine sponges contain a lot of bioactive compounds that are potential for drug development. These compounds produce chemical compounds with useful pharmaceutical properties such as antitumor, anti-infective and anti-inflammatory properties. Sponges can also produce antiviral compounds that can be used to develop new antiviral drugs against viral diseases such as HIV. Compounds produced from sponges can also function to defend against pathogenic bacteria, algae, sponges and other potential predators (Sagar et al., 2010).

Many antibacterial agents have been identified from marine sponges and up to 800 antibiotics have been isolated from them (Torres et al. 2002). Studies on screening of marine sponges for antibacterial activity in 2009 showed that 10 out of 12 sponge species have antibacterial activity within a large spectrum of action. 7 of these 12 sponge species were shown to be active against resistant bacteria such as S. aureus (MRSA strain). This led to the isolation and characterization of a number of different active substances that may contain promising therapeutic leads. Crude extracts of marine sponges consist of a high incidence of antibacterial activity against pathogenic bacteria (Laport, C. S. Santos, and Muricy 2009).

Marine sponges are a source of new alkaloids with antibacterial activity. Axinellamines B-D have a bactericidal activity against Helicobacter pylori (Urban et al. 1999). Oceanapia species is another substance that is also active against H. pylori and an inhibitor of the enzyme aspartyl

semialdehyde dehydrogenase. This enzyme is important for bacteria because it is involved in the production of 25% of all amino acid residues for the protein synthesis step. Inhibition of the aspartyl semialdehyde dehydrogenase enzyme is therefore an important target for the development of antibacterial agents (Carroll et al. 2005).

1.3 Aim

The aim of the project is to screen the antibacterial activity of the sponge extracts by bio-assay guided fractionation followed by Minimum Inhibitory Concentration assay. The effect of salts towards the inhibition of anti-bacterial activity was determined by Solid Phase Extraction (SPE) for selected extracts.

2. Experimental

2.1. Chemicals

Acetonitrile (AcN) HPLC grade ≥99.9% was purchased from VWR, Sweden. Trifluoroacetic acid HPLC grade ≥99.0% was purchased from VWR, Sweden. Milli-Q water was from Merck Millipore. Tryptic soy broth (TSB) was purchased from VWR, Sweden. Trizma base was purchased from Sigma-Aldrich, Sweden. Methanol (MeOH) HPLC grade ≥99.9% was purchased from VWR, Sweden. Dichloromethane (DCM) was purchased from VWR Chemicals, Sweden. Hexane (C_6H_{14}) was purchased from Sigma-Aldrich, Sweden. Sodium hydroxide solution (NaOH) was purchased from Sigma-Aldrich, Sweden. Hydrochloric acid (HCL) was purchased from Sigma-Aldrich, Sweden. Formic acid (FA) ≥99.9% was purchased from VWR Chemicals, Sweden.

2.2. Instrumentation

Analytical RP- HPLC: Shimadzu Prominence LC-20AD UFLC Stack HPLC System: Shimadzu DGU-$20A_{5R}$ Degassing unit, Shimadzu LC-20AD Pump, Shimadzu CTO-20AC Column Oven, Shimadzu CBM-20A Communication Module, SPD-20A Detector UV/vis Detector, SIL-20AHT AutoSampler and Column: Kinetex® 5 µm, XB-C18 100 Å, LC Column 250 x 4.6 mm was used for micro fractionation**.** Scientific Industries Vortex-Genie 2 was used for mixing the extracts. Sartorius ME235S Analytical Balance was used for weighing the extracts. Heraeus Universal 320 R Centrifuge was used to centrifuge the extracts.

Minimum Inhibitory Concentration assay: WPA S800 Diode Array Spectrophotometer was used to measure the absorbance. Savant SpeedVac Plus SC110A was used to dry the microtiter plates. 2540 ML Autoclave Tuttnauer was used to autoclave the micropipette tips and chemicals.

Sterilisator Memmert UFE-400 was used to incubate the microtiter plates. Microcentrifuge, MiniStar silverline was used to centrifuge the samples.

Solid Phase Extraction: Column: Supel^{TM}, bed wt. 60 mg, volume 3ml, pk of 50 and ISOLUTE® SI 200mg/10ml (3ml XL) were used. Flexi-Dry MP freeze dryer was used to freeze-dry the fractions.

2.3. Sample collection

All the 6 specimens were collected by snorkelling from the Northern coastal line of Sri Lanka. The specimens *Stylissa massa* (figure 1), *Paratetilla bacca* (figure 2), and 200220 # 01-10 (figure 3) were collected at the Silavathurai in Mannar with the depth of 1.5-5 m and with the coordinates 8˚ 48′ 02.81 ” N, 79˚ 50′ 59.33 ” E. These specimens were collected on 20th February 2020.

The specimens 210220 # 02-7 (figure 4) and 210220 # 02-5 (figure 5) were collected at the South Bar in Mannar with the depth of 2-2.5 m and with the coordinates 8˚ 55′26.54 ” N, 79˚ 47′09.26 ” E. These specimens were collected on 21st February 2020.

The specimen 230220 # 05-1 (figure 6) belongs to a subclass called *Keratosa* and were collected at the Kayts Island in Jaffna with the depth of 0-1 m with the coordinates 9˚ 42′ 09.27 ” N, 79˚ 51′ 50.13 ” E. These specimens were collected on 23rd February 2020.

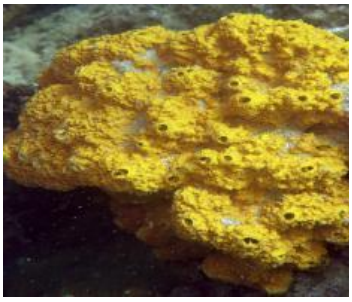

Figure 1. *Stylissa massa*

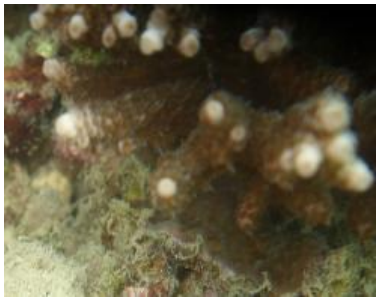

Figure 2. *Paratetilla bacca*

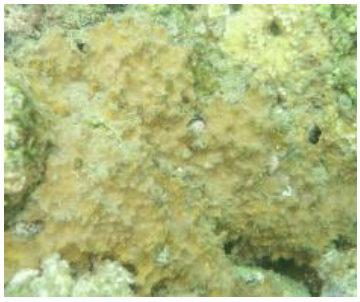

Figure 3. 200220 # 01-10

Figure 4. 210220 # 02-7

Figure 5. 210220 # 02-5

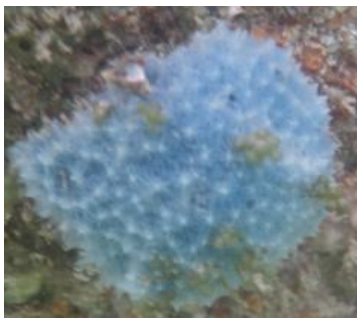

Figure 6. 230220 # 05-1

2.4. Small scale extraction

The specimens were preserved in 96% ethanol until they were brought to the laboratory. Before preparing the extracts, the specimens were cut into pieces, washed with water, air dried for 3 to 5 days at room temperature (30 °C) and separately pulverized by using a domestic grinder. Two extracts (aqueous and ethanolic) were prepared for each specimen depending on the amount of biomass available. The aqueous extract was prepared using 60% methanol (MeOH), it was left in a shaker overnight, filtered and rotar evaporated to get the dried crude extract. The ethanolic extract was prepared using 96% ethanol (C_2H_5OH) and rotar evaporated to obtain the crude extract.

2.5. Chromatographic methods

2.5.1. Sample preparation

An amount of 2 mg from each extract was dissolved in a volume of 200 microliter of 1% AcN. Centrifugation was carried out after mixing the extract using the digital vortex mixer.

2.5.2. Analytical HPLC

Analytical RP-HPLC using a Shimadzu LC20-AD HPLC System fitted with a Kinetex® C18 column (250 x 4.6 mm id., 5 µm, 100 Å). Fractions from Analytical RP-HPLC were collected 1 mL/min rate for 55 minutes yielding 45 fractions using a linear gradient of 5-97% B (solvent A: 0.05% TFA in 5% CH_3CN and solvent B: 0,05 TFA in 97% CH_3CN).

2.6. Minimum Inhibitory Concentration assay (MIC assay)

2.6.1. Preparations

100 µL from the 45 fractions collected above was transferred to a 96 well U bottom microtiter plate (VWR, Sweden) and the solvent was evaporated using the speed vac (medium heat) in order to test for the antibacterial activity. All tests were conducted in duplicate and were tested against *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922). 3% TSB in Milli Q water, which was the growth medium and Tris buffer (10mM, pH 7.8 at 20 °C) in Milli Q water were autoclaved at 121 °C for 15 min. 20% TSB in Milli Q water was autoclaved at 100 °C for 45 minutes. Ciprofloxacin (2 mg in 1mL of 20% DMSO) was used as the positive control.

2.6.2. Growth of bacteria

First 10 µL of bacterial stock solution was transferred to a conical flask containing 70 mL of 3% Tryptic soy broth and was incubated overnight approximately for 8 hours at 37°C.

2.6.3. Washing the bacterial pellet

10 ml of the bacterial suspension prepared above was transferred to a 15 ml falcon tube and was centrifuged at 2100 rpm for 8 minutes. The bacterial pellet was observed at the bottom of the falcon tube. The bacterial pellet was washed twice using tris buffer and was re-suspended in tris buffer.

2.6.4. Preparation of the required concentration of bacteria

The concentration of the bacterial suspension was measured at 600 nm using the spectrophotometer. The bacterial suspension was diluted in order to make the final concentration of bacterial cells as 50,000 in a total volume of 100 µl per each well.

2.6.5. Preparation of the plate

First 50 µL of tris buffer was added to the dried plate containing the fractions as well as the positive control and 100µL of tris buffer was added to the wells containing the negative control. 50 µL of Ciprofloxacin was added to the first well containing the positive control. Using serial dilution method, a serial dilution was carried out only for the wells containing the positive control to get a MIC value.

Then 50 µL of diluted bacterial suspension described above was added to all the wells except the negative control. 20% TSB was added to each well after 5 hours incubation at 37°C. The visual observation of the growth inhibition was carried out after incubation for 6 to 12 hours depending on the growth rate of the bacteria.

2.7. Solid Phase Extraction (SPE) to avoid the effect from salts

A similar purification protocol was carried out for the aqueous and ethanolic extracts. 2 specimens having both aqueous and ethanolic types of extracts were selected to carry out SPE to avoid the effect from salts. The specimens selected were *Stylissa massa* and 210220 # 02-05.

2.7.1. Sample preparation

2 mg of the aqueous and ethanolic extracts were dissolved in 300 µL of Milli Q water.

2.7.2. MIC assay for crude extracts before SPE

MIC assay was carried out on the aqueous and ethanolic extracts of the 2 specimens mentioned above. A MIC value was obtained using the serial dilution method described above.

2.7.3. SPE of the aqueous and ethanolic extracts

The column (Supel^{TM}, bed wt. 60 mg, volume 3ml) was equilibrated using 3 ml of 1% FA in Milli Q water after conditioning. Then the column was washed with 18 ml of Milli Q water after loading the extract in order to wash away the salts. The elution was carried out using 3 ml of 1% FA in 30% AcN and 3 ml of 1% FA in 60% AcN and both fractions were combined and freeze dried.

2.7.4. MIC assay for the extracts after SPE

MIC assay was carried out using the serial dilution method described under the section 2.6.5 for the combined aqueous and ethanolic fractions after SPE to obtain a MIC value. Then microfractionation followed by MIC assay was carried out again for the combined fractions after SPE to determine the effect of bacterial growth after the removal of salts.

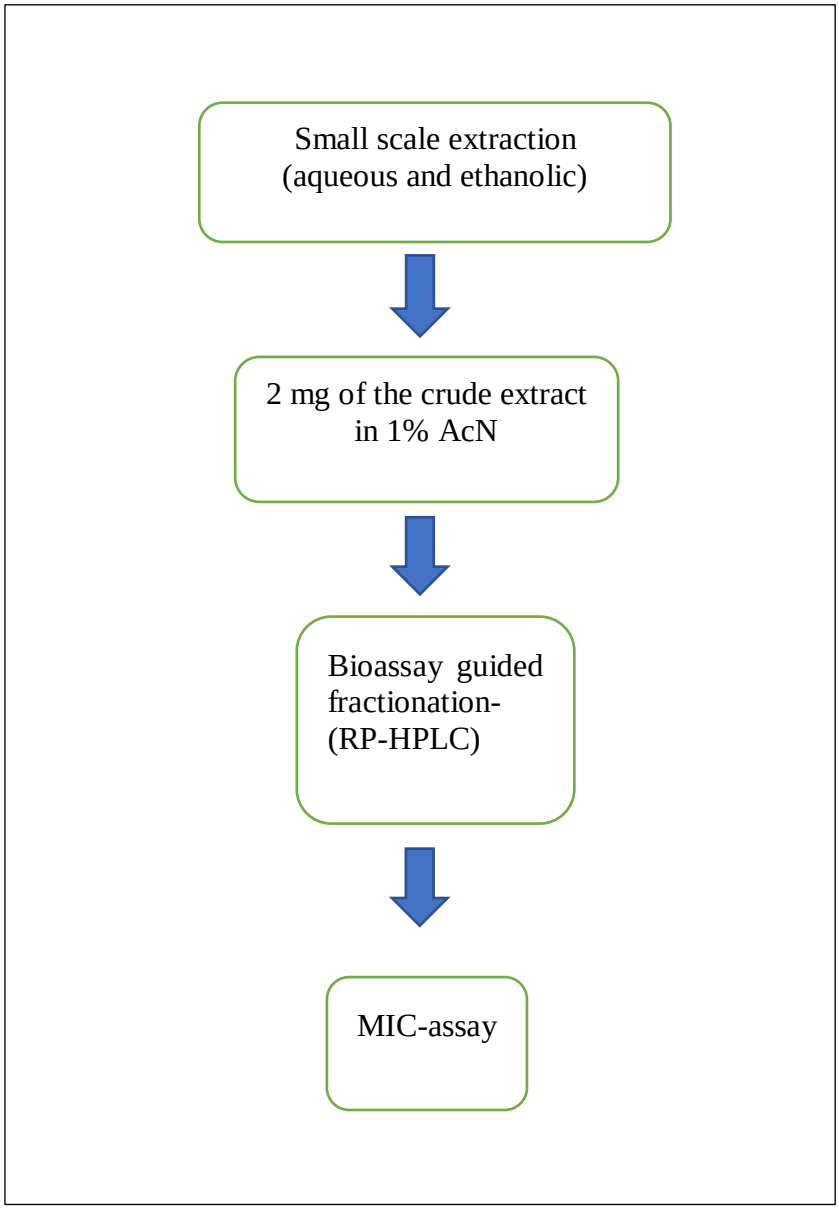

Figure 7. Flow chart for the micro fractionation before SPE.

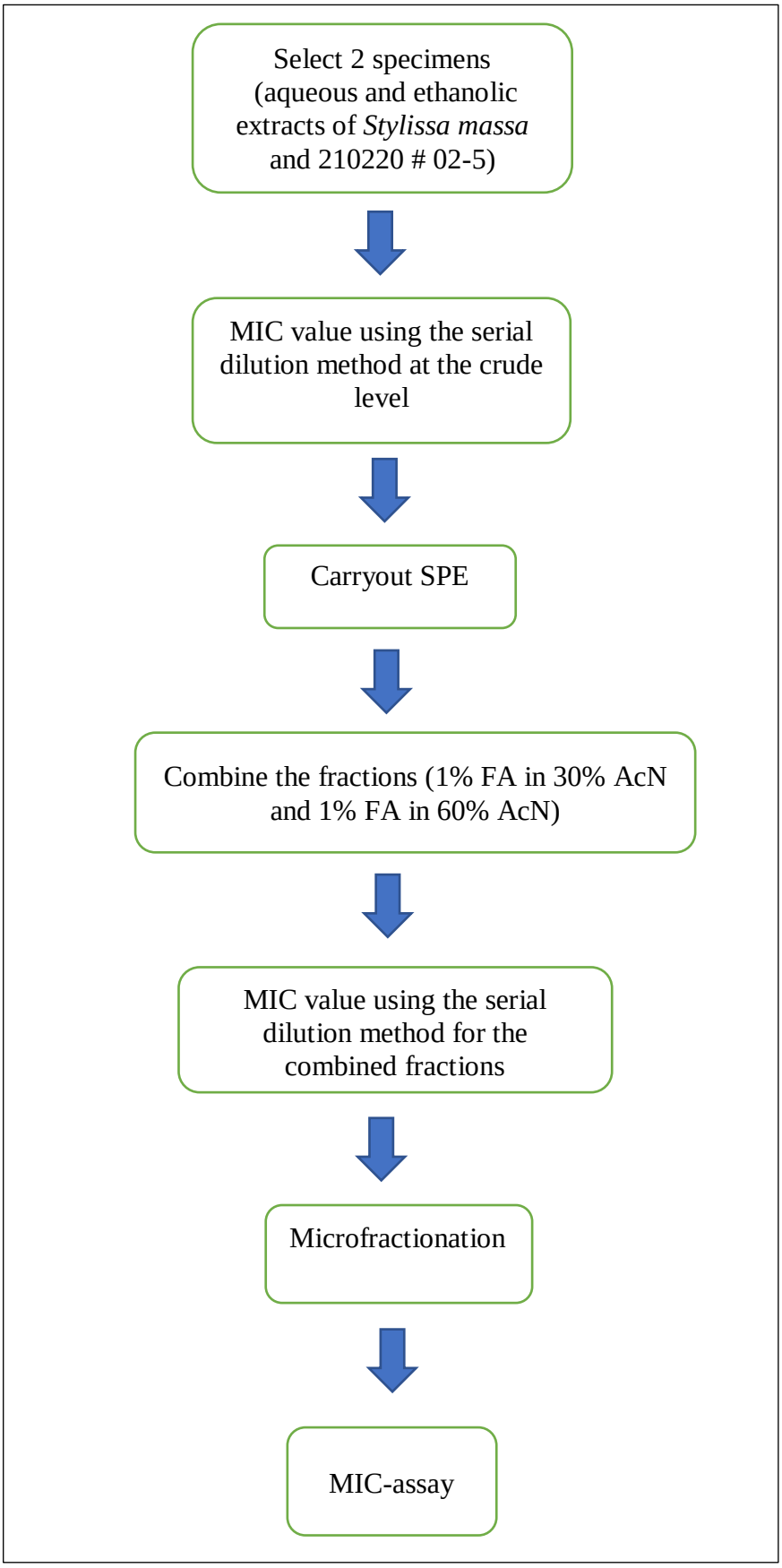

Figure 8. Flow chart for SPE for the two selected extracts.

3. Results

3.1. Summary

Bioassay guided micro fractionation was carried out using Analytical RP-HPLC for a total 8 extracts from six sponge specimens (table 1) to investigate their antibacterial activity. Minimum Inhibitory Concentration assay was used to screen the antibacterial activity. Further, Solid Phase Extraction followed by Minimum Inhibitory Concentration assay was carried out for four selected extracts from two specimens to determine the effect of salts present in the extract on the antibacterial activity of the extracts.

Table 1. Summary of the extracts used before and after SPE

Sample name	Before SPE		After SPE	
	AQUEOUS	ETHANOLIC	AQUEOUS	ETHANOLIC
Stylissa massa	√	√	√	√
Paratetilla bacca	√			
200220 # 01-10	√			
210220 # 02-5 (*Keratosas subclass)*	√	√	√	√
210220 # 02-7	√			
230220 # 05-1		√		

3.2. Results from RP-HPLC before SPE

All eight extracts (table 1) were fractionated using analytical RP-HPLC to a 96 deep well plate (VWR, Sweden). Using a gradient of 5% AcN 0.05% TFA to 97% AcN 0.05% TFA facilitate the better separation of both hydrophilic and hydrophobic compounds. The chromatograms can be used as a guide to understand the polarity of the compounds present in each extract.

In figure 9, a wide distribution of peaks between the gradient from 10 % AcN 0.05% TFA to 50 % AcN 0.05% TFA indicates the richness of the chemical composition in between the wavelength range 200 to 800 nm. Further, the highly intense peaks were localized between 10 to 20 minutes at the gradient of approximately 20 to 30 % AcN 0.05% TFA.

In Figure 10, a wide distribution of peaks between the gradient from 10% AcN 0.05 %TFA to 40% AcN 0.05% TFA indicates the richness of the chemical composition between the wavelength range 200 to 800 nm. Further, the highly intense peaks were localized between 10 to 15 minutes at the gradient of approximately 10 to 25% AcN 0.05 TFA%.

Figure 11 shows clear peaks at around 22.5 and 36 minutes. The chromatogram showed several peaks before 5 minutes, indicating that the sample contained several side products as salts and therefore needed to be purified.

Figure 12 showed two intense peaks at around 15 and 33 minutes. There is not much background noise despite the solvent peak in the beginning of the chromatogram. This indicates the absence of a considerable number of compounds within the wavelength range 200 to 800 nm.

In most of the chromatograms, peaks with a high intensity were localized in the gradient between 0 to 5% AcN 0.05% TFA signifying the presence of more hydrophilic compounds such as salts in most of the extracts.

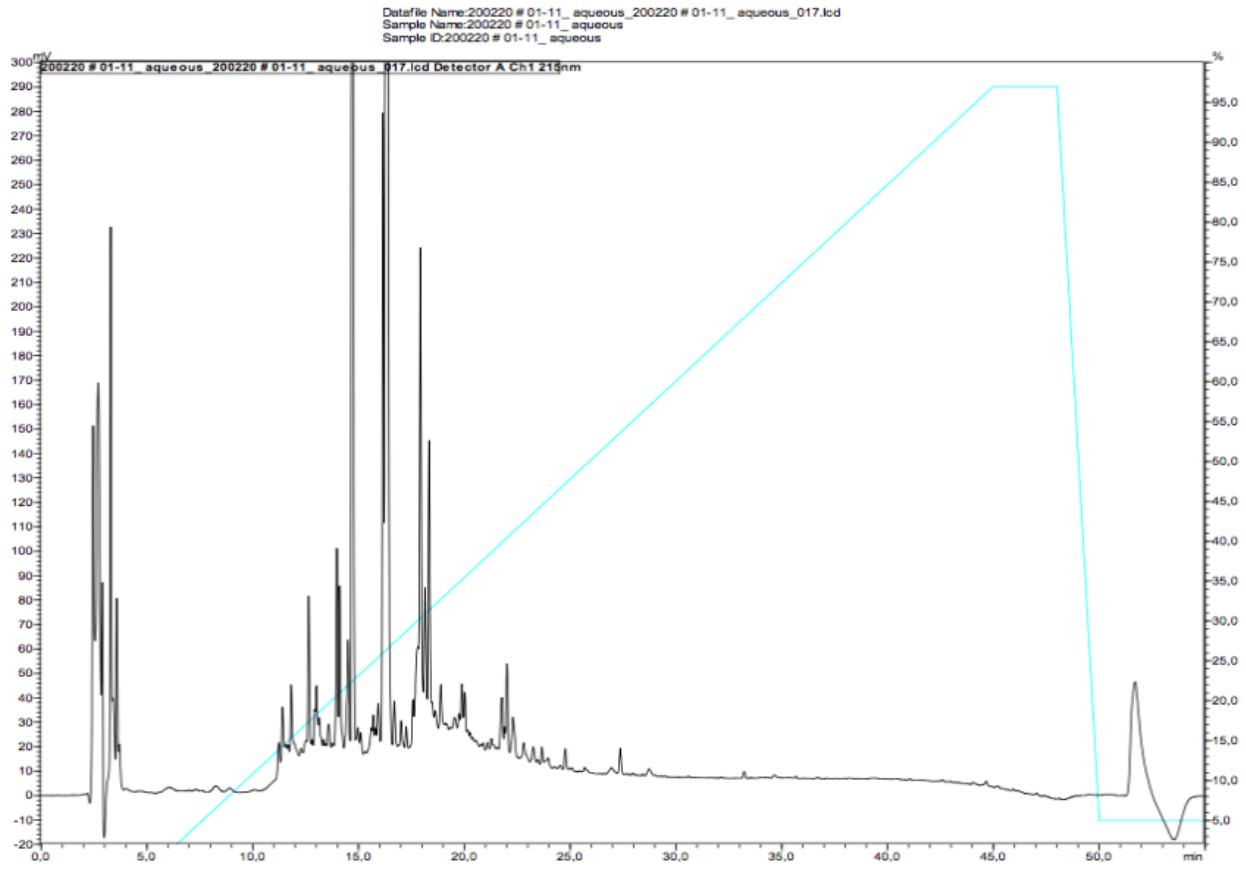

Figure 9. Analytical RP-HPLC chromatogram of the aqueous extract of *Stylissa massa*. Gradient 5-97% B and runtime 55 min.

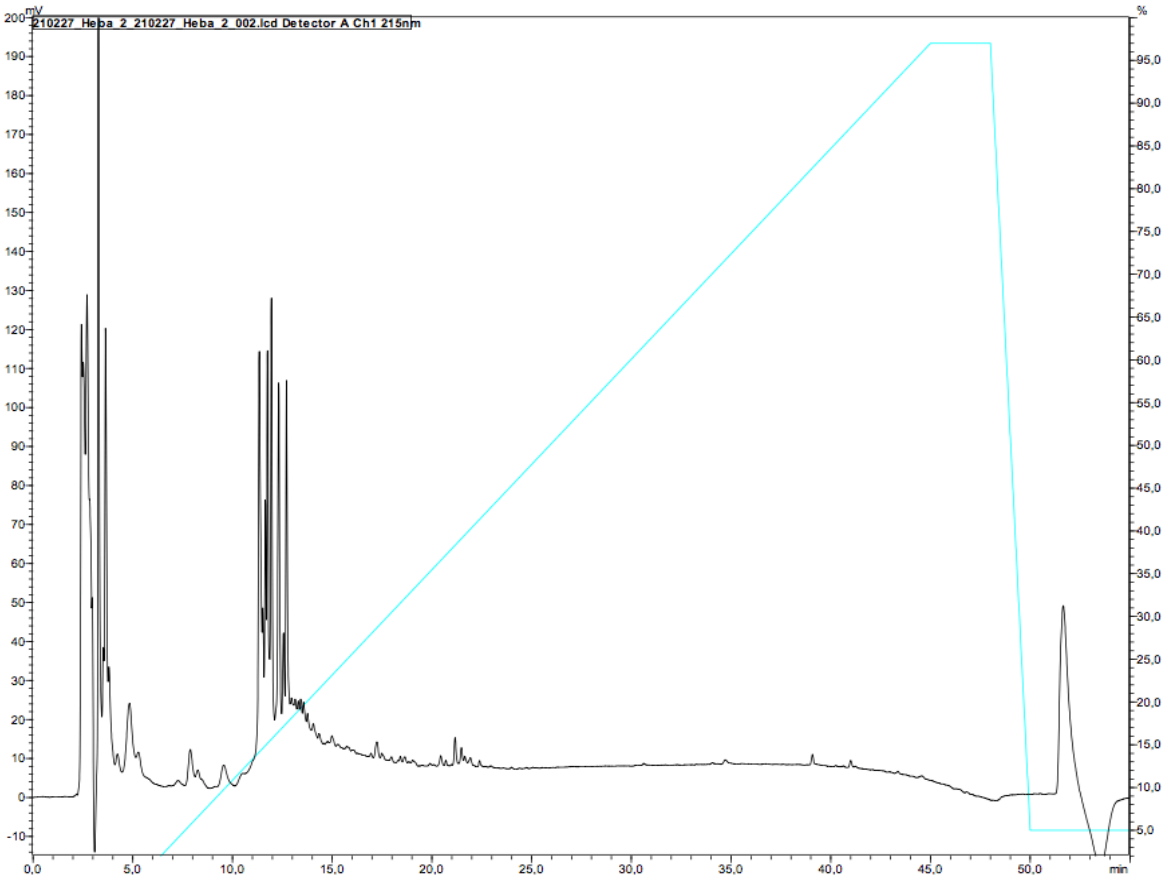

Figure 10. Analytical RP-HPLC chromatogram of the ethanolic extract of *Stylissa massa*. Gradient 5-97% B and runtime 55 min.

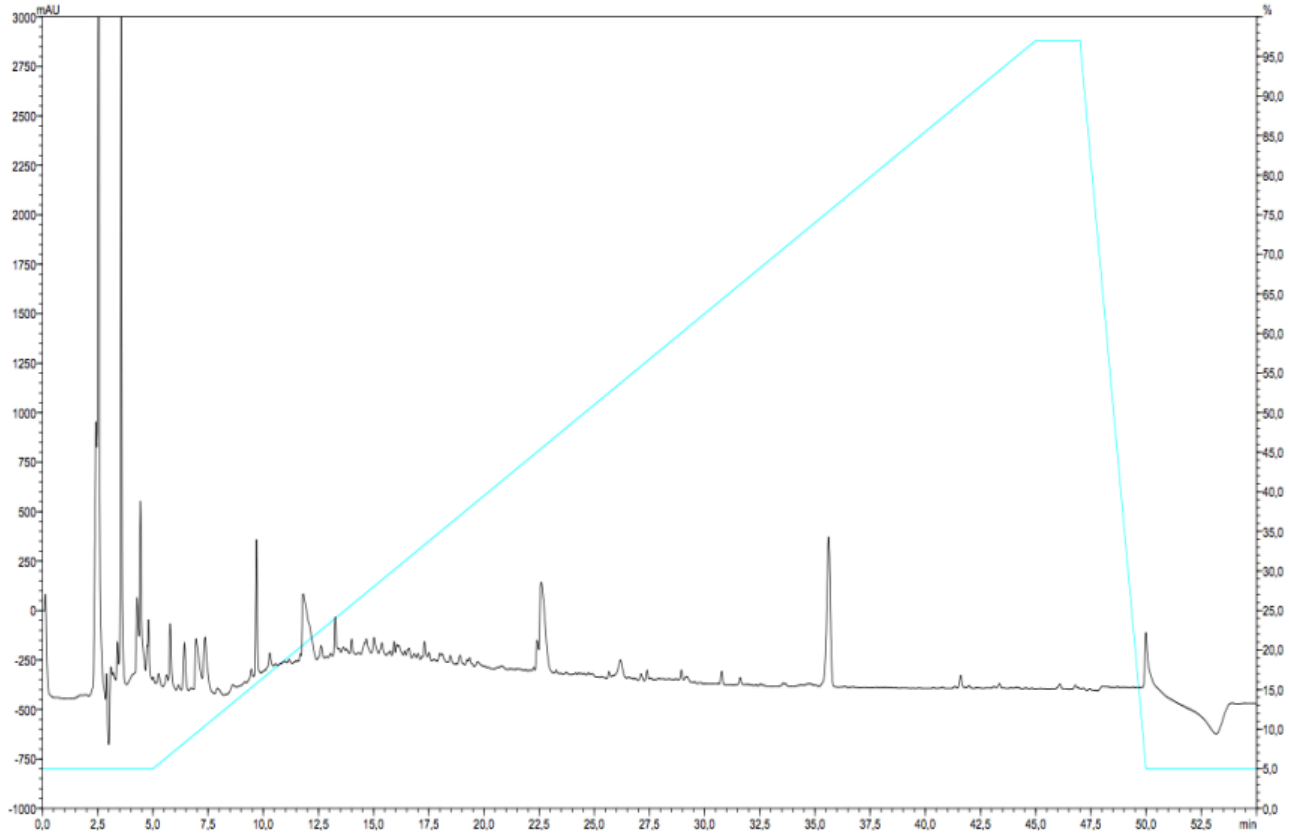

Figure 11. Analytical HPLC chromatogram of extract 210220 # 02-5 in aqueous. Gradient 5-97% B and runtime 55 min.

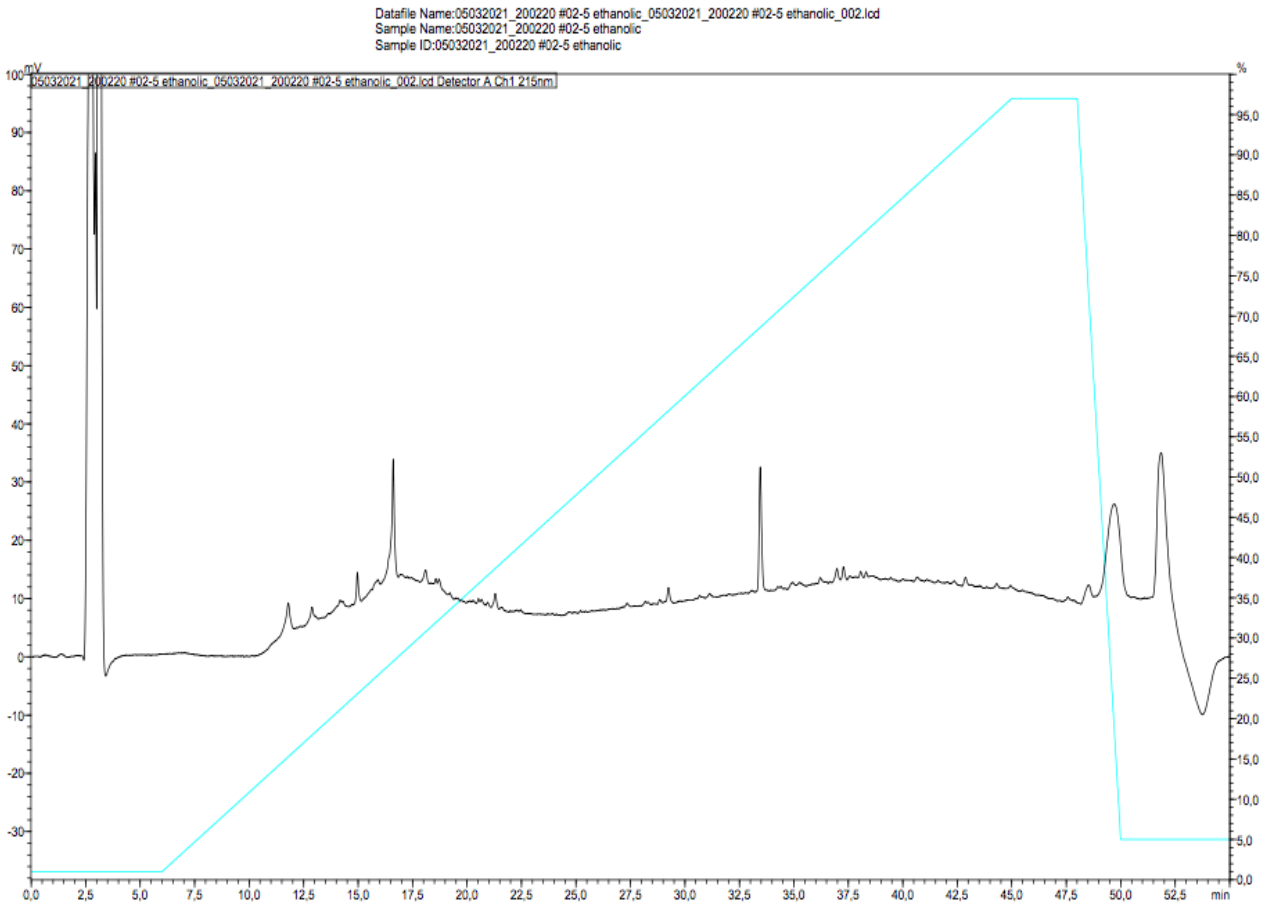

Figure 12. Analytical HPLC chromatogram of extract 210220 # 02-5 in ethanolic. Gradient 5-97% B and runtime 55 min.

3.3. Results from RP-HPLC after SPE

Micro fractionation was carried out using analytical RP-HPLC to the 4 extracts from two selected specimens after SPE (table 1). The chromatograms were obtained using a gradient of 5% AcN 0.05% TFA to 97% AcN 0.05% TFA.

Figure 13 showed a wide distribution of peaks between the gradients from 10% AcN 0,05% TFA to 40 % AcN 0,05% TFA, which is similar to the distribution of peaks in the chromatogram before SPE (figure 10). Figure 14 showed a distribution of peaks between the gradients from 20 % AcN 0,05% TFA to 40 % AcN 0,05% TFA, which is similar to the distribution of peaks in the chromatogram before SPE (figure 9). This signifies that there is no effect for the chemical composition even after carrying out SPE as a purification step.

In figure 15 and 16, there were less number of intense peaks which were similar to figures 12 and 13 respectively.

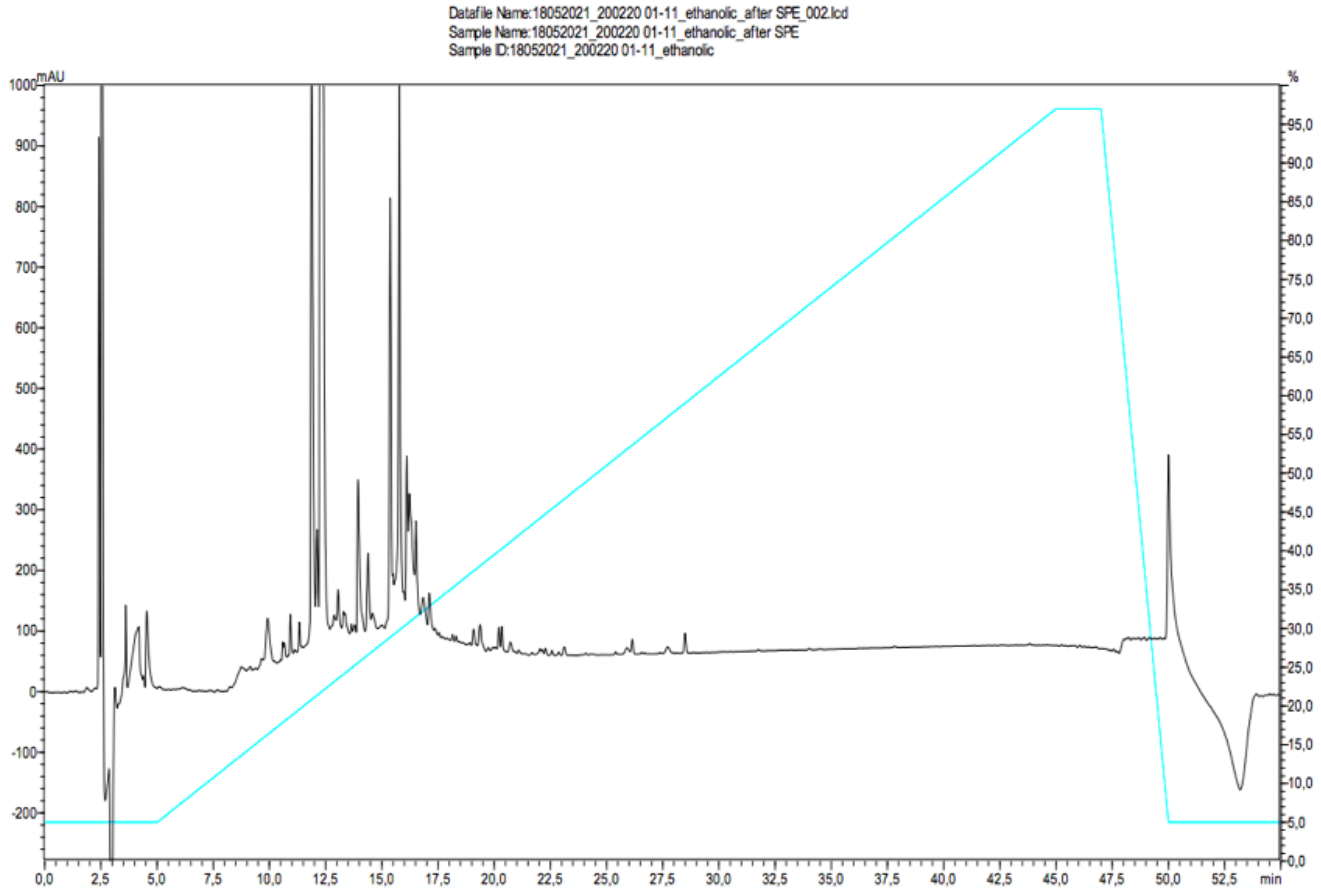

Figure 13. Analytical RP-HPLC chromatogram of the ethanolic extract of *Stylissa massa* after SPE.

Gradient 5-97% B and runtime 55 min.

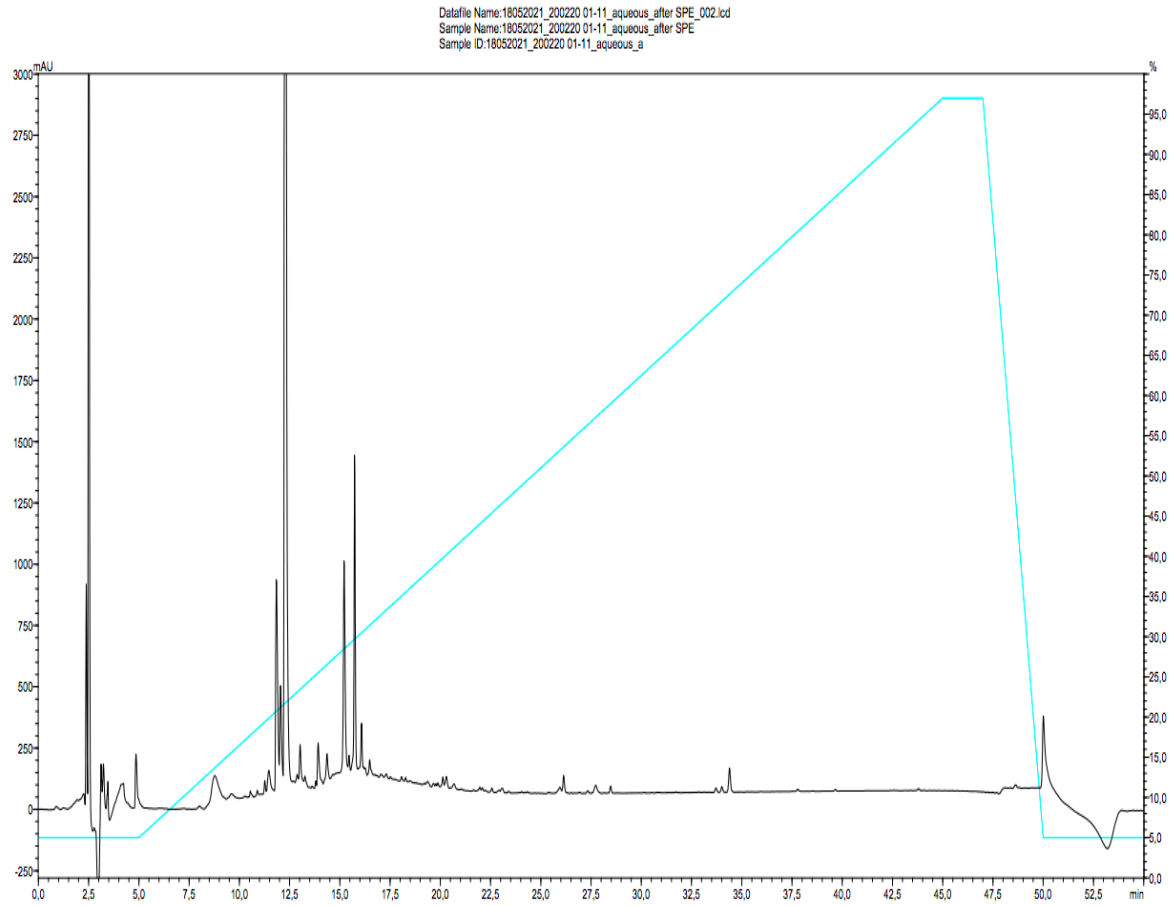

Figure 14. Analytical RP-HPLC chromatogram of the aqueous extract of *Stylissa massa* after SPE. Gradient 5-97% B and runtime 55 min.

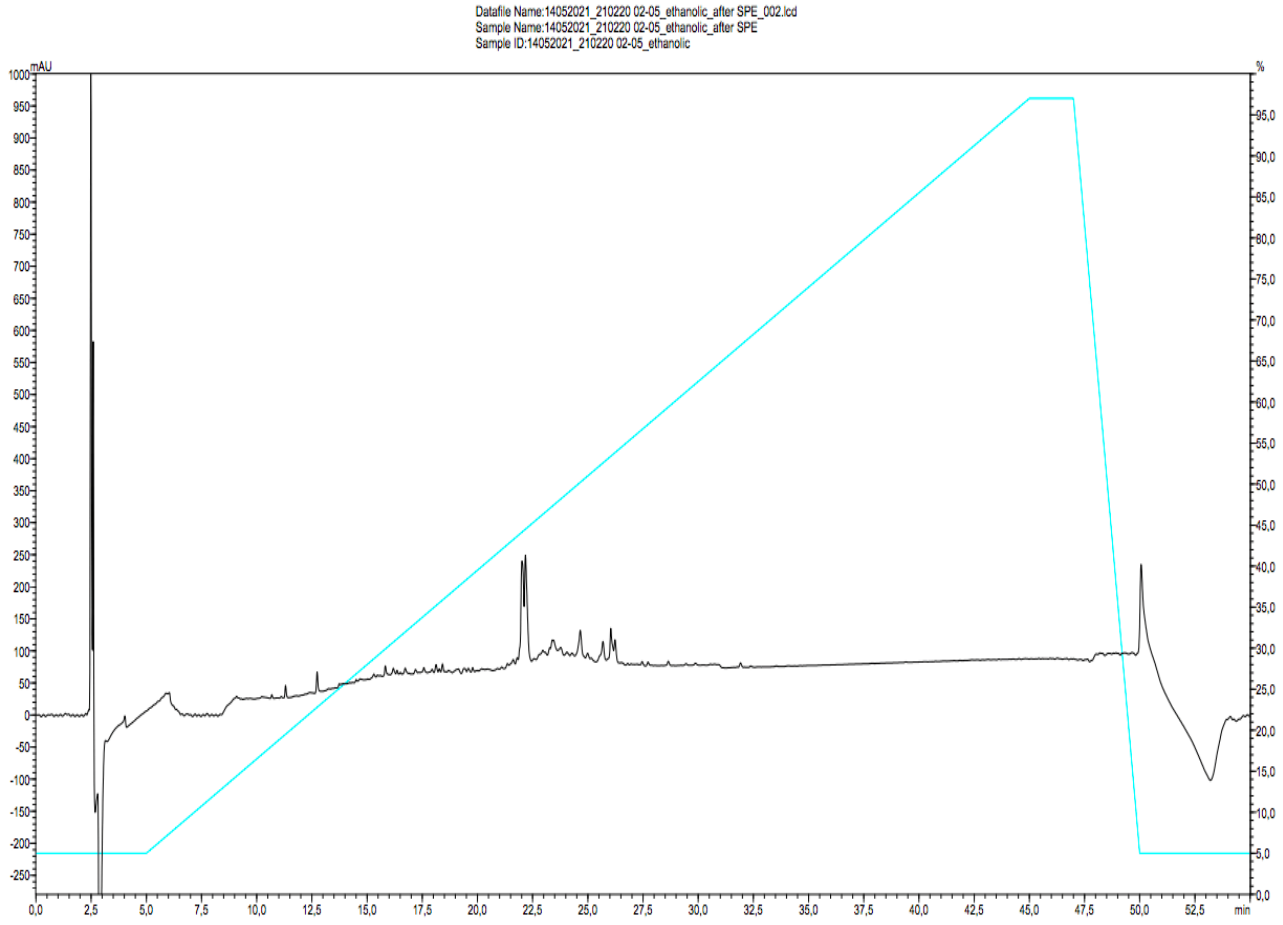

Figure 15. Analytical RP-HPLC chromatogram of the ethanolic extract of 210220 # 02-5 after SPE. Gradient 5-97% B and runtime 55 min.

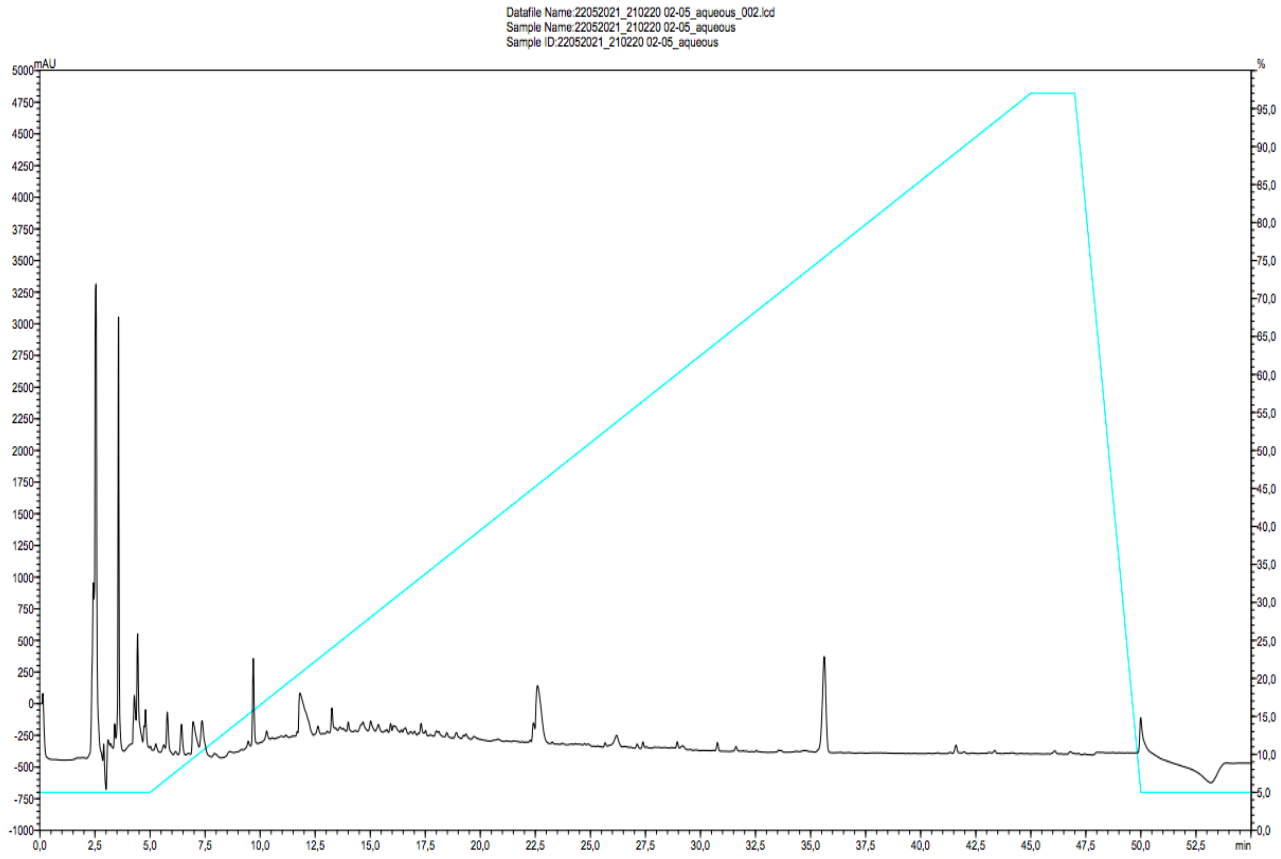

Figure 16. Analytical RP-HPLC chromatogram of the aqueous extract of 210220 # 02-5 after SPE. Gradient 5-97% B and runtime 55 min.

3.4. Screening of the micro fractionated aqueous and ethanolic extracts for its antibacterial activity

The MIC assay was performed on the fractions obtained after analytical RP-HPLC in order to determine the antibacterial activity of the eight different sponge extracts. The dried U bottom microtiter plate containing the 45 fractions from each extract were tested against the two bacterial strains, *S.aureus* and *E-coli*. None of the extracts showed an antibacterial activity against *E-coli* or *S.aureus* up to 2 mg/mL or below. The positive control, Ciprofloxacin, has a MIC value between 1 to 2 µg/mL for the both bacterial strains *E-coli* and *S .aureus*. Therefore, SPE were carried out for two selected extracts to wash the salts present in the extract and to determine its effect for the antibacterial activity.

Figure 17. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract *Stylissa massa*. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *E.coli*.

Figure 18. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract *Stylissa massa*. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *S.aureus.*

Figure 19. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract 210220 # 02-5. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *E.coli.*

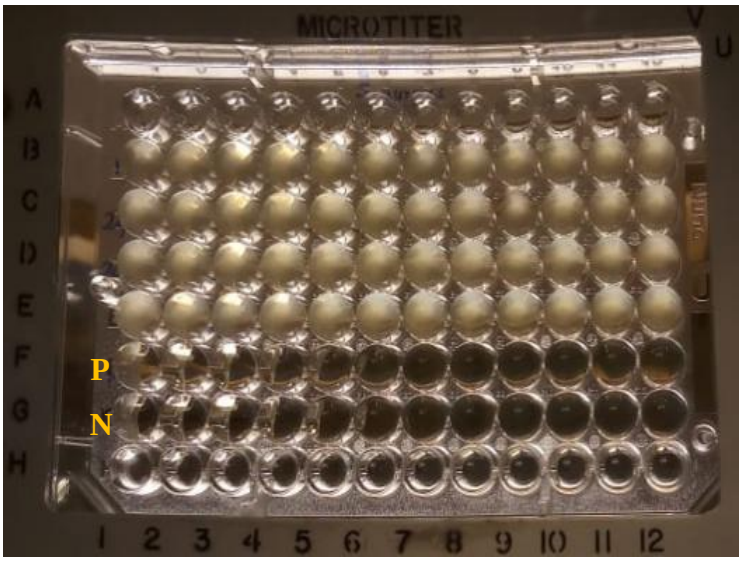

Figure 20. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract 210220 # 02-5. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *S.aureus.*

3.5. MIC values of the crude extract before and after SPE Screening

According to figure 21, a visual growth inhibition against *S.aureus* can be seen only in the first well of row 4 and up to the second well of row 5, corresponding to the ethanolic extract of *Stylissa massa* before SPE and after respectively. The MIC value of the ethanolic extract of *Stylissa massa* against *S.aureus* before SPE is 0.5 mg/mL and 0,25 mg/mL after SPE. Both the aqueous and ethanolic extract of 210220 # 02-5 and the aqueous extract of *Stylissa massa* have a MIC value of above 0.5 mg/mL against both *E.coli* and *S.aureus.*

Figure 21. Serial dilution method for the selected crude extracts. A visual growth inhibition up to one well in row 4 for the ethanolic extract of *Stylissa massa* before SPE. A visual growth inhibition up to two wells in row 5 for the ethanolic extract of *Stylissa massa* after SPE.

Table 2 – MIC values of the two selected specimens before SPE

Name of the extract	**MIC value of the aqueous extract/ mg/mL**		**MIC value of the ethanolic extract/ mg/mL**	
	E-coli	***S.aureus***	***E-coli***	***S.aureus***
Stylissa massa	>0.5	>0.5	>0.5	0.5
210220 #02-5	>0.5	>0.5	>0.5	>0.5
Ciprofloxacin	1-2 µg/mL		1-2 µg/mL	

Table 3 – MIC values of the two selected crude extracts after SPE

Name of the extract	**MIC value of the aqueous extract/ mg/mL**		**MIC value of the ethanolic extract/ mg/mL**	
	E-coli	***S.aureus***	***E-coli***	***S.aureus***
Stylissa massa	>0.5	>0.5	>0.5	0.25
210220 #02-5	>0.5	>0.5	>0.5	>0.5
Ciprofloxacin	1-2µg/mL		1-2µg/mL	

3.6. Micro fractionation followed by MIC assay after SPE

Figure 22 shows a total growth inhibition of all the 45 wells containing the 45 fractions after micro-fractionation. Such a total growth inhibition was observed for the duplicates prepared for aqueous as well as the ethanolic extracts of both *Stylissa massa* and 210220 # 02-05. Interestingly, a visual growth inhibition was observed up to the first 10 wells and a growth was observed in the rest for the positive control after the incubation period. Also a visual growth was not observed in any well containing the negative control.

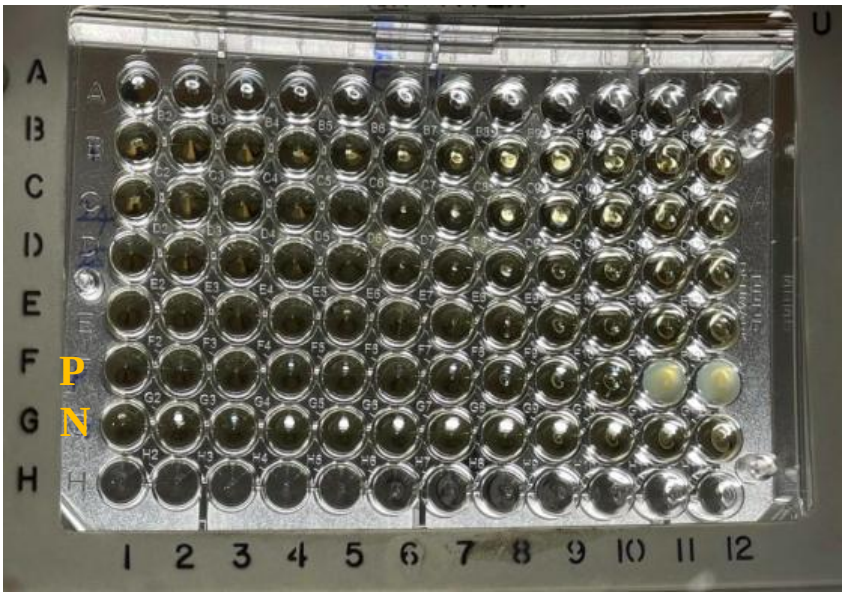

Figure 22. Micro fractionation of 2mg /ml of ethanolic extract of *Stylissa massa*. this plate was subjected for MIC assay against *S.aureus.*

4. Discussion

The main focus of this project was on screening the antibacterial activity of 8 different sponge extracts, where two of them are ethanolic and rest aqueous. Bioassay guided micro fractionation followed by MIC assay was used to determine the extracts having antibacterial properties against *E. coli* and *S. aureus*. Further SPE was carried out as a rapid purification step to remove the salts present in sponges, thereby to determine the effect of salts for the inhibition of antibacterial activity.

The RP-HPLC chromatograms showed a rich chemical composition for all the 8 extracts. But none of the 45 fractions after micro fractionation of each extract showed any antibacterial activity against *E.coli* or *S.aureus* up to 2mg/mL or below. But a MIC value of 0.5 mg/mL was indicated for the ethanolic extract of *Stylissa massa* against *S.aureus* at crude level before micro fractionation. This observation motivated us to carry out an additional SPE step for two selected specimens including *Stylissa massa*. A lower MIC value of 0.25 mg/mL was shown by the ethanolic extract of *Stylissa massa* against *S.aureus* after the SPE step. An unexpected observation of a total inhibition of bacterial growth was visualized when carrying out MIC assay after SPE for the two selected extracts.

4.1. RP-HPLC

The analytical RP- HPLC was used as a technique to separate the compounds present in an extract according to its polarity under a high pressure. Fractionation of the crude extract to 45 fractions would also be helpful to enhance the concentration of the chemical composition in each well rather than in the crude extract without fractionation. The PDA detector would detect the compounds within a wide wavelength range from 200 to 800 nm.

The distribution of intense peaks between the gradient from 5% AcN 0.05% TFA to 55% AcN 0.05% TFA in the aqueous extract of *Stylissa massa* indicates the richness of the chemical composition in between the wavelength range 200 to 800 nm. The most intense peaks were limited to a narrow gradient in the remaining eight extracts. Importantly, the HPLC chromatogram only gives signals for compounds that are absorbed between 200 to 800 nm and also only for compounds that absorb UV light. This means that all other compounds that absorb UV light and/or that are absorbed above 800 nm are not shown on the chromatograms.
In figure 9, highest absorption was between 10 to 20 minutes at the gradient of approximately 20 to 30 % AcN 0.05% TFA. This signifies the presence of polar compounds.
Micro fractionation of the extracts using RP-HPLC would also be a guide to identify the bioactive metabolites in the sponge extracts which can further function as a tool for isolating in large-scale. RP-HPLC micro fractionation has a great advantage as it requires only a small amount of sponge extracts (2mg). This would be a sustainable as well as environmentally friendly approach especially to focus on collection of specimens with potential bioactivities in large scale as a subsequent step once a promising species is identified. The method can enable rapid screening of large numbers of samples at a time. This resulted in a time-efficient screening of a large number of sponges for bioactivity with very small amounts of extracts.

4.2. SPE

The column (Supel^{TM}, bed wt. 60 mg, volume 3ml) is specifically developed for small scale extraction of a broad range of compounds from aqueous samples. The stationary phase of this column consists of hydrophilic modified styrene polymer. This type of column has a higher recovery rate of around 70 to 80% which is useful to recover most of the chemical constituents after passing through. By using a gradient of 30% AcN, 1% FA, 60 % AcN and 1% FA, a range of compounds with different polarities can be recovered. SPE would be a rapid

purification step to remove the salts present in sponges at a high concentration, thereby to determine the effect of salts for the inhibition of antibacterial activity.

4.3. MIC-assay

The sponge extracts after micro fractionation were subjected to screen their antibacterial activity. The two-fold serial dilution method can be used to calculate a MIC value to each extract having a visual growth inhibition of bacteria, thereby giving a significance for its antibacterial activity compared to the positive control. The antibacterial activity of the ethanolic extract of *Stylissa massa* against *S.aureus* before SPE was not significant compared to the positive control having a MIC value of 1-2 µg/mL. It could be due to the presence of active substances in a minute quantity. Interestingly, the MIC value after SPE for the extract discussed above was 0.25 mg/mL, which was lower compared to the MIC value before SPE, but not significant compared to the positive control. This signifies that the removal of salts might have an effect on the inhibition of antibacterial activity. This could be one of the reasons for all the 8 extracts to be non-antibacterial after micro fractionation without SPE. Another possible factor for this phenomenon is that several compounds might act synergistically in the crude extracts to give the observed bioactivity.

Once these are separated, each individual compound activity of the individual compounds might be below the sensitivity threshold, once they are separated (Mohotti et al. 2020).
The protocol of the MIC assay is specifically developed for peptides. The main goal of this protocol is to avoid the inhibition of the antibacterial activity due to the presence of interfering compounds in the highly rich growth media. In the absence of peptide like compounds in the 8

sponge extracts, but particularly other chemical compounds such as alkaloids, terpenoids and steroids could also be a reason to observe no antibacterial activity in the 8 extracts tested.

A proper explanation for the total growth inhibition of the micro-fractions after SPE cannot be described. An inhibition of the growth in the first 10 wells as well as a significant growth in the rest of the wells containing the positive control signifies the usual behavior of bacteria. Showing a usual bacterial growth inhibition of the negative control signifies that it could not be due to contamination while carrying out the assay. The 96 deep well plates, the U bottom plates used in this work were the same when before and after SPE. As the 45 fractions were in the dried form, there was no effect from the solvents used for extraction with respect to the inhibition. As the micro fractionation was carried out to the extract with a starting concentration of 2 mg/mL, an inhibition due to high concentration of the extract in the wells is also not acceptable.

Based on the above results, the ethanolic extract of *Stylissa massa* is a suitable and promising sample to carry out large scale isolation.

4.4. Limitations and improvements

To the best of the knowledge, no mistake happened when carrying out the MIC assay after SPE. It is required to repeat the entire assay as it showed false positive results. The extracts having an antibacterial activity can be tested for a wide range of bacterial strains including other pathogenic bacterial strains. Fractions having an antibacterial activity can be further purified to isolate compounds as well as to determine their structures by NMR.

5. Conclusion

During this study, antibacterial activity against *S.aurus* and *E.coli* was screened for a total of 8 extracts from six different sponges. The ethanolic extract of *Stylissa massa* showed an antibacterial activity against *S. aureus* after SPE, but not significant compared to the positive control. The Micro fractionation of the extracts using RP-HPLC is important to identify the bioactive extracts in small scale, which can be used as a tool when selecting extracts to study in large-scale. The removal of salts using SPE didn't have a significant effect on the inhibition of antibacterial activity. More investigations are needed to achieve this goal in the future. Overall, marine organisms are a great source for new drug candidates that can lead to interesting agents that could have an effect on different human diseases.

6. Acknowledgements

I would like to thank all the members in the pharmacognosy research group for the tremendous help. A special thanks to my supervisors, Sunithi Gunasekera and Lakmini Kosgahakumbura for the constant guidance and support through the entire project. It is a great opportunity to work with the exciting world of natural products from marine sponges specially collected from Sri Lanka, a biodiverse island in the Indian ocean.

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8. Appendix A

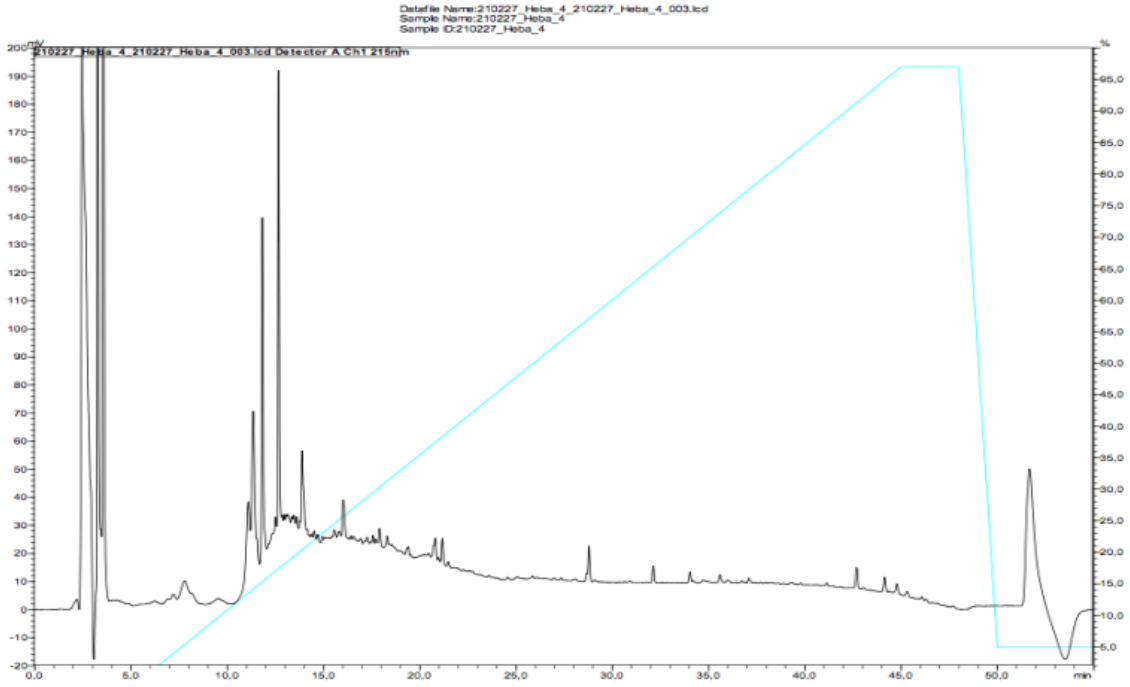

Figure A1 . Analytical HPLC chromatogram of extract 200220 # 01-10 in aqueous. Gradient 5-97% B and runtime 55 min.

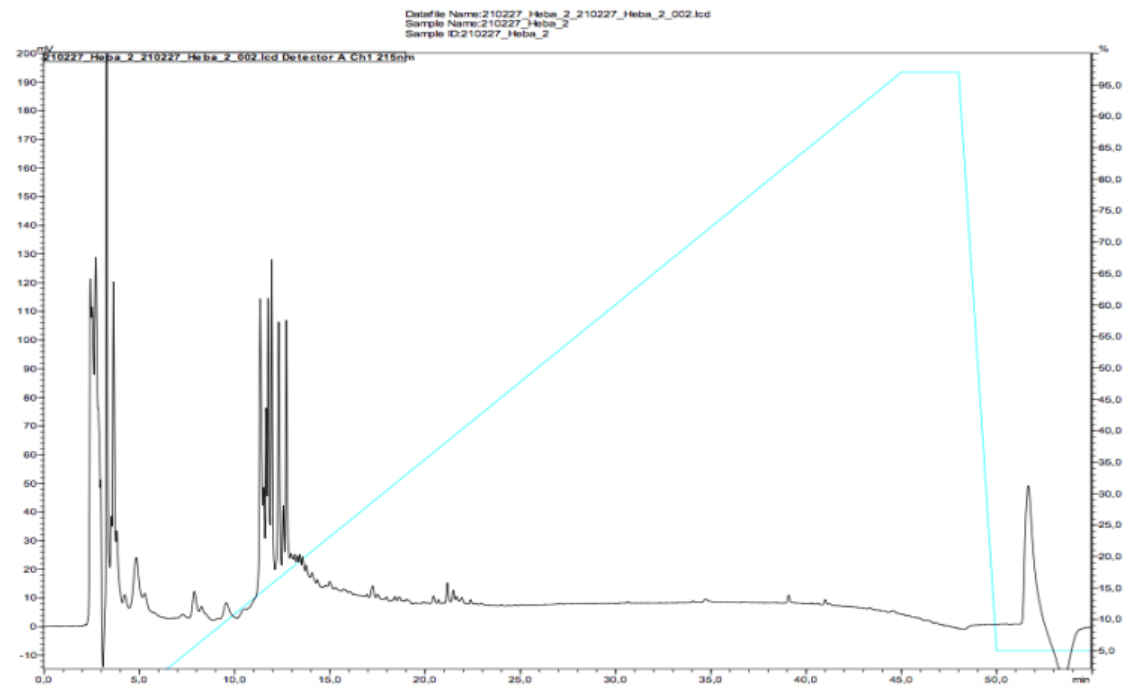

Figure A2 . Analytical HPLC chromatogram of extract *Paratetilla bacca* in aqueous. Gradient 5-97% B and runtime 55 min.

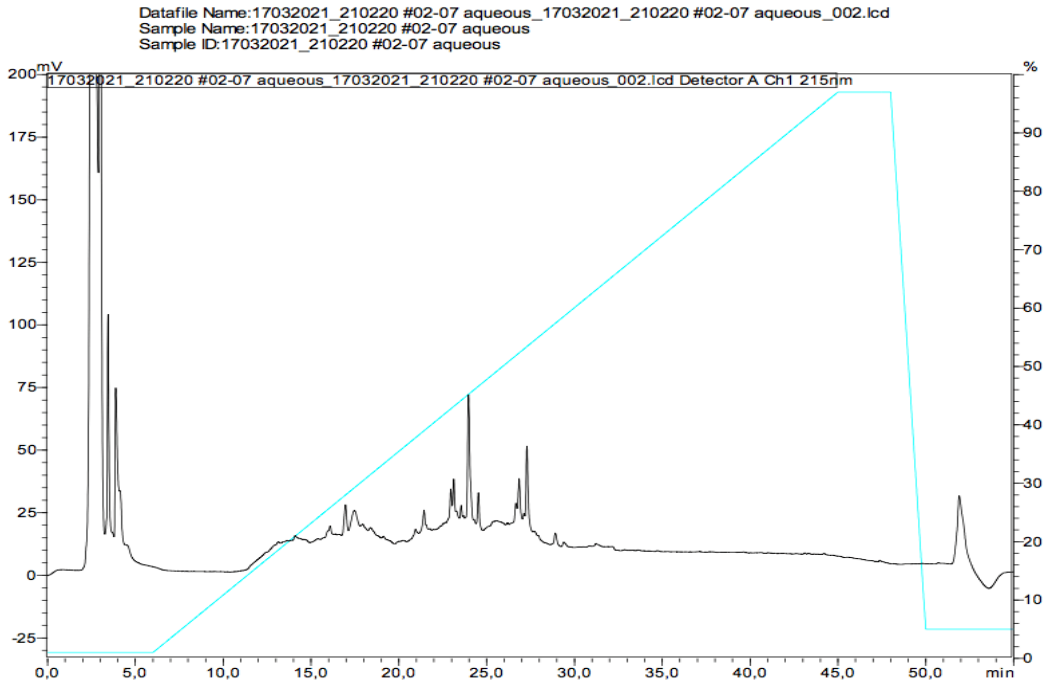

Figure A3. Analytical HPLC chromatogram of extract 210220 # 02-7 in aqueous. Gradient 5-97% B and runtime 55 min.

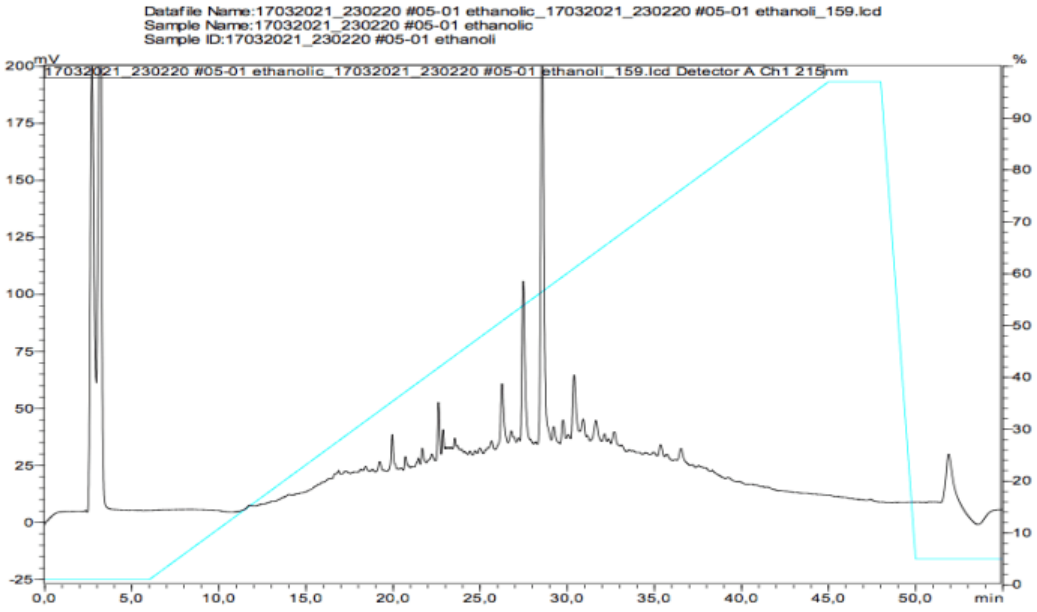

Figure A4. Analytical HPLC chromatogram of extract 230220 # 05-1 in ethanolic. Gradient 5-97% B and runtime 55 min.

9. Appendix B

Figure B1. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract *Paratetilla bacca*. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *S.aureus.*

Figure B2. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract *Paratetilla bacca*. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *E.coli.*

Figure B3. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract 200220 # 01-10. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *S.aureus.*

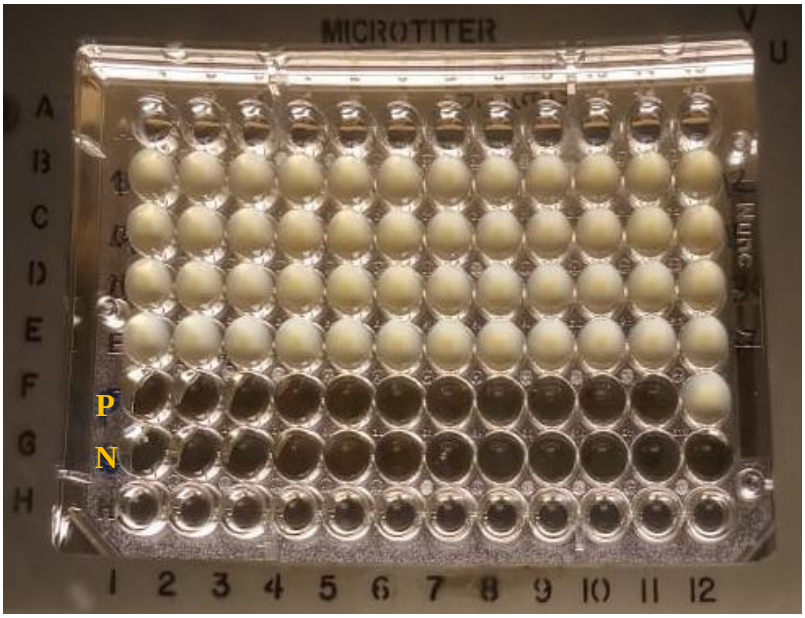

Figure B4. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract 200220 # 01-10. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *E.coli.*

Figure B5. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract 210220 # 02-7. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *E.coli*.

Figure B6. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract 210220 # 02-7. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *S.aureus.*

Figure B7. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract 230220 # 05-1The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *E.coli.*

Figure B8. The U bottom plate containing the 45 fractions after micro fractionation of 2 mg/mL of the extract 230220 # 05-1. The growth of bacteria in the rows from 2 to 5 indicate the absence of its antibacterial activity against *S.aureus.*