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Chemical nature of specialised defensive metabolites from seven common intertidal marine sponges found at Pulau Hantu, Singapore

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Abstract. Sponges are considered as one of the top spatial competitors on reef ecosystems and their success is due partly to the strong chemical defences against pathogenic microbial attacks, predators and other benthic dwelling competitors. This is the first study on the investigation of chemical defences of common intertidal marine sponges, including *Pseudoceratina purpurea*, *Spheciospongia* cf. *vagabunda*, *Halichondria cartilaginea*, *Haliclona* sp., *Stelletta clavosa*, *Clathria reinwardti* and *Hippospongia* sp., found at the sponge garden at the central lagoon of Pulau Hantu, Singapore. The chemical defence of the sponge-derived fractions was evaluated in the brine shrimp (*Artemia salina*) toxicity assay. In general, high brine shrimp toxic properties were found in the relatively polar VLC-derived fractions. At least one polar fraction from each sponge species showed toxicity above 50% when tested at 100 ppm, due to the presence of specialised metabolites. In addition, the polar fractions from *Hippospongia* sp. showed significant cytotoxicity against MOLT-4 leukemic cancer cell lines, revealing them to be potential sources of anticancer agents. Diverse molecules of different structural classes, including known and new metabolites, were detected in the toxic sponge-derived fractions using ^{1}H-NMR spectroscopy and metabolomic-based molecular networking platform. For instance, molecular families related to the fatty acid amides, ceramides and bromotyrosine alkaloids were dereplicated in the molecular network clusters of fractions from *S.* cf. *vagabunda* and *P. purpurea*. Triangulation of information based on the chemoinformatic database, Marinlit, along with ^{1}H NMR spectral data, revealed the hippolide class of molecules to be the major constituents in fraction E of *Hippospongia* sp. as well as being responsible for the observed cytotoxic activity. This study shows that the brine shrimp toxic nature of sponge extracts could confer chemical defences to certain intertidal sponge species, resulting in their abundance and occurrence at P. Hantu.

Key words. brine shrimp toxicity, chemical defence, cytotoxicity, marine sponges, metabolomic, ^{1}H NMR spectroscopy, molecular networking

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INTRODUCTION

Marine sponges are members of the phylum Porifera and are multicellular organisms with over 8,500 living species currently recognised (van Soest et al., 2012). They can be found widely across different ecological habitats from shallow to deep seas, the poles to the tropics and even salt to freshwater regions. Sponges are known to be one of the top spatial competitors for reef-building scleractinian corals and they appear to fulfil a similar ecological role to hard corals, such as offering unique refugia for recruitment, growth, reproduction, feeding and breeding of many reef fish species (Schönberg & Fromont, 2012; McMurray et al., 2015). Their increased abundance and proliferation can be partly attributed to their ability to feed on a wide range of nutritional sources, low energetic costs of filter-feeding activities (Gili & Coma, 1998) as well as chemical defences for protection (Pawlik et al., 2015). In addition, these non-reef-building taxa are able to cope better with anthropogenic stressors, including eutrophication, sedimentation, disease prevalence and climate change, resulting in their increased abundance (Bell et al., 2013). Furthermore, studies found that sponges can be released from top-down control due to overfishing and can undergo uncontrolled growth due to absence of predators (Slattery et al., 2014; Loh et al., 2015).

Sponges are known to be prolific sources of secondary metabolites (i.e., specialised compounds) among benthic marine organisms with more than 5,000 currently described (Calado et al., 2022). Historically, the main interest in the natural products of sponge chemistry has been their utility as potential drug agents. Research focusing on the biomedical applications of these metabolites found them to possess significant anti-inflammatory, anticancer, neuroprotective and anti-infective properties (Hong et al., 2022). This led to the discovery of myriad sponge-derived lead molecules with novel chemical structures in drug development efforts. However, starting in the early 1980s, collaborations between

chemists and marine biologists began to examine the ecological functions of these specialised metabolites from benthic invertebrates (Pawlik et al., 2013).

Based on terrestrial plant defence theories, sponge defences can be classified broadly into three strategic categories, namely constitutive, activated and induced defence, each being optimised in response to a specific threat (Thoms & Schupp, 2007). Constitutive defence is thought to be favoured when sessile benthic marine organisms, such as sponges, are under constant predation pressure and the dominant predators are highly motile. Certain sponges, such as those belonging to the genus *Aplysina*, use an activated defence where precursors of bioactive compounds are produced and stored. Upon wounding by predators, these precursor molecules are converted quickly, usually within seconds/minutes, to potent defensive metabolites via enzymes. Lastly, induced defence in sponges is defined as the 'de novo' production of defensive compounds upon predator attacks. These molecules are biosynthesised on demand and benefit sponges by saving resources and increasing their chemical variability. However, the main disadvantage of induced defences is the prolonged process, usually several days to weeks, of defensive compound production. Such induced defences are therefore favourable under slow and variable grazing by mesopredators, including small crustaceans or gastropods, on sponges.

Sponges have developed a vast diversity of defensive compounds, such as alkaloids, polyketides and terpenoids, to ward off predators due to their apparent lack of physical defence and immobility (Rohde et al., 2015). As sponges harbour diverse symbiotic microbial communities, such as bacteria, archaea, microalgae and fungi, they can also be involved in the biosynthesis of these defensive molecules (Li et al., 2023; Tan, 2023). These molecules help to prevent fouling organisms and other pathogenic microorganisms, resulting in sponge competitive dominance over other benthic organisms (Kelly et al., 2003; Pawlik et al., 2007). For instance, a bromotyrosine compound, ianthelline, isolated from the sponge, *Ailochroia crassa*, was found to possess antimicrobial activity against environmental marine bacterial strains (Kelly et al., 2005). In another study, the bromopyrrole alkaloid, oroidin, from *Agelas tubulata* showed potent antimicrobial activity against the marine bacterium, *Serratia marcescens* (Abraham et al., 2022). Other sponge defensive molecules, such as *N*-methyldibromoisophakellin (from *Stylissa caribica*) and clionapyrrolidine A (from *Cliona tenuis*), were found to have antifeedant properties and ward off coral competitors respectively (Assmann et al., 2001; Chaves-Fonnegra et al., 2008). Moreover, certain sponge compounds, e.g., triterpene glycosides from two Caribbean sponges, *Erylus formosus* and *Ectyoplasia ferox*, were found to possess multiple defensive roles, including deterring predation, microbial attachment and fouling by invertebrates and algae (Kubanek et al., 2002).

While several studies have been conducted in Singapore on the biodiversity of sponge communities in Singapore reefs, their chemical ecology has not been investigated despite their abundance and ecological importance (Lim et al., 2008; Lim et al., 2009; de Voogd & Cleary 2009; Lim et al., 2012). Since the description of the first sponge species, *Cliona* (as *Spongia*) *patera* (Hardwicke, 1822) from Singapore in the 19th century, more than 190 sponge species have been recorded in local waters (Lim et al., 2009; Lim et al., 2012; Lim et al., 2016). The first sponge diversity survey, with voucher specimens, by Hooper et al (2000) listed about 80 species. This was followed by a sponge diversity and community composition study by de Voogd & Cleary (2009) where a total of 82 species were identified. At the same time, a publication by Lim et al (2009) recorded 62 fouling sponge species found on navigational buoys. These publications provided mainly qualitative information on sponge diversity and no quantitative data related to sponge species-specific abundance were measured. In addition to sponge diversity studies, at least six new sponge species, including *Tethycometes radicosa* (Lim & Tan, 2008), *Suberites diversicolor* (Becking & Lim, 2009), *Theonella laena* (Lim & Tan, 2016), *Forcepia* (*Forcepia*) *vansoesti* (Lim et al., 2012), *Clathrina sororcula* and *Anamixilla singaporensis* (van Soest & de Voogd, 2015), have been described from Singapore.

Research on sponge-derived natural products in Singapore is largely related to the pharmaceutical importance of specialised metabolites. For instance, three publications provided detailed information on the anticancer and antimicrobial activities as well as the chemical structures of several sponge metabolites, including spongolipid, racemic xestospongin D and isoaaptamine from *Spongia* cf. *hispida* (Pettit et al., 1997), *Niphates* sp. (Pettit et al., 1996) and *Hymeniacidon* sp. (Pettit et al., 2004), respectively. Interestingly, *Hymeniacidon* sp. is not listed in any recent publications on Singapore sponge biodiversity. This is surprising as more than 500 kg of this sponge species were originally collected by Pettit's research group in 1989 and 1992 from Terumbu Pemalang Besar (Pettit et al., 2004), which is currently within the bounds of the Semakau Landfill. To date, marine chemical ecology studies conducted in Singapore mainly involve octocorals and investigation on the allelopathic effects of macroalgal extracts on coral larvae (Koh et al., 2000; Koh et al., 2002; Fong et al., 2019). For instance, antifeedant/toxic activities were found in extracts prepared from eight gorgonian species (Goh & Chou, 1998; Koh et al., 2000) while extracts from three gorgonian species exhibited antifungal properties against marine fungi previously isolated from these benthic invertebrates (Koh et al., 2002). The extracts of three macroalgae including, *Bryopsis* sp., *Endosiphonia horrida* and *Lobophora* sp., caused mortality of *Pocillopora acuta* coral larvae while extracts from *E. horrida* and *Lobophora* sp. significantly decreased larval settlement (Fong et al., 2019).

The choice of an ecologically relevant and non-vertebrate predator for toxicity assays is a challenge for the assessment of defensive compounds derived from marine sources since fish are often the dominant predator in marine ecosystems (Barrera-Oro, 2002; Steneck, 2012; Ellingsen et al., 2015; Saunders et al., 2019). Several chemoecological studies have employed the use of the brine shrimp (*Artemia salina*) toxicity/lethality assay to provide preliminary evaluation of

extract/compound toxicity (Muniain et al., 2008; Zhao et al., 2011; Xu et al., 2013; Giordano et al., 2017; Winters et al., 2018; Kamyab et al., 2020; Winters et al., 2022). Recent research on the validity of using the brine shrimp toxicity assay in ecological studies showed that nudibranch extracts that are toxic to brine shrimp larvae were also toxic to the blue-green damselfish, *Chromis viridis* (Chan et al., 2021). As such, it has been suggested that the brine shrimp assay can be a reasonable method to assess the potential toxicity of extracts to fish. However, researchers need to be aware of the fundamental differences in physiology between brine shrimp larvae and vertebrates and ecologically relevant field-based assays are required to validate lab-based data (Hermann & Thaler, 2021). There are several benefits of using such an assay as it is an easy, rapid and inexpensive method to test for general toxicity of extracts/compounds. In addition, ethical issues associated with the use of ecologically relevant vertebrate predators in toxicity studies can be circumvented by the brine shrimp toxicity assay. Moreover, brine shrimps are highly sensitive crustaceans, particularly when exposed to crude sponge extracts (Richelle-Maurer et al., 2002; Harvell et al., 2007).

In this study, we provide the first assessment of chemical defences in seven common intertidal sponge species, namely *Pseudoceratina purpurea*, *Spheciospongia* cf. *vagabunda*, *Halichondria cartilaginea*, *Haliclona* sp., *Stelletta clavosa*, *Clathria reinwardti* and *Hippospongia* sp., at a sponge garden located in the central lagoon of Pulau [=island] Hantu, Singapore. The chemical defences of these sponges were assessed based on the brine shrimp (*Artemia salina*) toxicity assay, which has been validated recently to be a reasonable method to evaluate the ecological function of marine-derived specialised metabolites (Chan et al., 2021). In addition, the chemical nature of sponge fractions that show significant brine shrimp toxic activity has been revealed by ^{1}H NMR spectroscopy as well as the MS-based metabolomic approaches. Lastly, to investigate the biomedical potential of these sponges, the bioactive fractions were further evaluated for anticancer activities in a cytotoxicity assay using the MOLT-4 leukemic cancer cell lines.

MATERIAL & METHODS

A total of seven sponge samples obtained from *Pseudoceratina purpurea*, *Spheciospongia* cf. *vagabunda*, *Halichondria cartilaginea*, *Haliclona* sp., *Stelletta clavosa*, *Clathria reinwardti* and *Hippospongia* sp. were collected at the intertidal area of Pulau Hantu, Singapore, in May 2021 (Fig. 1). Small samples, each measuring about 1.5 cm × 1.5 cm × 1.5 cm, were excised from each sponge species at the collection site and stored in Ziploc bags. The Ziploc bags were then placed in a cooler box and transported to a laboratory at NIE and stored in a −20 °C freezer prior to further processing. The morphology of sponges was photographed during surveys and compared against photographs and descriptions of sponges from taxonomic publications (Lim et al. 2008; Lim & Tan 2008; Lim et al. 2012). Photographs/samples were also examined by sponge taxonomy expert, Lim Swee Cheng, at the St. John's Island National Marine Laboratory, Singapore, for confirmation of taxonomic identifications.

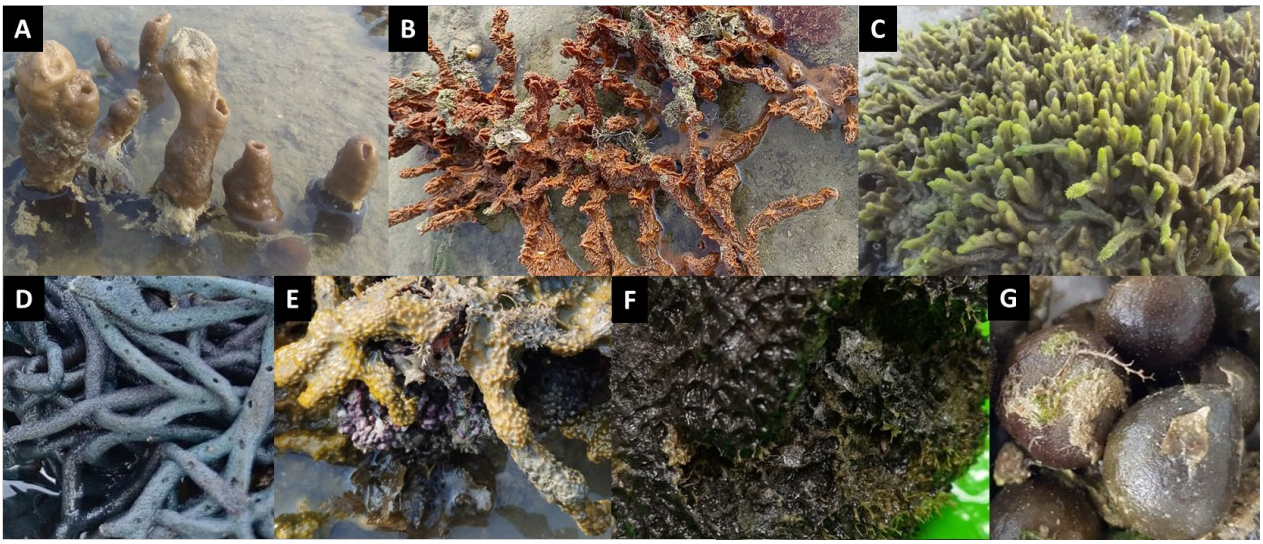

Fig. 1. Seven relatively common intertidal sponge species from P. Hantu used in the study: A, *Spheciospongia* cf. *vagabunda*, B, *Clathria reinwardti*, C, *Halichondria cartilaginea*, D, *Haliclona* sp., E, *Pseudoceratina purpurea*, F, *Hippospongia* sp. and G, *Stelletta clavosa* (Photograph by: Lik Tong Tan).

Sponge samples were allowed to thaw and were thoroughly washed with artificial seawater to remove debris before extraction. Each sponge sample was cut into smaller pieces using a knife and placed into a 50 mL beaker. The extracting solvent, methanol (MeOH), was added to the beaker till the sponge pieces were fully covered. The beaker was placed on a 50 °C hot plate for 30 min and stirred at 10 min intervals. After 30 min, the mixture was filtered through a cheese cloth

on the Buchner funnel and the filtrate was collected in a flask. Extraction was repeated three times for each sponge species. The organic solvent in the pooled filtrate was removed in vacuo using a rotary evaporator.

Sponge extracts were subjected to further fractionation using vacuum liquid chromatography (VLC). The main purpose of fractionation is to increase the concentration of possible bioactive metabolites for bioassay testing. Dry loading of extracts was carried out for the VLC where each sponge extract was redissolved in a small volume of MeOH and 10 g of celite was added. The solvent in the extract-celite slurry was dried down in vacuo to powder form. This extract-celite powder was loaded onto a packed NP silica column and eluded with solvents of increasing polarity from hexane to EtOAc to provide five fractions (A to E). Each fraction was dried down in vacuo and reconstituted in EtOAc to give a stock solution with concentration of 10 mg/mL. A total of 35 VLC-derived fractions were prepared from seven sponge extracts.

The toxicity of all 35 VLC-derived fractions was evaluated using the brine shrimp (*Artemia salina*) lethality assay following the method by Chan et al (2021) with modifications. Each fraction was tested at concentrations of 100 ppm and 10 ppm and together with controls, were prepared in duplicates. Briefly, appropriate amounts of stock solutions were pipetted into separate glass vials from stock solutions and solvent removed in vacuo before adding about 20 actively swimming first instar nauplii into each vial. Surviving brine shrimp nauplii instar II/III were counted using a dissecting microscope after 24 h incubation. Percentage toxicity was calculated using Abbott's formula (Abbott, 1925): % toxicity = (% mortality in treated – % mortality in control)/ (100% – % mortality in control) × 100.

The cytotoxicity of the VLC-derived fractions was accessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromine] bioassay based on MOLT-4 (T lymphoblast; acute lymphoblastic leukaemia) cancer cell line over a 3-day procedure. Prior to the assay, the cancer cell lines were maintained and cultured in 10 mL of medium, consisting of RPMI medium, foetal bovine serum, streptomycin and penicillin, until a cell viability of 90% and above was achieved. On the first day, two 96-well microliter plates were used to contain 100 ppm of each sponge fraction, conducted in triplicate. Appropriate amounts of stock solutions from each sponge fraction were reconstituted to 0.08 mg/mL with 10% DMSO. To each fraction, 10 µL of fraction was mixed with 70 µL of cancer cells. The plates were incubated for 24 h at 37 °C with 5% CO_2 incubator. After 24 h, 20 µL of MTT solution was added into each well and left to incubate for an additional 3 h. After which, 100 µL of lysing buffer was added into each well and left to incubate overnight. After incubation, the absorbance values were measured using a UV spectrophotometer at OD570 nm and the results were tabulated.

Fractions that showed significant biological activities in the brine shrimp lethality and cytotoxicity assays were profiled using ^{1}H NMR spectroscopy recorded in $CDCl_3$ on a 400 MHz Bruker NMR Spectrometer (400.13 MHz ^{1}H) using residual solvent signals as internal references (referenced to residual $CDCl_3$ observed at δH 7.24) with chemical shifts given in ppm downfield from TMS.

About 1mg of bioactive sponge fractions were sent to Thermo Fisher Scientific, Singapore, for acquiring of LC-MS/MS data to generate molecular networking clusters. Fractions were first filtered over C18 SPE cartridges by application of a 1.0 mL sample (1 mg/mL) and elution with 3 mL CH_3CN. Solvent was removed in vacuo using a rotary evaporator before being redissolved in 1 mL CH_3CN, vortex mixed for 5 min and transferred into separate Eppendorf tubes. Tubes were then centrifuged at 10,000 rpm at 4 °C for 10 min, and the supernatant was aliquoted and diluted with CH_3CN to 10,000 × dilution. One-and-a-half microliters of each diluted sample was subjected to LC-HRMS/MS (Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionisation (H-ESI) probe) performed with a Thermo Scientific Hypersil GOLD (C18 50 mm × 2.1 mm, 1.9 mm) column and maintained at a column temperature of 40 °C and a sample temperature of 4 °C using a gradient elution program of 0.1% aq. HCOOH (mobile phase A) and 98% CH_3CN in 0.1% aq. HCOOH (mobile phase B) at a flow rate of 0.5 mL/min. The gradient program began at 10% and increased to 50% of mobile phase B within 2 min and was held at 50% of mobile phase B for 2 min. It was then increased to 100% of mobile phase B within 6 min and was held at 100% of B for 0.5 min before reconditioning back to the starting composition in 0.5 min, and it was held at the starting composition of 10% of B for 3 min, bringing the total runtime to 14 min. All mass spectra were collected in the positive ion and data-dependent acquisition mode, where the first five most intense ions of each full-scan mass spectrum (mass range: m/z 100–1500) were subjected to tandem mass spectrometry (MS/MS) analysis: an MS scan time of 0.25 s over 14 min; an MS/MS scan time of 0.06 s; and a 3-step normalised collision energy of 25, 35 and 55 eV. The MS/MS data files were converted from RAW into mzXML files using the MSConvert software and uploaded to the Global Natural Product Social Molecular Networking (GNPS) server, and the molecular networking was performed using the GNPS data analysis workflow employing a special spectral clustering algorithm.

RESULTS & DISCUSSION

A. Assessment of Chemical Defenses of Intertidal Sponge Species Based on the Brine Shrimp (*Artemia salina*) Toxicity Assay

In this study, the chemical defense of seven common intertidal sponge species, including *Pseudoceratina purpurea*, *Spheciospongia* cf. *vagabunda*, *Halichondria cartilaginea*, *Haliclona* sp., *Stelletta clavosa*, *Clathria reinwardti* and *Hippospongia* sp., were evaluated in the brine shrimp toxicity assay. As seen from Fig. 2, relatively polar sponge fractions, namely fractions D and E, tend to exhibit higher toxicity especially at 100 ppm. Fractions D and E were obtained using elution solvents of hexanes/EtOAc (1:4) and 100% EtOAc, respectively. Based on average percentage toxicity, eight relatively polar fractions from four sponge species, including *S.* cf. *vagabunda* (fractions C to E), *Stelletta clavosa* (fractions D and E), *C. reinwardti* (fractions D and E) and *Hippospongia* sp. (fraction E), exhibited more than 80% mortality rate. Of these species, the polar fractions from *S.* cf. *vagabunda* showed the highest incidence of toxicity as all three fractions C to E gave more than 80% lethality at 100 ppm. When the threshold of 50% toxicity is considered, at least one polar fraction from each sponge species gave percentage toxicity above this threshold value. At lower concentration of 10 ppm, the average percentage toxicity dropped significantly with only two fractions, i.e., fraction E from *C. reinwardti* and *Hippospongia* sp., exhibited more than 50% percentage toxicity. The significant drop in average percentage toxicity for the majority of the fractions could be due to solubility issues of compounds in artificial seawater.

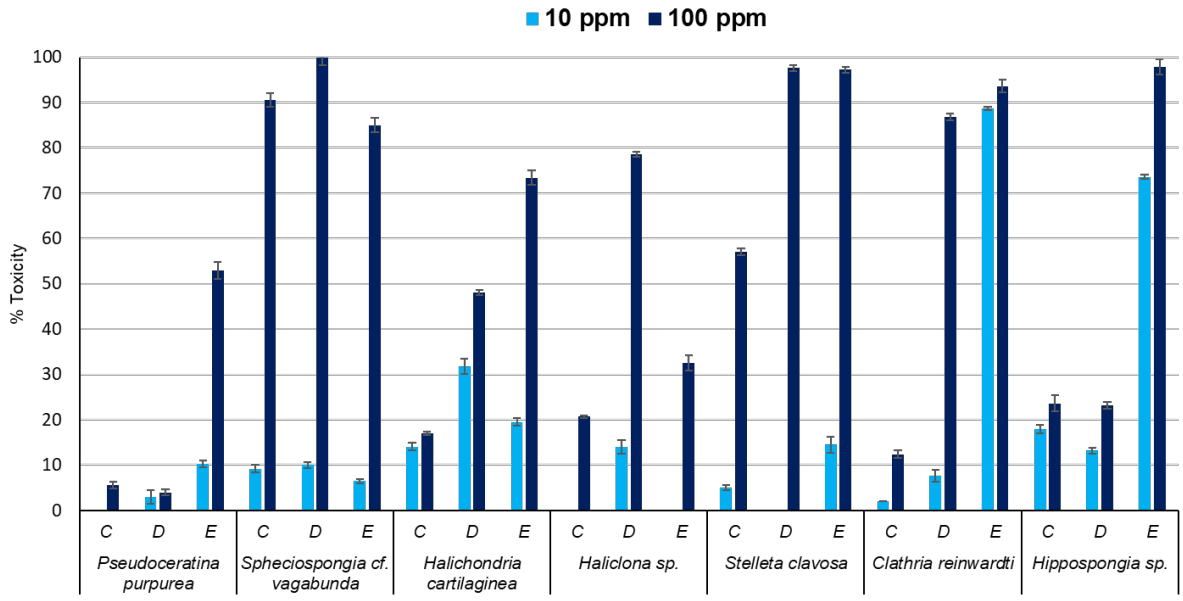

Fig. 2. Percentage brine shrimp toxicity of the polar fractions obtained from seven sponge species.

As studies have shown relationships between the toxicity of brine shrimp assays and the potential antitumour activities of extracts (Chavez et al., 1997; Carballo et al., 2002; Soapi et al., 2013), these polar fractions were considered for further cytotoxic evaluation, tested at 100 ppm, in the MTT assay based on MOLT-4 leukemic cancer cell lines (Fig. 3). The MTT assay is a colourimetric method used to test cell viability via the mitochondria ability to metabolise MTT where it could be reduced to purple formazan in living cells (Vistica et al., 1991). Lysing buffer is used to dissolve insoluble formazan into a coloured solution which would be measured at an absorbance of 570 nm in an UV spectrometer. Solutions with deeper violet colour (higher absorbance), would indicate lower cytotoxicity. There was no clear correlation between fractions that showed significant brine shrimp toxicity with those that exhibited cytotoxic properties in the MTT assay. In fact, all fractions, except fractions derived from *Hippospongia* sp., revealed percentage cytotoxicity below 50% (Fig. 3). Only one fraction, namely fraction E from *Hippospongia* sp., showed consistent significant activity of above 80% toxicity in both the brine shrimp toxicity (at 100 ppm in Fig. 2) and MTT assays (Fig. 3). The main reasons for the inconsistency could be due to compound solubility, permeability of sponge metabolites into the cancer cells and differences in physiology on the effects of compounds on toxicity in whole brine shrimp larvae and cancer cells. However, if the trend in cytotoxicity is considered, generally the most bioactive fractions tested in the brine shrimp assay seem to correlate with the highest cytotoxicity in the MOLT-4 based MTT assay, with the exception of *C. reinwardti*. More importantly, the MTT assay revealed potential sources of anticancer specialised metabolites that could be further explored in subsequent purification and structural determination steps. It is interesting to note that fraction E from *C. reinwardti* exhibited slight negative cytotoxicity, indicating that there could be presence of tumour promoting agents. This is not unusual as there are several sponge-derived metabolites, such as okadaic acid and calyculin A, that have been shown to possess tumour promoting properties (Suganuma et al., 1990; Fujiki et al., 1999). However, based on Marinlit search,

there are currently no tumour promoting agents reported from sponges of the *Clathria* genus. Marinlit is a database of marine natural products and consists of highly curated chemical and biological information.

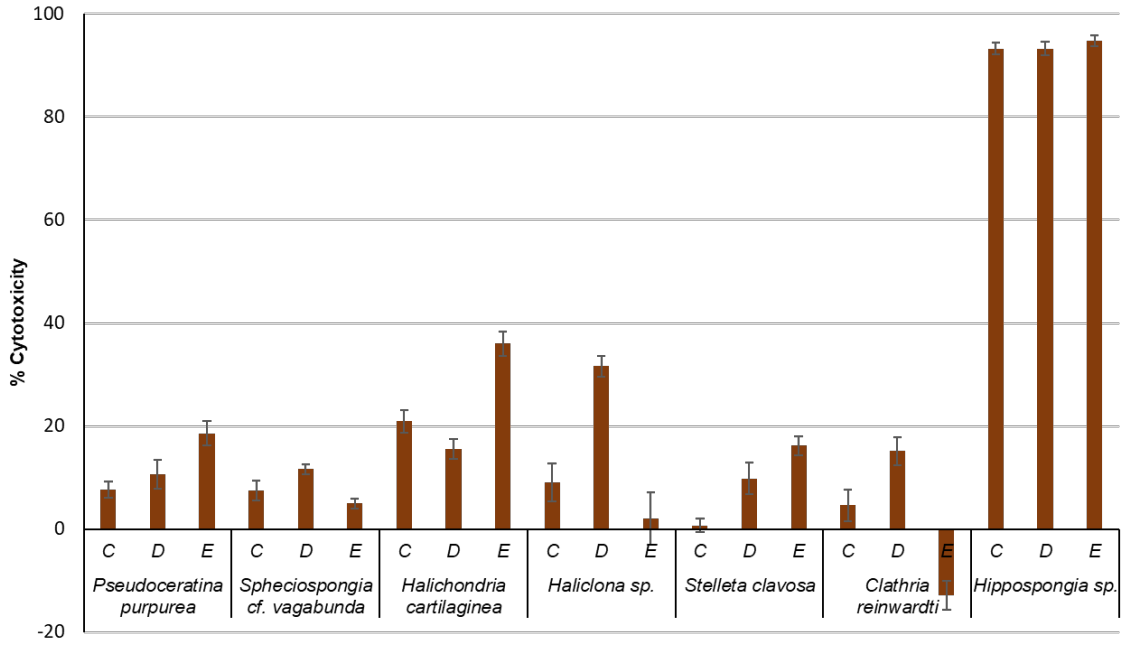

Fig. 3. Percentage cytotoxicity against MOLT-4 cancer cell lines of the VLC-derived polar fractions from seven sponge species tested at 100 ppm.

B. Determine the Chemical Nature of Specialised Metabolites Using ^{1}H NMR Spectroscopy and MS-Based Molecular Networking

Based on the above brine shrimp toxicity assay results (Fig. 2), several fractions with significant brine shrimp toxicity have been identified and this led to subsequent investigations into their chemical nature using ^{1}H NMR spectroscopy and the MS-based molecular networking platform. These two techniques will allow insights into the possible structural classes of natural products as well as the range of compounds present in the bioactive fractions. NMR is a highly useful tool for analysis of compounds as it is non-destructive, unbiased, quantitative, does not require separation or derivatisation and is amenable to molecules that are difficult to analyse using MS methods. The ^{1}H NMR spectra of selected brine shrimp toxic fractions, including fraction D from *S.* cf. *vagabunda* and *Haliclona* sp. as well as fraction E from *P. purpurea*, *H. cartilaginea*, *C. reinwardti* and *Hippospongia* sp. revealed similarities and differences in chemical shifts (Fig. 4). All ^{1}H spectra revealed complex mixtures of major and minor chemical components as well as similar high field chemical signals between 0.5 and 3 ppm. This is to be expected since fractions contain organic compounds that share common functional moieties, such as saturated hydrocarbons. It is the lower field signals, particularly between 4.0 and 7.0 ppm that showed signal differences. For instance, olefinic protons are present in fractions E (from *P. purpurea* and *Hippospongia* sp.) and D (*Haliclona* sp.). Closer examination within this olefinic region revealed slight differences in proton signals and splitting patterns among the fractions, indicating different substitution patterns. In addition, there could be presence of minor compounds containing aromatic protons (between 6.8 and 7.0 ppm) in fraction E of *Hippospongia* sp. (Fig. 4). Fractions could contain the presence of specific classes of specialised metabolites found in certain sponge genera. For instance, the sponge genus *Pseudoceratina* is known to contain bioactive bromotyrosine derivatives (Peng et al., 2005; Niemann et al., 2015). As such, the low field olefinic proton signals (between 6.1 and 6.7 ppm) observed in fraction E of *P. purpurea* are likely due to protons associated with the spirocyclohexadienyl-isoxazoline ring system found in bromotyrosine alkaloids reported from this genus (Fig. 4).

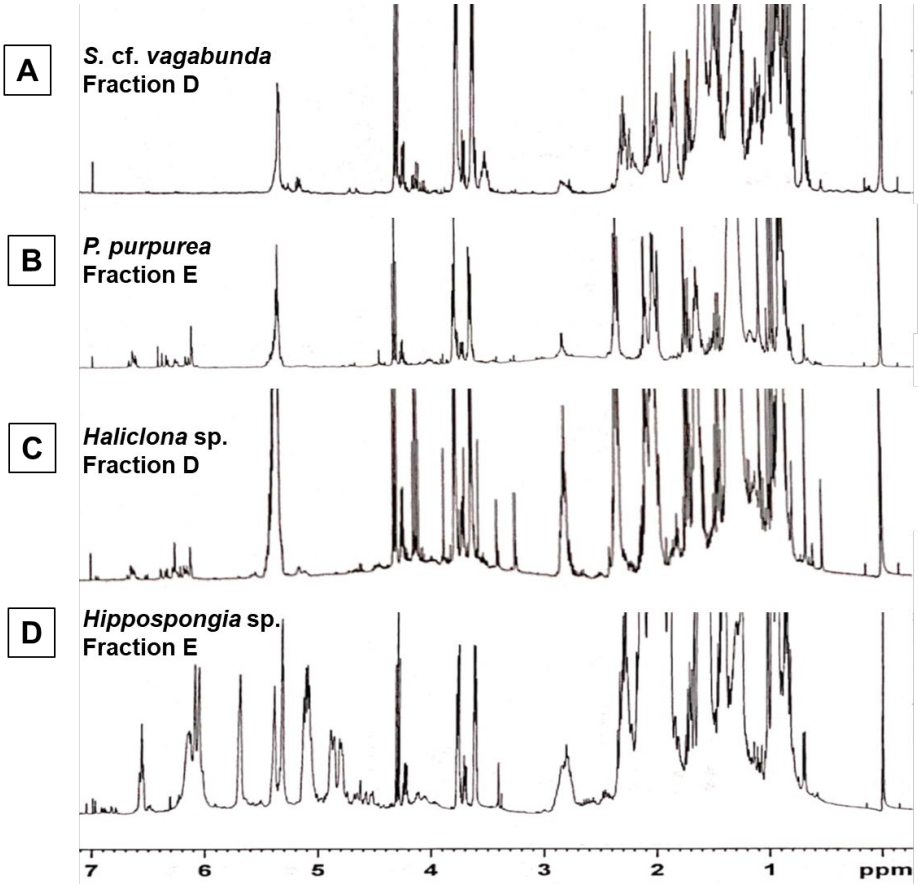

Fig. 4. ^{1}H NMR spectra, measured in $CDCl_3$, of selected brine shrimp toxic VLC-derived sponge fractions, including A, fraction D from *S.* cf. *vagabunda*, B, fraction E from *P. purpurea*, C, fraction D from *Haliclona* sp. and D, fraction E from *Hippospongia* sp.

Molecular networking is an effective analytical workflow for untargeted MS/MS-based metabolomic studies through the organisation of similar MS/MS spectra to a network map (Quinn et al., 2017). Currently, this technique is used in forensics, food chemistry, environmental science, plant science, natural products research for compound dereplication and chemical ecology (Paul et al., 2019; Qin et al., 2022). In this study, seven bioactive fractions, each from different sponge species, were profiled using molecular networking for detection of known and unknown metabolites (Fig. 5). More than 1,700 precursor ions were detected across all seven brine shrimp toxic sponge fractions with the top three highest number of precursor ions detected in fraction E from *Hippospongia* sp., fraction E from *P. purpurea* and fraction D from *Haliclona* sp. As seen in Fig. 5, there are ionisable ions that are present in all fractions while certain molecular families/nodes are detected solely in specific sponge fractions. Based on the molecular networking analysis, the bioactive fraction D of *Haliclona* sp. has one of the highest numbers of unique precursor ions detected only from this fraction and a majority of these ions did not match with the MS/MS spectra of known compounds in the GNPS database.

Certain common precursor ions, such as fatty acid amides, are found in all fractions (Fig. 6). For instance, fatty acid amides, including 9-octadecenamide, octadecanamide and palmitamides, were present in all sponge fractions. Interestingly, 9-octadecanamide was isolated from a sponge-associated (*Dendrilla nigra*) marine actinomycete *Nocardiopsis dassonvillei* MAD08 and was found to have antimicrobial property (Selvin et al., 2009). This C-18 fatty acid amide was also detected in a bioactive EtOAc extract prepared from a marine sediment-derived fungal strain, *Penicillium* sp. ArCSPf, isolated from the continental slope of the Eastern Arabian Sea (Farha & Hatha, 2019). The biosynthetic source of these bioactive fatty acid amides found in the fractions could be from symbiotic microbes and could also be incorporated as precursors for formation of complex bioactive lipids, such as ceramides, in sponge holobionts. For instance, cytotoxic ceramides, glycosylceramides as well as methoxylated fatty acids have been isolated from three *Spheciospongia* species, *S. cuspidifera*, *S. vesparia* and *S. vagabunda* (Carballeira & Alicea, 2002; Costantino et al., 2005; Costantino et al., 2008; Eltamany et al., 2015). In addition, one of the cytotoxic ceramides reported from S. vagabunda contained the octadecanamide moiety (Eltamany et al., 2015). In this study, the precursor ions related to fatty acid amides were detected in all sponge fractions (Fig. 6). However, the molecular family related to ceramide-related molecules were detected in only fractions derived from four sponge species, i.e., *S.* cf. *vagabunda*, *P. purpurea*, *Haliclona* sp. and *C. reinwardti* (Fig. 6). Moreover, due to the apparent lack of low field signals below 5.5 ppm in the ^{1}H NMR spectrum of fraction D from *S.* cf. *vagabunda* (Fig. 4), it could be suggested that the majority of the compounds consisted of fatty acid amides and ceramides. Determining whether or not these specific compounds are responsible for the brine shrimp toxic activities observed in fraction D of *S.* cf. *vagabunda* requires further validation.

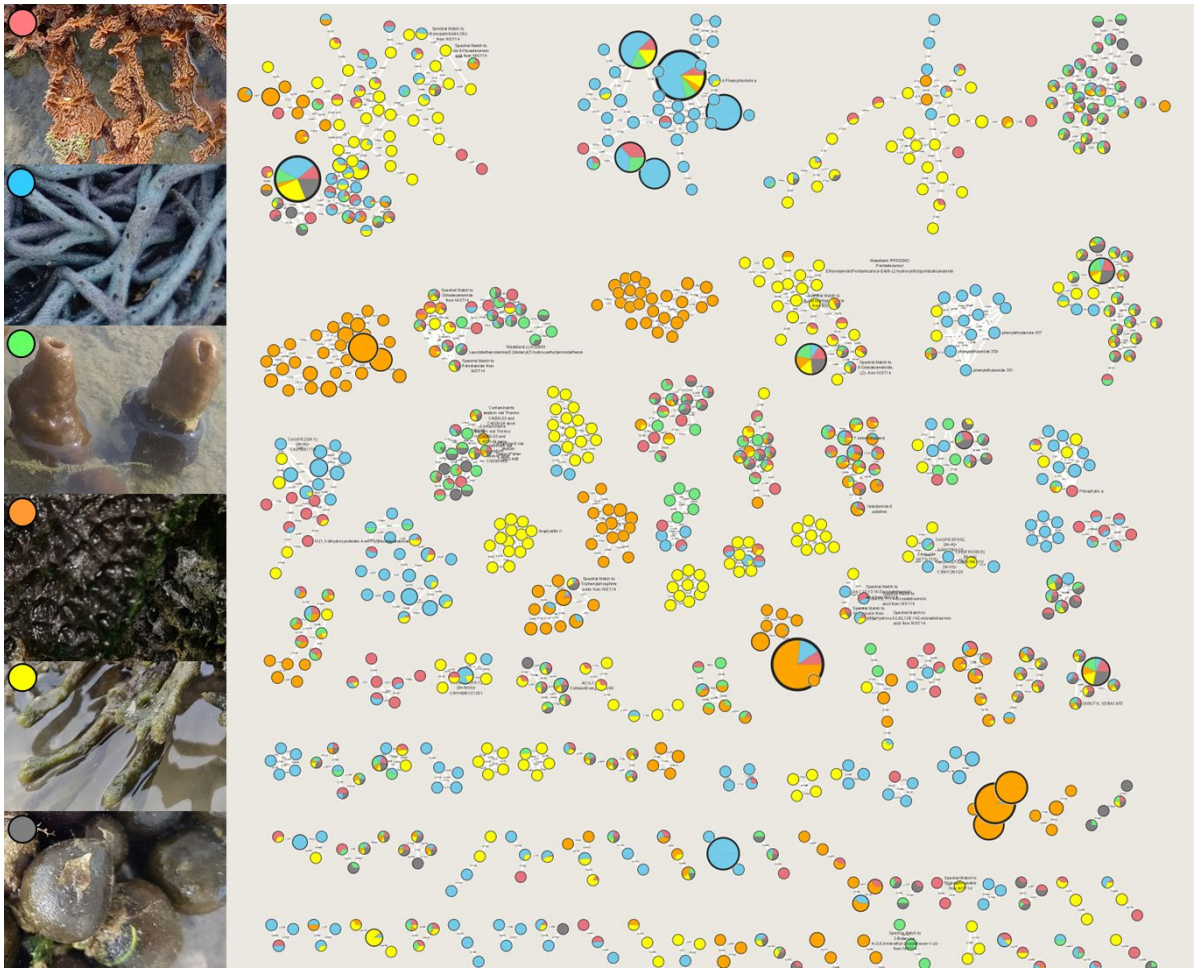

Fig. 5. Sponge fraction derived MS-based molecular network with a cosine cut-off of 0.7. Nodes unique to a specific sponge species are colour-coded as in left photo panel; *Clathria reinwardti* (fraction E, red), *Haliclona* sp. (fraction D, blue), *Spheciospongia* cf. *vagabunda* (fraction D, green), *Hippospongia* sp. (fraction E, orange), *Pseudoceratina purpurea* (fraction E, yellow) and *Stelletta clavosa* (fraction E, grey). A molecular network was created using the online workflow at GNPS. The spectra in the network were searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 4 matched peaks. Majority of the single occurring nodes have been omitted for clarity. Molecular networking of *H. cartilaginea* has been omitted due to maximum of up to six mzXML files on GNPS.

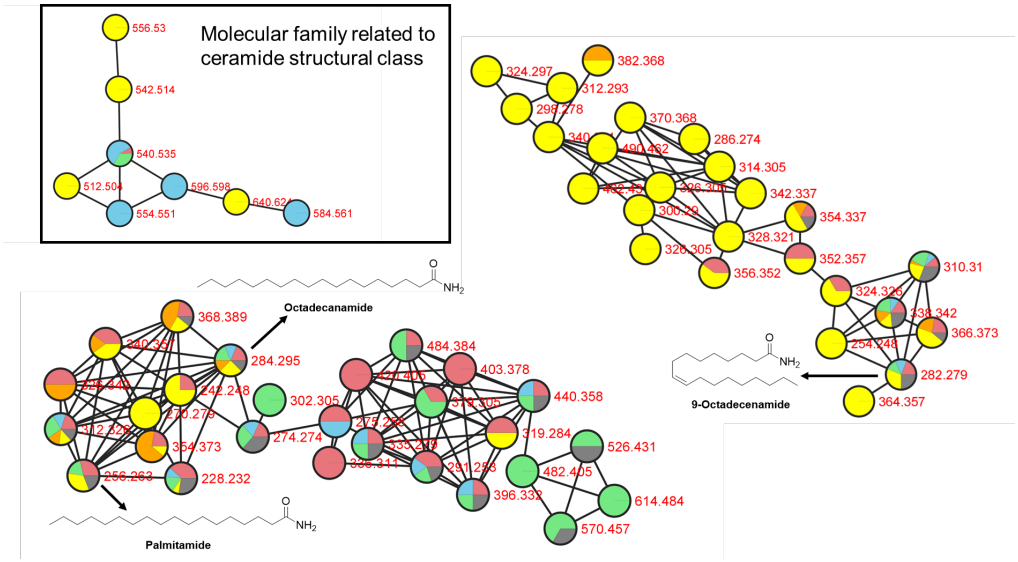

Fig. 6. Molecular network clusters of precursor ions, indicated in red numbers, found across all sponge derived fractions: *Clathria reinwardti* (fraction E, red), *Haliclona* sp. (fraction D, blue), *Spheciospongia* cf. *vagabunda* (fraction D, green), *Hippospongia* sp. (fraction E, orange), *Pseudoceratina purpurea* (fraction E, yellow) and *Stelletta clavosa* (fraction E, grey).

In Fig. 5, it is also observed that network clusters/nodes are detected solely from specific sponge fractions. A scan of the network clusters revealed that sponge fractions with the highest number of unique precursor ions are from *Hippospongia* sp. (fraction E), *P. purpurea* (fraction E) and *Haliclona* sp. (fraction D) while fraction D from *S.* cf. *vagabunda* has the lowest number of unique precursor ions with about 27 ions (Fig. 5). For instance, there are at least three unique network clusters in fraction E from *P. purpurea* that are assigned to the molecular families related to bromotyrosine alkaloids (Fig. 7). The known compound, araplysillin II, was dereplicated based on the GNPS MS/MS spectra libraries and it is clustered as monoisotopic m/z with a network containing 14 precursor ions (Fig. 7). A recent investigation to the chemistry of this polar fraction led to the isolation of six bromine-containing alkaloids, including two new anti-quorum sensing bromotyrosine alkaloids having an isotopic pattern that corresponded to four bromine atoms (Goh, 2023). The precursor ions of the known alkaloid and one of the new brominated alkaloids were detected in a separate network cluster from that of araplysillin II network cluster (Fig. 7). The presence of these bromotyrosine alkaloids in fraction E of *P. purpurea* detected in the molecular network is also consistent with the observation of low field olefinic proton signals, in the ^{1}H NMR spectrum, associated with the spirocyclohexadienyl-isoxazoline ring systems. Bromotyrosine alkaloids have been isolated from *P. purpurea* from other geographical regions and possess diverse biological activities, including antifouling, antibacterial, antifungal and cytotoxic properties (e.g., Tsukamoto et al., 1996; Takada et al., 2001; Jang et al., 2007; Hertiani et al., 2009). It could be that the presence of numerous bioactive bromotyrosine alkaloids within the sponge species provides an effective chemical defence strategy. In addition, certain marine slugs, such as *Tylodina corticalis*, are known to sequester selected bromotyrosine alkaloids from sponges, possibly for chemical defense (Gotsbacher & Karuso, 2015). Despite the extensive research previously conducted on several sponge genera, such as *Haliclona* and *Pseudoceratina* (Niemann et al., 2015; Zhu et al., 2019), molecular network analysis in this study detected the presence of possible new/novel specialised metabolites. This is due to only a very small fraction of the sponge chemistry having been described based on the comparison of the sponge metabolomes against known natural product MS spectral libraries. This was also observed in a study by Paul et al., (2019) where the majority of the chemistry of the well-studied Dysideidae sponges remains undescribed, as revealed by molecular networking.

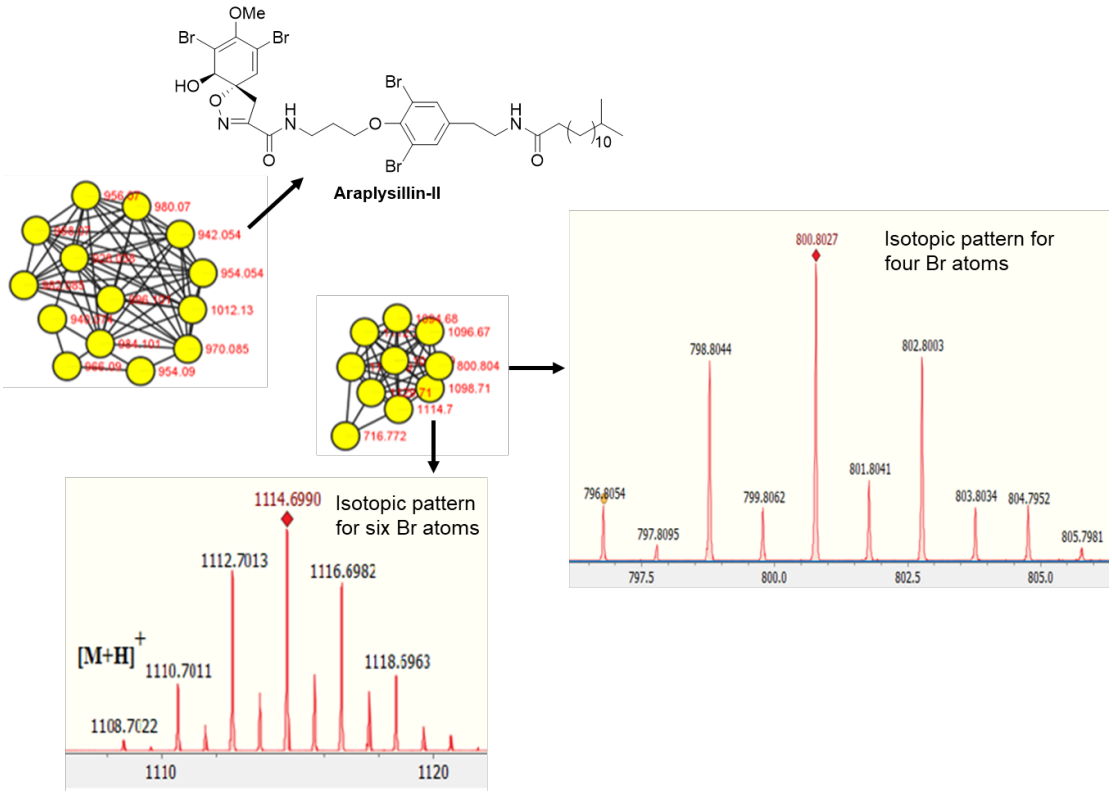

Fig. 7. Molecular networking of precursor ions, indicated as red numbers, detected in brine shrimp toxic fraction E from *P. purpurea* with detection of araplysillin-II and two related analogs containing four and six bromine atoms. Majority of single/cluster nodes present in the molecular networking have been omitted for clarity.

In spite of the exquisite brine shrimp toxicity (Fig. 2) and cytotoxicity (Fig. 3) of fraction E from *Hippospongia* sp., the dereplication of the molecular network of this fraction did not reveal any useful information regarding unique structural classes. We proceeded to perform chemoinformatic analysis on Marinlit for known specialised metabolites isolated from *Hippospongia* genus. The search on Marinlit (https://marinlit.rsc.org, accessed 7 August 2023) revealed about 100 metabolites reported from this genus, belonging to three main structural classes, including terpenoids (e.g., hippolides), hydroxylated sterols and fatty acid derivatives. Closer examination of the ^{1}H NMR spectrum of fraction E (Fig. 4D) and comparison with the ^{1}H NMR spectra of several sesterterpenoids from *H. lachne* led to the conclusion that fraction E

contains hippolide-related molecules (Piao et al., 2011; Piao et al., 2014; Jiao et al., 2017). Evidence is provided by the presence of multiplet olefinic proton signals and signals due to protons of methyl groups attached to sp^2 carbons at about 5.1 and 1.6 ppm, respectively. These proton chemical shifts are only found in the ^{1}H NMR spectral data of fraction E and are attributed to the farnesyl side chain of hippolides. Moreover, a low field singlet proton signal at 9.4 ppm is attributed to aldehyde protons which are present in several hippolide-related compounds, including hippolide D and derivatives (Piao et al., 2011; Piao et al., 2014). There are at least two unique major clusters of molecular families found in the molecular network of fraction E, each with more than 25 precursor ions. These clusters could be attributed to sesterterpenoid/sterols class of compounds due to the range of m/z values shown, which are typical of known sesterterpenoids/polyhydroxysterols from the genus *Hippospongia*. Moreover, the significant cytotoxicity of fraction E is probably due to sesterterpenes, as certain hippolides are known to be cytotoxic against cancer cell lines, such as hippolide A exhibiting cytotoxicity against A549, HeLa and HCT-116 cell lines with IC_{50} values of 5.22×10^{-2}, 4.80×10^{-2} and 9.78 μM, respectively (Piao et al., 2011).

This study uncovers the high prevalence of chemical defences, based on the brine shrimp toxicity assay, in common intertidal marine sponges found at the sponge gardens of P. Hantu (Fig. 2). A similar study recently conducted by Helber et al. (2018), using a panel of bioassays, including the brine shrimp toxicity assay, suggested that sponge chemical defences are a possible mechanism for high sponge abundance found on reefs at Zanzibar (Helber et al., 2018). The sponge community at P. Hantu is being dominated by sediment and low-light resistant species (de Voogd & Cleary, 2009). As there are many factors contributing to the abundance of sponge species, one such factors could be due to the sponge chemical defences. For instance, preliminary belt transact survey on sponge community at P. Hantu revealed *S.* cf. *vagabunda* to be the most abundant sponge species (Lim et al., 2023). The main factor for its abundance is due to environmental conditions which facilitated the occurrence of the endopsammic *S.* cf. *vagabunda* on sandy substrata. Chemical defences could be another factor since their polar fractions exhibited high brine shrimp toxicity levels (Fig. 2) and the presence of unique specialised metabolites, detected by molecular networking (Fig. 5), could deter/minimise predators, such as spongivorous fishes, including *Chatodontoplus mesoleucus* (Daisuke Taira, personal communication, 14 July 2023) and pufferfish (Zeehan Jaafar, personal communication, 14 July 2023) commonly found at P. Hantu reefs. Moreover, *P. purpurea* can produce a diverse range of bioactive molecules, structurally related to the bromotyrosine alkaloids, as chemical defenses and this could explain its persistence and wide occurrence in Singapore waters. In fact, *P. purpurea* is found in 11 of the 18 southern island sampling sites in a sponge community study by Lim et al. (2012). Furthermore, several researchers found related bromotyrosine alkaloids, including ceratinamides A and B, psammaplysins A and E, ceratinamine, moloka'iamine, 5-bromoverongamine and aplyzanzines C–F, from *Pseudoceratina* sp. to exhibit activities against fouling micro- and macro-organisms (Tsukamoto et al., 1996; Thirionet et al., 1998; Tintillier et al., 2020).

In conclusion, preliminary data from this study revealed the prevalence of brine shrimp toxicity in VLC-derived fractions from seven selected common intertidal sponge species found at P. Hantu. At least one polar fraction from each sponge species gave percentage toxicity above 50% when tested at 100 ppm. In addition, the toxic nature of these fractions is due to the presence of specialised metabolites as shown using ^{1}H NMR and mass spectrometric based metabolomic analyses, coupled with the Marinlit database. For instance, several unique classes of possible defensive molecules, including ceramides, bromotyrosine derivatives and sesterterpenoids, are detected in *Haliclona* sp., *S.* cf. *vagabunda*, *P. purpurea* and *Hippospongia* sp. Furthermore, polar fractions from *Hippospongia* sp. showed significant cytotoxicity against MOLT-4 leukemic cancer cell lines at 100 ppm and could be due to hippolide class of molecules. Moreover, metabolomic analysis showed that the natural products of several common intertidal sponge species, including *Haliclona* sp. and *H. cartilaginea*, are understudied. Metabolomic methods are effective analytical tools and increasingly being integrated into workflow for discovery of unique specialised metabolites from natural sources (Khushi et al., 2023). This study continues to support a recurrent selection for chemical defences in sponges using diverse specialised metabolites as a general life-history strategy (Thoms & Schupp, 2007).

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